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Teaser This review describes progress in respiratory system model development and key applications of culture inserts, artificial and decellularized scaffolds, and microfluidic chips as platforms for development of near-to-native lung tissue.

Options for modeling the respiratory system: inserts, scaffolds and microfluidic chips

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The human respiratory system is continuously exposed to varying levels of hazardous substances ranging from environmental toxins to purposely administered drugs. If the noxious effects exceed the inherent regenerative capacity of the respiratory system, injured tissue undergoes complex remodeling that can significantly affect lung function and lead to various diseases. Advanced near-to-native *in vitro* lung models are required to understand the mechanisms involved in pulmonary damage and repair and to reliably test the toxicity of compounds to lung tissue. This review is an overview of the development of *in vitro* respiratory system models used for study of lung diseases. It includes discussion of using these models for environmental toxin assessment and pulmonary toxicity screening.

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Introduction

The World Health Organization (WHO) estimates that ~7 million deaths were caused by air pollution exposure in 2016; air pollution is the world's greatest single environmental health risk [1]. Diseases like acute lower respiratory disease, chronic obstructive pulmonary disease (COPD), lung cancer, ischemic heart disease and stroke are attributable to the combined effects of air pollution. Moreover, the International Classification of Diseases category 'Respiratory conditions due to other external agents' includes drugs and external agents as disease causes [2]. Exposure to air pollutants and other harmful airborne substances, as well as side-effects of some drugs, is therefore the reason why the respiratory system is subjected to extensive examination during environmental toxin assessment and drug screening. Development of reliable and efficient *in vitro* models is a priority.

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Animal models can be used for respiratory toxicity testing and drug screening. However, even considering the principles of reduction, refinement and replacement (i.e., the 'three Rs') that guide the use of animals in experiments [3], part of the scientific community believes that animal models are not valid representations of the complexity of the human body. For example, Greek *et al.* thoroughly discussed the roles of animal models and the methodologies used [4]. They concluded that animal model results revealed drug side-effects but were insufficient for predictions of outcomes in humans because animals are completely different complex systems.

The human respiratory system has two main components; one is used for conductance of airflow and filtration and one is used for respiration (i.e., gas exchange) [5]. The entire system is continuously exposed to hazardous substances that can cause lung injury [5,6]. The subsequent remodeling results in a compromised lung function. Lung injury and repair mechanisms can be studied using models of specific parts of the respiratory system. Inhaled toxins and other allogeneic particles first contact the cells of the respiratory mucosa [5]. The size of the inhaled substance is a crucial factor to include in an investigation [6]. Larger particles are captured in mucus and then moved to the pharynx via ciliary clearance [5]. Particles <2–5 μm can reach the alveoli and undergo phagocytosis by the resident macrophages [6]. Gas diffusion follows Fick's law, as gases diffuse through the alveolar-capillary barrier directly into the bloodstream [7].

Lung histology

An in-depth knowledge of lung histology is necessary to develop a functional and credible model of the respiratory system. Here, we provide a basic overview of human lung histology that includes the fundamental nomenclature required to understand this component of the respiratory system. The conducting component of the respiratory system (Fig. 1b) is lined with respiratory mucosa covered with pseudostratified ciliated columnar epithelium, with or without an underlying submucosa [5]. Collapse is prevented by underlying bone or cartilage. Secretions produced by different types of glands ensure proper moisturization and capture of particles and pathogens. As the bronchioles branch in a distal direction, the epithelium gradually decreases in thickness and cartilage and glands disappear.

The respiratory bronchioles provide a transition to the respiratory part of the system. The cuboidal epithelium and solitary alveoli that extend from the respiratory bronchiole walls have functional roles in air conductance and gas exchange. Alveolar ducts and alveolar sacs open to the alveoli, which are terminal structures specialized for respiration. There are ~400 million alveoli per human respiratory system; alveoli diameters range from 100 to 300 μm. Alveoli have thin walls that contain numerous blood capillaries and an epithelial lining (Fig. 1a). This epithelium consists of type I and type II alveolar cells (AT I and AT II cells, respectively; or pneumocytes I and II, respectively). AT I cells have key roles in gas exchange and alveolar–capillary barrier formation.

