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Update on Molecular Biology of Lung Development - Transcriptomics

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Abstract

This chapter will highlight some of the many significant advances in our understanding of lung developmental biology that have been made over the last few years, which challenge existing paradigms and are relevant to a fundamental understanding of this processes. Additional comments will address how these new insights may be informative for chronic lung diseases that occur or initiate in the neonatal period. This is not meant to be an exhaustive review of the molecular biology of lung development. For a more comprehensive, contemporary review of the cellular and molecular aspects of lung development, readers can refer to recent reviews by others [1–6] [7].

Keywords

epithelium; mesenchyme; airway; alveoli; progenitor cells

Introduction to Lung Development

Historically, the process of lung development has been conceptualized as a linear set of stages, typically including 4 or 5 discrete parts, aligned with the age of the organism. These stages were defined largely upon histological and morphological changes in lung structure that occur during fetal development. The embryonic stage of lung development is recognized at encompassing the initiation of lung formation and, as a reference, occurs from 4 to 6 weeks post-menstrual age (PMA) in humans, and embryonic day 9.5 (E9.5) to E10.5 in mice. This stage involves budding of a patch of ventral foregut endoderm, located between the thymus and liver, to form a distinct organ primordium. A central role for retinoic acid (RA) in this process has been appreciated, and recent studies uncovered a Wnt/Tgfbeta/Fgf10 regulatory network controlled by RA to drive the formation of the lung bud [8].

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The pseudoglandular stage of lung development largely involves establishment of the airway structure of the mature rodent and occurs from 6 to 16 weeks PMA in the human, and E10.5 to E16.5 in the mouse. Formation of the airways results from recursive branching morphogenesis, similar to that which occurs in other glandular organs with a branched tubular structure (e.g., salivary, mammary). Branching morphogenesis in the lung appears to be regulated locally by FGF10/FGFR2 and BMP4/Shh signaling, to promote tube elongation or branch-point formation, respectively. Some investigators have suggested conversely, that FGF10 controls epithelial differentiation [9]. While it was largely presumed that this local regulation occurred stochastically, seminal studies by Metzer et al demonstrated that these events are programmed in both time and space [10, 11].

Once the major airway architecture of the lung has been established during the pseudoglandular stage, the canalicular stage of lung development involves initiation of formation of the functional (acinar) portion of the lung and initiation of differentiation of distinct respiratory cell types. This stage occurs from 16 to 26 weeks PMA in the human, but for some reason is much more condensed in mice, occurring from E16.5 to E17.5. The establishment of a proximal-distal differentiation pattern of lung epithelium has been described [12, 13], and appears to be regulated by a complex set of regulatory molecules and transcription factors driven by activation of Wnt/b-catenin signaling. The emergence of morphologically distinguishable alveolar epithelial cell types, which are essential for facilitating gas exchange, also begins at this stage. As discussed below, although it is now clear that mesenchyme undergoes analogous processes to specify various cell types [14], and can play a direct role in developmentally-associated lung diseases [15, 16], an understanding of how this process is regulated for mesenchymal cells is less clear.

The saccular stage of lung development involves the formation of frank, functional airspace capable of gas exchange. This is associated with the emergence of expression for numerous cell type-specific markers, and expansion of the density of the alveolar capillary bed. In the human, the saccular stage takes place entirely in utero, initiating at approximately 26 weeks PMA and continuing through 36 weeks PMA. In the mouse, for reasons that are not entirely clear, this stage spans birth, initiating at E17.5 and continuing through approximately the first 4–7 postnatal days (P4-7). The initiation of functional pulmonary surfactant production and surfactant secretion is a key physiological event that occurs during this stage. Functional surfactant is essential to maintain airspace patency, and its absence is a major cause of morbidity and mortality in babies born prior to this time [17].

