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Review article

hPSC-derived lung organoids: Potential opportunities and challenges

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ABSTRACT

Three-dimensional hPSC-derived lung organoids resemble the fetal lung stage, making them an excellent model for studying human lung development. However, current hPSC-derived lung organoids remain incomplete as they lack native lung components such as vasculature, neurons and immune cells. This highlights the need to generate more complex hPSC-derived lung organoids that can faithfully mimic native human lungs for studying human lung development, regeneration, disease modeling and drug screen. In this review, we will discuss the current studies related to the generation of hPSC-derived lung organoids, highlighting how hPSC-derived lung organoids can contribute to the understanding of human lung development. We further focus on potential approaches to generate more complex hPSC-derived lung organoids containing native cellular components. Finally, we discuss the present limitations and potential applications of hPSC-derived lung organoids in the future.

1. Introduction

The lungs are a very complex organ of tree-like airways and blood vessels that unite at the most distal part, the alveoli, for gas exchange [1,2]. In recent years, generation of human pluripotent stem cell (hPSC)-derived lung epithelial cells have changed from two-dimensional (2D) monolayer cell-based differentiation to three-dimensional (3D) organoid differentiation [1,3–14] (Fig. 1A).

Organoid is a 3D *in vitro* organ-like model system providing the microenvironment for cell-cell and cell-extracellular matrix (ECM) interactions [5,15,16]. Organoids often possess structural organization similar to the native organ and cell types from multiple germ layers, making them a physiologically complex system to investigate organ development, drug screening and disease modeling [5,11,17–19]. However, hPSC-derived lung organoids usually remain incomplete as they lack cellular components found in the native lung, including vasculature, neurons, and immune cells, which play very important roles in organ development, maturation, and function maintenance [20,21] (Fig. 1A).

In this review, we highlight the recent advances in hPSC-derived lung organoids and how these advances provide insights into human lung development. Furthermore, we focus on future challenges for the field that must be overcome in order to establish a better human lung organoid and methods to generate more complex hPSC-derived lung organoids containing native cellular components.

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2. Generation of hPSC-derived lung organoids

Generation of hPSC-derived lung organoids follow embryonic lung developmental cues by temporarily introducing a variety of growth factors and small molecules to sequentially generate definitive endoderm (DE) stage, anterior foregut endoderm (AFE) stage, lung progenitor stage and finally lung organoids (Fig. 1B) [1,3,5,7,9,13,14,22,23].

DE forms during gastrulation and possesses the capability to further give rise to the epithelial lining of the respiratory system and digestive tracts [24]. Studies have shown that high level of Nodal, a member of the transforming growth factor-β family, is the most important signal for high-efficiency induction of pluripotent stem cell (PSC) into DE endowed with the potential to further differentiate into downstream endodermal lineages [24–26]. Activin A has been widely used to mimic the Nodal signaling activity to induce hPSC determination into the DE stage at the concentration of 100 ng/ml [1,5,8,24], and the addition of GSK3β inhibitors such as CHIR-99021 or McX-928 has been shown to be effective in producing DE cells (GSC+/SOX17+/FOXA2^{high}/CXCR+) [1,25,27]. A recent study showed that crotonate could promote a very robust hPSC-derived DE cell differentiation that only needs a low concentration of Activin A (25 ng/ml) [28]. Given the relative high price of Activin A, a combination of low concentration of Activin A and crotonate can make DE cell induction cost less.

During the further development, DE cells form the gut tube which subsequently undergoes a period of regional determination resulting in AFE cells, which is the precursor for further lung bud formation [29,30]. It has been reported that DE cells will further differentiate into AFE cells by dual inhibition of TGF-β and bone morphogenic protein (BMP) signaling [5,31,32]. Human lung development begins at approximately day 28 of gestation and originates from AFE, and is characterized by the expression of thyroid transcription factor-1 (NKX2.1; also known as Titf1) [33,34]. Previous studies have shown that CHIR99021, BMP4, and retinoic acid (RA) were sufficient for the specification of NKX2.1+/FOXA2+ lung progenitors from human AFE cells (SOX2+/FOXA2+/NKX2.1−) [32,35] (Table 1). These NKX2.1+/FOXA2+ lung progenitors were then encapsulated in a 3D extracellular matrix or synthetic hydrogel to provide a 3D growth microenvironment to finally generate lung organoids (Table 1) [1,3,5,13,36].

