Developmental mechanisms and adult stem cells for therapeutic lung regeneration

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Abstract
Chronic degenerative lung diseases are essentially untreatable pathological conditions. By contrast, the healthy lung has numerous mechanisms that allow for rapid repair and restoration of function following minor acute injuries. We discuss the normal endogenous processes of lung development, homeostatic maintenance and repair and consider the research strategies required for the development of methods for human therapeutic lung regeneration.

Introduction
One definition of regeneration is the ability of an organ to regrow a fully functional replacement following catastrophic loss; classical regenerative organs include newt limbs and zebrafish hearts. Lungs are slow-turnover organs that are highly quiescent at steady-state. However, the lung has a tremendous ability to repair epithelial damage following acute injury and contains multiple, highly plastic, stem cell populations. Following partial pneumonectomy (removal of one entire lung lobe) in a mouse the remaining lung lobes undergo compensatory growth to restore gas exchange capacity; meeting a functional definition of regeneration (Fehrenbach et al., 2008; Voswinckel et al., 2004; Young et al., 2015). In humans there is clinical evidence for adult lung regrowth following partial pneumonectomy, or severe flu infection (Butler et al., 2012; Toufen et al., 2011). In spite of this intrinsic ability to repair and regenerate, chronic degenerative lung disease in humans is an essentially untreatable condition, significantly affecting length, and quality, of life. The World Health Organisation predicts that Chronic Obstructive Lung Disease (COPD) will become the third leading cause of death worldwide by 2030. The only treatment for many end-stage chronic lung diseases is transplantation and there is an acute shortage of suitable donor lungs.

If the lung has a capacity for repair and regrowth, how is chronic lung disease such a problem? Current hypotheses suggest that COPD, in which alveolar units are often lost, and pulmonary fibrosis, in which alveoli are clogged with scar tissue, are the end result of repeated episodes of injury which have exhausted the lung's endogenous repair capacity; likely coupled to a sensitive genetic background (Gunther et al., 2012; Kheirallah et al., 2016). Therapeutic lung regeneration is therefore a long-term goal of many research programmes. Approaches for therapeutic lung regeneration include growing new lungs in vitro and the addition of exogenous stem cells to damaged lungs (Weiss, 2014). An

alternative approach would be to pharmacologically manipulate surviving healthy cells to restore diseased lungs. Given the intricate and complex structure of the lung the concept of activating endogenous developmental, or repair, mechanisms is very attractive. We discuss our current understanding of the normal developmental/homeostatic and repair/regeneration mechanisms employed by the mammalian lung and consider the research strategies required to allow the manipulation of these processes for therapeutic regeneration.

Lung structure and organisation
The adult lung is composed of branching networks of epithelial tubes and blood vessels which meet at the alveoli. Air enters the nasal passages, passes down the trachea and successively finer branches of the conducting airways (bronchioles) until it reaches the alveolar network where gas exchange occurs (Fig. 1). The major airways are supported by C-shaped cartilage rings and smooth muscle and lined by a pseudostratified columnar epithelium consisting of basal, secretory (predominantly mucous-secreting goblet cells in human, but club cells in mice) and ciliated cells. Rarer chemosensory epithelial cells include neuroendocrine and brush cells. Airway epithelial composition depends on proximal-distal and dorso-ventral position; it is also likely that there is more epithelial heterogeneity than currently appreciated. Distally, the airways open out into the alveoli at the bronchoalveolar duct junctions in the mouse; or via respiratory bronchioles, which can comprise multiple alveolar ducts, in the human. In these regions the epithelium transitions to a more squamous morphology and the alveoli are lined by surfactant-secreting alveolar type 2 (AT2) cells and attenuated alveolar type 1 (AT1) cells which provide a minimal barrier for gas exchange.

The adult lung mesenchyme is much less-well characterized than the epithelium. Cartilage, and airway and vascular smooth muscle, are easily distinguished by morphology and position, but the various fibroblast populations are not. Fibroblasts are found throughout the airways and alveoli and can be loosely categorized based on position (tracheal, bronchiolar, alveolar, vessel-associated). Fibroblasts have been most carefully characterized in the alveolar region where, in mice, they are typically divided broadly into alveolar myofibroblasts (matrix-secreting, alpha smooth muscle actin; αSMA^+) and lipofibroblasts (roles in lipid trafficking and retinoid storage). However, the composition of lung fibroblasts also changes during development and there are clearly additional sub-types that require further characterization (Branchfield et al., 2016a; Endale et al., 2017; Moiseenko et al., 2017; Ntokou et al., 2015; Rock et al., 2011a).

The lung contains two separate branches of the blood circulatory system, plus a lymphatic network. The bronchial branch of the systemic circulation supplies lung cells with oxygen and nutrients and removes waste. Whereas, the pulmonary circulation carries deoxygenated blood to the alveoli for gas exchange. Vascular and lymphatic vessels are supported by a varying number of pericytes, smooth muscle cells and other fibroblasts depending on vessel size (Kool et al., 2014). Lung capillaries are particularly rich in pericytes which share a common basement membrane with endothelial cells and stabilise the capillary beds (Hung et al., 2017). The lung contains two readily-distinguishable types of resident macrophage. Alveolar macrophages are located within the lumen of the alveolus and have organ-specific functions in surfactant catabolism. Whereas, interstitial macrophages are found within the alveolar walls (Gibbings et al., 2017). Innervation from the vagal branch of the peripheral nervous system (PNS) controls respiration. Moreover, local environmental conditions are sensed via chemosensory cells which signal via the PNS to modulate local blood and air flow appropriately (Cutz et al., 2013). The whole lung is surrounded by the mesothelium, a thin layer of squamous epithelium that surrounds internal organs and lines the peritoneal cavity.

Lung development

Lung development is predominantly characterized using the mouse model. The tracheal and lung epithelium are derived from adjacent regions of the foregut endoderm (Domyan et al., 2011; Morrisey and Hogan, 2010; Que et al., 2007). As the trachea elongates it is initially lined by a Tp63$^+$, Sox2$^+$ progenitor population that differentiates into basal, ciliated, club and rare neuroendocrine and brush cells. Mechanisms controlling developmental cell fate allocation are less studied in the developing trachea than bronchioles. However, FGF Receptor (Fgfr) signalling and epigenetic mechanisms control the number of basal cells (Snitow et al., 2015; Volckaert et al., 2013b) and signalling from the underlying cartilage regulates epithelial fate (Hines et al., 2013).

