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Early View

Review

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Use of 3D organoids and lung-on-a-chip methods to study lung development, regeneration and disease

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Abstract:

Differences in lung anatomy between mice and humans as well as frequently disappointing results when using animal models for drug discovery emphasize the unmet need for in vitro models that can complement animal studies and improve our understanding of human lung physiology, regeneration and disease. Recent papers have highlighted the use of three-dimensional (3D) organoids and organs-on-a-chip to mimic tissue morphogenesis and function in vitro. Here, we focus on the respiratory system and provide an overview of these in vitro models which can be derived from primary lung cells, pluripotent stem cells, as well as healthy or diseased lungs. We emphasize their potential application in studies of respiratory development, regeneration and disease modeling.

Introduction

The lung is a complex organ, consisting in its most proximal part of a tree-like tubular branched system (bronchiolar airways) connected to a single tracheal tube. Towards the periphery, the bronchiolar airways become progressively smaller and branch into smaller tubes (bronchioles) which terminate into alveolar regions where gas exchange takes place [1, 2]. In addition, different types of mesenchymal cells, vasculature, cartilage and neurons surround this epithelial network and contribute to lung development and homeostasis (Figure 1) [1].

The complex composition and structure of the lung are established during early embryonic development. The respiratory system is specified from the anterior foregut endoderm at embryonic day (E) 9.5 in mouse (around week 4 in human) as two primary lung buds emerge on the ventral side, distal to the rudimentary trachea surrounded by mesoderm and a vascular network [2]. During the pseudoglandular stage, which takes place between E9.5 and E16.5 in mouse (week 24 in human), these buds undergo a process of branching morphogenesis, in which epithelial bud tips continuously bifurcate resulting in a proximal-to-distal hierarchical branched tree-like tubular network [2, 3]. It is well established that epithelial-mesodermal interactions play key roles in directing this process. Of particular interest is the mesenchyme located at the bud tips. These mesenchymal cells migrate proximally and at the level of the cleft differentiate into smooth muscle cells to stabilize the cleft formed between two daughter buds, thereby facilitating the branching process [4]. At the same time, mesenchymal-derived diffusible signals including Fgfs, Tgfb/Bmps, Wnts, Egfs and Hedgehog (Hh) orchestrate the expansion and lineage specification of the epithelial progenitor pools located at the proximal and distal airways [2, 5]. This developmental event in turn leads to the canalicular stage (E16.5-E17.5 in mouse and weeks 24-26 in human) and saccular stage (E17.5 to birth or weeks 26-36 in human), during

which the distal buds become progressively narrower and the distal progenitor cells at the epithelial tips begin to differentiate into alveolar epithelial cells that populate the distal sacs, such as alveolar epithelial type 1 (AEC1) and alveolar epithelial type 2 (AEC2) cells [6, 7]. Finally, the newly formed sacs continue to develop via a process referred to as alveolarization, which starts at birth till P20 in mice whereas in humans begins in week 36 and persists for up to 3 years. The sacs undergo a number of subdivisions by the ingrowing crests (septae) which consist of myofibroblasts, lipofibroblasts, endothelial cells and pericytes resulting into the formation of the alveoli [2]. These critical events have prompted studies into the molecular mechanisms that mediate lung development, as well as the elaboration of compatible in vitro and ex vivo tools.

In conditions of adult lung homeostasis, cell turnover is normally very low and the existing airway progenitor cells are quiescent. Remarkably, numerous animal studies have shown that a number of progenitor cells have the capacity to proliferate and differentiate into one or more cell types in response to a variety of injuries including exposure to infectious agents, tobacco smoke, high levels of oxygen and the chemotherapy drug bleomycin [1].

The regenerative ability of these progenitor cells has triggered the scientific community to search for drugs that could activate them in vivo or establish cell-based therapies which would allow to treat patients suffering from respiratory disease that arise from lung injury or aging. Unfortunately, the translational transfer of the results obtained using animal models, mostly mice, has so far been limited and is likely due to unappreciated differences in lung anatomy and physiology between mice and humans. Altogether, these observations emphasize the unmet need for lung model systems that can complement animal models, validate the results obtained in animals to humans as well as improve our understanding of human lung physiology, regeneration and disease.

In this review, we focus on two lung model systems, organoids and lung-on-a-chip, which are going to play a protagonist role in respiratory biomedical research. We discuss the applications of these approaches in the fields of stem cell differentiation, regenerative medicine, respiratory disease modeling and drug screening.

What are organoids?

Organoids are 3D self-organized aggregates of multiple cell types grown within gels made of a complex mixture of different extracellular matrix (ECM) proteins including laminin, fibronectin, collagen and heparin sulfate proteoglycans (Figure 2). The main commercial gel used to grow organoids is Matrigel, a protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells, which is liquid at low temperature and solidifies into a relatively soft gel at 37ºC. In addition to ECM proteins, Matrigel also contains a mixture of growth factors such as Tgfb, Egf and Fgf, which can impact the differentiation and proliferation of many cell types. The ECM gels can mimic the in vivo extracellular environment in part and allow some of the cells to display characteristics of stem cells, namely, self-renewal and multipotency. Once grown, these organoids can be reseeded for prolonged periods of time without changes in their karyotype. These organoids can also be cryopreserved without obvious alterations of the stem cells characteristics. When exposed to differentiating media, organoids will give rise to specific cell types observed in the corresponding adult tissue in vivo [8, 9].

These novel 3D cultures are generated from primary stem cells harvested from mouse or human lungs or even from progenitor cells derived from pluripotent stem cells (PSCs) (Figure 2), and they have revolutionized biomedical research by enabling studies of in vitro organ development, disease modeling and cell-cell interaction (Supplementary Figure 1).

Critical to the respiratory field, organoids have been successfully generated from epithelial progenitor or stem cells originating from mouse embryonic lung [10-13], human embryonic lung [14, 15], mouse adult lung [16-20], human adult lung [16, 19, 21], mouse pluripotent stem cells (mPSCs) [22] and human pluripotent stem cells (hPSCs) [23-28]. Further refinement of these approaches, with the scope of improving the differentiation efficiency, include co-culture of these epithelial progenitor cells with mesenchymal cells [20, 22, 23, 26, 29] or endothelial cells [18]. In addition to the epithelial-based organotypic cultures mentioned above, mesenchymal-based 3D cultures have been generated using fetal lung tissue or hPSCs and cultured in bioreactors. This approach resulted in the formation of a structural phenotype similar to that of native alveolar regions in vivo [30].

Differentiation of pluripotent stem cells into lung progenitors

Organoids derived from PSCs require an additional step compared to primary organotypic cultures, as PSCs have to undergo directed differentiation in 2D monolayer cultures using defined growth factor cocktails that attempts to mimic gastrulation, lung patterning and lineage commitment. These step-wise events use information gained from decades of animal model research and involves multiple signaling pathways that are controlled in a tight temporal and spatial manner in vivo.

For example, hPSC cultures are directed into the endodermal lineage using ActivinA, which mimics the Nodal signaling that has been shown to be critical for definitive endoderm (DE) specification in vertebrates [31]. DE formation is followed by acquisition of foregut identity by inhibiting both BMP and TGFβ signaling pathways using Noggin or small molecule inhibitors. However, the secreted WNT inhibitors that help to maintain foregut identity in vivo are not

necessary to induce foregut endoderm in vitro [23-25, 32-36]. In mouse, diffusible morphogen signals from cardiac and mesenchymal mesoderm orchestrate progenitor specification via activation of multiple signaling pathways [5]. Thereafter in hPSC cultures, lung progenitors expressing NKX2.1 have been successfully generated using growth factor cocktail containing FGF, BMP, WNT, Hedgehog (HH) and Retinoic acid (RA) [23-27, 32-35, 37-39]. Cell sorting methods, such as FACS, enable the constitution of homogeneous progenitor populations. Subsequently, these isolated cells are seeded in ECM gels to facilitate the development of a organoid culture and increase their differentiation towards conducting airway or alveolar epithelial cells. Given the unique contribution of epithelial progenitor cells to lung development and function, modeling this aspect using organoids from healthy or diseased lungs represents a powerful model for deciphering the underlying molecular mechanisms. Finally, such organotypic cultures are also a suitable tool for high-throughput drug efficacy testing, toxicity effect and small molecule screening studies.

