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REVIEW

Plasticity in the lung: making and breaking cell identity

Purushothama Rao Tata[1,2,3,*] and Jayaraj Rajagopal[1,2,3,4,5,‡]

ABSTRACT

In contrast to a prior emphasis on the finality of cell fate decisions in developmental systems, cellular plasticity is now emerging as a general theme in the biology of multiple adult organ systems. In the lung, lineage tracing has been used to identify distinct epithelial stem and progenitor cell populations. These cells, together with their differentiated progeny, maintain a stable identity during steady state conditions, but can display remarkable lineage plasticity following injury. This Review summarizes our current understanding of the different cell lineages of the adult mammalian lung and their responses to injury. In the lung, which is constantly exposed to infection and aerosolized toxins, epithelial plasticity might be more of a rule than an exception, and it is likely that different injuries elicit different facultative responses.

KEY WORDS: Cellular heterogeneity, Cellular plasticity, Lineage tracing, Lung injury repair, Lung regeneration, Lung stem cells

Introduction

The adult mammalian lung is organized into two major compartments: the airways, which function to conduct gases, and the alveoli where gas exchange occurs. The lung is thought to comprise as many as 40 different cell types (Franks et al., 2008), although an even greater diversity is very likely to exist. The lung arises in the embryonic foregut; the organ's epithelium is sourced from the endoderm, whereas the mesenchyme is of mesodermal origin. This Review focuses on the epithelial compartment of the murine lung because it is the epithelial cell lineages that have been the most well, albeit preliminarily, defined. Furthermore, the epithelial responses to injury have been more closely scrutinized than those of the other compartments of the lung, such as the endothelial, mesenchymal and neural compartments. It is important to note that the murine lineage-tracing studies described herein may indeed reflect many aspects of human lung biology, but it is also very likely that the human lung epithelium has many unique properties. This underscores the need to be conservative in drawing conclusions about human lung lineages based upon lineage-tracing studies performed in mice. Indeed, suggestions concerning the behavior of human lung epithelial lineages *in vivo* are currently largely based on inferences from a distantly related organism or based upon the behavior of human cells *in vitro*. As *in vitro* environments are known to elicit cell plasticity, the elucidation of the behavior of human lung cell lineages will require the application of new techniques, such as mitochondrial mutation tracing or computational single cell lineage reconstruction (Teixeira et al., 2013; Treutlein et al., 2014).

The airway epithelium serves as the luminal barrier of the tubes that conduct gases to the alveoli. Its functions include sensing the environment, secretion, regeneration, repelling infection, processing toxins and removing debris. Secretory cells produce mucins and antimicrobial peptides and metabolize toxins, whereas ciliated cells use their cilia to propel debris out of the lung (Jeffery and Li, 1997). More proximal regions of the murine airway epithelium possess basal cells, which act as epithelial stem/progenitor cells to replenish lost secretory and ciliated cells. Neuroendocrine cells are thought to be involved in sensing activities, and they communicate with the immune system and the nervous system. The alveolar epithelium, on the other hand, contains thin type 1 cells that permit gas exchange, as well as type 2 cells that produce the surfactant necessary to prevent alveolar collapse and that subtend an alveolar progenitor cell function. In addition to the roles described above, these major epithelial cell types are likely to possess other functions at steady state and after injury. Indeed, less frequent cell types, such as M cells and brush cells are already known to exist, and even their physiological functions are still being interrogated (Branchfield et al., 2016; Krasteva et al., 2012; Reid et al., 2005; Song et al., 2012; Teitelbaum et al., 1999). In some of the aforementioned functional cell types, such as secretory cells or type 2 cells, subsets of cells are thought to possess differing progenitor cell activities even under steady state conditions (Barkauskas et al., 2013; Guha et al., 2014; Reynolds et al., 2002) and much more is likely to be learned about this in the coming decade.

The steady state lung is viewed as a low turnover tissue that possesses quiescent stem/progenitor cells. These cells possess enormous reparative potential, which is unleashed following injury. However, recent studies have pointed to alternative facultative sources of cells that participate in repairing the damaged lung (Herriges and Morrisey, 2014; Hogan et al., 2014; Kotton and Morrisey, 2014). In this Review, we discuss our current and incomplete understanding of the diversity of epithelial stem and progenitor cells in the lung, as well as the surprising cellular plasticity of certain differentiated cells. Herein, we use the term plasticity to refer to the ability of cells to undergo lineage conversions not characteristic of steady state tissue maintenance. For example, a mature 'terminally differentiated' cell might de-differentiate into a stem cell following injury. Alternatively, one differentiated mature cell might transdifferentiate into another differentiated cell of a distinct lineage following injury. We further discuss some of the factors that determine cellular plasticity in the lung, such as maturation state and neighboring cell-to-cell interactions. Reflective of the field, the majority of the findings discussed in this Review draw from studies on the murine lung. Where possible, we attempt to relate these findings to the little that is known about the human lung. In the main, however, save pointing

[1]Center for Regenerative Medicine, Massachusetts General Hospital, 185 Cambridge Street, Boston, MA 02114, USA. [2]Harvard Stem Cell Institute, Cambridge, MA 02138, USA. [3]Departments of Internal Medicine, Division of Pulmonary and Critical Care Medicine, Massachusetts General Hospital, Boston, MA 02114, USA. [4]Massachusetts General Hospital for Children, Pediatric Pulmonary Medicine, Boston, MA 02114, USA. [5]Division of Otology and Laryngology, Massachusetts Eye and Ear, Boston, MA 02114, USA.
*Present address: Department of Cell Biology, Duke University Medical School, Durham, NC 27710, USA.

‡Author for correspondence (jrajagopal@mgh.harvard.edu)

P.R.T., 0000-0003-4837-0337; J.R., 0000-0003-3190-8647

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out the apparent differences in the organs of the two species, our understanding of the human lung remains mysterious and much of what can be said is inferential.

Cellular diversity and lineage in the mammalian lung

The developmental origin of the lung epithelium

In mammals, the lung epithelium originates from the anterior endoderm, which also gives rise to the epithelia of other organs including the esophagus, thyroid, pancreas, liver and intestine (Cardoso and Lü, 2006; Herriges and Morrisey, 2014; Okubo and Hogan, 2004; Wells, 2015). The lungs evaginate from the primitive endodermal tube and distinct regions of the adult organ are patterned along the newly forming proximodistal axis of the growing organ, in a process referred to as branching morphogenesis (Alanis et al., 2014; Que et al., 2009). Initially, the trachea and larynx derive from a distinct region of the gut tube known as the laryngotracheal groove, whereas the rest of the lung derives from two small pouches emanating from the distal part of the laryngotracheal groove (Que et al., 2006, 2007). The embryonic distal lung bud or 'tip' epithelial progenitors are derived from these pouches and divide rapidly to form new epithelial cells. These epithelial tips also divide iteratively and sequentially to lay out the primary bronchial airways, then the secondary bronchi, and so on until the terminal bronchi are formed. At this point, the distal tip epithelial cells begin forming alveolar structures. Thus, the adult lung epithelium derives from a small number of founding endoderm cells that ultimately generate the remarkable diversity of epithelial cells that occur at distinct locations along the mature respiratory tree. The adult lung possesses a single large airway known as the trachea, which is derived from the proximal embryonic laryngotracheal groove. The trachea is directly connected to the larynx, which is housed in the neck. In mice, the tracheal tube is lined by a pseudostratified epithelium on its luminal inner surface. This epithelium is, in turn, surrounded and protected by dense connective tissue and discontinuous c-shaped cartilage rings that are sealed by bands of posteriorly located smooth muscle. In the mouse, the pseudostratified epithelium extends to the first and largest division of the airway known as the bronchi (Fig. 1). Here, the epithelium consists of basal cells located directly atop the basement membrane, luminal secretory and ciliated cells, and a smattering of pulmonary neuroendocrine cells (PNECs) (Rock and Hogan, 2011; Rock et al., 2010).

