Lung organoids: current uses and future promise

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ABSTRACT

Lungs are composed of a system of highly branched tubes that bring air into the alveoli, where gas exchange takes place. The proximal and distal regions of the lung contain epithelial cells specialized for different functions: basal, secretory and ciliated cells in the conducting airways and type II and type I cells lining the alveoli. Basal, secretory and type II cells can be grown in three-dimensional culture, with or without supporting stromal cells, and under these conditions they give rise to self-organizing structures known as organoids. This Review summarizes the different methods for generating organoids from cells isolated from human and mouse lungs, and compares their final structure and cellular composition with that of the airways or alveoli of the adult lung. We also discuss the potential and limitations of organoids for addressing outstanding questions in lung biology and for developing new drugs for disorders such as cystic fibrosis and asthma.

KEY WORDS: Lung organoids, Stem cells, Lung progenitors, Plasticity

Introduction

The main function of the lungs is to enable efficient gas exchange between the air and the blood. For this purpose, they are composed of a complex three-dimensional (3D) system of tubes that terminate in hundreds of millions of highly vascularized distal sacs (Fig. 1). During development, the lungs arise from the anterior foregut as two small rudimentary endodermal buds surrounded by mesoderm and a vascular plexus (Morrisey and Hogan, 2010). The epithelium undergoes extensive branching morphogenesis to give rise to the conducting airways known as bronchi (if they are supported by cartilage) and bronchioles (if they are not). The bronchioles open into the air sacs, known as alveoli, where gas exchange takes place. The epithelium lining the airways is composed mainly of multiciliated cells and secretary cells, including Club and goblet cells. Together, these specialized components produce a thin surface layer of liquid that contains mucins and glycoproteins and serves to moisten the air, provide antimicrobial activity, and move particles directionally out of the lungs. In the larger airways of the mouse lung, and throughout most of the human lung the so-called mucociliary epithelium contains basal cells that function as progenitors of the multiciliated and secretary populations. By contrast, the air sacs are lined by two other distinct cell types: specialized alveolar type II cells (AEC2s) that secrete surfactants and other proteins; and very thin, delicate type I cells (AEC1s) that provide an extensive surface area for gas exchange with the surrounding capillaries. The mesoderm of the embryonic lung gives rise to numerous specialized cell populations that interact closely with the conducting airways, such as cartilage, smooth muscle, and fibroblasts, the alveolar epithelium, which includes myofibroblasts and lipofibroblasts, and the vasculature, which includes pericytes and vascular smooth muscle cells. Other important cell populations of the lung are the outer mesothelial layer and immune cells. The latter comprises T cells, mast cells, eosinophils, dendritic cells and distinct populations of macrophages that either reside permanently in the alveoli or interstitium or that traffic in and out of the lung in response to injury or infection (Tan and Krasnow, 2016). Immune cells are not the only source of pro- and anti-inflammatory cytokines in the lung; the epithelial cells themselves are known to produce numerous cytokines directly in response to injury or pathogens and they contribute to the impressive innate immunity functions of the lung (Whitsett and Alenghat, 2015).

Under ideal environmental conditions, such as those encountered by laboratory mice in specific pathogen-free units, cell turnover in the lung is very low. However, in real life, the human lung is directly exposed to many airborne hazards. Among these are pollutants, such as tobacco and biofuel smoke, and pathogens such as bacteria, mycobacteria and viruses. These agents, as well as others such as the anticancer drug bleomycin and X rays, can inflict considerable damage on the lungs. Consequently, respiratory diseases, as well as lung cancer, are a major cause of morbidity in vulnerable human populations (www.who.int/respiratory/en/). Fortunately, there are innate mechanisms that can be called into play to repair epithelial damage, and in laboratory animals these are usually remarkably efficient. Over the past few years there have been exciting advances in our understanding of the regenerative processes activated in different regions of the lung in response to various injuries, and of the relative roles of either undifferentiated stem/progenitor cells or specialized cells that can proliferate and undergo phenotypic reprogramming (transdifferentiation) (Hogan et al., 2014; Tata and Rajagopal, 2017). Despite these advances there is still much to be learned, in particular about the identity of stem/progenitor cells in the human lung and how deficient repair may contribute to pathological conditions such as chronic obstructive pulmonary disease (COPD), emphysema, familial and idiopathic pulmonary fibrosis (IPF) and bronchiolitis obliterans syndrome (BOS). As we shall see, organoids hold great promise in this area and in the quest for new drugs and therapies that enhance endogenous repair. Lung organoids also have considerable potential in the search for new treatments for diseases such as asthma, in which there is an overabundance of mucus-secreting cells as a result of the chronic release of cytokines in response to allergens (see www.nature.com/ni/multimedia/lung), and cystic fibrosis (CF), a genetic condition that leads to an increase in the viscosity of the mucus layer over the surface of the epithelium and to greater risks of bacterial infection and cellular stress.

