Review

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The molecular and cellular choreography of early mammalian lung development

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Abstract: Mammalian lung development starts from a specific cluster of endodermal cells situated within the ventral foregut region. With the orchestrating of delicate choreography of transcription factors, signaling pathways, and cell–cell communications, the endodermal diverticulum extends into the surrounding mesenchyme, and builds the cellular and structural basis of the complex respiratory system. This review provides a comprehensive overview of the current molecular insights of mammalian lung development, with a particular focus on the early stage of lung cell fate differentiation and spatial patterning. Furthermore, we explore the implications of several congenital respiratory diseases and the relevance to early organogenesis. Finally, we summarize the unprecedented knowledge concerning lung cell compositions, regulatory networks as well as the promising prospect for gaining an unbiased understanding of lung development and lung malformations through state-of-the-art single-cell omics.

Keywords: organogenesis; embryonic pattern formation; cell-to-cell interaction; lung malformation; single-cell analysis

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Mammalian lung development: fascinating voyage to the first breathe

Until the WHO chief declares an end to COVID-19 as a global health emergency, the pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV2), continues to inflict devastating impacts to daily lives of human [1]. The proper operation and development of the respiratory system, which is usually exposed to the external environment, hold pivotal importance in safeguarding us from harmful intrusions. Thus, it's of great significance to advance the comprehension of the mammalian lung development.

The mammalian respiratory system is a sophisticated network that encompasses organs and tissues to breathe. Generally, it is composed of the airway, lung, blood vessels as well as muscles powering the lungs. The major biological function of the mammalian respiratory system is gas exchange, during which oxygen is delivered throughout the body and waste gases like carbon dioxide are cleaned out [2]. Alveoli within the distal lung tissues are acinar structures where gas exchange occurs. Meanwhile, the conducting airways, including the trachea, bronchi and bronchiole, are proximal branching tubules that connect the external environment to alveoli and remove various harmful particles, such as viruses and bacteria, to protect alveoli from invasion [3]. Developmental defects in the respiratory system can result in life-threatening breathing disorders at birth [4].

The embryonic development of the respiratory system starts just at the early organogenesis stage, and can be divided into five sequential stages. To specify, the embryonic stage (embryonic week 3–7 in humans and embryonic day 8.0–9.5 in mice), the lung primordium is specified within the foregut during this stage; the pseudo-glandular stage (embryonic week 7–17 in humans and embryonic day 9.5–16.5 in mice), following the primary lung bud forms, extensive airway branching occurs at this stage; the canalicular stage (week 17–27 for human embryos and day

16.5–17.5 for mouse embryos), bronchioles and alveolar epithelium evolves at this stage; the saccular stage (embryonic week 27–36 in humans and embryonic day 17.5 to postnatal day 5 in mice), the alveolar duct and air sacs keep develop at this stage; and the final alveolar stage, which is characterized by the maturation of the alveoli, and this stage usually takes years from the late fetal life to childhood age [5–8] (Figure 1).

Pioneering studies have underscored the crucial roles of systematic coordination of signaling pathways and transcription factors in orchestrating the precise development of the respiratory system. An increasing understanding of the molecular pathways important for lung development also sheds light on exploring postnatal lung regeneration, as many of these pathways and processes are recapitulated during injury and regeneration [9, 10]. Notably, it is recently demonstrated that *in vitro* differentiation of pluripotent stem cells into self-renewal epithelial progenitors under the instruction of developmental morphogens represents a promising therapy for diseases that result from alveolar damage [11].

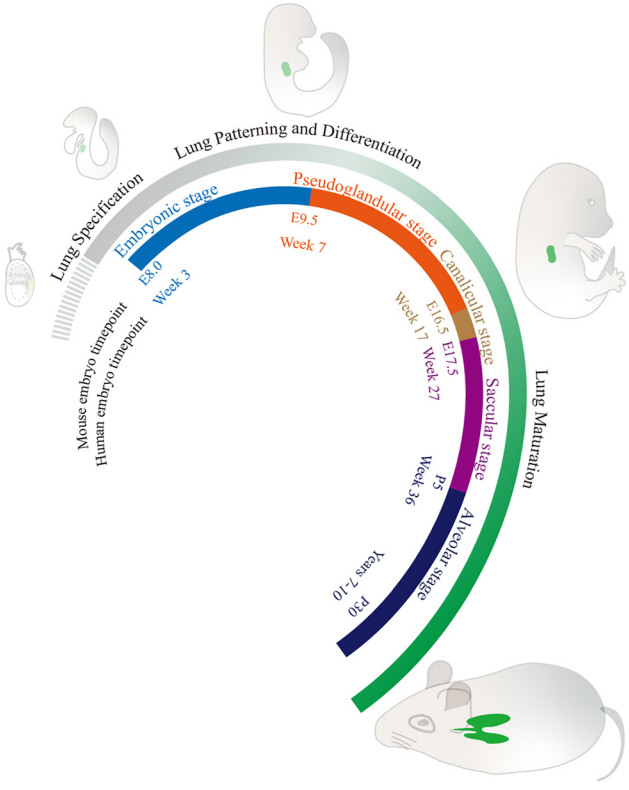

Figure 1: The developmental timeline of mammalian lung. Mammalian lung development starts from the early organogenesis stage to early post-natal stage. Five sequential stages could be divided as highlighted in different colors. The specific developmental timepoints for both mouse and human embryos were also included.

Here, in this review, we will mostly focus on the current progresses concerning the molecular mechanisms underlying the mammalian lung development, especially for the early developmental stage, and also explore the developmental cues for the congenital lung disease. We also appreciate the forthcoming explosion of knowledge in the field of mammalian lung development using the cutting-edge technologies of single-cell or spatial transcriptomics. We believe with all these efforts, proper development of the respiratory system must be ready for new lives when they come into the world, take their first breath, and let their first cry out.

