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Lung Stem/Progenitor Cells: Regulatory Mechanisms of Behavior, Development and Regeneration

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Abstract

New insights have been added to identification and characterization of stem and progenitor cells in the lung over the last few years. The exploration of endogenous lung stem and progenitor cells holds promise for advancing our understanding of the biology of lung repair and regeneration mechanisms after injury. This will also help in the future use of stem cell therapy for the development of regenerative medicine approaches for the treatment of different lung diseases. In this concise review, we describe the main types of lung stem/progenitor cell populations as well as summarize recent research progress and accumulated information regarding the behavior, development and function of stem/progenitor cells in the lung, and factors controlling their repair/regeneration after injury as well as molecular mechanisms regulating lung stem/progenitor cell behavior during development.

Keywords: Progenitor cells; Regeneration; Self-renewal

Introduction

Stem cells are undifferentiated or 'blank' cells found in the human body that have the potential to self-renew and develop into many different cell types that carry out different functions and, therefore, are multipotent source of multiple cell lineages. While such cells are essential for development and growth through childhood, pools of adult stem cells are hypothesized to be the source of the often somewhat limited tissue regeneration and repair in adults. Stem cells are defined as cells with the ability both to self-renew to preserve the stem cell pool and to differentiate into specialized cells, in response to appropriate signals. Due to their self-renewal capacity and pluripotency, stem cells are essential players during development, tissue repair and regeneration after injury, and healthy homeostatic cell turnover. Therefore, stem cells are a critical driving force for fast-growing fields of regenerative medicine and functional tissue engineering.

Currently, there is limited knowledge about the existence of specific self-renewing cells in the lung. Similarly, little is known about whether such putative stem/progenitor cells in the lung conform to classic or non-classic stem cell hierarchies and whether a single stem cell suffices to generate the more than 40 distinct cell types that are required for mature lung function. There are at least five putative epithelial stem/progenitor cell niches in adult murine airway [1,2] in addition to both airway smooth muscle stem cells and endothelial stem cells in the pulmonary vasculature. Another source of stem cells in the lung is circulating stem/progenitor cells that may take up residence in the lung. Unlike other epithelial tissues that undergo rapid regeneration such as the skin and gastrointestinal tract, the lung has a much slower turnover, which has hampered the identification and characterization of putative lung progenitor cells. Other factors also hampered progress in the field of lung stem cell research, including the lack of stem/progenitor cell markers and clonality assays to identify and isolate them. In this concise review, we discuss recent research progress and accumulated information regarding the development and function of stem/progenitor cells in the lung, and factors controlling their repair/regeneration after injury as well as molecular mechanisms regulating lung stem/progenitor cell behavior during development.

Types of Lung Stem/Progenitor Cells

Prenatal endogenous embryonic epithelial progenitors and postnatal alveolar epithelial progenitor cells

Localization and Characterization: There is considerable recent evidence that, at least during the pseudoglandular stage of lung development, the distal tips of the branching tubules contain a population of undifferentiated multipotent epithelial progenitor cells, which characteristically expressed several genetic markers (Figure 1). In the adult lung, putative endogenous epithelial stem/progenitor cells have been located in the basal layer of the upper airways, within or near pulmonary neuroendocrine cell rests as well as at the bronchoalveolar junction and in the alveolar epithelium [3-10]. In addition, Rawlins et al. [11] has shown that the distal-most epithelial cells are a multipotent progenitor cell population during branching morphogenesis of the lung. A recent model suggests that, during epithelial branching, descendants of the distal tip progenitors are left behind in the stalks, where they begin to differentiate, whereas the self-renewing stem/progenitor cells remain within the epithelial budding tips. Several lines of evidence have been recently shown to strongly support this hypothesis. For example, distal epithelial cells have a unique pattern of gene expression (Figure 1), including high levels of the transcription factors: Sox 9 (sry box containing gene 9), Id2 (inhibitor of differentiation 2), N-myc (v-myc myelomatosis viral related oncogene, neuroblastoma-derived), and Etv5/ERM (ets variant gene 5). Moreover, distal epithelial cells are exposed to and affected by high levels of activity of the SHH, FGF, BMP, and Wnt signaling pathways [12-14]. The activity of these genes and

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El-Hashash Fig. 1

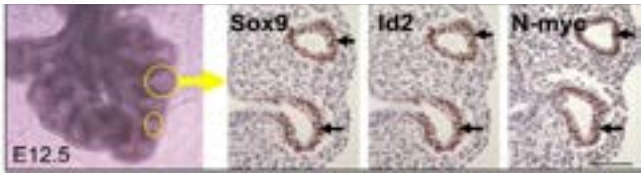

Figure 1: Lung epithelial stem/progenitor cell markers. External appearance (left hand panel) and immunoperoxidase staining of day (E) 12.5 embryonic lung show that lung epithelial stem/progenitor cells localized to distal epithelial tips (yellow circles) and expressed Sox9, Id2 and N-myc proteins (arrows). Scale bar: 100 μm.

