Stem cells in pulmonary alveolar regeneration

Huijuan Wu¹ and Nan Tang¹,²,*

ABSTRACT

The lungs are constantly exposed to the external environment and are therefore vulnerable to insults that can cause infection and injury. Maintaining the integrity and barrier function of the lung epithelium requires complex interactions of multiple cell lineages. Elucidating the cellular players and their regulation mechanisms provides fundamental information to deepen understanding about the responses and contributions of lung stem cells. This Review focuses on advances in our understanding of mammalian alveolar epithelial stem cell subpopulations and discusses insights about the regeneration-specific cell status of alveolar epithelial stem cells. We also consider how these advances can inform our understanding of post-injury lung repair processes and lung diseases.

KEY WORDS: Alveolar regeneration, Intermediate cell state, Niche, Stem cells

Introduction

The essential function of the lungs is to exchange oxygen in the air with carbon dioxide in the blood. At the same time, this exposure means that the lungs are vulnerable and constantly exposed to insults from the external environment that can cause infection and injury. Respiratory diseases are now leading global causes of death and disability, so elucidating the mechanism of lung regenerative responses mediated by tissue resident stem/progenitor cells is crucial for understanding lung repair post-injury. Deeper understanding of these processes will almost certainly facilitate the development of new therapeutic strategies for treating respiratory diseases.

In this Review, we provide an overview of alveolar epithelial stem cells in the lung. In light of several recently published excellent review papers on some of these topics, which we strongly encourage readers to peruse, our present Review focuses on recently identified alveolar epithelial stem cell subpopulations and discusses insights about the regeneration-specific cell status of alveolar epithelial stem cells in the mammalian lung (Basil et al., 2020; Parimon et al., 2020; Whitsett et al., 2019; Zepp and Morrisey, 2019). We also consider how these advances can inform our understanding of post-injury lung repair processes, as well as the pathogenesis of lung diseases. In addition, we discuss how the integration of mouse genetics, biophysical analysis and live imaging will provide insights into alveolar stem cell regulation during lung regeneration.

Overview of lung epithelial cell populations

Until birth, the mother provides all the nutrients needed for growth, as well as oxygen, to the fetus. Upon birth, the acquisition of required oxygen is completely dependent on the gas-exchange function of lungs. The lung is a complex, multi-cellular organ that is composed of multiple epithelia that line the inside of airways and that also includes various types of stromal cells, blood and lymphatic endothelial cells, and immune cells (Hogan et al., 2014; Morrisey and Hogan, 2010). It is known that different regions of the mammalian lung contain different populations of epithelial cells and stromal cells (Hogan et al., 2014; Morrisey and Hogan, 2010).

Epithelial cell populations in proximal airways

The proximal airways of the lung consist of the trachea, a pair of primary bronchi and many bronchioles of various sizes, generated through a branching morphogenesis process (Metzger and Krasnow, 1999) (Fig. 1). The epithelium lining the trachea and bronchi is pseudostatified, primarily consisting of three types of epithelial cells, including basal cells, club cells and ciliated cells, as well as a small number of neuroendocrine cells, goblet cells, ionocytes and tuft cells (Table 1; Fig. 1) (Hogan et al., 2014; Montoro et al., 2018; Plasschaert et al., 2018; Zepp and Morrisey, 2019). It is generally accepted that basal cells function as proximal airway epithelial stem cells. During physiological cell turnover or after injury, basal cells can self-renew and differentiate into various airway epithelial cell types for maintaining the epithelial integrity of proximal airways (Rock et al., 2011b, 2009). Specifically, basal cells are a group of cells that tightly attach to the basal layer of the airway and specifically express transformation related protein 63 (Trp63 or P63), keratin 5 (Krt5) and nerve growth factor receptor (Ngfr). Human lung basal cells are distributed throughout the main trachea to the terminal bronchioles, whereas mouse basal cells are mainly distributed in the main trachea (Fig. 1) (Hogan et al., 2014; Morrisey and Hogan, 2010; Nikolić et al., 2018).

Studies have established that the epithelial cell turnover rate in the trachea of adult animals and humans is low under normal conditions (Hegab et al., 2012; Hong et al., 2004; Rock et al., 2011b, 2009). After tracheal epithelial injury, however, basal cells can rapidly proliferate and differentiate into multiple cell types such as secretory, goblet and multi-ciliated cells (Hegab et al., 2012; Hong et al., 2004; Rock et al., 2011b, 2009). In the bronchioles of mouse lungs, club cells and ciliated cells are the two major types of epithelial cells, as well as a small number of goblet cells. The intrapulmonary airways of mouse lungs lack basal cells, highlighting a clear difference from human intrapulmonary airways. At this region of the mouse lung, club cells respond to injury by proliferating and then differentiating into ciliated cells to support the repair of a damaged epithelium (Rawlins et al., 2009b). Club cells can also dedifferentiate into basal cells when basal cells are specifically ablated in the mouse trachea (Tata et al., 2013). A recent study of COVID-19 patients identified that a majority of proliferating cells in small airways (<0.5 mm) of these patients are KRT5* cells (Fang et al., 2020). An interesting
future direction will be identifying and comparing the stem cells/progenitor cells that participate in intrapulmonary airway regeneration processes in human lungs after various injuries.

Two types of alveolar epithelial cells
Oxygen passes through the bronchial airways and eventually enters the alveoli, which are the basic structural units performing the gas exchange functions of the lungs. There are ~300-700 million alveoli in adult human lungs, and the total surface area of the entire alveoli complement ranges from 75-100 square meters (Ochs et al., 2004).