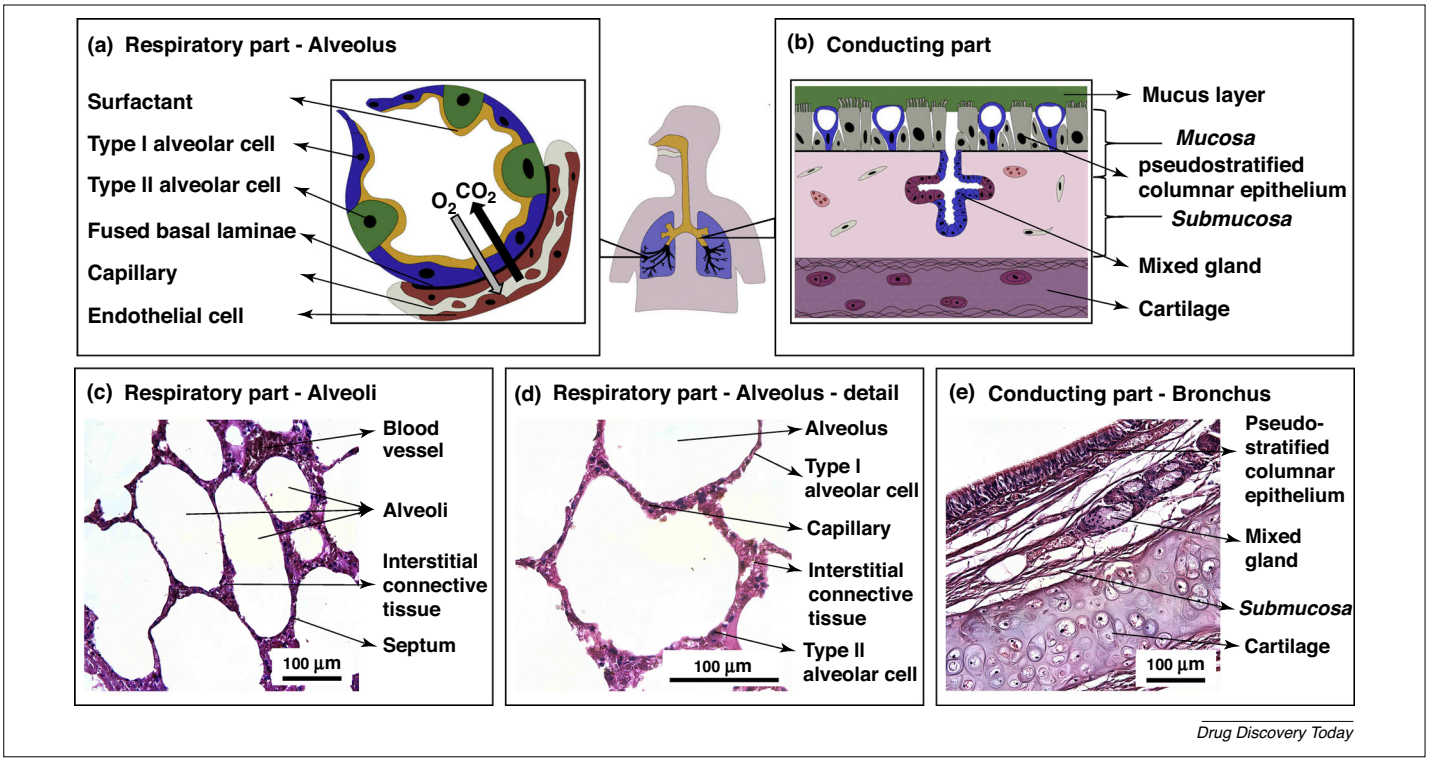

FIGURE 1

Schematic illustrations and histological sections of human respiratory system —respiratory and conducting part. **(a)** Respiratory part is responsible for the exchange of oxygen (O_2) and carbon dioxide (CO_2). This exchange takes place within alveoli as gases diffuse across the alveolar–capillary barrier. Alveolar–capillary barrier is composed of thin surfactant layer, type I alveolar cells, fused basal laminae and endothelial cells of the capillary. **(b)** Conducting part is composed of respiratory mucosa, submucosa and cartilage. Secretion of glands ensures proper moisturizing and pathogen or particle capture. **(c)** Histological section of human lung tissue showing respiratory part with multiple alveoli. **(d)** Histological section of alveolus. **(e)** Histological section of human bronchus representing conducting part. Scale bar: 100 μm.

AT II cells produce surfactant, replace themselves and AT I cells upon injury and play a part in fluid resorption and immunomodulation [5,6]. The alveolar–capillary barrier is thus composed of a thin surfactant layer, AT I cells, the fused basal laminae of the alveolar and endothelial cells and the endothelial cells of the capillaries. The alveolar–capillary barrier varies from 0.1 to 1.5 μm in thickness [5]. The pulmonary extracellular matrix (ECM) consists of basal laminae and interstitial ECM. Basal laminae consist of type IV collagen, laminin, fibronectin and proteoglycans. Interstitial ECM consists mainly of type I and type III collagen, elastin, glycosaminoglycans and proteoglycans [8,9].

The vascular and lymphatic circulation have key roles in tissue nutrition and immune responses. The nerve supply is important for the reflexive responses of air passages and blood vessels [5]. Not all of these components are routinely incorporated into *in vitro* models of the human lung. The complexities associated with the intricate branching of bronchi, blood vessels and nerves force scientists to omit structures from a model or to simplify the structures. However, crucial structures and functional aspects of the respiratory system (e.g., air–liquid interface, breathing motions and dynamic flow) can be created *in vitro*.

Cells: a key factor of every successful *in vitro* model

Of the factors that must be considered before creating an *in vitro* model of the respiratory system, the most important is choosing the proper cell type. Primary cells isolated from healthy donors, disease-specific primary cells or cancer cell lines are used for *in vitro* experiments. Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) can be differentiated into the desired cell types and used for *in vitro* lung tissue modeling as well [10–13].

Normal human bronchial epithelial cells [14], the A549 human lung carcinoma epithelial line of cells [15] and the NCI-H441 (see Glossary) human papillary adenocarcinoma lung line of cells [16] are the cells most commonly used for respiratory system modeling. Compared with primary cells, cancer-derived cells can cause misleading results owing to differences in metabolic capacity [17]. Use of carcinoma cell lines risks nondisclosure of the differences between their origin-dependent abnormalities and the phenomenon under study. Specific cell types should be carefully selected with respect to the morphological and functional aspects of the intended experimental goal. The access to tissue samples for primary cell isolations presents a challenge for the investigator. Additionally, as the lung is an organ with very low cell turnover rates [18], use of progenitor cells differentiated from the hESCs or patient-derived hiPSCs offers a promising source of human cells with respect to their relatively good accessibility, easy expansion and noncarcinoma origin.

Technical constraints: inherent limitations of inserts, scaffolds and microfluidic chips

In vitro models of the human respiratory system can be divided into two categories: static culture systems and dynamic culture systems. Static culture systems are typically designed in a planar or 3D arrangement. They are the most universally used systems and include typical cell monolayers in culture wells, dishes or flasks, as well as more-complex culture systems represented by inserts and 3D scaffolds. Dynamic culture systems are represented by bioreactors and microfluidic chips. Bioreactors are mechanical devices with pumps that provide dynamic culture conditions to 3D scaffolds, which represent the actual cell carriers. The source of dynamic culture conditions (i.e., the device with pumps) is combined with the actual place for cell adhesion and growth (i.e., the microfluidic channel) in microfluidic chips.

GLOSSARY

A549 A549 cell line
NCI-H441 NCI-H441 cell line
SPCA-1 human non-small-cell lung cancer cell line
NKX2-1$^+$/SOX2$^+$ early proximal lung progenitor cell
HUVEC human umbilical vein endothelial cell
HL-60 neutrophil cell line
HFL1 human lung fibroblast cell line
hESCs human embryonic stem cells
hiPSCs human induced pluripotent stem cells
AT I type I alveolar cells
AT II type II alveolar cells

The technical constraints inherent to each type of *in vitro* respiratory system model limit the numbers and types of analytical methods that can be used. For example, standard planar culture systems, including inserts, do not allow for dynamic culture conditions nor for more-sophisticated motion simulation, such as breathing motions. These systems also do not offer any 3D spatial simulation, and mimicking the ECM is limited to coatings. Consequently, the standard planar culture systems mimic the *in vivo* state the least. By contrast, low cost, easy handling, facile microscopic visualization and good quantity and purity of cellular extracts are the benefits of using them. Inserts, moreover, enable establishment of cell interfaces, including the air–liquid interface.

3D scaffolds, either artificial or decellularized, have the ultimate benefit of providing 3D spatial stimulation and a close ECM mimic. Decellularized scaffolds contain preserved vessel and bronchi branching. However, of note is that the decellularization process can pose a risk of loss of specific ECM components during the scaffold preparation. The level of ECM loss can be attenuated by automated decellularization systems as compared with manual decellularization processes [19]. An air–liquid interface can be established with scaffolds as well. Static culture conditions are gold standard, whereas dynamic culture conditions can be established using special bioreactors. Breathing motions are harder to recreate but certainly possible. Especially with decellularized scaffolds, where *in vivo* transplantation of 'whole organ construct' (i.e., decellularized scaffold seeded with cells) can be performed. Scaffold manufacturing and/or decellularization is undoubtedly more technically challenging and expensive than the typical planar culture on dishes or inserts. Confocal microscopy is typically used for morphological assessment of 3D scaffolds. Quantity and purity of isolated cellular extracts limit the use of PCR, western blotting and ELISA for scaffold-based modeling.