Foregut morphogenesis and specification of the respiratory lineage: prepare to be competent lung progenitors

During mouse embryo development, following gastrulation, gut endoderm cells, which are derived from the definitive endoderm and intermediate visceral endoderm cells [12–15], endure complex morphogenetic movements and finally form the primitive gut tube. According to the cellular location and prospective fates, the gut tube can be majorly dissected into the foregut, which represents the most anterior region of the gut tube, and the midgut and hindgut, which locate progressively at more posterior regions [16]. Generally, the mammalian respiratory system is specified at the anterior foregut region, marked by the specific expression of the transcription factor NKX2-1 (TTF1) [4, 17, 18] and the gradual formation of an endodermal diverticulum in the ventral wall of the foregut [19]. Actually, the earliest morphological appearance of the lung primordium is not apparent until a pair of lung buds form at E9.25–E9.5 [20]. However, the presence of *Nkx2-1* transcript can be detected as early as the E8.25 stage [21–23]. Notably, the earliest expression of Nkx2-1 is not only restricted in the lung progenitors but also in the forebrain [24] and thyroid cells [25]. Thus, the determination and characterization of specific respiratory markers are needed to more accurately depict the early events of respiratory specification.

To trace the earliest lung fate during mouse embryogenesis, Perl et al., have established an inducible tracing system by preparing *SP-C-rtTA/tetO-Cre/ZAP* (*Sftpc* promoter driven rtTA expression plus Dox dependent temporal control) mouse embryo to identify the timing of cell fate restriction and lineage relationships among cells that serve as progenitors of the respiratory epithelium during lung

morphogenesis [26]. They found that progenitors of the distal respiratory system (peripheral lung tubules) may be set aside before or coincident with gastrulation [26]. Recently, single cell analyses of both molecular and cellular phenotypes have been revealed as crucial to delineate and interrogate the process of lineage commitment [27–31]. Interestingly, a sub-cluster of foregut (Foregut 2) at the stage of E8.25 and E8.5 was identified as the potential lung precursors, which express lung-associated genes *Ripply3* and *Irx1* but with limited *Nkx2-1* transcript abundance [23]. However, whether this earlier cell type truly represents the lung fate-restricted cells still requires further experimentation through strict lineage tracing and functional analyses. Moreover, two developmental origins of gut endoderm cells can be identified as the extra-embryonic endoderm and embryonic endoderm [12–14, 23]. The specific contributions to foregut, especially to lung fate restricted foregut, from these two progenitors derived gut progeny remain to be clarified.

Moreover, previous studies have indicated that epigenetic priming usually precedes the transcriptional transition that directs the acquisition of a new cellular identity during embryogenesis [32–34], and proper activity of regulatory elements could serve as ideal lineage progenitor markers with spatial-temporal specificities [35, 36]. In fact, a specific *Tbx4* enhancer has been used as a specific marker to track the developmental trajectory of lung mesenchyme [37]. Geusz et al. found that FOXA1 could bind to a subset of priming lung enhancers (class I enhancers) [38], which may be candidates for determining the lung progenitors during early development. Therefore, strict lineage tracing and systematic evaluation of lung development with broader dimensions, including both transcriptional and epigenetic signatures, are required to determine and validate the earliest lung primordium and related regulators.

Complex gene regulatory networks have been revealed to control respiratory lineage induction. As previously mentioned, *Nkx2-1* acts as one of the available early lung markers. However, mouse with genetic mutation of *Nkx2-1* still developed normal primary lung bud, even though further branching and trachea development process were largely compromised [22]. This result indicates that NKX2-1 may be only responsible for a subset but not all lung cells, or acts later than the lung cell fate specification. Transcriptome profiling of the Nkx2-1 mutant mouse embryo revealed that an *NKX2-1*-independent transcriptional program exists within the developing trachea and esophagus [39]. In contrast, embryos with double mutation of *Wnt2* and *Wnt2b* or endoderm-specific deletion of *β-catenin* lead to complete lung agenesis but without abnormalities in other endoderm derived organs [40]. Thus, Wnt2/2b-β-catenin signaling pathway seems to function as the master regulator of the lung fate specification prior to *Nkx2-1*. Given Wnt2/2b are mostly derived from the splanchnic mesoderm (SM) surrounding the gut tube, the coordinations that synchronize the development of mesenchyme and epithelial lineages should be the key regulatory factors during the formation of functioning lung, and a systematic measurement of the endoderm as well as the surrounding mesoderm and ectoderm cells will promote a comprehensive understanding of the lung development.

To chart the inductive signals for *Nkx2-1* expression, Rankin et al., isolated foregut tissues and arranged three explant culture combinations: (1) intact explants containing the endoderm with adjacent mesoderm, and non-neural ectoderm; (2) endoderm-only explants, in which the mesoderm and ectoderm were removed; and (3) mesoderm and ectoderm-only explants (referred to as mesoderm) [41]. They found that only co-culture of both endoderm and mesoderm explants could induce the expression of respiratory marker-*Nkx2-1*. Mechanistically, they revealed that RA signal pre-patterns the lateral plate mesoderm and then promotes Hedgehog (Hh) ligand expression in the foregut endoderm; then, Hh subsequently signals back to the pre-patterned mesoderm to induce the expression of the lung-inducing ligands Wnt2/2b and Bmp4; and finally, retinoic acid (RA) regulates the competence of the endoderm to activate the Nkx2-1 positive respiratory program in response to the mesodermal WNT and BMP signals [41]. Ikonomou et al., directly compared the transcriptome of E9.0 NKX2-1 positive lung cells, E9.0 NKX2-1 positive forebrain cells, and E13.5 NKX2-1 positive thyroid cells, and they revealed that Wnt, Hedgehog, Tgf-β superfamily, and Hippo pathways were exclusively upregulated in lung primordial progenitors upon specification [42]. Moreover, pharmacological interruption and genetic manipulation were further applied to validate the critical roles of these signals during lung fate specification [42]. Actually, the critical inductive role for the mesenchyme in gut tube organogenesis was firstly established in the 1960s, where it showed that SM transplanted from different anterior-posterior regions of the embryo could direct the adjacent epithelium to adopt the organ identity consistent with the original SM position [43, 44]. Amanda et al. were able to identify that it is cardiac mesoderm derived fibroblast growth factor (FGF) signals are required for the patterning of ventral foregut cells into lung cells by using embryo tissue explants [45]. Furthermore, FGF signaling plays a dosage-dependent role during lung specification, in which high concentration of FGF can induce the expression of Nkx2-1 in the original midgut dorsal endoderm cells [45]. Recent study using 3D organoid system further demonstrate that the cell fate of the

