Developmental Biology 420 (2016) 278–286

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Review article

Organogenesis of adult lung in a dish: Differentiation, disease and therapy

Jinwook Choi[a], Elhadi Iich[a], Joo-Hyeon Lee[a,b,*]

[a] Wellcome Trust/Medical Research Council Stem Cell Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK
[b] Department of Physiology, Development and Neutabroscience, University of Cambridge, Cambridge CB2 3DY, UK

ARTICLE INFO

Keywords:
Organoid
Lung
Stem cells
Niche
Self-organization

ABSTRACT

The remarkable regenerative capacity of the lung suggests that stem cells could be of therapeutic importance in diverse lung diseases; however, the successful exploitation of lung stem cell biology has long been hampered by our inability to maintain and expand adult lung stem cells while retaining their multi-lineage potential *in vitro*. Recently, advances in our understanding of stem cell niches and the role of key signalling modulators in controlling stem cell maintenance and differentiation have fuelled the development of new *in vitro* three-dimensional (3D) culture technologies that sustain the stem cell-driven formation of near-physiological, self-organizing structures called organoids. Here we review basic approaches to organoid model systems and highlight recent achievements in the generation of organoids from adult stem and progenitor cells of both the murine and human lungs. We evaluate current applications in studying cellular changes in proliferation, differentiation, plasticity, and cell polarity, and cellular and molecular crosstalk of epithelial cells with stroma. Advantages and limitations of organoids for clinical use are also discussed.

1. Introduction

The lung is a complex organ composed of numerous types of interconnected epithelial cells, stromal cells, including vascular cells and immune cells, and extracellular matrix (ECM) that synergize to maintain lung integrity. In the last decade there has been significant progress in understanding the organization of stem and progenitor cells in the adult lung. The analysis of lung injury models, combined with *in vivo* lineage-tracing techniques, has identified various potential epithelial stem and progenitor cells that respond to local injury and replace the damaged epithelial cells. These achievements have opened up a new era for the potential exploitation of adult stem cells in clinical applications for degenerative lung diseases arising from the impairment or depletion of specific cell types. However, historically it has been challenging to maintain and expand these stem cells and to direct their lineage specification to reproduce such physiological functions as airway patency, mucociliary clearance, and gas exchange *in vitro*. This is partly due to the limited reproduction of multi-cellular organization of the lung *in vitro*.

For decades, researchers have attempted to find suitable *in vitro* model systems that can recapitulate *in vivo* functions and processes, from molecular and cellular levels to whole tissue, and organ functions. Two-dimensional (2D) model systems, such as monolayer cell cultures, have been used to assess the clonogenicity of adult progenitor cells and to induce lineage differentiation of pluripotent stem cells. However, the lack of structural and physical supports provided by stromal components including the ECM, has been a barrier to understand cellular and molecular functions under physiological and pathological conditions. Recent progresses in isolating numerous types of cells, including epithelial progenitor cells and stromal cells, and defining niche factors that are important for lung development has led to the establishment of an *in vitro* three-dimensional (3D) lung culture system, termed lung organoid cultures. In organoids, epithelial stem and progenitor cells, cultured in ECM supplemented with either a mixture of growth factors or stromal cells, self-organize into complex structures retaining clusters of multi-lineage epithelial cells. Lung organoids recapitulate various features of the lung including heterogeneous cell composition, spatial organization and retention of a stem cell population harboring the capacity for both self-renewal and differentiation (Fatehullah et al., 2016). Importantly, lung organoids provide an *in vitro* model system for studying regenerative mechanisms of epithelial stem and progenitor cells proposed from *in vivo* studies.

In this review, we will focus on recent advances in the murine and human organoids derived from adult primary lung epithelial cells. This review will provide an overview of the potential of adult lung stem and progenitor cell that are responsible for lung homeostasis, regeneration, and enable the generation of organoids retaining multi-lineage epithelial cell types. The value of lung organoids as potent model systems for understanding tissue regeneration, lineage specification, and disease modelling will be evaluated. Future challenges for clinical application

* Corresponding author at: Wellcome Trust/Medical Research Council Stem Cell Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK.
E-mail address: jhl62@cam.ac.uk (J.-H. Lee).

http://dx.doi.org/10.1016/j.ydbio.2016.10.002
Received 27 July 2016; Received in revised form 29 September 2016; Accepted 2 October 2016
Available online 03 October 2016

Table 1
Adult lung stem and progenitor cells capable of generating organoids in mouse and human.

location	Cell type	Markers for Isolation	Differentiation ability in 3D organoids
Tracheobronchiolar region	Basal cells	$Krt5^+$, $Ngfr^+Itg\alpha6^+$, $GSI\beta4^+$ (mouse) $Ngfr^+Itg\alpha6^+$ (human) $Ngfr^+CD166^+CD44^+$ (human)	Basal cells (Trp63+, Krt5+, Krt14+, Ngfr+, Pdpn+) Club cells (Scgb3a2, Splunc) Goblet cells (Muc5AC, Muc5B) Ciliated cells (Foxj1, Acetylated tubulin) (Rock et al., 2009, 2011a, 2011b; Hegab et al., 2012; Tata et al., 2013; Tadokoro et al., 2014; Danahay et al., 2015; Butler et al., 2016)
Bronchiolar region	Club cells	$Scgb1a1^+$ (mouse)	Club cells (Scgb1a1) Goblet cells (Muc5AC) Ciliated cells (Foxj1, Acetylated tubulin) AT2 cells (Sftpc)
	Bronchioalveolar stem cells (BASCs)	$EpCAM^{hi}Itg\alpha6^+\beta4^+CD24^{low}$ (mouse) $CD45^-CD31^-CD34^-$ (Lin^-) $Sca\text{-}1^{low}$ (mouse) $EpCAM^+CD24^{low}Sftpc\text{-}GFP^{neg/low}$ (mouse) $EpCAM^+Sca1^+$ (mouse)	(McQualter et al., 2010; Teisanu et al., 2011; Chen et al., 2012; Lee et al., 2014)
Alveolar region	AT2 cells	$Sftpc^+$ (mouse) $EpCAM^+Sca1^-CD24^-Sftpc\text{-}GFP^{hi}$ (mouse) $EpCAM^+Sca1^-$ (mouse) $EpCAM^+HTII\text{-}280^+$ (human)	AT2 cells (Sftpc) AT1 cells (Pdpn, Hopx, Ager) (Chen et al., 2012; Barkauskas et al., 2013; Lee et al., 2014; Jain et al., 2015)

will also be discussed.

2. Adult lung stem and progenitor cells

The adult lung is a highly quiescent tissue with a slow turnover rate (< 1% per day) of airway and alveolar epithelia (Bowden, 1983; Goss, 1966; Kotton and Morrisey, 2014). However, following tissue damage, lungs demonstrate extraordinary regenerative capacity to repair tissue damage and restore function. Recent advances in cell lineage tracing, flow cytometric analyses, and cell culture techniques have identified the specific stem and progenitor cells responsible for these extraordinary feats. In this review, we will focus on stem and progenitor cells capable of generating lung organoids. A brief overview is provided in Table 1.

2.1. Epithelial stem and progenitor cells of the adult mouse lung

Within the mammalian lung, there are a large number of epithelial cells along the pulmonary axis, stretching from the proximal to the distal end. This axis can be sub-divided into three regions, the tracheobronchial, bronchiolar, and alveolar region. Each region is lined by specialized epithelial cell types that are maintained by regional epithelial stem and progenitor cells with potential to generate lung organoids.

2.1.1. Basal Cells

Basal cells expressing Trp63, Krt5, and Ngfr lie close to the basal lamina and comprise ~30% of the pseudostratified mucociliary epithelium lining the trachea region. Lineage tracing studies have shown the maintenance of this region by basal and basal luminal precursor cells with a slow turnover during steady state (Ghosh et al., 2011; Mori et al., 2015; Mou et al., 2016; Rock et al., 2009; Watson et al., 2015). Following injury, basal cells demonstrate extensive proliferative and self-renewal capacity to differentiate into the mucous- and serous-secreting cells as well as ciliated cells, secretory goblet, and club lineage cells (Borthwick et al., 2001; Ghosh et al., 2011; Hegab et al., 2011; Hong et al., 2004a, 2004b; Rock et al., 2009). The Notch signalling pathway has been highlighted as playing a critical role in regulating the fate decision of basal cells between secretory lineage cells and ciliated cells (Carraro and Stripp, 2015; Pardo-Saganta et al., 2015; Rock et al., 2011b). Recently, club cells have been shown to revert into the lost basal cells post genetic ablation of basal cells *in vivo* suggesting the contribution of cellular plasticity to regenerative process (Pardo-Saganta et al., 2015; Tata et al., 2013). However, the molecular mechanisms how these differentiated cells work together with stem or progenitor cells to maintain lung integrity still remain to be fully elucidated.