Microfluidic chips are the most advanced of the presented models. They enable air–liquid interfaces, dynamic culture conditions and simulation of various physiological movements (breathing motions, liquid plug formation). Multi-organ-on-a-chip platforms are also rapidly emerging, which increases clinical validity of obtained data. However, the chips developed to date are mainly based on synthetic porous membranes that lack *in-vivo*-like ECM stimulation. An ECM mimic can be achieved by coating or by

TABLE 1

Advantages and disadvantages of culture inserts, scaffolds and microfluidic chips

	Air–liquid interface	Mimicking ECM	Spatial stimulation	Preserved vessel and bronchi branching	Static culture conditions	Dynamic culture conditions	Breathing motions
Culture insert	xx	(x)			xx	(x)	
Artificial scaffold	xx	xx	xx		xx	x	x
Decellularized scaffold	xx	xx	xx	xx	xx	x	x
Microfluidic chip	xx	x			xx	xx	xx

Advantages and disadvantages of culture inserts, scaffolds and microfluidic chips are indicated. Blank space indicates 'not possible'; (x) indicates 'problematic, but possible'; x indicates 'possible with special equipment'; xx indicates 'very well performed'.

novel approaches, which are discussed later. Analyses of microfluidic chips are highly design-dependent. Owing to the smaller chip sizes and the small fluid volumes passing through the chambers, light microscopy and highly sensitive biochemical methods (e.g., PCR, ELISA) are used for the analyses. These constraints still pose a bit of a challenge for application of microfluidic chips in high-throughput studies. The benefits and limitations of inserts, scaffolds and microfluidic chips are summarized in Table 1.

Planar culture systems

Standard planar culture systems are usually set as single cell type monolayers submerged in cell culture medium in culture wells, dishes or flasks. The parameters that can be modified in these systems are restricted to functionalization of culture substrate using specific coatings (e.g., proteins, proteoglycans), substrate stiffness, cell seeding density and culture medium composition.

Inserts

To increase the complexity of planar culture, specific membrane inserts are used to establish interfaces between cells. These inserts are designed as hanging or standing tools that are placed into a cell culture well (Fig. 2). The porous membrane is located at approximately one-half of the well depth. Cells can be grown on both sides of the culture insert membrane. This flexibility allows co-culture and spatial separation of two cell types, which is beneficial for study of paracrine signaling. An air–liquid interface can be established by eliminating the medium from the upper part of the insert. The apical sides of the cells are then exposed to air, which allows for simulation of the alveolar part of the alveolar–capillary interface [20] and the inner tracheobronchial surface [21,22].

There are several examples of culture inserts used to model the alveolar–capillary barrier. Hermanns *et al.* developed an alveolar-capillary barrier model by seeding the permeable insert with cells

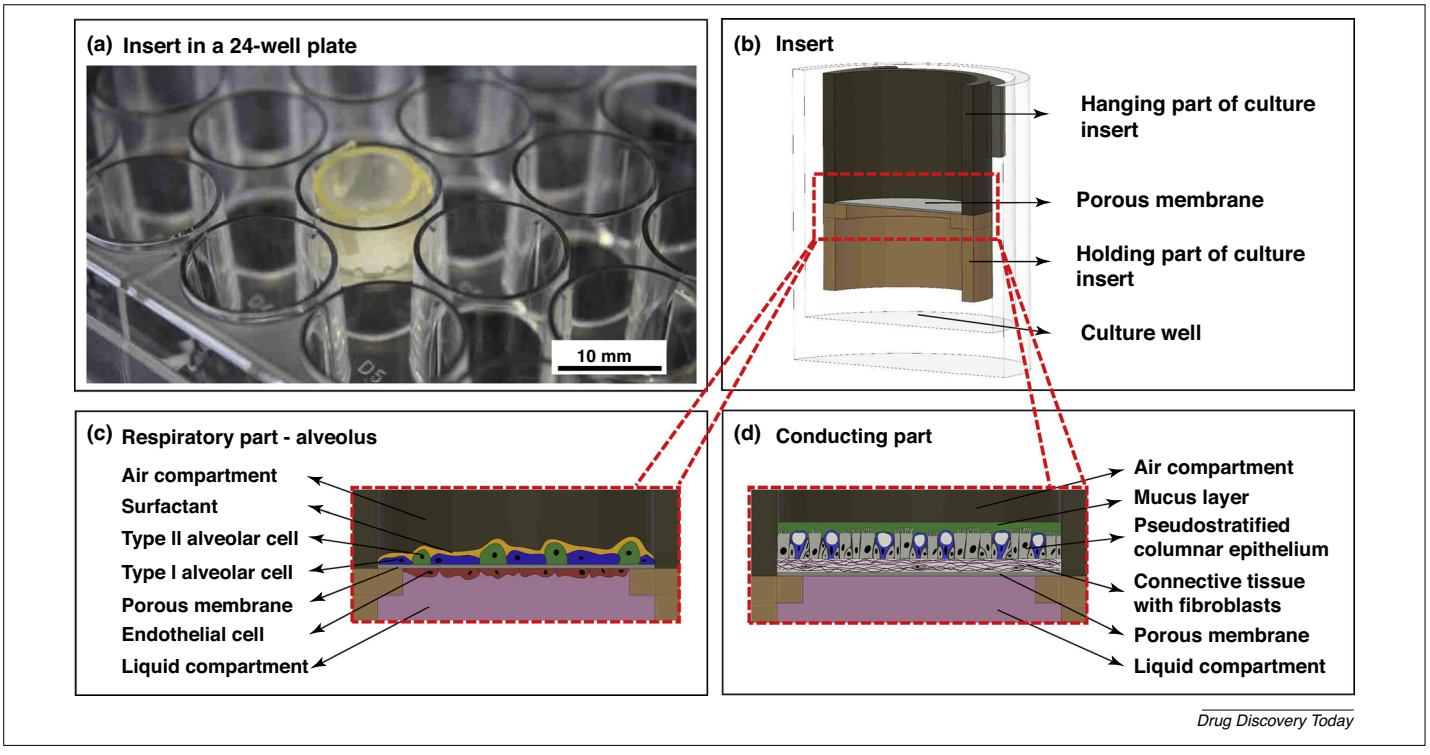

FIGURE 2

Cell culture insert. **(a)** Culture well plate with cell culture insert integrated into a hanging tool. Scale bar: 10 mm. **(b)** Schematic illustration of cell culture insert with its hanging part for emplacement into culture well, porous membrane and holding part that holds the porous membrane. **(c)** Schematic illustration of modeling the respiratory part (alveolus) on a porous membrane of an insert. **(d)** Schematic illustration of modeling the conducting part on a porous membrane of an insert.

of human lung carcinoma cell lines (A549 and NCI-H441) and primary human pulmonary microvascular endothelial cells [16]. After dexamethasone stimulation, the NCI-H441 cells form intercellular junctions that are measured using transepithelial electrical resistance. The NCI-H441 cell line is suitable for human distal lung epithelia transport studies [20], which can be used in basic pharmacological and toxicological experiments. *In vitro* modeling of the alveolar–capillary barrier advanced further via use of exposure to proinflammatory cytokines and nanocarriers [23] and incorporation of immune cells into triple cell co-culture models intended for studying cell–cell interactions and toxicology [15]. In this triple cell model, epithelial cells are co-cultured on transparent inserts with two immune cell types (macrophages and dendritic cells).