Basal cells

Basal cells are morphologically characterized by their small height relative to adjacent luminal cells, as well as their position hugging the basement membrane (Fig. 1; Fig. 2A) (Evans and Plopper, 1988). At the molecular level, basal cells are characterized by the expression of the N-terminus-truncated isoform of TRP63 (hereafter referred to as p63), cytokeratin 5 (KRT5), nerve growth factor receptor (NGFR) and podoplanin (PDPN) (Hogan et al., 2014; Rock et al., 2009). Early studies using xenografts of purified basal cells suggested that these cells could self-renew and were multipotent, meaning that they could generate not only more basal cells, but also secretory and ciliated cells (Liu et al., 1994; Rock et al., 2010). More recently, single basal cells have been used to grow 3D organoid cultures and air-liquid interface (ALI) cultures (Rock et al., 2009; Tata et al., 2013). Furthermore, the identification of clones of airway epithelial cells containing the same mitochondrial DNA mutation in humans has provided some initial evidence that human basal cells are similarly multipotent *in vivo* (Teixeira et al., 2013). The ability of basal cells to restore normal numbers of relatively rare cell types, in addition to secretory and ciliated cells, has only recently received attention. Of the rare cell types in the airway, neuroendocrine cells have been shown to be the progeny of adult basal cells *in vivo* (Watson et al., 2015).

Different injury models, including those that employ gaseous toxins such as sulphur dioxide and chlorine, as well as those that employ infectious agents such as influenza, have been used to study the regeneration of pseudostratified airway epithelium (Matalon and Maull, 2010; O'Koren et al., 2013). It is interesting to note that the kinetics of cell proliferation and differentiation are almost identical in the different injury models when the basal cells are left intact. During the process of repair, basal cells generate a common intermediate cell that co-expresses basal (KRT5) and luminal (KRT8) cell markers. These cells then differentiate into mature ciliated and secretory cells (Mori et al., 2015; Rock et al., 2011a). Recent studies have also demonstrated that, under steady state conditions, the basal cell population is functionally heterogeneous. Indeed, a small population of basal cells expresses the differentiation marker KRT8 (Watson et al., 2015). Similarly, small basal cell populations express activated Notch2 intracellular domain (Notch2ICD) or c-myb (Myb), early differentiation markers for secretory and ciliated cells, respectively (Pardo-Saganta et al., 2015a). These data suggest that basal cells are not only heterogeneous, but that they might occur in a 'primed' state to facilitate the rapid differentiation of appropriate numbers of secretory and ciliated cells following injury. Whether such a hypothetical primed state is reversible or irreversible remains to be studied, although prior work has suggested that particular subpopulations of basal cells have more limited potential, in the sense that they can only give rise to unipotent or bipotent lineages of epithelial cells *in vitro* (Ghosh et al., 2011; Tata et al., 2013). How these *in vitro* results are mirrored by *in vivo* behavior remains unresolved. Remarkably, the number of basal cells expressing c-myb and Notch2ICD increases immediately following injury, suggesting that injury-induced signals can enhance the early segregation of 'primed' basal cell subpopulations, or that the loss of suppressive signals unleashes an intrinsic tendency towards differentiation of previously 'committed' basal cells (Pardo-Saganta et al., 2015a).

The mechanisms that regulate injury-induced segregation remain obscure. We speculate that there might be epigenetic differences amongst basal cell subpopulations that predict their future behavior, as has been shown in other systems such as the intestine (Kim et al., 2014). It has been recently suggested that dynamic reactive oxygen species levels in basal cells can regulate their symmetric versus asymmetric cell division (Paul et al., 2014). It would be interesting to assess whether and how reactive oxygen species levels regulate injury-associated basal cell heterogeneity (Paul et al., 2014).

In mice, basal cells, at the population level, are primarily present in the trachea and main stem bronchi (Hogan et al., 2014; Rock and Hogan, 2011; Rock et al., 2009). However, in humans, basal cells extend distally into the small bronchioles (Fig. 1). Whether there are molecular or functional differences in the basal cells of the trachea versus the smaller airways has not been extensively studied. Indeed, the murine small airway, which lacks significant numbers of basal cells, and where the majority of disease modeling has occurred, histologically mirrors only a small fraction of the most distal human airway (Fig. 1). Based largely on the presence of basal cells, the murine trachea has been posited as a better model for the study of human airways disease (Hogan et al., 2014); however, it remains unclear how human airway cell diversity corresponds to its murine counterpart. Indeed, different regions of the mouse airway may

Development (2017) 144, 755-766 doi:10.1242/dev.143784

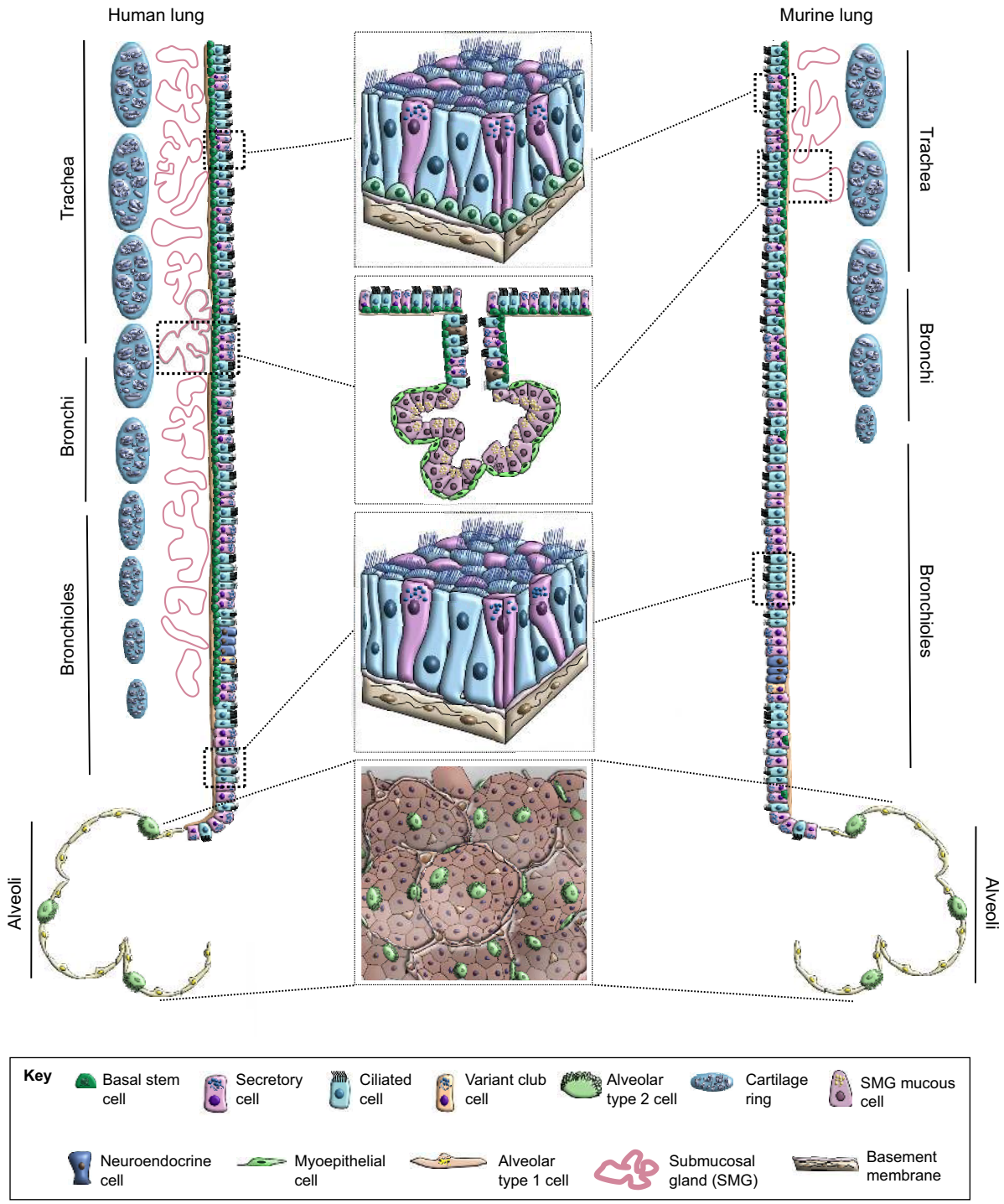

Fig. 1. Anatomical and cellular differences between murine and human lung. The respiratory tree consists of two distinct regions: airways and alveoli. Within airways, distinct compartments contain different cell populations that vary along the proximodistal axis. In human airways, basal cells extend throughout the small airways, whereas in mouse, basal cells extend only up to the mainstem bronchi. In humans, cartilage rings span several generations of airway, whereas in mouse, cartilage rings are present only in the trachea and mainstem bronchi. In humans, submucosal glands are present throughout many small airways, whereas in mice submucosal glands are restricted to the proximal domains of the trachea. The distribution of other various cell types is also indicated in the schematics. Notably, whereas much of the small airway in mouse is composed of a cuboidal epithelium consisting mainly of ciliated and secretory cells, in the human such epithelium is restricted to only the very most distal cells of the bronchioalveolar duct junction.