2.1.2. Club cells

Club cells expressing secretoglobin family 1a member 1 (Scgb1a1, also known as CC10 or CCSP) are columnar epithelial cells comprising the majority of the bronchiolar epithelial region. Given their capacity for both self-renewal and differentiation into ciliated cells over the long-term, club cells have been considered stem cells in the bronchiolar epithelium at steady state (Rawlins et al., 2009). Following the administration of naphthalene to selectively ablate club cells, naphthalene-resistant variant club cells near to neuroendocrine bodies (NEBs) and bronchioalveolar duct junctions (BADJs) have been revealed to replenish the bronchiolar epithelium (Giangreco et al., 2002; Hong et al., 2001; Rawlins et al., 2009; Reynolds et al., 2000; Stripp et al., 1995). By exploiting the bleomycin- and influenza-induced alveolar injury models, club cells have been demonstrated to produce alveolar lineage cells (Rock et al.., 2011b; Tropea et al., 2012; Vaughan et al., 2015; Zuo et al., 2015). At BADJ regions, bronchioalveolar stem cells (BASCs) expressing both Scgb1a1 and Sftpc, which are the markers for club and alveolar type II cells (AT2) respectively, have been suggested to be stem cells that can expand after naphthalene- and bleomycin-induced injury *in vivo* (Kim et al., 2005). These cells also show multi-lineage differentiation into bronchiolar and alveolar lineages *in vitro* in the 3D organoid culture system (Lee et al., 2014). Endothelial derived Thrombospondin-1 has been suggested to induce alveolar lineage differentiation of BASCs during bleomycin-induced alveolar damage repair. In response to cytokines such as IL-13, club cells can also generate goblet cells (Atherton et al., 2003; Wills-Karp et al., 1998; Zhu et al., 1999). Despite of these advances in identifying the multi-potent differentiation capacity of club cells, the heterogeneic response of club cells to different injuries and the molecular mechanisms regulating bronchiolar- and alveolar-lineage differentiation remain to be uncovered.

2.1.3. AT2 cells

The distal alveolar region is lined by surfactant-producing AT2 and gas-exchanging AT1 cells. Lineage tracing studies have shown that AT2 cells function as stem and progenitor cells that self-renew and give rise to AT1 cells in the steady state and during regeneration after injury

(Barkauskas et al., 2013; Desai et al., 2014). A recent study performing partial pneumonectomy-induced damage has suggested an unexpected plasticity of the terminally differentiated AT1 cells expressing homeodomain only protein x (Hopx). These rare AT1 cells have the potential to undergo reprogramming and become AT2 cells during alveolar regrowth (Jain et al., 2015). Such findings and further understanding of molecular mechanisms underlying cell fate changes are important for respiratory disorders such as idiopathic pulmonary fibrosis which affect alveolar structures and function.

2.2. Epithelial stem and progenitor cells of the adult human lung

In the human lung, the epithelium containing basal cells extends to the terminal bronchioles, while this region is restricted to the trachea in mice. In contrast to studies using mouse models, the limited availability of normal human lung tissue has impeded the characterisation of adult stem and progenitor cells in the human lung. A number of studies have provided evidence for the existence of human lung progenitor cells that may share similar characteristics to their mouse counterparts. Recently, Teixeira et al. nicely showed the clonal pattern of accumulated mutations that occur in the mitochondrial genome in the upper airways of human lung tissues suggesting the presence of clonal multi-potent progenitor cells within basal cells (Teixeira et al., 2013). In addition, various approaches have been applied to isolate and grow normal human lung epithelial cells *in vitro*. Dissected normal tissue from lung tumor resections or discarded airways from transplants or cadavers have been used as main sources of adult human lung cells. Nasal epithelium, bronchial brushing or endobronchial biopsies are also used as sources for isolating human respiratory basal cells (Butler et al., 2016; Hackett et al., 2011; Hegab et al., 2012; Mori et al., 2015; Oeztuerk-Winder et al., 2012; Randell et al., 2011; Rock et al., 2009). We will highlight the current achievements to suggest the existence of stem and progenitor cell populations in human lungs using organoid cultures in the following sections.

3. *Ex vivo* organoid culture systems

The scant knowledge of cellular composition in the tissue and niche environment surrounding stem and progenitor cells has limited our progress in identifying the molecular and cellular processes of organogenesis. Recent advances in our understanding of the stem cell microenvironment, including biological signalling pathways and ECM, have enabled the establishment of stem cell-derived organoid model systems. Using this new system, the direct observation of dynamic cellular changes including proliferation, differentiation, and interactions within the self-organizing structures has become possible.

3.1. Development of the culture platform for lung organoids

To investigate cellular differentiation and physiological function using a simple and reproducible *in vitro* culture system, various cell and tissue culture systems have been established from the lung (Fig. 1)..

3.1.1. 2D culture

2D monolayer culture systems are useful to observe and manipulate mammalian cells and tissues. However, 2D cultures do not completely recapitulate the *in vivo* behaviour of cells and the developmental organization of tissue. In particular, lung epithelial cells in 2D monolayer cultures have been shown to maintain their differentiated status for only a few days (Chander et al., 1983; Diglio and Kikkawa, 1977; Douglas et al., 1979). Even in co-culture with feeder cells such as irradiated mouse embryonic fibroblasts (MEFs) (Kim et al., 2005), lung epithelial stem cells including BASCs and AT2 cells lose expression of lung lineage markers such as Scgb1a1 and Sftpc in 2D monolayer culture (unpublished data). These model systems fail to represent 3D morphogenesis, long-term culture capacity and differentiation into other lineages.

3.1.2. 2D air-liquid interface (ALI) culture

The air-liquid interface (ALI) culture recapitulates a more realistic lung environment and drives airway epithelial cells to proliferate and differentiate *in vitro* (Berube et al., 2010; Vaughan et al., 2006; Wong et al., 2012). In this culture system, cells are seeded on the apical surface of the permeable membrane of transwell inserts, followed by placing media in both of upper and lower chamber. After reaching confluency, the media is removed from the upper chamber to induce differentiation. The apical side of the cells is exposed to air while the basolateral side is exposed to media through the transwell membrane, thus mimicking the pseudostratified mucociliary microenvironment observed *in vivo*. Under these conditions, cells develop tight junctions and a differentiation state similar to that reached *in vivo*, with ciliated, basal, and secretory cells. For example, using ALI cultures, human bronchial epithelial cells (HBEC) have been shown to differentiate into mucociliary cells (Bals et al., 2004; Fulcher and Randell, 2013; Neuringer et al., 2005; Pageau et al., 2011; Whitcutt et al., 1988). Despite these advantages, lack of tissue architecture with multi-cellular differentiation resulted in developing 3D culture system.

3.1.3. 3D-submerged culture

Organ cultures with fetal lung explants demonstrated branching of the bronchial tract and lobules, yet no further cellular differentiation was observed (Alescio and Colombo Piperno, 1967; Zimmermann, 1987). Adult stem cells actively interact with their microenvironment and the behaviour of stem cells is finely regulated by extrinsic factors derived from the niche. Thus, engineered niche systems are absolutely required to promote the tissue-specific differentiation of epithelial stem cells. The basement membrane extract (known as BME or Matrigel) was first established from mouse tumor as a crude mixture composed of laminin, fibronectin, and collagen. It liquefies at 4 °C, gels under physiological conditions (24–37 °C), and provides a physical framework similar to *in vivo* tissue (Benton et al., 2014; Peerani and Zandstra, 2010; Vazin and Schaffer, 2010). Organoids embedded in 3D ECM have been widely used for the study of branching morphogenesis of several epithelial cell types including those from the lung (Fata et al., 2007; Liu et al., 2004; Qiao et al., 1999; Simian et al., 2001). The cells are suspended with growth factor reduced Matrigel and loaded onto multi-well plates, followed by adding media to cover organoid droplets. Alternatively, prior to seeding, the chamber bottom was coated with 25–100% Matrigel that was allowed to gel. Mixture of cells with 2–5% Matrigel in media is layered on pre-coated chamber (Rock et al., 2011b; Rock et al., 2009; Tata et al., 2013). Organoids are derived from single cells and grow clonally. However, establishing long-term expanding 3D organoids has required tissue-specific modifications that reflect the niche microenvironment for proper *in vitro* expansion of progenitors as well as differentiation of specific cell types.