In the context of this Review, “lung organoids” refers to self-assembling structures generated from lung epithelial progenitor cells cultured in 3D, with or without mesenchymal support cells. These organoids do not yet recapitulate all of the complex structures...
and cellular interactions of the different regions of the lung, especially the highly vascularized and delicate alveolar region. Nevertheless, over the past decade they have become an indispensable tool for basic and translational research. This Review highlights discoveries and advances made using lung organoids derived from three of the epithelial stem/progenitor cell populations of the adult lung: basal cells, airway secretory Club cells (previously known as Clara cells), and AEC2 cells. We also briefly review the current status of lung organoids derived from embryonic and induced pluripotent stem cells. These contain both lung epithelium and mesoderm and, together with cell lines derived from human fetal lung, have the potential to provide important information about human lung development as well as disease. We consider how lung organoids can be used to address questions in lung biology, such as the mechanisms by which endogenous lung progenitors effect repair, and how these might be enhanced by small molecules or drugs. Finally, we discuss some of the major limitations in lung organoid culture and how they might be overcome in the future.

**Basal progenitor cells**

Basal cells make up ~30% of the pseudostratified mucociliary epithelium, lining most of the conducting airways of the human lung and the trachea and main stem bronchi in the mouse. Basal cells adhere closely to the basal lamina and do not extend to the lumen, unlike the more columnar multiciliated and secretory cells, and the minor populations of neuroendocrine and tuff cells that make up the rest of the epithelium (Fig. 1) (Hogan et al., 2014). The luminal cells are connected apically by junctional complexes and play a crucial role in forming a selectively permeable barrier between the external and internal environments of the lung. Genes characteristically expressed with normal histology (Ghosh et al., 2011; Rock et al., 2009; Watson et al., 2015). However, following cell damage by agents typically used experimentally – for example naphthalene, which kills secretory Club cells, or SO₂ gas, which kills all luminal cells – or viral infection there are rapid changes in the behavior and proliferation of the basal cells so that they quickly regenerate the epithelium and restore barrier function. Recently, genetic techniques have been used to kill very selectively most basal cells. In response, some Club secretory cells undergo reprogramming to become Krt5⁻ Trp63⁻ basal cells that can function as stem cells in vivo (Pardo-Saganta et al., 2015; Tata et al., 2013). Taken together, these injury/repair studies have revealed remarkable and rather unexpected flexibility in the way in which basal cells, luminal precursors and differentiated secretory cells can work together to maintain and repair the pseudostratified mucociliary epithelium of the mouse airway. Organoids provide an in vitro model for the regeneration of the mucociliary epithelium from basal cells. They can therefore be used to test regenerative mechanisms proposed from in vivo studies and to screen for drugs, small molecules and molecular pathways that can regulate cellular plasticity and lineage outcomes, as well as crucial epithelial cell functions.

In the human lung, TRP63⁻ KRT5⁻ basal cells are present throughout the airways, extending down to bronchioles of ~1 mm in diameter. There can be considerable variation in their abundance and organization between and within lungs, even from normal donors, with regions of hyperplasia and metaplasia interspersed with normal histology (Ghosh et al., 2011; Rock et al., 2010). Genetic lineage tracing is not possible in the airways of the human
lung. Nevertheless, a very elegant substitute has been developed, based on analysis of the size and cellular composition of clonal pieces of cells carrying mutations in the gene for mitochondrial cytochrome oxidase (Teixeira et al., 2013). The results predict the existence of a multipotent progenitor population of basal cells that maintains the secretory and ciliated cell populations through the stochastic replacement of lost cells. Various methods have been developed for isolating and growing these basal cells from different regions of the normal human respiratory system, including nasal epithelium, ‘large airways’, which include the trachea, primary bronchi and intralobular bronchi down to about the third or fourth generation, and from bronchial brushings (Hackett et al., 2011; Randell et al., 2011). The most efficient methods for expanding and cloning TRP63+ KRT5+ human basal cells involves culturing them either on irradiated mouse 3T3-J2 fibroblasts in the presence of the Rho kinase inhibitor Y-27632 (Butler et al., 2016; Kumar et al., 2011; Suprynówicz et al., 2012), or with a Rho kinase inhibitor together with inhibitors of Smad-dependent signaling through the BMP and TGFβ pathways and an activator of Wnt signaling (Mou et al., 2016). The progenitor properties and differentiation capacity of these basal cells can then be followed in organoid cultures.

**Organoids from mouse basal cells**

The first organoids derived from mouse tracheal basal cells were called tracheospheres. These were clonal, as shown by mixing basal cells constitutively expressing red or green fluorescent proteins (Rock et al., 2009). Typically, flow cytometry is used to isolate the cells from protease-dissociated tissue, based on the surface expression of Ngfr, Itga6 or a carbohydrate that binds the lectin GSLβ4 (Rock et al., 2011; Tata et al., 2013). The cells are seeded into medium containing growth factor-reduced Matrigel and cultured in either transwell inserts or multiwells under conditions in which they do not adhere to the substrate. This can be achieved using a relatively high concentration of Matrigel (50%) or by suspending the cells in a low concentration (2-5%) of gel on top of a cushion of higher concentration (25-40%) (Fig. 2). In the latter condition the cells sink into the lower layer, and some spheres may fuse and therefore not be clonal.