signaling is not restricted to the lung because many of them, including sox9, etv5, and FGF signaling are also associated with stem/progenitor cells in other endodermally derived organs such as the pancreas [15,16]. Another characteristic of distal epithelial cells compared with the rest of the epithelium is that they have different cell cycle kinetics; a higher proportion of them incorporate the thymidine analog bromodeoxyuridine (BrdU) during a short pulse [17].

Alveolar epithelial cell repair and regeneration: Recently it has been suggested that the failure to regenerate and repair that inevitably occurs with ageing may be due to endogenous stem cell failure. These studies suggest that a large number of alveolar epithelial cells must function as a "ready reserve" to repair damaged alveolar surface. For example, Driscoll et al. [18] has demonstrated that the expression of telomerase, a stem/progenitor cell marker, after acute oxygen injury is strongly up-regulated in Alveolar Epithelial Cells (AEC) during the recovery phase. These findings suggest that alveolar epithelial cells either contain a relatively large subpopulation of progenitors, or that the majority of alveolar epithelial cells can undergo reactivation into a progenitor-like state in response to injury cues [18]. In addition, Broncho Alveolar Stem Cells (BASC), which possess stem cell characteristics, are resistant to naphthaline injury and proliferate in response to airway or alveolar injury [9]. These BASC cells express both alveolar (SP-C) and airway (CC10) epithelial cell markers, as well as co-expressing Sca-1, and appear to reside near bronchiolar-alveolar junctions. BASC cells are capable of self-renewal and differentiation into Clara cells and alveolar cells, and are multipotent in clonal assays *in vitro*. In addition, some studies identified the variant Clara cell as an endogenous lung stem cell, which infrequently proliferate during steady state but are felt to be responsible for repopulating the distal airway epithelium in response to injury [19]. These variant Clara cells characteristically express Clara cell secretory protein but unlike the more abundant Clara cells they survive naphthalene injury. During the early postnatal period, Clara cells both self-renew and can act as progenitors for ciliated cells, based on the kinetics of cell labeling after a short pulse of tritiated [3H] thymidine as the lung continues to grow in size [20,21]. This is further supported by recent lineage labeling data by Perl et al. [22]. However, further investigation is needed to determine whether all Clara cells have this capacity. Additionally, alveolar epithelial type II cells have been shown to proliferate and give rise to type I cells after injury of the adult alveoli, and probably this also occurs during postnatal growth [23]. The presence of these several types of putative endogenous alveolar stem cell populations may thus provide a target for directed regenerative therapies in the lung.

Regulatory mechanisms during development, repair and regeneration: Several transcription factors are important for lung epithelial progenitor cell development, repair and regeneration (intensively reviewed in reference [24]. For instance, recent studies from our laboratory have identified Eya1 and Six1 as essential transcription factors for the maintenance of lung epithelial stem/progenitor cells. Mice deficient for *Eya1* or *Six1* gene show loss of epithelial progenitor cell markers with increased expression of differentiation markers in the lung. They also show severely hypoplastic lung phenotype with reduced epithelial branching and increased mesenchymal cellularity [25,26]. Similarly, E74-like transcription factor-3 (Elf3) plays an important role in the regulation of lung cell proliferation and differentiation during repair of the injured bronchiolar airway epithelium following Clara cell-specific injury [27]. Furthermore, a recent study has postulated that TTF-1 has an influential role in modulating, and possibly initiating, the early phase of compensatory lung growth [28].

Growth factors also play a critical role in lung epithelial progenitor cell development and alveolar cell protection against lung injury, including members of FGF family of growth factors. FGF10, for example, plays a pivotal role in maintaining epithelial progenitor cell proliferation [29]. Moreover, FGF10 has a protective effect against lung injury and fibrosis [30]. In addition, several studies have evaluated FGF7 *in vivo* as a treatment to enhance resistance to alveolar injury in animal models [31,32]. A recent hypothesis suggests that enhancement or protection of alveolar progenitor cell function may be a viable therapeutic option that could possibly be evaluated in clinical trials using small molecules such as inosine. In support of this hypothesis, Buckley et al. [33] has shown that both FGF7 and inosine treatment can ameliorate DNA damage in alveolar epithelial cells as well as enhancing mitochondrial protection and the ability of AEC to migrate and repair in an *in vitro* scratch assay. Moreover, inosine has protective properties against oxygen injury, including glutathione repletion, mitochondrial protection, decreased apoptosis, and increased VEGF expression [34]. Other studies demonstrated that epithelial FGF9 primarily affects epithelial branching, whereas mesothelial FGF9 and mesenchymal WNT2A are principally responsible for maintaining mesenchymal FGF-WNT/β-catenin signaling [35].