Incorporation of immune cells into *in vitro* models of the respiratory system is not a unique approach. Lung epithelial cells, human primary monocytes and macrophages are also co-cultured on fibroblasts embedded in collagen matrix placed on an insert [24]. The culture is then infected with *Mycobacterium tuberculosis*. The epithelial cells stratify and secrete mucus under the air–liquid interface. The immune cells then develop into the early granuloma stages typical of *M. tuberculosis* infection. The granuloma formation using this human lung tissue model is significant because animal models of *M. tuberculosis* infection do not develop true granulomas and it is not possible to follow the complex disease pathophysiology.

A microfluidic insert with a track-etched porous membrane enabling constitution of small microflows was developed to quantify spatial drug concentration gradients [25]. To prove the concept, the researchers exposed neutrophil cell line HL-60 to a chemotactic peptide *N*-formyl-Met-Leu-Phe gradient. The authors found evidence of peptide gradient-associated induced cell migration.

Models of respiratory passages using culture inserts seeded with cells from healthy or diseased tissues are commercially available for drug and toxicity screening [26–28]. There are many examples of applications of culture inserts used to study lung cell interactions, and normal and pathological behavior. Culture inserts are cost-effective and relatively easy to manipulate [29–31] but lack the spatial support required for complex 3D cellular organization. Use of 3D culture systems that more accurately resemble the *in vivo* environment of the human lung is one potential solution.

3D culture systems

3D scaffolds are porous constructs that provide structural support for 3D cell organization, adhesion, proliferation and differentiation. Scaffolds can be categorized by origin into artificially manufactured scaffolds and decellularized scaffolds isolated from native tissues. 3D artificial scaffolds are prepared from natural or synthetic substances processed using various methods (e.g., lyophilization [32], 3D printing [33], salt leaching [34], electrospinning [35]). Decellularized matrices are prepared from cadaver organs or tissues using one or more physical, chemical or enzymatic agents to remove cells from the ECM while preserving the complex 3D structure of the tissue [36].

Artificially prepared scaffolds

Natural substances are often used to prepare artificial scaffolds. Collagen and elastin are highly abundant in lung ECM and are crucial for lung function. Collagen provides structure and support, and elastin increases lung tissue flexibility [8]. Cells use integrin receptors to bind to integrin recognition motifs present in natural ECM macromolecules. Synthetic biocompatible and biodegradable polymers (e.g., polyesters, polyanhydrides, polyurethanes) are also used to prepare scaffolds [37].

An early human bronchial mucosa model consists of collagen gel with cells isolated from healthy human donors or donors with asthma [38]. The fibroblasts and bronchial epithelial cells from the respective donors are included. The air-liquid interface is introduced via a porous polycarbonate filter attached to the bottom of the system. This simple model allows formation of the histological structure of the cells and the ECM that are similar to the *in vivo* composition of healthy mucosa and mucosa affected by asthma-associated inflammation. This model also proves to be functionally relevant to study cellular interactions and inflammation because the addition of T lymphocytes promotes extensive interleukin (IL)-5 production only when cultured using an asthmatic bronchial mucosa model [38].

Tissue remodeling of the airway is a characteristic of asthma. The subsequent airflow obstruction is probably caused by the mechanical strain generated by the smooth muscle cell response [39]. A collagen-based bronchial model containing human fetal lung fibroblasts and human bronchial epithelial cells includes a built-in strain applicator that induces 50% lateral strain [40]. This unique application of mechanical strain, together with the activated eosinophils, promotes the epithelial thickening and ECM remodeling found in samples from patients with asthma, which was not achieved on planar models developed before.

The model was later improved by adding a computer-controlled, motor-driven mechanical arm to apply dynamic strain of various amplitudes and frequencies to further simulate physiologically relevant phenotypes [41]. This change resulted in altered deposition of ECM proteins (e.g., type III and IV collagen, fibronectin and matrix-metalloproteinases). The results correlate with the tissue remodeling that occurs in diseased human airways. The final model supports differentiation of epithelial cells to ciliated cells, mucus-secreting cells and basal cells, and can be maintained for >4 weeks [42].

An even more sophisticated model was designed in the form of a bioreactor system with cylindrical airways [43]. This system maintains the engineered bronchial mucosa under mechanical stimulation and humidified airflow. The bioreactor contains thin silicone rubber tubing covered by collagen matrix with incorporated normal human lung fibroblasts. Small airway epithelial cells are seeded inside the tube and smooth muscle cells are seeded onto the peripheral part of the tubing to mimic the *in vivo* bronchioles. To mechanically stimulate the tissue, pulsed airflow is introduced to the system via a peristaltic pump. This bioreactor system offers a unique opportunity to study various aspects of airway remodeling, cellular interactions and drug screening applications using precisely driven manipulation of individual system components.

An *in vitro* platform using nanofibers was introduced to simulate the fibrous architecture of lung basal lamina [44]. Electrospun nanofibers were prepared from polycaprolactone (PCL) modified with polyethylene glycol (PEG). This approach enables nanofiber biofunctionalization through conjugation of peptides to the nanofiber surface. Epithelial and endothelial cells seeded on each side of the nanofiber layer recapitulate *in vitro* the structural and func-

tional characteristics of the alveolo–capillary barrier. This co-culture model was further improved by the introduction of inflammatory cells (human leukemia monocyte cell line) [45]. This improvement increased its validity for use for pathophysiological studies. The biological properties of PCL nanofibers can also be improved using other changes. For example, PCL modification with hexaamino-*cyclo*-triphosphazene significantly improved cell adhesion, spreading, metabolism and proliferation of NCI-H441 cells and adipose-derived stromal cells [46]. The same modification was used to produce poly-l-lactic acid nanofibers with similar properties.

Lung tissue morphogenesis studies were performed using models based on 3D scaffolds seeded with mouse or rat lung cells. A collagen-based tissue construct containing mouse fetal pulmonary cells (a mix of epithelial, mesenchymal and endothelial cells) has been used to investigate the effects of different fibroblast growth factors (FGFs) on pulmonary epithelial branching and proliferation [47]. The same fetal pulmonary cell mixture is suspended in Matrigel® and injected into the abdominal wall of adult mice. The cells facilitate neovascularization and formation of epithelial ducts, especially after addition of basic FGF (FGF2) [48]. Neovascularization and formation of alveolar-like structures is also achieved using implantation of gel-foam sponges containing fetal rat lung cells into adult rat lung parenchyma [49]. Chen *et al.* used collagen type-I-glycosaminoglycan sponge-like scaffolds seeded with dissociated normal healthy fetal rat lung cells [50]. After 3 weeks of culture, the cells were organized into alveolar-like structures. Some of the epithelial cells differentiated into ciliated pseudostratified epithelium; other cells expressed α-smooth-muscle actin.