**Step 1: Tissue dissociation into single cells**

**Step 2: Isolation of stem/progenitor cells**

**Step 3: 3D culture with or without stromal cells**

**Step 4: Analyses**

Most of the various culture media used to date (Table 1) are not chemically defined but consist of a medium with ~1 mM calcium and supplements such as bovine pituitary extract (BPE), insulin, transferrin and selenium (ITS), cholera toxin (CTX) and retinoic acid (RA). The most important additive is epidermal growth factor (EGF), which promotes growth. In some protocols the medium is switched after a few days to one with a lower concentration of EGF to slow proliferation and promote differentiation. Rho-associated protein kinase (ROCK) inhibitor Y-27632 is usually added for the first 48 h to promote cell survival. After ~7 days, each sphere has developed a single lumen and there is evidence for differentiation of luminal cells (Fig. 3A). Colony forming efficiency (CFE), which is calculated as the number of spheres that grow compared with the total number of starting progenitor cells, is typically ~3-10%. At 14 days, which is when the spheres are typically harvested, sphere diameters range from 150-500 µm. Immunohistochemistry shows that the majority of spheres of >300 µm diameter have an outer layer of Trp63+ Krt5+, Krt14+, Ngfr+ basal cells and an inner population of Krt8+ columnar ciliated and secretory cells (Fig. 3B,C). At this time, about half of the luminal cells have cilia and express the ciliated cell-specific transcription factor FoxJ1. Although the cilia are motile, there is no evidence for coordination between cells, and manifestation of planar cell polarity (Vladar et al., 2012) has not been explored. The other half of the differentiated luminal cells are secretory cells. For unknown reasons these only express very low levels of the secretoglobin Sgb1a1 (also known as CCSP or CC10), which is normally expressed by secretory Club cells at high levels in vivo. They do, however, express other proximal Club cell markers, namely Sgb3b2 and an antimicrobial peptide known as Saplnc1 (palate, lung and nasal epithelium clone; officially known as Bpifa1) (Musa et al., 2012; Tadokoro et al., 2014) (Fig. 3B,C). Importantly, expression of Saplnc1 and Muc5AC can be dramatically upregulated at the expense of ciliated cell-specific genes by addition of the cytokine IL13 to the culture medium (Fig. 3D,D'). Thus, there is no doubt that single basal cells can give rise to both ciliated and secretory cells in this assay. Differentiation of neuroendocrine cells is rarely seen.

At present it is unclear whether all basal cells and their proposed lineage-biased progenitors (Watson et al., 2015) can potentially give rise to tracheospheres with the same probability, or whether only a
subtlety has this capacity. In the future, live imaging of developing tracheospheres, already shown to be feasible (Rock et al., 2011) (Fig. 2), could be used to follow in real time the asymmetric versus symmetric divisions of individual basal cells and their progeny, coincident with dynamic changes in the activity of Notch and other key signaling pathways.

Mouse tracheospheres have been used to screen for small molecules and drugs that regulate basal cell proliferation and differentiation. Such screens are highly relevant to finding new therapies for lung diseases in which the proportion of ciliated versus secretory cells is disturbed. For example, in patients suffering from asthma, COPD and CF, all of which are associated with inflammation, immune cytokine production and cellular stress, the proportion of mucus-producing cells is greatly increased at the expense of multiciliated cells (Rock et al., 2010). Mucus-secreting cells can be generated in two ways: either directly from Scgb1a1+ Club cells without cell proliferation (Evans et al., 2004; Pardo-Saganta et al., 2015) or from basal cells. Using organoids opens up the possibility of high-throughput screening for compounds that regulate the fate of basal cells, and whether they differentiate into ciliated versus secretory lineages. Although this screening is possible using air-liquid interface (ALI) cultures, in which both multiciliated and secretory cells are generated from basal cells, many more samples can be assayed quickly and quantitatively in a multiwell format. One of the first such screens involved using basal cells from multiwell format. One of the first such screens involved using basal cells from the trachea (tracheospheres) or large airways (bronchospheres). As with mouse basal cells, the culture media for human basal cells are not yet fully defined (Table 1), but contain EGF as the major mitogen.

Table 1. Compendium of protocols for growing organoids from basal cells

<table>
<thead>
<tr>
<th>Basal cell source</th>
<th>Supporting cells</th>
<th>Medium</th>
<th>Matrigel concentration</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Mouse trachea</td>
<td>None</td>
<td>Days 1-7, MTEC*: DMEM/F12 supplemented with ITS, EGF, BPE, CTX, 5% FBS, antibiotics; RA freshly added; Y-27632 (ROCK inhibitor) for the first 2 days</td>
<td>50% mixed with cells</td>
<td>Barkauskas et al., 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Days 7-14, MTEC serum-free medium: DMEM/F12 supplemented with ITS, EGF, BPE, CTX, BSA, antibiotics; RA freshly added</td>
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</tr>
<tr>
<td>Human trachea and large airways</td>
<td>MRC5 human cell line</td>
<td>Air-liquid interface medium: 50:50 DMEM-H and LHC basal medium supplemented with BPE, insulin, EGF, transferrin, hydrocortisone, triiodothyronine, epinephrine, epinephrine, RA, zinc sulfate, phosphorylethanolamine, ethanalamine, antibiotics</td>
<td>20% on the bottom, 2% mixed with cells</td>
<td>Tata et al., 2013</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>50:50 BEBM (Lonza) and DMEM supplemented with BEGM supplements (minus triiodothyronine, gentamycin, amphotericin and RA); 100 nM RA freshly added</td>
<td>Thin layer of 100% on the bottom, 50% mixed with cells</td>
<td>McQuailet al., 2010</td>
</tr>
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<td></td>
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<td>Differentiation medium (Lonza): B-ALI medium supplemented with BPE, insulin, hydrocortisone, GA-1000, transferrin, triiodothyronine, epinephrine and RA</td>
<td>25% on the bottom, 5% mixed with cells</td>
<td>Chen et al., 2012</td>
</tr>
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ITS, insulin, transferrin and selenium; BPE, bovine pituitary extract; BSA, bovine serum albumen; CTX, cholera toxin; EGF, epidermal growth factor; FBS, fetal bovine serum; GA-1000, 30 µg/ml gentamicin and 15 ng/ml amphotericin; RA, retinoic acid.

