Cell and Tissue Research (2022) 390:317–333
https://doi.org/10.1007/s00441-022-03686-x

REVIEW

Lung organoids: current strategies for generation and transplantation

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Received: 20 September 2021 / Accepted: 8 September 2022 / Published online: 30 September 2022

Abstract
Lung diseases occupy a leading position in human morbidity and are the third leading cause of death. Often the chronic forms of these diseases do not respond to therapy, so that lung transplantation is the only treatment option. The development of cellular and biotechnologies offers a new solution—the use of lung organoids for transplantation in such patients. Here, we review types of lung organoids, methods of their production and characterization, and experimental works on transplantation in vivo. These results show the promise of work in this direction. Despite the current problems associated with a low degree of cell engraftment, immune response, and insufficient differentiation, we are confident that organoid transplantation will find it is clinical application.

Keywords Lung organoids · Spheroids · Differentiation · Orthotopic transplantation · Ectopic transplantation

Introduction

Chronic respiratory diseases (CRDs) are one of the main causes of disability and death. According to the Global Burden of Diseases, Injuries, and Risk Factors Study, 544.9 million people worldwide had a CRD in 2017, a 39.8% increase from 1990 (Soriano et al. 2020). As stated by WHO, chronic obstructive pulmonary disease (COPD) was the third leading cause of death in 2019, after ischemic heart disease and stroke. By 2040, premature mortality from many noncommunicable diseases, including COPD and lung cancer, will increase by more than 70% by 2040 (Foreman et al. 2018). Oxygen therapy, pulmonary rehabilitation, and pharmacological and surgical treatments (lung volume reduction surgery and lung transplantation) are current strategies for CRD (Keen et al. 2017; Siddiqui et al. 2018). Cell therapy and tissue engineering are modern and actively developing methods for treatment of lung diseases (Geiger et al. 2017; Sun et al. 2018; Kadyk et al. 2017). As of December 2020, ClinicalTrials.gov reported eleven completed clinical trials on transplantation of various types of cells (hematopoietic stem cells, mesenchymal stem cells (MSCs), bone marrow mononuclear cells, bronchial basal cells, and endothelial progenitor cells) for the treatment of lung diseases. Intravenous administration of MSCs was reported to be safe in patients with moderate to severe acute respiratory distress syndrome (ARDS) and COPD (Zheng et al. 2014; Wilson et al. 2015; Matthay et al. 2019; Weiss et al. 2013). Infusion of autologous bone marrow mononuclear cells in patients with COPD was also safe (Stessuk et al. 2013; Ribeiro-Paes et al. 2011). In 2020, a review article was published that summarized clinical data on the feasibility, safety, and tolerability of infusion of MSCs derived from bone marrow or umbilical cord in Severe Acute Respiratory Syndrome, ARDS, and Middle East Respiratory Syndrome (Majolo et al. 2020). Only three stem cell-related clinical trials were considered complete, of which two were in Phase 1 and one was in Phase 2. Cell therapies were shown to cause no complications in gas exchange, spirometry, quality of life, cardiopulmonary circulation, and immune system of those suffering from the lung disease. However, there exist some obstacles such as low mobilization of transplanted MSCs at the site of injury and their low survival rate.

Lung transplantation is often the last chance for patients with various lung conditions. Recently, a new method of transplantation was developed: the lungs undergo decellularization following recellularization (Peloso et al. 2015;

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Tsuchiya et al. 2014; Tebyanian et al. 2019; Calle et al. 2017). Recellularization is carried out using various types of cells, for example, alveolar epithelial cells, endothelial cells, MSCs, embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and other stem/progenitor cells. Currently, decellularization protocols are being standardized and recellularization methods are being optimized.

The use of three-dimensional (3D) cell structures, such as organoids, is a new and dynamically developing direction in cell therapy. Organoids, according to Huch and Koo, are defined as a 3D structure derived from either PSCs, neonatal tissue stem cells, or adult progenitor cells, in which cells spontaneously self-organize into properly differentiated functional cell types and progenitors, and which resemble their in vivo counterparts and recapitulate at least some of the functions of the organ (Huch et al. 2015). Organoids are used for study of intercellular interactions, disease modeling, drug screening, and regenerative medicine (Nikolić et al. 2017b; Kim et al. 2020; Bartfeld et al. 2017). Lung organoids recapitulate lung development and may serve as a useful tool for modeling lung disease (Chen et al. 2017a, b). In this review, we shall discuss the main type of lung organoids and how to obtain them. We shall also focus on in vivo lung organoid transplantation studies to analyze the viability, engraftment, and maturation of organoids, as well as the effectiveness of the treatment of pulmonary diseases.