foregut and hindgut can be switched by the regional niche factors, including FGFs and RA [46].

Beyond the mesoderm-derived signals can regulate the cell differentiation of gut tube, signals derived from endoderm cells can also pattern the surrounding splanchnic mesoderm. Han et al., dissected the foregut cells that are spatially located between the posterior pharynx and the midgut from three embryonic stages spanning the period of early patterning and lineage induction: E8.5 (5–10 somite), E9.0 (12–15 somite), E9.5 (25–30 somite), and then applied single cell transcriptomic profiling of these foregut single cells [44]. Based on the established transcriptomic atlas, they have identified the diversity of transcriptome features of distinct organ-specific epithelium related splanchnic mesoderm lineages, and inferred a spatiotemporal signaling network of endoderm-mesoderm interactions that orchestrate foregut organogenesis. These results indicate that the signaling roadmaps of both the foregut epithelium and splanchnic mesoderm are vital for the lung primordium specification and following development.

These studies highlight the essential roles of mesenchyme-derived signals during the initial development of lung fate and the developmental plasticity for gut cells in response to SM-derived signals along rostral-caudal axis.

Lung patterning and differentiation: step-by-step process through integrating both extrinsic commands from the surrounding mesenchyme and intrinsic regulators of epithelium

Appropriate body axes formation at the individual level or correct tissue patterning at the organ or tissue level relies on the major morphogenetic events controlled by intricate molecular networks during mammalian embryo development [47]. Following the specification of anterior gut endoderm into the lung primordium, the patterning of three major orthogonal axes (dorsal-ventral axis, proximal-distal axis, and left-right axis) starts, which finally build the structural basis for a functioning lung (Figure 2).

Dorsal-ventral patterning

During early mouse lung development, signals from surrounding tissues, including the notochord and splanchnic mesoderm, begin to establish a dorsal-ventral pattern in the gut tube [48, 49]. The correct dorsal-ventral (D-V) patterning of the single foregut tube is related to the future separation of the esophagus (dorsal) and the trachea (ventral) [50, 51]. Failure of normal D-V patterning is associated with common birth defects, including esophageal atresia and tracheoesophageal fistula [52].

Molecular dissection of the respiratory-specified gut tube along the dorsal-ventral axis revealed a complementary pattern between NKX2-1 and SOX2 [53], which indicates the molecular establishment of the original D-V axis for the lung gut tube. The dorsal-enriched SOX2 orchestrates the separation of the trachea and esophagus and regulates subsequent epithelial morphogenesis. Meanwhile, SOX2 could also interact with other transcription factors and signaling pathways to modulate the proliferation and differentiation of the lung epithelium [54]. Molecularly, analyses of the direct target genes for the transcription factor SOX2 and NKX2-1 by ChIP-seq indicate that SOX2 and NKX2-1 shared some common target genes while also regulating a unique set of targets [55, 56]. The transcription factors of SOX2 and NKX2-1 seem to function reciprocally during the early D-V patterning of the mouse lung. In the mutant esophagus of Sox2 compound mutant embryos ($Sox2^{EGFP/COND}$ mouse), strong nuclear staining for NKX2-1 could be detected in the dorsal region; meanwhile, in the NKX2-1 null embryo, an elevated level of SOX2 exists in the abnormal foregut [57]. Phenotypic analyses of the Sox2 compound mutant embryo also identified that about 60 % of mutant embryos exhibit distal tracheoesophageal fistula, with proximal esophageal atresia (EA), and nearly 100 % of P0 mutant mice show labored breathing and die with air in the stomach [57]. To precisely pinpoint the roles of Sox2 in early lung morphogenesis, Machiko et al. generated the mouse line with endoderm-specific deletion of *Sox2* ($Foxa2^{EGFP-CreERT2}Sox2^{flox/flox}$ mouse) [58]. They confirmed the aberrant overexpression of *Nkx2-1* at the dorsal foregut tube region. Besides, they found that airway epithelia in the absence of SOX2 showed normal tissue growth and branching patterns identical to wild-type embryos [58]. These results indicate that the balance of NKX2-1 and SOX2 play crucial roles in demarcating the D-V axis of the foregut tube and following physical separation between the future esophagus and the trachea, but may be dispensable for the early lung morphogenesis. Additional markers for the dorsal-ventral patterning were also identified in the single-cell RNA-seq atlas, such as *Klf5*, *Lrig1*, *Krt19*, and *Dcn* [39]. Ventral foregut endoderm enrichment of *Shh* [59] also plays a crucial role in the dorsal-ventral compartmentalization of the foregut [49]. Mice with genetic mutation of *Shh* or the core components of Hedgehog signaling