3.1.4. 3d-ALI combined culture

Recent studies introduced the combination of these two systems, Matrigel (3D) and ALI, into the adult stem/progenitor derived-lung organoid culture (Barkauskas et al., 2013; Chen et al., 2012; Lee et al., 2014; McQualter et al., 2010). The cells are mixed at a 1:1 ratio with growth factor reduced Matrigel in media and plated on top of transwell inserts. Media is placed in the lower chamber where the nutrients and signalling molecules are delivered through the semipermeable membrane. Importantly, 3D-ALI combined culture promotes organoid formation including increased colony forming efficiency with multilineage differentiation (Fig. 2A), possibly by creating a supportive microenvironment for expansion, polarization and differentiation of adult distal lung progenitor cells. However, it still remains to elucidate whether these cells sense a gradient of airs or nutrients to create epithelial polarity which may affect lineage differentiation within

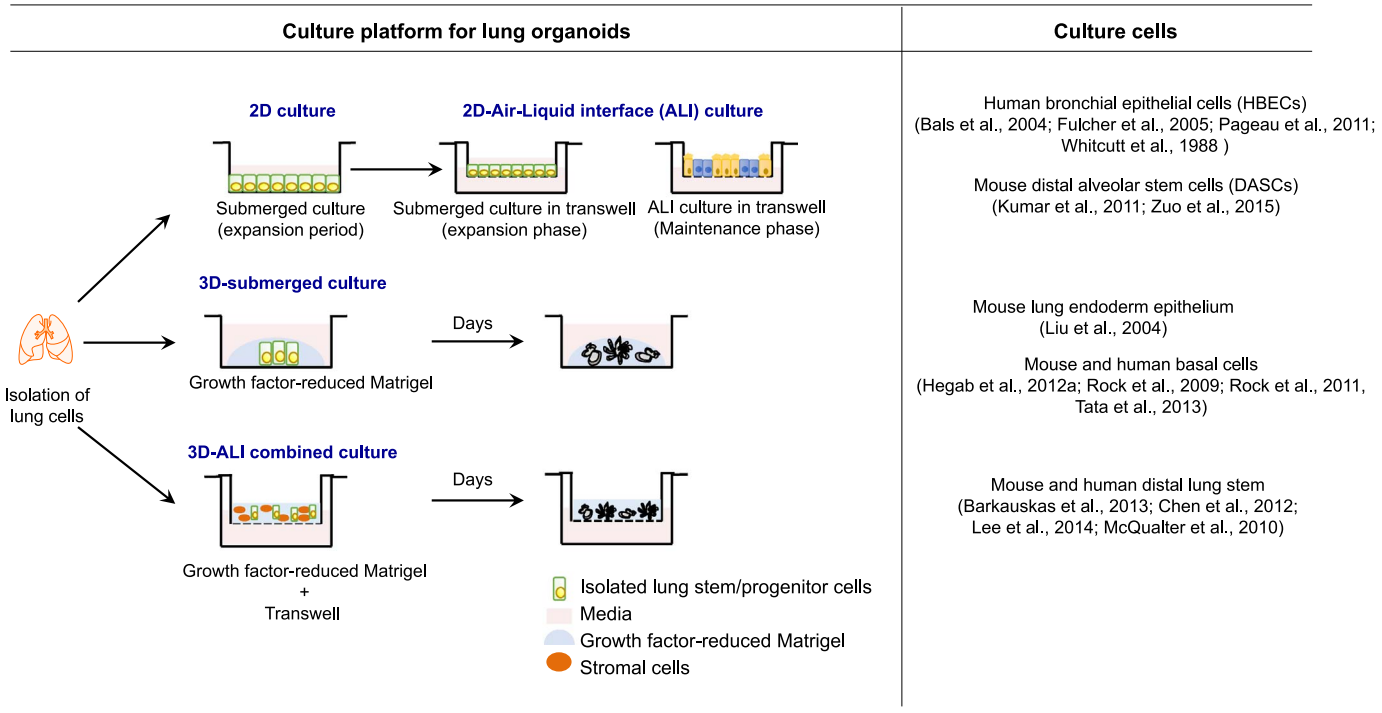

Fig. 1. Culture platform for lung organoids. (Top) Primary airway epithelial cells are placed on standard tissue culture plates prior to passage and introduction to differentiation condition in air-liquid interface (ALI) culture. Expansion phase is followed by ALI culture, which drives differentiation and exhibits characteristic properties of airway cells, including mucus secretion, ciliary motility and formation of tight junction. (Middle) Primary airway epithelial cells are mixed with a gel of extracellular matrix (ECM) protein (Matrigel) and placed on standard tissue culture plates. ALI media to induce lineage differentiation are fed in every other day. Airway stem and progenitor cells clonally grow to form 3D spheres. (Bottom) A mixture of primary airway epithelial cells with Matrigel is placed on transwell insert and is exposed to medium in ALI, which allows nutrient diffusion without submersion. Further advanced co-culture system has been employed by adding stromal cells in this mixture.

organoids. Furthermore, this system enables the addition or removal of components that constitute the microenvironment of lung tissues, such as stromal cells or secreted molecules which can be added either to the mixture of cells and Matrigel or to the lower chamber of plates in media. Taken together, 3D-ALI cultures make it easier to recapitulate a more realistic microenvironment to understand the crosstalk between adult lung stem cells and their niches..

3.2. *Establishment of lung organoids from adult epithelial stem cells*

Lung organoids derived from adult stem and progenitor cells have been successfully established from several groups and have proven to be a powerful culture system that enables the prolonged long-term growth and expansion of adult lung stem and progenitor cells whilst preserving their differentiation potential. Thus, it provides a useful *in vitro* tool to study regenerative potential of these cells that have been identified by *in vivo* studies. Here we will discuss currently available advanced 3D organoid culture systems for proximal and distal adult lung stem and progenitor cells in mouse and human (Table 1).

3.2.1. *Lung organoids derived from tracheal and proximal airway stem and progenitor cells*

For lung organoids derived from mouse basal cells, primary Krt5$^+$, Ngfr$^+$Itga6$^+$ or GSIβ4$^+$ cells isolated from the trachea are cultured in mTEC/Plus growth media in either 3D-submerged culture or 3D-ALI combined culture (the precise composition of the culture medium is described in Table 2 (Rock et al., 2011b, 2009; Tata et al., 2013). Despite broad uses of mTEC/Plus media in basal cell culture, it is not defined media but basic media containing various growth factors including EGF which is critical for proliferation of cells. Basal cells develop multi-cellular spheres with ciliated and secretory cells surrounding a single, central lumen structure. Trp63$^+$ basal cells are present in the outer layer of spheres and luminal cells including ciliated cells are found in the inner layer of spheres (Fig. 2B). Observation of variation in size of spheres (diameter, 150–500 μm) and proportion of differentiated cells within spheres suggests the heterogeneity of sphere-forming cell populations in isolated basal cells. After 14 days in culture, spheres can be collected to be re-seeded at a 1:10 dilution in Matrigel following by either enzymatic digestion or mechanical dissociation for the next round culture or fixed in fixatives for immunostaining analysis. For lung organoids derived from human basal cells, freshly isolated Ngfr$^+$Itga6$^+$ cells cultured in 1:1 Matrigel/ALI media (Table 2) have been shown to generate spheres containing Krt14$^+$Trp63$^+$ basal cells and ciliated cells (Rock et al., 2009). Similarly, Ngfr$^+$CD166$^+$CD44$^+$ epithelial cells isolated from human trachea and submucosal glands are able to form luminal structures comprising Krt5$^+$Krt14$^+$ basal cells, mucous- and serous-secretory epithelial cells in the same 1:1 mixture of Matrigel and mTEC/Plus medium (Hegab et al., 2012; You and Brody, 2013; You et al., 2002). Similar to mouse basal cell culture, culture media for human basal cells are not fully defined. Two basic media have been used for basal cell culture; a basal media for expansion and ALI media for differentiation. Together, these studies strongly suggest that the 3D organoid culture system is an efficient and powerful *in vitro* system to maintain the regenerative capacity of basal cells from both mouse and human. Despite these remarkable achievements, the prolonged expansion of basal cells whilst maintaining genetic stability and functional activity still remains challenging, in part due to a lack of the defined media which can be achieved by understanding molecular mechanisms underlying regulation of cell fate changes. A recent study reported the retardation of luminal cell differentiation with expansion of basal cells when TGFβ/BMP/SMAD signalling was inhibited (Mou et al., 2016). However, better culture conditions for preserving cellular function in these cells will be the next step on which to focus. In addition, the variation in cellular compositions and organization between and within human lung tissue samples needs to be considered for evaluating cellular changes and functional properties of these cells in culture.

