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Review

Vascularized Lower Respiratory-Physiology-On-A-Chip

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Abstract: Recently, respiratory systems are increasingly threatened by high levels of environmental pollution. Organ-on-a-chip technology has the advantage of enabling more accurate preclinical experiments by reproducing in vivo organ physiology. To investigate disease mechanisms and treatment options, respiratory-physiology-on-a-chip systems have been studied for the last decade. Here, we delineate the strategic approaches to develop respiratory-physiology-on-a-chip that can recapitulate respiratory system in vitro. The state-of-the-art biofabrication methods and biomaterials are considered as key contributions to constructing the chips. We also explore the vascularization strategies to investigate complicated pathophysiological phenomena including inflammation and immune responses, which are the critical aggravating factors causing the complications in the respiratory diseases. In addition, challenges and future research directions are delineated to improve the mimicry of respiratory systems in terms of both structural and biological behaviors.

Keywords: respiratory system; organ-on-a-chip; vascularization; biofabrication; extracellular matrix

1. Introduction

The incidences of acute and chronic respiratory diseases, including chronic obstructive pulmonary disease (COPD), asthma, and cancer, are gradually increasing because of various environmental causes and genetic disorders. Hundreds of millions of people suffer from respiratory diseases, approximately 4,000,000 of whom die prematurely each year [1]. To overcome this challenge, numerous medications have been developed to treat respiratory diseases. Animal models have been used to test drug safety and efficacy for the treatment of respiratory disease. However, these models are costly and time-consuming to establish the disease model, and maintenance of the animal facility is another critical factor increasing cost during the experimental period. In addition, it is difficult for the animal model to represent the phenomena in the human system because of the variation of species [2,3].

The development of organ-on-a-chip (OoC) increases the potential to fill the gap between preclinical studies and the real world. Various studies on OoC are currently being conducted to understand tissue development and the underlying mechanisms of disease progression, and are utilized to test drugs and treatment options. In particular, the OoC can facilitate low-cost and short-term evaluation of drug efficacy. The chip has the advantage of enabling more accurate preclinical experiments to replace animal experiments by reproducing actual function of tissue and organs, and these technologies can increase likelihood of clinical success [4,5].

Extracellular matrix (ECM) has been studied to recapitulate the microenvironment closed to the native tissue and organ [6,7]. Basically, cells are surrounded by a complex ECM for biochemical and mechanical interactions. It regulates cellular behaviors such as cell adhesion, growth, migration, differentiation, and function by controlling the focal adhesion and cytoskeletal dynamics [8]. ECM contains various proteins including fibronectin, collagen, and elastin. To mimic the ECM's role, biocompatible polymers like natural and synthetic polymer have been used [9].

OoC are fabricated through various technologies to simulate the function of the tissue. There are critical effects on cell behaviors, so the trend is to implement the OoC in three dimensional (3D) rather than two dimensional (2D) [10]. Among the 3D fabrication technologies, the most widely used approach is the use of microfluidic chips to control the flow of fluid and air to provide OoC with a mechanical environment similar to that of the actual tissue [11].

Vasculature plays an important role in mimicking living tissues. The vasculature is the pathway to transport nutrients, toxic substances, and body waste and they affect all cells in the body. The presence of appropriate vasculatures is critical to investigate complicate pathophysiological phenomena including inflammation and immune responses [12,13]. In addition, by generating a flow in the vasculatures, a physiological motion can be provided to mimic the tissue fluid environment [14]. Accordingly, research on the fabrication and application of OoC incorporated vasculatures are being actively pursued [15].

In this sense, we delineate the strategic approaches to develop respiratory-physiology-on-a-chip that can recapitulate respiratory system in vitro. The state-of-the-art biofabrication methods and biomaterials are considered as major contributions to building the chips. We also explore the vascularization strategies to investigate complicated pathophysiological phenomena including inflammation and immune responses, which are the critical aggravating factors that cause the complications in the respiratory diseases. In addition, challenges and future research directions are delineated to improve the mimicry of respiratory systems in both structural and biological behaviors.

2. Lower Respiratory Microenvironment

2.1. Lower Respiratory

The respiratory system can be separated into the upper respiratory tract, comprising the nose, nasal cavity, paranasal sinuses, pharynx, and larynx, and the lower respiratory tract comprising the trachea, bronchus, and lungs. Based on the physiological role, from the nasal cavity to bronchus can be referred to as the conducting zone. The bronchiole and the alveoli facilitate the passage of air and promote gas exchange, and pulmonary vasculature is an essential factor in determining the physiological characteristics of the lungs [16]. The entire respiratory tree is lined with specialized and continuous epithelial cells that are essential for the normal functions of the respiratory system. These layered epithelial cells serve as a protector of the respiratory system through various defense mechanisms, including mucociliary transportation, production of anti-infectious substances, secretion of ions, and maintenance of homeostasis. In addition, physical defense is ensured by tightly packed junctions that protect against infiltrations of pathogens or toxic substances. The respiratory epithelial layers are morphologically and functionally unique for each component leading to the lower respiratory tract. The tracheal epithelium consists of ciliated, goblet, and basal cells. In the bronchioles, the simple cuboidal epithelium contains mucin-secreting cells, including clara cells. The alveolar epithelium is composed of alveolar type I and II cells. These epithelial cells are connected to endothelial cells by the basement membrane to form the gas exchange barrier [17–19].

2.2. Lower Respiratory Extracellular Matrix Composition and Role

2.2.1. Compositions of Extracellular Matrix and Its Roles

ECM is associated with specific mechanical properties and is essential for the biological, physical, and biochemical features of the lungs respiratory system. In normal lungs, the ECM is a complex mixture of glycoproteins, collagens, and polysaccharides that are clearly assembled to maintain tissue integrity and to separate the epidermal and mesenchymal cell layers from the inner tissues (Table 1) [20]. In addition, lung cells usually regulate the production and deposition of pulmonary ECM during lung development [21]; thus, interactions between lung cells and the surrounding ECM are important for normal lung development. Therefore, the pulmonary ECM not only facilitates the signaling of lung cells, but also provides the necessary physical support for lung cells. Abnormalities in the pulmonary ECM result in lung disorders such as idiopathic pulmonary fibrosis (IPF), emphysema, and lung cancer [20,22].