Types of lung organoids

The respiratory system consists of the upper and lower respiratory tracts. The upper respiratory tract comprises the nasal cavity, pharynx, and larynx. The lower respiratory tract consists of the trachea, primary bronchi leading to the bronchioles, and alveoli. The epithelium in the lower respiratory tract is mainly composed of basal cells, goblet cells producing mucus, and ciliated cells required for mucociliary clearance (Chang et al. 2008; Klein et al. 2011). In the bronchioles, the epithelium consists of more cuboidal-shaped cells with shorter cilia and secretory club cells; in the alveoli, the epithelium consists of alveolar type I and II cells. Alveolar type II cells perform the functions of surfactant producers or differentiate into alveolar type I cells. Thus, the most common classification of lung organoids is based on the cell types present in organoids (Fig. 1):

- airway organoids (include tracheospheres, bronchospheres, and nasospheres) consisting of ciliated, goblet, basal, club, tuft, and pulmonary neuroendocrine cells;
- alveolar organoids consisting of alveolar type I and II cells;
- lung organoids consisting of cell types characteristic of both airway and alveolar organoids.

Airway, alveolar, and lung organoids can be obtained from both adult cells and pluripotent stem cells, including ESCs and iPSCs (Table 1).

Airway organoids

Airway organoids derived from adult cells are often classified according to the location of the biopsy used to isolate these cells, namely, tracheospheres, bronchospheres, and nasospheres. Such organoids are characterized by the expression of markers of basal cells (KRT8 +, KRT14 +, and p63 +), ciliated cells (AC-TUB + and FOXJ1 +), mucosecretory or goblet cells (MUC5AC + and MUC5B +), and club cells (CC10 +) (Rock et al. 2009; Lee et al. 2020; Tesei et al. 2009; Kumar et al. 2011).

All protocols for obtaining airway organoids from adult cells are similar to each other with some variations (Fig. 2). The first protocol for obtaining mouse tracheospheres was published in 2009 and involved mechanical separation and enzymatic digestion of a piece of mouse trachea, fluorescence-activated cell sorting (FACS), 1:1 mixing with Matrigel and further cultivating on the Transwell inserts at the air–liquid interface (ALI) for 26 days (Rock et al. 2009). The ALI-culture method consists of seeding cells onto a permeable membrane of a cell culture insert, with the basal side of the cells in contact with liquid culture medium, whereas the apical side is exposed to air. This initiates the differentiation of cells into a mature polarized pseudo-stratified epithelium consisting of functional basal, ciliated, and secretory cells (Choi et al. 2020; Kumar et al. 2011; Usui et al. 2000). Another method called culturing self-assembled spheres (SAS) uses seeding of cells after their digestion onto ultra-low attachment plates (Tesei et al. 2009). With nasal spheroids, ALI cultures were shown to stratify into basal cells and suprabasal differentiated cells, while SAS cultures remain a monolayer of ciliated and goblet cells and lack basal cells (Kumar et al. 2011). And one of the most popular methods of organoid cultivation is the use of the extracellular matrix. One of the most common matrices for cultivation is Matrigel. Matrigel is xenogenic; it is derived from the basement membrane matrix secreted by Engelbreth–Holm–Swarm mouse sarcoma cells. Matrigel is extremely complex: it contains more than 1,800 unique proteins (Hughes et al. 2010). The concentrations of growth factors and mechanical and biochemical properties may vary from batch to batch in Matrigel, which may lead to uncertainty in cell culture experiments and lack of reproducibility (Aisenbreyet al. 2020). Many synthetic matrices based on polyacrylamide and polyethylene glycol, as well as natural matrices based on decellularized tissues, are currently being developed (Aisenbreyet al. 2020; Kozlowski et al. 2021).

Airway organoids from embryonic stem cells are obtained in the same way as from adult cells. Nikolić M. Z. et al.