Taken together, these findings reveal the potential for artificial complex lung tissue formation. They clearly indicate the potency of the use of artificial matrices for modeling various parts of the respiratory system for tissue engineering, drug screening and lung disease modeling studies. However, there are also limitations associated with artificial scaffolds. In general, they do not have the same chemical and topographical distribution of ECM components as native tissue and are not convenient for use in high-throughput studies.

Scaffolds from decellularized tissue

Use of decellularized native tissue is a promising option for engineering functional lung tissue. Decellularization removes the tissue's cellular content. This change results in 3D scaffolds that preserve the chemical and physical complexity of ECM (Fig. 3). Physical, chemical and enzymatic methods are used to decellularize mouse, rat, goat, sheep, pig, non-human primate and human-origin lung tissue [36,51–56].

A breakthrough in the field of tissue decellularization was achieved in 2008 [57]. A 30-year-old woman with end-stage bronchomalacia was effectively treated by replacing her left main bronchus with an artificial graft. A decellularized human cadaveric trachea scaffold was seeded with autologous bronchial epithelial cells and chondrocytes 6 weeks before grafting. Because of the treatment, the patient avoided undergoing a left pneumonectomy. Follow-up results indicated that the tissue-engineered trachea was well-vascularized, well-epithelialized, had normal mucociliary clearance and the entire lumen length was maintained [51]. This work supported the trend of using tissue-engineering approaches in clinical applications.

Decellularization is a powerful lung-tissue-engineering tool. After construction of the air-conducting component, the discipline moved to bioengineering the entire organ using elimination of the original cells and subsequent scaffold reseeding. Human-sized porcine lungs could be generated after automatization of the procedure [57]. Automatization is achieved by linking the decellularization chamber with fluid reservoirs, peristaltic pumps, valves and a computer with customized software control.

It is even possible to bioengineer an entire functional lung that can survive and continue to develop after it is transplanted into a porcine model. The animal lung is decellularized and reseeded with autologous cells. The bioengineered lung develops vascular perfusion via collateral circulation within 2 weeks after transplantation [58].

The coordination of circulatory and respiratory systems is crucial to ensure functional gas exchange. To examine the gas exchange ability of an *in vitro* recellularized model, rat lung was decellularized using a sodium dodecyl sulfate (SDS) detergent-

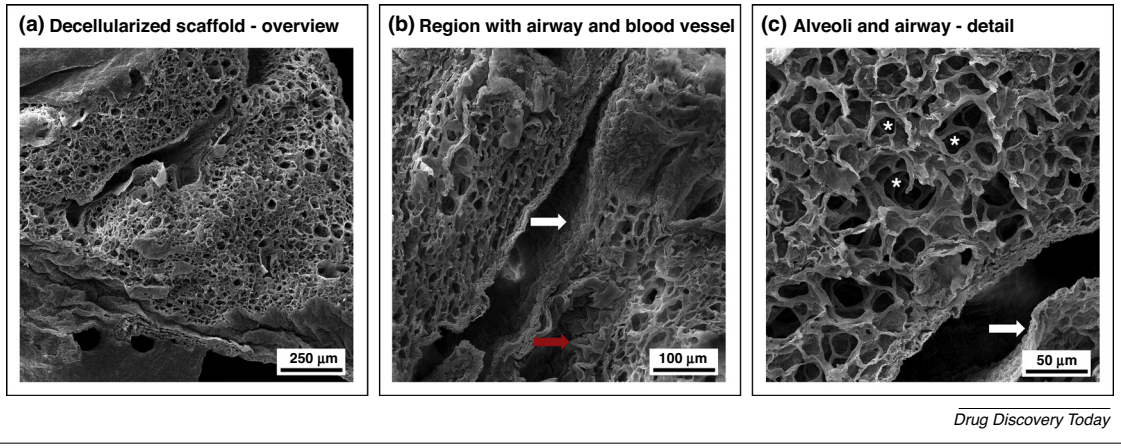

FIGURE 3

Microarchitecture of scaffold from decellularized lung tissue. **(a)** Representative scanning electron microscopy image of decellularized scaffold from mouse lung. Scale bar: 250 µm. **(b)** Region with decellularized airway and blood vessel showing denuded airway wall (white arrow) and denuded vessel wall (red arrow). Scale bar: 100 µm. **(c)** Region with alveoli (white asterisks) and decellularized airway with denuded airway wall (white arrow). Scale bar: 50 µm.

based protocol [59]. Decellularized scaffolds were seeded with rat fetal lung cells obtained during the canalicular development period and with human umbilical vein endothelial cells. After left orthotopic lung transplantation in rats, this recellularized lung tissue participated in *in vivo* gas exchange for 6 h. In the next *in vivo* experiment, whole adult rat lungs were decellularized using a protocol that included 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) detergent [60]. An ECM scaffold with well-preserved branches of airways and vessels was produced. Neonatal rat lung epithelial cells were injected into airways and microvascular lung endothelial cells were seeded into vessels. Efficient repopulation was achieved, and the artificial lungs had mechanical qualities analogous to native lungs. The engineered lungs participated in gas exchange for 45–120 min after left orthotopic lung transplantation. In another study, SDS and Triton™ X-100 were used to decellularize scaffolds from rat lungs [56]. The scaffolds were then seeded with rat fetal alveolar cells and human umbilical vein endothelial cells (HUVECs). These engineered lungs successfully participated in *in vivo* gas exchange for 7 days.

Advances in the field of decellularization have led to new questions for investigation. One study examined whether decellularized lungs from patients with chronic lung illnesses can be recellularized and used for subsequent transplantation to the same extent as decellularized lungs from healthy individuals [61]. The results indicated that scaffolds with pathologically remodeled ECM structure support initial cell binding, but not long-term cell survival (i.e., >1 week). By comparison, lung scaffolds from healthy individuals support growth of human cells (e.g., bronchial epithelial cells, endothelial progenitor cells, lung fibroblasts) for up to 1 month.

To examine whether decellularized lung matrix has the capacity to direct differentiation of lung progenitor cells, scaffolds made of decellularized ECM from rat lung can be seeded with definitive endoderm cells (cKIT+/CXCR4+) derived from embryonic stem cells [62]. The scaffolds direct these cells to NKX2-1+/SOX2+ early proximal lung progenitor cells and later to mature airway epithelial cells. Differentiation depends on the presence of matrix heparan sulfate proteoglycans and associated factors, which remain in the scaffold after decellularization.

Use of dynamic culture conditions also positively affects the fate of cells cultured on decellularized lung scaffolds. Use of this approach results in a significant decrease in cell apoptosis and increases in cell proliferation, and enhances scaffold recellularization [63,64].

The hiPSC is a suitable cell type for use in personalized medicine because patient-specific iPSCs can be generated and differentiated *in vitro* into the desired cell type. In one study, rat and human decellularized lung scaffolds were seeded with alveolar epithelial cells derived from human iPSCs [65]. These cells grew on the scaffold and had markers of differentiated pulmonary epithelium. Another group used a similar protocol to prepare grafts of recellularized scaffolds [64]. After orthotopic transplantation, these grafts were perfused and ventilated by the host vasculature and airways, respectively.