In addition, pulmonary ECM proteins (e.g., hyaluronan, tenascin C, and decorin) have been shown to affect immune cell activation and cytokine expression, function as a reservoir of growth factors, and affect autophagy [23,24]. Recent studies have investigated the role of pulmonary ECM proteins (e.g., collagen, fibronectin and elastin) in regulating tissue stiffness and their effects on cellular behavior. Increased lung stiffness due to mechanical stimulation and collagen deposition during tissue fibrosis results in changes in cellular function [25].

Table 1. Composition of the pulmonary extracellular matrix (ECM). Reproduced with permission from [26]. Copyright © 2018 Elsevier.

ECM Glycoproteins		Collagens	Proteoglycans
5430419D17RIK	LGI3	COL10A1	ACAN
ABI3BP	LTBP1; LTBP2; LTBP3; LTBP4	COL11A1; 11A2	ASPN
ADIPOQ	MATN1; MATN2; MATN4	COL12A1	BGN
AEBP1	MFAP2; MFAP4; MFAP5	COL13A1	CHAD
AGRN	MFGE8	COL14A1	DCN
AW551984	MGP	COL15A1	FMOD
BMPER	MMRN1; MMRN2	COL16A1	HAPLN1
CILP	NDNF	COL17A1	HAPLN3
CILP2	NID1; NID2	COL18A1	HAPLN4
COLQ	NPNT	COL19A1	HSPG2
COMP	NTN1; NTN3; NTN4	COL1A1; 1A2	IMPG1
CRISPLD2	PAPLN	COL22A1	LUM
DPT	PCOLCE; PCOLCE2	COL23A1	OGN
ECM1; ECM2	POSTN	COL24A1	PODN
EFEMP1; EFEMP2	PXDN	COL25A1	PRELP
EGFEM1	RELN	COL27A1	PRG2
ELN	SBSPON	COL28A1	PRG3
EMID1	SLIT3	COL2A1	VCAN
EMILIN1; EMILIN2	SNED1	COL3A1	
FBLN1; FBLN2; FBLN5	SPARC; SPARCL1	COL4A1; 2; 3; 4; 5; 6	
FBN1; FBN2	SPON1	COL5A1; 5A2; 5A3	
FGA; FGB; FGG	SRPX; SPRX2	COL6A1; 2; 3; 4; 5; 6	
FGL2	SVEP1	COL7A1	
FN1	TGFBI	COL8A1; A2	
FRAS1	THBS1; THBS2; THBS3	COL9A1; 9A2; 9A3	
GLDN	THSD4		
HMCN1; HMCN2	TINAG; TINAGL1		
IGFALS	TNC; TNXB		
IGFBP6; IGFBP7	VTN		
IGSF10	VWA1; 3A; 5A; 5B1; A9		
KCP	VWF		
LAMA1; A2; A3; A4; A5; B1; B2; B3; C1; C2; C3	WISP2		

2.2.2. Pulmonary Extracellular Matrix Composition in Disease

Pulmonary ECM proteins are associated with branching, angiogenesis, alveolar maturation, tissue repair after injury as well as pathological processes leading to acute and chronic lung diseases such as asthma, COPD, and IPF [26]. Recent studies have shown that damage to elastin fibers upon increased fibronectin and tenascin levels in COPD airways alters ECM stiffness [27]. Similarly, a high level of collagen deposition in fibrotic disorders (e.g., IPF) is associated with a marked increase in lung stiffness [28]. The ECM in normal lungs forms a loose network of fibronectin, collagen, and elastin

that is maintained by the activity of fibroblasts and that adheres to the basement membrane of the epithelial cell layer. However, the properties of the pulmonary ECM show substantial changes under diseased condition.

The following describes pathological changes of the pulmonary ECM in lung disease. (1) In IPF, fibroblasts are converted to myofibroblasts, which deposit pulmonary ECM proteins into the cytoplasm, and increased collagen–elastin enzymatic covalent crosslinking dramatically enhances pulmonary ECM stiffness. (2) In COPD, a characteristic pathological change in the pulmonary ECM is the extensive destruction of elastic fibers by ECM-degrading enzymes released by inflammatory cells. This increases levels of hyaluronan and tenascin C and decreases the deposition of decorin, and destruction of the epithelial cell layer results in air space expansion (pulmonary emphysema). (3) In pulmonary arterial hypertension (PAH), remodeling of the ECM in the arterial wall is characterized by an increase in elastin and collagen, fibronectin, tenascin C, and the proliferation of smooth muscle cells (SMCs). (4) In asthma, pulmonary ECM alterations and enrichment occur below the bronchial epithelial basement membrane, SMCs proliferate and increase their deposition of collagen, fibronectin, hyaluronan, and decorin. (5) In cancer, TGFβ function is modulated by the decorin and Syndecan-4, and it drives proinvasive EMT in cancer cells, and conversion of stromal fibroblasts to contractile cancer-associated fibroblasts (CAFs). CAFs modulate collagen matrix stiffness via interactions through integrins, and matrix stiffness also affects mobility of tumor-infiltrating leukocytes (TILs). Primary and metastatic tumors are surrounded by a wide range of stiff substrates containing highly crosslinked collagen and high levels of fibronectin, hyaluronan, and tenascin C [29,30].

2.3. Construction of the Pulmonary Extracellular Matrix Environment in Lower Respiratory-Physiology-On-A-Chip

2.3.1. Natural Polymers

The pulmonary ECM contains various proteins including collagen, elastin, and fibronectin, that provide structural and biochemical support. For example, collagen and fibronectin are mainly used as materials to help cell growth and adhesion in culture dishes or OoC. In addition, they have been used to mimic 3D actual environments in vitro or to produce scaffolds for regenerative studies. Thus, these biocompatible proteins are mainly used to mimic the pulmonary ECM environment in the lower respiratory-physiology-on-a-chip (Table 2). However, these proteins are associated with problems that are yet to be solved. Most currently used materials are extracted and commercialized from only a single ECM component. This is associated with various limitations in the generation of tissue- or organ-specific functions. To overcome this problem, more studies are using combinations of various materials. However, it is not easy to mimic the composition and structure of the actual ECM, which has not yet been fully identified [31–37]

Table 2. Investigations on natural-derived coating materials used for lower respiratory-physiology-on-a-chip.