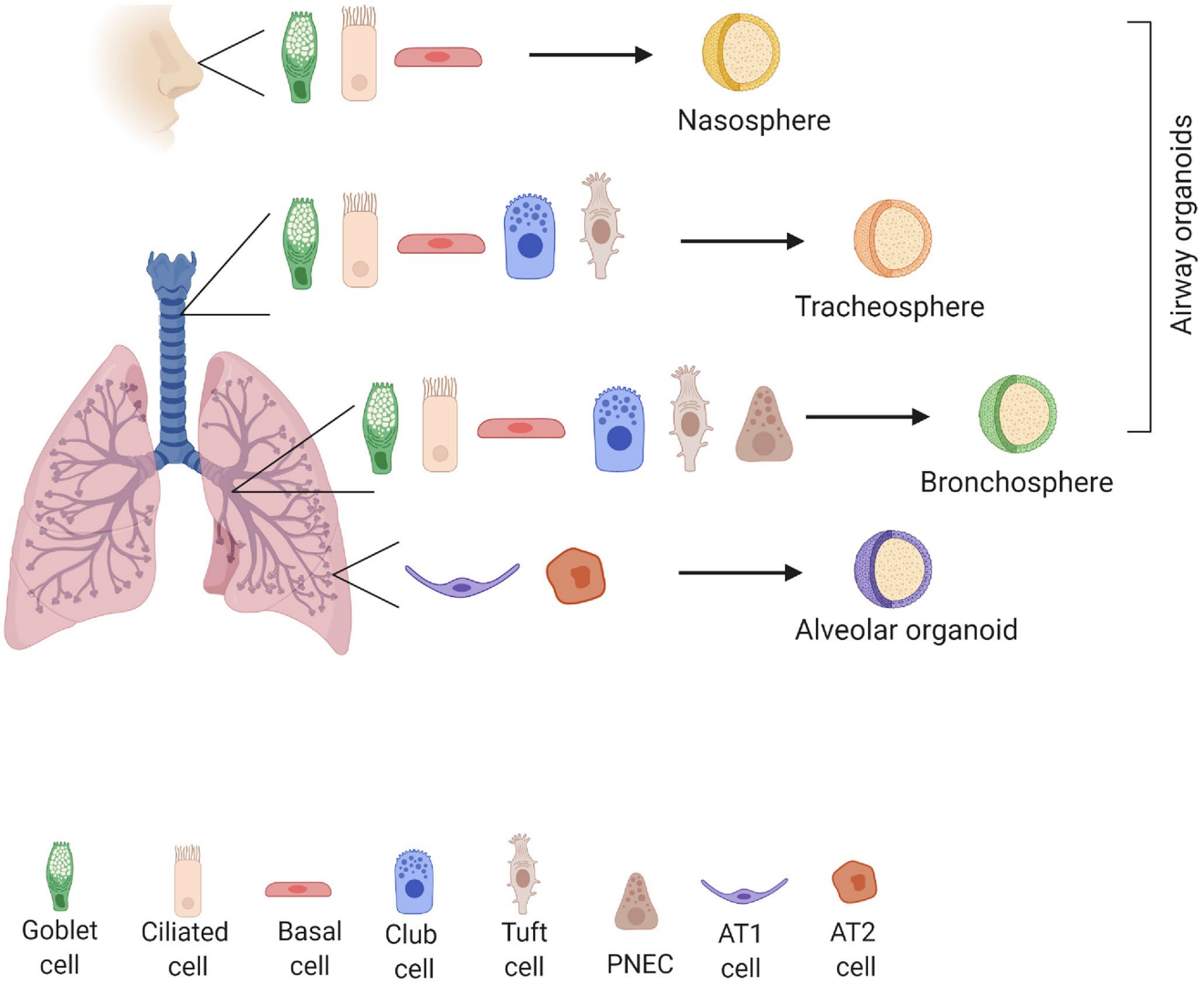

Fig. 1 Types of lung organoids. PNEC—pulmonary neuroendocrine cells, AT1—alveolar type 1 cells, AT2—alveolar type 2 cells. All figures are created with BioRender.com

showed that human embryonic lung distal tip epithelium comprises a multipotent progenitor cell population with the capacity of self-renewal and differentiation (Nikolić et al. 2017a). Human lung epithelial tips from 5 to 9 weeks post-conception were microdissected, digested, mixed with Matrigel, and transferred into a low-attachment plate. Then, the tip-derived organoids were differentiated into bronchiolar and alveolar lineages. The bronchiolar organoids at high passages contained mostly goblet cells (MUC5AC+), while at lower passages, the organoids contained basal cells (KRT5+).