Tracheal and Bronchial Epithelial Stem/Progenitor Cells

Localization and characterization

Recent studies have identified several candidate endogenous stem or progenitor cells in the trachea and bronchial epithelium using models of lung injury and repair. For instance, Rawlins et al. [11] has shown that Scgb1a1+ Clara cells have the capacity to self-renew and proliferate in response to tracheal injury but are not the main source for tracheal regeneration. In addition, Hong et al. [36] have reported that subsets of keratin-14 (K-14)-expressing basal cells in the trachea have the capacity for restoration of a differentiated epithelium after injury and are distinct from basal cells in the bronchi. Moreover, keratin-14–positive cells can act as progenitors and that ciliated cells cannot, as demonstrated by several lineage-tracing studies in the adult mouse lung and trachea [36,37].

Tracheal/bronchial epithelial progenitor cell repair and regeneration

A leading study from Hogan laboratory proposed that distinct distal populations of epithelial stem/progenitor cells are essential for the maintenance of the alveoli in the lung [38]. Taking advantage of the restricted expression of *CC10*/*Scgb1a1*, Rawlins et al. [38] generated a "knockin" transgenic mouse with a tamoxifen (TM) inducible *Cre-recombinase* (*ScgB1a1-CreER*™) that lineage-tags Clara cells of the airway. By varying the dose and timing of TM administration,

they further demonstrated that reconstitution of the epithelium in the bronchioles involves Clara cell self-renewal and differentiation into ciliated cells, and thus they concluded that Clara cells can thus contribute to tracheal repair. Conversely, Rawlins et al. [38] discovered that another special and putative subpopulation of stem cells, named as Bronchio Alveolar Stem Cells (BASCs) that co-express *CC10* and *SP-C,* which have been proposed to contribute descendants to both bronchiolar and alveolar epithelium, has no apparent function during postnatal lung growth, adult homeostasis, or repair.

Another lineage tracing study from Hogan laboratory focused on the function of a population of Basal Cells (BCs) as progenitors in the trachea [39]. Since the pseudostratified epithelium of the mouse trachea and human airways contains a population of BCs expressing cytokeratin 5 (*Krt5*), this study used *Krt5-CreER*^{T2} transgenic mouse line for lineage tracing, and demonstrated that BCs of the murine trachea function as progenitor cells, during postnatal growth and in the adult at steady state, as well as in a tracheal epithelial repair of experimentally-induced SO_2 damage. Rock et al. [39] has used a clonality assay to demonstrate that BCs of both mouse and human airways can self-renew and differentiate into both mucus and ciliated cell lineages in the absence of stroma or columnar epithelial cells, which further support their importance in the lung. Another most recent study has shown that grainyheadlike 2 (GRHL2) transcription factor is important for the development of bronchial epithelial cells, both as undifferentiated progenitors and as they differentiate [40].

Other studies also showed that there is a mixed population of pluripotent cells in the lower respiratory tract, which express molecular markers of airway and mesenchymal origin and characterized as a Hoechst dye effluxing Side Population (SP) cells [5]. In addition, Hackett et al. [41] found that $CD45^-$ SP cells that are isolated from human tracheobronchial epithelium have proliferative potential and increased in number in asthmatic airways. They, therefore, suggest that dysregulation of pluripotent cells may play a role in the pathogenesis of Asthma [41]. Moreover, another study has shown that some of these SP cells, which are $CD45^{++}$ and $CD45^-$ have endothelial progenitor cell potential in response to hyperoxic exposure in the developing lung [42].

Relatively little is known about glandular stem/progenitor cells and their niche(s). Lu et al. [1] suggested that the submucosal gland ducts in the proximal airway are likewise suspected to contain stem cells; whereas Borthwick et al. [43] suggested that the regenerating surface airway tracheal epithelium in a naphthalene injury model arises from cells migrating from gland ducts. In addition, a recent study has demonstrated the role of the airway Sub-Mucosal Gland (SMG) duct cells in regeneration of both the SMG tubules and the Surface Epithelium (SE) after severe hypoxic-ischemic injury. Using a novel method to isolate SMG duct cells from the airway, Hegab et al. [44] used *in vitro* and *in vivo* stem cell model systems and lineage tracing to show that the SMG duct cells have the ability to self-renew and differentiate into SMGs and SMG duct cells as well as to form the SE adjacent to the sub-mucosal duct area. They concluded that SMG duct cells are a multipotent stem cell for airway epithelial repair, which is of importance to the field of lung regeneration as determining the repairing cell populations could lead to the identification of novel therapeutic targets and cell-based therapies for patients with airway diseases [44].