Polymer Composition	Cell Populations	Features	Ref.
Collagen I	Primary human airway epithelial cells, human lung microvascular endothelial cells, neutrophils	Porous membrane sandwiched microchip, secretion of the inflammatory cytokines	[33]
	Primary human airway epithelial cells obtained from healthy donors or COPD patients	Porous membrane sandwiched microchip, smoke-induced pathological microenvironment	[34]
Collagen I/Fibronectin	Human alveolar epithelial cells, endothelium cells, neutrophils	Porous membrane sandwiched microchip, alveolar-capillary barrier	[32]
Collagen I/Matrigel	Calu-3, human bronchial smooth muscle cells	Multilayer PMMA chip	[35]
Collagen I/Matrigel/Chitosan	Human alveolar epithelial cells, HUVECs	Multichannel microfluidic chip, perfusion culture	[36]
Collagen I/Fibronectin/Gelatin	Bronchial epithelial cells, primary human pulmonary alveolar epithelial cells, HUVECs	Porous membrane sandwiched microchip, mechanical stress induced by respiration movements	[31]
Fibronectin	A549, primary murine alveolar epithelial cells	Porous membrane sandwiched microchip, surface tension forces	[37]

2.3.2. Decellularized Pulmonary Extracellular Matrix

Decellularized extracellular matrix (dECM) is defined as materials prepared from various types of native tissues and organs by specific removal of cellular components [38], which overcomes

the limitations of incomplete biometric of natural polymers. The pulmonary ECM consists of 3D combinations of various structural and functional molecules secreted by different cells. Pulmonary ECM proteins such as collagen and elastin determine the physical properties of tissues, and glycoproteins such as fibronectin and laminin play a role as adhesives for attaching cells to the pulmonary ECM [39,40]. However, the characteristics, functions, and molecular components of the pulmonary ECM have not yet been fully elucidated, and thus it is impossible at present to manufacture them artificially. In order to maintain the physiological characteristics of tissue ECM, the ECM should be manufactured to retain as much of physiologically active substances as possible. In addition, some studies have used whole ECM to make a suitable ECM environment for the desired tissue at the OoC in which all cytoplasm and nuclear material is removed and the major components of ECM are preserved [28,41,42].

3. Lower Respiratory-Physiology-On-A-Chip

3.1. 2D Lower Respiratory-Physiology-On-A-Chip

Traditional preclinical models are divided into animal models and 2D in vitro models. One of the weaknesses of animal models is that they are difficult to acquire and maintain. Comparably, 2D in vitro models are widely used in various tissue engineering and medical fields because they are easily accessible through the plating cells onto coated plastic culture dished [43]. However, cells are grown in a single layer and adhered to the surface of plastic culture dishes, resulting in a different environment compared to where cells proliferating in the body. This affects the differences in cell morphology and cellular behavior differently from residual cells in vivo [44].

3.2. 3D Lower Respiratory-Physiology-On-A-Chip

In fact, there are many reports showing that the drug responses of cells differ between 2D and 3D cell culture models [45]. 2D in vitro models have obvious limitations in their ability to mimic 3D in vivo environments. Recently, lower respiratory models have been studied to overcome these challenges and increase the similarity to 3D environments in vivo. In 3D models, cells show high differentiation rates and improved drug metabolism. These models also show increased cellular responsiveness to biomimetic culture method compared to 2D models [44–46]. In particular, respiratory organs are located in an environment in which airborne particulate matter affects cells and tissues during respiration. Therefore, the epithelial cells must be cultured in a specific environment known as the air–liquid interface (ALI) [47]. A transwell chamber has been fabricated for the convenient application of cell culture composed in the ALI condition. However, it is not easy to mimic the tissue environment. Therefore, a microfluidic chip has been developed that can provide functions such as biochemical and mechanical stimulation of tissue environments.

3.2.1. Transwell-Based Air–Liquid Interface Respiratory Model

Pezzulo et al. [48] compared and evaluated the similarity to the in vivo airway epithelium when human airway epithelial cells (Calu-3) and primary human airway epithelial cells (hAEC) were immersed at the ALI and a plastic surface, respectively. This study found that ALI cultures with the use of primary cells have similar transcription profiles to epithelium in vivo. Therefore, transwell-based 3D cultures have been used recently as a standard culture protocol to characterize the newly developed experimental cell sources. Rayner et al. [49] evaluated the characteristics of primary normal human bronchial epithelial (NHBE) cells using transwell-based ALI culture conditions to understand the effect of culture conditions on cell growth, epithelial phenotypes, and function. Primary NHBE cells were cultured for up to 6–8 passages in the ALI environment, and it was confirmed that bronchial epithelial phenotype, cell integrity, differentiation, and ion channel function were well maintained. In addition to primary hAEC, many groups used mouse organ epithelial cells (MTECs). However, MTECs has the disadvantage of requiring a large number of mice for cell isolation since it is difficult to grow in vitro. Thus, Evelien et al. [50] developed ALI culture conditions and a new combination of medium

components to maintain the differentiation capacity and proliferation of MTECs. Recent studies have also demonstrated that ALI culture conditions are important factors for the directed differentiation of induced pluripotent stem cells into airway and alveolar epithelial cells [51,52]. Primary hAEC ALI culture provides a platform for investigating the mechanism of differentiation and dynamic recovery process after airway epithelial injury, as well as a method for studying a fully epithelialized epithelial layer.

