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Review

Alternative lung cell model systems for toxicology testing strategies: Current knowledge and future outlook

Joana A. Moura [1], Kirsty Meldrum [1], Shareen H. Doak, Martin J.D. Clift *

In Vitro Toxicology Group, Swansea University Medical School, Singleton Park Campus, Swansea, Wales SA2 8PP, UK

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ABSTRACT

Due to the current relevance of pulmonary toxicology (with focus upon air pollution and the inhalation of hazardous materials), it is important to further develop and implement physiologically relevant models of the entire respiratory tract. Lung model development has the aim to create human relevant systems that may replace animal use whilst balancing cost, laborious nature and regulatory ambition. There is an imperative need to move away from rodent models and implement models that mimic the holistic characteristics important in lung function. The purpose of this review is therefore, to describe and identify the various alternative models that are being applied towards assessing the pulmonary toxicology of inhaled substances, as well as the current and potential developments of various advanced models and how they may be applied towards toxicology testing strategies. These models aim to mimic various regions of the lung, as well as implementing different exposure methods with the addition of various physiologically relevent conditions (such as fluid-flow and dynamic movement). There is further progress in the type of models used with focus on the development of lung-on-a-chip technologies and bioprinting, as well as and the optimization of such models to fill current knowledge gaps within toxicology.

1. Introduction

The respiratory tract is composed of three different regions. The nasal/tracheal/pharangyeal (trachea and bronchi), the upper airways (bronchioles) (i.e., the conducting airways (no gas exchange)) and the lower airways (i.e., the alveoli, or the respiratory airways (gas exchange region)). The entire lung is the first point of exposure for numerous inhaled chemicals, particles, bioaerosols and gaseous compounds that humans are exposed to either therapeutically, environmentally, or occupationally [1]. Though, from a toxicological perspective, it depends upon the physical and chemical attributes of the inhaled compound of interest as to the specific region of the airways that may be exposed and thus studied [2,3]. This also has an importance towards the potential for systemic effects of these exposures, and thus additional biological models implemented (i.e., beyond the lung). Thus, a physiologically relevant model for each section of the airway is necessary for elucidating the toxicology of any inhaled substance.

Different regions of the lung are constituted by a variety of different cells (Fig. 1), with changing phenotypical, structural and functional components in order for them work collectively to enable tissue/organ homeostasis. The normal epithelial layer in the large airways is composed of goblet cells as well as club cells. Goblet cells are secretory cells that produce mucus, which contributes to the construction of a physical barrier to the outside world and a major part of the innate defense system [4]. Club cells are precursors to ciliated cells, and are the predominant cell in the airways arising from either basal or secretory cells [5]. Each ciliated cell has in the region of 200 cilia allowing sufficient beating to power the mucociliary escalator and clearance of debris out of the airways [6]. Ciliated cells form a segment of the mucociliary transport system and through their beating they enable the transport of foreign bodies trapped in the mucus out of the respiratory system [7]. On average, 30% of the large airway epithelium in the human lung is made up of basal cells. These cells protect the underlying stroma from the external environment [8]. In contrast, 9% of the small airway epithelium is constructed of club cells which produce multifunctional uteroglobin/club cell secretory protein (CC10), antiproteases such as secretory leukoprotease inhibitor, and other molecules that are essential towards the lung's defense, as well as being important

* Correspondence to: In Vitro Toxicology Group, Institute of Life Science, Swansea University Medical School, Swansea, Wales SA2 8PP, UK.
E-mail address: m.j.d.clift@swansea.ac.uk (M.J.D. Clift).

[1] Contributed equally.

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components of lung surfactant [9]. In the alveolar region, type II alveolar epithelial cells can be characterized very easily by their distinctive cuboidal shape, numerous microvilli and secretory lamellar bodies which contain and secrete lung surfactant (surfactant proteins A, B, and D) [10]. They are also progenitors of type I cells. Type I alveolar epithelial cells (which are also progenitor cells) which line 95% of the epithelial surface are characterized by their very thin cytoplasm and facilitate gas exchange and passage of small molecules across the membrane [11].

Each of the different types of epithelial cells of the whole lung are capable of interacting with, and activating, cells of the innate immune system, as well as the adaptive [15], through the release of various inflammatory mediators and by interaction with certain cell dependent receptors on the cell surface [16]. As well as playing a role in host defense against various pathogens, the epithelium plays an important role in the activation of the adaptive immune response across different disease states [17]. They are capable of releasing cytokines and chemokines that bridge the gap between the innate and adaptive responses [18], as well as attract B cells and different T cell types [17,19].

Any fault in these mechanisms has the potential to disrupt the airway homeostasis. This barrier also stops the interaction of various environmental particles with the surface of the epithelium. Surfactant is produced by type II alveolar epithelial cells. Lung surfactant consists of 90% lipids and 10% proteins. This allows pathogens *etc.* to then be cleared *via* mucociliary clearance mechanisms [20]. Thus, based upon these subtle, yet significant differences in the cellular and tissue structure of the human lung it is imperative to either create, and/or implement a lung model that considers the physiological and anatomical structure lung region being investigated related to the inhaled substance of interest, and where it is considered to deposit within the lung. Based on this, models of the human lung need to attempt to mimic the human airway and should include as many of the various cells relevant to the region of interest as possible. For example, when considering inhaled airborne particles this region depends on their (aerodynamic) size, shape, and charge, as based upon these characteristics these particles will deposit at and interact with different places along the respiratory tract [21–27]. The health status of the population of interest must also be considered when determining which model to choose to determine the (mechanistic) toxicological effects of the compounds of interest may illicit. Various avenues allow this to be achieved *in vitro*. For example, this may be acheived by using donor cells with the specific disease of interest, or *via* implementation of tissue explants to grow these cell regions.

2. Considerations of *in vivo*

Historically, mammals (i.e., monkeys, dogs, rabbits, and (Guinea) pigs) were used for elucidating the impact of inhaling xenobiotics [28]. Whilst there are many reviews that highlight the specific differences between *in vivo* (non-human), *in vitro* and humans, it is important to note some specific lung-related points, especially in relation to human lung vs. rodent lung (since rodents are the most commonly used, non-human *in vivo* lung model). There are key differences between murine and human airways that may cause varying responses and therefore cause the murine model to be more obsolete than previously thought. Regarding the anatomy of the respiratory-tract, the differences start on the total number of branching generations; there are twenty-three for human [29] and sixteen for mouse [30]. Further, the pattern of bronchial branching also varies between these species [31,32], leading to different deposition patterns of inhaled substances [30]. Also, differences arise in the methods of breathing. Specifically, mice are predominantly nasal breathers, while humans are oronasal. There are also significant differences in the immune responses between the two airways [33,34]. At the cellular level in the mouse the number of club and goblet cells are inverse to the numbers found in humans [35,36]. In the mouse, in the terminal bronchioles reside a population of bronchioalveolar stem cells which express bronchiolar club cell marker, CCSP (club cell secretory protein [Scgb1a]), and the alveolar type 2 cell marker, SPC (pro-surfactant protein C) [37]. However the presence of