As with the mouse, organoids from human basal cells have been used to screen for cytokines and other proteins that affect the ratio of ciliated and secretory cells and might therefore be potential therapeutic agents for disorders in which the balance is disrupted, for example chronic asthma. One such study involved plating cells in 384-well trays and analyzing almost 5000 different compounds (Danahay et al., 2015). The results identified a number of proteins that promote mucus cell production, including IL13, and showed that antibodies to NOTCH2 were very effective in inhibiting the proportion of secretory relative to ciliated (FOXJ1+) cells. Currently, screens using human rather than mouse organoids are limited by the paucity of easily scored fluorescent reporters for assaying gene expression. This should change as it becomes more feasible to manipulate basal cells genetically using CRISPR/Cas9.

In assays with human basal cells, it is important to recognize that there is considerable variability in the kinetics of proliferation and apoptosis between different organoids. For example, some organoids may show increased proliferation and apoptosis in response to certain cytokines, while others may show decreased proliferation and increased apoptosis. This variability can make it difficult to interpret the results of screens for compounds that affect basal cell proliferation and differentiation.

Organoids have been obtained from basal cells isolated and expanded from human lungs (see Box 1). Different names have been given to the organoids depending on whether the basal cells are derived from the trachea (tracheospheres) or large airways (bronchospheres). As with mouse basal cells, the culture media for human basal cells are not yet fully defined (Table 1), but contain EGF as the major mitogen. CFE is ~10% and can be increased to ~20% by adding Rho kinase inhibitor and/or a human fibroblast cell line such as MRC5 cells (Fig. 3E). Under standard conditions the organoids contain TRP63+ KRT5+ basal cells, functional multiciliated cells and secretory goblet (MUC5AC+, MUC5B+) cells (Butler et al., 2016; Danahay et al., 2015; Hild and Jaffe, 2016; Rock et al., 2009) (Fig. 3F). Since basal cells also exist in the nasal epithelium, it should be possible to derive organoids, or ‘nasospheres’, from these cells, which would be a particularly convenient approach for generating organoids from patients for eventual drug screening. It is likely that these organoids would give variable results, depending on where in the nose the basal cells are isolated from, since in one published case the luminal cells were reported to differentiate into squamous epithelial cells (Kumar et al., 2011).

As with the mouse, organoids from human basal cells have been used to screen for cytokines and other proteins that affect the ratio of ciliated and secretory cells and might therefore be potential therapeutic agents for disorders in which the balance is disrupted, for example chronic asthma. One such study involved plating cells in 384-well trays and analyzing almost 5000 different compounds (Danahay et al., 2015). The results identified a number of proteins that promote mucus cell production, including IL13, and showed that antibodies to NOTCH2 were very effective in inhibiting the proportion of secretory relative to ciliated (FOXJ1+) cells. Currently, screens using human rather than mouse organoids are limited by the paucity of easily scored fluorescent reporters for assaying gene expression. This should change as it becomes more feasible to manipulate basal cells genetically using CRISPR/Cas9.

In assays with human basal cells, it is important to recognize that there is considerable variability in the kinetics of proliferation and
differentiation of basal cells from different lung donors. Samples from at least three to five different donors are therefore typically used in quantitative assays. Taking this variability into account is important when organoids are used to address outstanding questions in human lung biology. Among these questions are whether the chronic inflammatory conditions prevalent in disorders such as asthma, smoking-associated COPD, and CF result in epigenetic changes in basal cells. Such changes might make them inherently more likely to differentiate into secretory rather than multiciliated cells, or into squamous versus mucociliary epithelium, even when pathological conditions revert to normal (Shaykhiev et al., 2013). Another question under investigation is whether basal cells isolated from different positions along the proximal-distal axis of the human airways have inherently different potentials to give rise to ciliated versus secretory lineages, or even alveolar lineages under certain conditions (Kumar et al., 2011).