The lung grows by branching morphogenesis. Genetic analysis of branching has revealed an underlying network of reciprocal epithelial-mesenchymal signals; current challenges are to understand network connectivity and how signalling controls specific cell behaviours (Herriges et al., 2015; Lin et al., 2017; Short et al., 2013; Tang et al., 2011). The branching tip epithelium comprises a Sox9$^+$, Id2$^+$ progenitor population which self-renews during embryogenesis and produces all lung epithelial lineages (Alanis et al., 2014; Rawlins et al., 2009a). This tip progenitor population is not maintained postnatally. Whilst the bronchiolar tree is being laid down (~E11-15 in mice) cells that leave the Sox9$^+$ tip progenitor compartment become Sox2$^+$ airway progenitors and subsequently differentiate into ciliated, club and neuroendocrine cells in a Notch-dependent manner (Morimoto et al., 2010; Morimoto et al., 2012). Active cell migration can occur within the developing airways; newly-specified neuroendocrine cells migrate to form clusters at airway branch points (Kuo and Krasnow, 2015; Noguchi et al., 2015). From ~E16-18 the tip progenitors produce Sox2$^-$ alveolar-fated descendants. The signals that induce bronchiolar to alveolar fate-switching in tip epithelium are not completely defined, but include Glucocorticoid and Stat3 (Alanis et al., 2014; Laresgoiti et al., 2016). Alveolar progenitors initially co-express AT1 and AT2 markers (bipotent progenitors) and are hypothesised to undergo further differentiation by down-regulating markers of one lineage whilst up-regulating the other (Desai et al., 2014; Treutlein et al., 2014) in a Notch-independent process (Tsao et al., 2016).

Following AT1 and AT2 cell differentiation, alveolar saccules are subdivided by secondary septation and the microvasculature is remodelled (alveolarization). This increases the gas exchange surface area and decreases diffusion distance (Schittny, 2017). Secondary septa formation requires PDGFA signalling to induce elastin secretion by the alveolar myofibroblasts (Bostrom et al., 1996; Lindahl et al., 1997; Wendel et al., 2000), but detailed cellular and molecular mechanisms await characterization. Notch2 activity in differentiated AT2 cells induces PDGFA thus signalling to local alveolar myofibroblasts (Tsao et al., 2016). Although, given that PDGFRα expression is widespread in the lung mesenchyme at this developmental stage, there is clearly more specificity to be discovered (Branchfield et al., 2016a; Ntokou et al., 2015). Interestingly defective Notch signalling has been implicated as a pathogenesis mechanism in the development of the human condition bronchopulmonary dysplasia (BPD), which is likely to be a disease of defective neonatal lung development (Sucre et al., 2016). During mouse alveolarization, the AT1 cells elongate, flatten and remodel their junctions such that one AT1 cell comes to line multiple alveoli in a TGF-β-dependent mechanism (Wang et al., 2016; Yang et al., 2016). Moreover, a sub-set of mouse AT2 cells become Wnt responsive at around postnatal day 4 and likely function as stem cells for alveolar growth having greater self-renewal and clonal output than non-Wnt responsive AT2s (Frank et al., 2016). Pathological Wnt signalling has also been associated with human

BPD development (Sucre et al., 2017). Normal human postnatal lung samples are very difficult to come by, but the continued development of human embryonic lung organoid cultures should ultimately allow the function of these pathways to be tested during normal human alveolar development (Nikolic et al., 2017; Wilkinson et al., 2016).

The mesenchymal progenitors which surround the initial lung buds are mostly Tbx4^{+}, but are highly heterogeneous in their clonal output (Kumar et al., 2014; Zhang et al., 2013). Within this Tbx4^{+} mesenchyme, lineage-labelling of Gli1^{+} (Hh-responsive) and Axin2^{+} (Wnt-responsive) mesenchymal progenitors showed that both contribute significantly to airway and vascular αSMA[+] smooth muscle and alveolar αSMA[+] myofibroblasts (Li et al., 2015; Moiseenko et al., 2017). However, many αSMA^{-} mesenchymal cells were also labelled in these experiments and clonal analysis to determine the heterogeneity of these progenitor populations is required. Lineage-tracing has also shown that FGF10^{+} mesenchymal cells can generate at least four different mature mesenchymal lineages, although fascinatingly in these studies many of the lineage-labelled cells could not be identified using known markers suggesting they comprise unknown cell types (El Agha et al., 2014). A Foxd1^{+} mesenchymal progenitor present from ~E11-13 has been shown to give rise to at least 15% of lung pericytes, but also to arteriole smooth muscle and other mesenchymal lineages (Hung et al., 2013). It is likely that other mesenchymal progenitor populations exist. The composition of the lung mesenchyme changes extensively during normal development, therefore the time of analysis will affect the out-come of all of these lineage studies (Endale et al., 2017). FGF, Parathyroid hormone receptor (PTHR), PDGF, Thy-1, TGF-β and Wnt signalling contribute to differentiation of the alveolar fibroblast sub-types (Al Alam et al., 2015; Cohen et al., 2009; Li et al., 2015; Nicola et al., 2009; Rubin et al., 2004; Varisco et al., 2012), but cell type-specific knockouts for mechanistic analysis are currently lacking.

There are two known additional sources of lung mesenchymal progenitors: the mesothelium and a cardiopulmonary mesoderm progenitor (CPP) population located in the developing heart. Mesothelial contribution to smooth muscle is likely to be relatively minor, although it may be more significant for other fibroblast populations (Moiseenko et al., 2017; Que et al., 2008; von Gise et al., 2016). Exit from the mesothelium is regulated by Hh signalling and the epigenetic regulator Ezh2 (Dixit et al., 2013; Snitow et al., 2016). Clonal analysis is still required to determine if a single multipotent mesothelial progenitor enters the lung, or if there is heterogeneity. By contrast, the CPP population generates a significant proportion of cardiomyocytes, lung vascular and airway smooth muscle, proximal lung vascular endothelium and pericytes (Peng et al., 2013). Clonal lineage analysis showed that the CPP includes a common progenitor of these four distinct lung mesoderm lineages. It is not yet clear when CPP progenitors enter the lung; they may already be contained within the Tbx4^{+} mesenchyme as lung buds initiate. Interestingly, the alveolar capillary endothelium is not derived from the CPP and likely originates from separate endothelial precursors present in the lung bud at E8.5 (Parera et al., 2005; Schwarz et al., 2009).

Macrophages are known to play important roles in developing tissues, including in branching of the mammary gland, kidney and pancreas (Wynn et al., 2013). Lung macrophages (alveolar and interstitial in the adult) are known to enter the lung in at least three waves, (Mass et al., 2016; Schulz et al., 2012; Tan and Krasnow, 2016). The developmental importance of lung macrophages was confirmed by work in which Roundabout signalling was disrupted in developing neuroendocrine cells leading to excessive macrophage recruitment and, consequently, alveolar simplification (Branchfield et al., 2016b). However, normal macrophage functions in lung development are not yet defined.