model different aspects of human cell biology and disease to varying degrees, despite the histological similarity of murine tracheal and human airway epithelium. Recent studies in mice have also demonstrated the presence of small numbers of basal-like progenitor cells in the distal airways, even at steady state (Kumar et al., 2011; Vaughan et al., 2015). Remarkably, the number of such cells is increased in the small airways and alveoli following severe injury caused by H1N1 influenza injury or bleomycin (Vaughan et al., 2015; Zuo et al., 2015). Finally, it is likely that each of the cell types of the airway epithelium have additional functions beyond that

Development (2017) 144, 755-766 doi:10.1242/dev.143784

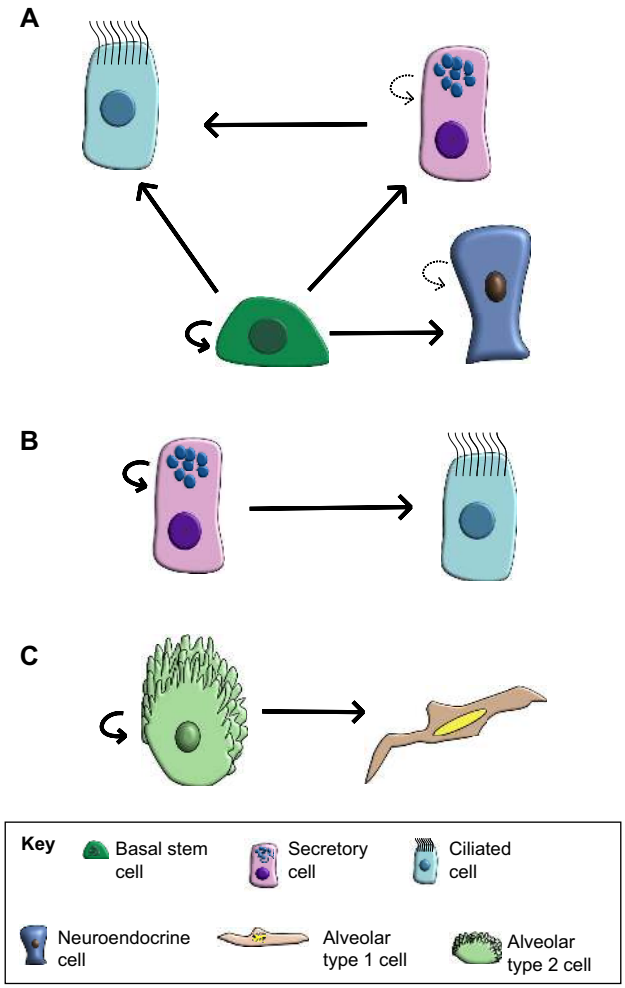

Fig. 2. Lineage hierarchies in lung epithelial tissues. (A-C) Epithelial lineages in the large airways (A), in the small airways (B) and in the alveoli (C). Bold curved arrows indicate high self-renewal potential whereas dotted curved arrows indicate limited self-renewal potential.

indicated by their description as ciliated, secretory or basal stem cells. For example, in addition to their ability to self-renew and generate other epithelial cells, it has been suggested that basal cells recruit immune cells to the airways by secreting IL33, and hence may subtend an important immunomodulatory function (Byers et al., 2013). Other cell types are undoubtedly involved in further immune-epithelial interactions as discussed below, including phagocytosis (Juncadella et al., 2013).

Secretory cells

Secretory or club cells (formerly known as Clara cells) are characterized by their dome shape and also by the presence of a cytoplasm filled with secretory granules (Hogan et al., 2014; Rawlins et al., 2009a; Stripp, 2008). These cells are present in the murine trachea, bronchi and bronchioles, and are scattered throughout much of the human airway epithelium in smaller numbers. In the murine pseudostratified epithelium, lineage-tracing studies using *Scgb1a1* promoter-driven *CreER* transgenic mice have shown that, at a population level, murine secretory cells can self-renew and that they can differentiate into ciliated cells (Rawlins et al., 2009a) (Fig. 2B). The secretory cell population at large is molecularly characterized by the expression of SCGB1A1. However, recent studies indicate that these cells are a highly heterogeneous population. In the large airway, using SSEA1 (FUT4), a surface antigen that marks some secretory cells, in combination with a transgenic green fluorescent protein reporter driven by the *Atp6v1b1* promoter (hereafter referred to as B1-eGFP), which marks cells with mature functional secretory vesicles, three subsets of secretory cells can be discriminated based upon their maturity, with $SSEA1^{+}B1\text{-}eGFP^{-}$ cells being the least mature, followed by double-positive cells, and then by the most mature $SSEA1^{-}B1\text{-}eGFP^{+}$ cells (Tata et al., 2013). Other studies point to a self-renewing population of tracheal secretory cells with a different division pattern compared with putative primed tracheal secretory cells which might be destined to differentiate specifically into ciliated cells (Rawlins et al., 2009a). It is interesting to note that, although progenitor cells are often thought of as 'undifferentiated' cells, secretory cells are functional differentiated cells that also retain a progenitor cell activity (Rawlins et al., 2009a). Whether the subsets of more and less mature tracheal secretory cells also possess differing progenitor properties remains to be clearly defined (Tata et al., 2013; Watson et al., 2015).

In the small airways, two subsets of secretory cells were first identified based on their susceptibility to naphthalene-induced injury. Following naphthalene administration in murine models, the majority of secretory cells are lost. However, a small subset of secretory cells that reside predominantly at the branch points of small airways, adjacent to neuroendocrine bodies, resists injury (Hong et al., 2001). These naphthalene-resistant secretory cells, termed 'variant club cells', then replicate and repopulate the damaged airway epithelium. Secretory cell subsets have also been identified based on the differential expression of secretoglobin family proteins such as SCGB3A1 and SCGB3A2 (Reynolds et al., 2002). More recently, these subsets of secretory cells were prospectively identified based on the presence or absence of expression of Upk3a, a family member of the uroplakin proteins with unknown function in the lung (Fig. 1) (Guha et al., 2012). Secretory cells that express Upk3a are present immediately adjacent to PNECs, and these cells are thought to represent the variant club cells (Guha et al., 2012; Hong et al., 2001) (Fig. 2B). They are characterized by low levels of SCGB1A1 expression, in contrast to their conventional secretory cell counterparts. Lineage-tracing studies have shown that the population of $Scgb1A1^{+}$ cells in the small airways self-renews and generates ciliated cells in the absence of a basal stem cell compartment. In the large airway, basal cells can directly form ciliated cells (Fig. 2A) (Pardo-Saganta et al., 2015a); however, it has been suggested that most ciliated cells in the large airway are a product of secretory cell differentiation under steady state conditions, thus mimicking the pattern seen in the small airways (Rawlins et al., 2009a). Further studies must be performed using single-cell RNA sequencing followed by lineage-tracing analysis to prospectively identify and characterize different subsets of secretory cells along the proximodistal axis.