The alveolar epithelium comprises two types of alveolar epithelial cells: alveolar type I (AT1) and alveolar type II (AT2) cells (Table 1; Fig. 1). AT1 cells are large squamous cells that function as the epithelial component of the air-blood barrier. AT1 cells comprise more than 95% of the gas exchange surface of the lung, and these AT1 cells are intimately associated with the underlying endothelial capillary plexus to form the thin gas-diffusible interface (Weibel, 2015). Helping to promote efficient gas exchange, the thickness of the barrier between alveoli and blood vessels is only 0.2-2.5 μm (Weibel, 2015).

AT2 cells are cuboidal in shape and occupy ~5% of the total alveolar surface. One of the known essential functions of AT2 cells is to produce and secrete a large amount of surfactant into the alveolus (Andreeva et al., 2007; King, 1982). The surfactant, 90% of which is composed of lipids, covers the entire inner surface of the alveoli and helps to reduce the surface tension as the alveoli mechanically expand during breathing movements (Andreeva et al., 2007; King, 1982). AT2 cells also participate in innate immune responses in the lung (Qian et al., 2013). Unlike the stem cells in the epidermis, intestine and hematopoietic system, which are characterized by a high rate of cell turnover, AT2 cells in homeostatic conditions are quiescent and seldom proliferate (Barkauskas et al., 2013; Desai et al., 2014). However, upon damage to the alveolar epithelium, AT2 cells can quickly proliferate and differentiate into AT1 cells, thereby maintaining the integrity of the alveolar epithelium cells (Barkauskas et al., 2013; Desai et al., 2014; Hogan et al., 2014; Katsura et al., 2019; Khatri et al., 2019; Liu et al., 2016; Rock et al., 2011a).

AT2 cells are alveolar epithelial stem cells
In this section, we discuss how AT2 cells develop, the mechanisms that control their proliferation and what is known about their capacity to regenerate alveoli. To date, the majority of direct evidence showing in vivo alveolar regeneration has been collected from mouse models. There is no experimental evidence that new alveolar structures can be created in the adult human lung. However,
employing live-imaging approaches in mice, a study found that a mouse alveolar development (Li et al., 2018). Specifically, of AT2 cell fate determination and AT1 cell differentiation during fetal breathing movements has been identified as an essential driver either AT1 cells or AT2 cells (Rawlins et al., 2009a). Recently, the Alveolar epithelial cell development' to show that the patient assessment of lung microstructure and analyses of lung function over a period of 15 years. This study relied on MRI-based undergone pneumonectomy (PNX) (Box 1) had alveolar growth in 2012, a medical study showed that a 33-year-old woman who had shown that the use of human serum as an addition to the culture organoid culture system (Barkauskas et al., 2017). A recent study AT1 cell differentiation has been observed in such a human AT2 cell of human alveolar repair. However, one of the challenges is that little AT2 cells will help us to gain insight into the regulatory mechanisms of human alveolar repair. However, one of the challenges is that little AT1 cell differentiation has been observed in such a human AT2 cell organoid culture system (Barkauskas et al., 2017). A recent study showed that the use of human serum as an addition to the culture medium can significantly promote the differentiation of human AT2 cells (Katsura et al., 2019; Khatri et al., 2014; Hogan et al., 2014). In other injury models that involve minimal, likely due to very little loss of AT1 cells in such an injury model (Barkauskas et al., 2013). In other injury models that involve the loss of alveolar number or AT1 cells, such as bleomycin-, virus-, and diphtheria toxin (Box 1), the proliferation of AT2 cells increases significantly, yet the differentiation of AT2 cells remains minimal, likely due to very little loss of AT1 cells in such an injury model (Barkauskas et al., 2013). In other injury models that involve the loss of alveolar number or AT1 cells, such as bleomycin-, virus-, and PNX-induced injuries (Box 1), AT2 cells show both robust proliferation and differentiation (Barkauskas et al., 2013; Desai et al., 2014; Hogan et al., 2014; Katsura et al., 2019; Khatari et al., 2019; Liu et al., 2016; Rock et al., 2011a). Although the exploration of the mechanisms regulating the differentiation of AT2 cells is still in its infancy, the mechanisms that regulate the proliferation of AT2 cells have been extensively studied. Multiple studies have demonstrated that several developmental signaling pathways regulate the proliferation of AT2 cells. Using a co-culture system with rat primary AT2 cells and fibroblasts, one such study found that the keratinocyte growth factor (KGF) signaling pathway and the large subset of alveolar progenitor cells could protrude from the airway epithelium toward the mesenchyme, leading to myosin Bingle and Bingle, 2011; Dye et al., 2016; Yu et al., 2010