Several studies identified potential surface markers for NKX2.1+ lung progenitors based on their own differentiation protocol (Table 1). It has been reported that carboxypeptidase M (CPM) is expressed in NKX2.1+ "ventralized" anterior foregut endoderm cells (VAFECs) [35], and the CPM^{high} cells harbor the potential to generate alveolar organoids (Table 1) [7,37]. On the other hand, a cell surface phenotype, CD47^{high}/CD26^{neg}, has been used to prospectively isolate hPSC-derived NKX2.1+ human lung progenitors [38,39] (Table 1). CPM lacks lung specificity and is also detected in the NKX2.1− hepatic cells, which emerge in hPSC-derived lung directed-differentiation protocol [39]. In this regard, it suggests that CD47^{high}/CD26^{neg} can act as a more specific NKX2.1+ lung progenitor marker than CPM during hPSC-derived lung differentiation.

Given the multiple differentiation capabilities of the NKX2.1+ human lung progenitors, such surface markers anticipate that access to pure lung progenitors will facilitate studies on human lung development and regeneration in the future.

3. hPSC-derived lung organoids for human lung development

An extensive literature has demonstrated that hPSC-derived organoids resemble the fetal tissue stage, making them an excellent tool for studying organ developmental events [1,15,22,40–42]. hPSC-derived lung organoids faithfully recapitulate many key processes in human lung development, some of which are unique to humans.

During lung development, the most striking difference between mouse and human bud tip progenitor is in SOX2, the mouse bud tip progenitor cells are SOX2−/SOX9+ throughout lung development [43]. However, human bud tip progenitor cells are SOX2+/SOX9+

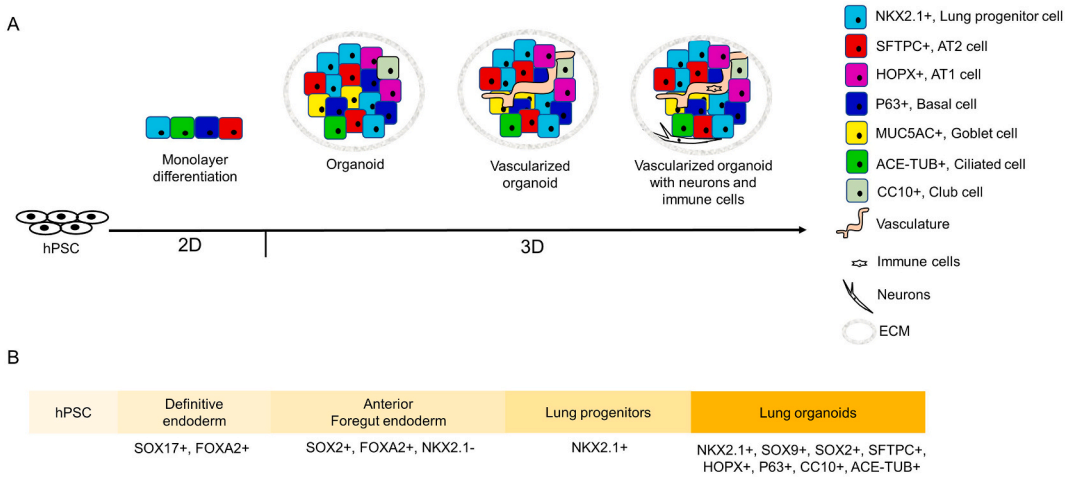

Fig. 1. Overview of hPSC-derived lung organoids. (A) Generation of human pluripotent stem cell (hPSC)-derived lung cells in 2D monolayer cell-based differentiation or in three-dimensional (3D) organoid differentiation. (B) The process of generating hPSCs derived lung organoids consecutively generate definitive endoderm (DE), anterior foregut endoderm (AFE), lung bud progenitor stage, and lung organoid stage.

Table 1
Overview of hPSCs derived lung organoid model systems.