The peripheral innervation of the lung is wholly derived from the neural crest (Langsdorf et al., 2011). Interactions between epithelium and nerves are crucial for salivary gland embryonic branching and adult regeneration (Knox et al., 2013; Knox et al., 2010). Similarly, lung branching morphogenesis relies on parasympathetic innervation, but not neurotransmission (Bower et al., 2014). Moreover, neurological control of respiration is a highly active area of research (Chang et al., 2015; Nonomura et al., 2017).

Mechanisms of lung homeostasis and repair
In adulthood the lung contains mature epithelial and mesenchymal cell types and the embryonic progenitor populations are no longer maintained. Current evidence suggests that during postnatal growth, adult epithelial patterns of stem cell division are used (Desai et al., 2014; Rawlins et al., 2009b; Rock et al., 2009). However, it is likely that the rates and types of stem cell divisions (self-renewing versus differentiating) differ between postnatal growth and adult maintenance. In the steady-state adult the lung is relatively quiescent with little cell turn-over, particularly when compared with rapidly dividing tissues such as the intestine and skin (Bowden, 1983; Kauffman, 1980). This seems paradoxical when the lung has a tremendous capacity to repair following acute injury. One explanation is that there is no impetus for cell division at steady-state; an alternative hypothesis is that there are signalling mechanisms that actively maintain quiescence. Active maintenance of steady-state quiescence and morphology is an emerging theme from several recent studies and has implications for the initiation of repair/regeneration and the return to steady-state quiescence. The concept of active maintenance of quiescence is particularly exciting for the therapeutic induction of lung regeneration as the reactivation of quiescence mechanisms could provide a method for inhibiting any induced process and preventing cancer development.

During repair many lung cells can acquire plasticity with the severity of the injury apparently dictating which cell responds (Tata and Rajagopal, 2017). Such plasticity provides the lung with flexible, robust, repair mechanisms, but has caused confusion and debate amongst stem cell researchers. Questions that now need to be addressed are: what mechanisms regulate the transition of cells from quiescent to dividing and back again? How do distinct cell types sense different injuries? Which cell populations have the flexibility to de-differentiate and when are these complementary repair mechanisms used?

Trachea and large airways
In the basal cell-containing airways of human and mouse, basal cells function as the stem cells during homeostasis and repair: self-renewing and giving rise to new ciliated, club and neuroendocrine cells (Rock et al., 2009; Teixeira et al., 2013; Watson et al., 2015). Two equally-distributed populations of basal cells have been identified at steady-state, one of which is the long-term self-renewing stem cell. The other, a long-lived precursor that is already committed to differentiation, is morphologically indistinguishable from the stem cell (Mori et al., 2015; Watson et al., 2015). The presence of pre-committed cells in the basal layer may be a feature of homeostasis that has been adapted to facilitate a rapid repair response. Indeed, following chemical injury approximately 50% of remaining basal cells have readily detectable club or ciliated cell markers and rapidly differentiate (Pardo-Saganta et al., 2015a).

Mouse tracheal club cells also self-renew and can generate ciliated cells (Rawlins et al., 2009b). During repair it is likely that basal cells directly produce ciliated cells, but asymmetric division of club cells is the major source of new steady-state ciliated cells

(Pardo-Saganta et al., 2015a; Watson et al., 2015). When club cells survive tracheal injury they increase their proliferation rate and produce greater numbers of new club and ciliated cells (Rawlins et al., 2009b). Club cells also display plasticity and, following genetically-mediated ablation of basal cells, they can de-differentiate to basal cells which are able to function as stem cells for the long-term maintenance of the tracheal epithelium (Tata et al., 2013). However, the same study also demonstrates that where basal cells survive they are the preferred progenitor for repair. Ciliated cells do not divide following injury, or at steady-state (Rawlins and Hogan, 2008; Rawlins et al., 2007).

The adult mouse tracheal epithelium is relatively quiescent at homeostasis with basal stem cells dividing approximately every 11 days and ciliated cells being replaced every 6 months (Rawlins and Hogan, 2008; Watson et al., 2015). Paradoxically maintenance of this quiescent epithelium requires multiple active signalling mechanisms. Steady-state BMP and Fgfr1 signalling inhibit basal cell proliferation (Balasooriya et al., 2016; Tadokoro et al., 2016). Yap, likely in complex with Tp63, maintains steady-state basal cell identity and prevents differentiation (Zhao et al., 2014). Basal cells signal via Notch2 to their club cell daughters to actively inhibit transdifferentiation of club cells into ciliated cells (Lafkas et al., 2015; Pardo-Saganta et al., 2015b). Levels of *Bmp* transcription decrease following tracheal injury to allow proliferation to occur (Tadokoro et al., 2016), but how the activity of other pathways is modified to facilitate repair is not known.

FGFR2 signalling is required for basal cell self-renewal, implying that FGF levels control the number of basal stem cells at steady-state (Balasooriya et al., 2017). EGFR and Wnt signalling have both been linked to basal cell proliferation during repair (Brechbuhl et al., 2011; Brechbuhl et al., 2014; Giangreco et al., 2012; Lu et al., 2013; Vermeer et al., 2003). Club versus ciliated cell identity is affected by Bmp, Fgfr1, Notch and Wnt signalling (Balasooriya et al., 2016; Brechbuhl et al., 2011; Cibois et al., 2015; Giangreco et al., 2012; Pardo-Saganta et al., 2015a; Rock et al., 2011b). It is not clear how these signalling inputs are integrated. Additionally, the emergence of cells from the basal layer into the columnar epithelium is likely to be a regulated process at steady-state. Integration of a basal cell into the columnar epithelium of *Xenopus* skin requires force generated by actin within the emerging cell (Sedzinski et al., 2016). Nothing is known about the steady-state maintenance, or repair, of the smooth muscle and other tracheal fibroblast lineages although these are assumed to be self-renewing populations.

Small airways
Mouse lungs have many small airways lined predominantly by club, ciliated and neuroendocrine cells; similar regions are extremely rare in human airways (Rock et al., 2010). In these small airways, club cells are stem cells and capable of long-term self-renewal to generate new ciliated cells (Giangreco et al., 2009; Rawlins et al., 2009b). Airway club cells can be specifically depleted by systemic administration of naphthalene which they convert to cytotoxic metabolites via Cytochrome p450 (Cyp2f2). Cyp2f2$^-$ club cells located at airway branch points and adjacent to neuroendocrine bodies survive naphthalene injury and proliferate to repopulate the bronchial epithelium (Giangreco et al., 2009; Giangreco et al., 2002; Reynolds et al., 2000a). Whether these surviving club cells are a distinct stem cell population, and if the neuroendocrine cell provides a specific microenvironment, have been controversial points and require further work (Li and Linnoila, 2012; Reynolds et al., 2000b; Song et al., 2012). However, there is club cell heterogeneity at steady-state and new evidence suggests that Uroplakin3a$^+$ (Upk3a$^+$) club cells, which are somewhat enriched near

neuroendocrine bodies, are more likely than the bulk club cell population to self-renew and produce ciliated cells (Guha et al., 2017; Guha et al., 2014).