In vitro toxicity studies of nanoparticles (NPs) that enter the respiratory tract and accumulate in the lungs are typically performed under immersion conditions, in which NPs are suspended in the cell culture medium; therefore, it is difficult to determine the adverse effect of NPs on the accumulation in cells appropriately. Moreover, NPs easily changed their morphology (e.g., precipitation and aggregation) under immersion condition. The cellular response to NPs under immersion culture conditions is completely distinct from those observed in the ALI environment. To mimic the actual pathological features associated with inhalation, Panas et al. [53] exposed human lung epithelial cells (A549) to aerosols (silica NP) at the ALI culture conditions. They found a lower cellular sensitivity to silica NPs under ALI culture conditions for cytotoxic and inflammatory responses compared to the conventional immersion culture results.

Although it is well-known that cigarette smoke (CS) affects the innate immune function of airway epithelial cells and epithelial repair, direct assessments of the impact of CS have not yet been fully understood. Most studies applied an aqueous extract of CS to an undifferentiated primary airway epithelial cells, which is difficult to reflect local conditions of the smoker's lungs. Amatngalim et al. [54] exposed CS to an ALI culture model of primary bronchial epithelial cells (ALI-PBEC) to investigate the effects of CS on airway epithelial repair. The results suggested that CS adversely affect the immune response for repair of damaged airway epithelial cells and contribute to the development and progression of COPD. Thus, the ALI respiratory model has been used in numerous respiratory studies for its ability to more closely simulate the actual in vivo environment compared to the 2D or 3D immersion models. However, limitations remain in simulating the actual respiratory environment that facilitates gas exchange and nutrient supply through the blood flow.

3.2.2. Microfluidic Chip-Based Air–Liquid Interface Respiratory Model

Appropriate tissue and organ models are needed to accurately represent the structure and function of the human respiratory microenvironment, but most in vitro respiratory models do not properly reproduce important in vivo microenvironmental characteristics (such as biological, physical properties). Transwell membrane inserts are widely used to establish co-cultures of epithelial cells grown on membranes of the ALI and airway constituent cells such as SMCs or vascular endothelial cells. However, transwell membranes are made of synthetic materials such as polyester or polyethyleneterephthalate, which cannot mimic the stiffness and properties of natural pulmonary ECM. Fluid flow simulations involving air and blood circulation in respiratory systems have limitations of being difficult to reproduce in conventional culture dishes or transwell culture methods. Recently, the focus has been on creating more complex in vitro respiratory models with greater physiological similarity to the actual in vivo environment. In addition, research on respiratory system is being conducted using microfluidic devices, which can utilize laminar flow to provide nutrients and various flow-induced physiological stimuli to cells. Nalayanda et al. [55] cultivated human alveolar basal epithelial cells in a transwell and microfluidic system. They compared the ability to maintain monolayer integrity of epithelial cells and to produce surfactants between ALI culture and immersion culture. In that study, epithelial cell junctions formed more tightly in ALI cultures than immersion cultures, and mechanical stimulation by fluid flow in the microfluidic system ALI cultures showed an improvement in epithelial monolayer formation and reduced surface tension because of increase surfactant production in epithelial cells compared to conventional transwell ALI cultures. In addition, Humayun et al. [35] developed microfluidic respiratory chips for more complex lung microenvironments, such as intercellular and cell–ECM interactions, through co-culture of airway epithelial cells (EC) and airway SMCs. They adjusted the

mixing ratio of type 1 collagen and Matrigel to establish an optimal EC-SMC co-culture condition for long-term culture of cells more than 31 days in the microfluidic chips. Recently, these microfluidic chips have been used in various studies including efforts to mimic respiratory diseases, mechanistic studies, and drug response. Huh et al. [56] reproduced the physiological and pathological liquid plug flow found in the human airway epithelium using a microfabricated airway system integrated with computerized air–liquid biphasic microfluidics. They demonstrated that the propagation and rupture of liquid plugs, which simulated the reopening of closed airways in the absence of pulmonary surfactant, cause mechanical stress on harmful fluids, resulting in serious damage to small airway epithelial cells; in addition, explosive pressure waves generated by plug rupture were shown to facilitate detection of mechanical cell damage using a stiff sound. In addition, Punde et al. [57] proposed a biomimetic microsystem that reconstructs the lung microenvironment to monitor the role of eosinophil cationic protein (ECP) in lung inflammation. ECP induces airway epithelial cells to express CXCL12 and stimulates the migration of fibroblasts toward the epithelium. By reproducing the flows that mimic the native physiological action, they were able to demonstrate that CXCL12-CXCR4 stimulation mediates the extracellular outflow of ECP-induced fibroblasts in lung inflammation. Benam et al. [34] developed a smoking airway-on-a-chip by connecting a device allowing the system to "breathe" cigarette smoke across a small airway-on-a-chip made from cultured living human bronchial epithelial cells from normal and COPD patients, and used it to study the association between smoking and COPD. Recently, as the incidence of respiratory diseases related to microparticles are increasing, research on the use of ALI microfluidic chips to efficiently detect the cytotoxicity of microparticles also continues to increase. Dong et al. [58] developed an ALI microfluidic system for screening PM 2.5-mediated cytotoxicity in a human lung epithelial cell line (BEAS-2B). A strong association among inflammation-, apoptosis-, and microbial infection-related pathways was identified using this system, which provides information on PM 2.5-induced cytotoxicity and the molecular mechanisms that can threaten health. However, these microfluidic chip-based respirator models still have limitations. It reproduces blood flow through perfusion only without vascular endothelial cells, and cannot explain the interaction between epithelial cells and vascular cells in the respiratory microenvironment.

4. Vascularized Respiratory-Physiology-On-A-Chip

4.1. Need for Vascularization

Because most organs are surrounded by vessels, vasculature formation is important for accurately mimicking in vivo contexts. Vasculatures are associated with cancer, inflammatory reactions, and various complications. Typically, cancer induces angiogenesis in the surrounding blood vessels to acquire oxygen and nutrients. Since angiogenesis causes invasion and metastasis, many attempts have been made to treat cancer by preventing angiogenesis. In addition, microangiopathy is a microvascular occlusive disorder that occurs when endothelial cells exhibit abnormal behavior. Microangiopathy causes a variety of diseases, including obesity, pulmonary diseases, and diabetes complications. Furthermore, neurotoxic thrombin-induced endothelial cells have been shown to increase the risk of Alzheimer's disease. Besides, the vasculature plays an important role in various organs through features such as selective permeation and long-term regeneration. In vitro vascularization models using microfluidic chips are very helpful in understanding the physiological and pathological phenomena associated with vasculatures. These vasculature structures are fused with organ chips, which greatly help to mimic the characteristics of each organ [59–63].