The development of the lungs consists of the following stages: embryonic (appearance of the definitive and anterior foregut endoderm), pseudoglandular (formation of the bronchial tree), canalicular (expansion of the respiratory tree), saccular (specification of the alveolar epithelium), and alveolar (maturation of the alveoli and microvessels). During embryogenesis, the definitive endoderm (DE) develops and transforms into a gut tube located along the anterior–posterior and dorsal–ventral axes (Zorn et al. 2009). As the development proceeds, the anterior foregut endoderm (AFE) is formed, which gives rise to the esophagus, trachea, stomach, lungs, thyroid, liver, biliary system, and pancreas. Then, lung specification begins with the expression of the thyroid transcription factor-1 (NKX2.1) on the ventral side of the AFE (Goss et al. 2009). Thus, obtaining airway organoids from iPSCs includes differentiation into DE, then into AFE, and finally into NKX2-1+lung epithelial progenitors which are purified by sorting cell surface markers and cultured in Matrigel with WNT signaling inhibitors to form 3D epithelial-only airway organoids, since McCauley K. B. et al. showed that the inhibition of WNT signaling efficiently induces proximal lung progenitors, while

Table 1 Main studies on generation of lung organoids

Organoid types	Cell sources	Methods of generation	Cell types in the organoids (day from start of differentiation)	Expressed markers	References
Adult cells					
Nasospheres	Human nasal epithelial stem cells	SAS	Ciliated cells (15d) Goblet cells (15d) Squamous epithelial cells (21d)	AC-TUB + MUC5A + KRT10 +	Kumar et al. (2011)
		Matrigel	Squamous epithelial cells (21d)	Involucrin +, KRT10 +	
Tracheobronchial spheres	Human tracheal airway stem cells	Matrigel	Squamous epithelial cells (21d)	KRT10 +	
Distal airway spheres	Human distal airway stem cells	Matrigel	Alveolar type II cells (21d) Alveolar cells (21d)	PDPN + 4C10 +	
Bronchospheres	Human bronchial epithelial cells	Matrigel	Basal cells (0–14d) Goblet cells (5d, 7d) Ciliated cells (7d)	p63 + MUC5AC +, MUC5B +, FOXA3 + FOXJ1 +, DNAI2 +	Hild et al. (2016)
	Human lung cells	SAS	Mesenchymal stem cells (n/a) Epithelial cells (n/a) Basal cells (n/a) Alveolar type II cells (n/a) Club cells (n/a)	CD90 +, CD105 + KRT8 +, KRT18 +, KRT19 + KRT5 + SP-A + CC10 +	Tesei et al. (2009)
Tracheospheres	Mouse tracheal basal cells	Matrigel	Basal cells (9d) Luminal cells (9d) Ciliated cells (20d)	p63 +, KRT14 + KRT8 + AC-TUB +	Rock et al. (2009)
Alveolospheres	Human alveolar type 2 cells combined with human fetal lung fibroblast cell line	Matrigel and Transwell insert	Alveolar type II cells (14d)	SFTPC +, HTII-280 +	Barkauskas et al. (2013)
	Mouse alveolar type 2 cells combined with mesenchymal cell populations		Alveolar type II cells (16–17d) Alveolar type I cells (16–17d)	SFTPC +, T1a + AQP5 +, HOPX +	
Alveolar organoids	Human alveolar type II cells (HTII-280 +) or alveolar epithelial progenitor cells (HTII-280 +, TM4SF1 +) combined with human fetal lung fibroblast cell line	Matrigel and Transwell insert	Alveolar type II cells (21d) Alveolar type I cells (21d)	SFTPC + AQP5 +	Zacharias et al. (2018)
Lung organoids	Human lung cells	Matrigel	Alveolar type II cells (n/a) Basal cells (n/a) Ciliated cells (n/a) Goblet cells (n/a) Club cells (n/a)	SFTPB +, SFTPC + KRT5 + AC-TUB + MUC5AC + CC10 +	Tindle et al. (2021)
Embryonic cells					
Alveolospheres	Human embryonic stem cells	Matrigel	Lung epithelial cells (30d) Alveolar type II cells (30d)	NKX2.1 + proSFTPB +, proSFTPC +	Jacob et al. (2017)

Table 1 (continued)