Organoids to model early stages of lung development

The establishment of the initial proximo-distal epithelial patterning begins with the first respiratory stem cell population marked by the expression of the transcription factor Nkx2.1 in the ventral anterior foregut endoderm. In mouse, this domain is denoted by two main endodermal stem/progenitor pools marked by proximal Sox2 expression or distal Sox9 and Id2 co-expression [2, 5]. However, in contrast to mice, human progenitor cells located at the distal tips co-express SOX2 and SOX9 during pseudoglandular stage and become single SOX9 positive during the canalicular stage [14, 15, 40]. $Sox2^{+}$ progenitor cells generate the conducting airway epithelium, including secretory cells, multi-ciliated cells, neuroendocrine cells and basal

cells, and are shown to be regulated by intracellular Notch signaling [41-43]. Sox9/Id2^{+} progenitor cells are initially capable of generating both airway and alveolar epithelial cell types[44]. Following branching morphogenesis after E13.5, Sox9/Id2/Bmp4/Spc^{+} progenitors are restricted in their fate and only generate AEC1 and AEC2 cells[44]. In an effort to grow and maintain embryonic progenitor cells in vitro, a number of groups used serum-free organotypic cultures derived from mouse [13, 15] and human embryonic tissue [14, 15]. Following a chemical screen, Nichane et al observed that a growth factor cocktail containing Fgf9, Fgf10, Egf, and inhibitors for Gsk3, Tgfβ, Rock and p38 pathways are essential for long-term mouse progenitor maintenance [13]. Moreover, Nikolic et al were able to maintain human embryonic lung epithelial tip progenitors for long-term in vitro in the presence of 7 factors: EGF, FGF7, FGF10, NOG (Noggin), RSPO1 (R-Spondin 1), CHIR99021 and SB431542. [14]. Unfortunately, these conditions were unable to maintain long-term self-renewal of undifferentiated mouse lung tip epithelial progenitors in vitro, suggesting differences of functional significance between the human and mouse distal progenitor cells [14]. In contrast, Miller et al demonstrated that FGF7, CHIR-99021 and RA support mouse and human embryonic epithelial lung tip growth and maintenance in vitro. Importantly, using the same culture conditions, they were also able to mimic human embryonic lung development in vitro by inducing hPSC-derived NKX2.1 ventral foregut cells into a tip-like progenitor state [15]. Using hPSC derived from patients suffering from respiratory disease together with such culture conditions could benefit studies aiming to model respiratory disease in a dish.

Given the transcriptomic significant similarities of the human iPSC-derived Lung bud Organoids (LBOs) to the second trimester of human gestation samples, the use of hPSCs to study human lung development remains the gold standard [28]**.** A number of studies have reported the

establishment of 3D cultures able to self-renew and generate airway epithelial cells [24, 25, 27, 28, 32-35, 45-48]. Of note, Konishi et al used carboxypeptidase M (CPM) as a surface marker of NKX2.1^{pos} ventralized anterior foregut endoderm cells (VAFECs), in order to generate proximal airway progenitor cell spheroids able to give rise to functional multi-ciliated cells with beating cilia [24]. Work utilizing directed airway and distal PSC differentiation revealed that the WNT pathway regulates proximodistal patterning of purified NKX2.1 lung epithelial progenitor; WNT activation using CHIR99021 promotes distal epithelial differentiation while CHIR99021 withdrawal prompts rapid proximal patterning [27].

Distal organotypic cultures have also been generated from hPSCs applying similar concepts used to derive airway cultures mentioned above [23, 25, 26, 28, 38, 39, 47-50]. PSC-derived alveolospheres are successfully generated from enriched CPM^{pos} VAFECs when co-cultured with human fibroblasts under alveolar-related growth factor culture conditions. Most studies have shown that a combination of Dexamethasone, CHIR99021, FGF7, cAMP and IBMX is sufficient to drive alveolar differentiation and generate SFTPC^{pos} epithelial cells [23, 26, 28, 48, 50]. To derive distal epithelial cells and partly overcome the maturity limitation displayed by PSC-derived epithelial cells, some groups have employed prolonged in vitro cultures [25, 28], co-culture with fetal lung mesenchyme [48], seeding into acellular lung matrices [38], differentiation in a rotating bioreactor culture system [49] or transplantations under the murine kidney capsule [28, 34, 51]. Together, data from these approaches suggest that mechanical forces of the ECM, as well as signals from the extracellular matrix or from mesenchymal cells (e.g., reconstituting epithelial-mesenchymal cell interactions) all play a key role in promoting alveolar epithelial fate.

Although the branching morphogenesis stage during embryonic lung development has been well characterized, the diversity of cellular events during the sacculation stage are still poorly understood. Using organotypic cultures derived from minced E17.5 fetal mouse lungs, Mondrinos and colleagues demonstrated how exogenous FGF signals drive the action of diverse endogenous mediators (SHH, VEGF-A, TN-C) during de novo fetal pulmonary alveolar tissue morphogenesis [10, 11].

Lung homeostasis, regeneration and disease

Lineage-tracing experiments during lung development or regeneration/repair after acute injury of distinct parts of the lung (tracheobronchial airways, bronchiolar airways or the alveoli) have allowed the identification of region-specific epithelial stem/progenitor cells (Figure 3). More recently the identity of the corresponding stromal niches allowing self-renewal and differentiation of these epithelial cells towards distinct airway and/or alveolar epithelial lineages has also been unraveled.

Organoids for modeling airway regeneration and disease

Several progenitors are also produced during lung development from the Sox2-positive ($Sox2^{pos}$) early endoderm progenitors. These include basal stem cells (BSCs), which express transcription factor Trp63 (p63) and cytokeratin 5 (Krt5), and are considered to be long-term stem/progenitor cells. BSCs are able to generate multi-ciliated and secretory epithelium of the trachea and proximal bronchiolar region during post-natal growth, homeostasis and after airway injury in adult mice (Figure 3A) [16]. The first organoids derived from adult tracheal basal cells were called tracheospheres. Single mouse Krt5-GFP^{pos} basal cells were able to self-renew and

generate luminal cells, including differentiated ciliated and secretory cells [16]. The transcriptional profile of Krt5^{pos}/Isolectin β4^{pos} (GSIβ4^{pos}) cells identified two cell-surface markers, Ngfr and Itga6, which were used to isolate human lung basal cells by flow cytometry and successfully generate human tracheospheres. As with the mouse system, human tracheospheres are able to self-renew and generate luminal daughter cells [16, 52, 53]. In multiple reports from the Hogan group using combined *in vivo* and tracheospheres approaches, the integrative role of key transcription factors and Notch, Bmp, Wnt and Il6 signaling was demonstrated during basal cell proliferation and differentiation [17, 54-57]. In addition to tracheospheres, human bronchiospheres composed of functional multi-ciliated cells, mucin-producing goblet cells and airway basal cells have been derived from human airway basal cells [58].

In addition to BSCs, lineage tracing studies showed that secretory cells that express secretoglobin 1a1 (Scgb1a1), but lack (or have reduced) expression of cytochrome P450 family 2 subfamily f (Cyp2f2), are able to self-renew and re-epithelialize the airway epithelium after injury (Figure 3A) [44, 59-61]. Recently, lineage tracing approaches revealed a key crosstalk between airway Scgb1a1^{pos} Club secretory cells and specific mesenchymal population in the adult lung during injury repair [57]. In this study, Lee and colleagues identified that Lgr6^{pos} mesenchymal cells specifically support Club cell proliferation and differentiation via a paracrine Wnt-Fgf10 signaling network [57]. Three independent studies demonstrated the use of stromal cells as support cell populations while generating mouse secretory club cell organoids derived either from a minor stem cell population isolated on the basis of being either Cd45^{neg}Cd31^{neg}EpCAM^{high}Itga6^{pos}Itgb4^{pos}Cd24^{low} or Scgb1a1^{pos} cells [20, 29, 57]. Furthermore, neuroendocrine (NE) cells are also able to proliferate and generate secretory and ciliated cells

after naphthalene-induced loss of secretory cells (Figure 3A). However, depletion of NE cells following injury does not impede regeneration of secretory cell, suggesting that their contribution maybe limited and/or compensated by other progenitors [62].