The alveolar stage is the ultimate stage of lung development, and it initiates at 36 weeks PMA in humans, and at approximately P4-7 in mice. The major events of the alveolar stage include a dramatic expansion of surface area, through secondary crest formation and elongation, and reorganization of the alveolar capillary bed to ensure close apposition of the blood supply to alveolar surfaces. Although acinar units are sometimes referred to as alveoli, this term is appropriately applied only to fully mature airspaces derived from secondary crest elongation. The complexity of alveolar formation, essentially the processes that guide secondary crest formation and elongation, is widely appreciated, is poorly understood relative to the preceding stages, and has been a focus of recent investigation. Among the complexity of this process, the organized deposition of elastin fibers at alveolar entrance

rings, at a location consistent with elongating secondary crests, appears to be among the most critical. Processes that block events leading up to the formation of these fibers, either through disruption of signaling events [18] or emergence of cell types [19, 20], or that interfere with proper elastin fiber organization [19] or function [21], block or attenuate proper alveolar size and number. As such, this stage is arguably most important to human health, since abnormalities in this process are compatible with life (while abnormalities in earlier stages are much more likely to lead to defects that are not survivable), but can have sustained impacts upon long-term health and susceptibility to disease.

There is some debate as to the timing at which the alveolar stage ends, and when the formation of new alveoli ceases. In the mouse, it is widely appreciated that alveolar formation peaks in the first week of life, and is complete by the end of the first month of life, at the time of animal maturity. The precise timing for the end of mouse alveolar stage has apparently not been rigorously evaluated. In humans, it was long held that “development” occurred in utero, and that all or most of alveoli were formed at or shortly after the time of birth. However, this dogma has been reprised over the past decade, by careful morphometric analyses in humans and other primates, which suggest that alveoli are formed after birth [22]. It is now appreciated that alveolar formation continues in human through the first decade of life. As in the case of the mouse, these data support a termination of the alveolar stage at about the time of maturity.

Molecular Stages of Development

Numerous recent studies have codified an appreciation of the overly simplistic nature of this “liner stage” model of development, and underscored the heterogeneity of both the processes and cell types involved. As an example, a “physiological “ model of lung development would focus on the binary nature of the organ prior to and after its necessity for functional gas exchange at birth. Similarly, ontological models of lung development, focusing upon biological processes that occur during and across overlapping stages, have been proposed for decades. More recently, genome-wide molecular expression analysis of lung development has helped to understand how the complexity of lung development can be explained from an alternate, molecular perspective. Consistent with a functional, “physiological” model of development, early transcriptomic studies suggested that the regulation of processes associated with preparing for, during and after the time of birth exhibited the greatest molecular impact on lung development [23]. Furthermore, although gene expression differences between species could be readily identified, this signature of “time to birth” was conserved.

These observations should not be interpreted to suggest that “histological” stage-specific genome-wide expression patterns were not recognizable, or of significant importance. Prior to the recognition of this molecular signature of “time to birth”, analysis demonstrated a substantial impact of initiation of the alveolar stage upon the developing mouse lung transcriptome [24]. Indeed, secondary to birth, alveolar formation appears to have the greatest influence upon gene expression patterns across development in the mouse [23]. These data provide for an alternate means to identify process- and stage-specific gene expression patterns, and regulatory networks that may contribute to these processes.

Analagous studies in the human thus far have been restricted to the earlier epochs of development, prior to the end of the canalicular stage, and do not provide an equivalent degree of clarity [25]. However, these studies do clearly demonstrate that global genome-wide expression during human lung development follows patterns that are not entirely correlated with classic “histological” stages. In early human lung development, there appear to be two temporal demarcations in the transcriptome. Interestingly, one of these demarcations occurs exactly at the time of transition from the pseudoglandular to the canalicular stages of development. However, another occurs in the middle of the pseudoglandular stage, and appears to demarcate an early from a late “phase” of pseduglandular lung development. Unlike the case where demarcations in the mouse lung transcriptome have identifiable biological correlates, the biological processes that these demarcations in human lung development represent are currently not entirely clear. Regardless, cumulatively these data support the appreciation of “molecular phases” of lung development, which integrate stages and processes, and can be considered analogous to classical “histological” stages.