Decellularized lung scaffolds have also been compared with artificial 3D scaffolds [55]. Using the same approach as for decellularized lung tissue, type I collagen hydrogel, gel-foam, and Matrigel® scaffolds were seeded with murine embryonic stem cells. Compared with the artificial scaffold, use of the decellularized lung scaffold resulted in cell differentiation toward a greater number of lung cell types. The study also revealed expression of epithelial, endothelial and smooth muscle cell markers commonly expressed in lung tissue [e.g., thyroid transcription factor-1, prosurfactant protein C (SP-C), PECAM-1/CD31, cytokeratin 18, α-actin, CD140a, platelet-derived growth factor receptor-α (PDGF-Rα) and club cell protein (CC16)] in cells cultured on the decellularized scaffolds.

Melo *et al.* developed an upper respiratory tract model that uses decellularized porcine luminal trachea membrane placed in a culture insert [66]. They used three cell types (i.e., human bronchial epithelial cells, human lung fibroblasts and microvascular endothelial cells) to seed the scaffold. Analysis of cell–cell interactions indicated that the presence of fibroblasts and endothelial cells in the culture results in greater numbers of tight junctions between the bronchial epithelial cells, and thus promotes functional barrier formation.

The natural 3D organization of decellularized lung tissue is a valid platform for *in vitro* tissue modeling. ECM has the properties required of a potent stimulator of progenitor differentiation. After reseeding with cells, decellularized lung tissue participates in gas exchange *in vivo*. Decellularized lung tissue thus has properties that appear useful for tissue engineering applications. However, environmental toxin assessment and pulmonary toxicity screening remains a challenge in decellularized scaffolds. A solution to combine the decellularized tissue or other culture systems with the determinant factors for routine toxicity testing can be found in a microfluidic chip.

Microfluidic chips

In vitro culture systems usually involve static conditions with a regular exchange of culture medium. Because bioreactors and microfluidic devices (Fig. 4) are designed to support the cellular microenvironment [67] and provide continuous nutrient and waste exchange, dynamic culture conditions provide tissue models with more-natural conditions [68]. Such systems also enable mimicking of *in vivo* conditions and tissue functions such as dynamic liquid and airflow and tissue expansion and shrinkage, respectively [69,70]. Microfluidic chips can contain biosensors based on optical or electrochemical sensors that enable monitoring of various parameters (e.g., pH, temperature, secretion of target molecules, intercellular junction integrity) [71]. Therefore, they are prospectively suitable for high-throughput drug and toxicity screening [72–74]. The multi-organ-on-a-chip platform is one rapidly emerging application [71]. Microfluidic chip use for lung tissue modeling includes modeling the alveolar–capillary barrier [75,76], airway tissue [77,78], inflammatory processes [78,79], diseases, functional phenomena like oxygen gradients and particle transport [80–82]. The research facilities and pharmaceutical industry use these models to test the primary and side-effects of drugs [81,83,84].

Modeling the alveolar–capillary barrier

One microfluidic design incorporates a multilayer molded assembly of polydimethylsiloxane (PDMS) to suspend a polyethylene terephthalate membrane that provides the culture surface [85]. Researchers seeded the surface with A549 cells, human microvascular endothelial cells and primary mouse fetal pulmonary cells and examined outcomes using media flow rates, seeding densities

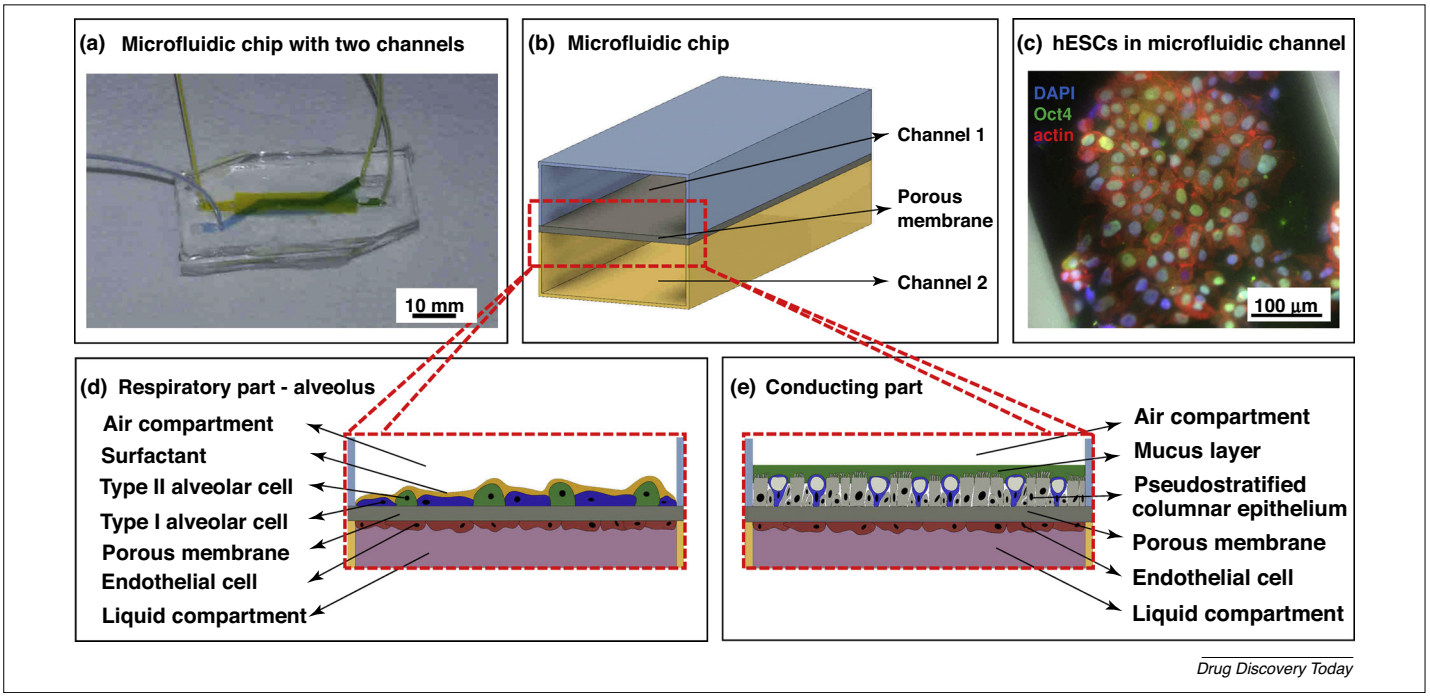

FIGURE 4

Microfluidic chip. **(a)** Microfluidic chip with two channels and tubing. Scale bar: 10 mm. **(b)** Schematic illustration of microfluidic chip, which contains two channels for cell insertion and media exchange. Channels are separated by a porous membrane. **(c)** Human embryonic stem cells (hESCs) cultured in a microfluidic channel for 24 h with continual flow of culture medium. Nuclei are shown in blue (DAPI), marker of pluripotency Oct4 is shown in green (immunofluorescence), cytoskeletal actin is shown in red (Phalloidin Rhodamine). Scale bar: 100 µm. **(d)** Schematic illustration of modeling the respiratory part (alveolus) on a porous membrane of a microfluidic chip. **(e)** Schematic illustration of modeling the conducting part on a porous membrane of a microfluidic chip.

and surface coatings. Production of a stable A549 cell morphology and surfactant protein secretion that closely mimics the *in vivo* condition resulted from this study.