Recently, human tracheospheres were used to screen molecules that affect the differentiation ratio of ciliated versus secretory cells from basal cells [53, 55, 56]. This work led to the identification of a pivotal role for IL-13 in promoting mucus cell production and Notch signaling in inhibiting the proportion of secretory relative to ciliated cells, consistent with previous observations [17, 42, 43, 63-69] . In contrast, a similar assay reported the inhibitory effect of BMP signaling during basal cell differentiation [55]. Together these studies highlight new potential therapeutic targets for conditions like chronic asthma in which the epithelial balance is disrupted.

Another respiratory disease that leads to chronic bacterial infection and secretion of pro-inflammatory cytokines is cystic fibrosis (CF). hiPSC derived from CF patients were used to evaluate the function of CFTR protein in airway epithelial cells and rescue the CF phenotype following treatment with small compounds [27, 39, 70, 71] or using zinc-finger nuclease (ZFN)-mediated gene editing approach [27, 71]. This is another promising in vitro system that facilitates lung-specific disease modeling and drug screening.

Modeling alveolar stem cells and stromal niche interaction

Stem/progenitors also exist in the alveolus, the basic respiratory unit composed of AEC1 and AEC2 cells, capillary endothelial cells and associated distinct mesenchymal lineages. The bronchioalveolar duct junction (BADJ), between the bronchioles and the alveolar sacs, consists of a small number of putative bronchioalveolar stem cells (BASCs) marked by Scgb1a1 and

surfactant protein C (Sftpc) co-expression, which are able to give rise to secretory and alveolar epithelial cells *in vivo* (Figure 3B) [17, 72, 73]. Notably, a cooperative interaction between Gata6 and Wnt signaling is essential for the expansion of BASCs after naphthalene-induced airway injury [74], whereas endothelial-derived Bmp4-Nfatc1-Tsp1 signaling is crucial for BASCs cell differentiation during lung regeneration [18]. In addition to BASCs, $Sftpc^{neg}/\alpha6\beta4^{pos}$ progenitors (called lineage-negative epithelial progenitors LNEP) also display regenerative potential (Figure 3B) [75]. Although their regenerative capabilities have been reported to be mostly towards the alveolar lineage, it remains to be tested whether these cells can also contribute to proximal airway lineages. Finally, cell lineage tracing evidence show that $Sftpc^{pos}$ AEC2 act as progenitor cells able to self-renew during homeostasis in vivo and differentiate into AEC1 cells in vitro when grown as organoids (alveolospheres) in combination with stromal cells (Figure 3B) [19, 57, 76-78]. These 3D structures are composed of AEC1 in the lumen side and AEC2 cells on the outside. Of particular interest, a balance of IL-6/Stat3, Bmp and Fgf signaling proved important in AEC2 self-renewal and AEC1 differentiation [78]. Moreover, in an in vivo (kidney capsule) transplantation assay, isolated $Sftpc^{neg}/\alpha6\beta4^{pos}$ cells gave rise to $Scgb1a1^{pos}$ club cells and $Sftpc^{pos}$ AEC2 cells [75]. On the other hand, AEC1 cells, which are thought to be terminally differentiated cells in the postnatal lung, can proliferate and transdifferentiate into AEC2 after partial pneumonectomy (Figure 3B) [77]. It is unclear so far how heterogeneous the AEC1 population is and whether it displays enhanced stem cell characteristics. More recently, two AEC1 cell subtypes marked by $Hopx^{neg}$ $Igfbp2^{pos}$ and $Hopx^{pos}Igfbp2^{neg}$ have been identified in the postnatal lung exhibiting distinct cell fates during alveolar regeneration following pneumonectomy [79].

In addition to alveolar stem cells, different types of resident stromal cells (which include

mesenchymal and endothelial cells) are required for both homeostasis and regeneration after injury. The identification of these populations is facilitated by co-culture with epithelial lung progenitors in organoid settings. The characterization of the cellular and molecular features of these stromal cells during repair and/or regeneration in vivo are so far elusive. It is unclear for example if these interactions are solely based on growth factors secreted by the stromal cells and/or on direct cell-to-cell contact which may or may not involve extracellular matrix components. In the alveolar niche of the adult murine distal lungs, several mesenchymal lineages expressing Fgf10, Gli1, or Axin2 function as multipotent cells able to self-renew and give rise to smooth muscle and fibroblast-like cells [80-83]. Notably, mouse fibroblast have been shown to support the growth of distal epithelial stem/progenitor cells, emphasizing the importance of epithelial-to-mesenchymal interactions in close proximity [19, 29, 84]. In this regard, the Kim and Morrisey groups recently investigated the effect of distinct mesenchymal cell subtypes in promoting Sftpc^{pos} AEC2 cell self-renewal and differentiation in AEC1 cells [57, 78]. Using single-cell RNA sequencing, lineage tracing and organoid cultures have identified mesenchymal lineages with distinct spatial distribution and unique niche regulatory function [57, 78]. For example, Lgr5^{pos} alveolar mesenchymal cells are able to induce alveolar lineage differentiation of epithelial progenitors (Scgb1a1^{pos} or Sftpc^{pos} cells) by secreting Wnt ligands [57]. In addition to Lgr5^{pos} population, the Axin2^{pos}/Pdgfra^{pos} alveolar mesenchymal lineage is capable of promoting AEC2 self-renewal via Il-6 and Fgf7 signaling [78]. An additional challenge lies in determining whether these alveolar mesenchymal populations are two distinct mesenchymal cell subtypes.

Given the close proximity of the pulmonary capillary endothelial cells (PCECs) to the alveolar epithelial cells in vivo, endothelial-derived membrane-bound and soluble angiocrine factors have

significant input for alveolar stem cell differentiation [18, 85-87]. AEC1 and AEC2 cells are lined with PCECs that are positive for Cd31, Cd34, Fgfr-1, Vegfr-1, Vegfr-2, negative for Cd45 [85]. *In vivo* studies using unilateral pneumonectomy as a model for neo-alveolarization demonstrated that platelet-derived Sdf-1 leads to the upregulation of Mmp-14 in PCECs, which in turn triggers alveolar stem cell proliferation by unmasking the cryptic Egf-like fragment in Hb-Egf and the γ2 chain of Laminin-5 [85, 87]. Mouse organotypic study of epithelial stem cells ($EpCAM^{pos}$ $Sca1^{pos}$ $Cd31^{neg}$ $Cd45^{neg}$) that were co-cultured with primary mouse lung endothelial cells (LuMECs) support a model in which angiocrine signals regulate alveolar stem cell proliferation and differentiation into AEC1 and AEC2 cells [18]. LuMECs have been shown to produce Tsp-1, which fosters lung stem cell differentiation to the alveolar lineage.

For disease modeling, Chen and colleagues established the first proof-of-principle hPSC-based modeling of Hermansky-Pudlak syndrome (HPS) which leads to pulmonary fibrosis (PF) with abnormalities in AEC2 cells and surfactant proteins secretion in vivo [88]. To study the effect of the gene *HSP1* gene in human development in vitro, they generated lung bud organoid from RUES2 hPSCs with CRISPR-Cas9 gene editing induced deletion of HPS1 [28]. Notably, this study provided consistent evidence [88, 89] that mutant epithelial cells are responsible for the accumulation of mesenchymal cells and increased ECM deposition [28].

Development of human organs-on-chips

Recent advances in the development of microengineered cell culture systems provide new opportunities to present cultured living cells with physiologically relevant biochemical and mechanical cues with high spatiotemporal precision (Figure 4). These biomimetic in vitro models collective known as organs-on-chips are constructed in microfabricated devices that

often consist of two or more layers of cell culture chambers made out of optically transparent and biocompatible polymeric materials such as poly(dimethylsiloxane) (PDMS) using soft lithography-based replica molding techniques. This widely used multilayered design provides compartmentalized environments for co-culture of multiple cell types under dynamic flow conditions to engineer various kinds of tissue-tissue interfaces and three-dimensional multicellular structures that mimic the functional units of living human organs (Figure 2 and Figure 4A) [90]. In vitro approaches based on organ-on-a-chip technology have proven instrumental in modeling the complexity and dynamic nature of the respiratory system (Supplementary Figure 1). Here we discuss representative examples of such systems to demonstrate the proven utility and potential of lung-on-a-chip technology.