<table>
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<th>Location</th>
<th>Epithelial cell population</th>
<th>Markers</th>
<th>References</th>
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<tr>
<td>Airway</td>
<td>Basal cells</td>
<td>TRP63, KRT5, NGFR, PDPN</td>
<td>Hackett et al., 2011; Rock et al., 2009; Teixeira et al., 2013</td>
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<td>Multi-ciliated cells</td>
<td>Acetylated tubulin, FOXJ1</td>
<td>Dye et al., 2016; Gao et al., 2015; Look et al., 2001</td>
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<td>Club cells</td>
<td>SCGB1A1, SCGB3A1, SCGB3A2, PLUNC</td>
<td>Dye et al., 2016; Khoor et al., 1996; Nakajima et al., 1996; Reynolds et al., 2002</td>
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<td></td>
<td>Goblet cells</td>
<td>MUC5AC, MUC5B, SPDEF</td>
<td>Bingle and Bingle, 2011; Dye et al., 2016; Yu et al., 2010</td>
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<td>Neuroendocrine cells</td>
<td>ASCL1, CGRP, chromogranin A, GRP, NCAM, substance P</td>
<td>Cutz et al., 2013; Miki et al., 2012</td>
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<td></td>
<td>Ionocytes</td>
<td>CFTR, FOXI1</td>
<td>Montoro et al., 2018; Plasschaert et al., 2018</td>
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<td></td>
<td>Tuft cells</td>
<td>DCLK1, GNAT3, TSLP, IL25</td>
<td>Krasteva et al., 2011; Bankova et al., 2018; Montoro et al., 2018</td>
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<td></td>
<td>Bronchioalveolar stem cells (BASCs)</td>
<td>SCGB1A1/pro-SFTPC double positive</td>
<td>Kim et al., 2005; Lee et al., 2014; Liu et al., 2019; Salwig et al., 2019</td>
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<td>H2-K1high cells</td>
<td>SCGB1A1, H2-K1high, MHChigh</td>
<td>Kathiriya et al., 2020</td>
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<td></td>
<td>Lineage-negative epithelial progenitors (LNEPs)</td>
<td>KRT5+, TRP63+, SOX2+</td>
<td>Kanegai et al., 2016; Kumar et al., 2011; Vaughan et al., 2015; Xi et al., 2017; Zuo et al., 2015</td>
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<tr>
<td>Alveolus</td>
<td>Type I alveolar epithelial cells</td>
<td>AQP5, HOPX, PDPN, RAGE, HTI-56 (human specific)</td>
<td>Dobbs et al., 1999; Fujino et al., 2012; Nikolic et al., 2017; Shirasawa et al., 2004</td>
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<td></td>
<td>Type II alveolar epithelial cells</td>
<td>ABCA3, LAMP3, LPCAT1, SFTPC, SPA, SPB, HTI-280 (human specific)</td>
<td>Barkauskas et al., 2013; Cau et al., 2016; Gonzalez et al., 2010; Khoor et al., 1993; Khoor et al., 1994; Nikolic et al., 2017; Phelps and Floros, 1988; Stahlman et al., 2007</td>
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Table 1. Summary of epithelial cell populations in mouse and human

AT2 cell proliferation
It is now clear that, although key mechanisms of regeneration are conserved between different types of repair in mice and humans, regenerative behaviors of AT2 cells may vary in response to different types of injuries. For example, when AT2 cells are experimentally depleted by diphtheria toxin (Box 1), the proliferation of AT2 cells increases significantly, yet the differentiation of AT2 cells remains minimal, likely due to very little loss of AT1 cells in such an injury model (Barkauskas et al., 2013). In other injury models that involve the loss of alveolar number or AT1 cells, such as bleomycin-, virus-, and PNX-induced injuries (Box 1), AT2 cells show both robust proliferation and differentiation (Barkauskas et al., 2013; Desai et al., 2014; Hogan et al., 2014; Katsura et al., 2019; Khatari et al., 2019; Liu et al., 2016; Rock et al., 2011a). Although the exploration of the mechanisms regulating the differentiation of AT2 cells is still in its infancy, the mechanisms that regulate the proliferation of AT2 cells have been extensively studied. Multiple studies have demonstrated that several developmental signaling pathways regulate the proliferation of AT2 cells. Using a co-culture system with rat primary AT2 cells and fibroblasts, one such study found that the keratinocyte growth factor (KGF) signaling pathway and the

Alveolar epithelial cell development
During the alveolar development process, alveolar progenitor cells positioned at the tip of distal airways subsequently differentiate into either AT1 cells or AT2 cells (Rawlins et al., 2009a). Recently, the mechanical force generated from the inhalation of amniotic fluid by fetal breathing movements has been identified as an essential driver of AT2 cell fate determination and AT1 cell differentiation during mouse alveolar development (Li et al., 2018). Specifically, employing live-imaging approaches in mice, a study found that a

in 2012, a medical study showed that a 33-year-old woman who had undergone pneumonectomy (PNX) (Box 1) had alveolar growth over a period of 15 years. This study relied on MRI-based assessment of lung microstructure and analyses of lung function to show that the patient’s vital capacity and alveoli number increased significantly since the PNX (Butler et al., 2012). Although this provides the first compelling evidence that alveoli can regenerate in adult human lungs, alveolar regeneration in adult humans is still poorly characterized, mainly because of the lack of post-injury lung samples and regeneration-specific molecular markers. Considering the opportunities of single cell RNA sequencing (scRNA-seq) techniques, it is likely that ongoing work is cataloguing the gene expression changes in AT2 cells in both mouse and human lungs. Such work can be expected to identify regeneration-specific markers that can increase our ability to monitor regeneration processes, both experimentally and clinically. Organoid culture systems using human AT2 cells will help us to gain insight into the regulatory mechanisms of human alveolar repair. However, one of the challenges is that little AT1 cell differentiation has been observed in such a human AT2 cell organoid culture system (Barkauskas et al., 2017). A recent study showed that the use of human serum as an addition to the culture medium can significantly promote the differentiation of human AT2 cells into AT1 cells during organoid culture (Katsura et al., 2020). Further study of the mechanisms that regulate the differentiation of the human AT2 cells in organoids will definitely give us more opportunities to explore the regenerative behavior of human AT2 cells.