4.2. The Pathophysiological Importance of Pulmonary Vasculature

Changes in pulmonary vascular structure and function lead to serious complications of lung diseases associated with gas exchange dysfunction and reduce to survival. For example, pulmonary hypertension (PH) is a common complication of COPD, and pulmonary edema (PE) is characterized by damage to the capillary endothelium [64–67]. Furthermore, cytokines and chemokines release

from abnormal pulmonary vessels mediates the abundance of inflammatory cells, such as monocytes, T and B lymphocytes [68]. In addition, the role of pulmonary endothelial cell cytokines, including TGF-β, TNF-α, ET-1, IL-1 and IL-8, is associated with the pathogenesis of pulmonary fibrosis [69,70]. According to the increased interest in changing pulmonary vascular characteristics of lung diseases, the National Heart, Lung, and Blood Institute (NHLBI) held a workshop on strategic plans to improve the understanding of pulmonary vascular structure and advance the development of pulmonary vascular studies in 2010 [71], but there remains a lack of understanding between pulmonary disease and the vasculature. For this reason, recapitulating pulmonary vasculature is an important factor in respiratory disease research.

4.3. Vascularized Respiratory-Physiology-On-A-Chip

4.3.1. Lung-On-A-Chip

Vascularized lung-on-a-chip research has been actively pursued for almost 10 years since the first microfluidic device was presented by Huh and colleagues in 2010. The Huh et al. [32] developed a microfluidic device consisting of two superimposed microfluidic channels separated by a microporous membrane. In this system, the upper chamber (ventilation) supports the growth of human lung alveolar epithelial cells, while the lower chamber (perfusion) cultivates pulmonary microvascular endothelial cells to mimic vasculature and provide a laminar flow. In addition, the membrane was periodically swelled and relaxed by hollow microchannels on both sides of the cell culture chamber to generate the mechanical strain by respiratory movements. Lung-on-a-chip-based research has demonstrated that periodic mechanical strain enhances the uptake of silica nanoparticles into epithelial and endothelial cells, which facilitates transport into the lower microvascular channels and increases lung toxicity and inflammatory responses. This model describes a biomimetic microsystem that reconstructs the alveolar–capillary interface of the human lung, providing particular utility by reproducing the integrated organ-level responses to inflammatory cytokines and bacteria infiltrated to the alveolar space. Stucki et al. [31] developed a lung-on-a-chip that mimics the microenvironment of the lung parenchyma by regenerating the alveolar barrier using a thin and flexible membrane co-cultured with epithelial and endothelial cells (Figure 1a). Primary human lung epithelial cells from patients and a bronchial epithelial cell line were used to demonstrate that mechanical stress significantly affects epithelial barrier permeability. In addition, when primary human lung alveolar epithelial cells were cultured in dynamic condition, the metabolic activity and expression of the inflammatory marker IL-8 were found to be significantly higher than cultured in static condition. This is the first case to investigate the effect of mechanical modification on primary cells from patients.

The same group [72] successfully co-cultured patient-derived human primary alveolar type I and II-like epithelial cells and pulmonary endothelial cells on this respiratory lung-on-a-chip for over several days (Figure 1b). This has implications for the ability to mimic the in vivo environment using actual patient-derived lung epithelial cells. Recently, Jain et al. [73] proposed an alveolar-on-a-chip that mimics pulmonary thrombosis (Figure 1c). This microfluidic alveolar-on-a-chip reproduces the role of platelets and endothelial cells in vivo, and it was shown that toxins do not act directly on the endothelium but indirectly stimulate vascular thrombosis by activating the alveolar epithelium. In addition, endothelial activation and inhibition of thrombosis by protease-activated receptor-1 (PAR-1) antagonists were analyzed to demonstrate the applicability of the system for new antithrombotic drug development studies. The vascularized lung-on-a-chip clearly mimics the pathological and biological characteristics of human lungs, and it is valuable as a tool for developing preclinical models to replace animal experiments as well as for screening new drugs related to lung disease (Table 3).