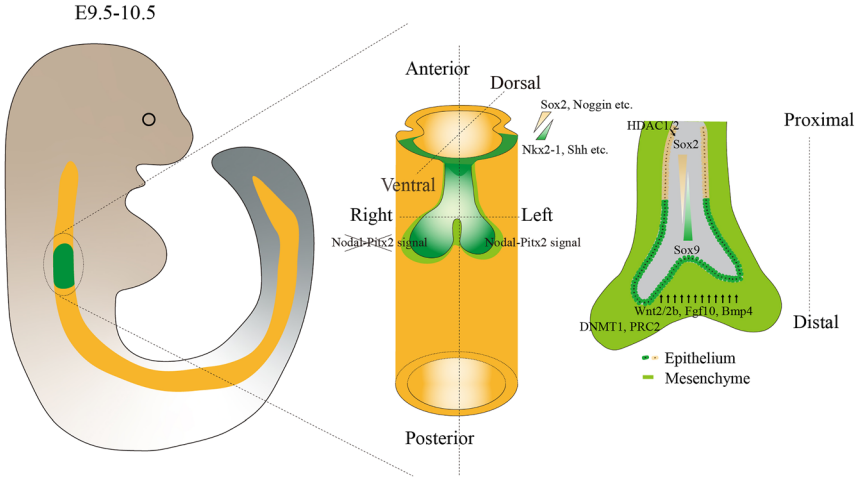

Figure 2: Spatial patterning of the mouse lung. The morphological distinctions for the mouse lung appear following the extension of the specified lung foregut into the surrounding mesenchyme. Crucial developmental signaling pathways and regulators play roles in the spatial patterning of the lung.

Gli2 or *Gli3* exhibit typical phenotypes of abnormal separation or hypoplasia between the trachea and esophagus [49, 60].

It has been revealed that the D-V pattern depends on signals from the surrounding mesenchyme including BMPs, Fgfs, and Wnts. In the E10.5 mouse embryos, FGF10 is expressed in the mesoderm surrounding the ventral foregut and phospho-ERK1/2 is specifically distributed in the ventral gut endoderm [57]. Moreover, *ex vivo* treatment of the embryo foregut with 50 ng/mL FGF10 led to a significant downregulation of *Sox2* expression. Bmp signaling as well as Wnt signaling were also found to participate in the regulation of dorsal-ventral patterning of the foregut tube [40, 61, 62]. BMP4 and BMP7 are the predominant BMPs expressed in the foregut mesenchyme region [63]. Meanwhile, the BMP antagonist Noggin and Chordin derived from the notochord counteracts with BMPs, and directs the dorsal-ventral patterning of the gut tube [63, 64].

Proximal-distal patterning

During mouse lung morphogenesis, NKX2-1 positive endoderm cells give rise to the lung epithelium and are patterned in a proximal-distal fashion in part through a process called branching morphogenesis [65]. Throughout this temporally regulated process, early endodermal precursors undergo fate decisions along the highly patterned proximal-distal (P-D) axis. As the primary buds evaginate from the ventral foregut into the surrounding splanchnic mesenchyme, the elongating distal lung buds launch a series of reiterated branching processes, lining the airways to a diverse population of specialized epithelial cells that differentiate along the P-D axis, and finally form the stereotypic morphology of the pulmonary tree [4]. Two levels of the P-D axis could be dissected as to the P-D axis of the mouse lung. One level of the P-D axis exists in the early embryonic stage manifested as the distinction between the trachea (proximal) and the lung (distal), this forms just after the emergence of the Nkx2-1 positive lung epithelium diverticulum in the foregut region of the mouse embryo; and the other P-D axis exists in the mature lung manifest as proximal bronchiolar epithelium and the distal respiratory epithelium [20, 66].

Generally, proximal epithelial precursors, marked by expression of *Sox2*, will give rise to airway lineages, such as ciliated and secretory cells. The distal epithelial precursors, marked by *Sox9*, will ultimately give rise to alveolar lineages, alveolar type 1 (AT1) and type 2 (AT2) cells, which form the surface for gas exchange and produce pulmonary surfactant, respectively [67]. A number of genes encoding signaling molecules and transcription factors, such as *Tgfβ2*, *Id2*, *Sox9*, and *Etv5*, have been identified to be differentially expressed in the epithelium and mesenchyme of the distal buds [68, 69]. Meanwhile, genes, such as the Clara cell 10-kDa protein (*CC10*) and *Foxj1*, are expressed exclusively in the proximal epithelium [70]. Through conditional control of *Sox2* expression, Gontan et al. found that *Sox2* deletion in mouse lung results in an increase of club cells, ciliated cells, and basal cells, whereas its overexpression leads to elevated levels of committed progenitor cells, including p63+ cells and neuroendocrine cells [71]. Meanwhile, interruption of *Sox9* expression leads to branching defects with decreased domain branches and the presence of terminal cystic structures [72, 73].

Through *in vitro* grafting experiments, Shannon et al. found that epithelial cells of embryonic lung were pluripotent, displayed plasticity, and proposed that their fate toward a proximal or distal phenotype was dictated by lung mesenchyme [74, 75]. The counteracting equilibrium

between extracellular secreted signals and epithelium-derived antagonists was instrumental to the P-D patterning of lung epithelium. The BMP antagonist gremlin is specially enriched at the proximal region of lung epithelium [70]. Lung-specific overexpression of the *Gremlin* gene would lead to proximalization of the distal lung tubules [70]. Moreover, the BMP signaling target gene-*Id2*, was identified to specifically distribute in the distal epithelial tip region. Lineage tracing by genetic knocking-in the CreERT2 expression cassette into the *Id2* enhancer locus, Rawlins et al., successfully labeled the distal epithelium tip and found that Id2 labeled epithelium cells not only maintain the capacity of self-renewal but also are able to contribute to both the bronchiolar and alveolar compartments during the pseudoglandular stage [76]. Wnt/β-catenin signaling is also involved in the regulation of proximal-distal differentiation of airway epithelium. Inhibition of Wnt/β-catenin signaling, either by expression of Dkk1 or by tissue-specific deletion of β-catenin, results in disruption of distal airway development and expansion of proximal airways [77, 78].