Early demonstrations of human lung-on-a-chip

Lung-on-a-chip studies focused on demonstrating the possibility of using multilayered microfluidic devices to enable the formation of differentiated airway epithelium with morphological and secretory phenotypes matching those found *in vivo* [91-96]. For example, a human small airway-on-a-chip model developed by the Takayama group permitted long-term (>3 weeks) air-liquid interface (ALI) culture of primary human small airway epithelial cells (SAECs) to produce a differentiated airway epithelium with structural integrity and barrier function [94]. More importantly, this culture system was integrated with a microfluidic plug generator capable of creating microscopic liquid plugs that were actuated to propagate over the epithelial surface and rupture to mimic closure and reopening of small airways due to mucus plugs in diseased lungs. This dynamic airway-on-a-chip model showed that plug propagation and rupture can generate abnormally large mechanical forces and harm the epithelium in a dose-dependent manner [94]. Furthermore, the development of bifurcating microchannel network

enabled to investigate the dynamics of plug rupture events in independent subnetworks, which then evolve independently of one another as demonstrated by the work from Baroud's lab [97-99]. Combined with engineering novelty of the system, this new physiological insight provided strong evidence for the advantages and potential of lung-on-a-chip systems as a novel research platform.

This demonstration was followed by another major study that showed for the first time the feasibility of reconstituting complex and integrated organ-level physiological responses of the lung in a microengineered device [100]. This system was designed to mimic the mechanically active alveolar-capillary unit of the living human lung. The device was created in a multi-compartment microfluidic system in which human alveolar epithelial cells were cultured in close apposition to primary human pulmonary microvascular endothelial cells on a thin porous elastomeric membrane to form a barrier tissue reminiscent of the alveolar-capillary interface *in vivo*. Inspired by the mechanism of breathing in the lung, the authors also devised a novel mechanical actuation scheme based on controlled application of vacuum to cyclically stretch the microengineered alveolar-capillary barrier and to mimic physiological breathing motions (Figure 4). Importantly, this study demonstrated the novel capability of the human breathing lung-on-a-chip model to mimic complex integrated organ-level responses. For example, when the alveolar epithelium was exposed to pathogenic bacteria in this microdevice, the epithelial cells released inflammatory cytokines and activated the microvascular endothelial cells on the opposite of the membrane, inducing them to express high levels of adhesion molecules such as ICAM-1. Primary human neutrophils circulating in the lower capillary channel recognized these activated endothelial cells and established firm adhesion to the endothelium, after which the adhered neutrophils underwent transmigration across the alveolar-capillary barrier.

In addition, the ability of this model to recapitulate dynamic mechanical activity of the lung led to the discovery of previously unexplained adverse effects of physiological breathing-induced mechanical forces on inflammatory and injury responses. For example, in nanotoxicology studies using silica nanoparticles that simulated air pollutants, cyclic breathing motions in the lung-on-a-chip system substantially increased endothelial expression of pro-inflammatory adhesion molecules and intracellular production of reactive oxygen species, suggesting the promotive effects of physiological breathing on acute toxic responses to environmental particulates.

This advanced lung-on-a-chip platform was used in the follow-on study to test the feasibility of engineering specialized models of lung diseases. To this end, the authors created a microengineered model that simulated the development and progression of pulmonary edema induced by dose-limiting toxicity of a chemotherapeutic drug, interleukin-2 (IL-2) [101]. Furthermore, this mechanically active model capable of mimicking physiological breathing motions revealed the promotive effects of breathing-generated mechanical forces on IL-2-induced tissue injury, which was effectively inhibited by potential drug candidates such as angiopoietin-1 and a newly developed transient receptor potential vallinoid 4 (TRPV4) ion channel blocker. In another study, Long et al used mathematical modeling to optimize and control gas concentrations within the gas and liquid sides of alveolus-capillary interface, thus enhancing the performance of the system for drug and toxicity studies [102]. Another "breathing" lung-on-a-chip system was developed by Guenat's lab to mimic the alveolar system of the lung (Figure 4B) [103]. This model consists of a PDMS cell culture well and an underlying chamber separated by a thin PDMS membrane with microscopic pores. In this multilayered device, primary human pulmonary alveolar epithelial cells and endothelial cells

were cultured on the opposite sides of the membrane to form a bi-layer tissue structure reminiscent of the alveolar-capillary interface. Using mechanical actuation inspired by the mechanism of respiration in the lung, this system also made it possible to simulate breathing-induced three-dimensional deformation of the alveolar air sacs. Specifically, the lower chamber of the device was equipped with a membrane-based actuation system where negative pressure was applied to induce the deflection of a diaphragm membrane, which led to downward deformation of the engineered alveolar-capillary barrier (Figure 4B). Importantly, this study demonstrated that breathing-induced mechanical strain influences barrier permeability, the metabolic activity and the cytokine secretion (IL-8) of primary human pulmonary alveolar epithelial cells obtained from patients [103].

Recently, Davies' Lab developed a 3D in vitro model by co-culturing human epithelial cell line and human umbilical vein endothelial cells (HUVECs) at the air-liquid interface [104]. This system was applied to dissect the temporal crosstalk between epithelial and endothelial barriers in response to viral infections. While this work successfully recapitulated human pulmonary viral infection *in vitro*, additional progress is needed to evaluate the co-culture model using fully differentiated primary airway/bronchial epithelial and microvascular endothelial cells. Another example of microengineered human lung models can be found in the work of Sellgren et al. that developed vertically stacked and individually accessible 3D culture compartments for culture of primary human airway cells [105]. This platform allows for co-culture of trachea-bronchial epithelial cells, lung fibroblast, and microvascular endothelial cells at the upper, middle, and lower compartments, respectively. The authors observed well-differentiated epithelial cells that exhibited physiological tissue-specific phenotypes including barrier function.

Development of alveolated microchannels

Inspired by the innate acinar morphology of the alveoli, researchers have explored the possibility of using single or bifurcating microchannel networks combined with cylindrical alveolar cavities (Figure 4C) to study airflow mixing in the pulmonary acinus and to predict the fate of inhaled aerosols in the distal airway [106-109]. For example, Sznitman's lab created a model that mimics the alveolar morphology, flow patterns, and other important physiological characteristics aspects of fetal lungs developing in utero [106]. Furthermore, this platform enabled time-resolved imaging of inhaled particle flight trajectories and ensuing respiratory acinar flows [109]. This study demonstrated the complexity of acinar particle dynamics that result from the coupling between intrinsic transport mechanisms (gravity and diffusion) and the complex local alveolar airflow patterns.

Recent advances in lung-on-a-chip technology

Early investigations described above exemplify an array of opportunities enabled by lung-on-a-chip technology for prediction and mechanistic investigation of complex physiological responses of the human lung. Building upon the success of these studies, researchers have recently reported several lung-on-a-chip systems that model various aspects of lung physiology and pathophysiology. Here we describe representative examples of these models.

Small airway-on-a-chip

Lung-on-a-chip model systems enable unprecedented study of the synergistic crosstalk between multiple cell types that drive pathological processes in the human lung as demonstrated by the work of Benam et al. [110]. In this study, the research team engineered a human 'small airway-

on-a-chip' to study the influence of epithelial-endothelial crosstalk on inflammatory cytokine production and neutrophil recruitment in the small airways of patients with diseases such as asthma and chronic obstructive pulmonary disease (COPD). The upper microfluidic channel was seeded with human small airway epithelial cells later cultured at the air-liquid interface to facilitate mucociliatory differentiation while the lower microfluidic channel was seeded with lung microvascular endothelial cells and perfused to create a physiological milieu capable of modeling organ-level inflammatory disease processes. First, to model goblet cell hyperplasia, cytokine hypersecretion and mucociliatory dysfunction seen in the small airways of asthmatics, supraphysiological concentrations of IL-13 were added to the small airway-on-a-chip via the medium perfused in the microvascular channel. The use of COPD patient-derived cells in this device recapitulated key disease features including selective cytokine secretion, increased neutrophil recruitment, and exacerbation-like increases in these responses upon introduction of viral and bacterial pathogens. This system was also treated with IL-13 to create a disease model that recapitulated certain features of asthma, which was used to demonstrate quantitative measurement of clinically-relevant outputs upon treatment with anti-inflammatory compounds, including reduced cytokine production, decreased goblet cell hyperplasia, and recovery of normal ciliary beat frequency.