Ciliated cells

Ciliated cells are also present throughout the large and small airways (Fig. 1). Ciliated cells are characterized by the presence of multicilia on their apical surface, and are molecularly characterized by their expression of the nuclear transcription factor FoxJ1. Lineage-tracing studies combined with bromodeoxyuridine incorporation assays indicate that ciliated cells are post-mitotic terminally differentiated cells (Rawlins and Hogan, 2008). This is consistent with the presence of innumerable centriole-like structures in the basal bodies of cilia, which probably precludes effective cell division (Rawlins and Hogan, 2008; Rawlins et al., 2007). As noted above, in the large airways

Development (2017) 144, 755-766 doi:10.1242/dev.143784

under steady state conditions ciliated cells are largely replenished by secretory cells, but it is likely that there are small numbers of ciliated cells that are also directly produced from basal cells (Pardo-Saganta et al., 2015a). By contrast, following injuries that cause the loss of secretory cells, ciliated cells are produced directly from basal cells in large numbers (Pardo-Saganta et al., 2015a). In the small airways, ciliated cells are produced by secretory cells alone (Rawlins et al., 2009a). Ciliated cell damage in the small airways engenders a replicative response in secretory cell neighbors and subsequent differentiation to restore ciliated cell numbers (Reynolds et al., 2000a). In contrast, surprisingly, ciliated cell ablation in the large airways does not engender increased secretory or basal cell replication or their differentiation into ciliated cells (Pardo-Saganta et al., 2015b). Whether this differing response to ciliated cell loss is due to differences in the ciliated cells themselves or in secretory cells, or due to the presence or absence of basal cells remains unclear. Ciliated cells do vary in morphology, and cilia length decreases along the proximodistal axis of the murine airways (Toskala et al., 2005). Thus, ciliated cell heterogeneity remains an interesting open question, including whether the heterogeneity is based on cell-autonomous factors specific to the ciliated cells themselves, or whether the heterogeneity is due to some local environmental signal.

Neuroendocrine cells

PNECs can be found singly or as organized clusters that are in close contact with nerve fibers and surrounded by 'variant' secretory cells. PNECs are marked by the expression of CGRP (calcitonin gene-related peptide; CALCA), chromogranin A and ASCL1 (achete-scute homolog 1) and are present in mouse in both large and small airways and are enriched at the branch points of airways (Guha et al., 2012; Reynolds et al., 2000b; Song et al., 2012). They derive from the same tip progenitors that produce the other epithelial cell types during branching morphogenesis. Using live imaging and lineage tracing, it has been demonstrated that, during lung organogenesis, individual PNECs migrate towards one another in a process referred to as 'slithering', and then they coalesce as clusters adjacent to airway branch points (Kuo and Krasnow, 2015; Noguchi et al., 2015). PNEC clusters become innervated whereas singlet neuroendocrine cells do not. Lineage-tracing studies have also indicated that PNECs self-renew, but do not contribute to other epithelial cell lineages under steady state conditions (Song et al., 2012). PNECs of the bronchiolar epithelium are also thought to serve as niche cells for naphthalene-resistant variant club cells (Guha et al., 2012; Hong et al., 2001). Several studies have suggested that PNECs perform many other functions including oxygen sensing, mechanotransduction, immune cell recruitment, and chemosensing (Branchfield et al., 2016; Lembrechts et al., 2012; Pan et al., 2002). Some of these functions are presumably mediated in part through their connections with nerve fibers.

Alveolar epithelial type 2 and type 1 cells

During murine embryogenesis, both alveolar and airway epithelial cells derive from $ID2^+$ $Sox9^+$ multipotent distal tip epithelial progenitor cells (Alanis et al., 2014; Perl et al., 2002; Rawlins et al., 2009b; Rockich et al., 2013). Mature alveolar epithelium consists of cuboidal surfactant-producing alveolar type 2 cells and thin gas-exchanging alveolar type 1 cells (Evans et al., 1975). Using elegant high-resolution single-cell imaging methods, type 1 cells in the mouse lung have been shown to form their flat extensions via a non-proliferative two-step process involving a flattening step and then a subsequent folding step (Yang et al., 2016). Recently, single-cell RNA sequencing analysis of lung epithelial cells at embryonic day 18.5 identified a bipotent progenitor cell population that expresses both type 2 and type 1 cell markers (Treutlein et al., 2014). These cells are likely to represent part of the developmental sequence of the aforementioned $ID2^+$ tip progenitor cells at embryonic day 18.5. In the adult, recent lineage-tracing analysis has demonstrated that type 2 cells maintain the homeostatic turnover of type 1 cells and also contribute extensively to the type 1 population following bleomycin-induced lung injury (Barkauskas et al., 2013; Desai et al., 2014; Rock et al., 2011b). Of note, these experiments also demonstrated that type 2 cells not only generate type 1 cells, but also clonally generate more type 2 cells, thus qualifying these cells as stem/progenitor cells of the adult lung alveoli (Barkauskas et al., 2013) (Fig. 2C). How these cells compare with embryonic bipotent alveolar progenitor cells is unclear. In a recent clonal lineage analysis of surviving type 2 cells following extensive ablation, residual lineage-tagged type 2 cells clonally generated large regions of alveolar epithelium that included both type 1 and type 2 cells. Hypothetically, this suggests that there could be a unique population of type 2 cells that participate in alveolar recovery. Alternatively, this potential to restore alveoli may be evoked in 'ordinary' type 2 cells after ablation (Barkauskas et al., 2013). Along these lines, a type 2 cell subset, characterized by Wnt-responsive Axin2 expression, has recently been identified, and these progenitors have a particularly robust capacity to generate alveolar epithelium (Frank et al., 2016). It is likely that further subsets of type 2 epithelial cells will emerge with further scrutiny.

Bronchioalveolar duct junction

The zone of transition from the bronchioles to the alveolar region is referred to as the bronchioalveolar duct junction (BADJ). In the mouse, rare epithelial cells in this region co-express markers of both secretory cells ($SCGB1A1^+$) and type 2 alveolar cells ($Sftpc^+$) and have been referred to as bronchioalveolar stem cells (BASCs) (Kim et al., 2005). BASCs were originally identified based on the observation that cells in the BADJ region proliferate after bleomycin injury (Kim et al., 2005). These cells have been proposed to function as stem cells that contribute to both the airways and the alveoli, although stringent tracing with unique markers, which would confirm their status as bona fide stem cells, has not yet been performed. Furthermore, their function as a dual progenitor *in vivo* has not been reconciled with lineage-tagging data in a hypoxia-induced lung injury model (Rawlins et al., 2009a). The identification of unique markers of BASCs followed by lineage tracing needs to be completed to clarify any possible role of BASCs as stem cells. An alternative possibility is to perform intersectional promoter-based lineage tracing using *Sftpc* and *Scgb1a1* promoters acting in concert to uniquely label and follow BASCs and their progeny. However, a further complexity in this line of inquiry occurs because there are dual SCGB1A1- and Sftpc-positive cells within alveoli that are distant from the BADJ where putative BASCs are thought to reside. For example, occasional type 2 cells throughout mouse alveolar regions have been shown to express both SCGB1A1 and Sftpc (Rawlins et al., 2009a; Rock et al., 2011b). Consequently, *Scgb1a1* lineage tracing does not entirely distinguish airway secretory cells (including putative BASCs and secretory cells) from rare type 2 cells that are also traced using SCGB1A1-driven Cre tagging. Of note, lineage-tracing studies of both embryonic bipotent alveolar progenitors and mature type 2 cells mark $SCGB1A1^+$ SPC^+ double-positive cells located at the BADJ, but these cells proliferated little, if at all (Desai et al., 2014). This stringent analysis argues against a BASC stem cell activity. Lineage tracing at the single-cell level with unique markers or

Development (2017) 144, 755-766 doi:10.1242/dev.143784

combinations of markers and in multiple injury models injuries will be necessary to support the contention that BASCs are stem cells. Currently, the preponderance of the evidence would indicate a minimal role for BASCs as stem cells during normal lung homeostasis and the limited set of injuries that have been stringently analyzed thus far. Intriguingly, and more importantly, the *ex vivo* production of clonal spheres that can produce both alveolar and airway cells does suggest that some cells have the ability to execute both airway and alveolar differentiation *in vitro* (Kim et al., 2005; Lee et al., 2014), which casts a very important light on distal progenitor cell plasticity. As a final note, BASC cells in the human airways have not been clearly identified (Hogan et al., 2014; Kotton and Morrisey, 2014).