There also exists regionalized enrichment of proliferating cells along the P-D axis. Okubo et al., have found that cells in the distal tips of the lung bud possess a higher proliferation index than the proximal counterpart, as revealed by a higher level of BrdU incorporation and *N-Myc* expression. Mechanistically, they found that lung tissue-specific over-expression of *N-Myc* could specifically enhance the ratio of Sox9-positive cells while conditional deletion of *N-Myc* led to defective proliferation and differentiation of the lung epithelium and mesenchyme [79].

Epigenetics are also involved in the regulation of the P-D patterning of the lung. Enzymes critical for the writing, maintaining, or erasing the epigenetic modifications are responsible for the differences in gene expression patterns along the proximal-distal axis. Distinct members of the histone deacetylases (HDACs) family have been reported to execute factor-specific functions. To specify, HDAC1 and HDAC2 are responsible for the maintenance of *Sox2* expression in the proximal lung epithelium [80]. Meanwhile, HDAC3 plays a role in the regulation of AT1 cell spreading and the subsequent distal alveolar maturation [81, 82]. The core component of Polycomb repressive complex 2 (PRC2), EZH2, seems to play an essential function in repressing the basal cell lineage and restricting the smooth muscle lineage during lung development [83]. Apart from the histone modification level, DNA methylation also plays specific roles in the proximal-distal patterning of mammalian lung development. Lung endoderm-specific deletion of *Dnmt1* through Shh^{Cre}:$Dnmt1^{Flox/Flox}$ leads to the expansion of the distal epithelium and a concomitant loss of proximal endoderm cell fate [67].

It is worth mentioning that the exact molecular demarcation of the P-D axis in the lung epithelium is not exactly conserved between rodents and primates [73]. Different from mouse lung epithelium, SOX2 and SOX9 are co-expressed in the human distal lung epithelium during early morphogenesis [84]. Currently, it was found that SOX2 and SOX9 double-positive human distal lung epithelium cells are crucial for the P-D patterning [85, 86]. The molecular and cellular differences along the P-D axis between human and mouse indicate that there may exist species-specific regulatory mechanisms for lung morphogenesis as well as the P-D axis defects-related lung disease pathogenesis [87, 88]. However, limited knowledge could be acquired for the embryonic development of human lung tissue at the current stage. Therefore, future exploration of the native human lung tissues or human embryonic stem-cell based lung organoids will be of great significance.

Left-right patterning

Globally, the internal mammalian body plan is laterally asymmetric with a consistent handedness, and the organs as well as vasculature are conspicuously L-R asymmetric in their positions and patterns [89]. For example, the mesoderm-derived organ-heart, loops asymmetrically during development and ultimately acquires a leftward position in the chest. Meanwhile, the endoderm-derived organs-the stomach and pancreas sit to the left and the liver to the right in the abdomen [90]. Abnormal left-right asymmetries of organs, known as heterotaxy, are usually associated with severe developmental defects.

Interestingly, for the endoderm-derived branched organ-lung, the left-right asymmetry is apparently shown as the disparate lobation patterns of the left vs. right lungs (fewer lung lobes on the left than the right). Murine lungs characteristically have one lobe on the left and four lobes on the right [18], while primate lungs have two lobes on the left and three lobes on the right. Despite the differences in lobe numbers between left and right, there also exist some other anatomical differences as well as functional differences between the left and right lung lobes. Unbiased alveoli counting based on Euler characteristic and fractionator sampling design reported that the right lobes contained 47 % more alveoli than the left in adult rats [91]. Tsai et al., also reported that there are significant differences in P_T (the total power), F_{50} (equally divided the power spectrum of lung sounds), and $R_{I/E}$ (the ratio of inspiration power to expiration power) between the left and right lungs detected by dual-channel auscultation [92]. A significantly higher blood flow was also found through the left upper

pulmonary zone than the right counterpart of normal subjects [93]. These results indicate that there may also exist functional variations between the left and right lung lobes beyond the anatomical differences. In fact, through experimental infection of Pb18 yeast cells, Tristão et al. found that the left lungs were preferentially targeted by Pb18 yeast cells, while the right lungs showed increased production of nitric oxide and interferon-c (IFN-c) when facing infection [94]. Thus, measurement of the lung development, anatomy, and functions in both normal and disease conditions will broaden the understanding of the pulmonary system.

The left-right asymmetry of the lung can be developmentally traced as early as E9.5 to E10, when two primary lung buds (left and right lung anlage) have begun to form and extend caudally [95]. The two original lung buds have shown size differences with the right lung bud larger than the left [4]. Currently, it is well known that the left-right organ asymmetries are instructed by the early organogenesis stage-established left-right molecular axis (*Nodal-Pitx2* molecular cascade). Enormous progress has been made in the understanding of the early-stage left-right axis establishment. Both the newly identified gastrulation-stage symmetry-breaking event in the proximal mesenchyme and lateral mesoderm cells [96] and node-centered Nodal flow [97, 98] play roles in the molecular finalization of the left-right asymmetry in the lateral plate mesoderm (LPM). Genetic mutations of key components of early left-right patterning-related molecular pathways usually lead to defects of organ laterality [99–104]. In the lung, these defects commonly manifest as isomerism, the presence of equal numbers of lobes (with either right or left pattern) on both sides [18]. For example, the *iv* mice, in which the dynein gene-*iv* was mutated, exhibit severe organ situs defects such as left pulmonary isomerism (both left and right side develop one lobe) and right pulmonary isomerism (both left and right side develop four lobes) [99]. Interestingly, most of these left-right axis-related genes are only transiently expressed (E8.0–E8.5) in the splanchnic mesoderm, except for *Pitx2* [18]. And even for *Pitx2*, only the specific isoform-*Pitx2c* seems to play roles in the regulation of lung laterality [105]. Previous study also demonstrated that different organs exhibit organ-specific responsiveness to LPM-enriched *Pitx2* [105]. Thus, how does each organ primordium interpret and transfer the left-right signal, and what are the organ-specific downstream cellular mechanisms responsible for the asymmetric morphological patterns remain as open questions. Pioneering work has proposed an accelerator-brake mechanical model of *Pitx2* function in gut tilting and rotation [106]. However, whether this model could be applied to explain the molecular mechanisms of lung morphogenesis remain unknown. Considering the distinct abundance of lung lobes between left and right is apparently different from the gut tilting and rotation issue, more work is required in elucidating this fascinating biological event.