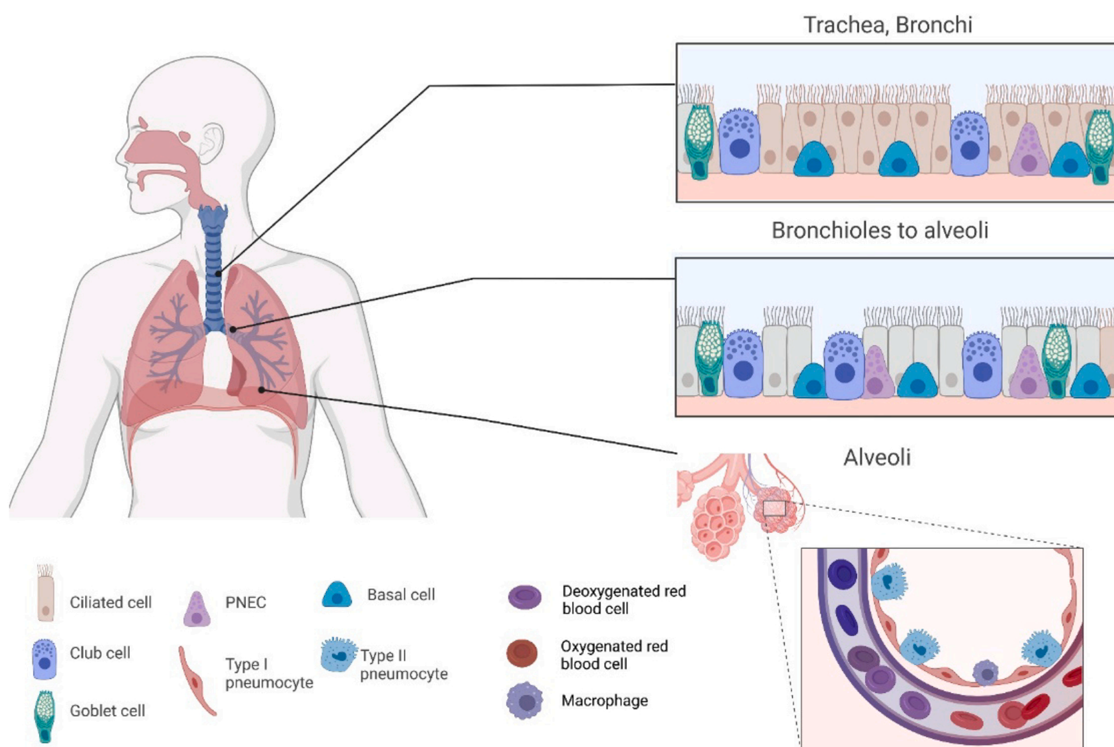

Fig. 1. Lung cell composition of the human lung within the different sections of the airways. Within this figure, the airways have been split into the trachea, bronchi (upper airways); the bronchioles to the alveoli and then the alveoli unit itself. Further anatomical detail is outlined in *Gehr* [12]*, Gehr, Hof* [13] *and Chang, Crapo* [14]*. Created with BioRender.*

this stem cell population in the human lung remains a matter of debate [38,39].

Another method of reducing the number of animals used for these studies is using tissue already obtained and reliably stored. This use of precision cut lung slices can be sections from either human tissue (healthy or diseased) or murine tissue. By using tissue from disease models, these contain the physical changes of the disease, as well as having relevant cells present within the section [40]. These sections are maintained *ex vivo* and have been known to constrict when exposed to various stimuli, but their responses reduce over time [41]. These sections however only give a "snapshot" of what the lung (and not the whole organism) looks like at the exact moment of fixation [40]. This would therefore only give an indication of what a potential "whole system" response may be, and thus can be considered as a scientific 'middle ground' between established *in vitro* models and *in vivo* models.

With the 3Rs (reduction, refinement, and replacement) becoming ever more important in toxicology studies, it is important to develop *in vitro* models that can be used to try and replace these models and allow extrapolation back to the human exposures. There is a need to move away from rodent models and implement models that are not only human based, but also mimic characteristics that are important in lung function.

In the following review, it is the objective to address the models that are currently being created (as previously outlined [42]), tested and put-forward for the reduction and potential replacement of *in vivo* models. Furthermore, discussion will surround the identification of the adaptations required to enable these lung models to truly mimic the imperative human physiological components necessary for them to be widely adopted across all stakeholder communities. All of these methods are introduced in Fig. 2 and then expanded upon throughout the present review.

3. Advancing alternative models

3.1. 2D monolayers

In the early stages of lung cell cultivation is the work, which by taking human nasal or bronchial brushings, collected ciliated cells [43]. The submerged cultures of the ciliated cells were used to check for structural and functional abnormalities [43–45]. The work had a major impact since it helped understand the key role on particle clearance and helped in diagnosing cilia associated disease such as, primary ciliary dyskinesia, cystic fibrosis, chronic obstructive pulmonary disease and asthma [46]. These experiments were the primary work of a 2D nasal/bronchi monoculture model that helped to identify each disease state. It is important to use this foundation of 2D models and use the differentiation of these models as building blocks for more advanced 3D models.

One of the first concerns when implementing a new lung model is what type of cells should be incorporated. Nowadays there are numerous available cell lines with standardized and well-defined characteristics (referred in this review as a *standard cell line*) as well as immortalized cell lines. A previous literature review [47] includes a list of the common lung and endothelial cell lines used for mono- and co-culture *in vitro* lung systems.

Naturally, physiological relevant cells isolated from human tissue are an alternative to cell lines. These cells are naïve (i.e. without chronic mutation *via* cell culture, or non-specifically activated) and are patient specific, which is especially important when considering disease models. However low tissue availability and donor heterogeneity makes it a challenge when trying to develop high-throughput models and compare research across different laboratories (i.e. in the sense of creating standard operating procedures [48]). As alternative cell source, there are human pluripotent stems cells (hPSCs), which include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). These cells have the potential to be differentiated into a variety of different cell types which can be subdivided multiple times which can then be implemented within these lung models. They do however come with their negatives,