Human 3D cultures are well suited to exploit CRISPR/Cas9 gene editing technology to identify genes that regulate important airway functions such as barrier formation, selective permeability, fluid transport, innate immunity and ciliogenesis (Chu et al., 2015; Gao et al., 2015). Recent studies, for example, identified a central role for the transcription factor grainyhead-like 2 (GRHL2) in coordinating barrier function and differentiation, and identified the transcription factor ZNF750 as a new component of the ciliogenesis pathway in the human lung (Gao et al., 2015). In these studies, however, basal cells were not cloned after transfection, and cell populations carrying a mixture of different mutant GRHL2 alleles were tested. Although conditions have been developed in which single basal cells can be cloned in 2D culture (Mou et al., 2016), it remains to be rigorously tested whether each clone retains full differentiation capacity in organoid culture after expansion. Finally, there is great potential in using nasospheres to screen for small molecules and drugs that may regulate or compensate for the activity of mutant forms of the cystic fibrosis transmembrane conductance regulator (CFTR), as measured by fluid transport and sphere diameter. Since nasal basal cells can be isolated with minimal invasion, such an approach might be used in the future to individualize the treatment of patients suffering from CF.
Box 1. Obtaining human lung samples
Human lung epithelial cells (mostly TRP63+ basal cells) isolated from large and small airways are commercially available from companies such as Lonza or Epithelix. The cells will have been expanded over a few passages from primary cultures. Samples from diseased lungs (COPD, asthmatic, CF) are also available. Investigators should obtain as much information as possible about their origin, including donor sex, age, smoking history, time since diagnosis, medications, and disease classification (in the case of COPD). Investigators should also be aware that there is considerable variability in cell growth rates and efficiency of differentiation even among cells from normal donors, and at least three to five different lots should be tested. An alternative to obtaining human lung epithelial cells commercially is to obtain donated normal lung tissue with institutional review board approval directly from hospital clinics, in particular academic centers with large lung transplant programs. Investigators should be aware of variability in handling, for example the time the sample is kept in ice-cold saline before processing, and should also know whether the samples come from donor lungs deemed unsuitable for lung transplant (in which case some areas may be contaminated, infected or otherwise damaged) or from trimmings of transplanted lungs. In either case, there can be variability between donors. If desired, diseased samples can be obtained from a number of sources: (1) lung explants; (2) bronchial brushings or endobronchial biopsies performed during bronchoscopy; or (3) lung resection samples. In the case of lung explant, investigators should be aware that this tissue comes from patients with end-stage disease and could be very different to that from an individual with earlier stage disease. Regardless of the source there can be tremendous regional variability within the lung, and stem cells isolated from a less affected region may have different properties than cells isolated from a more severely affected area. This is also true for samples obtained from the nasal passages by either brushing or curettage. Depending on the position from which the samples are taken and the disease status, samples may be more likely to undergo squamous versus mucociliary differentiation. In all cases with donated human lung samples, a consistent and proscribed isolation protocol should be followed.

Airway secretory cells
‘Secretory cells’ refers here to the columnar, non-ciliated, non-neuroendocrine cells present in the airway epithelium of the lung. The two main classes are Club cells and goblet cells (see Fig. 1). Mature Club cells synthesize proteins such as secretoglobin (Scgb1a1, Scgb3a2) and Splunc1, which are stored in apical electron-dense granules. Goblet cells, which are much more numerous in the human lung than in that of the laboratory mouse, synthesize mucins such as Muc5AC and Muc5B, and these are stored in large electron-lucent vesicles. The proportion of Club, goblet and ciliated cells varies somewhat along the proximal-distal axis of the mouse intralobar airways, with more ciliated and goblet cells proximally than distally.

Lineage-tracing studies in the mouse have shown that, at steady state, cells in the bronchioles that express Scgb1a1 can self-renew over the long term and give rise to ciliated cells, establishing their credentials as a stem cell population (Rawlins et al., 2009). Club cells can also directly differentiate into mucus-secreting goblet cells in response to cytokines such as IL13, especially in more proximal regions of the lung. Importantly, as summarized briefly in the legend to Fig. 1, there is extensive evidence that airway Club cells are a heterogeneous population that displays considerable phenotypic plasticity in response to viral and bacterial infections and agents that damage either the airway or alveolar epithelium. For example, lineage-tracing studies after damage to the alveolar region by the chemotherapeutic drug bleomycin have shown that Scgb1a1-expressing cells in the distal bronchioles proliferate and give rise to progeny in the alveoli with characteristics of AEC2s and AEC1s (Rock et al., 2011; Barkauskas et al., 2013; Tropea et al., 2012).

In such pathological conditions, which involve the production of numerous inflammatory cytokines as well as hypoxia, the contributions of different signaling pathways to changes in cell behavior are hard to disentangle. Theoretically, organoid culture provides a model system for testing the effect of individual cytokines and growth factors on the proliferation and differentiation of secretory cells, and for identifying subpopulations of Club cells with enhanced regenerative potential — that is, a higher CFE and with greater plasticity. Such populations could be exploited for therapeutic purposes. This ideal, however, is confounded by the current paucity of surface markers that can be used to both rigorously purify subsets of Club cells and to localize them unambiguously to specific regions of the mouse and human lung.