The molecular mechanisms that control steady-state club cell turn-over are not well defined: FGF10, HGF, TGF-β, p53 and Yap are all implicated (Lange et al., 2015; McConnell et al., 2016; McQualter et al., 2010; Teisanu et al., 2010). Similar to the larger airways, active signalling mechanisms repress bronchiolar steady-state turnover. In this case, Shh produced by the club cells inhibits proliferation of the underlying mesenchyme, which in turn inhibits epithelial proliferation via unknown signals (Peng et al., 2015). Hh signalling is down-regulated following injury, but returns to normal levels as quiescence is restored. In addition. following small airway injury surviving epithelial cells secrete Wnt7b. This stimulates proliferation and FGF10 expression in underlying airway smooth muscle. In turn, FGF10 from muscle promotes proliferation of surviving epithelial club cells (Volckaert et al., 2013a; Volckaert et al., 2011).

Bronchoalveolar stem cells (BASCs), which co-express markers of club and AT2 cells, were identified at the mouse bronchoalveolar duct junction as the putative cell of origin of adenocarcinomas (Kim et al., 2005). However, these cells have not yet been identified in humans and there is no evidence that this sub-population of cells makes a significant contribution to either airway or alveolar epithelial homeostasis, or repair following mild injury such as hyperoxia (Desai et al., 2014; Rawlins et al., 2009b). The number of BASCs is observed to increase in specific injury conditions, but lineage labelling is still required to definitively determine any BASC functions. Interestingly, the Upka3$^+$ club cells (which are distinct from BASCs) can produce differentiated alveolar epithelial descendants following more severe alveolar injury (Guha et al., 2017).

It is typically assumed that adult airway smooth muscle is a self-renewing population, as it is in the late stages of embryogenesis (Moiseenko et al., 2017). Consistent with this, Lgr6-expressing airway smooth muscle cells have recently been reported to proliferate at steady-state and after genetic depletion (Lee et al., 2017). Very little is known about turn-over, or repair, of other airway mesenchymal lineages (Seyed-Razavi et al., 2013).

Alveoli
In the alveolar region of the lung turn-over is extremely slow at steady-state and it is likely that mechanisms that promote quiescence also exist here (Peng et al., 2015). However, lineage-labelling experiments have shown that AT2 cells both self-renew and produce new AT1 cells in the adult mouse. Moreover, the rate of this expansion is much higher after alveolar injury by bleomycin, hyperoxia, or genetic ablation of AT2 cells (Barkauskas et al., 2013; Desai et al., 2014). It is not yet known if all AT2 cells are stem cells, or if there are sub-fractions that act as preferential stem cells during homeostasis and repair. There is a suggestion that AT1 cells located near arterioles are replaced more rapidly than in other parts of the lung, maybe hinting at a perivascular niche (Desai et al., 2014). However, proper quantitation in wholemount samples is required to confirm this hypothesis. PDGFRα$^+$ lipofibroblasts are sufficient to support clonal expansion of AT2 cells in vitro and were hypothesized to provide a niche (Barkauskas et al., 2013). More recently these alveolar mesenchymal cell populations have been dissected by single-cell sequencing and image analysis further defining a Wnt-responsive PDGFRα$^+$ sub-population as the alveolar epithelial niche cell (Zepp et al., 2017). EGFR, or FGFR, activation is sufficient to induce AT2 proliferation in vitro, or in vivo, respectively (Desai et al., 2014; Yano et al., 2000).

However, in vivo loss of function analysis, preferably analysed at clonal level, is necessary to test their roles in steady-state maintenance and repair.

Little is known about the cellular mechanisms of alveolar mesenchymal homeostasis, although it is typically assumed that the various lineages self-renew. The cell types that make up the vasculature, including endothelium, smooth muscle, and pericytes, are thought to self-renew at steady-state. Lineage-tracing of c-Kit$^+$ alveolar vascular endothelial cells for 3 months supported self-renewal, although quantitative analysis was lacking (Liu et al., 2015). Similarly, small clones of lung pericytes suggestive of self-renewal were observed following short-term (3-4 weeks) steady-state lineage-labelling (Rock et al., 2011a). There is strong evidence that alveolar macrophages are maintained by self-renewal at steady-state and also following ablation (Hashimoto et al., 2013; Murphy et al., 2008). However, parabiosis experiments have distinguished some replacement of interstitial macrophages from progenitors in the circulation (Tan and Krasnow, 2016). In addition to steady-state cellular turnover, the extra-cellular matrix is also renewed and it has been estimated that 5% of total collagen is replaced every week in the normal adult mouse lung (McKleroy et al., 2013).

Recent injury studies have shown that, in addition to AT2 cells, there are multiple progenitors that can be recruited to repair the alveolar epithelium following severe injury (Fig. 2). Intratracheal administration of bleomycin is often used as an alveolar injury. It causes a transient disruption of alveolar structure and fibrosis which is largely resolved over time. Following bleomycin, club cells in the terminal bronchioles generate AT2 cells (Guha et al., 2017; Rock et al., 2011a). The cellular and molecular mechanisms that induce alveolar differentiation of club cells remain largely unknown. Crosstalk between Sca1$^+$ airway epithelial stem cells and endothelial cells via Bmp4-Thrombospondin-1 has been reported to contribute to alveolar differentiation, although in vivo lineage-tracing is still required (Lee et al., 2014). The emergence of clusters of cells expressing Krt5 has been reported in the mouse alveolar epithelium following H1N1 influenza virus infection, and a likely analogous cell population also identified in the lungs of humans with Idiopathic Pulmonary Fibrosis and Scleroderma (Vaughan et al., 2015). This rare population of progenitors, termed lineage-negative epithelial progenitors (LNEP), or distal airway stem cells (DASCs), was suggested to migrate from the airways to the inflamed interstitial space where they proliferate and form pods of densely-packed epithelium in the alveoli (Vaughan et al., 2015; Zuo et al., 2015). However, their specific contribution to alveolar repair was debated. Subsequent work supports the idea that resident Sox2$^+$ airway progenitors (but not club or AT2 cells) generate Krt5$^+$ clusters that move from the airway into the alveoli post-infection (Ray et al., 2016) and that these include a very rare cell population that is p63$^+$ at steady-state (Xi et al., 2017). It is not yet clear how many sub-populations of LNEPs there are. However, new data show that they can either regenerate alveolar epithelium, or form pod-like structures in which the alveoli are not repaired, and that these fate decisions can be regulated by hypoxia and Notch and Wnt signalling (Xi et al., 2017). It is still unclear whether recruitment of these LNEPs is part of the normal damage-repair response, or whether it represents an emergency regenerative response to severe injury. It will be interesting to explore whether AT1 and 2 cells derived from these alternative progenitor pools (Upka3$^+$, or other, club cells; LNEPs, possibly BASCs) after injury have distinct characteristics compared to the resident alveolar cells. Future studies should also interrogate the precise mechanisms inducing cell fate changes, or migration of cells into the alveolar compartment. Is there a requirement for extracellular matrix remodelling for migration to proceed? How do the inflammatory and stromal cells influence the various epithelial progenitors? Amphiregulin (Areg) produced from innate lymphoid cells (ILC) has been reported to promote lung restoration after

influenza infection, although it is unclear whether Areg or ILCs directly impact on stem cell behaviour (Monticelli et al., 2011).