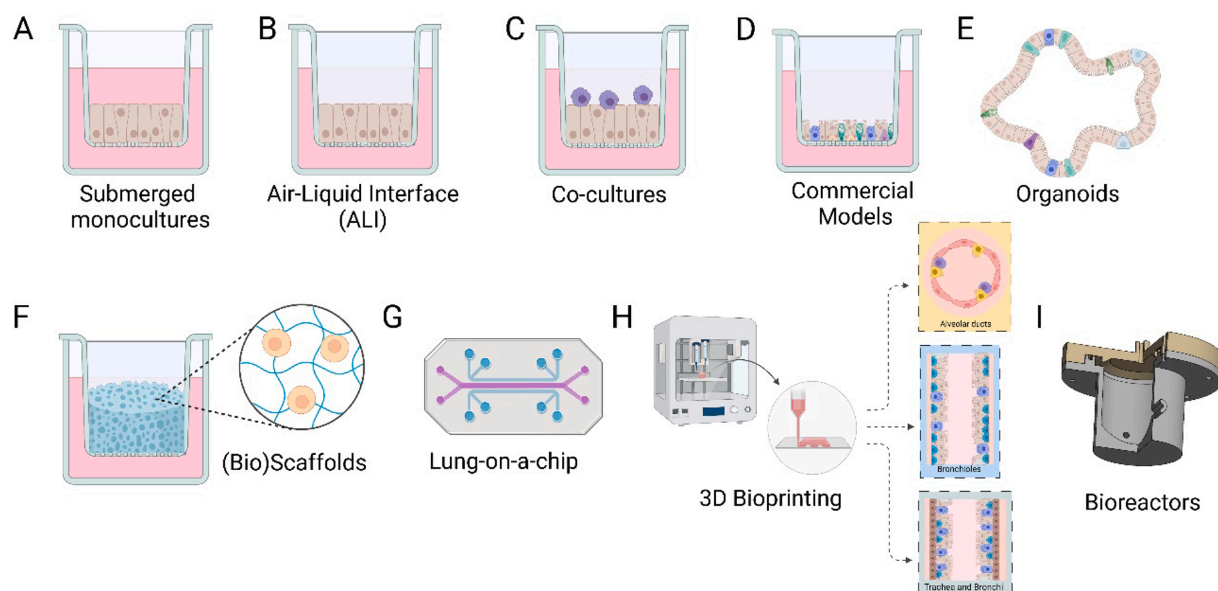

Fig. 2. Advancement of the lung cell model systems discussed within this review. A. Submerged monocultures – tends to be an epithelial cell layer with liquid above and below the cells. B. An air-liquid interface (ALI) culture. A layer of cells grown on a transwell insert with medium on the basal side and air on the apical side. C. co-culture models, these are also cultured at the ALI, but instead of a monolayer of epithelial cells, they also tend to contain immune cells or endothelial cells. D. commercial models - contain a bigger diversity of cells and are delivered to the end user ready to be used. E. organoids - can be made up of various cells with a round structure. F. (Bio)Scaffolds - help advance the cell culture models by giving the cells a structure to grow upon. G. Lung-on-a-chip models - aim to replicate the lung including fluidics at a much smaller scale. H. 3D Bioprinting - can "print" specific cells layer upon layer to mimic a specific area of the lung. I. Bioreactors - allow the addition of both fluid-flow and breathing mechanics to a culture that was previously static. *Created with BioRender.*

including patient-to-patient variability (including epigenetic differences) and ethical considerations [49].

Besides the types of cell to use, a further limitation in the future success of any *in vitro* system is the number of cells that would be required. For example, to re-create an entire human lung after decellularization, it is estimated that an adult lung has 250 billion cells, 26% are found in the conducting airways and 74% in the respiratory airways [50]. To address this problem, bioreactors are consider valuable platforms capable of producing large amounts of cells, such as stem cells [51] (please consider the following reviews of bioreactors used for stem cell expansion and differentiation [52–56]).

Nonetheless, 2D models are the only OECD regulated models that are currently available. There are currently no 3D model implemented in regularly toxicology testing [57]. These 2D models do have their place, that are practical and inexpensive, however, they are only useful for yes/no approaches and do not allow for the detail of response that has been reported as observed in 3D model systems, and pertinent to what would occur *in vivo* (human) [57]. Yet, any lung model composition is about balance. A good *in vitro* model could be built on a poor scaffold that replicates the lung but would remain more advantageous than a poor *in vitro* model on a very good scaffold. There are many variables that must be considered when choosing a model.

Currently, 2D monolayers also help to investigate lung cell differentiation. The early work of D'Amour, Agulnick [58] was able to show formation of a definitive endoderm by differentiation of human embryonic stem cells [58]. Then, the same was proven by using human embryonic stem cells [59]. Furthermore, the research of Longmire, Ikonomou [60] demonstrated the purification and directed differentiation of primordial lung and thyroid progenitors derived from mouse embryonic stem cells. The same was achieved by using patient-specific cystic fibrosis iPSCs by Mou, Zhao [61]. Lung epithelial cells are derived by the embryonic endoderm, inner germ layer of the embryo, however ectoderm contributes to the innervation of the lung and the mesoderm to the generation of blood vessels, fibroblasts, smooth muscle and cartilage. As lung embryonic development requires the three germ layers, it makes it an extra challenge to mimic embryonic development of the lung *in vitro*. The work of Huang et al., elucidates the presence of mesoderm layer to derive lung epithelial [62,63]. In their work, the authors claim to have been able to achieve *in vivo* and *in vitro* differentiation of ESC into basal, goblet, clara, ciliated, type I and type II alveolar epithelial cells [62,63]. However, they also found *in vivo* the contamination with mesoderm tissue surrounding the airways, which may indicate that the endoderm recruited it [62,63]. These findings are in alignment with the previous work of Blanc, Coste [64], who showed that the lung branching in mice is spatially controlled by the mesoderm, where lung buds fill the available space left by the mesenchyme tissue.

3.2. Culturing at an air-liquid interface (ALI)

The first ALI system was introduced by Whitcutt [65]. In this work, bronchial epithelial cells cultured at air liquid interface showed a mature cilia formation and furthermore increased mucus secretion after 3 weeks of culture. In contrast no signs of ciliogenesis or mucus was found in submerged cultures at any time course [66]. The ability to have both ciliated and mucus producing cells enabled the recreation of the mucociliary clearance mechanism. The improvement of cell morphology and characteristic secretome is also observed in alveolar cultures. The early work of Dobbs [67] showed that type II alveolar cells isolated from rats cultured under submerged conditions presented squamous phenotype, lacked surfactant proteins and the respective mRNA. Comparatively, at the ALI the cells acquired a cuboidal morphology, contained lamellar bodies, secreted surfactant proteins A, B and C together with the mRNA expression of the same proteins. The authors also observed a switch when submerged cultures were cultured to the ALI, which induced surfactant production [67]. ALI has also been shown to help restore mucus and surfactant production within *in vitro* models, both fluids very important for particle entrapment and clearance [68]. For reviews on mucus refer to [69,70] and for surfactant consider [71–74]. Since an early stage the introduction of ALI cultures has shown improved cell morphology and function, which helps to justify its widespread implementation in current research activities [75].

3.3. Co-cultures at an ALI

Whilst the previous section highlighted the advantages of culturing 2D cell systems at the ALI, emphasis in recent years has been upon how ALI can assist in supporting more physiologically relevant lung models, specifically co-culture systems. Notably, models of the alveolar region in the human lung have received most attention. Cultures can contain multiple cells on the basal or apical side of the membrane and do allow cells to be grown onto an established monolayer (for example, macrophages seeded onto an epithelial layer). Models can focus on the addition of immune cells, with models containing dendritic cells on the basolateral side of the membrane and on the apical contain alveolar type II and macrophages [76]. A triple cell co-culture model with alveolar type II cells (A549 cell line) and with human macrophages (THP-1 cell line) on the apical layer and human lung fibroblasts (MRC-5 cell line) on basolateral compartment can also consider additional structural cells (such as fibroblasts) [77]. Other examples of alveolar co-cultures include type II cells in the apical and endothelial cells in the basolateral side of the membrane [78]. It is important to understand the research question that needs to be answered before deciding which cell types to culture together.

3.4. Commercial models at an ALI

Human airway models are now commercially available and can be bought ready to be used for toxicity testing. The commercially available skin model from MatTek has been approved for OECD testing [79], but to date no other commercial model has been OECD approved. The EpiAirway™ provided by MatTek is a human mucociliary airway epithelium model that has been extensively used, such as within disease modeling [80], drug discovery [81], and toxicology testing [82,83]. The Epithelix detain several products: MucilAir™, and SmallAir™ [84]. The MucilAir™ contains mucus, ciliated, goblet and club cells. These systems provid higher data reproducibility due to lower batch-to-batch variability and the possibility to make repeat dosage for toxicity testing. However they do also have their limitations such as flexibility of cell and disease types [86]. As human airway models from these companies are cultivated in inserts, with standard dimensions, this enables them to be suitable for further use in most commercially available exposure systems. For example, the AlveoliX (AX lung-on-a-chip) system can now be implemented within the VitroCell$^{(R)}$ exposure system.