Submucosal glands

In the lung, submucosal glands lie beneath the luminal surface epithelium and are embedded within the connective tissue of the cartilaginous airways. Submucosal glands consist of two compartments: ducts and acini. Ducts are tubular tissues that connect acini to the surface epithelium (Liu and Engelhardt, 2008; Lynch and Engelhardt, 2014). The epithelium in the ducts is very similar to the surface epithelium, including the presence of basal, secretory and ciliated cell types. On the other hand, acinar tissues contain two unique cell types: basally located flat myoepithelial cells, which express KRT14, αSMA (ACTA2) and KRT5, and luminal cells, which are characterized by the expression of KRT8 (Fig. 1). Developmentally, the appearance of submucosal glands varies from species to species. For example, in mice, submucosal gland primordium first appears on postnatal day 1, whereas in humans, submucosal gland placodes first appear during the third trimester (Lynch and Engelhardt, 2014). In addition to the differences in their relative developmental timing, the abundance and the location of submucosal glands vary between rodents and other mammals. In mice they are restricted to the proximal trachea (up to cartilage rings 2-3), whereas in humans they extend all the way from the trachea to the lobular small airways (Fig. 1). Interestingly, it has been noted that aged mice develop gland-like structures that span beyond cartilage ring 3 and extend to the mainstem bronchi. These gland-like structures do not possess myoepithelial cells and they are more reminiscent of cysts than conventional glands. These have been termed age-related gland-like structures (ARGLSs) (Wansleeben et al., 2013). However, the mechanisms regulating the development of submucosal glands and the cellular origin of ARGLSs are yet to be determined. It also remains unclear how the cells of the submucosal glands themselves are maintained, both during homeostasis and following injury, as the necessary lineage studies using drivers specific for luminal and basal myoepithelial cells in both duct and acini have not yet been performed under identical circumstances.

Lineage plasticity following injury

De-differentiation of airway cells

Recent findings have suggested that the strict lineage hierarchies that characterize development and homeostatic tissue turnover are not necessarily obeyed following injury (Blanpain and Fuchs, 2014; Tata and Rajagopal, 2016a; Tetteh et al., 2015). Indeed, multiple recent studies in various tissues indicate that cellular plasticity is a common phenomenon encountered in the injury repair process (Rompolas et al., 2013; Tata et al., 2013; Tetteh et al., 2016; van Es et al., 2012; Yanger et al., 2013). In the lung, early studies suggested that sorted airway non-basal epithelial cells generated all the major cell types of the pseudostratified epithelium when they were grafted onto denuded trachea (Liu et al., 1994). This study provided the first suggestion that tracheal non-basal epithelial cells could generate basal cells after injury. Another study using a secretory cell-specific *CreER* driver provided the first line of evidence that luminal secretory cells can generate basal cells after injury (Rawlins et al., 2009a). In this experiment, the authors noted a very small fraction of labeled basal cells that were derived from lineage-labeled secretory cells following sulphur dioxide-induced airway injury (Rawlins et al., 2009a).

Additional evidence for robust plasticity has come from cell ablation experiments. In the tracheal epithelium, fully mature secretory cells have been shown to de-differentiate into bona fide basal stem cells following diphtheria toxin-induced stem cell ablation (Tata et al., 2013). Surprisingly, secretory cells began to replicate when more than 80% of the basal cells were ablated. Such a proliferative response was not noted when fewer basal cells were ablated. How secretory cells 'sense' the lack of threshold numbers of basal cells remains an interesting mystery. Lineage tracing using quadruple transgenic mouse models to simultaneously label secretory cells, followed by the ablation of basal cells, demonstrated that labeled secretory cells replicate and also de-differentiate into basal stem cells both *in vivo* and *ex vivo* (Fig. 3A) (Tata et al., 2013). De-differentiated basal cells were molecularly and morphologically indistinguishable from normal basal cells. Furthermore, the cells participated in the injury repair process and generated basal, secretory and ciliated cells following physiological forms of injury induced by either influenza infection or sulphur dioxide exposure. In addition, this study also suggested that there is an inverse relationship between the maturity of secretory cells and their ability to de-differentiate. Using an organoid culture system, it was shown that secretory cells could de-differentiate into stem cells when cultured singly in Matrigel (Tata et al., 2013). However, the ability of secretory cells to de-differentiate was blocked when these cells were plated in close proximity to basal cells. This finding suggests that basal stem cells suppress secretory cell plasticity through a very short range or a contact-mediated form of cell-cell communication (Fig. 4). The misexpression of Yap (Yap1) in secretory cells mimics some aspects of de-differentiation and generates pyramidal basally located cells with incomplete features of basal cells (Zhao et al., 2014). Furthermore, this reprogramming event is partial and depends upon the continuous expression of the Yap transgene. Hence the mechanisms underlying de-differentiation remain obscure.

In alveoli, recent studies using *Hopx-CreER*-based lineage labeling indicate that type 1 cell-like cells can replicate and generate type 2 stem cells *in vivo* following pneumonectomy-induced lung regeneration (Fig. 3B) (Jain et al., 2015). Recent studies also indicate that a bidirectional signaling loop involving fibroblast growth factor receptor activation in alveolar type 2 cells and VEGFR (KDR) signaling activation in endothelial cells is essential for the pneumonectomy-induced compensatory lung growth (Ding et al., 2011). However, the signals that regulate plasticity mechanisms per se are yet to be determined. In addition, it remains unclear whether de-differentiating cells represent a special subfraction of type 1 cells. Again, the need for single-cell analysis comes to the fore as a means to clarify these issues.

Transdifferentiation and transdetermination of airway cells

In addition to de-differentiation, other experiments have suggested that stem cells from one region of the lung can convert into stem cells from another region of the lung. Such plasticity has often been referred to as transdifferentiation, although when one stem cell converts into another, the appropriate terminology with reference to

Development (2017) 144, 755-766 doi:10.1242/dev.143784

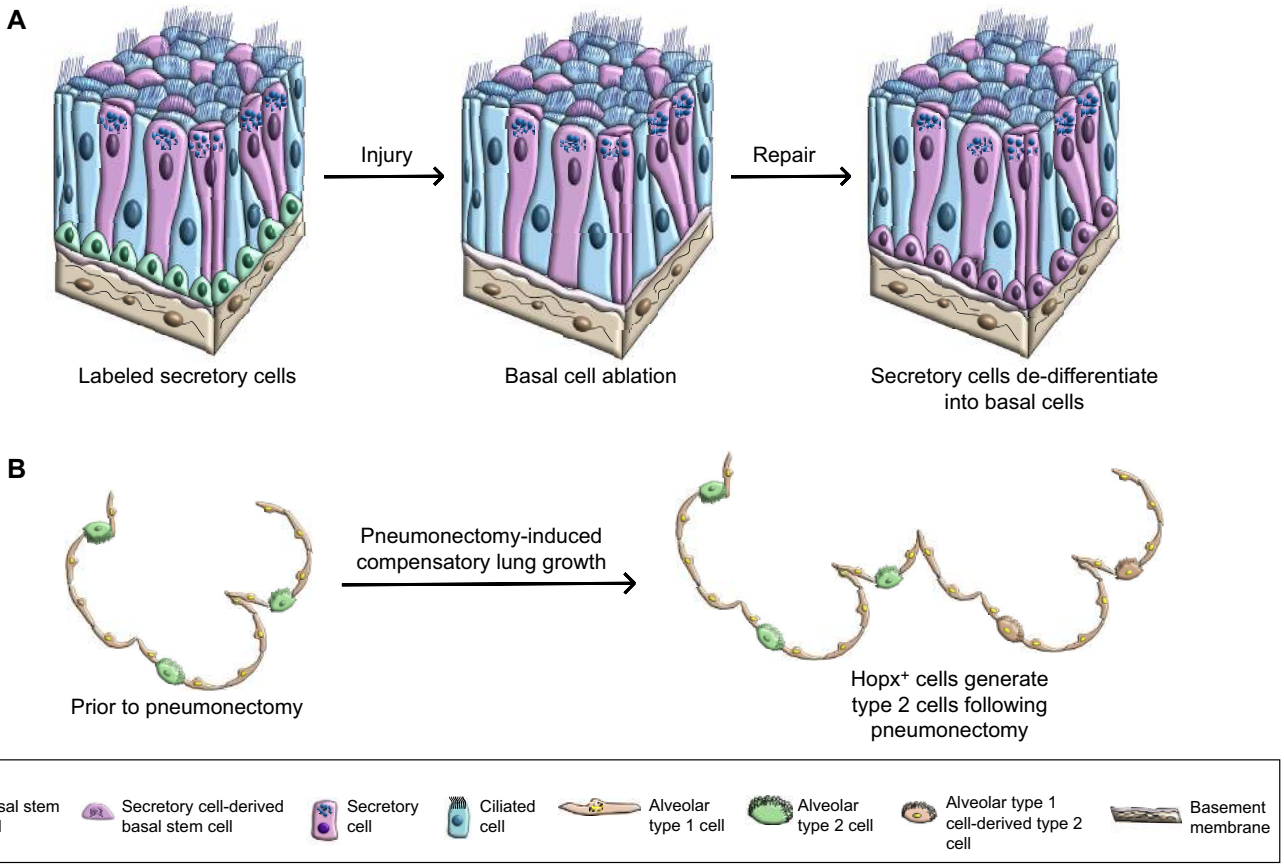

Fig. 3. Injury-induced lineage plasticity of differentiated cells. (A) In the airways, following ablation of the basal cell population (green), lineage-labeled secretory cells (purple) undergo de-differentiation into basal cells, thereby repopulating the stem cell compartment. (B) In the alveoli, Hopx$^+$ alveolar type 1 cells replicate and generate alveolar type 2 cells following pneumonectomy.