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AT2 cell differentiation during alveolar regeneration

In various lung injury mouse models (Box 1), lineage-tracing studies have demonstrated that AT2 cells are capable of proliferating and differentiating into AT1 cells (Barkauskas et al., 2013; Desai et al., 2014; Hogan et al., 2014; Katsura et al., 2019; Khatri et al., 2019; Rock et al., 2011a; Wu et al., 2020). However, a remaining question concerns whether all AT2 cells, or only a particular subset of AT2 cells, have this regenerative capacity. Two studies have reported that mouse AT2 cells expressing AXIN2\(^+\), a transcriptional target of WNT signaling, may represent the dominant population of alveolar stem cells during post-injury alveolar regeneration (Nabhan et al., 2018; Zacharias et al., 2018). These AXIN2\(^+\) AT2 cells have been deemed alveolar epithelial progenitors (AEPs) (Zacharias et al., 2018). In both an influenza virus-induced lung injury model and a bleomycin-induced lung injury model (Box 1), these AXIN2\(^+\) AT2 cells are mainly found at sites adjacent to the most severely damaged alveolar regions. These AXIN2\(^+\) AT2 cells are highly proliferative, and can further differentiate into a large number of AT1 cells (Nabhan et al., 2018; Zacharias et al., 2018). Nabhan and colleagues have reported that only \(\sim1\)% of the AT2 cells are AXIN2\(^+\) in homeostasis, and almost all AT2 cells become AXIN2\(^+\) in response to injury (Nabhan et al., 2018). Conversely, Zacharias and colleagues have reported that \(\sim20-30\)% of AT2 cells are AXIN2\(^+\) in homeostasis, and these pre-existing AXIN2\(^+\) AT2 cells function as a major cell source for alveolar regeneration after injuries (Zacharias et al., 2018). Although the percentage of such AXIN2\(^+\) AT2 cells in the lung varies between these two studies, both studies indicate that canonical WNT signaling functions in alveolar regeneration.

Other studies have provided clear evidence that NOTCH, WNT, BMP and YAP-mediated signaling function in the differentiation of AT2 cells and alveolar progenitor cells (Chung et al., 2018; Finn et al., 2019; LaCanna et al., 2019; Lange et al., 2015; Liu et al., 2016). For example, a study using an AT2 cell organoid culture system and a PNX mouse lung injury model has shown that reducing BMP signaling in AT2 cells impairs AT2 cell differentiation, whereas increasing BMP signaling in AT2 cells promotes AT2 cell differentiation (Chung et al., 2018). In addition, NOTCH signaling is activated in AT2 cells after bacterial-induced alveolar injury (Box 1). Notably, such activation of NOTCH signaling must be inhibited to enable a complete AT2-AT1 cell transition: disrupting DLK1, an inhibitor of NOTCH signaling, impairs AT2-AT1 cell transition. There is also evidence from various lung injury models supporting the view that YAP/TAZ in AT2 cells is essential for AT2 cell differentiation (Finn et al., 2019).

Interestingly, after specific elimination of some AT2 cells with diphtheria toxin (Box 1), the remaining AT2 cells are able to proliferate and restore the number of AT2 cells in alveoli (Barkauskas et al., 2013). Unlike other lung injury models that involve a reduction in the number of AT1 cells, such as bleomycin-, virus-, bacteria- and PNX-induced injuries (Box 1), diphtheria toxin-induced AT2 cell elimination does not involve significant loss of alveolar units and no new AT1 cells have been observed in this injury model (Barkauskas et al., 2013). In the bleomycin-induced or PNX-induced injury models, the effective respiratory units are reduced; this may lead to elevated mechanical tension in the remaining respiratory units. It is therefore tempting to speculate that the differentiation of adult AT2 cells may also be controlled by the increased mechanical tension on the alveolar epithelium. In one of the studies from our group, we tested this hypothesis by inserting a prosthesis that imitates the shape and size of the removed lung lobe after PNX (Liu et al., 2016). The lack of newly formed AT1 cells in

hepatocyte growth factor/scatter factor (HGF/SF) signaling pathway are essential for the proliferation of AT2 cells, characterized by DNA synthesis (Panos et al., 1993). Additional studies have also demonstrated that β-catenin signaling can regulate AT2 cell proliferation (Zemans et al., 2011). More growth factor-mediated signaling in regulating AT2 cell proliferation is discussed below.

A recent study has provided new evidence that mechanical tension is an important regulator for AT2 cell proliferation (Liu et al., 2016). In a PNX mouse model (Box 1), loss of alveoli significantly increases the mechanical tension of the remaining alveolar epithelium (Liu et al., 2016; Wu et al., 2020). Moreover, this increased mechanical tension enhances actin polymerization in AT2 cells, which in turn promotes both the nuclear translocation of YAP in AT2 cells and the subsequent YAP-dependent proliferation of AT2 cells (Liu et al., 2016). YAP is a transcription coactivator that functions as a nuclear effector of mechanical tension to promote cell proliferation in multiple epithelial systems (Dupont et al., 2011). An important question concerns the as-yet-unknown identity of any sensors for mechanical force in AT2 cells. Claudin 18 (CLDN18), a lung-specific tight junction protein, regulates the activity of YAP in AT2 cells and participates in alveolar regeneration (Zhou et al., 2018); therefore, it will be interesting to investigate any function(s) of tight junction proteins in regulating AT2 cell responses to mechanical forces. These studies together indicate a complicated and fine regulatory signaling network involved in AT2 cell self-renewal.

**Box 1. Models of lung injury used in studies of lung stem cells.**

**Pneumonectomy (PNX).** The surgical removal of lung tissue is a procedure used in the treatment of lung diseases in the clinic. In various species, including mice, dogs and humans, compensatory growth and new alveolar formation occurs in the remaining lung after PNX (Butler et al., 2012; Hsia et al., 1994; Thane et al., 2014). Mechanical stress has been reported as one of the most important initiating factors for such post-PNX alveolar regeneration (Liu et al., 2016; Thane et al., 2014; Wu et al., 2020).