Organoid types	Cell sources	Methods of generation	Cell types in the organoids (day from start of differentiation)	Expressed markers	References
Alveolar organoids	Mouse fetal epithelial tips	Matrigel	Proximal epithelial cells (0–6d) Distal epithelial cells (6d) Ciliated cells (6d) Basal cells (6d) Alveolar cells (2–6d)	SOX2 + SOX9 + FOXJ1 + KRT5 + SFTPC +, RAGE +	Gkatzis et al. (2021)
Lung epithelial tip organoids	Human embryonic lung distal tip cells	Matrigel	Bronchiolar cells (70–98d) Lung epithelial tip cells (70–98d)	SOX2 + SOX9 +, HMGA2 +, ETV5 +, HNF1B +	Nikolic et al. (2017a)
Lung epithelial stalk organoids	Human embryonic lung epithelial stalk cells		Bronchiolar cells (42d) Lung epithelial tip cells (42d)	SOX2 + SOX9 +	
Lung bud organoids	Human embryonic lung epithelial cells	SAS/ Matrigel	Mesodermal cells (25d) Lung epithelial cells (25d) Alveolar type II cells (70d and > 170d) Goblet cells (70d) Proximal epithelial cells (70d and > 170d) Distal epithelial cells (70d and > 170d)	PDGFR + KRT8 +, NKX2.1 +, FOXA1 +, P63 + SFTPC +, SFTPB +, ABCA3 + MUC5B +, MUC5AC + SOX2 + SOX9 +	Chen et al. (2017a, b)
Lung organoids	Rat fetal distal lung epithelial cells combined with CD31 + rat endothelial cells	Matrigel and ALI-culture	Alveolar type II cells (15d) Club cells (15d) Epithelial cells (15d)	RT2-70 + CC10 + EPCAM +	Laube et al. (2021)
iPSCs					
Airway organoids	Human iPSCs	Matrigel	Secretory cells Goblet cells Basal cells	SP-B + /NKX2.1 + ; SCGB3A2 + / NKX2.1 + MUC5AC + / NKX2.1 − NKX2.1 + /P63 + / KRT5 +	McCauley et al. (2017) McCauley et al. (2018)
Alveolospheres	Human iPSCs	Matrigel	Alveolar type II cells (n/a) Lung epithelial cells (n/a)	proSFTPB + NKX2.1 +	Jacob et al. (2017)
Alveolospheres	Human iPSCs combined with human fetal lung fibroblasts	Matrigel and Transwell insert	Lung epithelial cells (n/a) Alveolar type I cells (n/a) Alveolar type II cells (n/a)	NKX2.1 + AQP5 + /PDPN + SFTPC +, SFTPB +	Gotoh et al. (2014) Yamamoto et al. (2017)

Table 1 (continued)

Organoid types	Cell sources	Methods of generation	Cell types in the organoids (day from start of differentiation)	Expressed markers	References
Alveolar organoids	Human pluripotent stem cells	SAS	Alveolar progenitor cells (27d) Alveolar type I cells (27d) Alveolar type II cells (27d) Mesenchymal cells (27d)	EPCAM +, CPM +, NKX2.1 + AQP5 +, T1α + SFTPC + Vimentin +	Kim et al. (2021)
Lung bud organoids	Human iPSCs	SAS/ Matrigel	Lung epithelial cells (70d) Alveolar type II cells (70d and > 170d) Proximal epithelial cells (70d and > 170d) Distal epithelial cells (70d and > 170d)	NKX2.1 + SFTPC +, SFTPB + SOX2 + SOX9 +	Chen. (2017a)
Lung organoids	Human iPSCs	Matrigel	Proximal epithelial cells (2, 6, and 16 weeks) Distal epithelial cells (n/a) Club cells (n/a) Goblet cells (n/a)	NKX2.1 +, SOX2 + SOX9 + SCGB1A1 + MUC5AC +	Miller et al. (2018)
	Human iPSCs	Matrigel	Basal cells (n/a) Club cells (n/a) Goblet cells (n/a) Alveolar cells (n/a)	KRT5 + SCGB3A2 + MUC5AC + SP-C +, SP-B +, HTII-280 +, AGER +	Leibel et al. (2020)
	Human iPSCs	Matrigel	Proximal epithelial cells (15d) Basal cells (65d) Distal epithelial cells (15d, 65d) Ciliated cells (65d) Club cells (65d) Alveolar type I cells (65d) Alveolar type II cells (65d)	SOX2 + P63 + SOX9 + FOXJ1 +, AC-TUB + CC10 + PDPN +, HOPX + SFTPB +, SFTPC +	Dye et al. (2015)