Ontogeny of Lung Epithelial Cells

The hierarchical nature of cell ontogeny during lung development has not been completely defined. And while tremendous diversity in pulmonary cell types has been appreciated for more than half a century [26], the last decade has seen a tremendous growth in characterization of cellular heterogeneity. For a large part, this progress has focused upon epithelial cells, while progress on the mesenchyme has lagged, as is the case for most of our understanding of the pulmonary system. Arguably, a new appreciation of this diversity among the major epithelial cell types was stimulated by observations regarding the differential sensitivities of airway epithelial cells to toxins, and their differing capacity for regenerating the airway following injury; the identification and characterization of so-called “variant” Club cells that contribute to repopulation of the airway [27]. Another watershed discovery was the identification of bronchio-alveolar stem cells (BASCs), that reside at the entrance to alveolar ducts, and have the capacity to produce progeny of an alveolar (AT2/AT1) or airway (Club cells) fate. There is little evidence that these BASCs actually give rise to alveolar lineages during development. However the paradigm that airway epithelial progenitor cells can contribute to alveoli following injury or during lung regeneration has been further supported by the laboratories of McKeon and Chapman. Chapman and colleagues have reported that epithelial cells located in the proximity of the BADJ (and/or within the alveolus) that express Itgb4, but not Sftpc, can differentiate to alveolar epithelial cell types in a fibrotic model [28]. Furthermore, distal airway stem cells co-expressing p63 and Krt5 are essential for alveolar repair in models of severe viral-derived or cytotoxin-mediated respiratory destruction [29–31]

Classically, alveolar epithelial cells were believed to exist in two forms; the squamous type I epithelial cell (AT1) that allows apposition of airspace and vasculature, and the cuboidal type II cell (AT2) that is responsible for production and secretion of pulmonary surfactant. Markers of AT2 can be observed at the earliest time-points of lung formation [32], and for decades these cells have been described as the progenitors for AT1 during development and in response to injury. A seminal study from Desai and colleagues [33] used lineage tracing

and molecular analysis to demonstrate that AT1 and AT2 arise from a common bipotential progenitor during lung development. A follow-up study by Treutlein et al described using single cell transcriptional profiling of epithelial cells isolated from the developing lung to construct hierarchical relationships between AT1 and AT2 and their progenitors, and their genomic profiles [34]. Also included in this analysis were airway ciliated and secretory cell types. These studies expanded, by an order of magnitude or more, the number of cell type-specific markers associated with individual lung epithelial cell types. These studies also support a model where frank bi-potential progenitors, defined by the co-expression of Sftpc and Pdpn, emerge around E18.5 and are responsible for the formation of both mature alveolar epithelial cell types. The work by Desai et al, also clearly showed, as had long been held, that "fully differentiated" AT2 give rise to AT1 in mature lungs following injury. However, this capacity was apparently restricted to a sub-set of long-lived AT2 that were also capable of self-renewal [33]. It remains unclear whether these mature AT2 "progenitor" cells are programmed or stochastically determined. Regardless, the data clearly indicate a distinction in the origin of alveolar epithelial cells during development and during homeostasis following injury/repair.

With regard to epithelial cell diversity in the pseudo-stratified airway of the lung, basal cells are believed to be progenitors for differentiated secretory and ciliated cells, both during lung development and during repair responses to injury. Two recent reports have further clarified how these airway progenitors are specified during development, restricted from the distal lung, and function in homeostatic maintenance [35, 36]. The Hippo/Yap pathway appears to be central to controlling the specification of airway, and the progenitor capacity of basal cells, at least in part by driving the expression of the cardinal airway epithelial cell transcriptional regulator Sox2. Intriguingly, the ability of Hippo signaling to specify airway is associated with sub-cellular redistribution of Yap from a nuclear to a cytoplasmic pool [36]. Furthermore, Yap is necessary and sufficient to maintain the pseudo-stratified airway epithelium, and form the appropriate distribution of ciliated and secretory cells [35]. This ability of Yap to promote/maintain basal progenitors occurs through the coordinated regulation of a p63-dependent transcriptional program.