**Bleomycin.** A chemotherapeutic drug that can cause DNA strand breakage and oxidative stress, leading to transient inflammation and fibrosis of the lungs (Lown and Sim, 1977; Sauvage et al., 1976). The drug is instilled into the murine lungs, typically via the trachea. Finally, the lung fibrosis and structural destruction caused by the drug can be recovered (Moeller et al., 2008; Moore and Hogaboam, 2008).

**Diphtheria toxin.** An exotoxin derived from bacteria that can trigger cell death (Mitamura et al., 1995; Murphy, 1996). Combined with genetic manipulations, diphtheria toxin is often expressed in a cell-specific manner to induce the selective ablation of a targeted cell type (Barkauskas et al., 2013; Mitamura et al., 1995; Murphy, 1996). Naphthalene. An aromatic hydrocarbon that is used to induce ablation of club cells (Van Winkle et al., 1995). Club cells express the cytochrome P450 enzyme, CYP2F2, which can convert naphthalene to a cytotoxic product. Thus, naphthalene exposure of mouse lungs leads to massive loss of club cells (Reynolds et al., 2000; Stripp et al., 1995).

**Murine infectious lung injury models.** These have been developed to support discoveries about pathological mechanisms related to the human lung infectious diseases. These models include: (1) intratracheal administration of bacteria or bacterial products (e.g. lipopolysaccharide, LPS), into mouse lungs (Vernooy et al., 2002; Yanagihara et al., 2001); (2) delivering a murine-adapted influenza virus into mouse lungs (Kumar et al., 2011; Nakajima et al., 2012). Both models can induce widespread damage to airways and alveoli, which is marked by epithelial cell death and immune cell infiltration. Sometimes, the structural destruction of infectious mouse lungs can be recovered over several months.
prosthesis lungs supports the hypothesis: increased mechanical tension is indeed essential for the differentiation of adult AT2 cells into AT1 cells (Liu et al., 2016). However, when AT1 cells are experimentally depleted by diphtheria toxin (Chung and Hogan, 2018), loss of AT1 cells may not associated with increased alveolar mechanical tension. The differentiation of AT2 cells into AT1 cells in the AgerCreERT2;DTA model may be triggered by a mechanism that is independent of mechanical tension.

It will be interesting to investigate the relationships between mechanical force-mediated signaling and other above signaling pathways. Although determining the level of mechanical tension that is required for AT2 cell differentiation in vivo is still a big challenge, increased integration of biophysical modeling and further in vivo mechanical analyses are likely to contribute to future breakthroughs about the signaling networks through which mechanical force regulates alveolar regeneration.

**An intermediate AT2-AT1 cell state exists during alveolar regeneration**

Using various sets of molecular markers obtained from scRNA-seq analysis, an intermediate AT2-AT1 cell state during alveolar regeneration has been identified (Choi et al., 2020; Jiang et al., 2020; Kobayashi et al., 2020; Riemondy et al., 2019; Strunz et al., 2020; Wu et al., 2020). For example, in a lipopolysaccharide-induced alveolar regeneration mouse model (Box 1), three distinct development states of AT2 cells have been identified in the lung: a proliferating state of AT2 cells, a cell cycle arrest state of AT2 cells, and a transitional AT2 cell state (Riemondy et al., 2019). In addition, a dynamic differentiation process for AT2 cells has been carefully characterized in a bleomycin-induced alveolar regeneration mouse model (Box 1; Strunz et al., 2020). This work showed that some AT2 lineage cells in post-bleomycin injury lungs exhibit significantly reduced expression of AT2 cell canonical marker genes (e.g. Sftpc, Sftpa1 and Cxcl15), yet do not express AT1 cell biomarker genes at elevated levels. Going some way to explain these observations, an RNA velocity analysis has suggested that these AT2 cells apparently exist in an intermediate cell state between AT2 cells and AT1 cells (Strunz et al., 2020).

The transient expression of several molecular markers, including claudin 4 (Cldn4), stratifin (Sfn) and keratin 8 (Krt8), can specifically define this intermediate AT2-AT1 cell state (Fig. 2). Lineage tracing experiments in mice have demonstrated that cells at the intermediate AT2-AT1 cell state are able to subsequently differentiate into AT1 cells, further confirming that the intermediate AT2-AT1 cell state can be understood as a normal differentiation stage that occurs while AT2 cells differentiate into AT1 cells (Jiang et al., 2020; Kobayashi et al., 2020; Riemondy et al., 2019; Strunz et al., 2020; Wu et al., 2020). Together, these studies have established a suite of molecular markers that facilitates time-resolved characterization of the alveolar stem cell regeneration process in human lung specimens.

Recent studies have found that many AT2 cells in idiopathic pulmonary fibrosis (IPF) lungs persistently express these intermediate cell markers (Kobayashi et al., 2020; Strunz et al., 2020), supporting the hypothesis that AT2 cells in IPF patients do not have the capacity to further differentiate into AT1 cells (Jiang et al., 2020; Wu et al., 2020). Furthermore, several studies have revealed that cells at the intermediate state are enriched for many molecular and cellular features commonly found in IPF lungs (Kobayashi et al., 2020; Wu et al., 2020). Using combined approaches including AT2 cell organoid cultures, scRNA-seq and mouse models, one study has shown that cellular senescence signaling and TP53 signaling are significantly enriched in cells at the intermediate state (Kobayashi et al., 2020). Another study has found that the TGFβ signaling is transiently activated in cells at such
an intermediate state using scRNA-seq analysis (Wu et al., 2020). Our recent study has shown that a progressive lung fibrosis phenotype occurs in Cdc42 AT2-null mice post PNX (Wu et al., 2020). The differentiation of Cdc42 null AT2 cells is impaired, which leads to sustained exposure of AT2 cells to elevated mechanical tension and the persistent activation of a TGFβ signaling loop in the AT2 cells at the intermediate state. Specifically, mechanical tension activates TGFβ signaling in these AT2 cells at the intermediate state, which leads to increased TGFβ ligand expression and further constitutively activated TGFβ signaling in these AT2 cells (Wu et al., 2020). Together, these studies have supported the interesting concept that the differentiation state of AT2 cells – in addition to the subpopulation of AT2 cells per se – should be a focus for investigating alveolar regeneration, lung diseases and perhaps even development of novel therapeutic approaches.