Endogenous Mesenchymal Progenitors

In contrast to epithelial stem/progenitor cells, little is known about endogenous mesenchymal stem/progenitor cells. However, it is well reported that signals from lung mesenchyme play a critical function in epithelial branching morphogenesis. FGF signaling is primarily responsible for regulating mesenchymal proliferation, whereas β-catenin signaling is a required permissive factor for mesenchymal FGF signaling [35]. In addition, FGF9 that is produced by mesothelium, activates and controls FGF10 signaling from the peripheral mesenchyme to the epithelium by assembly of a signaling complex comprising FGFR2b, SHP2, Grb2, Sos, and Ras in the epithelium as well as Sprouty2, which is an inducible negative modulator of this signaling pathway [45-48]. Moreover, it has been recently shown that Glycogen synthase kinase-3β/β-catenin signaling regulates the differentiation of neonatal lung mesenchymal stromal cells into myofibroblasts [49].

In addition studies in our laboratory showed that Six1 transcription factor and its co-activator *Eya1* (Six1/Eya1 signaling) act to optimize the expression level and activity of SHH in distal lung mesenchymal progenitors. Thus Six1/Eya1 signaling acts to maintain SHH signaling, which is negative regulator of FGF10, at a normal level required for proper lung development. In the absence of *Six1* or *Eya1*, SHH expression increases above normal levels and the ectopic SHH expression acts to inhibit FGF10 signaling activity, leading to severe defects in the lung mesenchyme and epithelial branching morphogenesis [25,26]. Other transcription factors are also important for lung mesenchymal progenitors. A recent study by Lüdtke et al. [50] has shown that lungs of mice deficient for Tbx2 transcription factor are markedly hypoplastic and exhibit severely decreased mesenchymal cell proliferation, while mesenchymal differentiation into fibrocytes was prematurely induced.

There are different types of endogenous mesenchymal progenitors: smooth muscle progenitors and vascular progenitors that are discussed below.

Smooth muscle progenitors

Several studies suggest that the peripheral mesenchyme cells that expresses *Fgf10* also serves as a progenitor cell population for peripheral airway smooth muscle, which occurs very early in development. Using lineage tracing studies with *Fgf10*^{lacz}, several investigators [29,51,52] found that airway smooth muscle progenitors begin as *Fgf10*-expressing cells that, as the airway grows outwards, become distributed along the elongating peripheral airway, much as a sock goes up the leg as one puts it on. They also found that SHH and BMP4 signals, which are expressed proximal to the very tip of the airway regulate the trans-differentiation of airway smooth muscle progenitors to express alpha–smooth muscle actin fibers. Another study by Shan and collaborators [53] has shown that another population of airway smooth muscle progenitors arises in the proximal mesenchyme and advance peripherally. Wnt signaling is critical for these stem cells because the activation of the airway smooth muscle program is dependent on Wnt2 signaling, which regulates myocardin/Mrtf-B and Fgf10 expression in the lung [54].

Vascular progenitors

Little is known about the progenitor cells of the lung microcirculation. A study by Que et al. [55] has shown that the mesothelium overlying the lung contains a progenitor population that gives rise to pulmonary vascular (but not epithelial) smooth muscle cells during embryonic development. Conversely, vascular endothelial progenitor cells may arise from endogenous vascular wall progenitors or from circulating bone marrow derived progenitors. Furthermore, a recent study has utilized a transgenic reporter mouse line harboring a BMP-responsive eGFP reporter allele to demonstrate that during the pseudoglandular stage, when branching morphogenesis characterizes lung development,