Similar to the epithelium, there is evidence for flexibility in the phenotypes of mesenchymal cells following lung injury. For example, lineage-tracing studies in hypoxia-induced pulmonary hypertension in mice have shown that smooth muscle cells in the remodelled vessels derive from pre-existing smooth muscle by undergoing differentiation, migration, and proliferation (Sheikh et al., 2014). The hypotheses for the cellular origin of activated myofibroblasts in fibrotic lungs post-bleomycin injury, include pericytes/perivascular cells, resident fibroblasts, bone-marrow-derived circulating fibrocytes, or epithelial cells via epithelial-to-mesenchymal transition (EMT) (Hoyles et al., 2011; Kim et al., 2006; Marriott et al., 2014; Phillips et al., 2004). However, due to the lack of cell-specific markers and lineage-tracing these hypotheses have remained controversial. By contrast, lineage-tracing studies have implicated endogenous lung mesenchyme including lipofibroblasts and mesothelium, but not circulating cells, pericytes, AT2 cells, or pre-existing myofibroblasts as the source of activated myofibroblasts following bleomycin injury (El Agha et al., 2017; Kramann et al., 2015; Rock et al., 2011a; von Gise et al., 2016; Xie et al., 2016). However, even the lineage-tracing data are somewhat contradictory and it is possible that the differing claims are due to varying doses of bleomycin and/or differing mouse strains. Alternatively, there could also be more than one source of abnormal fibroblasts, possibly depending on the severity of the injury. Wnt signalling has been reported to have protective or deleterious effects in bleomycin-induced fibrosis depending on the extent of Wnt activity (Henderson et al., 2010; Tanjore et al., 2013). Similarly, circulating monocytes and macrophages can worsen, or ameliorate, bleomycin-models of fibrosis depending on the stage of the process (Gibbons et al., 2011; McCubbrey et al., 2017). Current data also suggest that FGF, TGF-β and PTHrP signalling contribute to conversion of fibroblast sub-types (Hogaboam, 2017; Yan et al., 2014).

Lung regrowth and regeneration
There is increasing evidence for remodelling and growth of the adult lung after partial pneumonectomy (PNX) (Butler et al., 2012; Hsia et al., 1994; Thane et al., 2014). Following PNX the remaining lobes expand; there is alveolar growth and remodelling and progressive increase in gas exchange function. This size increase is particularly noticeable in the accessory lobe (Voswinckel et al., 2004). This compensatory growth requires coordinated proliferation and rearrangement of numerous epithelial and stromal cell types. Mechanical strain has been proposed to trigger the regenerative process, although it is unclear how the quiescence mechanisms are overcome. A recent report showed that increased external mechanical tension post-PNX activates Yap in AT2 cells and promotes their proliferation (Liu et al., 2016). By contrast, other studies have suggested that activation of endothelial cells is required for post-PNX expansion of BASCs and/or AT2 cells (Ding et al., 2011; Rafii et al., 2015). Interestingly, lineage-tracing has suggested that the normally terminally-differentiated AT1 cells may have the flexibility to proliferate and generate AT2 cells during alveolar regrowth following PNX (Jain et al., 2015). It is thus likely that there are multiple mechanisms which initiate epithelial proliferation post-PNX.

Local proliferation of pre-existing macrophages, and recruitment of circulating macrophages to the regenerating lung, have been reported following PNX (Chamoto et al., 2012; Chamoto et al., 2013). A more recent study has shown that lung macrophages originating from the bone marrow modulate both the proliferation and lineage differentiation of AT2 cells after PNX (Lechner et al, 2017). This paper provides the first evidence of direct effects of immune

cells on the regenerative capacity of epithelial stem cells in adult lungs. However, the precise mechanisms underlying control of macrophage identity, and macrophage to epithelial signalling, remain to be determined. Dynamic expression levels of PDGFRα and αSMA have been reported in mesenchymal cells during alveolar regrowth following PNX (Chen et al., 2012). Moreover, it is likely that this plasticity in resident fibroblast populations facilitates lung regeneration (Green et al., 2016). However, the signals that regulate plasticity mechanisms in all cell compartments are yet to be determined. Multiple lineage-labelling approaches, coupled with single-cell analyses, should be powerful methods to investigate this.

How do we achieve therapeutic lung regeneration?
The endogenous mechanisms of lung development and regeneration are increasingly well-characterized. Although recent surprises, such as the multiple active signalling mechanisms required to maintain homeostasis, show us how much is still to be learned. In spite of its intrinsic ability to repair, the human lung is subject to degenerative disease in which the homeostatic and reparative mechanisms discussed are not adequate to maintain its structure. Such conditions are likely to be a result of repeated external insults on a sensitive genetic background. If our fundamental knowledge is to be translated into results for such patients we need to develop strategies to halt the progression of degenerative lung disease, or even to therapeutically regenerate the lung. There are three main therapeutic lung regeneration strategies that being actively pursued (Fig. 3). Firstly, tissue engineering new organs in vitro (Calle et al., 2014).

Secondly, transplantation of healthy stem or progenitor cells into an injured lung. Lung cell transplantation has been achieved in mice following severe injury by influenza infection, or by naphthalene injury followed by systemic irradiation (Rosen et al., 2015; Vaughan et al., 2015; Zuo et al., 2015). Or, more recently, following a low dose of bleomycin (Nikolic et al., 2017). These techniques are in the early stages of development and it is not clear if the cell transplant results in improved lung function, or for how long grafted cells can be maintained. For a cell transplantation strategy to be employed therapeutically in humans there are a number of questions to be answered: will it be possible to graft the cells without acutely injuring the endogenous lungs? Will the underlying disease mechanisms destroy grafted cells? How many cells would be required for human lung grafts and could cell production be safely scaled up to this level? Obtaining enough cells for human lung grafts may require improvements in the in vitro differentiation of lung cells from pluripotent stem cells (Chen et al., 2017; McCauley et al., 2017).