Congenital lung disorders: developmental disasters resulted from interruption of the highly regulated lung patterning process

Congenital lung disorders refer to abnormal conditions that usually affect the lung development of babies during pregnancy. The fascinating but highly regulated lung development process involves cell proliferation, cell fate differentiation, as well as intricate cell–cell interactions. Disruption at any step in these processes can lead to congenital malformations of the foregut and later lung organ. A summary of congenital lung malformations has been listed in previous reviews [107–109] or by referencing the public database like RespiRare. Here, we will list several congenital lung diseases related to early development, the current pathological understanding of these diseases, and briefly discuss the potential developmental cues for the diseases.

Bronchogenic cysts

Bronchogenic cysts, also known as foregut duplication, are generated from an abnormal budding or lesion of the ventral foregut [109]. Bronchogenic cysts characteristically exhibit clinical and radiological polymorphism [110]. The location of a bronchogenic cyst depends on the embryological stage of development when the anomaly occurs. When the abnormal budding happens during early development, the cyst occupies the tracheobronchial tree. Cysts that arise later are more peripheral and may involve the lung parenchyma [111]. The cysts could be filled with fluid or air. Even though many of them are asymptomatic, however, frequent symptoms of cough, fever, dyspnea, or even malignancy have also been reported in patients with bronchogenic cysts [110]. Even though the bronchogenic cysts form during embryogenesis, the causes of bronchogenic cysts are largely

unknown. Bronchogenic cysts are not generally associated with genetic or chromosomal differences.

Tracheoesophageal fistula

Tracheoesophageal fistula (TEF) refers to an abnormal connection between the airway and the gastrointestinal tract [112]. For patients with TEF, swallowed liquids or food can be aspirated (inhaled) into the lungs of patients with TEF. Feeding into the stomach directly can also lead to reflux and aspiration of stomach acid and food. These will further lead to the symptoms of coughing while feeding and frequent lung infections. Moreover, TEF usually occurs with a related condition called esophageal atresia (EA). Esophageal atresia with or without tracheoesophageal fistula (EA/TEF) is the most common congenital malformation of the upper digestive tract [113]. It's estimated that TEF affects one in every 3,000 to 5,000 births in the United States.

The major developmental causes for TEF could be partially attributed to unsuccessful separation between the trachea and the esophagus. During mammalian embryogenesis, both the trachea and esophagus are developed from one single gut tube [52]. Following the dorsal-ventral patterning of the foregut, the fetus' trachea and esophagus will be separated into two distinct tubes. Mutations of genes involved in the dorsal-ventral patterning and the formation of tracheoesophageal septum are highly related to TEF, such as SOX2 [114], MYCN [115], NOGGIN [116], FOX family genes [113].

Pulmonary agenesis

Pulmonary agenesis (PA) is a rare congenital anomaly, defined as the absence of the lung parenchyma, bronchus, and pulmonary vessels [117]. The estimated prevalence is about 24–34 per 1,000,000 live births, and one per 10,000–15,000 autopsies with a slight preponderance of females [118]. Anomalies in the cardiovascular, gastrointestinal, genitourinary, or musculoskeletal systems are also frequent in cases with lung agenesis [118]. More than 50 % of children with lung agenesis die within 5 years of birth [118]. Classically, PA can be classified into three types: type 1 (agenesis) with a phenotype of complete absence of the lung and bronchus and no vascular supply to the affected side; type 2 (aplasia) with a phenotype of rudimentary bronchus with the complete absence of pulmonary parenchyma; type 3 (hypoplasia) presents as the presence of variable amounts of bronchial tree, pulmonary parenchyma, and supporting vasculature [119, 120].

This deleterious phenotype is usually caused by the failure of the foregut to specify the lung primordium and give rise to the two primary lung buds (left and right lung buds). Disruptions of regulators involved in lung foregut cell fate specification, proliferation and branching, are highly related to the occurrence of lung agenesis/aplasia/hypoplasia [121]. The right lung is reported to be more severely affected by agenesis than the left lung [122, 123]. Right lung agenesis is usually associated with the displacement of the heart and mediastinum rightwards accompanied by a distortion of the bronchial and vascular structures, which worsens the prognosis [118].

Pulmonary isomerism

As mentioned in the above section, the mammalian lung shows a typical left-right asymmetric morphological pattern, manifesting as different numbers of lobes between the left and right sides. Pulmonary isomerism is an anomaly of the number of lung lobes. The anomaly in pulmonary isomerism is frequently associated with situs inversus [109]. Besides, left isomerism is often associated with polysplenia, and right isomerism is often associated with asplenia [124]. As revealed in both model animal experiments [101, 125] and clinical studies [124, 126, 127], genetic mutations of the Nodal-Pitx2 cascade as well as the primary ciliary function should be attributed to the incidence of isomerism. Beyond the genetic cues, it was recently reported that the infection of SARS-CoV2 is associated with an increment of situs inversus during early gestation [128]. They reported over four times increment of fetal situs inversus in China since the "zero-Covid" policy was lifted. Even though the contribution of genetic abnormalities related to primary ciliary dyskinesia remains verifying, this report also suggests environmental factors, such as SARS-CoV2 infection here, may also play roles in the correct development of organ situs.