ABCA3, ATP-binding cassette, sub-family A; *AC-TUB*, acetylated tubulin; *AGER*, advanced glycosylation end product-specific receptor; *AQP*, aquaporin; *CC10*, Club cell 10kD protein; *CD*, cluster of differentiation; *d*, day; *DNAI*, dynein intermediate chain 1; *EPCAM*, epithelial cell adhesion molecule; *ETV5*, ETS variant gene 5; *ETS*, ERM transcription factor; *FOXA2*, forkhead box protein A2; *FOXJ*, forkhead-box J1; *HMGA2*, high mobility group AT-hook protein 2; *HNF1B*, hepatocyte nuclear factor 1; *HOPX*, homeodomain-only protein; *HTII-280*, a 280–300 kDa protein specific for human alveolar type II cells; *KRT*, keratin; *MUC5AC*, mucin-5 subtype AC; *MUC5B*, mucin-5 subtype B; *NKX2.1*, NK2 homeobox 1; *p63*, transformation-related protein 63; *PDGFR*, alpha Platelet-Derived Growth Factor receptor; *PDPN*, podoplanin; *proSFTPB*, pro–surfactant protein B; *RAGE*, advanced glycosylation end product-specific receptor; *SAS*, self-assembled spheres; *SCGB3A2*, secretoglobin family 3A member 2; SFTPC, pulmonary-associated surfactant protein C; *SOX*, SRY (sex determining region Y)-box; *SP-A*, surfactant protein A; *SP-B*, surfactant protein B; *n/a*, not available

the activation of WNT leads to a significant increase in distal patterning (McCauley et al. 2017, 2018). The airway organoids obtained using this protocol contain all cells characteristic of this type of organoids (basal, ciliated, goblet, and club cells). Figure 3 summarizes different protocols for differentiation of iPSCs into airway, alveolar, and lung organoids.

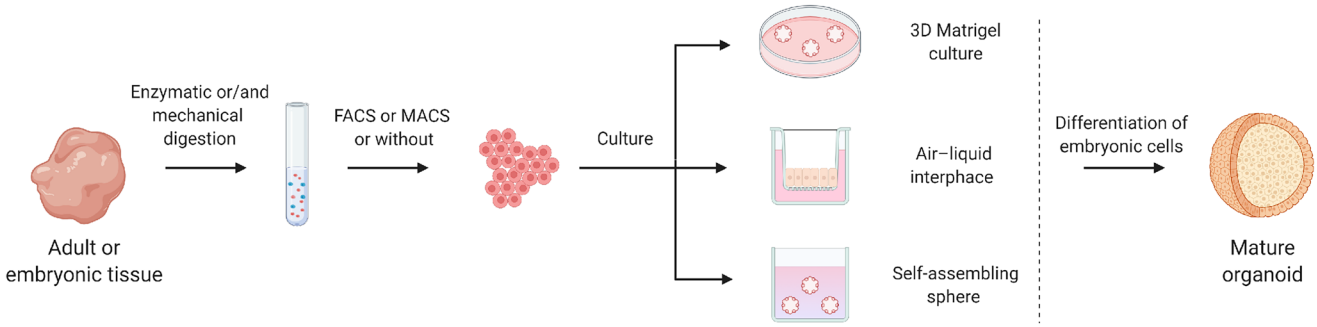

Fig. 2 Scheme for obtaining lung organoids from adult and embryonic cells. FACS—fluorescence-activated cell sorting, MACS—magnetic-activated cell sorting, 3D—three-dimensional

Alveolar organoids

Alveolar organoids can be derived from adult tissue, embryonic progenitors, and induced pluripotent stem cells; they are characterized by the expression of markers of AT2 cells (SFTPC+, ProSPC+, AQP5+, HT2-280+) and alveolar type 1 (AT1) cells. In research, AT2 cells are isolated from adult or embryonic tissues, since these cells behave as facultative stem cells, they are capable of self-renewal and differentiation into AT1 cells (Evans et al. 2020). For isolation of AT2 cells, it is necessary to enzymatically digest and mechanically dissociate whole lungs or transbronchial samples (Barkauskas et al. 2013, 2017; Dinh et al. 2017; Nikolić et al. 2017a; Zacharias et al. 2018). Dissociated AT2 cells are isolated by FACS (EPCAM+, HT2-280+or TM4SF1-, APC+), mixed with fibroblasts and Matrigel and ALI cultured (Zacharias et al. 2018). Dinh P. U. C. suggested an alternative protocol according to which tissue explants are cultured on a fibronectin-coated plate for 17–25 days (Dinh et al. 2017). After that, cells are seeded into an ultra-low attachment flask and in 5–7 days reseeded onto fibronectin-coated surfaces to produce alveolar organoids. Recent studies showed that alveolar organoids derived from both murine and human AT2 cells are capable of differentiating into AT1 cells in vitro (Chen et al. 2021; Katsura et al. 2020). Obtaining alveolar organoids containing AT1 and AT2 cells from iPSCs is reduced to the differentiation of NKX2.1+cells using a combination of CHIR99021, FGF10, KGF, and DAPT, which are plated in Matrigel and cultured with or without lung fibroblasts. Co-cultivation with fibroblasts proved to be advantageous for the induction and stable expansion of SFTPC+cell populations while maintaining their stem cell properties, which suggests that the niche provided by epithelial–mesenchymal interaction may be crucial for maintaining the progenitor properties of AT2 cells (Yamamoto et al. 2017; Kim et al. 2021).