The third therapeutic strategy is pharmacological manipulation of endogenous pathways that underpin lung development and repair. This is very attractive as drugs could be delivered directly to the lungs to enhance naturally-occurring processes that normally function extremely efficiently for development, homeostatic maintenance and repair of acute injuries. The concern about pharmacological manipulation of repair is that unless proliferation and differentiation are very closely balanced the long-term result will be neoplasia. However, this may be less of a concern if we are able to build on our new understanding of active homeostatic mechanisms to properly understand how steady-state proliferation is normally inhibited. It is possible that ultimately a combination of cell engraftment and pharmacological manipulation will be required to provide a source of new, healthy stem cells that can be induced to undergo normal morphogenetic processes by pharmacological manipulation.

Tremendous progress has been made towards lung regeneration by studying development, homeostasis and acute injury-repair in mice. While continuing to be very powerful for

elucidating fundamental mechanisms, these approaches will need to be supplemented by additional techniques if we are to make real progress towards human lung regeneration. It is becoming apparent that there are many mouse-human differences and that the use of human organoids will be necessary to identify human-specific mechanisms (Nikolic et al., 2017). However, the study of in vitro organoids will not be sufficient to allow us to test therapeutic strategies for human regeneration. We need improved in vivo models that are more reflective of chronic human disease for both exploratory and pre-clinical work. One relatively new model which aims to mimic chronic human disease is repeated bleomycin challenges. This results in impaired proliferation and differentiation of AT2 cells leading to sustained fibrosis (Degryse et al., 2010). Investigation of the injury mechanisms at work in this model has already raised the possibility of targeting endothelial Notch ligand expression to promote repair of chronic lung disease (Cao et al., 2016). It is likely that further models that more specifically mimic particular human conditions will soon be developed. The current ease of genetic manipulation raises the possibility of developing mouse models that incorporate both chronic injury and sensitised genetic backgrounds. Such approaches, coupled to continued progress in elucidating fundamental mechanisms, will bring the possibility of therapeutic human lung regeneration much closer.

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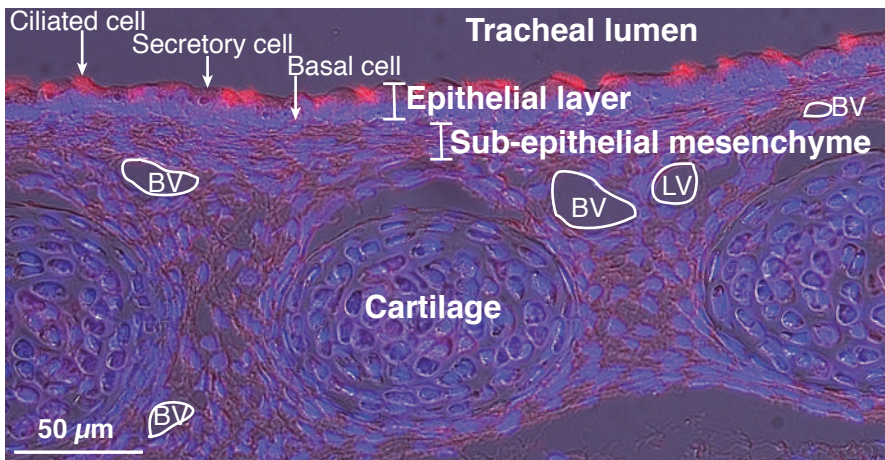
Figure Legends

Figure 1. Mammalian lung structure. A. Section of mouse tracheal epithelium with stained cilia (acetylated tubulin protein; red). The trachea is lined by an epithelial layer containing ciliated, secretory and basal cells. Underlying the epithelium is a layer of sub-epithelial mesenchyme, predominantly consisting of ill-characterized fibroblasts. The trachea is supported by cartilage rings (seen in cross-section) and smooth muscle (not visible; only present on the dorsal side) which are embedded within a more loosely-packed layer of fibroblasts. Blood vessels (BV) and lymphatic vessels (LV) are out-lined in white. B. Human airway sections stained to show ciliated cells (nuclear FOXJ1 protein: red), or basal cells (nuclear TP63 protein; green). C. Section of mouse lung showing a large airway and alveolar region with club cells and alveolar type 2 cells labelled (Fgfr2 protein; red). The airway is lined by an epithelium containing secretory and ciliated cells. Underlying the airway epithelium is a thin layer of mesenchyme, predominantly consisting of smooth muscle cells. The alveoli contain alveolar type 2 cells and type 1 cells, capillaries and multiple types of fibroblast. D-F. Adult human alveolar sections. D. Auto-fluorescence (green and yellow) outlines the alveolar structure with elastin fibres being particularly prominent. E. Alveolar type 2 cells (LPCAT1 protein; green) are dotted throughout the alveolar surface which is supported by various fibroblasts and capillaries. F. The membrane of alveolar type 1 cells (HOPX protein; red) lines the entire alveolar surface. Nuclei of fibroblasts and capillary populations can be seen within the septae.

Figure 2. Cells involved in alveolar development and repair. A. Schematic of the early saccular stages (left panel) and the adult lung (right panel) showing the cell composition of the alveolar compartment. Towards late gestation, the differentiation of distal Sox9$^+$/Id2$^+$ progenitors into alveolar type 2 cells (AT2) and type 1 cells (AT1) is an important step in the process of generating functional alveolar gas exchange units. During alveologenesis the saccules, formed during the saccular stage of lung development, are subdivided by the ingrowth of ridges or crests known as secondary septae and both myofibroblast progenitors and vascular endothelial cells migrate into these crests. As the alveoli mature the septae become thinner, the interstitial myofibroblast disappears and the capillaries remodel to form one unit with the endothelial cells becoming tightly apposed to the AT1 cells, allowing for efficient gas exchange. The process of alveologenesis involves precise temporal and spatial coordination of multiple cell lineages. B. Bleomycin damages multiple alveolar cell types resulting in the exposure of denuded basal lamina and matrix (dashed lines) and influx of immune cells (left panel). Various mesenchymal cells expand and give rise to myofibroblasts, although the cell origin of myofibroblasts is not fully defined (right panel). Both AT2 cells and club cells in the terminal bronchioles (including BASCs) proliferate and generate the majority of AT2 and AT1 cells in the fibrotic regions. Krt5$^+$/p63$^+$ pod cells are increased in the small airway and alveoli following bleomycin exposure, suggesting an alternative source of reparative AT2 cells. Surrounding vascular endothelial cells promote AT2 cell expansion and promote the fibrotic process upon bleomycin damage. In mouse models where a single dose of bleomycin is used the fibrosis usually resolves over time. C. Following the lung resection, the remaining lobes expand asymmetrically, associated with alveolar tissue regrowth, remodeling, and progressive functional compensation (left panel). In experimental pneumonectomy (PNX), the question of whether compensatory lung growth and consequent increases in lung volume are primarily due to the formation of new alveoli (right upper panel) or the expansion of pre-existing alveoli (right lower panel) is still controversial. Recent notable studies suggest an equal contribution of both phenomena to compensatory lung growth (Fehrenbach et al., 2008). This compensatory growth requires the coordinated
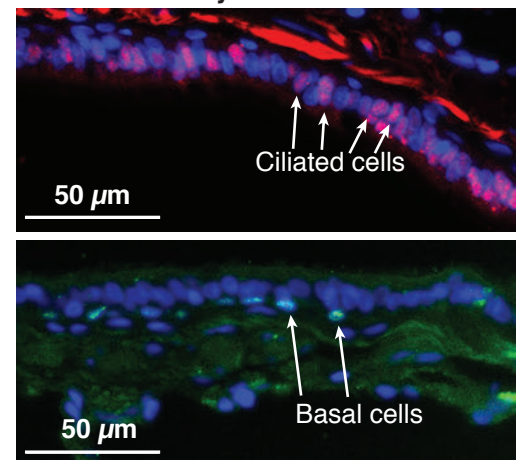
proliferation and rearrangement of numerous epithelial (AT2 cells, AT1 cells, and BASCs) and stromal cell types (fibroblasts, endothelial cells, and immune cells).