Organotypic lung-on-a-chip model for the study of host-pathogen interactions

Lung-on-a-chip technology provides new opportunities to model pathophysiologically-relevant host-pathogen interactions by allowing for co-culture of lung cells with living bacteria, virus, or fungi in a controlled manner. A recent example of modeling host-pathogen interactions in the lungs can be found in the work of Barkal et al. that established a microengineered model of the

human bronchiole used to study the early stages of *Aspergillus Fumigatus* infection in human lungs [111].

A central lumen was seeded with small airway epithelial cells to form the bronchiole, and microvascular endothelial cells were added to the two remaining lumens to form perfusable vessels. Additionally, fibroblasts were embedded in the surrounding collagen hydrogel to model the stromal tissue compartment. To model the small airway response to inhaled pathogens, the central airway lumen was exposed to *Aspergillus Fumigatus*, a common fungal pathogen responsible for respiratory infections. Following exposure, the fungus traversed the epithelial barrier and formed typical fungal hyphae structures in the surrounding collagen hydrogel. To model the first line of host defense, human neutrophils were added to the perfused microvascular lumens where some cells were shown to enter the surrounding hydrogel and migrate toward fungal hyphae structures.

Leveraging the inherent capacity of microengineered models to physically separate and fluidically connect different types of cells or microbes, the authors created a closed device containing separate chambers of *Aspergillus Fumigatus* and the bacterium *Pseudomonas Aeruginosa* along with three human bronchiole modules to facilitate volatile communication between the pathogens and the airway epithelial cells via open airway lumens. While this work successfully recapitulated human pulmonary fungal infection *in vitro*, much additional progress is needed to realize clinically-relevant models of pulmonary infection that will help inform a better understanding of how to boost the early host response to pathogen exposure.

Alveolus-on-a-chip model of intravascular thrombosis

Although pulmonary thrombosis is a significant cause of patient mortality, there exist no

traditional *in vitro* models of microvascular thrombi formation. Additionally, animal models of pulmonary microvascular thrombosis fail to capture the complex hemodynamic behavior of human lungs. To address these limitations, Jain et al. presented a microfluidic alveolus-on-a-chip model that recapitulated the organ-level pathophysiological effects of pulmonary thrombosis [112].

Primary human alveolar epithelial cells were cultured at the air-liquid interface along the membrane in the upper compartment, and HUVECs lined all four sides of the lower compartment to form a continuous lumen. This platform enabled the perfusion of whole blood through the lower vascular compartment without thrombus formation nor platelet adhesion in healthy engineered microvessels. Following stimulation with tumor necrosis factor-α (TNF- α), however, the system exhibited thrombus formation and rapid platelet recruitment. The aggregating platelets in this condition also formed a teardrop shape that mirrored *in vivo* observations, which is absent in ECM-coated microfluidic devices traditionally used to study thrombus formation. Additionally, the system was used to study lipopolysaccharide (LPS) endotoxin-induced thrombus formation, revealing that tissue-tissue interactions between the alveolar epithelium and vascular endothelium mediate the prothrombotic response through an epithelium-generated cytokine cascade. Lastly, the application of the alveolus model in preclinical drug development was shown by demonstrating the cytoprotective and antithrombotic activity of permodulin-2 (PM-2), a potent inhibitor of inflammation-mediating protease activated receptor-1 (PAR-1), in the presence of LPS.

Challenges and future directions

Organoid and lung-on-a-chip technologies are able to recapitulate several basic functions of the

lung, but a number of obstacles need to be tackled as the field develops into preclinical applications. Despite the obvious benefit of organoids in recapitulating cellular heterogeneity and physiologically relevant complexity, it is still unclear how useful organoids will be for studying dynamic interactions under physiological vascular shear. In this particular case, the field will more likely overcome this limitation through synergistic engineering between organoid and lung-on-a-chip approaches (organoid-on-a-chip) [113]. Thus, it is important to accelerate the combined engineering and stem cell expertise among the scientific community. Unlike organoids, the lung-on-a-chip system is able to reproduce key dynamic processes, including immune-endothelial cell interactions under blood flow and inhalation/exhalation-associated airflow. Taking it a step further, this technology can be used to reconstitute disease-specific biological responses to smoke or fine particles and identify molecular signatures that can serve as potential therapeutic targets or diagnostic biomarkers [114]. Given the complex composition of the fluid in organoids' lumen and its challenging accessibility, it is currently unclear whether the organoid system can be adapted to similar air stimuli. Furthermore, the primary concern regarding the use of hPSC derived organoids is that current differentiation protocols often mimic the second trimester of fetal development. This concern is a major drawback, especially when organoids are used for modeling adult diseases and challenging clinical applications such as drug development, screening and personalized medicine. As mentioned above, mechanical forces of the ECM, flow, and inputs from the surrounding mesenchymal-endothelial cells might play a key role in promoting such maturation events. The development of organoid-on-a-chip could overcome some of these limitations and generate fully differentiated human epithelial cells. In line with this idea, the use of hPSCs as a source of cells for lung-on-a-chip system has a number of advantages. For example, hPSCs provide a potentially unlimited source of different cell types

which can be a cost effective and more robust source than primary cells. Furthermore, given the major challenges in obtaining human lung tissues from patients and healthy individuals, the use of hPSCs combined with gene editing technologies (e.g. CRISPR) to generate disease-causing mutations can eliminate the need for invasive procedures, such as biopsy and bronchoscopy brushing. One of the most difficult obstacles to overcome when using organotypic cultures is the challenge of generating organoids of a uniform size and controlling cell ratios during self-organization. In contrast, the lung-on-a-chip approach where cells are cultured in a defined orientation and in a uniform manner represents a more reproducible cell culture system. This organoid variation makes it hard to incorporate microsensors for critical control and functional parameters (e.g. barrier integrity, cell migration, flow, oxygen, glucose, fluid pressure), which has been proved to be enormously useful in organ-on-a-chip systems [115-117]. Despite the remarkable progress in lung-on-a-chip technology, another challenge to overcome is that the ECM-coated PDMS membranes that mimic tissue interface may have different transport and structural properties from those seen in vivo.

In summary, both organoid and lung-on-a-chip approaches offer a wealth of opportunities not only to investigate mechanisms involved in lung development, regeneration and disease but also to screen for drugs which may eventually lead to more efficient treatments of debilitating lung diseases.

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Figure legend

Figure 1. **Human and mouse lung architecture. Left panel**: The walls of the human conducting airways consist of pseudostratified epithelium with basal, multiciliated, and secretory cells surrounded by supporting smooth muscle, fibroblasts, cartilage, vasculature and neurons extending along the airways. The epithelium lining the bronchi (Br) is predominately composed of mucous secreting goblet cells, scattered neuroendocrine (NE) cells, and neuroendocrine bodies (NEBs), whereas the bronchiolar epithelium (Brl) is lined by club cells and an increased number of NE cells and NEBs. **Right panel**: In mouse, only the pseudostratified epithelium of the trachea (Tr) and bronchi (Br) are surrounded by cartilage. The bronchioles (Brl) are lined by a simple epithelium that consists of multiciliated and club cells, a small number of NE cells, NEBs, and branchioalveolar stem cells (BASCs). In the distal alveolar region (Alv), the epithelium is highly vascularized and composed of flat alveolar type 1 cells (AEC1), flanked by cuboidal alveolar type 2 cells (AEC2), which form a 600nm-2μm thin barrier and provide an extensive surface area for gas diffusion [118-120]. Bronchioalveolar duct junction (BADJ)

Figure 2. Derivation and use of organoids and lung-on-a-chip.