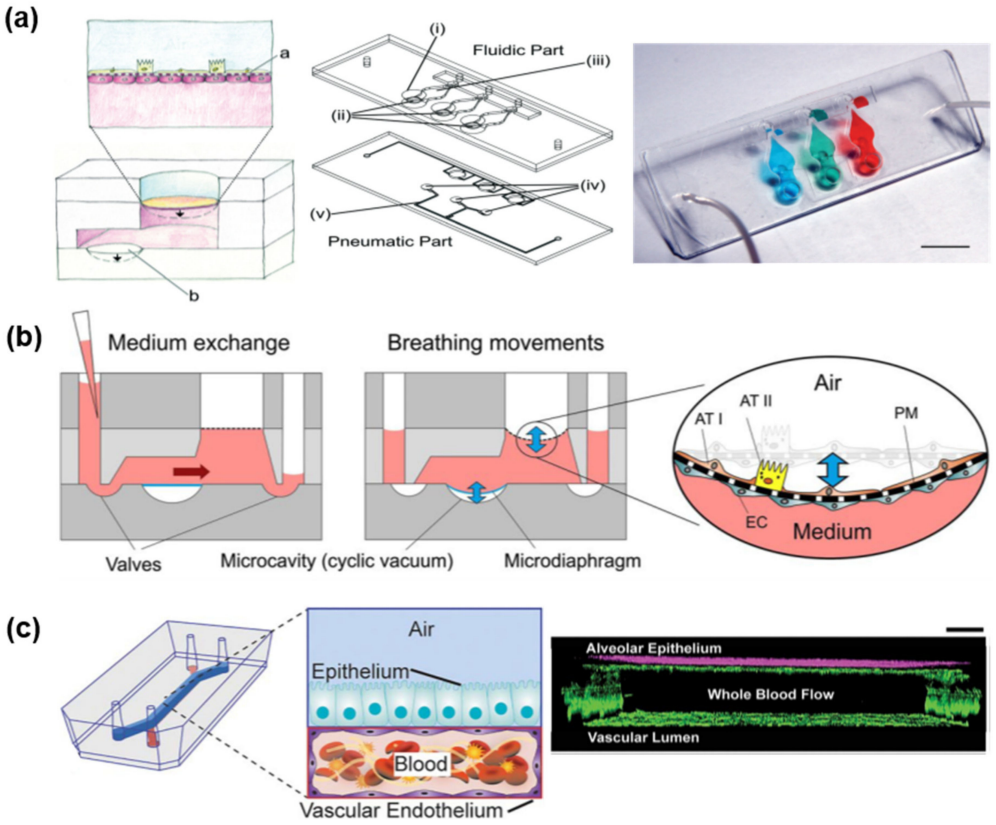

Figure 1. Vascularized lung-on-a-chip. All chip with two channels cultured of epithelial and endothelial cells that are separated by a stretching porous membrane. (**a** and **b**) Successfully co-cultured patient-derived human primary alveolar type I and type II-like epithelial cells and pulmonary endothelial cells on this lung-on-chip for over several days. This system is meaningful for the ability to mimic the in vivo environment using actual patient-derived lung epithelial cells. (**c**) In order to mimic the human lung, the upper chamber supports the growth of human lung alveolar epithelial cells and was filled with air, while the lower chamber cultivates pulmonary microvascular endothelial cells and was filled with liquid. (**a**) Reproduced with permission from [31]. Copyright © 2001 ROYAL SOCIETY OF CHEMISTRY (**b**) Reproduced with permission from [72]. Copyright © 2018 Springer Nature. (**c**) Reproduced with permission [73]. Copyright © 2017 John Wiley and Sons.

Table 3. Vascularized lung-on-a-chip.

Coating Material	Cell Population	Features	Ref.
Fibronectin or collagen	Human alveolar epithelial cells and microvascular endothelial cells	Stretchable microfluidic system	[32]
Fibronectin or gelatin and collagen I	Bronchial epithelial cells and primary human pulmonary alveolar epithelial cells, primary HUVECs	Stretchable microfluidic system	[31]
Fibronectin and collagen IV/ I	Bronchial epithelial 16HBE14o- cells, primary human alveolar epithelial cells, primary human lung microvascular endothelial cells	Stretchable microfluidic system	[72]
Fibronectin and collagen I	Human umbilical vein endothelial cells from pooled donors, primary alveolar epithelial cells containing type I and II cells	Microfluidic system	[73]

4.3.2. Airway-On-A-Chip

The trachea is a tubular structure that connects the larynx to the bronchi. The trachea branches vertically down the front of the esophagus, splitting into the left and right bronchus. These are the pathways that takes air in and out. In addition, when a substance from outside enters, the airway cilia function to discharge the extrinsic substance.

Benam et al. [33] succeeded in mimicking the structure and functions of the human airway in vitro and modeling disease and inflammatory responses. They used soft lithography to fabricate

a microfluidic device that included an upper airway channel along with a microvascular channel. To mimic the physiological microenvironment of the native tissue, human alveolar epithelial cells were cultured in the upper airway and human pulmonary microvascular endothelial cells were used on opposite sides. They were also able to independently control various system parameters to reproduce complex integrated organ-level responses. The mechanical strain increases the uptake of nanoparticles and stimulates their transport into microvascular channels. Lenke et al. [74] used 3D printing to fabricate an airway-on-a-chip comprising the human alveolar epithelial type II cell line A549 and the EA.hy926 hybrid human cell line derived by fusing human umbilical vein endothelial cells (HUVEC) with A549 cells on the commercial transwells (Figure 2). The use of 3D bioprinting technology enables the automated and reproducible creation of thin and homogeneous cell layers and complex cell–cell interactions. These studies showed that the vascularized airway-on-a-chip by 3D bioprinting more faithfully reflects the structural and functional resemblance of the actual tissue. Park et al. [75] applied 3D bioprinting to simply and quickly construct a chip. To mimic the microenvironment of a trachea, decellularized trachea ECM bioink was used for printing. The primary human tracheal epithelial cells (hTEpCs) were seeded onto the vitrified membrane by decellularized trachea ECM on a transwell. Human extracellular microvascular endothelial cells (hDMEC) and lung fibroblasts were encapsulated and printed within designated polycarprolactone (PCL) frames using decellularized trachea ECM bioink. This model promoted the differentiation and function of the airway epithelial cell by the incorporation of vasculatures. In particular, the freedom of design control has enabled the use of various cell types and flow conditions (Table 4).

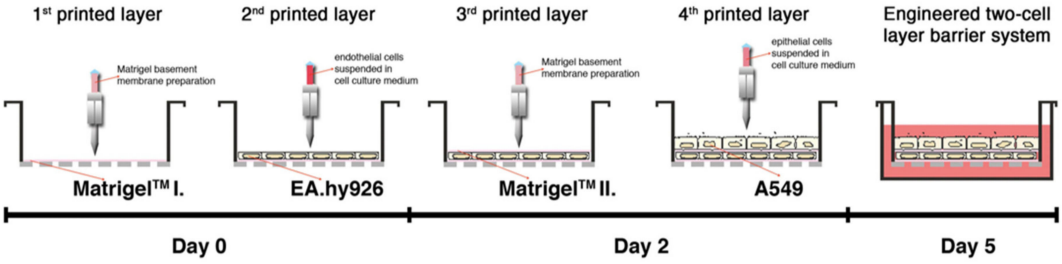

Figure 2. Vascularized airway-on-a-chips. Diagram of the timeline process for 3D bioprinting cell pattering of the cell-layer barrier. Reproduced with permission from [74]. Copyright © 2015 Springer Nature.

Table 4. Vascularized airway-on-a-chip.