The human lung is a dynamic system with typical mechanical properties. Development of a microdevice that mimics breathing motion has also been achieved [75]. This device is fabricated from PDMS containing two central microchannels separated by a microporous membrane, and two side chambers. Cyclic application of vacuum to the side chambers is used to produce cyclic mechanical stretching of the membrane. In one study, the membrane was coated with collagen gel or fibronectin and cells were seeded on each side of the membrane. Human pulmonary microvascular endothelial cells were placed in the vascular channel and NCI-H441 cells were placed in the alveolar channel. This alveolar–capillary barrier model under cyclic mechanical strain and supplemented with immune cells in the vascular channel was used to successfully reproduce complex tissue responses [e.g., pulmonary inflammation with cytokine release initially stimulated by tumor necrosis factor (TNF)-α introduction into the alveolar channel, and lung bacterial *Escherichia coli* infection].

These same authors also modeled multiple physiological functions that occur in the whole breathing lung [76]. To develop a disease model of pulmonary edema induced by IL-2, they created a microdevice and seeded it with human pulmonary microvascular endothelial cells and NCI-H441 cells. They found that mechanical breathing motions increase the effects of IL-2 in pulmonary edema induction. The study also revealed that an animal model could be replaced by this *in-vitro*-created functional microdevice. The authors applied the drugs angiopoietin-1, angiopoietin-2 and TRPV4 inhibitor GSK2193874 to the chip and found statistically significant correlations between the drug effects using the microdevice and the effects using a mouse whole-lung model.

Examination of surface tension is another important use of alveolar–capillary membrane modeling. Douville *et al.* revealed the individual and total effects of fluid and solid mechanical stress on alveolar cells [86]. They created 'alveoli-on-a-chip', which consists of two microchannels separated by a PDMS membrane. They then seeded human A549 cells or primary mouse alveolar epithelial cells into the air channel. They examined the alveolar cell response to surface tension stress evoked by cyclic propagation of the air–liquid interface through the channel and solid mechanical stress applied to the wall. This model is valid for the study of lung pathology associated with changes in surface tension (e.g., acute respiratory distress syndrome, neonatal respiratory distress syndrome). The model results were experimental evidence for the clinical observation that the final extent of cell damage in ventilator-induced lung injury is not caused solely by mechanical stress; it is also caused by surface tension stress. The clinically used application of lung surfactant to the damaged lung is supported by the experimentally confirmed decrease in numbers of detached cells and cell death when surfactant is introduced into the air channel.

Higuita-Castro *et al.* and Yang *et al.* modeled the fibrous architecture of lung basal lamina by incorporating an electrospun nanofiber membrane into a microfluidic chip [87,88]. Higuita-Castro *et al.* used electrospun nanofibers with different ratios of PCL and gelatin to replicate the changes in fiber stiffness and topography that occurs during some lung disorders [87]. Com-

pared with the polydimethylsiloxane porous membrane commonly used in microfluidic chips, the nanofiber mesh more closely reflects the fibrous architecture of native ECM. This system also allows investigation of epithelial–endothelial interactions within the *in vitro* alveolar–capillary barrier model. Using an analogous approach, Yang *et al.* used a different synthetic polymer (polylactic-co-glycolic acid) to electrospin a nanofiber membrane that was subsequently embedded into a chip. The researchers co-cultured A549 cells, human fetal lung fibroblasts and HUVECs on the nanofiber membrane and, using gefitinib, found that the model had validity for anticancer drug testing [88].

Intravascular thrombosis can also be modeled using *in vitro* conditions [89]. Use of the microfluidic chip to recapitulate *in vivo* responses, including platelet–endothelial dynamics, revealed that lipopolysaccharide endotoxin indirectly stimulates intravascular thrombosis by activating alveolar epithelium, rather than by direct crossing the endothelium. These models clearly demonstrate the broad spectrum of pathologies possible for investigation with alveolar–capillary barrier models.

Gradient studies

In vivo, chemical compound and oxygen concentration gradients are present within different body compartments. Because the level of oxygenation can increase or decrease the effect of a specific compound, development of gradient model devices is clinically relevant [90]. Research on the interactions between molecular gradients and corresponding cellular responses improves understanding of the mechanisms associated with human body functions [91].

In the past, integrated systems had challenges that limited their practical use in biological studies (e.g., control and prediction of oxygen gradients). In addition to use for two-liquid chemical gradients, microfluidic devices can be combined with pyrogallol to establish an oxygen gradient via spatial scavenging of oxygen [92]. In this study, anticancer drugs were tested under different oxygen tension conditions. Cytotoxicity of A549 cells was induced by applying tirapazamine, which is only activated to form a toxic radical at very low oxygen levels. The researchers also found cell migration toward higher oxygenated areas. These results further revealed the effects of different oxygen tensions for cell survival and drug efficiency.

Chip-based 3D co-culture for drug sensitivity screening and application during individualization of lung cancer treatment is another example of chemical gradient use [93]. One device consists of four chip subunits interconnected at the center. Each subunit has a concentration gradient generator with continuous medium flow. This design enables four simultaneous experiments using four drugs or their combinations. In the study, the device was filled with a cell-basement-membrane protein mixture as surrogate ECM and was seeded with human non-small-cell lung cancer cells (SPCA-1 cell line), human lung fibroblast cells (HFL1 cell line) and primary cancer and stromal cells from lung cancer tissue donated by eight patients. The study found that cells in a 3D environment have a different morphology compared with cells in a 2D environment. It also revealed that, compared with administration of one drug, administration of a drug combination is a more efficient treatment strategy, and that fibroblasts (as part of the tumor stroma) have a significant role in the response to anticancer drugs. There was also a significant difference in the anticancer drug response of primary cancer cells, compared with the cancer cell lines usually used for drug testing. More-accurate results derive from the use of primary cells; use of these cells is more appropriate for treatment individualization.

Oxygen gradients are also crucially important for respiratory infection treatment in cystic fibrosis patients who suffer from recurrent *Pseudomonas aeruginosa* infections [94]. Disease recurrence can be caused by reservoirs of bacteria residing in sinus cavities with high amounts of mucus and low levels of oxygen. Antibiotics have greater effects on metabolically active bacteria residing in highly oxygenated regions, which results in maintenance of survivors in areas with low oxygenation. A microfluidic model was used to examine antibiotic efficiency under different oxygen concentration. The model revealed that antibiotic effectivity against *P. aeruginosa* is highly dependent on oxygen tension. This microfluidic model has validity for testing antibiotic effectivity under different oxygen levels.