Scimitar syndrome

Scimitar syndrome is a rare congenital disease that usually affects the function of the heart and the lung [109]. Only about 1 to 3 out of 100,000 newborns are born with this disease [129]. For people who are diagnosed with scimitar syndrome, a typical anatomic feature that resembles a backsword can be detected in chest radiography [129]. The constant feature of scimitar syndrome is the partial or total anomalous pulmonary venous return to the inferior vena cava. Some variable features, including dextrocardia, hypoplasia of the right lung, as well as hypoplasia of the

right pulmonary artery, are also found in patients with scimitar syndrome. The higher frequency of right lung development defects indicates the molecular causes for scimitar syndrome may be related to early stage of lung specification and pattern formation. However, the specific genetic causes for scimitar syndrome remain largely unknown [130]. Surgery to directly reroute the pulmonary vein or remove the hypoplastic lung can efficiently alleviate the symptoms.

Although most congenital lung malformations are rare diseases, it has been reported that they account for up to 18 % of all congenital anomalies [109]. Moreover, the specific developmental or genetic cues for these disorders remain largely elusive. A brief summary of lung development-related key genes and their implications in lung diseases has been listed in Table 1. Efforts to identify the molecular architecture and novel regulators underlying lung morphogenesis, and determine the developmental and genetic causes for aberrant lung development should be made firmly.

Revisiting mammalian lung development at single-cell resolution

Since the first bona fide single-cell transcriptomics was developed [148], we have witnessed an exponential increment of versatile single-cell profiling technologies [149] and embraced the subsequent revolutionized new understanding of biology. As to the field of developmental biology, single-cell omic technologies have shown promising prospects in identifying new cell types, recognizing cellular heterogeneities, reconstructing the developmental trajectories, delineating the cellular regulatory networks, recapitulating the intricate cell–cell interactions, and determining the molecular abnormalities for anomalies [150–154].

Traditionally, the developmental stages and trajectories of the lung were largely based on histologically descriptive features [8] or a limited number of molecular signatures [20, 66]. Recently, through integrating the known biology with single-cell sequencing data, several previous unappreciated cell types, novel developmental patterns, and new regulators of lung cells were revealed [87, 155, 156]. The first single-cell study of lung development was published in 2014 [28], when the single-cell technology was in its infancy, and thus the sequence depth and throughput remained limited. Treutlein B et al. profiled the transcriptome of 198 cells from the distal lung epithelium tip, encompassing four developmental stages of alveolar differentiation [28]. The authors were able to identify the existence of Ciliated cells, Clara cells, AT1 cells, and AT2 cells within the distal lung epithelium [28]. Interestingly, they identified a group of bipotent cells with the co-expression of AT1 and AT2 markers (*Sftpc*$^+$/*Ptpn*$^+$) [28]. By characterizing the intermediates during AT1 and AT2 cell fate specification, they successfully reconstructed the lineage hierarchies of the distal lung epithelium [28]. Interestingly, a later study in 2019 combining both single-cell RNA-seq and strict lineage tracing revealed that the fate of the majority of AT1 and AT2 cells were specified from Nkx2-1 and Id2 double-positive cells before E13.5, and the bipotent alveolar cells reported by Treutlein et al. [28], that give rise to AT1 and AT2 cells, are a minor contributor to the alveolar epithelial population [157]. The differences in the characterization of bipotent cells may highlight the significance of sufficient cell coverage when using single-cell technologies. The more comprehensive single cell atlas of mouse lung morphogenesis were also published [158, 159]. Zepp et al. applied single-cell RNA-seq as well as single-cell ATAC-seq of the developing murine lung tissues, and they found that AT1 epithelial cells seem to function as a signaling hub with a pervasive expression of signal ligands [158]. Meanwhile, Negretti et al. also generated a single-cell atlas of the developing mouse lung ranging from the stage of E12 to P14. Based on this atlas, they were able to capture the cell type diversification process of epithelial, endothelial, as well as mesenchyme lineages. Moreover, they reported the asynchronous features of cell fate specification and differentiation process during lung development [159].

However, for the very early stage of organogenesis, systematic transcriptomic analyses by using single cell or limited cell number RNA-seq were usually challenging due to limited cell numbers of relevant tissues. It was not until 2020 that the single-cell surveys of the early embryonic stages of the lung were reported. Kuwahara et al. took advantage of the single-cell RNA-seq of the dissected foregut epithelial cells from E10.5 and E11.5 embryos [39]. Based on the transcriptome from normal control as well as multiple genetic mouse models, they identified several dorsoventral populations of the foregut and also found that the majority of the tracheal and esophageal transcriptome is NKX2-1-independent [39]. Meanwhile, Han et al., micro-dissected the early foregut tissues, which are located between the posterior pharynx and the midgut, at three time-points that span the period of early patterning and lineage induction: E8.5 (5–10 somites; s), E9.0 (12–15 s), and E9.5 (25–30 s). They found that an extensive diversification of the early splanchnic mesoderm into distinct organ-specific mesenchyme subtypes, the diversities of splanchnic mesoderm cell types are closely registered with the organ-specific

Table 1: The expression pattern, known functions, and typical mutant phenotype of key lung development-related genes.