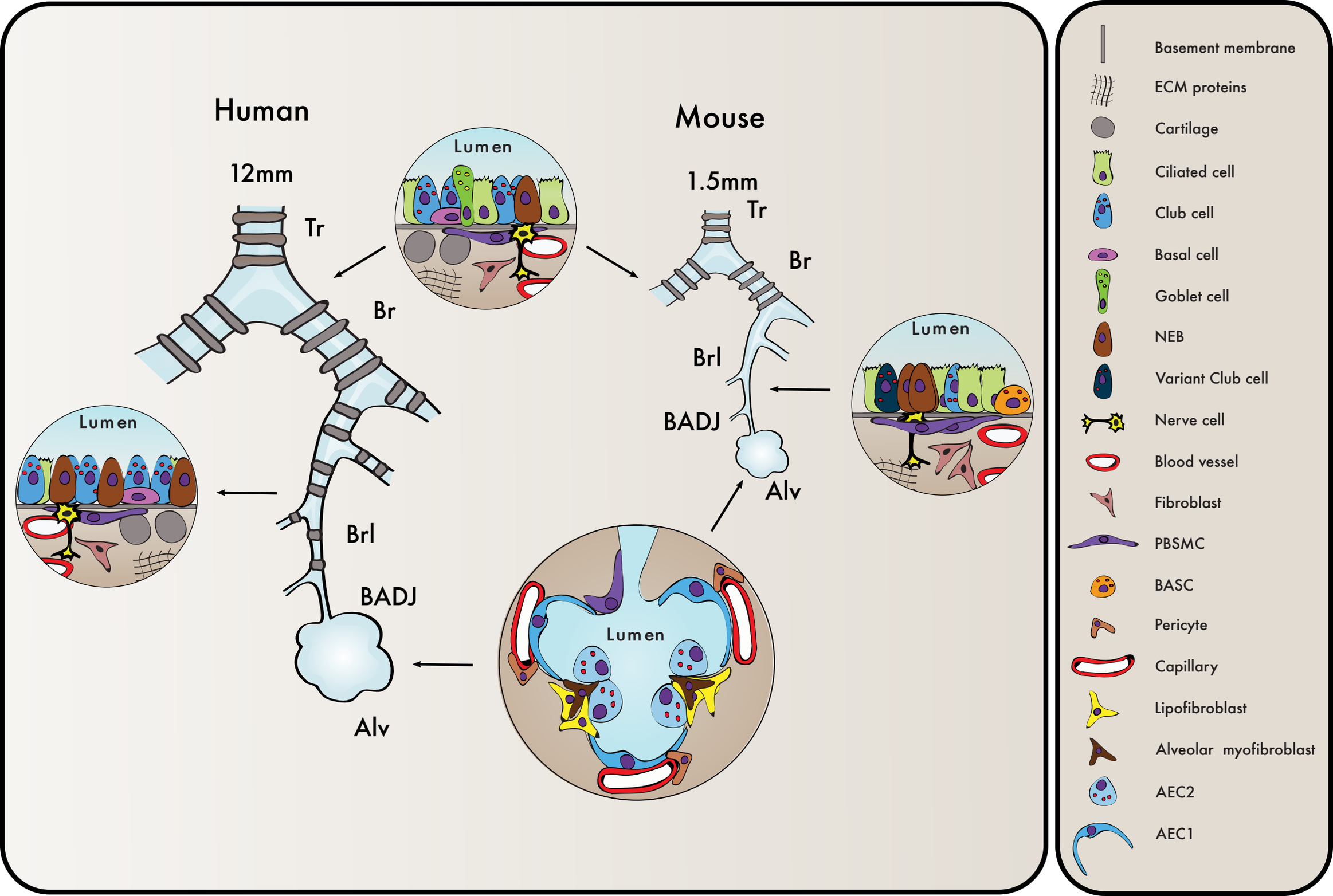
Human and mouse organoids have been generated from stem cell populations isolated from (A) healthy or diseased lung tissue and pluripotent stem cells (PSCs). (B) PSCs, such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), can be maintained in culture for a number of passages and differentiate into derivatives of the three germ layers. iPSCs can be generated from reprogrammed somatic cells isolated from healthy individuals or people who suffer from respiratory diseases. The reprogramming process is carried out by overexpressing the Yamanaka factors OCT3/4, SOX-2, KLF4 and C-MYC in somatic cells and expanding

individual iPSCs colonies under conditions that support PSCs self-renewal [121, 122]. (C) Gene editing approaches such as the CRISPR/Cas9 technology enable the generation, or correction, of mutations leading to respiratory disease, providing appropriate control cells for these studies. With the development of robust differentiation protocols, iPSCs can be used to generate airway and/or alveolar epithelial cells. (D) Further culture in Matrigel and specific growth factor cocktails can give rise to organoids which resemble distinct spatial regions of the lung. Organoids can also be grown from mouse stem cells isolated from the adult lung as well as from mouse PSCs. Depending on the origin of the stem cell population used, organoids are called tracheospheres (trachea), bronchospheres (large airways) or alveolospheres (alveolar region). (E) In the future, organoid biobanks of healthy and diseased lung tissues will be established. These biobanks can be a major source of organoids covering the range of genetic mutations known to lead to respiratory disorders. (F) Lung-on-a-chip have been generated from differentiated cells, such as airway and/or alveolar epithelial cells, fibroblast and endothelial cells derived from primary cultures or isolated from healthy or diseased human or mouse tissue. It remains to be tested whether hPSC derived cells can also mimic human biology and human disease in the lung-on-a-chip approach. Both, organoids and lung-on-a-chip systems are amenable to high-throuput set ups and have immediate application to model respiratory diseases, identify new drugs able to normalize the disease phenotype, evaluate the potency and toxicity of candidate drugs and identify biomarkers of drug interactions and drug induced injury in the lung. In addition, both approaches have possible applications in personalized medicine, for instance to reveal which patients may benefit from treatment with a specific drug.

Figure 3. Epithelial subtypes exhibiting stem cell properties during development and

repair. (A). Proximal progenitor cells. BSCs expressing Krt5, Krt14, Trp63 and Ngfr can give rise to Club (Scgb1a1^{pos}, Cyp2f2^{pos}), ciliated (Foxj1^{pos}, b-tubulin^{pos}), and goblet (Muc5ac^{pos}) cells. Neuroendocrine cells (Cgrp^{pos}) and Club cells can generate both goblet and ciliated cells. **(B)** Distal epithelial progenitors. BASCs co-expressing Sftpc and Scgb1a1 contribute to the generation of AEC2 and AEC1 cells. LNEP (Sftpc^{neg}/α6β4^{pos}) and AEC2 (Sftpc^{pos}) cells can also act as precursor cells able to self-renew and differentiate into AEC1 cells. Mouse cell type specific markers are shown.

Figure 4. Micro-engineered models of the human lung-on-a-chip. (A, Left panel) Schematic representation of cross-section through the lung-on-a-chip model containing two PDMS compartments separated by a thin porous membrane and surrounded by two vacuum chambers. The top "air" compartment is lined by human epithelial cells; the bottom "liquid" compartment is cultured with human endothelial cells. **(A, Right panel)** Vacuum can be applied to cyclically stretch the microengineered epithelial-endothelial barrier and to mimic physiological breathing motions. (B, Left panel) Schematic representation of lung-on-a-chip model containing two PDMS compartments separated by a stretchable membrane to form the alveolar barrier. The lower compartment is filled with culture medium and a PDMS membrane (microdiaphragm) at the bottom of the compartment is surrounded by a cavity. (B, Right panel) The alveolar barrier is stretched downwards a negative pressure applied in the cavity. (C) Alveolated microfluidic channel lined with regularly positioned cylindrical cavities replicating the in vivo acinar morphology. Image modified from [106].