historical usage is transdetermination (Tata and Rajagopal, 2016a). Earlier studies have suggested that submucosal gland duct cells proliferate and migrate to repopulate the damaged pseudostratified surface epithelium following severe injury (Duan et al., 1998; Lynch and Engelhardt, 2014). Recent studies have also suggested that submucosal gland ductal basal cells can regenerate proximal airway epithelium following hypoxic ischemic injury (Hegab et al., 2011, 2012). The authors developed methods to specifically isolate and purify submucosal gland duct basal cells, which were then able to generate airway surface epithelial cells in *ex vivo* cultures as well as in fat pad transplantation assays. To complement *ex vivo* observations, the authors also performed *in vivo* lineage tracing using an inducible *KRT14* promoter-driven *CrePR* mouse model, which suggested that submucosal gland ductal cells could generate surface epithelial cells after hypoxic ischemic injury. However, KRT14 is also expressed in a subset of surface basal cells, and therefore additional studies using a lineage-tracing approach that is specific to ductal basal cells is required in order to establish definitively whether surface epithelial cells arise from submucosal gland ductal cells. Furthermore, unique markers that distinguish duct epithelial cells from surface epithelial cells are needed to establish whether ductal basal cells are transdetermined to form surface basal stem cells. Interestingly, this form of potential plasticity harkens back to a very similar finding in which skin hair follicle cells can repopulate surface interfollicular epidermis following injury (Rompolas et al., 2013). Live-imaging studies are also very likely to be useful in the airway epithelia, as they have been in skin stem cell systems (Rompolas et al., 2013).

In contrast to the example of transdetermination noted above, other experiments suggest that mature cells of the lung, which are thought to be distinct based on putative lineage hierarchies, can actually interconvert, and are therefore described as being able to transdifferentiate (Tata and Rajagopal, 2016a). One such example occurs in the case of fully differentiated neuroendocrine cells. In the small airways, it has been reported that *Cgrp*$^+$ neuroendocrine cells can generate secretory cells as well as ciliated cells following naphthalene induced-injury (Song et al., 2012). However, the relative lineage contribution of PNECs to other cell types was very low. This raises the question of whether the observed cellular plasticity might be a result of low-level expression of the CGRP in secretory cells following injury.

Another example of possible transdifferentiation is suggested by data from H1N1 influenza-induced injury repair models. In humans with respiratory failure, influenza-induced injury causes severe acute injury to the airways and alveoli. Infected lungs display a striking decline in function and patients generally take months to regain normal function. A more recent study reported that distal airway p63$^+$/KRT5$^+$ cells regenerate damaged lung epithelium following H1N1 influenza infection in mice (Kumar et al., 2011). The authors referred to these cells as distal airway stem cells (DASCs) and to the injury-induced nests of cells as ‘pods’ (Fig. 5A). Remarkably, it was suggested that DASCs expand, migrate along the distal airways into the alveoli, and then form discrete regenerating ‘pods’ in the damaged areas of the lungs. The authors performed lineage-tracing analysis using *Krt14* promoter-driven CreER and concluded that KRT14 lineage-labeled cells

Development (2017) 144, 755-766 doi:10.1242/dev.143784

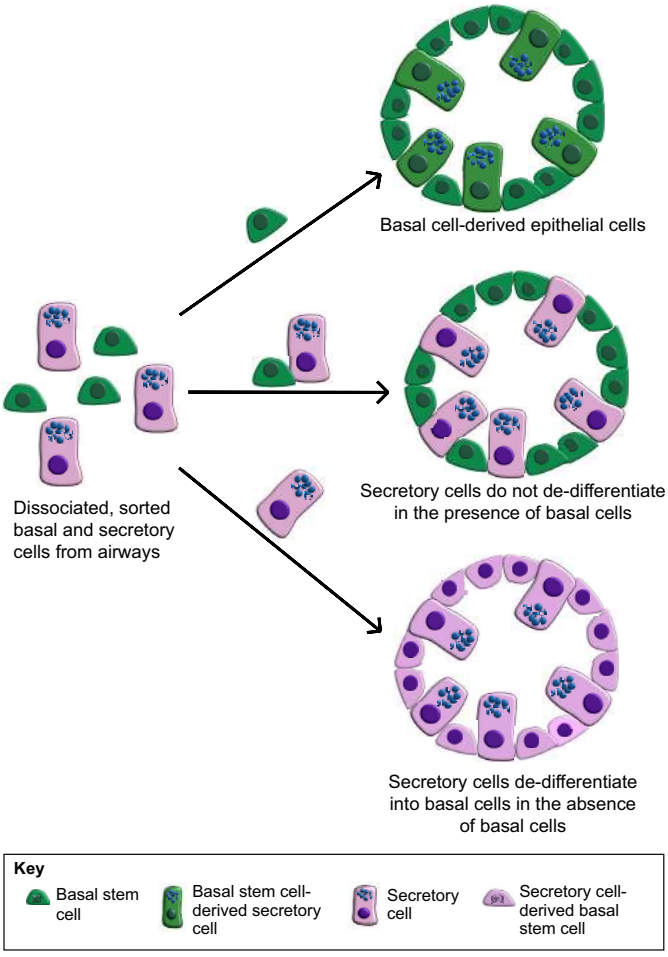

Fig. 4. Intercellular communication restricts lineage plasticity in the airway. Schematic of the role of cell-cell communication between parent and daughter cells, and its effect on daughter cell plasticity as demonstrated in *ex vivo* organoid assays. In the top scenario, organoids that originate solely from basal cells contain both basal and secretory cell types. In the middle scenario, organoids that originate from both basal and secretory cells contain both cell types, but the secretory cells do not de-differentiate in the presence of basal cells and basal cells do not differentiate into secretory cells. In the third scenario, organoids that originate solely from secretory cells contain both secretory and basal cell types demonstrating that secretory cells can de-differentiate into basal cells in this environment.

contribute to pods and that pod cells subsequently go on to differentiate into alveolar epithelial cells. However, in these lineage-tracing experiments the authors subjected the animals to injury prior to the tamoxifen-induced labeling of KRT14 cells (Kumar et al., 2011). This limits the conclusions one can draw about the contribution of pre-existing rare DASCs to alveolar regeneration because the *Krt14* promoter might be an injury-responsive transgene. In a follow-up study, the same group developed mouse models that bear a diphtheria toxin receptor under the control of *Krt6* gene promoter to ablate the highly proliferating *Krt6*-expressing basal-like pod cells. Using this model, the authors ablated *Krt6*-expressing cells and reported that the p63^{+}/KRT5^{+}/KRT14^{+}/KRT6^{+} cells that appear following influenza injury are essential for the regeneration of the damaged lung because they give rise to new alveolar epithelium sourced from the pod cells (Zuo et al., 2015). A second study suggested that pods may arise from both *Krt5*$^{+}$ lineage-labeled basal cells and *Scgb1a1*$^{+}$ lineage-labeled secretory cells (Zheng et al., 2013). However, this study was also called into question due to the caveat that tamoxifen-induced labeling might have persisted into the time period in which pod cells differentiated into basal and secretory cells. Thus, instead of demonstrating that pods cells were derived from secretory or pre-existing basal-like cells, it has been suggested that these authors simply labeled secretory and basal cells that had already differentiated from pod cells (Ray et al., 2016; Vaughan et al., 2015).