Definitive endoderm	Anterior Foregut endoderm	Lung progenitor	Lung organoid
Activin A, FBS, 4 days	Noggin, SB, SAG, FGF4, CHIR, 5–6 days	FGF7, RA, CHIR, 14 days	Bud tip progenitor organoids, ID2+, NKX2.1+, SOX2+, SOX9+, ProSFTPC+ [3].
Activin A, FBS, 4 days	Noggin, SB, SAG, FGF4, CHIR, 5–6 days	FGF7, RA, CHIR, 14 days	FBS, FGF10, 50–85 days. Lung organoids, NKX2.1+, P63+, Foxj1+, HOPX+, SOX2+, SOX9+, SCGB1A1+, SFTPC+, MUC5AC+ [3].
Activin A, CHIR, 3 days	Noggin, SB, FGF4, CHIR, 4 days	RA, BMP4, CHIR, 6 days, *then* CHIR, FGF10, FGF7, DAPT, 7 days	Dexamethasone, 8-Br-cAMP, IBMX, FGF7, CHIR, SB. Alveolar organoids, NKX2.1+, SOX2+, SOX9+, P63+, Foxj1+, SCGB1A1+, MUC5AC+, SFTPC+, AQP5+ [13].
Activin A, CHIR, 3 days	Noggin, SB, FGF4, CHIR, 4 days	FBS, 14 days	FBS, 20 days. Lung organoids, NKX2.1+, SOX2+, SOX9+, P63+, SCGB1A1+, MUC5AC+, SFTPC+, Hopx+ [1].
Activin A, CHIR, Sodium butyrate, 6 days	Noggin, SB, 4 days	CHIR, BMP4, RA, 4 days, *then* FGF10, 7 days; CPM+ cell sorting	8-Br-cAMP, IBMX, KGF, Dexamethasone, 10–14 days. Alveolar organoids, SFTPA+, SFTPB+, SFTPC+, SFTPD+, AQP5+, PDPN+ [35].
DE KIT, 3 days	Noggin, SB, 3 days (Y-27632 was added for the first 24 h)	CHIR, FGF10, FGF7, BMP4, RA, 9 days	FGF2, FGF10. Proximal airway organoids, P63+, SCGB3A2+, SCGB1A1+, MUC5B+, CFTR+, FOXJ1+, SFTPB. *OR* FGF2, FGF10, CHIR. Distal lung organoids, SFTPC+, ETV5+, ABCA3+, LPCAT1+ [49].
DE KIT, 3 days	Dorsomorphin, SB, 3 days	CHIR, FGF10, FGF7, BMP4, RA, 9 days; $CD47^{high}CD26^{low}$ cell sorting	CHIR, FGF10, FGF7, 7 days *then* CHIR, FGF10, FGF7, Dexamethasone, 8-Br-cAMP, IBMX. Lung organoids, NKX2.1+, MUC1+, SFTPB+, P63+ [39].
EB formation, CXCR4+ Sorting	Noggin, SB, 2 days	WNT3a, FGF10, FGF7, BMP4, EGF, 12 days	CHIR, FGF10, FGF7, BMP4, RA. Lung organoids, NKX2.1+, FOXA2+, SOX9+, SFTPB+, SFTPC+, ABCA3, MUC1+, HOPX+, MUC5AC+, SCGB3A2+ [23].

FBS, Fetal bovine serum; CHIR, CHIR99021; DE, Definitive endoderm; SB, SB431542; EB, Embryoid body; RA, Retinoic Acid.

during the pseudoglandular stage (5–17 post-conception weeks) and tips become SOX2−/SOX9+ during the canalicular stage (16–26 post-conception weeks) [2,43,44]. It has been confirmed that human lung bud tip progenitors (14–16 weeks of human fetal lung) are SOX2+/SOX9+ (Fig. 2A), and hPSC-derived foregut organoids (FOXA2+/NKX2.1+/SOX2+) could differentiate into lung