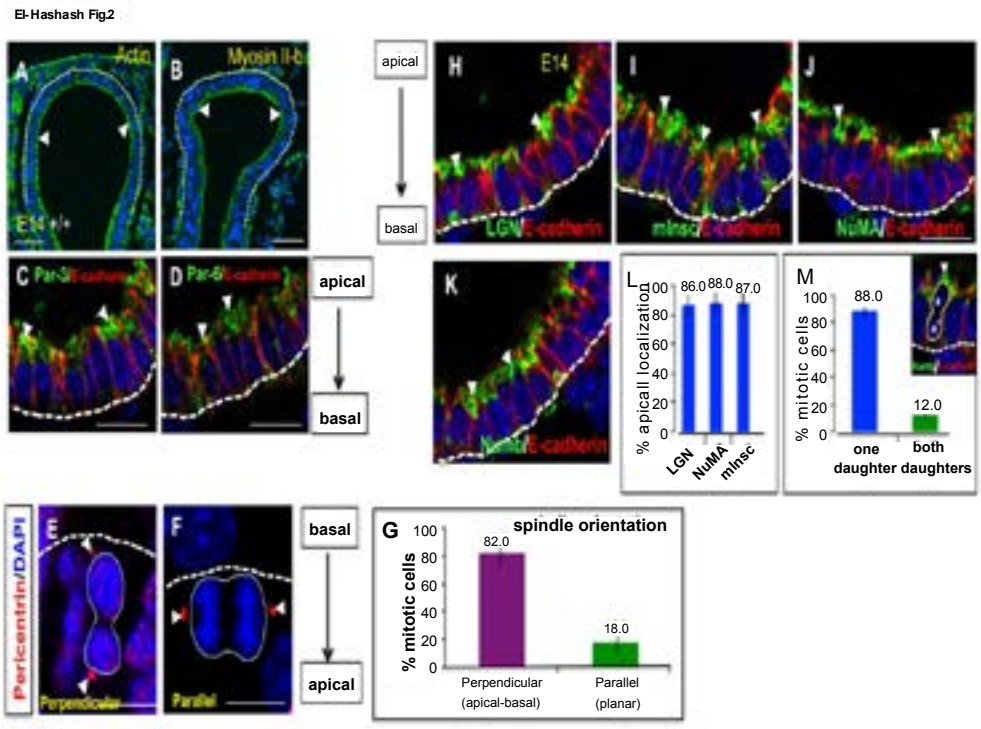

Figure 2: Cell polarity, perpendicular as well as asymmetric cell division in embryonic lung distal epithelial stem/progenitor cells. Panels (**A-M**), which are adapted from El-Hashash and Warburton (2011) show evidences of cell polarity and both perpendicular and asymmetric cell division in distal epithelial stem/progenitor cells of embryonic lungs (E14). (A-D) Immunofluorescence at E14 shows strong signals for actin (A), myosin II-b (B) as well as the polarity proteins: Par-3 (C) and Par-6 (D) proteins at the apical side of distal epithelial cells (arrowheads). Dashed line represents the collagen IV–stained basement membrane. (**E,F**) Most mitotic cells in the distal epithelium divide perpendicularly, as represented by the perpendicular orientation of pericentrin-stained centrosomes (E; arrowheads, arrows) relative to the basement membrane (dashed line; E,F), while only a few mitotic cells have their centrosomes aligned parallel to the basement membrane (F; arrowheads). (**G**) Quantitation of spindle orientations, expressed as a percentage of all divisions in the distal epithelium from the experiments shown in E,F (n=48). (**H-M**) Expression of the polarity proteins LGN, mInsc, NuMA, and Numb in lung distal epithelium, as shown by immunofluorescence (H-K). Note polarized apical localization of these proteins (H-K; arrowheads) relative to collagen IV–stained basement membrane (dashed line). (**L**) Quantitation of the apical localization of proteins shown in H-J, which is expressed as a percentage of all cells in the distal epithelium (n= 87). (**M**) Quantification of late mitotic distal epithelial cells, with Numb inherited by one(inset in M) or both daughter cells at E14. This is expressed as a percentage of all divisions in the distal epithelium (n=42). Scale bars . 50 **m**m.

canonical BMP pathway is active mainly in the vascular network and the airway smooth muscle layer [56].

The primitive capillaries, which surround the laryngotracheal groove as it buds from the foregut, can be visualized by the expression of β-galactosidase under the control of the Flk1 promoter, which is the earliest marker of hemangioblasts, at a very early stage of lung development. Two studies have demonstrated that these hemangioblasts differentiate into a stereotypic capillary network that surrounds the bronchial, lobar, and segmental branches of the airway under the stimulation of epithelial-derived VEGF [29,48]. Proper organization of this vascular plexus is likely to be essential for both correct airway branching and tissue perfusion. Thus, mesothelial-mesenchymal-epithelial-endothelial crosstalk matches epithelial and vascular progenitor function and will likely be essential if lung regeneration using endogenous or exogenous stem/ progenitor cells is to succeed [24]. More studies are needed to define the biochemical and functional phenotypes of both pulmonary endothelial cell and smooth muscle cells that might exist at different locations in the pulmonary vasculature [57].