Regulation of Diversity of Lung Mesenchyme

The field has been slow to appreciate that similar diversity exists for epithelial and mesenchymal cells in the lung, and the mesenchyme is sometimes referred to as relatively homogeneous, which is clearly not accurate. A preponderance of data in the literature over the last few decades quite clearly demonstrates significant diversity among frank smooth muscle cells, be it airway or vascular smooth muscle, muscularized and non-muscular, lipid-laden parenchymal fibroblasts, and various vascular support cells including pericytes. Kumar and colleagues have provided perhaps the most complete assessment of mesenchymal specification and airway smooth muscle cell diversity during lung development [14]. Using a strategy to target individual airway-associated mesenchymal cells during lung development, they characterized the location and dynamics of airway smooth muscle (ASM) progenitor niches. Unlike the case for epithelial progenitors, it seems that mesenchymal progenitor niches are not fixed in space or time. Conversely, an ASM niche "originates" along with each airway branch, "migrates" along with the developing airways, and cells from each

niche are restricted to that specific airway structure. Interestingly, the ASM progenitor niche appears to be localized around the tips of growing airway branches. Transcriptomic analysis revealed mesenchyme from these growing “tips” display a signature of Wnt activation, suggesting this ligand family may be a key factor in defining the ASM progenitor niche. Genome-wide profiling of ASM progenitors obtained from outside of the niche, treated with Wnt ligand in vitro, demonstrated regulatory effects ontologically associated with motility and migration, consistent with Wnt controlling the migration and differentiation of these cells. Wnt treatment of these cells also resulted in them assuming a phenotype more reminiscent of mesenchymal progenitors within the niche. It is unclear whether Wnt5a may be a key family member contributing to this process, and whether this partially explains the distal mesenchyme phenotype observed in the Wnt5a deficient mouse [37].

A Molecular Basis for Dysanapsis?

Dysanapsis, or the disproportionate growth of airways and lung parenchyma, has been put forth as an explanation for respiratory physiology deficiencies in certain disease states, most notably in the argument for a developmental origin for asthma [38–40]. However, little evidence exits for the theory of variability in airway growth or length. A paradigm-establishing study recently published by Chen and colleagues indicates that the length of airways, and the point at which the respiratory portion of the lung begins as demarcated by the bronchio-alveolar duct junction (BADJ), is genetically programmed [41]. This study describes two “waves” of regulatory control during lung development; the first associated with establishing Sox9 expression and generally defining lung patterning through branching morphogenesis, the second associated with establishing Sox2 expression and specifying the end of the conducting airway and the location of the BADJ. Importantly, genetic manipulation could alter the location of the BADJ, and thus airway length, independent of differentiation of the full complement of proximal and distal cell types.

Establishment of the location for BADJ formation was shown to be associated with changes in endogenous glucocorticoid signaling in vivo, and affected by exogenous glucocorticoids, but not retinoids, in an ex vivo model of lung development. Furthermore, excess glucocorticoids promoted precocious alveolar formation proximal to the default location of the BADJ. Transcriptomics analysis demonstrated this was associated with widespread increases in alveolar cell markers. It appears there exists a population of Sox2 negative cells in the developing terminal airway, capable of responding to hormonal cues and differentiating towards an alveolar fate, that will otherwise go on to form conducting airway. Given the importance of endogenous glucocorticoids to biochemical (e.g., surfactant) and structural (e.g., elastin) aspects of lung maturation, and the use of exogenous glucocorticoids to promote lung maturation in late fetal development, these novel observations have potential relevance to lung disease occurring in the perinatal period. Furthermore, by analogy with dysanapsis, these data indicate that airway length may be a clinically relevant structural parameter with implications for lung function and disease susceptibility.