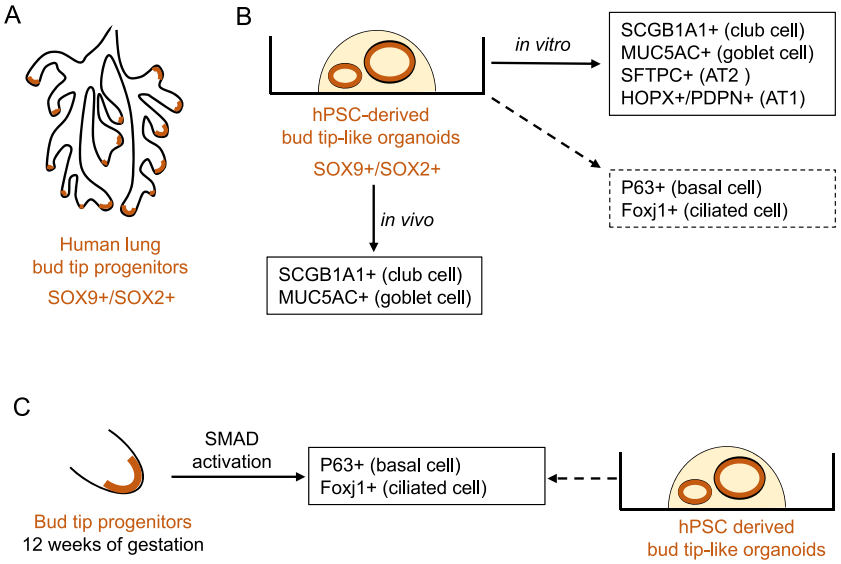

Fig. 2. Application of hPSCs derived lung organoids for lung development. (A) Bud tip progenitors from 14 to 16 weeks of human fetal lung. (B) hPSC derived bud tip-like organoids *in vitro* and *in vivo* differentiation into lung proximal and distal cell types except basal cell and ciliated cell. (C) Generation of basal cell and ciliated cell from human fetal lung bud tip progenitors and hPSC derived bud tip-like organoids.

proximal-like and bud tip-like domains, and this bud tip-like regions could further generate hPSC-derived bud tip-like organoids (SOX2+/SOX9+) (Fig. 2B) [22]. Based on the expression of SOX2 and SOX9 patterning, it suggests that hPSC-derived bud tip-like organoids resemble the human fetal lung at pseudoglandular stage.

In mice, lung bud tip progenitor cells give rise to all lung epithelial cell types [45,46]. hPSC-derived bud tip-like organoids could differentiate into club cell (SCGB1A1+), goblet cell (MUC5AC+/MUC5B+), alveolar type 2 (AT2) cell (SFTPC+/ABCA3+), and alveolar type 1 (AT1) cell (HOPX+/PDPN+) *in vitro*, but the markers of basal cell and ciliated cell were absent (Fig. 2B), and only club cells and goblet cells were observed when hPSC-derived bud tip-like organoids were transplanted into the airways of injured mouse (Fig. 2B) [22]. The basal cells of the human airways are capable of self-renewal and serving as precursors for specialized lung proximal epithelial cell types, including secretory cells and ciliated cells [47,48]. Previous studies revealed that low-Wnt conditions promote hPSC-derived lung progenitors (NKX2.1+) towards lung proximal epithelial progenitors (NKX2.1+/SOX2+) and their downstream basal cells, secretory cells or ciliated cells [12,49]. More recent studies have shown that small mothers against decapentaplegic (SMAD) signaling potently stimulates human fetal lung bud tip progenitors to differentiate into basal cells (Fig. 2C) [45]. The absence of basal cells and ciliated cells indicates that hPSC-derived bud tip-like organoid cells (SOX2+/SOX9+) might possess weaker differentiation capability than mouse bud tip progenitor cells and human fetal lung bud tip progenitors, or modified induction conditions such as low-Wnt or SMAD signaling stimulation are needed to optimize the differentiation capability of hPSC-derived bud tip-like organoid cells.

A possible reason is that current *in vitro* hPSC-derived lung organoid induction system is much simpler than that in native lungs and more complex hPSC-derived lung organoid system is needed in the future.

4. To generate vascularized hPSC-derived lung organoids

Despite hPSC-derived lung organoids tremendous achievements, current hPSC-derived lung organoids are still in its infancy with many limitations in recapitulating of *in vivo* cellular physiology.

On the one hand, hPSC-derived lung organoids do not fully recapitulate the complexity of the native human lung, lacking vasculature, resulting in limited oxygen and nutrient delivery to the inner-most part of organoids. Of note, vascularization of hPSC-derived lung organoids has been restricted to *in vivo* models, whereby hPSC-derived lung organoids are transplanted into highly vascularized regions of immunocompromised mice [1,3,50]. These vascularized hPSC-derived lung organoids could long-term survive and gain some adult structures. These studies suggest that vasculature may play a major role in overcoming the aforementioned limitations of hPSC-derived lung organoids.