Organoids from mouse airway secretory cells
Two different methods have been used to isolate secretory Club cells by FACS for organoid culture studies: isolation based on the expression of surface markers; and lineage tracing using an Sgb1a1-CreER knock-in allele (Rawlins et al., 2009) with a fluorescent reporter allele. Using the first approach, McQualter and colleagues sorted lung epithelial cells on the basis of being CD45 (Ppcreg), CD31 (Pecam1)reg, EpCAMhigh, CD49f(Itgα6)pos, CD104 (Itgb4)pos and CD24low (McQualter et al., 2010). This population includes some, but not all, Scgb1a1-expressing cells. When placed in 50% Matrigel in a relatively simple ‘basal’ medium, these cells gave rise to spheres, but only when co-cultured with primary EpCAMreg Sca1(Ly6a)+ lung stromal cells. The spheres were divided into three general categories based on morphology after ~14 days culture: large and rounded with a single lumen (type A, 46%); small, dense and lobular (type C, 35%); and ‘mixed’, with multiple bud-like protrusions (type B, 19%). Immunohistochemistry and RT-PCR studies showed that, in addition to Sgb1a1+ cells, type A and B colonies contained Trp63+cells, Foxj1+ ciliated cells, and Muc5AC+ secretory cells that were absent from type C colonies. By contrast, the type C colonies contained predominantly Sftp+ AEC2-like cells. Mixed colonies also contained Sftp+ AEC2 cells, predominantly at the tips of the buds. Broadly similar results were obtained by combining Sgb1a1-CreER lineage traced cells with a mouse lung stromal cell line (MLg) and SB431542, a TGFβ inhibitor, during the initial culture period (Chen et al., 2012). One drawback to both approaches is that it is not known whether the cells that gave rise to the large cystic spheres are normally located in a different region of the lung from those that gave rise to spheres containing AEC2s. This question was addressed in part by isolating EpCAMpos CD24low cells from mice carrying an Sftp-GFP transgene (Chen et al., 2012). GFPhigh cells, which gave rise predominantly to type C colonies, were assumed on the basis of the in vivo localization of GFP to be derived from the very terminal bronchioles, whereas the GFPlow cells that gave rise to type A colonies were all proximal. GFPhigh cells, which gave rise to mixed colonies, were assumed to come from distal bronchioles (Fig. 1). These results lend some support to the idea that there are intrinsic differences between subpopulations of Club cells in their ability to transdifferentiate into basal cells versus AEC2s.

Finally, organoid culture has been used to test the response of a small subpopulation of Sgb1a1+ Club cells in the distal bronchioles that also express Sftp (known as dual-positive, bronchiolalveolar stem cells or BASCs) to factors made by lung endothelial cells (Lee et al., 2014; Tropea et al., 2012). These experiments suggest that BASCs have the potential to differentiate into both AEC2s and airway cells, and that the alveolar differentiation can be specifically
enhanced by thrombospondin secreted by endothelial cells. Currently, the standard way to isolate BASCs involves FACS, and is based on their expression of EpCAM and Sca1. Going forward, new technologies should enable these cells to be more rigorously purified based on co-expression of the genes encoding Scgb1a1 and Sftpc, so that additional markers can be found to distinguish them from other Club cells. This will allow a more detailed comparison of their potentially unique responses to cytokines and other factors produced by both endothelial cells and fibroblasts following lung injury.

**Alveolar type II cells**

The alveolar epithelium is composed of two distinct epithelial cell types. Type II cells (AEC2s) are cuboidal and characterized by the production of pulmonary surfactant proteins (e.g. Sftp, Sftp) and the lamellar bodies and machinery associated with their production and secretion (e.g. Lamp3 and Lys2) (Fig. 1). By contrast, type 1 cells (AEC1s) are large squamous cells that cover most of the surface area of the alveoli and are closely apposed to a fine network of capillaries. AEC1s typically express advanced glycosylation end product-specific receptor (Ager), Pdpn and the transcription factor Sftpc, so that additional markers can be found to distinguish them from other cell populations. AEC1s expressing advanced glycosylation end product-specific receptor (Ager), Pdpn and the transcription factor Sftpc. AEC2s are defined as being propidium iodide staining (PI)neg, CD31neg, CD45neg, Sca1neg primary lung mesenchymal cells (McQualter et al., 2014; Jain et al., 2015). In addition to epithelial cells, alveoli contain multiple stromal cell types, capillary endothelium and associated pericytes, as well as interstitial and alveolar macrophages (Fig. 1).

Table 2. Mouse AEC2 isolation strategies

<table>
<thead>
<tr>
<th>Isolation</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>Genetic lineage labeled Surface markers Sftp-CreER&lt;sup&gt;&lt;b&gt;T2&lt;/b&gt; R&lt;/sup&gt; Rosa-tdTomato (Barkauskas et al., 2013)</td>
<td>Clearly defined locations in vivo and purity; can be combined with conditional alleles</td>
<td>Breeding is time-consuming</td>
</tr>
<tr>
<td>CD31&lt;sup&gt;+&lt;/sup&gt; CD45&lt;sup&gt;+&lt;/sup&gt; EpCAM&lt;sup&gt;+&lt;/sup&gt; (McQualter et al., 2010)</td>
<td>Complex genetic breeding is not necessary; potential to use magnetic beads to collect primary cells</td>
<td>Purity; location in vivo is not well defined</td>
</tr>
<tr>
<td>CD31&lt;sup&gt;−&lt;/sup&gt; CD45&lt;sup&gt;−&lt;/sup&gt; Sca1&lt;sup&gt;−&lt;/sup&gt; CD24&lt;sup&gt;−&lt;/sup&gt; Sftpc-GFP&lt;sup&gt;H&lt;sub&gt;up&lt;/sub&gt;&lt;/sup&gt; (Chen et al., 2012)</td>
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<td>CD31&lt;sup&gt;−&lt;/sup&gt; CD45&lt;sup&gt;−&lt;/sup&gt; EpCAM&lt;sup&gt;+&lt;/sup&gt; Sca1&lt;sup&gt;+&lt;/sup&gt; (Lee et al., 2014)</td>
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outside (Barkauskas et al., 2013; Jain et al., 2015) (Fig. 4D-F). This configuration does not strictly reproduce the structure of alveoli in the adult lung (Fig. 1) and it is still not clear how the AEC1s and AEC2s are polarized and interconnected by junctional complexes. To address these questions, the dynamics of AEC1 formation are being studied using live imaging to follow the morphogenesis of the spheres. In addition, an Ager-H2B:Venus knock-in allele is being used to quantify AEC1 differentiation under various conditions (Fig. 4E) and, in the long term, to develop high-throughput screens for small molecules and drugs that promote AEC2 differentiation. The long-term self-renewal of AEC2s can be quantified by dissociating spheres after 14 days of culture, resorting AEC2s and reseeding them with fresh stromal cells. As shown in Fig. 4G, this assay demonstrates that mouse AEC2s retain stem cell function for at least five passages.