Coating Material	Cell Population	Features	Ref.
Collagen I	Primary human small airway epithelial cells and primary human lung microvascular endothelial cells	Microfluidic system	[33]
Decellularized extracellular matrix bioink derived from porcine tracheal mucosa	Human dermal microvascular endothelial cells and primary human tracheal epithelial cells and lung fibroblasts	3D printing	[75]
Matrigel	human alveolar epithelial type II cell line A549 and the EA.hy926 hybrid human cell line	3D printing	[74]

5. Application as a Disease Model for Vascularized Lower Respiratory-Physiology-On-A-Chip

5.1. Cancer

Although many researchers are attempting to overcome cancer, it remains the second leading cause of death in the world, it is difficult to study because of its heterogeneity. In addition, the complex microenvironments surrounding cancers are important, but are not considered in many cancer studies. One of the most important factors for reproducing the cancer microenvironment in vitro is the presence or absence of vasculatures around the cancer. Interactions between cancer and vasculatures affect

the expression of target proteins, drug delivery, and clinical responses. In addition, vasculatures are known to play an important role in cancer metastasis.

As the importance of vascularized 3D cancer models increases, co-culture studies of cancer cells or cancer spheroids and vascular cells in transwell or 3D scaffold environments have been conducted in order to study the interactions of vasculatures and cancer within the chip; numerous researcher have focused on the characteristics of cancer in the presence of vasculature. In particular, the lung actively facilitates gas exchange between the alveoli and surrounding capillaries, and the ability to simulate peripheral vasculatures is becoming increasingly important for modeling the lung cancer microenvironment. Dereli-Korkut et al. [76] analyzed apoptosis through caspase-3 when non-small lung cancer cells (PC9) co-cultured with vascular endothelial cells (HMVEC) were exposed to four anticancer agents in a 3D microfluidic cell array (3D μFCA) (Figure 3a). Their results demonstrate the potential for high-throughput drug screening using vascularized microfluidic lung cancer models. Dynamic caspase-3 activity in PC9 cells showed that cancer cells have different drug reactivity in different culture platforms, including static 2D or 3D culture, static 3D co-culture, and structured 3D co-culture with vasculatures in 3D μFCA. This study shows the importance of a lung cancer model that can simulate vascularization, blood flow, and the air–liquid interface.

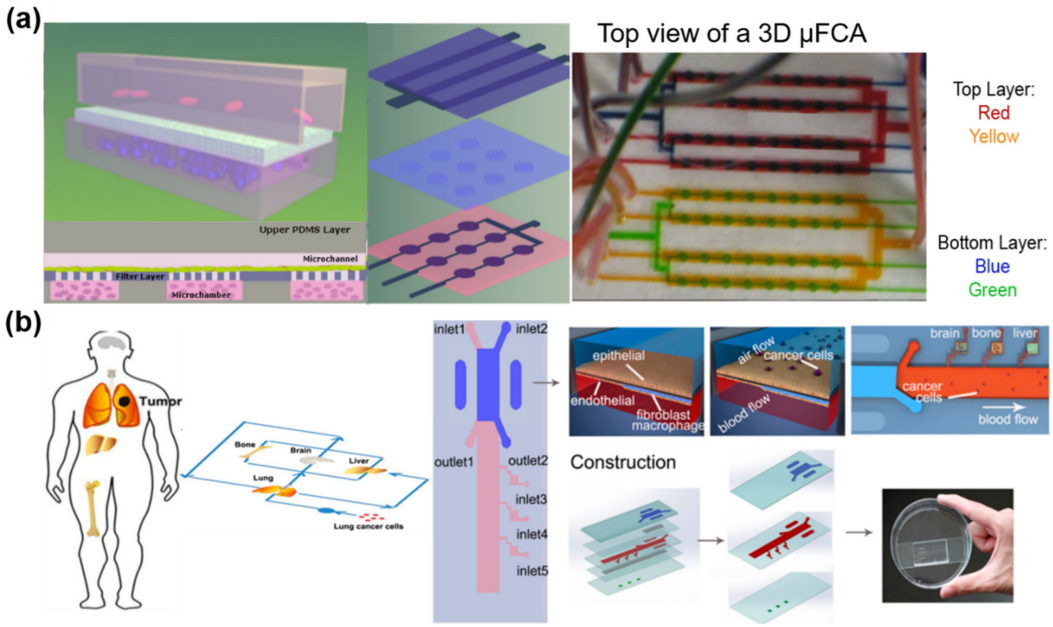

Figure 3. Vascularized cancer chip models. (**a**) Four anticancer agents were treated with non-small lung cancer cells (PC9) co-cultured with vascular endothelial cells (HMVEC) in a 3D microfluidic cell array (3D μFCA). This study shows the importance of a lung cancer model that can simulate vascularization, blood flow, and the air–liquid interface. (**b**) The device is a micro-engineered multi-organ microfluidic chip, that demonstrated lung cancer metastasis to multiple distant organs. This system includes an upstream lung and three downstream distant organs (brain, bone, and liver). (**a**) Adapted with permission from [76]. Copyright © 2016 American Chemical Society. (**b**) Adapted with permission from [77]. Copyright © 2014 American Chemical Society (https://pubs.acs.org/doi/abs/10.1021/ac403899j).

In order to overcome cancer, not only are drug reactivity studies important, but also research on cancer metastasis mechanisms is critical. Therefore, the development of a vascularized cancer metastasis model is essential. Recently, in order to reproduce the complex interactions of lung and distant organs, Xu et al. [77] designed a micro-engineered multi-organ microfluidic chip (Figure 3b). The device consists of one upstream chamber that mimics the lung and three downstream chambers for the brain, bone, and liver. The invasiveness of lung cancer cells was analyzed using RANKL for bone-specific metastasis, CXCR4 for brain-specific metastasis, and liver cell damage was evaluated by

measuring AFP. The lung metastasis microfluidic chip was shown to improve the reliability of results by simulating a lung cancer microenvironment resembling the actual environment via co-culture with vascular endothelial cells, fibroblasts, and immune cells.