The innermost layer of blood vessels is comprised of endothelial cells (ECs) that are heterogeneous in nature [51]. ECs are critical for supplying oxygen and nutrients to cells and tissues, and are involved in coagulation and generation of vasoactive substances,

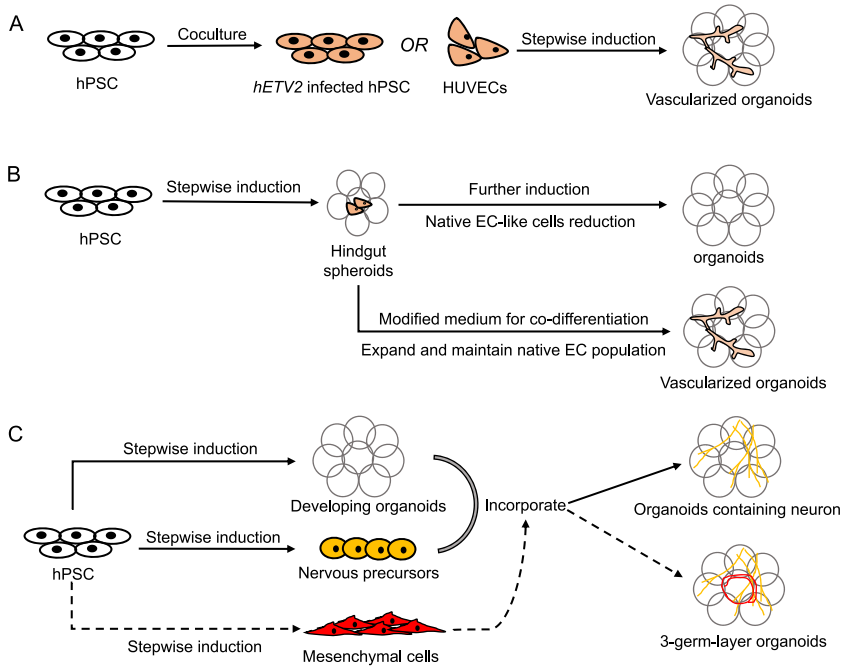

Fig. 3. hPSC-derived organoid containing native cellular components. (A) Generation of vascularized hPSC-derived organoid by coculture HUVECs and hPSCs *in vitro*. (B) Generation of vascularized hPSC-derived organoid by co-differentiation endogenous EC-like cells and developing organoids. (C) Generation of complex 3-germ-layer hPSC-derived gastrointestinal organoids by incorporating hPSC-derived nervous precursors and hPSC-derived mesenchymal cells into developing hPSC-derived gastric organoid.

hormones and growth factors [52]. There are three main lung ECs, pulmonary microvascular, pulmonary artery and pulmonary vein ECs [53]. Lung ECs express angiotensin converting enzyme (ACE), which is involved in regulating blood pressure, and several developmental regulators including osteoglycin and secreted frizzled-related protein 1 (sFRP1) [53,54]. In addition, abundant VEGF expressed by lung ECs maintains normal alveolar structure [55].

In recent years, many studies have tried to generate vascularized hPSC-derived organoids by adopting various methods *in vitro*, and these strategies might be used for generation of vascularized hPSC-derived lung organoids. The first type of strategy to generate vascularized organoids is to coculture with ECs or hPSC-derived EC-like cells (Fig. 3A). For example, Cakir and colleagues engineered hPSCs to ectopically express the transcription factor human ETS variant 2 (*hETV2*) and these ETV2-expressing cells contributed to form a complex vascular-like network in hPSC-derived cortical organoids [56] (Fig. 3A). Shi and colleagues cocultured hPSC with human umbilical vein endothelial cells (HUVECs) *in vitro* to generate a vascularized hPSC-derived cerebral organoids, which recapitulate human cortical development not only in cell type and cellular organization but also in circuit formation [57] (Fig. 3A).

The second type of strategy to generate vascularized organoids is to co-differentiate hPSC-derived ECs or EC-like cells along with the organoid induction (Fig. 3B). It has been reported that a rare population of native EC-like cells are generated during hPSC-derived intestinal organoid induction by single cell RNA sequencing analysis, but these resident EC-like cells are rare and are mostly lost along with hPSC-derived intestinal organoids differentiation [58] (Fig. 3B). Interestingly, such hPSC-derived EC-like cells can co-differentiate with hPSC-derived intestinal organoids under modified culture conditions, resulting in an increase of EC-like cells within intestinal organoids without impacting the other intestinal organoids cell populations induction, and supporting long-term survival of this population of ECs within intestinal organoids [58]. Furthermore, RNA sequencing analysis showed that such population of ECs was similar to the fetal intestine ECs, suggesting that hPSC-derived intestinal organoids possess intrinsic properties sufficient to induce native hPSC-derived EC-like cells to undergo proper organ-specific patterning [58]. It also indicates that the crosstalk of early EC progenitors and organoids play an important role in organ-specific EC differentiation.