The insert membranes that the air-liquid interface cultures are grown upon come in different pore sizes and material compositions. The benefit of developing lung models on inserts is that by using conventional laboratory plates one can introduce an ALI and the ability to seed different types of cells. In a simple set up, a multicellular approach can be created where the basal side of the membrane is submerged in culture media and the apical side, at the ALI, receives nutrients *via* diffusion. These membranes are very firm and are good for static models, however, due to the material they are made from, they are not appropriate for advancing the lung model with the addition of flex (this aims to mimic breathing) [87]. Other methods of advancement with these inserts can still be used, such as the addition of flow to the system (for example using the Kirkstall systems currently on the market) [88]. The integration of inserts on more advanced platforms can be seen in lung-on-a-chip, 3D bioprinting and bioreactors section.

3.5. Organoids

An organoid is defined as an *in vitro* 3D structure composed of

multiple cell types [89] these are summarized in Table 1. The cells spontaneously assemble, commonly exhibiting a degree of organ specific spatial organization and function [89]. Organoids are derived by self-organized primary lineages, such as embryonic stem cells (ESC), induced pluripotent stem cells (iPSC) or organ stem/progenitor derivatives [89]. In the homeostatic lung, basal cells have the capacity to self-renew and to differentiate into club, ciliated and pulmonary neuroendocrine cells (PNECs), also with a self-renewing capacity. Club cells (expressing Scgb1a1 and Cyp2f2) are also a self-renewing population with capacity to generate ciliated and goblet cells [10]. In the alveolar region, the alveolar type II epithelial cells give rise to the alveolar type II and I [90]. The use of embryonic stem cells or iPSC to derive lung organoids are dependent on direct differentiation, which has been previously discussed in the 2D section above.

Organoids are normally formed a commercial gel (e.g. Matrigel [91]) protein and growth factors mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells, which is liquid at low temperature and solidifies at 37 °C. Under differentiation media and growth factors, the pluripotent stem/progenitor cells start to migrate and organize, *via* two principles of self-organization: cell sorting and spatially restricted lineage commitment. The embedded cell suspension in Matrigel can be cultured in multiwells or inserts (to promote ALI), in the presence or [89] absence of mesenchymal cells [36]. Alveolar type II cells have to be combined with other supporting cell lines such as, fibroblasts [92], lung mesenchymal cells [93], the MLg cell line [94] or endothelial cells [95] to ensure cell viability. Table 1 contains a summary of lung organoid examples, divided into 3D constructs that present structure and cells from conducting or respiratory airways. Giving the self-organization and differentiation potential there are organoids that show some similarities of both of these airways (Table 1).

Lung organoids share important physical features with the *in vivo* scenario, making them potential tools to study normal and abnormal lung development. In addition, organoid viability is not affected by passage or freeze-thaw, making them attractive for culturing them for a long period of time, and thus potentially a tool for investigation of chronic disease progression, or low-dose, repeated measure toxicology assessments. Also they may be suitable for monitoring a long-term drug therapeutic effect or accumulative toxicology. The limited number of cells needed to create an organoid further makes them potentially favorable, due to shortage of established primary cell lines. Yet, concerns remain as to their ability to truly mimic the physiology and anatomy of all regions of the human lung.

Future work on the organoid field needs to address the lack of more defined ECM based material to promote organoid formation. Matrigel is commonly used for organoid culture however, limits the organoid used in clinical applications since it includes animal derived products that can

Table 1
Summarizes the state-of-the-art literature regarding organoid *in vitro* models from the conducting airways, respiratory airways and both. ESC: embryonic stem cell, iPSC: induced pluripotent stem cell, AT1: alveolar type 1, AT2: alveolar type II, h: human, EC: endothelial cells, BASCs: bronchioalveolar stem cells, HBECs: human bronchial epithelial cells, FB: fibroblasts, CF: cystic fibrosis, PDXs: Patient-derived xenograft models, PEG: polyethylene glycol, PLG: lactide-co-glycolide, PCL: scaffolds or polycaprolactone.

Anatomical Area	Type	Precursor cells	Present Cells	Matrix/Setup/Outcome	Ref.
Conducting Airways (trachea → bronchioles)	**healthy model/ organ maturation**	mouse primary basal cell	basal, ciliated, club	Matrigel /ALI	[34, 97,98]
		mouse primary basal cell	basal, ciliated, club	Matrigel	[99]
		hiPSC, hESC	basal, ciliated, club, goblet	Matrigel /ALI	[100]
		human primary basal cells	basal, ciliated, goblet	Matrigel	[101]
	disease model	human primary basal cell	ciliated, goblet, basal	Matrigel/ study excess mucus production	[102]
		iPSC normal and from fibrosis patient	basal, ciliated, club, goblet	Matrigel /ALI	[103]
		biopsy from healthy or cancer patient, broncho-alveolar lavage fluid from healthy and CF patient	Basal, ciliated, club	Matrigel/ALI/ Organoids passed for > 1-year mimic normal, cancer, CF and virus infection	[104]
		biopsy from bronchial airway	basal cells, ciliated cells, goblet cells and club cells	Matrigel/Organoid support parasite complete life cycle	[105]
	drug test	biopsy from healthy and 5 different cancer subtypes	healthy organoids show basal, club, ciliated cells, cancer organoids maintain the histology and genetic of original tissue	Matrigel/ different cancer drug response from 2D, PDXs and organoids.	[106]
Respiratory Airways (alveolar region)	**healthy model/ organ maturation**	hESC	AT2, AT1, after mice transplantation: mesenchymal, PNECs, vasculature, nerve fibers	Matrigel	[107]
		PDGFRAα+ stromal cells	AT1, AT2	Matrigel /ALI	[92]
	toxicology	hESC, hiPSC, primary AT2 and FB	AT2, AT1-like cells	Matrigel/submerged exposure with GNE7915 (5 μM) or amiodarone (10 μM)	[108]
Both Airways	**healthy model/ organ maturation**	hiPSCs	basal, alveolar progenitors, PNECs secretory	Matrigel	[109]
		hESC, mesenchymal stem cells	AT1, basal, ciliated, club	Matrigel	[110]
		mouse isolated EC, AT2, BASCs	club, ciliated, goblet, AT1, AT2	Matrigel	[95]
		hPSC	basal, ciliated, alveolar markers, mesenchymal markers	Matrigel	[35]
		hiPSCs, hESC	basal, immature ciliated, smooth muscle, club, goblet, fibroblasts, alveolar progenitors	Matrigel	[111]
		HBECs, EC, FB (10:7:2 ratio)	basal, club, goblet, ciliated, AT1, FB, AT2, EC	Matrigel	[112]
	disease model	hPSC, iPSC with CF	ciliated, club, basal, goblet, AT2, immature AT1	Matrigel	[113]
	branching model	HBECs, EC	branching structures with basal, AT2	Matrigel /ALI	[114]
		HBECs, FB	branching structures with basal, club, AT2,	Matrigel /ALI	[115]

create an immune response and transfer pathogen, as well as hinders the study of growth factors and signaling gradients [91,96]. The validation in human scenario still needs to be done. Organoids are mostly generated using cells from rodent origin and with final maturation in rodents [33]. Despite this, organoid technology holds prospective promise, since there remain cellular interactions that have not yet been recapitulated, specially the highly vascularised part of the alveolar region [36]. Also, the introduction of relevant cell types, such as immune cells has not been achieved yet. The viability, shape and size heterogeneity among organoids, make it necessary to track them individually. There is also a gap in the knowledge in understanding the impact of biomechanical forces during formation and maintenance of organoids [96].