Lung organoids

Lung organoids can also be derived from adult tissue, embryonic progenitors, induced pluripotent stem cells; they contain proximal and distal lung cells, such as basal, ciliated, club, AT1, and AT2 cells. Obtaining lung organoids from adult tissue consists of enzymatic digestion and 3D culturing (Nikolić et al. 2017a). Both for organoids derived from iPSCs and for organoids derived from embryonic cells, a similar protocol of differentiation into lung organoids is employed (Leibel et al. 2020). Leibel et al. described a protocol for the production of human lung organoids (HLOs) from PSCs by endoderm induction using activin A and CHIR99021, followed by induction of anterior foregut endoderm using inhibition of BMP and TGFβ signaling by SB431542 and dorsomorphin (Leibel et al. 2020). The anterior foregut cells were then cultured with BMP4, CHIR99021, and all-*trans* retinoic acid (ATRA). At the last stage, the cells were placed in Matrigel with FGF7, FGF10, EGF, and CHIR99021 to form organoids. The resulting organoids expressed airway (KRT5+, MUC5AC+, SCGB3A2+) and alveolar (SP-C+, SP-B+, HTII-280+, AGER+) markers. Dye et al. described a similar protocol for obtaining lung organoids from iPSCs, however, the authors obtained anterior foregut spheroids which were then placed in Matrigel with Noggin, SB431542, FGF4, CHIR99021, and 1% fetal bovine serum for maturation of lung organoids (Dye et al. 2015). The resulting organoids expressed proximal (SOX2+) and distal (SOX9+, HOPX+, SFTPC+) lung markers. HLOs cultured for more than 2 months had epithelial structures resembling the proximal and distal airways and expressing markers of basal (P63+), ciliated (FOXJ1+, AC-TUB+), club (SCGB1A1+), AT2 (SFTPC+, SFTPB+), and AT1 (PDPN+, HOPX+) cells.