Clarifying this confusing situation, a recent report using lineage labeling well prior to injury (so as to avoid mislabeling due to tamoxifen persistence) in combination with cell transplantation experiments, demonstrated the existence of novel rare cell populations that inhabit the distal airway and which respond to injury by generating pod cells following both H1N1 influenza virus infection-induced injury and bleomycin-induced lung injury (Vaughan et al., 2015). The authors referred to these cells as 'lineage negative epithelial progenitor cells' (LNEPs), as they lacked markers associated with other lung stem/progenitor cells (Vaughan et al., 2015) (Fig. 5A). In this case, the relevant LNEPs were marked by a *Krt5*-CreER driver, but lacked obvious KRT5 protein, thus making them 'lineage negative'. Strikingly, the authors proposed that LNEP-derived pods gave rise to a 'failed regenerative' process following H1N1 influenza infection (Kanegai et al., 2016; Vaughan et al., 2015). Indeed, the authors showed that these pods formed 'cysts' that persist long term (200 days) and generated structures that appear similar to the honeycomb lesions that characterize fibrotic human lungs (Fig. 5B). These pod-derived structures are also likely to be consistent with the pathological finding known as 'bronchiolization' in which new cystic airway structures are produced in lung disease. These disease-associated 'neo-bronchioles' are characteristically lined by a ciliated respiratory epithelium, as was the case in the corresponding murine experiments (Kanegai et al., 2016). Interestingly, following infection with a less virulent H3N2 influenza strain, X31, no pods were observed (Kanegai et al., 2016).

In compelling transplantation experiments, sorted LNEPs generated alveolar epithelial cells, but only when the Notch pathway was inhibited (Vaughan et al., 2015). This finding suggests that LNEPs could successfully participate in an effective *in vivo* repair process that includes differentiation into alveolar epithelia under some circumstances. This could occur following bleomycin injury in which, surprisingly, *Krt5*-CreER-labeled KRT5 protein-negative LNEPs produced mature alveolar cells. In LNEP transplants, the alveolar cells that are produced are indeed associated with low Notch signaling. The cystic airway structures produced following LNEP transplant, on the other hand, are associated with clear Notch target gene expression (Vaughan et al., 2015). How, when and whether appropriate alveoli-generating Notch downregulation occurs in human injury is an entirely open question. It also remains theoretically possible that pods, pathologically known as 'metaplasia', might themselves serve some protective function, even if they do not form alveolar epithelium. Finally, it is also possible that, under some circumstances, pods resolve into proper functional bronchioles that in turn connect to new alveoli or existing alveoli.

Following the initial report of LNEPs, a subsequent lineage-tracing study using multiple *CreER* driver lines attempted to trace the cells that contribute to the generation of pods following influenza injury (Ray et al., 2016). This study demonstrated that *Sox2-creER* lineage-traced cells are the predominant (75%) contributors to the pod lineage. *Krt5-creER* traced cells contributed to 15.5% of the pod cells, whereas lineage tracing using *Scgb1a1-creER* marked only 0.7% of pod cells. There was no

Development (2017) 144, 755-766 doi:10.1242/dev.143784

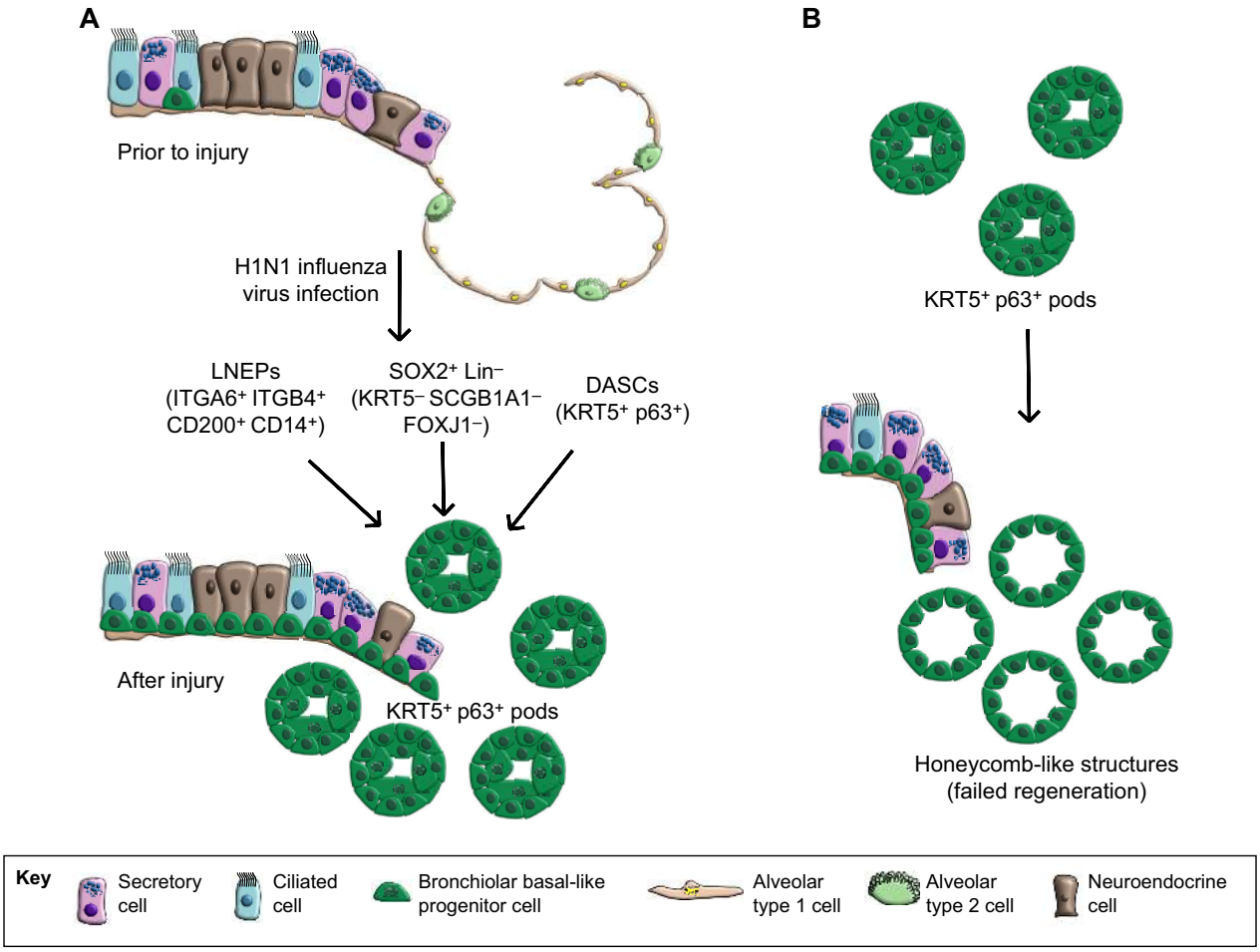

Fig. 5. Cellular responses of LNEPs/DASCs following H1N1 influenza-induced alveolar injury. (A) Schematic of the appearance of basal-like cells in pods following H1N1 infection in murine models. Three different cell populations, namely LNEPs, DASCs and Sox2$^+$Lin$^-$ cells, were recently proposed to respond to H1N1 influenza injury and form KRT5$^+$p63$^+$ epithelial pods in areas with alveolar damage. It is very likely that all these populations have a high degree of overlap, and furthermore that each is divisible into further subsets of progenitor cells. (B) Schematic of the fate of the KRT5$^+$p63$^+$ pod cells following H1N1 influenza injury. Pod cells do not regenerate alveoli following H1N1 infection.

contribution from type 1 or type 2 cells. Furthermore, this study also demonstrated the presence of *Sox2-creER* lineage-labeled cells that are negative for KRT5, SCGB1A1 and FOXJ1 expression (Ray et al., 2016). This suggests the presence of some rare pre-existing Sox2$^+$Lin$^-$ cells that are very likely to be the same cells as the previously identified LNEPs. As *Sox2* lineage tracing as well as secretory and type 2 cell lineage tracing does label putative BASCs, it can be concluded that BASC cells do not contribute to pods, just as the preponderance of current evidence suggests that they are not stem cells. It is possible that the Sox2$^+$Lin$^-$ cells might include neuroendocrine cells, brush cells, or some non-epithelial Sox2$^+$ cell type that remains to be identified. It will be interesting to see whether, as we suspect, Sox2$^+$Lin$^-$ cells are p63$^+$, thus representing some basal-like LNEP. Indeed, there could be a spectrum of distal plastic progenitor cells that have some overlap. The sorting and transplantation experiments suggest that there are p63$^+$ and p63$^-$ or p63-low LNEPs, and that these populations might have different functional properties. For example, *Krt5*-CreER-marked LNEPs do indeed make some contribution to pods, but when these cells are transplanted, they rarely make alveoli. Perhaps these cells are somewhat committed towards a KRT5$^+$ basal-like lineage that has a bias away from alveolar differentiation. Further lineage studies using a *p63*-driven CreER allele will be helpful to clarify the nature of the cells that give rise to pods. Ultimately, once again, single-cell sequencing will be necessary to resolve these issues. For now, we suggest that all three populations be referred to as LNEPs/DASCs (Fig. 5A).