Human
12mm
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Lumen
Basement membrane
ECM proteins
Cartilage
Ciliated cell
Club cell
Basal cell
Goblet cell
NEB
Variant Club cell
Nerve cell
Blood vessel
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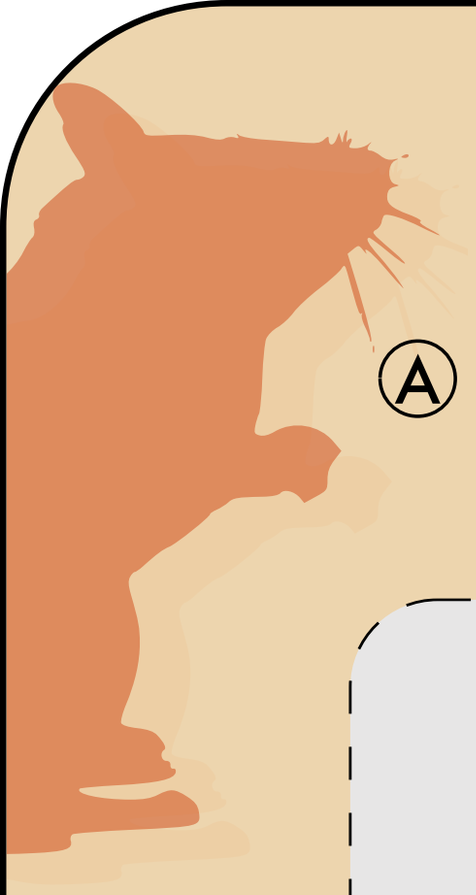
BASC
Pericyte
Capillary
Lipofibroblast
Alveolar myofibroblast
AEC2
AEC1