That could be a route to improve organoid architecture and maturity together with the adoption of bioengineering approaches, such as topographically structured scaffolds or control spatial positioning of cells (strategic positioning of PSC with mesenchymal or endothelial cells) [91]. Thus, the following sections show lung *in vitro* models using technologies such as (bio)scaffolds (highlighted in Table 2), organ-on-a-chip, 3D printing and bioreactors.

3.6. (Bio)scaffolds

In 2D cultures a fraction of the cells are in contact with other cells and ECM, while others are exposed to the culture media. This creates differences in cell polarization (abnormal uneven integrin biding events) having direct consequences on intracellular signaling. In addition, cells are subjected to a homogenous concentration of nutrient from the media which is also unnatural. *In vivo*, soluble factors have spatial gradients which influence cell function, migration and differentiation [116].

On the contrary, 3D scaffolds can introduce stiffness, local microstructure and curvature (though the effect of curvature in cell behavior has been overlooked [117]) (as highlighted in Table 2). The cells are no

Table 2
Summarizes the state-of-the-art literature on scaffold *in vitro* models from the nasal cavity, conducting airways and respiratory airway. Decellu.: decellularized, HBECs: human bronchial epithelial cells, HLF: human lung fibroblasts, ALI: air-liquid-interface, 16HBE14o⁻: human bronchial epithelial cell line, Wi-38: human lung fibroblast cell line, ISO-HAS-1: microvascular endothelial cell line, HBSMC: Human bronchial smooth muscle cells, PET: polyethylene terephthalate, TFA: trifluoroacetic acid, DCM: dichloromethane, Calu-3: epithelial lung cells, MRC-5: fetal lung fibroblast-like, 344SQ: lung metastatic murine cell line, MMP-5/− 9: matrix metalloproteinase 5/9, HUVEC: human umbilical vein endothelial cells, HVP: human vascular pericytes, NHBE: Normal human tracheobronchial epithelial cells, FB: fibroblasts, 393 P: lung non-metastatic murine cell line, h/m MSC: human/mouse mesenchymal stem cells, A549s: human epithelial cell lines, HpuVECs: human primary microvascular endothelial cells, NCO: isocyanate end groups, YIGSR: laminin derived peptide, NCI H441: AT2-like cell line, HPMEC: human pulmonary microvascular endothelial cells, CCL-210: fibroblast cell line, LAM: invasive lung disease lymphangioleiomyomatosis, HA: hyaloronic acid, NCI-H1299/ NCI-H446 human bronchial epithelial cells, SMC: smooth muscle cells.

Anatomical Area	Type	Cells	Matrix/Set up	Outcome	Ref
Nasal cavity	**Translational medicine/ cartilage repair**	human chondrocytes	low/high PEG content	After 4 weeks *in vitro* scaffold with more hydrophilic/ interconnected porous show enhanced cell differentiation and cartilaginous tissue formation	[131]
		Autologous chondrocytes	Porous porcine collagen I/III membrane (Chondro-Gide)	Positive results (breathing/ appearance/ pain) after 1-year patient implantation	[132]
		Human/rat nasal septal chondrocytes	Marine collagen	marine collagen proof of concept with rat implantation	[133]
Conducting Airways (trachea → bronchioles	**healthy model**	HBECs HLF	porous PE cylinder mold for collagen I supported on a transwell at ALI	Presence of HLF, ciliated cells, mucus secretion, collagen III/IV and fibronectin. Viable for 4w.	[134]
		Apical:16HBE14o⁻ Basolateral: Wi-38, ISO-HAS-1	Decellularized porcine trachea supported on a transwell	Microvilli, occludin, and b-catenin expression	[135]
		HBSMC	Electrospinned PLA and decellularized porcine lung	Cells showed contractile calponin 1 protein/ collagen I	[80]
	translational medicine	Autologous epithelial and MSC derived chondrocytes (human application)	decellularized trachea (from a donor)	After 4 months graft enabled functional airway and improved life quality (no immunosuppressive drugs needed)	[136]
		Primary fibroblasts and basal cells (mouse application)	Cells + collagen I dehydrated on RAFT absorbers grafted on pre-vascularized decellu. trachea	After one week, constructs had engrafted with signs of re-vascularization and keratin-positive cells.	[137]
	drug test	Calu-3 MRC-5 Dendritic cells from monocytes derivation	Electrospun scaffolds of PET, TA1, DCM 4 weeks at ALI	FB essential for epithelial barrier formation and recover from allergen exposure. Viable dendritic cells.	[81]
	disease model	344SQ, HUVEC and HVP cell line	PEG hydrogels with RGD, MMP-2 and MMP- 9 conjugations	Investigation of vasculature role in tumor growth.	[82]
		FB NHBE	Collagen/ PuraMatrix coated inserts (ALI optionally)	Suitable model to study mid/long term host-pathogen processes	[83]
		Patient-derived lung cancer cells, SMC NCI-H1299 and NCI-H446	Hyaluronic acid hydrogel with vitronectin/fibronectin mimetic peptide and MMP sensitive crosslinker	Hydrogel mimic the native niche of LAM	[138]
Respiratory Airways (alveolar region)	**healthy model**	HUVEC NCI-H441 HPMEC	Electrospinning nano fiber mesh formation with PLC and NCO-sPEG. Functionalized with RGDs and YIGSR.	Suitable model for evaluating pathological conditions, drug efficacy, pollutants, and nanotoxicology.	[139]
	disease model	CCL-210 A549 Primary AT2	Photodegradable PEG spheres containing the cells encapsulated in PEG	Faster migration rates when FB are cultured in co-culture as well as MMP activity. MMP can be a potential cancer target	[140]
		A549	Dextran-chitosan hydrogel	low hydrophobicity and protein absorption, promote A549s to cluster - microtumour	[141]
Lung	**healthy model**		Hydrogel from decellularized porcine lung + genipin crosslinker	Scaffold proof of concept with hMSC, mMSC, A549s, HpuVECs and HUVECs	[85]

longer submerged or at the ALI. In contrast, cells are embedded in a soft-solid scaffold where they can attach, proliferate, migrate, and contact with active ingredients, but also alter the material composition digesting it (through the work of metalloproteinases) and laying down new extracellular matrix. The 3D scaffolds are not passive vehicles but give essential biophysical and biochemical signals that determine cell fate. Studies show that cells are susceptible to substrate topography [117], with local geometry of the material dictating cells into proliferation or apoptosis [118,119], but also stiffness [120] and cellular chemistry [121].