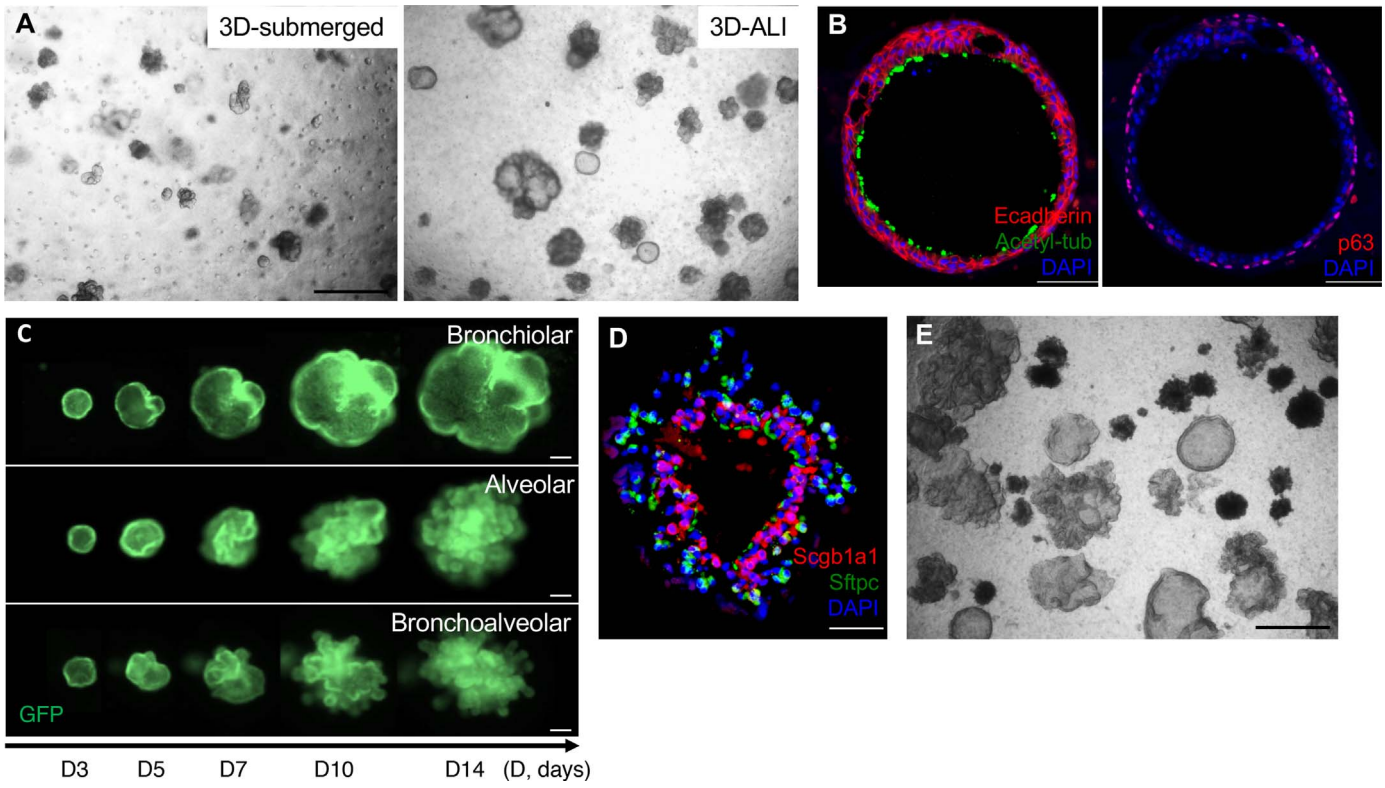

Fig. 2. Lung organoids grown *in vitro*. (A) Representative images of organoids derived from 5000 EpCAM^{+}Sca1^{+}BASCs in 3D-submerged co-culture (left) or 3D-ALI co-culture (right) with mouse lung endothelial cells (LuMECs) at 14 days in culture. Note better organoid formation of BASCs in 3D-ALI culture compared to one in 3D-submerged culture. Scale bar, 500 μm. (B) Representative immunofluorescence (IF) for lung cell markers in a basal sphere derived from Ngfr^{+} mouse basal cells; Ecadherin (red), Acetylated tubulin (green), and DAPI (blue) (left); Trp63 (red) and DAPI (blue) (right). Scale bar, 50 μm. (C) Representative images of GFP fluorescent organoids from 3D co-culture of EpCAM^{+}Sca1^{+} BASCs isolated from β-actin-GFP mice with LuMECs for 14 days in 3D-ALI culture. Starting with nearly spherical geometry, the organoids develop lumen-like structures (top), alveolar-like structures (middle), and mixed phenotype with distinct budding structures (bottom). Scale bar, 100 μm. (D) Representative IF for lung cell markers in a bronchioalveolar sphere derived from a single BASC; CCSP (red), SPC (green), and DAPI (blue). Scale bar, 50 μm. (E) The BASC organoids have been in continuous culture with bi-weekly passaging for 6 months and grow and differentiate normally. Scale bar, 500 μm.

Table 2
Compositions of culture medium for lung organoids.

Culture medium	mTEC/Plus medium	ALI medium	BEGM medium	3D basic medium
Culture cells	Mouse basal cells (Rock et al., 2011a, 2011b, 2009) Human basal cells (Hegab et al., 2012) Mouse AT2 cells (Barkauskas et al., 2013)	Human basal cells (Rock et al., 2009)	Human basal cells (Ghosh et al., 2011) Human AT2 cells (Barkauskas et al., 2013)	Mouse CD24^{low}Sftpc^{hi} cells (Chen et al., 2012; McQualter et al.,2010) Mouse Lin^{-}Sca-1^{low} (Teisanu et al., 2011) Mouse EpCAM^{+}Sca-1^{+} cells (Lee et al., 2014)
Components	DMEM-Ham's F-12 Penicillin/streptomycin 4 mM L-glutamine 10 μg/ml insulin 5 μg/ml ITS (bovine insulin/ transferrin/selenium) 0.1 μg/ml cholera toxin 25 ng/ml Epidermal Growth Factor (EGF) 25 ng/ml Fibroblast Growth Factor (FGF) 0.01 μg/ml retinoic acid (RA) 5% Fetal Bovine Serum (FBS)	DMEM-Ham's F-12 Penicillin/streptomycin 0.5 mg/ml Bovine serum albumin 0.87 μM insulin 0.21 μM Hydrocortisome 0.01 μM Triiodothyronine 0.5 ng/ml Epidermal Growth Factor (EGF) 2.7 μM Epinephrine 5×10^{-8} M retinoic acid (RA) 0.5 μM phosphorylethanolamine 0.5 μM Ethanolamine 3 μM Zinc sulfate	ALI medium (with 25 ng/ml EGF instead 0.50 ng/ml in ALI medium) + 50 μg/ml Gentamicin 0.25 μg/ml Amphotericin	DMEM-Ham's F-12 Penicillin/streptomycin 4 mM L-glutamine 1 mM HEPES 5 μg/ml ITS (bovine insulin/ transferrin/selenium) 10% Fetal Bovine Serum (FBS)
	You et al., 2002 **You and Brody, 2013**	**Fulcher et al., 2005;** **Fulcher and Randell, 2013**	**Fulcher et al., 2005;** **Fulcher and Randell, 2013**	**Lee et al., 2014**

3.2.2. Lung organoids derived from distal airway stem and progenitor cells

Three dimensional-ALI organoid cultures provide a reliable *in vitro* model system for the regeneration of distal airways and alveolar epithelium from endogenous airway stem and progenitor cells. Importantly, the limited ability of distal stem and progenitor cells to form organoids in the absence of supporting cells has led to developing a more advanced culture system, called organoid co-culture. Various methods have been developed to isolate club cells or subsets of club cells using surface markers or *Scgb1a1-CreER* knock-in allele which labels club cells. Distal EpCAM^{hi}Itgα6^{+}β4^{+}CD24^{low} lung epithelial cells have developed distinct morphological spheres only when co-cultured with EpCAM^{-}Sca-1^{+} lung mesenchymal cells followed by seeding onto the transwell insert in 1:1 Matrigel/3D basic media (Table 2; McQualter et al., 2010). After 14 days in culture, individual spheres in this culture were reported to retain airway and/or alveolar lineage cells. Co-culture of CD45^{-}CD31^{-}CD34^{-} (Lin^{-}) Sca-1^{low} epithelial cells or lineage-labelled club cells from *Scgb1a1-CreER*;*Rosa26-mT/mG* mice with an immortalized fibroblast cell line (MLg, neonatal mouse lung fibroblasts) in 3D basic media has also revealed subsets of distal epithelial progenitor cells producing airway and/or alveolar lineage cells (Chen et al., 2012; Teisanu et al., 2011). In this culture, TGFβ inhibitor (SB431542) was added to allow cell growth for the initial 7–10 days. A more elegant study has been done with cells isolated from transgenic *Sftpc-GFP* mice which allow for the segregation of airway epithelial cells located in the different regions based on expression levels of GFP fluorescence (Chen et al., 2012). GFP^{-} cells, located in the proximal airways, generated cystic spheres retaining airway lineage cells, whereas GFP^{hi} cells, located in the terminal bronchioles and alveoli, formed alveolar-like spheres retaining alveolar lineage cells. GFP^{low} cells, located in the bronchioles, revealed to retain progenitor cells that give rise to airway and alveolar lineage cells. More recently, isolated EpCAM^{+}Sca-1^{+} BASCs have been demonstrated to generate distinct spheres including bronchiolar, alveolar, and bronchioalveolar (mixe) spheres when co-cultured with mouse lung endothelial cells (LuMECs) (Fig. 2C; Lee et al., 2014). The spheres derived from a single EpCAM^{+}Sca-1^{+} BASCs retain multiple lineages including club cells, ciliated cells, goblet cells, AT2 and AT1 cells. Sftpc^{+} AT2 cells are found in the outer layer of branching spheres and airway cells including Scgb1a1^{+}club cells, Acetyl-tub^{+}ciliated cells, and Muc5ac^{+}goblet cells reside inside of spheres (Fig. 2D; Lee et al., 2014). Low incidence of this "bronchioalveolar" sphere formation from EpCAM^{+}Sca-1^{+} BASCs suggests the strong need of additional surface markers to isolate pure BASCs which may retain multi-potency in lung regeneration. At 14 days in culture, dissociated organoids are sorted by EpCAM and re-seeded with fresh stromal cells for the next round culture. Co-culture with LuMECs permits the clonal long-term expansion of BASCs whilst maintaining their differentiation potential for more than 6 months by bi-weekly passages (Fig. 2E). Moreover, bronchioalveolar spheres were able to form epithelial structures retaining Sftpc- and/or Scgb1a1-expressing cells when co-transplanted into recipient mice with LuMEC subcutaneously. Given the heterogeneity of airway epithelial stem and progenitor cells in their regenerative potential, tracing individual cell populations producing distinct spheres using real-time live imaging combined with a reporter assay could provide important clues to define the subsets of progenitor cells giving rise to specific lineage cells.