Table 3. Compendium of alveolosphere protocols

<table>
<thead>
<tr>
<th>AEC2 source</th>
<th>Supporting cells</th>
<th>Medium</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic lineage-labeled</td>
<td>PDGFRα+ lung lipofibroblasts</td>
<td>MTEC+: DMEM/F12 supplemented with ITS, EGF, BPE, CTX, 5% FBS, antibiotics; RA freshly added; Y-27632 (ROCK inhibitor) for the first 2 days</td>
<td>Barkauskas et al., 2013</td>
</tr>
<tr>
<td>SFTPC+ AEC2s</td>
<td>Lung mesenchymal cells (EpCAM−, Sca1+)</td>
<td>DMEM/F12 supplemented with ITS, 10% newborn calf serum, glutamine, sodium bicarbonate, antibiotics</td>
<td>McQualter et al., 2010</td>
</tr>
<tr>
<td>Surface markers</td>
<td>MLg</td>
<td>DMEM/F12 supplemented with ITS, 10% FBS, antibiotics; SB431542 for the first 7-10 days</td>
<td>Chen et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Lung endothelial cells (LUMECs)</td>
<td>DMEM/F12 supplemented with ITS, 10% FBS, 1 mM HEPES pH 7.5, glutamine, antibiotics</td>
<td>Lee et al., 2014</td>
</tr>
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</table>
A major limitation in using organoids to study gene function in alveolar epithelium is the fact that neither mouse nor human AEC2s can be expanded efficiently in culture before seeding in Matrigel. Because current protocols for CRISPR/Cas9 genome editing require 2D growth and expansion of cells, ideally combined with single-cell cloning of specific mutants, this technique has yet to be applied in the alveolar sphere culture system.

**Lung organoids derived from embryonic and induced pluripotent stem cells**

Lung tissues derived from human pluripotent stem cells (hPSCs), including embryonic stem cells and induced pluripotent stem cells (iPSCs), have the potential to make a powerful impact on our understanding and treatment of lung disease. Efforts are currently underway to generate populations of immature lung epithelial and mesenchymal progenitors that can be massively expanded – with the option to store in a cryobank – and then directed to differentiate efficiently into mature airway and/or alveolar tissue. There are many potential questions that could be addressed using such a resource. For example, airway epithelial cells could be produced from iPSCs derived from patients with chronic asthma or CF to test the idea that epigenetic changes in the progenitors affect their self-renewal and differentiation capacity (Mou et al., 2012; Vladar et al., 2016). CF iPSC-derived lung organoids could also provide a reliable and reproducible source of CF mutant cells for screening drugs that compensate for, or correct, patient-specific mutations (Wong et al., 2012). This would overcome the problem of variability in the behavior of primary lung progenitor cells derived from even healthy individuals.

In the case of alveolar tissue, the differentiation of hPSCs into distal lung progenitors would allow studies of mutations affecting surfactant genes (SFTPA, SFTPB, SFTPC) or telomerase (TERT) that in AEC2s can cause respiratory failure and interstitial lung disease (Whitsett et al., 2010). ‘Omics’ – genome, transcriptome, proteome, metabolome, and so on – profiling of healthy versus patient-specific hPSC-derived distal epithelium would enable a better understanding of how mutant cells become dysregulated over time (Grün et al., 2015). Finally, a source of progenitor cells that can be expanded after manipulation by CRISPR/Cas9 gene editing and still reliably differentiate would enable this powerful technique to be used to test the function of specific human genes in airway and alveolar cell specification during development. Although the molecular mechanisms that drive lung development and repair are likely to be conserved in general between mouse and human, work with early human embryos has already revealed interspecies differences in the transcription factors or ligands that are crucial at certain stages (Madisson et al., 2014). Therefore, it will be important to examine the expression and function of human genes in the lung in relevant models.

The primary challenge in realizing the above goals has been to direct the differentiation of hPSCs towards functional respiratory tissue that accurately resembles adult lung. The greatest progress in generating proximal and distal lung epithelial populations has been made by basing the differentiation protocols on the signaling pathways that direct embryonic lung development (Clevers, 2016; Dye et al., 2015; Huang et al., 2014; Longmire et al., 2012; Mou et al., 2012; Wong et al., 2012). A pioneering study first demonstrated the generation of anterior foregut endoderm (AFE) from hPSC-derived definitive endoderm (Green et al., 2011). To date, the most successful differentiation protocols first generate definitive endoderm, then AFE, followed by ventralization of the AFE via 3D culture using fibronectin or Matrigel substrates to yield immature, fetal-like lung and airway progenitors (Huang et al., 2015) (Fig. 5).