Common materials for developing ECM analogs are separated by synthetic and natural means, thus addressing a broad range of mechanical and chemical properties. Common use synthetic materials are poly-lactic acid (PLA), polycaprolactone (PCL), polyethylene glycol (PEG, and PEG diacrylate (PEGDA). The Arg-Gly-Asp (RGD) aminoacidic sequence can be added to synthetic scaffold to facilitate cell adhesion. The natural materials widely used are gelatin, alginate, fibrin, chitosan and collagen. Special attention has been given to decellularization approaches of tissues/organ/cell sheet, as they have been identified to improve cell adhesion, growth and differentiation. When working with decellularized tissue one should bear in mind that decellularization protocols need to be optimized for each organ or tissue and individual applications. Insufficient decellularization can lead to residual cell detritus and consequently cause an immunologic response. In contrast, aggressive decellularization will bleach growth factors and denature proteins [122,123]. After decellularization is completed the scaffold can be formulated into a hydrogel after pepsin digestion and pH correction. The following reviews are suggested to readers interested in

Table 3
Summarizes the state-of-the-art literature on organ on a chip *in vitro* models from the nasal cavity, conducting airways and respiratory airway. Cells seeded on: T: apical side of a membrane/top chamber, m: the middle chamber, B: the basolateral side of a membrane/bottom chamber, ES: External stimulation, ALI: air-liquid-interface, M: presence of dynamic media, A: presence of dynamic air, D: deformation of the cell substrate, PM: particulate matter, wi38: human lung fibroblast cell line, NHLF: normal human lung fibroblasts, pHNE: primary human nasal epithelial cells, hNECs: human nasal epithelial cells, FA: formaldehyde, NIH/3T3 cells: mouse fibroblast, 16HBE14o$^-$: bronchial epithelial cells, pHPAEC: primary human pulmonary alveolar epithelial cells, NHBE: normal human bronchial epithelial cells, PMMA: poly (methylmethacrylate), Calu-3: human airway epithelial cells, hBSMCs: human bronchial smooth muscle cells, hAECs: human airway epithelial cells, COPD: chronic obstructive pulmonary disease, FB: fibroblast, HBEC: primary human bronchial epithelial cells, SAECs: human small airway epithelial cells, IPF: Idiopathic pulmonary fibrosis, PC: polycarbonate.

Anatomical Area	Type	Cells	Design/Material	ES	Outcome	Ref.
Nasal cavity	**toxicology**	pHNE (T) NHLF (m), wi38 (m) HUVEC (B),	3 chambers PDMS separated by fibronectin coated PET porous (0.4 μm) membrane	M	Submerged exposure of PM showed suitability of the model to reproduce inflammation and cell membrane integrity	[159]
		Ciliated-derived pHNE cells, NIH/3T3 cells	transwell integration on PDMS device bounded to glass to allow microscopic inspection	ALI/M/A	Exposure to gaseous FA increased mucociliary function. The response was lost at higher FA level	[160]
Conducting Airways (trachea → bronchioles	**healthy model**	16HBE14o$^-$ HUVEC	2 PDMS plates separated by a porous (10 μm) and flexible PDMS membrane coated with fibronectin. Vacuum applied on lower plate deforms membrane	M/D	cyclic stretch affects the permeability, metabolism, and cytokine secretion of epithelial cells	[161]
		Bronchial epithelial cells (T), FB (T), EC (B)	2 PDMS plates separated by transwell membrane coated with decellularized ECM. With electrodes for TEER measurements	ALI/M	liver, heart, and lung on a chip for inter-organ responses to drug administration have showed efficacy in predicting side effects	[162]
		NHBE	Collagen IV coated transwell integrated on device. With electrodes for TEER measurements	ALI/M	Device supports lung and liver organ model crosstalk for up to 2 weeks.	[163]
		Calu-3 (T) hBSMCs (B)	3 chambers make of PMMA. Middle layer contains mix of collagen I and matrigel.	ALI/M	Viable coculture for > 31 days	[164]
		primary Tracheo-bronchial epithelial cells, FB, EC	3 overlapping channel on PDMS separated by a PTFE and a PET membrane	ALI/M	5 days viable model of a triculture with primary airway cells with mucociliary differentiation and barrier function	[165]
	disease model	2 PDMS plates separated by an infill of collagen + FB. The infill is perfused by 3 channels, 2 with EC lining and 1 with HBEC l lining cells		ALI/M	Volatile respiratory pathogen infection to simulate of lung-microbe complex interaction	[166]
		SAECs	2 PDMS plates separated by a porous membrane. The bottom layer contains the collagen and media channels. The top layer contains the airway channel. The collagen seeps into the membrane by constant compressing	ALI/M	Suitable model to replicate mucus plugs seen on obstructive pulmonary diseases	[167]
		16HBE14o$^-$ (T) HUVECs (B)	collagen coated transwell integration on PC device to allow media perfusion from the basolateral side of the membrane. The device allows to automated media sample collection	ALI/M	Epithelial cells secreted TNF-α after viral dsRNA infection. This led to chemoattractant and adhesion proteins release by the HUVECs. This reaction was not observed when TNF-α was neutralized	[168]
Respiratory Airways (alveolar region)	**healthy model**	pHPAEC HUVEC	2 PDMS plates separated by a porous (8 μm) and flexible PDMS membrane coated with fibronectin. Vacuum applied on lower plate deforms membrane	M/D	cyclic stretch affects the permeability, metabolism, and cytokine secretion of epithelial cells	[161]
	disease model	A549	PDMS channel bounded to glass with 3 inlets and outlets. The cells are cultured in the middle channel. Lateral channels used to gastric contents exposure	M	novel device to study gastric reflux effect as trigger for IPF	[169]
	particle distribution	acellular	5 generations of bifurcating alveolated ducts with periodically expanding and contracting walls in PDMS	A/D	physiological breathing motions on acinar airways on to study inhaled aerosol transport	[170–172]

decellularizations protocols [124–127] and specifically on lung tissue [128]. Recent advances towards biofabricated lungs are completed in bioreactors, therefore, more details are given further in the (bio)reactor section [129]. Synthetic materials offer higher flexibility, the degrees of substitution affect the pore size, rheology, and swelling behavior of the scaffold. They also contain a defined composition; however, they may need to be adapted to be biocompatible and bioactive. This problem is addressed in the natural materials or decellularized constructs, that contain endogenous factors, but have batch-to-batch variability. The mixture between the natural and synthetic material increase the scaffold possibilities and at the same time keep it biological active [130].

Despite understanding the need to culture cells in more physiologic conditions, such as in 3D biomaterials, there are several limitations that hinders cell viability. Currently there is limited understanding as to the best approach towards combining a scaffold with a network of channels that would mimic the role of the vasculature (transport of nutrients, dissolved oxygen and remove cellular waste products). In high metabolic tissues, cells are no more than 100 μm from a high oxygen source [116]. The porosity also has limitations, if it is larger than the dimensions of the cells then the scaffolds transform into a 2D matrix with a curvature. On the other hand, small porosity, brings diffusion limitations. Other constraints to consider when working with scaffolds is the heterogeneity of the material during polymerization or surface formation. Additionally, standard techniques of imaging or protein/RNA/DNA extraction are more challenging and more laborious with an increased number of steps and optimizations [116].