Several studies suggest the importance of lung Endothelial Progenitor Cells (EPCs), which depends for their mobilization and homing on Vascular Endothelial Growth Factor (VEGF), nitric oxide and erythropoietin, in a developmental disorder such as Broncho Pulmonary Dysplasia (BPD). For instance, Balasubramaniam et al. [58] reported that oxygen toxicity disrupts growth of both alveolar and vascular compartments, limiting the surface area available for gas exchange. In addition, this study shows that several related developmental changes occur after hyperoxia exposure in neonatal mice, including reduced expression of endothelial nitric oxide synthase, VEGF, and erythropoietin receptor as well as decreased number of EPCs in the blood, bone marrow [58].

II- Genes and Signals Regulating Stem/Progenitor Cell Behavior during Lung Development

Several recent studies from our laboratory and other laboratories suggest that the behavior of lung stem/progenitor cells, including self-renewal/proliferation and differentiation, is controlled both by both intrinsic factors and extrinsic signals. The intrinsic aspects of progenitor cell control provide the context in which extrinsic signals are interpreted. The role of these factors and signals in the regulation of progenitor cell behavior in the lung will be discussed in the following sections: control of progenitor cell self-renewal/proliferation and regulation of the patterning of their daughters.

Control of lung progenitor cell self-renewal/proliferation

In different organs, the balance of stem cell self-renewal and differentiation maintains tissue homeostasis. Excess of stem cell self-renewal may lead to tissue hyperplasia and/ or tumorigenesis; while excess of differentiation may lead to tissue degeneration and/ or tissue aging. Asymmetric stem cell division, producing one stem cell and one

differentiating cell, is a simple way to maintain the balance between stem cell and differentiated cell populations.

During embryogenesis, stem/progenitor cells undergo a mixture of symmetric and asymmetric divisions. It is currently difficult to distinguish between these types of divisions at the cellular level. One possibility to do this is by looking at differences in spindle orientation or differential inheritance of cytoplasmic or membrane-bound proteins such as cell fate determinant Numb and atypical PKC zeta [59-63] (Figure 2). Cells divide asymmetrically in response to either extrinsic or intrinsic fate determinants. In the case of extrinsic fate determinants (i.e. microenvironment), the daughter cells are placed in different microenvironments, thus the two daughters take on different fates. In the case of intrinsic fate determinants, cytoplasmic cell fate determinants (e.g. Numb) are asymmetrically localized within a cell and subsequently segregate differentially into the two daughter cells, thus the two daughters take on different fates (Figure 2); reviewed in [64]. Currently, we can only infer that certain types of molecules normally act to promote self-renewal or differentiation of progenitors indirectly by comparing the number of progenitor cells identified in mutant, and sibling control, lungs [65].

Studies in our laboratory demonstrate that the proper balance between self-renewal and differentiation of lung-specific stem/ progenitor cells at the distal epithelial tips is absolutely required for normal lung morphogenesis [62,63,66]. Cell polarity and mitotic spindle orientation play a critical role in the self-renewal and differentiation of epithelial cells and can impact normal physiological processes, including epithelial tissue branching and differentiation. Therefore, understanding the behavior of distal epithelial stem/progenitor cells of the embryonic lung could identify innovative solutions to restoring normal lung morphogenesis. Yet little is known about cell polarity, spindle orientation, and segregation of cell fate determinant in the distal embryonic lung stem/ progenitor cells. Recent findings from our laboratory showed that embryonic lung distal epithelial stem/ progenitor cells are polarized and highly mitotic with characteristic perpendicular cell divisions (Figure 2) [62,63,66]. Furthermore, we found that the cell fate determinant Numb is asymmetrically distributed at the apical side of distal epithelial stem/progenitor cells and segregated to one daughter cell in most mitotic cells, suggesting that distal lung stem/progenitor cells divide asymmetrically (Figure 2) [62,63,66]. In addition, we discovered that cell polarity and spindle orientation as well as the asymmetric segregation of the cell fate determinant Numb in embryonic lung stem/progenitor cells are dependent on the activity of Eya1 protein phosphatase [66].

Transcription factors and signaling mechanisms are known to control lung growth and therefore probably affect progenitor cell self-renewal/proliferation (see reference 24 for detailed review). For instance, expression of the transcription factor, Thyroid transcription factor 1 (Ttf-1/Nkx2.1) marks the lung lineage commitment in the early embryo. Ttf-1 is also critical for the development of distal lung progenitors [67]. Loss of *Ttf1 in vivo* results an abnormal murine lung phenotype with insufficient differentiation for survival [68]. Another factor, the HMG box transcription factor Sox9, is specifically and intensively expressed in the distal epithelial progenitors from E11.5 to E16.5; however, lung-specific conditional deletion of Sox9 has no apparent effect on progenitor cell behavior [22,69]. One hypothesis to explain Sox9 lung phenotype is that Sox9 may act redundantly with other, as yet unknown, regulators of progenitor cell proliferation. One candidate gene for this activity is the transcription factor N-myc that plays a critical role in the developing lung by maintaining a distal population of undifferentiated, proliferating progenitor cells, and probably promoting their self-renewing divisions [17].