The burden of proof for plasticity

In general, in all experiments suggesting plasticity, there is concern that many transdifferentiating cells are identified as such because the putative originating cell marker is actually an injury-responsive gene. Thus, the cell type that was erroneously thought to be produced by transdifferentiation might instead simply be tagged as a result of the induced expression of an injury-responsive gene, rather than a true conversion of one differentiated cell type into another. This might happen, for example, if mRNA encoded by the locus is very difficult to detect but Cre-mediated recombination is efficient. Alternatively, one might be misled if protein is absent in the setting of active transgene expression, thus causing antibody staining to be misleading. In this setting, careful Cre-recombinase antibody staining prior to lineage tracing might be needed to demonstrate that the Cre is active exclusively in the putative originating cell type. Furthermore, studies must be performed with stringent lineage-tracing analysis in which the temporal labeling of cell types is far enough removed from the injury itself to avoid the inappropriate

Development (2017) 144, 755-766 doi:10.1242/dev.143784

marking of cell types because of this issue of injury-responsive gene expression (Vaughan et al., 2015). Regardless of the outcome, such injury responses might, in and of themselves, prove very interesting. The induction of lineage-specifying injury response genes would represent an interesting mechanism for endogenous 'reprogramming'. Given the complexity of this field and the concerns about mislabeling cells and injury-responsive genes, demonstrating plasticity with different drivers, in multiple hands, and in different contexts will be helpful in the future as the community drills down into a complex and demanding problem with ever-evolving tools. Further complicating matters, progenitor cells in the lung might be so plastic that they are frequently oscillating between closely related states. If this proves to be the case, only clonal analysis can say anything about the aggregate behavior of an entire spectrum of cells.

Factors that influence cell plasticity

How does the maturation state of a cell influence its plasticity? In some cases, plasticity is only seen in cells that are in the process of undergoing differentiation, whereas in other cases fully mature differentiated cells can de-differentiate into stem cells. For example, in *Drosophila* ovary and testis, differentiating gonialblasts or spermatogonial cells can revert back to stem cells, but mature cells have not been shown to possess this ability (Brawley and Matunis, 2004; Kai and Spradling, 2004). In contrast, fully mature secretory cells in the murine trachea can de-differentiate into stem cells after stem cell loss (Tata et al., 2013). As described earlier, even in this case, the degree of maturity of differentiated cells seems to have an inverse relationship with their ability to de-differentiate (Tata et al., 2013).

In the large airway, epithelium regeneration can occur through conventional basal stem cell differentiation, but in the absence of residual basal cells, secretory cells can serve as facultative stem cells through a de-differentiation event in which secretory cells give rise to a new basal cell pool (Fig. 3A). Interestingly and mysteriously, such plasticity is not observed when more than 20% of the original native basal cells remain intact. To this end, it has been shown that parent basal cells send ligands to their own daughter secretory cells to activate Notch signaling in these daughter cells. This signal is in fact required for maintaining secretory cell identity and stability (Pardo-Saganta et al., 2015b). However, whether and how such signals regulate de-differentiation remains to be studied, and it could be that multiple signals are required. It is likely that very complex intercellular signaling processes control different cellular states to maintain tissue equilibrium (Tata and Rajagopal, 2016b). As noted above, secretory cell identity seems to require active maintenance by a tonic Notch ligand signal from basal cells; thus, the 'default' tendency of a secretory cell isolated from its basal cell parent is either to differentiate or to de-differentiate. This suggests that cell fate plasticity might be just as likely to occur in tissues as would cell fate stability. Furthermore, this plasticity could be just as functionally relevant as cell fate stability. Additionally, we note that the acquisition of a stem cell or progenitor cell state can be either transient or permanent. For example, following severe interfollicular epidermal injury, hair follicle stem cells initially contribute to the regeneration of epidermal wounds. However, long-term lineage tracing has shown that these epidermal cells do not persist over time (Ito et al., 2005). In the case of airway epithelium, lineage tracing has revealed that de-differentiated basal stem cells can persist and participate not only in the steady state maintenance of the epithelium, but also in injury repair (Tata et al., 2013).

Conclusions

The majority of direct and stringent evidence for lung epithelial cell plasticity *in vivo* has arisen through the use of murine lineage-tracing experiments performed in just a subset of possible injury settings. Different injuries are likely to evoke different forms of plasticity. In humans, our understanding of plasticity, and indeed normal differentiation, are inferential. So, although there is little doubt that *in vitro* results demonstrating the plasticity of human lung epithelial cells yield valuable information, the results might not reflect a plasticity seen in the living organism. Furthermore, given the fact that the heterogeneity of murine and human lung epithelial cell types is only now being established at the single-cell level, even our most simple foundations for thinking about cell plasticity are nascent, and will almost certainly undergo revision in the coming decade. We also note that murine experiments, even stringent lineage-tracing experiments, have almost always been performed in controlled environmental settings in which rodents and their lungs are not exposed to the normal environmental assaults present in the wild. Thus, our conclusions, even about simple issues such as the distribution of various cell types and the relative degree of turnover in our experimental models could be severely prejudiced by our practice of animal husbandry. Already, there is evidence that the murine immune system changes dramatically in wild versus housed mice (Beura et al., 2016). The interaction between immune and epithelial cell populations adds another tier of complexity to the interpretation of existing experiments, and is likely to have a particularly important bearing on injuries associated with pathogens. Furthermore, modern experimental demonstrations of plasticity, including ours, often invoke artificial, non-physiological forms of injury – for example diphtheria toxin-mediated cell ablation – and these experiments might have no physiologic correlate in nature at all. Finally, it is worthwhile noting that the degrees of plasticity evoked in the experiments described above, in small numbers of cells following extensive damage, might not be functionally significant. Even if this eventually proves to be the case, one might be able to make use of the phenomenon of plasticity as an explanatory model for various lung pathologies.

As noted throughout this Review, future studies will require a more thorough delineation of new cell types using new tools such as single-cell sequencing and computational lineage reconstruction in both murine and human models. Where possible, these lineage predictions will need to be verified by cell type-specific clonal lineage tracing using newly identified markers. If computational and genetic experiments in murine systems correspond, this will lend credence to the notion that our guesses about lineage based on computational reconstruction can be applied to *in vivo* human systems in which prospective lineage tracing is not possible. New methods for establishing clonality in humans, such as following mitochondrial mutations, are likely to be supportive of certain hypotheses, but it is unclear how the initial cell of origin question would be established in such experiments. It will also be interesting to see how *in vitro* and *in vivo* findings correspond. Can all *in vivo* plasticity be captured in a dish? Indeed, one might guess *in vitro* culture engenders more plasticity than a normal *in vivo* context would allow, but this remains conjecture. Alternatively, one might guess, based on Waddington's notion of canalization, that *in vitro* plasticity is just as constrained as its *in vivo* counterpart (Waddington, 1957). Oncogenesis might be predicted to increase plasticity, but in certain instances it is conceivable that the acquisition of an oncogene or a loss of a tumor suppressor might paradoxically lock or favor a particular cell state rather than promote plasticity. Additionally, we might find that identity is blurred

Development (2017) 144, 755-766 doi:10.1242/dev.143784

following injury, and that steady state discrete cell types are replaced by sequences of intermediate progenitors that simply cannot be identified by unique markers. If so, identifying and tracing these cells non-computationally will require either intersectional lineage analysis or indeed real-time visualization. Clonal epigenetic profiling is also likely to inform and clarify the potential of cells to acquire new cell states. Certainly, our emerging powerful new tools are poised to shed new light on the biology of cell state transitions, not only in the lung, but in many other tissues.

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Competing interests

The authors declare no competing or financial interests.

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