Importantly, through immunofluorescence staining analysis of human lung tissues, AT2 cells at the intermediate state have also been observed in lungs of acute respiratory disease symptom patients with diffused alveolar damage (Chen et al., 2020a; Strunz et al., 2020). Thus, it is clear that we need to better understand the physiological and pathological contributions of AT2 cells in diseased human lungs.

Moving forward, deeper mechanistic understanding of the molecular regulation of alveolar stem cell differentiation, and identification of additional regeneration-specific molecular markers, will help to better characterize alveolar regeneration in human lungs, potentially enabling much more accurate prognostication of human lung injury patient outcomes and/or informing clinical treatment decisions.

The AT2 cell niche

The alveolar epithelium is surrounded by various types of fibroblasts, pericytes, endothelial cells and immune cells (Fig. 3). Recent studies have begun to uncover the roles of these neighboring cells, which we now know can functionally impact the AT2 cell niche during alveolar regeneration.

**PDGFRα**+ lipofibroblasts function as the niche of AT2 cells

Direct and extensive contacts between AT2 cells and the surrounding mesenchymal cells are required for the stem cell behaviors of AT2 cells during homeostasis, as well as during post-injury alveolar regeneration (Barkauskas et al., 2013; Sirianni et al., 2003). Mesenchymal cells expressing platelet-derived growth factor receptor alpha (PDGFRα) mark a population of lipofibroblast-like cells that are spatially associated with AT2 cells (Fig. 3) (Barkauskas et al., 2013; Green et al., 2016; Zepp et al., 2017). A pioneering study has shown that the PDGFRα+ lipofibroblasts can support the growth and differentiation of AT2 cells in an alveolar organoid co-culture system, demonstrating that PDGFRα+ lipofibroblasts function as the alveolar stem cell niche (Barkauskas et al., 2013). Subsequently, many studies have provided useful information about how PDGFRα+ lipofibroblasts influence the regenerative behaviors of AT2 cells, both in the homeostatic condition and during post-injury alveolar regeneration (Chung et al., 2018; Green et al., 2016; Zepp et al., 2017). Briefly, using 3D alveolar organoids and mouse models, several groups have found that PDGFRα+ lipofibroblasts can maintain AT2 cell stemness or control AT2 cell self-renewal and differentiation into AT1 cells through mediating multiple signaling, including BMP, FGF and WNT signaling (Chung et al., 2018; El Agha et al., 2017; Green et al., 2016; Yuan et al., 2019; Zepp et al., 2017).

**Vascular endothelial cells promote alveolar regeneration**

In alveoli, AT1 cells maintain close contact with pulmonary capillary endothelial cells (PCECs) and several studies have demonstrated that PCECs function as an essential component of
the AT2 cell niche in supporting the proliferation and differentiation of AT2 cells.

After PNX, the proliferation of PCECs is strongly induced, which facilitates the generation of capillaries in new alveolar units. Furthermore, PCECs can promote AT2 cell proliferation via VEGFR2- and FGFR1-mediated signaling. We know that deletion of VEGFR2 or FGFR1 in PCECs reduces the proliferation of PCECs themselves and reduces AT2 cell proliferation. Mechanistically, this is controlled by the secretion of matrix metalloproteinase 14 (MMP14) from PCECs, which promotes the release of peptides with a cryptic EGF domain from the alveolar extracellular matrix that further bind to EGF receptors in the AT2 cells, promoting the proliferation of AT2 cells (Ding et al., 2011). A subsequent study has shown that this MMP14 effect is co-mediated through platelet activation of endothelial stromal cell derived factor 1 (SDF1) receptors (Rafii et al., 2015). Furthermore, PCECs have been shown to promote alveolar regeneration via HGF signaling (Cao et al., 2017) or sphingosine-1/-spingosine-1-phosphate receptor 2 (SIP/SIPR2) signaling (Chen et al., 2020b). Interestingly, fetal lung endothelial cells also support the preferential outgrowth of distal epithelial organoids through the production of thrombospondin 1 (Lee et al., 2014), suggesting that the function of PCECs in the niche may not be limited to regulating the proliferation and differentiation of AT2 cells.

Two groups have used scRNA-seq analysis to demonstrate the heterogeneity of PCECs (Ellis et al., 2020; Niethammer et al., 2020): both groups have identified a transcriptionally distinct EC subset, marked by a high level of carbonic anhydrase 4 (CAR4) (Ellis et al., 2020; Niethammer et al., 2020). Given that AT1 cells express high levels of VEGF, and considering that CAR4<sub>high</sub> endothelial cells express high levels of VEGFR, it is proposed that these CAR4<sub>high</sub> endothelial cells interact closely with AT1 cells, and may function in alveolar morphogenesis (Ellis et al., 2020). CAR4<sub>high</sub> endothelial cells may also contribute to alveolar regeneration, because these cells are known to be enriched in the regeneration regions following influenza- or bleomycin-induced lung injury. Interestingly, the same study has also identified a proliferating endothelial cell subset that has low CAR4 expression after influenza-induced lung injury (Niethammer et al., 2020), further highlighting the functional complexity of PCECs during alveolar regeneration. It has been further defined that the alveolar endothelium is made up of two intermingled cell types: aerocytes and general capillary cells. The aerocytes, which are specialized for the gas exchange function, express a high level of CAR4. The general capillary cells function as a progenitor cell in capillary homeostasis and repair. These two cell types develop from common, bipotent progenitors and are conserved between mouse and human lungs (Gillich et al., 2020). Any underlying molecular mechanisms that regulate the emergence of these endothelial subsets remain unknown. A better understanding of the various PCEC subsets will provide insight into the development of strategies for alveolar regeneration.