3.7. Lung-on-a-chip

Microfluidic devices, as the name indicates, are engineering micrometer platforms (see Table 3D). These devices come to light with the use of biocompatible, transparent elastomeric materials, such as poly (dimethylsiloxane) (PDMS). In combination with soft lithography-based replica molding techniques, researchers are able to create layers of compartmentalized chambers that help to recreate tissues interface (for further detail [142]). The microfluidic approach allows a network of channels that supply nutrients and oxygen to the cell system, whilst removing metabolic waste that can reside in static cultures. The benefits of perfusion flow compared with static conditions, in addition to shear stress stimulus from the constant/pulsatile flow, is that it exhibits a culture closer to the natural vasculature environment. In addition, working at the micro-level, the flow regime is often laminar (if the Reynolds number is less than 2300), which leads to the formation of linear gradients [143]. Another asset of working with microfluidic systems is the low number of cells and low volume of reagents needed, which contribute to lower running costs of the experiment. The ability to run several experiments in parallel contributes towards a high throughput approach. The incorporation of sensors has been widely explored to monitor and follow the status of the cell culture with the advantage of being label-free, as well as recording specific culture conditions, such as pH, oxygen or glucose levels [144]. Another advantage of microdevices is the ability to design multi-organ approaches, by combining different tissue representative models in different compartments of the device within a common nutrient supply [145,146]. These devices have been widely explored to tackle the insufficiencies of *in vitro* models, proposing them as novel research platforms.

Lung-on-a-chip models have been able to reproduce the interface between the alveolar and endothelial cells in the alveolar region by separating two chambers by a flexible porous membrane. The alveolar cells (seeded on the upper chamber) had contact with air and the endothelial (seeded on the lower chamber) with media. Together with the dynamic flow, the device allows stretching of the cellular membrane where cells are attached simulating breathing patterns (by applying a vacuum). The same device has been further used to investigate bacterial infection [147], silica nanoparticle exposure [147], pulmonary edema [148], lung inflammation [149], smoke inhalation [150], epithelial barrier function with TEER sensor integration [151], thrombosis [152] and lung cancer [153].

Despite the remarkable achievement with organ-on-a-chip technology, there are several obstacles to overcome to contribute to the development of the field. The fabrication of microdevices may require special facilities, machinery and expertize, which is not often available in all labs [144]. Although, the emergence of commercially available microdevices is now allowing such research to become widespread. A detail review on the current market strategies can be found by Zhang and Radisic [154]. The micro devices designs are very restricted to a porous membrane to recapitulate the air-blood barrier [155]. The homogenous seeding of the cells in the chambers, as well as ECM-analogs coating can be challenging as well. To address that, 3D printing improvement at the microscopic level would allow to lay material or material and cells together and have a temporospatial control at a smaller scale (consider review papers on the subject by Alizadehgiashi, Gevorkian [156], Bhushan and Caspers [157] and Knowlton, Yenilmez [158]).

3.8. 3D bioprinting

3D bioprinting, as a technology, is an emerging tool to fabricate organs or tissues for tissue engineering applications, including *in vitro* models but also organ/tissue transplant. It is also referred to as additive manufacturing, where through a computer-aided program, living and non-living materials are deposited layer-by-layer to assemble in a predesigned pattern [173]. To further enhance this technique, 3D bioprinting can also implement sacrificial removal of material to allow fluidics to be added to them [174]. Bio-ink is a coined term in this field for when cells and materials are deposited together [173]. This technique is considered one of the most promising tools to produce biomimetic organ/tissues *in vitro* models to reduce animal experimentation [175].

The bioprinting technology available nowadays fits into four major techniques: inkjet, extrusion stereolithography and laser-assisted. An extensive review on these types of 3D bioprinters is available [176,177]. In brief each bioprinter has its limitations, regarding surface resolution, cell viability due to nozzle geometry and compatible biological materials. Currently, several materials are used, both natural and synthetic. The natural polymers found in the literature in 3D bioprinting applications are Matrigel, extensively, but also alginate, gelatin, laminin, fibronectin, collagen, chitosan, fibrin, silk fibroin, hyaluronic acid and decellularized tissue [178]. The synthetic materials used for bioprinting that can be highlighted are PLC (polycaprolactone) and PEG (poly (ethylene glycol) or PEGDA (PEG-diacrylate).

3D printing has also gained relevance for surgical training, where surgeons can practice outside the patients [179]. But also for patient education, where the medical professional can more easily explain and show the procedure to the patient by using a real 3D model [180]. This technique opened the tissue engineering field to new possibilities, such as, rapid prototyping of organs or tissues, the ability to personalize implants to a patient specific anatomy and the customization of specific laboratory tools. In addition, as cells and materials are deposited simultaneously, cells can be precisely placed inside the biomaterials, which is a major advantage in comparison to the previous models. The major challenges in bioprinting are connected to the adaptation of the technique, more specifically, to the materials used to form the matrix involving the cells and the dispensable systems to provide the best conditions to the cells while being printed [177,181].

Despite a very promising technique, there are not many examples in the literature of 3D bioprinting lung representatives (as shown in Table 4). However, they have been utilized in both healthy and diseased models (such as an asthma model [182]), implementing both cell lines [183] and primary cells [182] throughout the airways.

Table 4
Summarizes the state-of-the-art literature on 3D bioprinting *in vitro* models from the nasal cavity, conducting airways and respiratory airway., ES: External stimulation, ALI: air-liquid-interface, M: presence of dynamic media, A: presence of dynamic air, D: deformation of the cell substrate, PCL: polycaprolactone, bMSC: bone marrow-derived mesenchymal stem cells, A549: AT2-like cells, EA.hy926: human umbilical vein cell line, IMR-90: fibroblasts, hMSC: human mesenchymal stem cells, dECM: decellularized extracellular matrix hydrogel, hDMECs: human dermal microvascular endothelial cells, hTEpC: primary human tracheal epithelial cells, MVECs: microvascular endothelial cells, LF: lung fibroblast.

Anatomical Area	Type	Cells	Printing technique	Material/ Geometry	ES	Outcome	Ref.
Conducting Airways (trachea → bronchioles	**trachea cartilage repair**	rabbit chondrocytes	dual head: PLC heat extrusion/ syringe injection unit for cells + hydrogel	PLC cylindrical ring infused with alginate and collagen I hydrogel	-	tracheal graft implanted in a rabbit. Inflammation and stenosis seen when chondrocytes are not separated from the tracheal lumen by an intervening membrane.	[184]
		rabbit epithelial cells, bMSC, chondrocytes derived bMSC	dual head: PLC heat extrusion/ syringe injection unit for cells + alginate	cylindrical ring with 5 layers, cells were printed in alginate separated by a PLC layer. To close the ring design another 2 PLC layers were deposit	-	tracheal graft implanted in a rabbit. Epithelialization and vascularization were observed in all grafts, but cartilage formation was only seen on chondrocytes derived bMSC implants	[185]
		hMSC	extrusion	CT scan to recreate pig 4 cm tracheal defect make of PLC-fibronectin/dECM coated	-	tracheal graft implanted in pig. Histologic evaluation showed respiratory mucosal coverage and vascularity of the graft.	[186]
	disease model	MVECs LF hTEpC	extrusion	Microfluidic device base with 7 channels with transwell integration on top. PLC+PDMS device backbone and porcine trachea ECM + cells as bioink	ALI/ M	high-content vascularized airway-on-a-chip platform for preclinical trials with asthmatic airway inflammation phenotype	[182]
Respiratory airways (alveolar region)	**healthy model**	A549 EA.hy926	matrigel dispensed by jetting, cells by contact dispensing	4 layers print supported on a transwell: matrigel, EA.hy926 with media, matrigel, A549 with media	-	Printed co-cultured show more thin and uniform distributed cells compared to manually assembled co-culture	[187]
		IMR-90 A549	stereolithography	Near alveolar sac design with 600 µm alveolus and 400 µm channels in diameter in PEGDA and GelMA	A/ M/D	Acellular models sustained oxygenation and flow of human red blood cells during tidal ventilation and distension. Co-Culture was kept submerged for 1 day.	[183]
	disease model	A549	extrusion	alginate, gelatin and matrigel mesh dispensed on a lattice geometry	-	model supported influenza A virus infection patterns only observed before *in vivo* (not in 2D cell culture)	[188]