5.2. Asthma, COPD, and PE

Asthma is a chronic inflammatory disease of the airways characterized by various forms of airway obstruction, airway hyperresponsiveness, and airway inflammation. The incidence of asthma has increased in recent years, despite the availability of various drugs, and the underlying disease pathogenesis remains unclear. Therefore, it is important to continuously investigate the pathophysiology and pathogenesis of asthma in humans and to develop new drugs based on these findings. Benam et al. [33] developed a small airway chip for human study that contains perfusable vasculatures comprising bronchial epithelium and vascular endothelial cells. They added interleukin-13 (IL-13), a commonly used experimental asthma inducer to the chip. They investigated the production of cytokines in the presence or absence of vasculatures on the small airway-on-a-chip. As a result, this effect appeared to be specific to the chip with vasculatures compared to the chip without vasculatures, which affected the secretion of RANTES, IL-6, and IL-10 under the presence of the polyinosinic-polycytidylic acid (poly (I: C)) condition. The poly (I: C) triggered inflammatory responses similar to in vivo. In addition, Park et al. [75] used 3D bioprinting to mimic the combination of airways with vascular networks in vitro. They developed a vascular platform via 3D printing of cell-containing dECM bioinks and also integrated them with epithelial models. In fact, the differentiation and function of epithelial cells were strongly apparent on the platform with vasculatures, along with the pathophysiological symptoms of asthma induced by IL-13. These results showed a strong increase in the number of goblet cells and reduced cilia cells. Expression of ZO-1, which is indicative of epithelial barrier function, was significantly decreased upon IL-13 treatment, and the transepithelial electrical resistance (TEER) value was also reduced. These results confirmed that the pathological symptoms occurring in the airway epithelium of asthma patients were highly reproduced in the airway platform with vasculatures.

COPD is a type of obstructive pulmonary disease with chronic airflow. It is common for the condition to worsen over time. Abnormal inflammatory reactions, such as the inhalation of harmful particles, gases, or smoking, reduce lung function more rapidly than others. Symptoms of COPD include coughing, sputum, and dyspnea during exercise. Chronic bronchitis and emphysema are commonly referred to as COPD because it is fundamentally difficult to distinguish their causes, symptoms, and treatment. Benam et al. [33] simulated the COPD environment by incubating human tracheal epithelial cells (HTEpC) from COPD patients on the vascularized small airway-on-a-chip. Normal or COPD epithelial cells in the chip were stimulated with LPS and poly (I: C), which increased the secretion of IL-8 and M-CSF on the COPD chips, respectively. This disease model did not show significant changes compared to the healthy model. M-CSF levels were increased only in the small airway-on-a-chip treated with poly (I: C). Their study confirmed that M-CSF may be a new biomarker of COPD exacerbation caused by respiratory virus infection. Small airway-on-a-chip with epithelial cells from patients with COPD reproduce the disease characteristics, such as selective cytokine hypersecretion, increased neutrophil recruitment, and clinical exacerbation by viral and bacterial infections.

In general, PE is caused by heart problems, and it is difficult to breathe because of the accumulation of excess lung fluid in numerous air sacs in the lungs. Huh et al. [78] induced PE in the microfluidic lung-on-a-chip. In these experiments, fluid leaked into the air chamber when interleukin-2 (IL-2) was added to the microvascular channel, and further damage to the lung barrier and a three-fold increased leakage occurred with the periodic mechanical modifications introduced with IL-2. These vascularized microfluidic lung-on-a-chip can replace animal experiments by not only mimicking changes in lung function by IL-2 and subsequent mechanical modifications, but also by successfully predicting new drug activity against PE.

6. Future Perspectives and Concluding Remarks

In this review paper, we present the lower respiratory-physiology-on-a-chip and describe related research advances. Because of the current levels of environmental pollution, respiratory organs are threatened more than ever. Lower respiratory-physiology-on-a-chip have been developed and researched for the prevention and treatment of respiratory diseases. The developed chips are being studied in efforts to mimic tissue such that results can be applied to humans rather than using animal experiments. Vascularization of organ chips, which allows the mimicking not only of structural and mechanical properties but also immune responses and the internal networking of tissue, are a good example of these efforts. The research on vascularized respiratory chips is ongoing, and the prospects are bright.

In order to manufacture a vascularized respiratory chip that more closely resembles human tissue, the following components are required. Most conventional respiratory chips have used cell lines or primary epithelial cell cultures. These have limited ability to reproduce the physiological characteristics of actual respiratory organs. It is necessary to mimic in vivo respiratory environments for disease modeling and drug testing. It should be possible to incorporate the structural, functional, and mechanical features of human respiratory epithelium along with the composition of various cells such as airway SMCs, fibroblasts, and immune cells. For this purpose, the utilization of patient-derived cells and organoids, an area of active research, may be considered a challenge.

In most respiratory chip studies, cells have been cultured using coating materials such as collagen and fibronectin. However, the ECM in vivo is composed of various proteins, and the use of a single material limits the ability of the system to mimic cell–cell and cell–substrate interactions. Therefore, to study the drug reactivity and molecular features similar to the in vivo context, tissue-specific decellularized extracellular matrices can be applied to the chip to mimic the tissue microenvironment more accurately.

The majority of lower respiratory-physiology-on-a-chip studies have fabricated chips using soft lithography. This method is advantageous in terms of mass production and precision, but it requires a high level of skill and time for mold making. For this reason, the use of 3D bioprinting technology that can produce chips in a shorter time period and facilitate flexibility in design changes represents an appealing alternative for the development of lower respiratory-physiology-on-a-chip.

Many researchers have investigated the relationships between respiratory risk factors and lung disease using lower respiratory-physiology-on-a-chip. However, these systems have limits in their ability to mimic the respiratory microenvironment, such as the lack of vasculatures or of an air–liquid interface environment. When humans inhale harmful respiratory risk factors, the particles are filtered several times, with only tiny particles reaching the alveoli. Therefore, there may be errors in the results of the toxicity test in chip, currently used in the respiratory field. Therefore, future developments with lower respiratory-physiology-on-a-chip require an advanced vascularized respiratory-physiology-on-a-chip that implements blood flow along with a structural simulation of the respiratory system that is similar to the actual human body.

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