**Immune response in alveolar regeneration**

After acute lung injury, pathogens and subsequent epithelial cell death can quickly change the immune microenvironment in the lung by activating and recruiting immune cells to alveoli, as well as altering the expression levels of various cytokines (Danahay et al., 2015; Katsura et al., 2019; Kuperman et al., 2002; Tadokoro et al., 2014; Xie et al., 2018). The immune response in the alveolar region can either damage alveolar epithelial cells or can promote alveolar regeneration by modulating the regenerative niche, depending on the types and phases of injury, as well as the specific immune cells involved in the injury responses (Byrne et al., 2015; Iwasaki et al., 2017; Tisoncik et al., 2012).

Both in vivo and in vitro studies have demonstrated that many cytokines directly affect the proliferation and differentiation of AT2 cells (Danahay et al., 2015; Katsura et al., 2019; Kuperman et al., 2002; Tadokoro et al., 2014; Xie et al., 2018). AT2 cells themselves can affect inflammatory responses in the lung: mice with Yap-Taz-null AT2 cells exhibit prolonged inflammatory responses after bacterial pneumonia. Impaired alveolar regeneration in Yap-Taz AT2-null mice leads to a failure to upregulate IkBα, the molecule that terminates NF-κB-mediated inflammatory responses (LaCanna et al., 2019).

Recently, work with a PNX mouse model has demonstrated that AT2 cells secrete the chemokine CCL2 and subsequently recruit CCR2<sup>+</sup> monocytes into the alveoli (Lechner et al., 2017). Genetic deletion of the bone marrow-derived CCL2 monocytes, or the innate immune cells thought to activate them, results in impaired PNX-induced alveolar regeneration (Box 1), suggesting that myeloid cells play a key role in promoting alveolar regeneration. Another study using the same PNX mouse model has demonstrated that platelets can secrete SDF1 to stimulate capillary endothelial cells to produce MMP14, which further releases EGF ligands from the extracellular matrix and subsequently promotes AT2 cell proliferation and differentiation (Rafii et al., 2015). All these lines of evidence have highlighted the contributions of, and revealed some underlying mechanisms for, immune cell functions in alveolar regeneration.

**Other stem cell populations in distal airways that participate in alveolar regeneration**

Although AT2 cells should be understood as primary contributors to regenerative responses to a variety of alveolar insults, increasing evidence is demonstrating that additional epithelial cell populations present in the distal airway can be activated upon severe lung injury (such as virus-induced injury) and can function as an alternative source of alveolar stem cells for alveolar regeneration (Barkauskas et al., 2013; Chapman et al., 2011; Xi et al., 2017). Club cells that are lineage labeled by secretoglobin family 1A member 1 (Scgb1a1) can function to replenish alveolar epithelial cells after bleomycin-induced mouse lung injury (Box 1) (Barkauskas et al., 2013; Rock et al., 2011a). In addition, we further discuss the distal airway stem cell populations that can participate in regenerative processes described to date.

**Bronchioalveolar stem cells**

A small population of cells located at the bronchoalveolar junction (BADJ) of mice has been shown to express both the canonical club cell marker SCGB1A1 and the canonical AT2 cell marker, surfactant protein C (SFTPC) (Kim et al., 2005; Lee et al., 2014). Based on their physical location and their possible multipotency, these cells are known as bronchioalveolar stem cells (BASCs) (Kim et al., 2005); however, whether such a population exists in humans has yet to be determined. Our knowledge about the lineages of BASCs has recently been improved significantly. Using different dual lineage-tracing mouse tools (Dre/Cre or split-Cre/split-ITA), two groups have demonstrated that BASCs are able to enter the alveoli to regenerate AT2 cells and AT1 cells after bleomycin-induced alveolar injury (Box 1) (Liu et al., 2019; Salwig et al., 2019). However, the BASCs rarely give rise to AT1 and AT2 cells during lung homeostasis, suggesting the regeneration process of BASCs into alveolar epithelial cells is triggered by lung injuries (Liu et al., 2019; Salwig et al., 2019). In summary, elucidating the mechanisms that regulate the differentiation of BASCs will provide insights into lung injury responses.
Lineage-negative epithelial progenitors/distal airway stem cells
Following influenza virus-induced or bleomycin-induced severe lung injury (Box 1), cells that express canonical basal cell markers such as KRT5 and TRP63 can be detected in alveolar regions (Kanegai et al., 2016; Kumar et al., 2011; Vaughan et al., 2015; Xi et al., 2017; Zuo et al., 2015). Lineage tracing has shown that these basal cell-like cells in alveoli are lineage-labeled by SOX2 and TRP63, but not KRT5; suggesting that KRT5 expression in these cells is somehow triggered specifically by injury (Ray et al., 2016; Yang et al., 2018). This population has been named lineage-negative epithelial progenitors (LNEPs) (Vaughan et al., 2015; Zuo et al., 2015), sometimes also referred to as distal airway stem cells (DASCs) (Kumar et al., 2011).