Airway modeling

Various models of airways were developed to closely mimic various pathologies that can affect the conducting part of the respiratory system. Sellgren *et al.* introduced a multicellular construct that closely recapitulated airway mucosa microarchitecture [95]. They used a model of larger airways with three chambers. The model was seeded with human primary cells (airway epithelial cells, fibroblasts and lung microvascular endothelial cells). Chambers containing cells were separated by synthetic nanoporous collagen-coated membranes. The chambers were stacked so that the endothelial cells and fibroblasts were seeded in the fluid channels and the airway epithelial cells were seeded in the air channel at an air–liquid interface. The researchers found that the airway epithelium was positive for mucin granules and constituted a functional cellular barrier. These results indicated that this model morphologically and functionally correlates with a simplified airway structure. Spindle-shaped fibroblasts represent the connective tissue layer and cobblestone endothelial cells represent vessel walls.

Mondrinos *et al.* addressed the native ECM mimic by using natural hydrogel membranes to separate the microfluidic channels instead of using synthetic membranes [96]. The hydrogel membranes were composed of type I collagen, type I collagen combined with Matrigel® or type I collagen with alginate. The membranes were positioned between the upper and lower microchannel to fabricate a two-channel microfluidic device. Several co-cultures were established using A549 cell line, human bronchial epithelial cells, human umbilical vein endothelial cells, human lung fibroblasts and mouse pericytes. Multiple models were thus created: air–liquid interface, epithelial–stromal interface and tumor spheroid model, respectively. The device stands out not only for using native ECM materials but also for its optical transparency, which enables easy visualization, and tunable membrane stiffness. The authors developed a near-to-native *in vitro* model valid for replication of physiological tissue interfaces and prospectively for drug and toxicity testing.

The presence of pulmonary surfactant significantly reduces surface tension. Therefore, surfactant dysfunction or deficiency is associated with various disorders [97,98]. Air–liquid instabilities lead to formation of liquid plugs that can block the airways and impair gas exchange. This impairment of airflow is clinically

associated with abnormal sounds – the respiratory crackles. One of the earliest small airway models investigated such liquid plug formation and successfully reproduced the associated crackling sounds *in vitro* [97]. Primary human small airway epithelial cells were cultured at an air–liquid interface and were subjected to liquid plugs. Plug propagation and rupture was associated not only with significant damage to the cells but also with formation of pressure waves, which were audible as the typical crackling sounds.

Tavana *et al*. investigated whether surfactant application can protect the cells from damage caused by plug propagation [98]. They seeded A549 cells onto a two-chamber chip with a porous polyester membrane; specialized channels for air and liquid conduction enabled development of liquid plugs. The authors found that surfactant application protects the epithelial cells from further injury and that this model has validity for investigation of various lung diseases.

Mucus plug formation causes occlusion of distal airways in many obstructive pulmonary diseases [99]. This study investigated mucus plug formation in a more natural manner — by compressing a hydrogel and allowing the fluid to exudate from it. Primary human small airway epithelial cells were cultured in an airway channel at an air–liquid interface. Simulated mucus plugs were formed by application of hydraulic pressure to a hydrogel present in a hydrogel chamber. This pressure forced liquid from the hydrogel to exudate through a semipermeable membrane into the airway channel, thus mimicking the *in vivo* exudation of liquid from the subepithelial compartment into the airway lumen. Additionally, plug movement (i.e., propagation speed) through the airway channel could be precisely regulated. Mechanical damage to the cells and inflammation were observed. This model, thus, closely recapitulates the acute epithelial injury during airway closure.

Because inflammation has an important role in many diseases, a double-layered microfluidic model with air and blood compartments was developed to study airway inflammation [100]. The study examined the contribution of eosinophilic cationic protein (ECP) to the pathogenesis of airway inflammation. ECP induced expression of CXCL-12 in the airway epithelium that led to the fibroblast migration toward the epithelium that occurs under *in vivo* conditions.

A 'small-airway-on-a-chip' is another microdevice intended to study inflammation [78]. Researchers first seeded the microchip with primary airway epithelium from healthy individuals or from patients with COPD. After full differentiation of these cells into mature mucociliary bronchiolar epithelium within the microfluidic channel, microvascular endothelium was seeded into the second channel to recreate functional small airway models. When the differentiated epithelium was exposed to a key cytokine associated with asthma and COPD pathogenesis, the IL-13, goblet cell hyperplasia, cytokine hypersecretion and decreased ciliary function responses occurred as they do in *in vivo* conditions. The model also proved to be efficient for testing of therapeutic responses to drugs.

Inflammation caused by inhaled pathogens was investigated by Barkal *et al*. [101]. The basic part of this bronchiole model was a collagen–fibrinogen gel with fibroblasts mimicking the connective tissue. Two different types of luminal compartments were present in the gel. One luminal compartment was seeded with primary human bronchial epithelial cells and constituted airway lumen. The other luminal compartment with primary human lung microvascular cells modeled a vessel. Spores of *Aspergillus fumigatus* were inoculated into the airway lumen. As *A. fumigatus* germinated, hyphae formed extensively and extended through the epithelium into the collagen–fibrinogen gel. Polymorphonuclear cells introduced into the vessel lumen responded to the fungal infection in a manner near-to-native. They extravasated and migrated toward the *A. fumigatus* hyphae. This model recapitulated the characteristic features of invasive aspergillosis. The authors also co-cultured *A. fumigatus* with *P. aeruginosa* in the bronchiole model. This co-infection is important for modeling infections of patients suffering from cystic fibrosis. These models further increase our understanding of disease pathogenesis and could help to define the most effective disease treatments.

Concluding remarks

Each model used to mimic the respiratory system has its own inherent technical advantages and challenges (Table 1) that must be considered during design of the *in vitro* model. Inserts for planar culture easily apply an air–liquid interface, are convenient for handling and affordable. Decellularized and artificial scaffolds provide the best ECM mimics and very efficient spatial cell organization. Decellularized scaffolds also have the advantages associated with preserved airways and vessel branches, which enables introduction of air and fluid into these compartments.

Microfluidic chip models are the most advanced of the presented models. They enable air–liquid interfaces, dynamic culture conditions and simulation of various physiological movements. New multi-organ-on-a-chip platforms are rapidly emerging. The chips developed to date are mainly based on porous synthetic membranes. ECM-like stimulation can be achieved by coating the membrane with natural proteins or via use of an emerging approach, incorporation of either nanofiber scaffolds or membranes from native ECM materials into the chips [87,88,96].

Although existing *in vitro* respiratory system models are facing many challenges, the advantages associated with incorporation of dynamic culture conditions, air–liquid interfaces, motion simulations and native ECM components might be combined soon to create even more-elaborate models. *In vitro* models of the human respiratory system are being used to develop tools for biomedicine, for toxicity testing and for routine patient-specific drug screening. Use of even more-advanced models is also expected in the field of *in vitro* disease modeling. Next, development should focus on the challenges of high-throughput devices, on combining the native 3D ECM signaling with physiological movements and on blood incorporation into the capillary compartments to simulate gas exchange more closely.

Conflicts of interest

There are no conflicts of interest to declare.

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