Air flow

Epithelium

Membrane

Endothelium

Fluid flow

F

Lung-on-a-chip

A

Modeling development and disease

Identification of Biomarkers

Drug screening

Toxicity test

Differentiation

Differentiation

Reprogramming

hPSCs

B

C

Gene editing

E

Biobanks

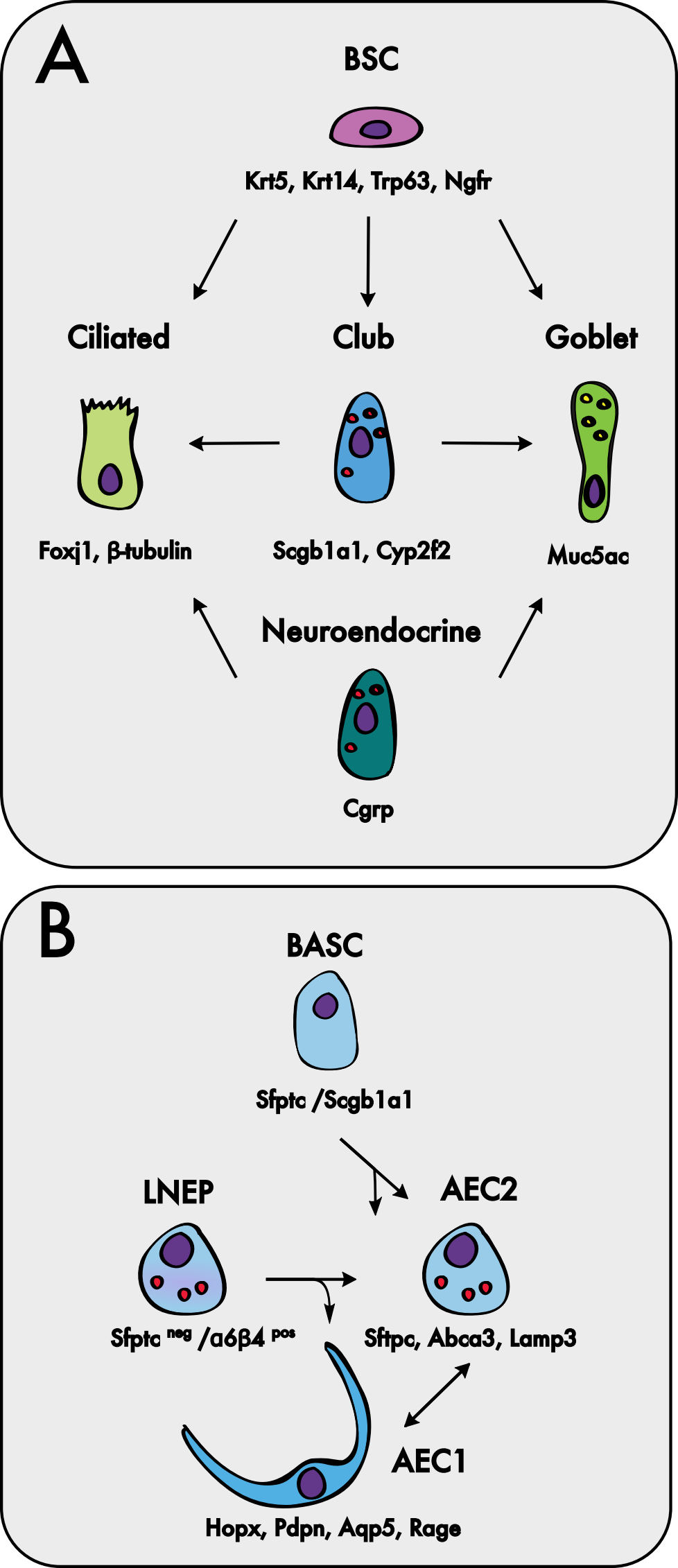
ECM

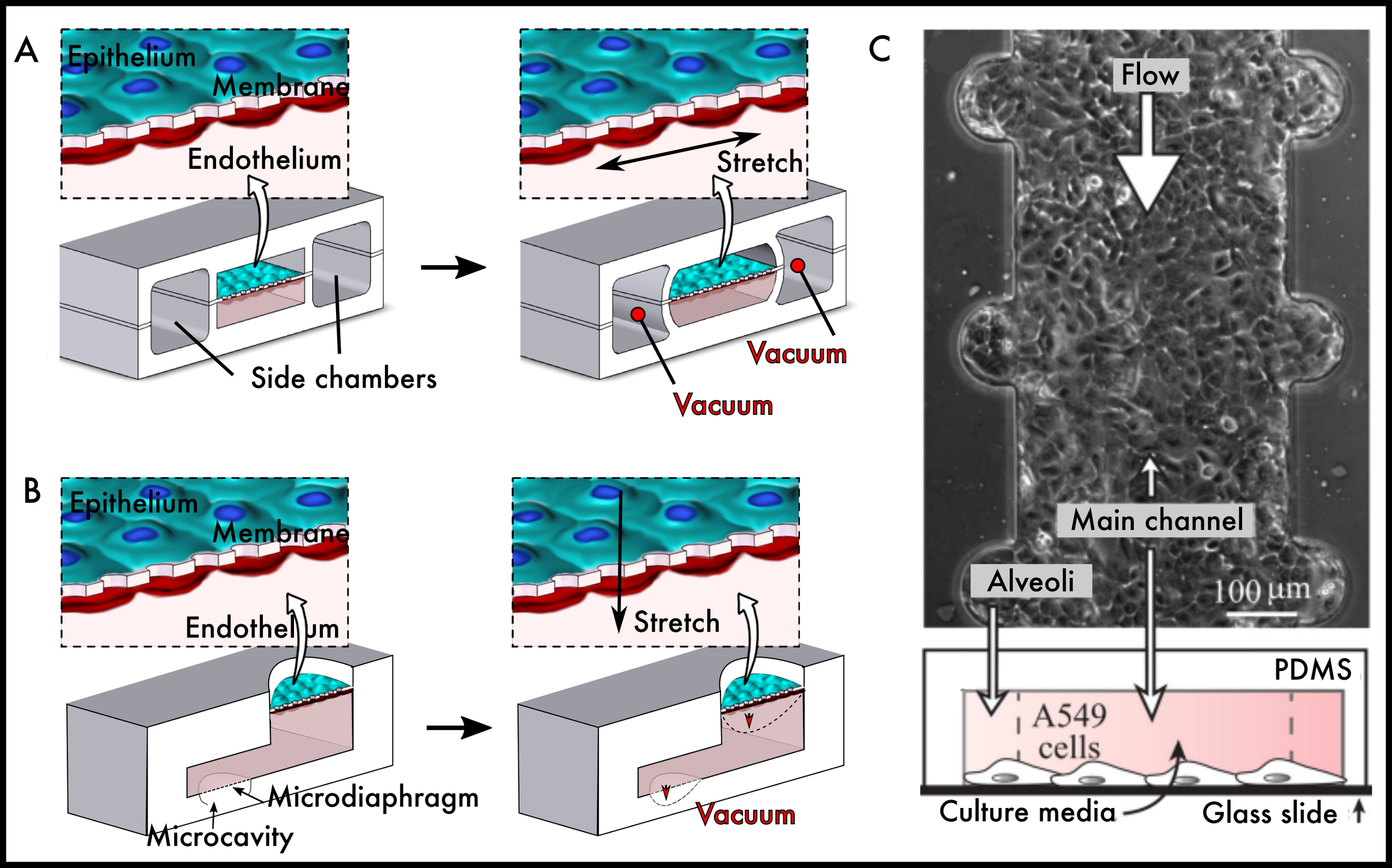
D

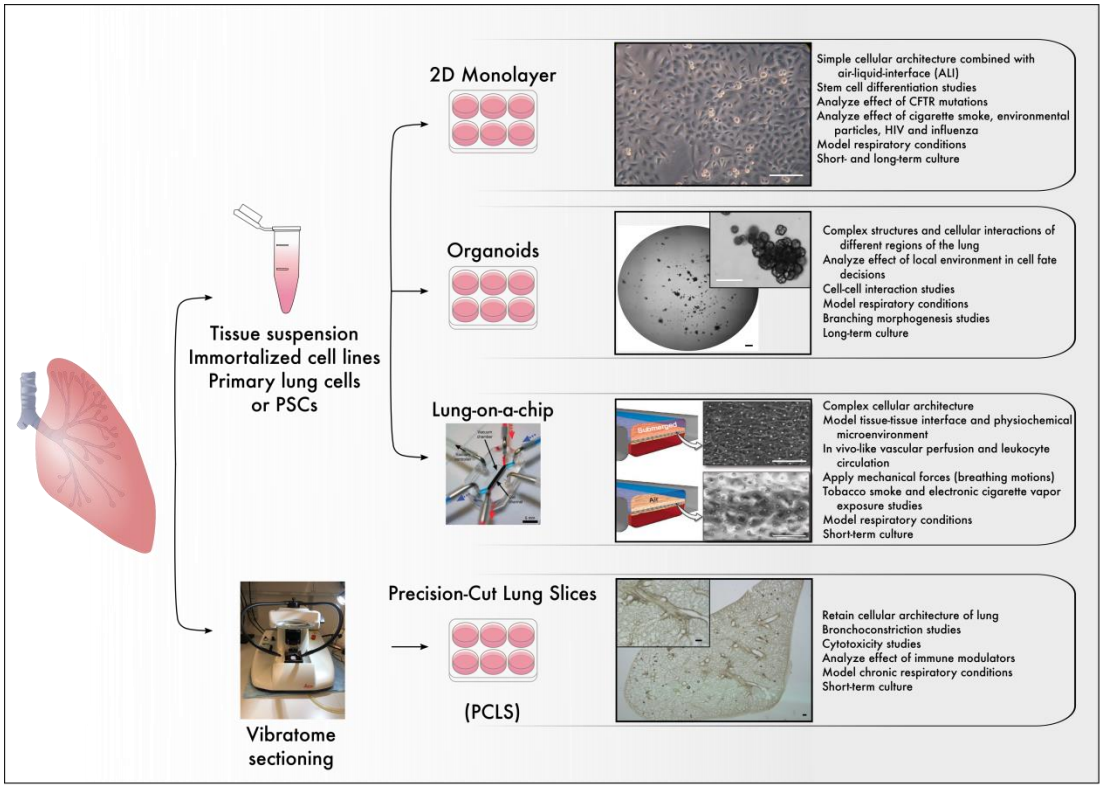
Organoid

A

Healthy / Affected Individual


A
BSC
Krt5, Krt14, Trp63, Ngfr
Ciliated
Club
Goblet
Foxj1, β-tubulin
Scgb1a1, Cyp2f2
Muc5ac
Neuroendocrine
Cgrp
B
BASC
Sfptc /Scgb1a1
LNEP
AEC2
Sfptc neg /α6β4 pos
Sftpc, Abca3, Lamp3
AEC1
Hopx, Pdpn, Aqp5, Rage


A
Epithelium
Membrane
Endothelium
Side chambers
Stretch
Vacuum
Vacuum
B
Epithelium
Membrane
Endothelium
Microdiaphragm
Microcavity
Stretch
Vacuum
C
Flow
Main channel
Alveoli
100 µm
PDMS
A549
cells
Culture media
Glass slide



Supplementary Figure 1. Current lung-related culturing techniques and their potential to model in vivo respiratory events. Scale bar, 75um.

Glossary

α6β4	Heterodimeric pairing of integrinα6 and integrin β4
AEC1	Alveolar type 1 cells
AEC2	Alveolar type 2 cells
BADJ	Bronchioalveolar duct junction
BASC	Bronchioalveolar stem cells
Bmp4	Bone morphogenetic protein 4
BSC	Basal stem cells
Cd	Cluster of differentiation
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator gene
CHIR-99021	GSK-3 inhibitor
COPD	Chronic obstructive pulmonary disease
CPM	Carboxypeptidase M
Cyp2f2	Cytochrome P450family 2 subfamily f
DE	Definitive endoderm
ECM	Extracellular matrix
Egf	Epidermal growth factor
EpCAM	Epithelial cell adhesion molecule
ESCs	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
Fgf	Fibroblast growth factor
Fgfr	Fibroblast growth factor receptor
Gata6	GATA binding protein 6
qPCR	quantitative Polymerase Chain Reaction
Gli1	Glioma-associated oncogene 1
GSIβ4	Griffonia simplicifolia isolectin beta4
GSK3	Glycogen synthase kinase 3
Hh	Hedgehog
HPS	Hermansky-Pudlak syndrome
Id2	Inhibitor of DNA binding 2
IHC	Immunohistochemistry
IL	Interleukin
IPF	Idiopathic pulmonary fibrosis
iPSCs	Induced pluripotent stem cells
Itga6	Integrin subunit alpha 6
Itgβ4	Integrin subunit beta 4
Krt5	Cytokeratin 5
Lgr	Leucine rich repeat containing G protein-coupled receptor 5
LBOs	Lung bud organoids derived from human embryonic stem cells
LPS	Lipopolysaccharide endotoxin
LuMECs	Murine lung endothelial cells
Mmp14	Matrix metallopeptidase 14
mPSC	mouse Pluripotent Stem Cells
NE	Neuroendocrine cell
Nfatc-1	Nuclear factor of activated T cell 1
Nkx2.1	NK2 Homeobox 1

Ngfr	Nerve growth factor receptor
Notch1	Neurogenic Locus Notch Homolog Protein 1
P38	Transcription factor Trp38
p63	Transcription factor Trp63
PCECs	Pulmonary capillary endothelial cells
Pdgfra	Platelet derived growth factor receptor alpha
PDMS	Poly-dimethylsiloxane
ROCK	Rho-associated, coiled-coil containing protein kinase
Poly (I:C)	Polyinosinicpolycytidylic acid
PSCs	Pluripotent stem cells
qPCR	Quantitative polymerase chain reaction
RA	Retinoic Acid
SAECs	Small airway epithelial cells
Sca1	Stem cell antigen 1
Scgb1a1	Secretoglobin 1a1
Sdf-1	Stromal cell-derived factor 1
Sftpc	Surfactant protein C
Shh	Sonic Hedgehog
Sox	SPY (sex determining region Y)-box
Stat3	Signal transducer and activator of transcription 3
Tgfβ	Transforming growth factor β
Tsp1	Thrombospondin-1
TN-C	Tenascin-C
Vegf	Vascular endothelial growth factor
Vegfr	Vascular endothelial growth factor receptor
VAFECs	Ventralized anterior foregut endoderm cells
Wnt	Wingless-related integration site