Morphologically, it is notable that these cells can become flattened; this flattening allows them to cover the basement membrane in extremely damaged alveolar regions. These appearance and expansion phenotypes for these cells have not been obvious when the lungs are infected with a less virulent influenza strain. In addition, these cells have limited potential to regenerate AT1 cells and AT2 cells: up to 200 days post-infection, these cells remained in ‘airway-like cystic structures’, but do not form functional alveolar structures (Kanegai et al., 2016).

Several signaling pathways can regulate the differentiation of these cells into alveolar epithelial cells (Ray et al., 2016; Vaughan et al., 2015; Xi et al., 2017). For example, NOTCH activation is required for the expression of TRP63 and KRT5 in these cells (Ray et al., 2016), and inhibition of NOTCH signaling by knocking down the NOTCH intracellular domain causes some of these cells to transform into AT2 cells (Vaughan et al., 2015). Furthermore, deleting hypoxia inducible factor 1 alpha (Hif1α) or promoting the sustained activation of WNT signaling using β-catenin gain-of-function (β-catenin(loxPlox)) mice in these cells also induces their differentiation into alveolar epithelial cells (Xi et al., 2017).

Upk3a* variant club cells
A subset of club cells expressing uroplakin 3A (Upk3a) is positioned near neuroendocrine bodies (NEBs) (Fig. 1) and the BADJs. In addition to their capacity to give rise to secretory and ciliated cells in a naphthalene-induced lung injury model (Box 1), these cells have also been shown to function in generating AT2 cells and AT1 cells in a bleomycin-induced lung injury model (Guha et al., 2017).

H2-K1high club cells
Through single cell transcriptomic profiling of the distal lung airway epithelium, one recent study has revealed the presence of club cell-like progenitor cells that express the canonical club cell marker SCGB1A1 (Kathiriya et al., 2020). These cells can proliferate to regenerate AT2 cells and AT1 cells after bleomycin-induced lung injury (Box 1) (Kathiriya et al., 2020). It is also notable that these cells express high levels of major histocompatibility complex (MHC) class I and class II genes, including H2-K1, and account for 5% of all Scgb1a1-CreER-lineage-labeled cells (Kathiriya et al., 2020). As H2-K1high progenitor cells can be expanded in vitro, transplanted into bleomycin-injured mouse lungs and successfully differentiate into both AT2 cells and AT1 cells, these cells may enable development of novel therapeutic strategies. Prerequisites for further investigation of the functions of these cells during post-injury alveolar repair will be the identification of specific markers for these cells and the development of new lineage-tracing mouse tools.

Many unanswered questions remain about these various rare stem/progenitor cell populations. First, investigations to characterize any overlap in the cell identities among these cell populations will strengthen our basic definitional understanding of the nature of these cells by integrating multiple lines of evidence from distinct experimental contexts. Second, it will be very interesting to elucidate the injury response signaling processes that specifically activate these individual stem cell populations. It could be mechanistically informative to determine how the distinct physical stresses encountered during normal lung function versus extremes from distinct injury types may interact with molecular repair signaling components resident within these cells to initiate AT1-related regenerative repair programs.

In addition, a small subgroup of AT1 cells can dedifferentiate into AT2 cells and thus participate in alveolar repair (Jain et al., 2015; Wang et al., 2018). Given the significant shape changes from AT1 cells to AT2 cells, in-depth characterization of these AT1 cells will guide future understanding of the mechanism(s) underlying AT2 cell differentiation. Further delineation of any differences in alveolar regeneration processes involving such cell populations – or perhaps discreet functions compared with AT2 cells alone – will provide insights into the function of physiological and pathological repair responses in different injuries.

Conclusions and future directions
We have gained significant knowledge of the mechanisms that regulate lung homeostatic maintenance and regeneration. Various lung stem cell populations and their niche cells have been identified, and many studies have characterized the regulated interactions that occur between stem cells and their niche cells. Together, these studies are collectively aiming to lay a solid mechanistic foundation for understanding lung regenerative processes to facilitate development of therapies to treat lung injuries.

It is very clear that the spatial and temporal relationships between stem cells and their niches, such as mechanical force, are complex and dynamic. scRNA-seq technology has opened a new door in this field and has enabled discoveries about previously unknown cellular players that function in lung regeneration. Such work has clearly deepened our understanding of the regulation of lung stem cells in the context of their respective niches. However, there are obvious limitations with scRNA-seq that inherently preclude their use for characterizing the in situ functions of stem cells and niche cells; most notably, the requirement for removing the cells from their native context. Thus, in vivo imaging technologies are essential complementary tools for monitoring the dynamic behaviors of stem cells in their three-dimensional niche. In vivo imaging allows us to gain cellular- and even tissue-level information that certainly impacts how alveolar stem cells maintain their stem cell identity or differentiate into other cells. Using an integrated combination of in vivo live imaging, new mouse lineage tracing tools and computational modeling that simulate complex cellular processes, we will be able to tackle questions at the interface of cell and developmental biology. For example: to elucidate how lung stem cells and their niche cells are dynamically coordinated when responding to physiological and pathological stimuli.

Finally, it is important to acknowledge that many organs, and especially the lung, have unique mechanical properties and are exposed to constant mechanical forces. Thus, any study seeking a comprehensive understanding must carefully consider the coordinated influences of both mechanical forces and molecular growth factors when investigating the mechanisms that control the development, dysregulation and regeneration of such organs. Live-imaging techniques are now enabling life scientists to properly...
consider questions about the interface of stem cell biology and biophysics in a rigorous manner.

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