Indeed, vascularized organoids can promote the cell survival in the inner-most part of organoids and can be used to study the dynamic EC-organ crosstalk during organ development. Because vasculature is originated from mesoderm, and endogenous EC population within organoids mostly have been reported in hPSC-derived kidney organoids [58–60]. In this regard, whether native EC-like cells are generated during hPSC-derived lung organoids still needs further investigation.

5. To incorporate neurons and immune cells into hPSC-derived lung organoids

On the other hand, current hPSC-derived lung organoids lack neurons and immune cells. Human lungs are one of major barrier tissues of our body, interface with the outer environment, with the responsibilities to face various stimuli, and to maintain homeostasis and integrity under changing conditions [61]. At the early stage of the lung development, a complex neuronal network is laid out [62]. Lungs are well innervated by peripheral nervous system that serves to detect stimuli, to respond and to regulate autonomic functions. Elimination of neural cells result in abolishment of lung branching and diminishment of endothelial and epithelial cell proliferation [1, 63]. Clusters of neuron-like cells have been observed in long-term transplanted hPSC-derived lung organoids, whereby organoids are transplanted under kidney capsule of immunocompromised mice [1]. Interestingly, the expression of protein gene product 9.5 (PGP9.5), a known neuronal marker [64], was mainly restricted in the SOX9+ region (lung distal progenitor marker) [1], which indicates that clusters of neuron-like cells might hold interactions with lung progenitor.

The major obstacle for generating hPSC-derived organoids containing neurons *in vitro* is lack of suitable culture system to meet the requirement for both neuron and organoid development. For example, hPSC-derived intestinal organoids containing neurons *in vitro* can be generated by incorporating hPSC-derived neural precursors into developing intestinal organoids, but incorporating neurons were underdeveloped and without neuroepithelial connections [65,66] (Fig. 3C). A more recent study showed that generation of complex 3-germ-layer hPSC-derived gastrointestinal organoids by incorporating hPSC-derived neural precursors and hPSC-derived mesenchymal cells into developing hPSC-derived gastric organoids, they found that neurons promote gastric mesenchymal development and glandular morphogenesis, and that the adequate amount of mesenchyme is essential for maintaining gastric reginal identity [67] (Fig. 3C). These studies suggest that the interaction of multiple germ layers is essential for survival, development and function of organoids *in vitro*.

It has been reported that several kinds of resident immune cells have been reported in human lungs including dendritic cells, macrophage and innate lymphoid cells [61]. Among different kinds of immune cells in the lungs, alveolar macrophages play an important role as the first line of defense against pathogens via phagocytosis function, cytokine secretion, and antigen presentation to T cells [68,69]. As that a rare population of endogenous EC-like cells are generated during hPSC-derived intestinal organoid induction mentioned above. A recent study showed that alveolar macrophage-like cells (CD68+/CD11C+/CD11B-) were also induced during hPSC-derived lung organoid differentiation, and RNA-Seq data revealed that immune cell-associated genes are expressed at a higher level in hPSC-derived lung organoids containing macrophage-like cells [69].

Collectively, studies mentioned above suggest that EC-like cells, neural precursors, mesenchymal cells, or immune cells might be incorporated into hPSC-derived lung organoids to generate more complex hPSC-derived lung organoids by using co-culture or co-differentiation protocol.

6. Conclusions and perspectives

It is well known that the interactions of different cellular components including mesenchymal cells, epithelial cells, ECM, vascular and neuronal network, promotes parenchymal and stromal cells, cross-communicate via signal transduction to trigger the maturation

of tissues finally enabling organ specific size, shape and function.

Notably, *in vitro* hPSC-derived lung organoid cultures reflect the human fetal lung stage, with adult-like structures or mature cell types generated by transplantation under the vascular sites within the murine host, suggesting that native stromal components mentioned above play a critical role in organoids survival and development. Current hPSC-derived lung organoids remain incomplete as they lack cellular components such as blood vessels, nerves, and immune cells. More studies are needed to investigate how to generate more complex hPSC-derived lung organoids that can faithfully mimic native human lungs, and such complex hPSC-derived lung organoids will be a real mini lung for studying human lung development, regeneration, disease modeling, and drug screen in the future.

Author contribution statement

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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