3.2.3. Lung organoids derived from alveolar stem and progenitor cells

For organoid cultures of alveolar epithelium, lineage-labelled AT2 cells from *Sftpc-CreER;Rosa-Tomato* mice or GFP^{hi} cells from *Sftpc-H2B-GFP* mice were placed into 3D-ALI co-culture with Pdgfrα^{+} stromal cells or lung fibroblasts (MLg), respectively (Barkauskas et al., 2013; Lee et al., 2013). Alternatively using cell surface markers, EpCAM^{med} or EpCAM^{+}Sca-1^{-} cells isolated from distal lungs were also cultured with EpCAM^{-}Sca1^{+} lung mesenchymal cells or LuMECs, respectively (Lee et al., 2014; McQualter et al., 2010). Alveolar-like structures were formed and consisted of alveolar lineage cells, including Sftpc-expressing AT2 cells in the outer layer of branching spheres and AT1 cells expressing T1α (Pdpn) and homeodomain only protein x (Hopx) within the spheres (Barkauskas et al., 2013). Notably, no proliferating spheres were seen when AT2 cells were plated in 3D-ALI culture without stromal cells, indicating the requirement of niche factors supplied in a direct or indirect manner for the stem cell activity of AT2 cells. EpCAM^{+}HTII-280^{+} AT2 cells derived from human lungs formed alveolar spheres in 3D-ALI co-cultures with human lung fibroblasts (MRC5). However, no cells morphologically resembling or expressing AT1 markers were detected in these spheres (Barkauskas et al., 2013). Together, these studies demonstrate the utility of organoid cultures in investigating the differentiation potential of distal lung stem cells. Furthermore, 3D-ALI organoid culture systems combined with co-cultures are able to answer specific questions about the function of the niche in the regulation of adult lung stem cells and the signalling involved in this process. In other hand, defining the niche factors required for distal epithelial organoid formation will lead to develop a stroma-free culture system which allow for the robust expansion of distal lung organoids and their genetic manipulation.

4. Applications of lung organoids

An additional advantage of adult stem cell-derived lung organoids is that they allow for the expansion of lung stem cell populations and the induction of differentiated cells from very limited amounts of starting material. These special characteristics of organoids provide us with an excellent model system for a wide range of applications both in basic and translational approaches such as drug screening, disease modelling and genetic screening. We will discuss current and potential applications of lung organoids.

4.1. Organoids for understanding lung tissue regeneration and niche function

The organoids derived from adult stem and progenitor cells reliably retain their *in vivo* regenerative programme *in vitro* thus provide detailed snapshots of tissue restoration after injury. For example, club cells maintain bronchiolar epithelium by self-renewal over the long-term, producing ciliated cells, and barely contribute to alveolar epithelium in the steady state (Rawlins et al., 2009). However, under certain conditions such as bleomycin-induced damage, club cells actively replace damaged alveolar epithelial cells (Rock et al., 2011a; Tropea et al., 2012). In 3D-ALI organoid culture of club cells or subsets of club cells including BASCs, clonal organoids retaining airway and/or alveolar lineage cells are generated from uninjured club cells. This suggests that *in vitro* organoid culture conditions promote the activation of the stem or progenitor programme, thereby providing a powerful model for studying the early reprogramming phases of the differentiated mature cells to an uncommitted state (Chen et al., 2012; Lee et al., 2014; McQualter et al., 2010). Moreover, recent studies took the advantage of this organoid system in screening factors that impact lineage specification of stem cells. IL-6, of which expression is changed in stroma during injury repair, revealed to induce basal cell differentiation toward a ciliated cell fate *versus* secretory club cells (Tadokoro et al., 2014). The bronchosphere from human basal cells respond to IL-13 which directs goblet cell lineage with a decline in ciliated cells (Danahay et al., 2015). Inhibition of BMP signalling using BMP antagonist promoted proliferation and clonal expansion of basal cells without changes in lineage specification (Tadokoro et al., 2016). It remains to be validated if differentiation of organoids gives rise to mature functional epithelial cells which can be used for transplantation.

In addition, lung organoids allow researchers to study the processes governing the homeostatic regulation of lung tissue and, most im-

portantly, to gain insights into the interaction and crosstalk between stem cells and their niche. Whereas our capacity to initiate the specific alteration either of stromal cells or ECM composition *in vivo* is limited, the organoid co-culture system makes it possible to precisely manipulate the components of the microenvironment in order to further determine their functional roles in modulating lung stem cells. Lipofibroblasts expressing Pdgfrα have been reported to support alveolar organoid formation from adult AT2 cells (Barkauskas et al., 2013). Fully differentiated AT1 cells were found in these organoids, confirming the regulatory role of the Pdgfrα signalling pathway in alveologenesis. A dedicated regulatory signalling pathway (BMP4-Bmpr1a-calcineurin/NFATc1-TSP1) that operates between BASCs/AT2 and endothelial cells has been demonstrated to drive alveolar lineage specification. (Lee et al., 2014). Notably, modification of LuMECs in co-culture of BASCs directed lineage specification of BASCs suggesting extrinsic regulation of epithelial differentiation. Further study is required to determine the importance of vascular cells and these signalling pathways in lung diseases where dysregulated vascular cells are involved in the pathogenesis. In addition, a great deal of evidence has been emerging for a complex co-ordination between epithelial and immune cells during repair and regeneration processes after lung damage. Two-photon analysis has shown migrating lymphocytes and myeloid cells into sites of damage after injury (Kreisel et al., 2010; Looney et al., 2011). Immediate damage is most likely a result of an acute inflammatory response from the myeloid-mediated breakdown of lung epithelial cells, however the protective role of immune response in tissue regeneration has also been reported (Johnson and Matthay, 2010; Levy and Serhan, 2014). Utilising co-culture system, cellular and molecular effectors of immune cells in stem cell regulation can be determined. Together, the organoid culture system allows the study of the network between stem cells and their niche, and in turn this knowledge can be used in the rational design of organoid cultures to more closely mimic the *in vivo* environment.

4.2. Organoids for disease modelling

Preclinical models for human disease are essential for the basic understanding of disease pathology and its translational application into efficient treatment for patients. Patient-derived organoid cultures from biopsies and/or surgical resections present a novel improvement to this end (Neal and Kuo, 2016). Disease modelling will likely be a primary focus of future organoid studies, encompassing developmental disorders, tumorigenesis, infectious disease and degenerative diseases.

4.2.1. Organoids for lung cancer

Lung cancer is a heterogeneous disease comprised of different histological subtypes. These tumors arise from sequential aberrations allowing uncontrolled proliferation and the gradual loss of differentiation, which may contribute to heterogeneity of tumor cells (Hanahan and Weinberg, 2011). Although the cell(s)-of-origin for lineage-specific lung cancers remains unclear, evidence suggests that the tumor cell-of-origin and the oncogenic mutations acquired during tumorigenesis together determine the pathological subtype and thence the required therapy. Adult primary epithelial cell-derived organoids combined with CRISPR/Cas-mediated genetic engineering technology may allow converting normal organoids into their respective malignancies in the dish by adding combinations of cancerous mutations as a sequential manner. The majority of lung cancer-driver mutations have been reported to occur early during tumorigenesis. Therefore, establishing a model system for functional validation of critical drivers that convert the organoids to a cancerous phenotype will advance the detection of early tumorigenic events which would be an important step towards an improved prognosis for lung cancer. In addition, driving tumorigenesis in diverse adult lung stem and progenitor cells, such as basal cells, BASCs and AT2 cells, which have been suggested as the cell-of-origin in non-small cell lung cancer (NSCLC) (Chen et al., 2014), will provide a model systems not only to recapitulate human lung cancer but also to further assess responses to combination drug therapy based on specific subtype and genotype vulnerabilities.