Figure 3. Mouse and human lung regeneration. Acute mouse lung injury is a research tool that has been successfully used to identify the multiple complementary cellular and molecular mechanisms the lung has at its disposal to effect efficient and rapid repair. By contrast, the human lung is subject to degenerative disease in which the homeostatic and reparative mechanisms discussed are not adequate to maintain its structure. Such conditions are likely to be a result of repeated external insults on a sensitive genetic background. On-going research should ultimately lead to the development of techniques for therapeutic human lung regeneration to either restore lung function, or halt the degenerative disease process. However, new research tools are required to progress rapidly to human lung regeneration. These include human cell-based organoids for the rapid elucidation of human molecular mechanisms in vitro and improved animal models that more closely mimic human degenerative disease.
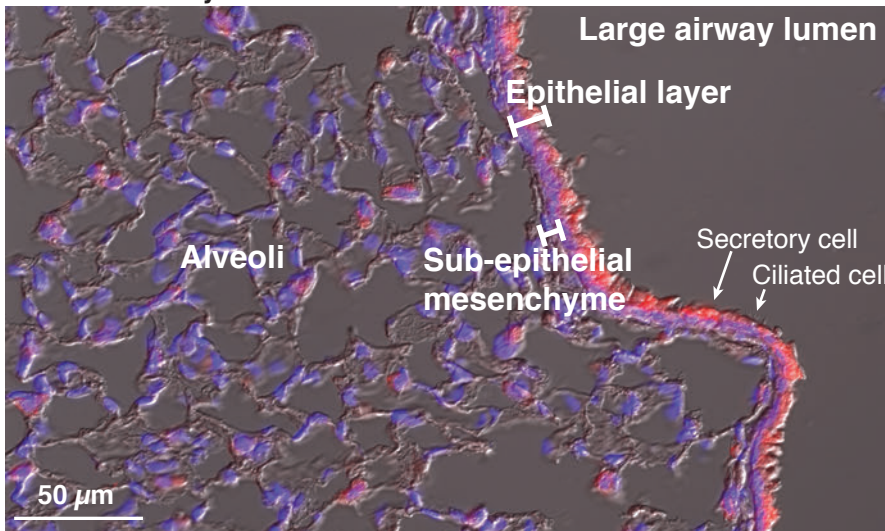
Lee and Rawlins Figure 1

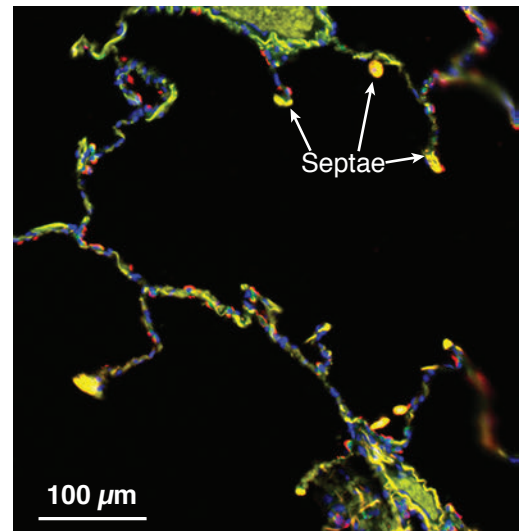**A. Mouse tracheal section.**

Ciliated cell
Secretory cell
Basal cell
Tracheal lumen
Epithelial layer
Sub-epithelial mesenchyme
BV
BV
LV
BV
Cartilage
50 μm
BV

B. Human airway sections.

Ciliated cells
50 μm

Basal cells
50 μm

C. Mouse airway and alveolar section.

Large airway lumen
Epithelial layer
Alveoli
Sub-epithelial mesenchyme
Secretory cell
Ciliated cell
50 μm