Furthermore, forkhead/winged helix (fox) family of transcription factors may play a critical role in the maintenance of lung progenitor cell population and promoting their self-renewing divisions. Members of fox family of transcription factors have mutant phenotypes, and may control epithelial progenitor cell proliferation in the lung. For example, a study by Wan et al. [70] showed that conditional deletion of both *foxa1* and f*oxa2* genes in the lung results in small lungs with decreased rates of cell division. Conditional deletion of both *foxp1* and *foxp2*, which are enriched in the distal epithelial progenitors, results in a similar lung phenotype to that shown for *foxa1* and f*oxa2* genes. Thus, in *foxp22/2*; *foxp11/2* double mutants, the lungs are hypoplastic, with inhibited proliferation, but normal proximal–distal patterning [71].

Several studies showed that a network of growth factors is essential for embryonic development. Basically, five key signaling molecules regulate many processes in embryonic development: Fibroblast Growth Factor (FGF), Hedgehog, Notch, Wnt and Transforming Growth Factor-b family (TGF-b). These signaling pathways are needed at some level and at some time to drive differentiation in many different stem cell lines, but it is still unknown what exactly it is that drives specific differentiation into the multiple cell types that have distinct roles in the lung. For instance, Wnt2/2b and beta-catenin signaling are necessary and sufficient to specify lung progenitors in the foregut [72]. Loss Wnt2/2b expression leads to complete lung agenesis and lack of the expression of Nkx2.1, which is the earliest marker of the lung endoderm. This phenotype is recapitulated by an endoderm-restricted deletion of beta-catenin, which suggests that Wnt2/2b signaling through the canonical Wnt pathway is important for the specification of lung endoderm progenitors within the foregut [72,73].

Several studies have identified an essential functional role for FGF in specification of the lung lineages distal to the trachea [29,74,75]. FGF10 is expressed by lung mesenchyme and functions as a chemotactic factor during branching morphogenesis as well as coordinates alveolar smooth muscle cell formation and vascular development [29]. Overexpression of FGF10 maintains epithelial progenitor cell proliferation and leads also to metaplastic differentiation of goblet cells [76]. Retinoic acid signaling also plays an essential role in the expansion of lung progenitors and for formation of primary lung buds, by affecting *Fgf10* expression through TGF-beta signaling [77].

Sonic hedgehog (Shh) signaling in the distal epithelium is essential for lung proliferation and branching morphogenesis, and may induce progenitor cell proliferation [78]. In addition, ectopic overexpression of Gli2, which is a mediator of Shh activity, in the lung mesenchyme results in an increase in both Shh activity and cell proliferation through regulation of cyclin expression in the lung [79]. Furthermore, it has been demonstrated that autocrine Bmp signaling is crucial for proliferation of the distal epithelial progenitor cell compartment [80]. Lung epithelium–specific deletion of Bmp receptor 1a (Bmpr1a) or the distal epithelium-specific Bmp ligand Bmp4 results in smaller lungs, with reduced rates of proliferation and decreased n-myc and foxa2 expression [80]. Likewise, Wnt5a, a prominent Wnt ligand is also highly expressed in and around the distal epithelial tips, and its deletion in mice leads to a general increase in levels of cell proliferation, and an additional branch of the conducting airways [81]; however, it is still unknown whether this phenotype is related to defects in progenitor cells. The general consensus is that these signaling pathways collaborate to control the proliferation of distal epithelial progenitor cells. However, the specific details of their interactions are still to be determined.

Furthermore, a recent study has shown that the RNA-Binding Protein (RBP) HuR is an essential regulator of mesenchymal responses during lung branching. Thus HuR binds and controls the Fgf10 and Tbx4 mRNAs; as a result its deletion abolished their inducible post-transcriptional regulation by the mesenchymal regulator FGF9, leading to blocking the morphogenesis of distal bronchial branches at the initiation of the pseudoglandular stage [82]. In addition, mesenchymal nuclear factor I B has recently been shown to regulate cell proliferation and epithelial differentiation during lung maturation [83]. Moreover, canonical Notch signaling in the developing lung is required for selection of Clara versus ciliated cell fate as well as determination of arterial smooth muscle cells [84].