4.2.2. Organoids for cystic fibrosis (CF)

Model systems for cystic fibrosis (CF) in mice containing mutations in the CFTR gene fail to sufficiently recapitulate the human symptoms of the disease (Cohen and Prince, 2012; Ratjen and Doring, 2003). Therefore, patient-derived organoids from both adult tissues and induced pluripotent stem cells (iPSCs) represent an alternative tool for studying the biology and pathology of cystic fibrosis. The expansion capacity of patient-derived organoids provides the opportunity for high-throughput analysis, including molecular pathogenesis, genetic screening and the reliable *ex vivo* study of cell signalling pathways in the disease environment. A recent study demonstrating the successful generation of CF patient-specific airway epithelium *via* non-invasive approaches such as bronchoscopic lavage or nasal brushing is expected to advance the application of patient-derived organoids in disease modelling (Mou et al., 2016). However, the partial loss of cellular physiological function including is gradual loss of CFTR-mediated chloride and ENaC-mediated sodium conductance in these *in vitro*-expanded epithelia will have to be resolved. Future work will be needed to better establish an organoid culture model that is amenable to genetic engineering, following assessment of physiological properties, in adult lung epithelial cells.

4.3. Organoids for drug development and therapeutics

Recent advances in growing adult epithelial stem cells allow patient samples to be maintained and grown in culture. This ability to rapidly generate organoids from individual patients enables the personalized testing of chemotherapies and targeted therapies. Recently, patient-derived organoids from human lung cancer biopsies have been established and have enabled the analysis of resistance mechanisms for targeted therapies in individual patients (Endo et al., 2013; Kimura et al., 2015). Lung tumor organoids derived from individual human samples were cultured in Matrigel with a high success rate. Importantly, it is possible to establish organoids derived from non-cancerous and cancer region from individual patient. In this study, the neuregulin 1 (Ngr1)-Her3 signalling pathway proved to be essential for the culture of organoids from NSCLCs. This lung patient-derived organoid model system has been utilized to examine the canonical sensitivity of Egfr-mutant lung cancer to the tyrosine kinase inhibitor (TKI) Erlotinib and to analyse the mechanism of resistance to Crizotinib in lung cancer patients harboring the Eml-Alk4 fusion. Interestingly, efficiency of tumor organoid formation varies in lung tumor subtypes; organoids from adenocarcinoma have the benefit of growth compared to one from squamous carcinoma. Identifying niche factor requirements for tumor organoids of each subtypes will provide insight into predicting the drug-resistance of specific tumor subtypes. Finally, the ability to expand organoids retaining stem cell populations enables the screening of small molecules or drugs that govern lineage specification as discussed previously. Such screens will provide the insight into developing new therapies for lung diseases in which the specific lineages are disrupted.

5. Future prospects in lung organoid systems

Knowledge about lung stem cells during homeostasis and regeneration has markedly increased through recent research. Advances in understanding the role of the stem cell microenvironment and the crosstalk between stem cells and their niche has allowed researchers to develop highly reproducible organoid systems that closely recapitulate *in vivo* conditions to study tissue regeneration. Using lung organoids, we can investigate the function of lung stem cells residing in distinctive regions of lung tissue, while also monitoring their behaviour in diverse

microenvironments. Although lung organoid culture systems are still in their infancy, it is becoming possible to extend their usage to biomedical applications such as in disease modelling and treatment. Generation of patient-derived organoids from biopsies provide a powerful resource for a wide range of translational and medical approaches. This system will be useful for studying drug toxicity and efficacy to finally realize the promise of personalized medicine. However, there are still a number of concerns that need to be addressed: (1) The physiological functions of differentiated cells need to be preserved over time in organoid culture. (2) Optimised culture conditions for the reproducible direction of lineage specification with high efficiency are essential to enable organoid cultures to have clinical impact. (3) The limited presence of niche components in the organoid system restricts the study of their role in lung disease modelling, such as during the inflammatory response to injury or drug administration. (4) Epigenetic modulation, which can result in genetic instability, needs to be evaluated in long-term cultures of lung organoids. (5) Variation in patient-to-patient needs to be considered for establishing patient-derived organoids which can be used for the therapeutic purpose. (6) A high-throughput platform for screening molecules and drugs in 3D organoids, already shown to be feasible (Danahay et al., 2015), and the analysis of downstream target gene expression should be established with better read-out methods. Such trials could represent a new generation of a drug screening for pharmacogenomics profiling that may lead early-phase clinical trials of novel drugs and rational therapeutic strategies.

Addressing the remaining issues described above in lung organoids would provide important advances towards the bioengineering of whole lung tissue and could significantly extend their utility in various arenas. The novel and rapid advances in lung organoid technology offer a promising perspective for studying lung development and for future clinical applications.

Acknowledgements

JC and JHL are supported by the Wellcome Trust Sir Henry Dale Fellowship that is jointly funded by the Wellcome Trust and the Royal Society (107633/Z/15/Z). EI and JHL are funded by European Research Council Starting Grant (679411). We would like to thank Christopher J. Hindley for helpful editing of the manuscript. We apologise to those scientists whose work could not be cited due to space restrictions.