Integrated Genomics Analysis of Development

While many comprehensive characterizations of lung development using high throughput methods have been published, relatively few have successfully integrated data sets to formulate a thorough understanding of regulatory processes. A notable success would be the studies of Bar-Joseph, Kaminski, Ambalavanan et al., who used a computational, systems biology approach to integrate genome-wide miRNA and mRNA expression patterns to identify dynamic regulatory networks [42]. This involved the development of a computational tool, which they named the MIRna Dynamic Regulatory Events Miner (mirDREM). Leveraging this probabilistic modeling approach, they were able to identify known and novel miRNA/transcription factor-regulated networks that are associated with specific stages and processes during lung development.

More recently, the same group used genome-wide transcriptomics and DNA methylation analyses to study relationships between chromatin modifications and gene expression in the perinatal period in both mice and humans [43]. In the mouse, a subset of genes with known roles in the regulation of lung development displayed an inverse correlation between DNA methylation and gene expression, including those associated with Wnt signaling, proximal-distal epithelial cell specification, capillary vascular formation and extracellular matrix formation. In the human lung, integration of DNA methylation patterns in normal development and expression profiles in chronic lung disease following preterm birth (bronchopulmonary dysplasia) identified genes/pathways suspected to play a role in defining susceptibility to disease. These data are consistent with the hypothesis that chromatin remodeling influences the regulatory processes controlling lung development in the perinatal period, and that failure of this coordination may be involved in associated disease states.

A Molecular Atlas for Lung Development

An ongoing NHLBI-supported multi-institutional, collaborative program (termed the developing LUNG Molecular Atlas Program, or LungMAP) is developing a map of human (and mouse) lung development at the structural, cellular and molecular levels, with a focus on the perinatal period including the saccular and alveolar stages. This active Program involves developing and leveraging high-throughput and multi-scale anatomical imaging, cellular and molecular methods to provide the research community benchmark data regarding normal developmental processes, which will aid in our understanding of abnormalities associated with diseased states. One product of the LungMAP is an interactive, web-based portal (www.lungmap.net), including the BREATH data repository (www.lungmap.net/breath/), to be used for frequent pre-publication data distribution. These resources were made available for public access in May 2015 in conjunction with the American Thoracic Society International Conference. Other products of the LungMAP will include high resolution, detailed ontologies to formalize terminology for describing the developing lung anatomy [44], including dynamic cell populations, and molecular functions important for the respiratory system.

Transcriptomics of BPD

For at least the last decade, microarray analysis has been used to characterize animal models of chronic lung disease initiated in the newborn period by exposure to excessive concentrations of oxygen [45, 46]. These data have provided critical insights into disease-associated mechanisms. Early studies in the mouse confirmed responses involved aberrant expression of chemokines and proteases, changes in ROS, and reductions in FGF- and VEGF-related signaling [46]. Other studies dissected the involvement of specific pathways, and for instances uncovered Nrf2 dependent and independent responses [45]. A more recent study suggested that neonatal hyperoxic lung injury in mice, which is associated with BPD-related phenotypes, centrally involves p21/Cdkn1 and Aryl-hydrocarbon receptor (Ahr)-related pathways [47].