3.9. Bioreactors

Bioreactors are defined as manufactured containers (vessels or chambers) that support biological/chemical processes often used in fermentations, wastewater treatment and pharmaceutical production. In a controlled, closed system (of temperature, pH, nutrients supply and waste removal) the bioreactors are able of automation and standardization, which is important in the future of *in vitro* models. These vessels sustain different modes of flow operation: continuous, batch or fed batch. Bioreactors can also contain features that can apply pressure (for compressive studies), known to be important in modulating cell physiology. The bioreactor can operate under agitation due to impellers, like spinner flasks, or can rotate as rotating-wall vessels, which promotes higher transport of mass (nutrients or oxygen). If an adherent substrate is required, pack bed or hollow-fiber bioreactors can be the solution, where media flows through the supporting material [189,190]. This is the case for lung models where cells need to be attached. Template to support cell attachment and proliferation can be some of the solutions presented before, such as bio-printed constructs, scaffolds, decellularized tissue, organoids or inserts. One example is the commercially available bioreactor (patent number WO 2010/013068 A2, invented by Arti Ahluwalia, Daniele Mazzei and Bruna Vinci from the University of Pisa, Italy in 2009) now commercially, and routinely supplied by Kirkstall [191,192]. The use of the Kirkstall bioreactor chamber for lung application was explored to investigate aspergillus infections in human bronchial or small airway epithelial under ALI and perfusion [193], branching morphogenesis [194], nanoparticle toxicity screening in endothelial cells [195,196] and the comparison between various exposure methods [88].

In this way, bioreactors are highly suitable platforms to sustain tissues in *ex vivo*, such as during cell implantation, growth, and differentiation or in future tissue engineering application for organ maturation. However, bioreactors are still time and laborious, which limits clinical practices [189]. Table 5 outlines the range of applications where bioreactors have been used, contributing to move forward the field on lung *in vitro* models.

4. Summary and future developments

Future solutions in the field of *in vitro* lung systems will have to combine several, if not all of the technologies discussed above. The advantages of each technique can be combined to increase the level of complexity of the designed tissue/organ analog. Transwells are able to reproduce the stratified organization of the lung from the trachea until the bronchus and simulate the physical barrier between the epithelial and endothelial cells in the alveolar region. The easy incorporation of inserts in the current laboratorial practices makes them widespread. The inserts act as an excellent support material that is often filled with scaffolds or integrated in organ-on-a-chip and at the same time enable essential physiological aspects, e.g. the ALI. Organoids are able to recapitulate lung embryonic development and are able to contain a diverse cell population with near human physiology and function, which makes them a potential tool to deliver cellular units for being integrated on organ-on-a-chip, bioprinted technologies or bioreactors. The major advantage of organ-on-a-chip technologies is the ability to recreate in a small apparatus inter-organ crosstalk, with the association of several microdevices, each representing a tissue. This is essential to evaluate all body response during novel therapeutics. The 3D bioprinted model, despite at its initial stage has showed through the work of Jordan S. Miller group[113] the potential of the technique in recreating in the

Table 5
Summarizes the state-of-the-art literature 3D bioreactors derived *in vitro* models: all lung approach, conducting airways and respiratory airway. HBECs: primary human bronchial epithelial cells, CF: cystic fibrosis, HMEC-1: human dermal microvascular endothelial cell line, SAECs: small airway epithelial cells, IPF: idiopathic pulmonary fibrosis.

Anatomical area	Type	Cells	Matrix	Type of reactor/ Operation mode	Outcome	Ref.
Conducting Airways (trachea → bronchioles	**disease model**	bronchial epithelial cells, FB	type I collagen matrix	dynamic compression reactor (15% strain at 0.1 Hz) of the cell substrate over 3 days	Cyclic strain, as in Bronchoconstriction, may promote transepithelial transport and enhance viral transgene delivery to epithelial and subepithelial cells.	[197]
		Fetal FB, HUVECs, SAECs, iPSCs	collagen I coated alginate beads	rotary commercial Synthecon bioreactor (4-ml HARV)	Model show morphologic scarring typical of IPF, not seen on 2D FB cultures.	[198]
Respiratory airways (alveolar region)	**healthy model**	iPSC-AT2, primary AT2	Collagen IV coated Millipore's Biopore Membrane	rotary reactor (0–100 rpm) exposes half of membrane to the media while the other half is at ALI	iPSC-AT2 and primary AT2 cultured in the reactor had higher levels of type I markers compared with the flask-grown treated with small molecules to induce differentiation.	[199]
	Drug test	Primary pulmonary FB, A549, HMEC-1	Silk collagen I	customized Flexcell commercial system allowing media perfusion and ALI	Model supports myofibroblast differentiation and antifibrotic drug test	[200]
All lung	**healthy model**	healthy/CF HBECs	decellularized murine lung	reactor simulated vascular perfusion (1 ml/min), liquid breathing and gassed every 24 h	at ALI the matrix supported HBECs differentiate into upper and lower airway	[201]
		normal/ immortalized isolated AT2, EC, trachea-bronchus cells	decellularized human adult/ paediatric lung	180°-view plastic aquarium sealed to HDPE plastic plate. 3 pump head enable perfusion of the pulmonary artery and trachea.	Reactor implantation for de/ recellularization shows vascular, alveolar–capillary junction formation, surfactant protein-C /D and collagen I.	[202]
		human EC and perivascular cells derived iPSC, HUVECs	Whole decellularized rat lung/human lobe	Custom-made bioreactor that allow perfusion for de/recellularization	~75% endothelial coverage in the rat lung scaffold relative to that of native lung. Efficient cell delivery, viability and establishment of perusable vascular lumens in human lung lobes.	[203, 204]
		-	Pig decellularized lung	Custom-made bioreactor: lungs are involved in a silicone membrane, contain an artificial diaphragm, negative pressure ventilation (pressure/ volume regulated flow), pulsatile perfusion	Novel system that provide a biomimetic mechanical environment	[205]

laboratory near-anatomy with vasculature integration constructs. The bioreactors are excellent providers of a barrier from the external world during de/recellularization protocols and in this way, giving a second use to unsuited organs for transplantation. This holistic approach is well represented in the work of Taniguchi, Matsumoto [206], where chondrocytes, endothelial cells and mesenchymal stem cells isolated from a rat, were further combined to form spheroids that were used to print in a trachea analog. The construct was maturated in a bioreactor before final implantation in rat [206].

If the aim of *in vitro* models is to one day reduce/replace animal experimentation they will need to be designed to enable both anatomical and physiological conditions, whilst adopting realistic exposure abilities. When investigating new therapeutic compounds to be delivered through the lung or study the potential harm of inhaled particles, the way the compounds reach the lung needs to be fully simulated. Therefore, developments in the *in vitro* models need to go hand-by-hand with advances in exposure systems. With all these additions towards the approach to mimic the physiological conditions as close as possible it is also important to consider the reproducibility within different labs, as the more complicated the model the harder it will be to replicate successfully. Thus, overall, a fine balance between complexity, reproducibility, and organ/tissue relevance is essential.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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