References

Alescio, T., Colombo Piperno, E., 1967. A quantitative assessment of mesenchymal contribution to epithelial growth rate in mouse embryonic lung developing in vitro. J. Embryol. Exp. Morphol. 17, 213–227.
Atherton, H.C., Jones, G., Danahay, H., 2003. IL-13-induced changes in the goblet cell density of human bronchial epithelial cell cultures: MAP kinase and phosphatidylinositol 3-kinase regulation. Am. J. Physiol. Lung Cell Mol. Physiol. 285, L730–L739.
Bals, R., Beisswenger, C., Blouquit, S., Chinet, T., 2004. Isolation and air-liquid interface culture of human large airway and bronchiolar epithelial cells. J. Cyst. Fibros. 3 (Suppl. 2), S49–S51.
Barkauskas, C.E., Cronce, M.J., Rackley, C.R., Bowie, E.J., Keene, D.R., Stripp, B.R., Randell, S.H., Noble, P.W., Hogan, B.L., 2013. Type 2 alveolar cells are stem cells in adult lung. J. Clin. Investig. 123, 3025–3036.
Benton, G., Arnaoutova, I., George, J., Kleinman, H.K., Koblinski, J., 2014. Matrigel: from discovery and ECM mimicry to assays and models for cancer research. Adv. Drug Deliv. Rev. 79–80, 3–18.
Berube, K., Prytherch, Z., Job, C., Hughes, T., 2010. Human primary bronchial lung cell constructs: the new respiratory models. Toxicology 278, 311–318.
Borthwick, D.W., Shahbazian, M., Krantz, Q.T., Dorin, J.R., Randell, S.H., 2001. Evidence for stem-cell niches in the tracheal epithelium. Am. J. Respir. Cell Mol. Biol. 24, 662–670.
Bowden, D.H., 1983. Cell turnover in the lung. Am. Rev. Respir. Dis. 128, S46–S48.
Butler, C.R., Hynds, R.E., Gowers, K.H., Lee, D.D., Brown, J.M., Crowley, C., Teixeira, V.H., Smith, C.M., Urbani, L., Hamilton, N.J., Thakrar, R.M., Booth, H.L., Birchall, M.A., De Coppi, P., Giangreco, A., O'Callaghan, C., Janes, S.M., 2016. Rapid expansion of human epithelial stem cells suitable for Airway Tissue Engineering. Am. J. Respir. Crit. Care Med..
Carraro, G., Stripp, B.R., 2015. A new notch for lung stem cells. Cell Stem Cell 16, 107–109.
Chander, A., Dodia, C.R., Gil, J., Fisher, A.B., 1983. Isolation of lamellar bodies from rat granular pneumocytes in primary culture. Biochim. Biophys. Acta 753, 119–129.
Chen, H., Matsumoto, K., Brockway, B.L., Rackley, C.R., Liang, J., Lee, J.H., Jiang, D., Noble, P.W., Randell, S.H., Kim, C.F., Stripp, B.R., 2012. Airway epithelial progenitors are region specific and show differential responses to bleomycin-induced lung injury. Stem Cells 30, 1948–1960.
Chen, Z., Fillmore, C.M., Hammerman, P.S., Kim, C.F., Wong, K.K., 2014. Non-small-cell lung cancers: a heterogeneous set of diseases. Nat. Rev. Cancer 14, 535–546.
Cohen, T.S., Prince, A., 2012. Cystic fibrosis: a mucosal immunodeficiency syndrome. Nat. Med. 18, 509–519.
Danahay, H., Pessotti, A.D., Coote, J., Montgomery, B.E., Xia, D., Wilson, A., Yang, H., Wang, Z., Bevan, L., Thomas, C., Petit, S., London, A., LeMotte, P., Doelemeyer, A., Velez-Reyes, G.L., Bernasconi, P., Fryer, C.J., Edwards, M., Capodieci, P., Chen, A., Hild, M., Jaffe, A.B., 2015. Notch2 is required for inflammatory cytokine-driven goblet cell metaplasia in the lung. Cell Rep. 10, 239–252.
Desai, T.J., Brownfield, D.G., Krasnow, M.A., 2014. Alveolar progenitor and stem cells in lung development, renewal and cancer. Nature 507, 190–194.
Diglio, C.A., Kikkawa, Y., 1977. The type II epithelial cells of the lung. IV. Adaption and behavior of isolated type II cells in culture. Lab Investig. 37, 622–631.
Douglas, W.H., McAteer, J.A., Smith, J.R., Braunschweiger, W.R., 1979. Type II alveolar pneumonocytes in vitro. Int. Rev. Cytol. Suppl., 45–65.
Endo, H., Okami, J., Okuyama, H., Kumagai, T., Uchida, J., Kondo, J., Takehara, T., Nishizawa, Y., Imamura, F., Higashiyama, M., Inoue, M., 2013. Spheroid culture of primary lung cancer cells with neuregulin 1/HER3 pathway activation. J. Thorac. Oncol. 8, 131–139.
Fata, J.E., Mori, H., Ewald, A.J., Zhang, H., Yao, E., Werb, Z., Bissell, M.J., 2007. The MAPK(ERK-1,2) pathway integrates distinct and antagonistic signals from TGFalpha and FGF7 in morphogenesis of mouse mammary epithelium. Dev. Biol. 306, 193–207.
Fatehullah, A., Tan, S.H., Barker, N., 2016. Organoids as an in vitro model of human development and disease. Nat. Cell Biol. 18, 246–254.
Fulcher, M.L., Randell, S.H., 2013. Human nasal and tracheo-bronchial respiratory epithelial cell culture. Methods Mol. Biol. 945, 109–121.
Ghosh, M., Brechbuhl, H.M., Smith, R.W., Li, B., Hicks, D.A., Titchner, T., Runkle, C.M., Reynolds, S.D., 2011. Context-dependent differentiation of multipotential keratin 14-expressing tracheal basal cells. Am. J. Respir. Cell Mol. Biol. 45, 403–410.
Giangreco, A., Reynolds, S.D., Stripp, B.R., 2002. Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction. Am. J. Pathol. 161, 173–182.
Goss, R.J., 1966. Hypertrophy versus hyperplasia. Science 153, 1615–1620.
Hackett, N.R., Shaykhiev, R., Walters, M.S., Wang, R., Zwick, R.K., Ferris, B., Witover, B., Salit, J., Crystal, R.G., 2011. The human airway epithelial basal cell transcriptome. PLoS One 6, e18378.
Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646–674.
Hegab, A.E., Ha, V.L., Darmawan, D.O., Gilbert, J.L., Ooi, A.T., Attiga, Y.S., Bisht, B., Nickerson, D.W., Gomperts, B.N., 2012. Isolation and in vitro characterization of basal and submucosal gland duct stem/progenitor cells from human proximal airways. Stem Cells Transl. Med. 1, 719–724.
Hegab, A.E., Ha, V.L., Gilbert, J.L., Zhang, K.X., Malkoski, S.P., Chon, A.T., Darmawan, D.O., Bisht, B., Ooi, A.T., Pellegrini, M., Nickerson, D.W., Gomperts, B.N., 2011. Novel stem/progenitor cell population from murine tracheal submucosal gland ducts with multipotent regenerative potential. Stem Cells 29, 1283–1293.
Hong, K.U., Reynolds, S.D., Giangreco, A., Hurley, C.M., Stripp, B.R., 2001. Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion. Am. J. Respir. Cell Mol. Biol. 24, 671–681.
Hong, K.U., Reynolds, S.D., Watkins, S., Fuchs, E., Stripp, B.R., 2004a. Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. Am. J. Pathol. 164, 577–588.
Hong, K.U., Reynolds, S.D., Watkins, S., Fuchs, E., Stripp, B.R., 2004b. In vivo differentiation potential of tracheal basal cells: evidence for multipotent and unipotent subpopulations. Am. J. Physiol. Lung Cell Mol. Physiol. 286, L643–L649.
Jain, R., Barkauskas, C.E., Takeda, N., Bowie, E.J., Aghajanian, H., Wang, Q., Padmanabhan, A., Manderfield, L.J., Gupta, M., Li, D., Li, L., Trivedi, C.M., Hogan, B.L., Epstein, J.A., 2015. Plasticity of Hopx(+) type I alveolar cells to regenerate type II cells in the lung. Nat. Commun. 6, 6727.
Johnson, E.R., Matthay, M.A., 2010. Acute lung injury: epidemiology, pathogenesis, and treatment. J. Aerosol Med Pulm. Drug Deliv. 23, 243–252.
Kim, C.F., Jackson, E.L., Woolfenden, A.E., Lawrence, S., Babar, I., Vogel, S., Crowley, D., Bronson, R.T., Jacks, T., 2005. Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell 121, 823–835.
Kimura, M., Endo, H., Inoue, T., Nishino, K., Uchida, J., Kumagai, T., Kukita, Y., Kato, K., Imamura, F., Inoue, M., 2015. Analysis of ERBB ligand-induced resistance mechanism to crizotinib by primary culture of lung adenocarcinoma with EML4-ALK fusion gene. J. Thorac. Oncol. 10, 527–530.
Kotton, D.N., Morrisey, E.E., 2014. Lung regeneration: mechanisms, applications and emerging stem cell populations. Nat. Med. 20, 822–832.
Kreisel, D., Nava, R.G., Li, W., Zinselmeyer, B.H., Wang, B., Lai, J., Pless, R., Gelman, A.E., Krupnick, A.S., Miller, M.J., 2010. In vivo two-photon imaging reveals monocyte-dependent neutrophil extravasation during pulmonary inflammation. Proc. Natl. Acad. Sci. USA 107, 18073–18078.
Lee, J.H., Bhang, D.H., Beede, A., Huang, T.L., Stripp, B.R., Bloch, K.D., Wagers, A.J., Tseng, Y.H., Ryeom, S., Kim, C.F., 2014. Lung stem cell differentiation in mice