Gene	Expression pattern (initial timepoint, regions)	Known functions (especially for early lung development)	Typical mutant phenotype, disease or trait annotations
1. Epithelial genes			
Nkx2-1	E9.0, lung epithelium	Transcription factor; regulates pattern specification of the lung primordium and following morphogenesis	Abnormal lung morphology, impaired branching involved in respiratory bronchiole morphogenesis [131, 132]; lung adenocarcinoma, neonatal respiratory distress etc.
Shh	E9.0, lung epithelium	Signal ligand; regulates epithelial tube branching and associated mesenchyme development during lung morphogenesis	Single-lobe hypoplastic lungs with decreased epithelium/mesenchyme; malformations of the trachea and tracheo-esophageal fistula [133] etc.
Sox2	E9.0, dorsal gut endoderm and proximal airway epithelium	Transcription factor; controls dorsal-ventral patterning of foregut tube, and following branch morphogenesis and airway differentiation	Tracheoesophageal fistula [114]; lung adenocarcinoma etc.
Fgfr2	E9.0, distal lung bud epithelium	Signal receptor; regulates proximal-distal patterning as well as lung branch morphogenesis [134]	Small epithelial outgrowths that arise arbitrarily along the main bronchi [134]; idiopathic pulmonary fibrosis, lung adenocarcinoma etc.
Bmp4	E9.5, distal lung bud epithelium and mesenchyme	Signal ligand; regulates lung bud outgrowth, extension and branching [135]	Abnormal foregut morphology, severe reduction in distal epithelial cell types and a concurrent increase in proximal cell types [136, 137] etc.
P63	E9.5, proximal lung bud and proximal airway epithelium	Transcription factor; plays critical roles in the development of a normal esophageal and tracheobronchial epithelium [138]	Abnormal esophageal epithelium morphology, increased lung adenoma incidence; lung adenocarcinoma etc.
Id2	E10.5, distal lung bud epithelium	Transcription factor; marks multipotent progenitors in distal tip lung epithelium [76]	Lung small cell carcinoma etc.
Etv5	E10.5, distal lung bud epithelium	Transcription factor; acts downstream of Fgf signaling and is essential for lung branch morphogenesis and the maintenance of alveolar type II cells [139]	Lung small cell carcinoma etc.
Sox9	E10.5, distal lung bud epithelium and proximal lung mesenchyme	Transcription factor; plays roles in the lung epithelium, balancing proliferation and differentiation and regulating the extracellular matrix [72]	Developmental abnormalities in the lung during branching morphogenesis [72], unable to breathe and died at birth, with noticeable tracheal defects [140]; neonatal respiratory distress, lung carcinoma etc.
N-Myc	E10.5, distal lung bud epithelium	Transcription factor; regulates lung progenitor proliferation and differentiation [79]	Reduced proliferation, epithelial differentiation and high levels of apoptosis in both epithelium and mesenchyme [79]
Wnt7b	E12.5, distal lung bud epithelium	Signal ligand; activates an autocrine and a paracrine canonical signaling cascade in adjacent pools of endoderm and mesenchyme [78]	Pulmonary hypoplasia, abnormal mesenchymal cell proliferation involved in lung development, and abnormal lung vasculature morphology [78, 141]
2. Mesenchymal genes			
Wnt2/2b	E9.0, lung bud mesenchyme	Signal ligand; specifies lung progenitors in the foregut [40]	Pulmonary hypoplasia, abnormal lung associated mesenchymal development [40]
Fgf10	E9.0, lung bud mesenchyme	Signal ligand; plays central roles in the formation of lung mesenchymal cells with dosage dependency [88]	Smaller lobes with a reduced number of branches [134, 142]; lung adenocarcinoma
Gli2	E9.5, proximal trachea and distal lung bud mesenchyme	Transcription factor; mediates Shh signaling and regulates cyclin expression during lung development [60, 143]	Hypoplastic lungs with severe patterning defects (single lobe right lung) and diminished epithelium/mesenchyme, mildly hypoplastic trachea and esophagus [60]
Gli3	E9.5, intermediate mesenchyme between lung buds	Transcription factor; mediates Shh signaling [60]	Hypoplastic lungs of decreased size and abnormal shape of the lobes [60]
Tbx4	E9.0, ventral lung mesenchyme	Transcription factor; regulates lung bud formation	Severely reduced lung branching [144]
Tbx5	E9.0, ventral lung mesenchyme	Transcription factor; initiates lung development [145]	A unilateral loss of lung bud specification and absence of tracheal specification [144]
Tbx2	E10.5, ventral lung mesenchyme	Transcription factor; controls lung growth and branch morphogenesis [146]	Pulmonary hypoplastic and reduced branching morphogenesis, decreased mesenchymal proliferation, and premature mesenchymal differentiation into fibrocytes [146, 147]

epithelium, and underscore the importance of endoderm-derived signals in mesoderm patterning [44].

It's noteworthy that the lung development process is not completely evolutionary conserved between the mouse model and humans [88, 160, 161]. It's also of great significance to establish a comprehensive molecular roadmap of human lung development. Due to continuous efforts from the scientific community, the direct delineation of the sequential dynamics of cellular compositions and molecular architectures during human lung development is now emerging [87, 156, 162–165].

These studies offer comprehensive insights into mammalian lung development from foregut specification to alveolarization, and provide novel information about the developmental trajectories of epithelial, endothelial, as well as mesenchymal lineages. Future endeavors aim to extensive survey of the inter-cellular interactions by expanding data dimensions to include the epigenome and spatial transcriptome, integrating data across multiple developmental stages and multiple species, and systematically comparing the molecular landscape with samples of defective lungs will largely boost the knowledge of mammalian lung development.

Conclusions

The investigation of mouse lung development has yielded crucial insights into the complex processes underlying mammalian lung morphogenesis and cellular differentiation. Through the dissection of key molecular pathways, cellular interactions, and morphogenetic events by integrating versatile toolkits, such as induced pluripotent stem cells, *in vitro* organoid system, genome editing technology, and next-generation sequencing, the molecular landscape and specific details of mammalian respiratory system development are increasingly getting clear. These foundational studies will lay a robust groundwork for our understanding of lung development and related diseases. Continued explorations in the future will offer opportunities to unravel the intricate complexities of lung disorders caused by diverse genetic mutations or environmental conditions, and hold promising prospects for the development of novel therapeutic approaches to tackle lung disorders.

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