D. Human alveolar section.

Septae
100 μm

E. Human alveolar section.

Alveolar Type 2
Fibroblasts/capillaries
50 μm

F. Human alveolar section.

Fibroblasts/capillaries
Alveolar Type 1
50 μm
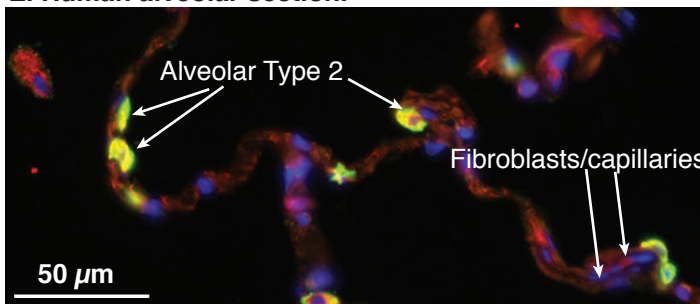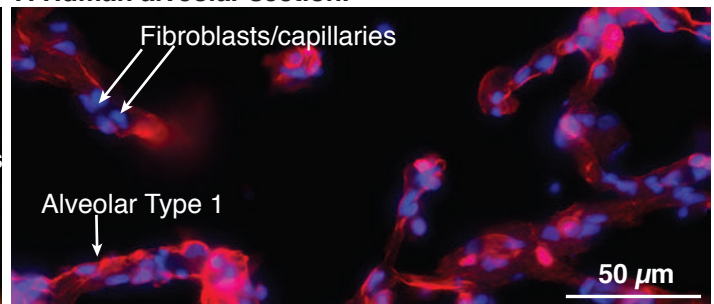
A. Development
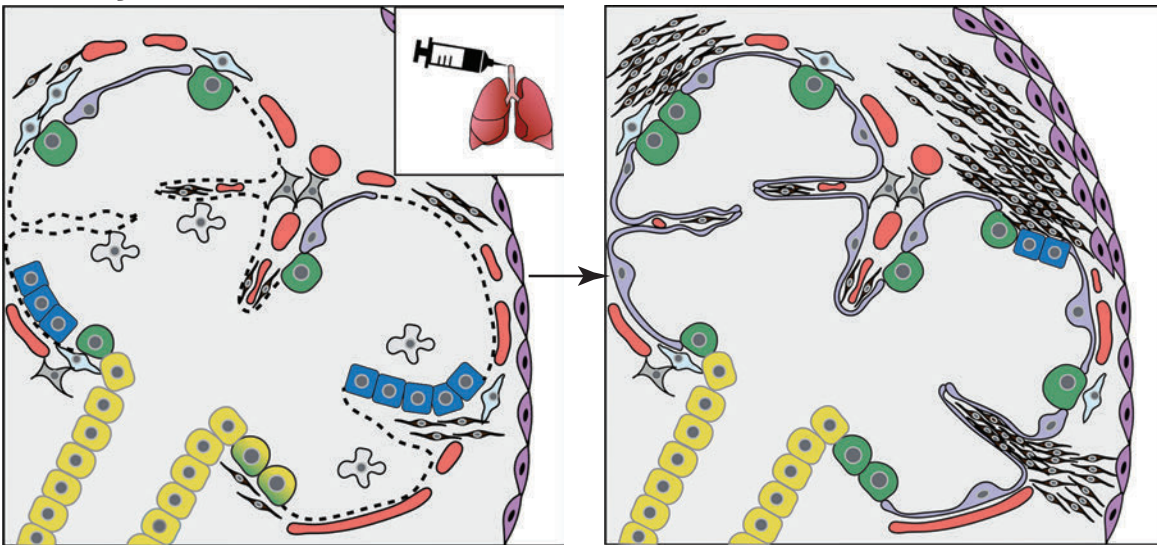Early saccular stage
Adult
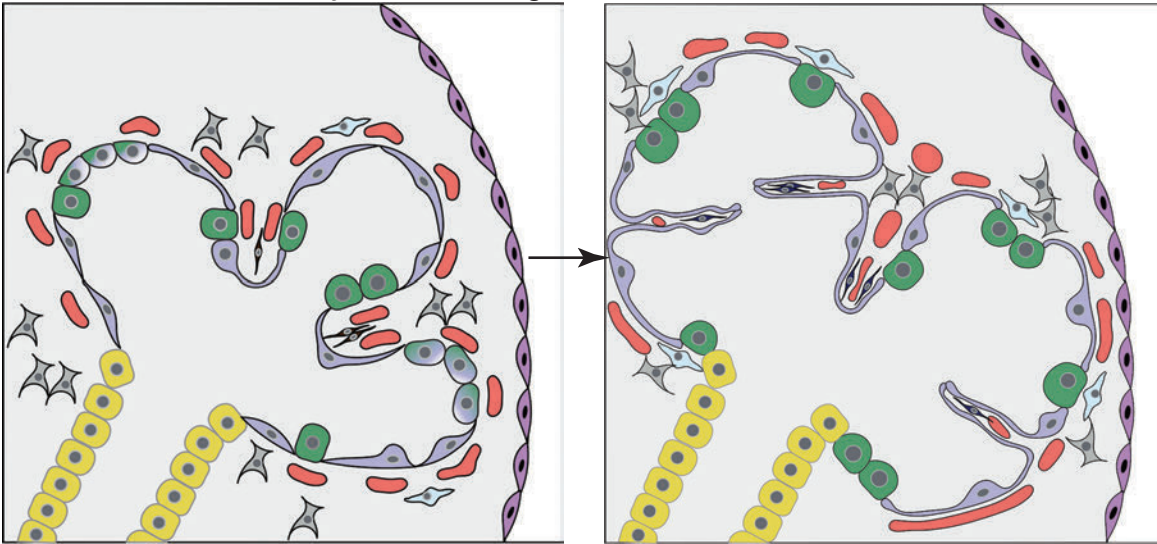
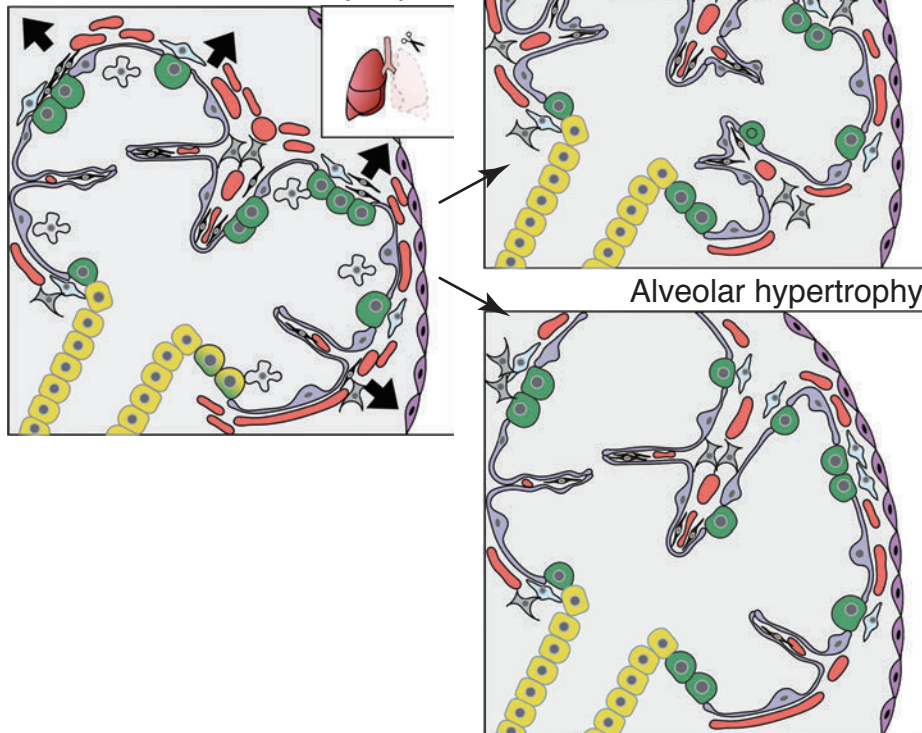**B. Bleomycin**
Early repair
Fibrosis

C. Pneumonectomy
Early repair
New septae
Alveolar hypertrophy

Differentiating type 1 and 2 cells

Alveolar type 1   Alveolar type 2

Bronchiolar cell   BASC

Fibroblast   Myofibroblast

Lipofibroblast   Capillary

Immune cell   Mesothelium

Excessive stretch   Krt5^{+} pod cells

Mouse

Partial Pneumonectomy
(or other acute injury)

Endogenous regeneration
(or repair)

Human

Unknown repeated insult(s)
and/or genetic sensitivity
cause degeneration

Treatment: pharmacological manipulation
of developmental or repair mechanisms;
providing exogenous cells

Aim: therapeutic regeneration
(or slowing of disease progression)