Moving forward, one of the greatest obstacles is the development of protocols in which mature airway and alveolar cells are efficiently generated from their corresponding immature progenitors. Recent studies have shown that hPSC-derived lung organoids grown in a Matrigel-coated scaffold (serving as a bioartificial niche) and subsequently transplanted into mice generate more mature airway epithelium than previous methods (Dye et al., 2016). However, this particular way of transplanting lung organoids to induce cell differentiation and maturation does not generate alveolar cells,

![Diagram](image_url)

*Fig. 5. Derivation of lung organoids from hPSCs.* Directed differentiation protocols vary as to the components of the growth medium, the extracellular coating, and the stages at which the cells are placed in a 3D environment. The schematic is based on results from three groups (Huang et al., 2014, 2015; Dye et al., 2015, 2016; Wong et al., 2012) (see main text). Human pseudoglandular and canalicular stage (weeks 6-19 of gestation) fetal lungs can also provide an epithelial cell source. A combination of in vitro growth and subsequent in vivo engraftment currently provides the best conditions for maturation of lung epithelium. Culture of ventral lung progenitors in 2D air-liquid interface transwells generates only proximal conducting airway epithelium. RA, retinoic acid; BEGM, bronchial epithelial growth medium (Fulcher et al., 2005).
indicating a need for both proximal and distal cell-specific engraftment protocols. Air-liquid interface culture can be used to generate mature, polarized, pseudostratified proximal airway epithelium (Wong et al., 2012). This method of 2D-transwell culture exposes the apical side of the epithelium to the atmosphere and is a useful model of the airway microenvironment, but lacks the facility for in vivo transplantation for study of disease.

Finally, an alternative to using hPSCs to derive multipotential lung epithelium is to start with the fetal lung itself. Human embryonic lung from the pseudoglandular or canalicular stage (6-19 weeks gestation) may serve as the best source of immature cells with the potential to differentiate into both airway and alveolar cell types (Mondrinos et al., 2014; Rosen et al., 2015). However, use of fetal-lung derived organoids for cell therapy faces similar roadblocks as for hPSCs, and benefits derived from this cellular source must be weighed against the current challenges of obtaining suitable tissue, at least in some countries.

Future directions
This Review has surveyed some of the methods used to derive 3D organoids from different epithelial cell populations of the adult lung, including basal cells, secretory Club cells and AEC2 cells, as well as hPSCs, and the impact that this culture system has made on our understanding of lung biology. We have also outlined some of the potential future uses of organoids, especially those made from human cells, for both basic and translational research, including models of human disease and drug screening. However, for this potential to be fully realized there are a number of improvements that must be made to overcome significant limitations. These have been mentioned in the preceding text but, to reiterate, we highlight the three major issues again here.

First, to date none of the culture media used to derive organoids is chemically defined and they often contain complex supplements such as BPE or fetal bovine serum (FBS). The effect of parameters such as glucose levels and oxygen tension has also not been rigorously tested. Thus, we do not yet have a precise definition of the growth factors and small molecules and metabolites required for the long-term self-renewal and directed differentiation of lung epithelial stem and progenitor cells. In addition to defining these factors, we need to identify their sources in vivo and show, for example, which molecules are made by neighboring epithelial cells and which by mesenchymal cells in the stem cell niche. Progress is being made in establishing organoid cultures in which multiple stromal cell types are combined, and this will help to tease apart how the cell types interact in vivo and how they are affected by injury, inflammation and aging. It is also likely that extracellular matrix components and physical forces play key roles in regulating stem cell behavior and these parameters are also beginning to be explored using organoid culture.

Hand-in-hand with defining the culture requirements of adult lung epithelial stem cells is the need to establish efficient methods for cloning and expanding the clonal populations in 2D culture, while still maintaining their complete capacity for differentiation. As we have discussed, it is necessary to fully exploit the powerful technology of genome editing using CRISPR/Cas9, particularly for studying the role of specific genes in the self-renewal and differentiation of human lung stem cells and in generating models of human respiratory disease. hPSCs are currently more amenable to genome editing and thus the organoids generated from them have great potential for translational research, including drug discovery. The most difficult obstacle to overcome at present is to obtain full differentiation of hPSC cultures into specialized lung cell types, in particular AEC1 cells, and alveolar-like cellular arrangements.

Finally, progress in the utility of lung organoids requires the identification of more surface markers and/or reporters for isolating and purifying subpopulations of stem and progenitor cells and stromal support cells, in particular from the human lung. This will be important in understanding the functional heterogeneity of these cells and in developing protocols for directed cell differentiation and maturation. There is also the possibility that new classes of stem cells and support cells will be discovered, and organoid culture will provide one quantifiable method by which they can be compared with known populations. In summary, the array of different organoids that can be used to model various aspects of lung development, homeostasis, regeneration and disease represents an exciting new avenue for pursuing outstanding questions in lung – especially human lung – biology. With vigorous persistence in overcoming the challenges and careful analyses to interpret the results, we expect that organoid culture will become an indispensable tool for both basic and applied lung research.

Acknowledgements
We thank Drs Tomori Tadokoro and Jason Rock for kindly providing unpublished data in Fig. 3 and Drs Purushothama Tata and Scott Randell for helpful discussion.

Competing interests
The authors declare no competing or financial interests.

Funding
B.L.M.H. acknowledges support from the National Institutes of Health (R37-HL071303, U01-HL110967) and the Ellison Medical Foundation. C.E.B. acknowledges support from the Burroughs Wellcome Fund Career Award for Medical Scientists and the National Institutes of Health (K08-HL122521), Deposited in PMC for release after 12 months.

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