directed by endothelial cells via a BMP4-NFATc1-thrombospondin-1 axis. Cell 156, 440–455.
Lee, J.H., Kim, J., Gludish, D., Roach, R.R., Saunders, A.H., Barrios, J., Woo, A.J., Chen, H., Conner, D.A., Fujiwara, Y., Stripp, B.R., Kim, C.F., 2013. Surfactant protein-C chromatin-bound green fluorescence protein reporter mice reveal heterogeneity of surfactant protein C-expressing lung cells. Am. J. Respir. Cell Mol. Biol. 48, 288–298.
Levy, B.D., Serhan, C.N., 2014. Resolution of acute inflammation in the lung. Annu Rev. Physiol. 76, 467–492.
Liu, Y., Stein, E., Oliver, T., Li, Y., Brunken, W.J., Koch, M., Tessier-Lavigne, M., Hogan, B.L., 2004. Novel role for Netrins in regulating epithelial behavior during lung branching morphogenesis. Curr. Biol. 14, 897–905.
Looney, M.R., Thornton, E.E., Sen, D., Lamm, W.J., Glenny, R.W., Krummel, M.F., 2011. Stabilized imaging of immune surveillance in the mouse lung. Nat. Methods 8, 91–96.
McQualter, J.L., Yuen, K., Williams, B., Bertoncello, I., 2010. Evidence of an epithelial stem/progenitor cell hierarchy in the adult mouse lung. Proc. Natl. Acad. Sci. USA 107, 1414–1419.
Mori, M., Mahoney, J.E., Stupnikov, M.R., Paez-Cortez, J.R., Szymaniak, A.D., Varelas, X., Herrick, D.B., Schwob, J., Zhang, H., Cardoso, W.V., 2015. Notch3-Jagged signaling controls the pool of undifferentiated airway progenitors. Development 142, 258–267.
Mou, H., Vinarsky, V., Tata, P.R., Brazauskas, K., Choi, S.H., Crooke, A.K., Zhang, B., Solomon, G.M., Turner, B., Bihler, H., Harrington, J., Lapey, A., Channick, C., Keyes, C., Freund, A., Artandi, S., Mense, M., Rowe, S., Engelhardt, J.F., Hsu, Y.C., Rajagopal, J., 2016. Dual SMAD signaling inhibition enables long-term expansion of diverse epithelial basal cells. Cell Stem Cell.
Neal, J.T., Kuo, C.J., 2016. Organoids as models for neoplastic transformation. Annu. Rev. Pathol. 11, 199–220.
Neuringer, I.P., Sloan, J., Budd, S., Chalermskulrat, W., Park, R.C., Stonebraker, J.R., O'Neal, W.K., Aris, R.M., Randell, S.H., 2005. Calcineurin inhibitor effects on growth and phenotype of human airway epithelial cells in vitro. Am. J. Transpl. 5, 2660–2670.
Oeztuerk-Winder, F., Guinot, A., Ochalek, A., Ventura, J.J., 2012. Regulation of human lung alveolar multipotent cells by a novel p38alpha MAPK/miR-17-92 axis. EMBO J. 31, 3431–3441.
Pageau, S.C., Sazonova, O.V., Wong, J.Y., Soto, A.M., Sonnenschein, C., 2011. The effect of stromal components on the modulation of the phenotype of human bronchial epithelial cells in 3D culture. Biomaterials 32, 7169–7180.
Pardo-Saganta, A., Law, B.M., Tata, P.R., Villoria, J., Saez, B., Mou, H., Zhao, R., Rajagopal, J., 2015. Injury induces direct lineage segregation of functionally distinct airway basal stem/progenitor cell subpopulations. Cell Stem Cell 16, 184–197.
Peerani, R., Zandstra, P.W., 2010. Enabling stem cell therapies through synthetic stem cell-niche engineering. J. Clin. Investig. 120, 60–70.
Qiao, J., Sakurai, H., Nigam, S.K., 1999. Branching morphogenesis independent of mesenchymal-epithelial contact in the developing kidney. Proc. Natl. Acad. Sci. USA 96, 7330–7335.
Randell, S.H., Fulcher, M.L., O'Neal, W., Olsen, J.C., 2011. Primary epithelial cell models for cystic fibrosis research. Methods Mol. Biol. 742, 285–310.
Ratjen, F., Doring, G., 2003. Cystic fibrosis. Lancet 361, 681–689.
Rawlins, E.L., Okubo, T., Xue, Y., Brass, D.M., Auten, R.L., Hasegawa, H., Wang, F., Hogan, B.L., 2009. The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium. Cell Stem Cell 4, 525–534.
Reynolds, S.D., Hong, K.U., Giangreco, A., Mango, G.W., Guron, C., Morimoto, Y., Stripp, B.R., 2000. Conditional clara cell ablation reveals a self-renewing progenitor function of pulmonary neuroendocrine cells. Am. J. Physiol. Lung Cell Mol. Physiol. 278, L1256–L1263.
Rock, J.R., Barkauskas, C.E., Cronce, M.J., Xue, Y., Harris, J.R., Liang, J., Noble, P.W., Hogan, B.L., 2011a. Multiple stromal populations contribute to pulmonary fibrosis without evidence for epithelial to mesenchymal transition. Proc. Natl. Acad. Sci. USA 108, E1475–E1483.
Rock, J.R., Gao, X., Xue, Y., Randell, S.H., Kong, Y.Y., Hogan, B.L., 2011b. Notch-dependent differentiation of adult airway basal stem cells. Cell Stem Cell 8, 639–648.
Rock, J.R., Onaitis, M.W., Rawlins, E.L., Lu, Y., Clark, C.P., Xue, Y., Randell, S.H., Hogan, B.L., 2009. Basal cells as stem cells of the mouse trachea and human airway epithelium. Proc. Natl. Acad. Sci. USA 106, 12771–12775.
Simian, M., Hirai, Y., Navre, M., Werb, Z., Lochter, A., Bissell, M.J., 2001. The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells. Development 128, 3117–3131.
Stripp, B.R., Maxson, K., Mera, R., Singh, G., 1995. Plasticity of airway cell proliferation and gene expression after acute naphthalene injury. Am. J. Physiol. 269, L791–L799.
Tadokoro, T., Wang, Y., Barak, L.S., Bai, Y., Randell, S.H., Hogan, B.L., 2014. IL-6/STAT3 promotes regeneration of airway ciliated cells from basal stem cells. Proc. Natl. Acad. Sci. U. S. A. 111, E3641–E3649.
Tadokoro, T., Gao, X., Hong, C.C., Hotten, D., Hogan, B.L., 2016. BMP signaling and cellular dynamics during regeneration of airway epithelium from basal progenitors. Development 143, 764–773.
Tata, P.R., Mou, H., Pardo-Saganta, A., Zhao, R., Prabhu, M., Law, B.M., Vinarsky, V., Cho, J.L., Breton, S., Sahay, A., Medoff, B.D., Rajagopal, J., 2013. Dedifferentiation of committed epithelial cells into stem cells in vivo. Nature 503, 218–223.
Teisanu, R.M., Chen, H., Matsumoto, K., McQualter, J.L., Potts, E., Foster, W.M., Bertoncello, I., Stripp, B.R., 2011. Functional analysis of two distinct bronchiolar progenitors during lung injury and repair. Am. J. Respir. Cell Mol. Biol. 44, 794–803.
Teixeira, V.H., Nadarajan, P., Graham, T.A., Pipinikas, C.P., Brown, J.M., Falzon, M., Nye, E., Poulsom, R., Lawrence, D., Wright, N.A., McDonald, S., Giangreco, A., Simons, B.D., Janes, S.M., 2013. Stochastic homeostasis in human airway epithelium is achieved by neutral competition of basal cell progenitors. Elife 2, e00966.
Tropea, K.A., Leder, E., Aslam, M., Lau, A.N., Raiser, D.M., Lee, J.H., Balasubramaniam, V., Fredenburgh, L.E., Alex Mitsialis, S., Kourembanas, S., Kim, C.F., 2012. Bronchioalveolar stem cells increase after mesenchymal stromal cell treatment in a mouse model of bronchopulmonary dysplasia. Am. J. Physiol. Lung Cell Mol. Physiol. 302, L829–L837.
Vaughan, A.E., Brumwell, A.N., Xi, Y., Gotts, J.E., Brownfield, D.G., Treutlein, B., Tan, K., Tan, V., Liu, F.C., Looney, M.R., Matthay, M.A., Rock, J.R., Chapman, H.A., 2015. Lineage-negative progenitors mobilize to regenerate lung epithelium after major injury. Nature 517, 621–625.
Vaughan, M.B., Ramirez, R.D., Wright, W.E., Minna, J.D., Shay, J.W., 2006. A three-dimensional model of differentiation of immortalized human bronchial epithelial cells. Differentiation 74, 141–148.
Vazin, T., Schaffer, D.V., 2010. Engineering strategies to emulate the stem cell niche. Trends Biotechnol. 28, 117–124.
Watson, J.K., Rulands, S., Wilkinson, A.C., Wuidart, A., Ousset, M., Van Keymeulen, A., Gottgens, B., Blanpain, C., Simons, B.D., Rawlins, E.L., 2015. Clonal dynamics reveal two distinct populations of basal cells in slow-turnover airway epithelium. Cell Rep. 12, 90–101.
Whitcutt, M.J., Adler, K.B., Wu, R., 1988. A biphasic chamber system for maintaining polarity of differentiation of cultured respiratory tract epithelial cells. In Vitro Cell. Dev. Biol. 24, 420–428.
Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T.Y., Karp, C.L., Donaldson, D.D., 1998. Interleukin-13: central mediator of allergic asthma. Science 282, 2258–2261.
Wong, A.P., Bear, C.E., Chin, S., Pasceri, P., Thompson, T.O., Huan, L.J., Ratjen, F., Ellis, J., Rossant, J., 2012. Directed differentiation of human pluripotent stem cells into mature airway epithelia expressing functional CFTR protein. Nat. Biotechnol. 30, 876–882.
You, Y., Brody, S.L., 2013. Culture and differentiation of mouse tracheal epithelial cells. Methods Mol. Biol. 945, 123–143.
You, Y., Richer, E.J., Huang, T., Brody, S.L., 2002. Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. Am. J. Physiol. Lung Cell Mol. Physiol. 283, L1315–L1321.
Zhu, Z., Homer, R.J., Wang, Z., Chen, Q., Geba, G.P., Wang, J., Zhang, Y., Elias, J.A., 1999. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J. Clin. Investig. 103, 779–788.
Zimmermann, B., 1987. Lung organoid culture. Differentiation 36, 86–109.
Zuo, W., Zhang, T., Wu, D.Z., Guan, S.P., Liew, A.A., Yamamoto, Y., Wang, X., Lim, S.J., Vincent, M., Lessard, M., Crum, C.P., Xian, W., McKeon, F., 2015. p63(+)Krt5(+) distal airway stem cells are essential for lung regeneration. Nature 517, 616–620.