Over the past 5 years, genome-wide transcriptional assays have been applied to gain a better understanding of expression changes associated with bronchopulmonary dysplasia in samples derived from human subjects. A focused, but multi-factorial analysis of angiogenesis related gene expression, in the lungs of preterm infants born at less than 27 weeks of gestation and receiving short-term ventilatory support identified a switch from pro-angiogenic factors to “anti-sprouting” regulators, consistent with deficiencies in alveolar vascularization [48]. In a study of samples collected at autopsy from preterm subjects with severe BPD leading to mortality, and preterm controls with no lung disease or no BPD, microarray analysis was used identify genes and pathways dysregulated in disease lung tissue [49]. In what appears to have been the first study to perform such a comprehensive assessment of the BPD transcriptome, some obvious disease-related pathways were identified including those involved in the regulation of cell proliferation, oxidative stress, control of vascular development and inflammation. The most remarkable finding was a predominant and consistent signature of mast cell accumulation in the lungs of these preterm infants dying of severe BPD. Importantly, this signature was not of the “typical” mucosal mast cells normally found within the respiratory system. The signature was clearly of connective tissue-type mast cells (CTMC), which are rarely observed in the lung. Validation studies clearly demonstrated a robust, albeit with variable magnitude, accumulation of CTMC in the airspaces in BPD lung tissue. In a preterm baboon model of hyperoxia-induced lung injury leading to BPD-like phenotypes, microarray analysis of lung tissue identified increased expression of genes related to chromosomal maintenance, proliferation, and differentiation [50]. Additionally, and of note, in this model there appeared to be an increase in genes associated with the inhibition of inflammation.

A more recent study characterized mRNA expression in peripheral blood mononuclear cells (PBMC) from preterm infants at risk for BPD [51]. In this study, peripheral blood was collected from 111 infants born at less than 32 weeks of gestational age, within the first week after birth, 2 weeks after birth or at one month of life, and PBMC RNA was interrogated by microarray analysis. When comparing those infants receiving a diagnosis of BPD (n=68) with those who did not develop BPD (n=43), approximately 10% of the genome was differentially expressed. These expression patterns were associated with a significant inhibition of the T cell receptor signaling pathway, as well as changes in cell proliferation. Equally of note, these data establish that disease-related “responses” are

detectable in peripheral samples that can be obtained with minimally invasive procedures, as can be seen in many other diseases.

Similar “transcriptomic” studies have attempted to understand miRNA responses in human BPD and in animal models of disease. In another study of the neonatal mouse hyperoxia model, 14 miRNAs displaying increased expression and 7 miRNAs displaying decreased expression were identified, some of which appear to target cell proliferation genes [52]. Another comparison of miRNA expression in peripheral blood mononuclear cell from 15 subjects with BPD and 15 controls, identified 4 miRNA (miR-152, miR-30a-3p, miR-133b, and miR-7) with aberrant expression levels. This included reduced expression of miR-152 and miR-30a-3p, and increased expression of miR-133b and miR-7 [53]. Results of a meta-analysis of BPD-related miRNA profiling identified four consistently up-regulated miRNAs (miRNA-21, miRNA-34a, miRNA-431, and Let-7f) and one consistently down-regulated miRNA (miRNA-335) in BPD lung tissues [54]. Additional miRNAs (miRNA-146b, miRNA-29a, miRNA-503, miRNA-411, miRNA-214, miRNA-130b, miRNA-382, and miRNA-181a-1*) appeared to be regulated in lung development and affected in BPD. Putative targets for these miRs included transcripts for genes previously demonstrated to show aberrant expression, such as HPGD and NTRK. Finally, at least one study has attempted to integrated comprehensive miRNA and mRNA expression profiles, using the neonatal mouse hyperoxia exposure model [55]. This study identified treatment-related effects upon cell proliferation, adhesion/migration, inflammation, and angiogenesis, and also implicated a significant role of miR-29.

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KEY POINTS

- The past two decades have witnessed tremendous growth in our understanding of fundamental regulatory processes and networks responsible for coordinating the development of the mammalian lung, and high-throughput, genome-wide analyses have facilitated this growth.
- Recent seminal observations regarding cellular heterogeneity and lineage relationships demonstrate we have much yet to learn.
- Recent developments in single cell transcriptome analysis are likely to transform our appreciation of cellular heterogeneity with the respiratory system, and help to determine whether this heterogeneity is programmed, stochastic or a combination.
- The precise role of environmental cues, both “normal” (e.g., oxygen) and “foreign” (e.g, microbes), in the coordinated regulation of lung development remains poorly defined.
- Multi-scale integration of molecular information, such as that defined by comprehensive profiling of miRNA and mRNA expression, will ultimately be necessary for a complete explanation of lung development.