Several studies have demonstrated the importance in lung epithelial cell development of micro-RNAs (miRNAs), which belong to a class of small, noncoding RNAs that regulate gene expression at a post-transcriptional level. For example, a dramatic increase in the number of highly proliferative epithelial cells expressing sox9 (i.e. multipotent progenitor cells), together with delay in epithelial differentiation, has been found after overexpression of the entire miR-17–92 cluster throughout the developing lung epithelium [85]. This suggests that miR-17–92 promotes self-renewal of progenitors at the expense of differentiated divisions. In addition, miR-17 and its paralogs, miR-20a, and miR-106b, which are highly expressed during the pseudoglandular stage, have a critical functional role during embryonic lung development [86] have demonstrated that simultaneous downregulation of these three miRNAs in explants of isolated lung epithelium altered FGF10 induced budding morphogenesis, an effect that was rescued by synthetic miR-17. Furthermore, E-Cadherin levels were reduced, and its distribution was altered by miR-17, miR-20a and miR-106b downregulation, while conversely, beta-catenin activity was augmented, and expression of its downstream targets, including Bmp4 as well as Fgfr2b, increased [86]. This study also identified Stat3 and Mapk14 as key direct targets of miR-17, miR-20a, and miR-106b. Simultaneous overexpression of Stat3 and Mapk14 mimics the alteration of E-Cadherin distribution observed after miR-17, miR-20a, and miR-106b downregulation, suggesting that mir-17 family of miRNA modulates FGF10-FGFR2b downstream signaling by specifically targeting Stat3 and Mapk14, hence regulating E-Cadherin expression, which in turn modulates epithelial bud morphogenesis in response to FGF10 signaling [86].

Embryonic lung progenitor cells and proximal-distal patterning

It is widely thought that both Wnt and Bmp signaling pathways regulate proximal–distal patterning in the lung. However, there is no current evidence to confirm that the effects of these signaling pathways are mediated through progenitor cells. For instance, a study by Shu et al. [12] showed that proximal-distal patterning of the lung depends on Wnt/b-catenin signaling and is mediated, at least in part, through downstream regulation of other signaling such as Bmp-4, N-myc and FGF signaling. In addition, potentiation of β-catenin signaling in the proximal airway leads to an arrested differentiation of immature bronchiolar stem cells, although β-catenin is not necessary for maintenance of the adult bronchiolar stem cell [87].

One advantage, which facilitates study of Wnt effect on progenitor cells at early stages of lung development, is that reporters of Wnt pathway activity are highly active in the distal epithelial tip cells. Two studies have taken this advantage and showed that Wnt signaling is likely regulate proximal–distal patterning and progenitor cell proliferation independently, and that Wnt signaling functions to promote distal airway fate at the expense of the proximal airways [12,88]. In one of these studies, Shu et al. demonstrated that reducing levels of Wnt pathway activity by overexpression of Dickkopf-1, a Wnt inhibitor, throughout the developing lung epithelium expands the proximal (conducting) airways at the expense of the distal, with no effect on total levels of cell proliferation [12] Similarly, Mucenski et al. [88] reported the lack of differentiation of distal airway epithelium after lung-specific deletion of β-catenin, which is required both for Wnt signaling and cell adhesion. On the other hand, Notch signaling that promotes progenitor cell identity at the expense of differentiated cell phenotypes in different organs [89,90] is also critical for lung epithelial progenitor cells. Notch1 expression is abundant in the distal epithelial progenitors during the pseudoglandular stage of lung development, and controls cell fates in developing airways [91,92]. In addition, Notch signaling also arrests the normal differentiation of distal lung progenitors before they initiate an alveolar program, and its misexpression in the distal lung prevents the differentiation of alveolar cell types [93]. Furthermore, expression of a constitutively active form of Notch3 throughout the developing lung epithelium prevents cell differentiation [94].

Another key signal pathway for lung development is BMP signaling, which is critical for lung epithelium development BMP signaling may promote distal, but repress proximal cell fate. This is supported by several studies showing that inactivation of Bmp signaling by overexpression of a dominant-negative Bmp receptor, or the Bmp antagonists Gremlin or Noggin results in proximalization of the lung epithelium [95,96]. These lung proximalization phenotypes can result from a reduction of Bmp or Wnt signaling [80,81]. Interestingly, a most recent study has shown that deficiency of histone deacetylases 1 and 2 (Hdac1/2) leads to a loss of Sox2 expression and a block in proximal airway development. This is mediated in part by derepression of Bmp4, which is a direct transcriptional target of Hdac1/2 [97].

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