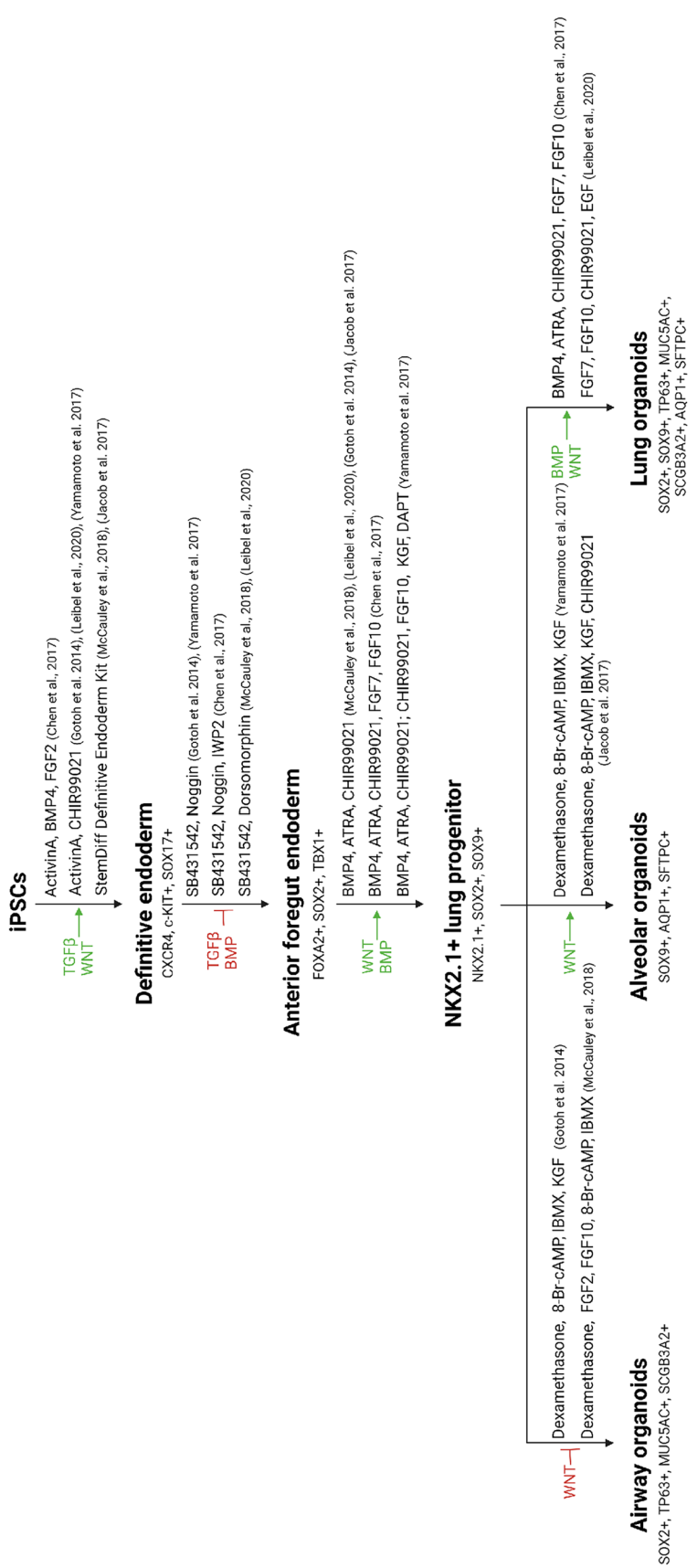

Fig. 3 Schemes of differentiation iPSCs into airway, alveolar, and lung organoids

Transplantation of lung organoids for maturation

Almost all of the above described organoids that were obtained in vitro have incomplete differentiation so far as they are not exposed to air (Schilders et al. 2016). Therefore, in some of the studies, the last stage of differentiation is transplantation which promotes cell maturation and formation of vascular and neuronal networks (Tan et al. 2017; Chen et al. 2018). It is also worth noting that «organoid transplantation» refers to both the transplantation of whole organoids and the transplantation of cell suspensions derived from organoids.

The most common in vivo approach is xenotransplantation, in which human organoids are transplanted into laboratory animals. Many studies showed that human lung organoids can be grafted to mammalian hosts. Dinh et al. successfully engrafted human lung spheroids into athymic nude mice; on days 1, 4, 7, 11, and 20 after transplantation, cells were found in the lungs and liver (Dinh et al. 2017). The cells expressed alveolar (AQP5 + and ProSPC +), secretory (CCSP +), and epithelial (EPCAM +) markers in the lung tissue, while they were absent in the liver. Thus, the authors showed that lung spheroid cells engrafted into mammalian hosts retain lung phenotype in orthotopic transplantation, in contrast to ectopic transplantation.

Orthotopic transplantation has important advantages, such as providing the best environment for cell survival and function, allowing to assess vascularization and neurogenesis, increasing the translational potential of organoid models, etc. However, in most cases, lung organoids are transplanted into an ectopic site, since this opens up the possibility of choosing a site with good access to blood supply and maintenance of vascularization of the grafted tissue without impairing the necessary functions of mammalian organ (Deward et al. 2014; Holloway et al. 2019). The site of transplantation was shown to have no effect on the engraftment of organoids (Dye et al. 2016). The authors transplanted HLOs grown in vitro from 1 to 65 days into the subcapsular pocket of the mouse kidney and into the greater omentum. They showed that alien sites of ectopic localization do not reliably support the survival or growth of the HLO lung epithelium in vivo.

Unlike ectopic localization, orthotopic graft localization provides the best environment for the cell and plays an important role in the maturation and differentiation of organoids. Chen et al. hypothesized that ectopic localization prevents organoids from definitive differentiation (Chen et al. 2017b). Human lung bud organoids (LBOs) were transplanted under the kidney capsule into immunodeficient NSG mice. After 7 months, LBOs were found at the transplantation site, but full phenotypic and architectural alveolar maturation was not achieved. However, (Nikolić et al. 2017a) showed (also in 2017) that 8 days after orthotopic in vivo transplantation of lung tip organoids (SOX2 +, SOX9 +) into NGS mice, cells retained the co-expression of SOX2 and SOX9, which means incomplete airway differentiation (Nikolić et al. 2017a). This result can be explained by the short observation period. The authors then performed an ectopic transplantation of lung tip organoids that were dissociated and mixed with a cell pellet formed from dissociated E13.5 mouse lungs under the kidney capsule of NSG mice. After 12 weeks, only some human cells expressed SOX9 at a very low level; thus, alveolar differentiation was either premature or ineffective. The authors concluded that human cells may be unable to respond efficiently to mouse differentiation signals, possibly because they require different signaling inputs.

As mentioned above, transplantation is in most cases considered the last stage in the differentiation of lung organoids, since lung organoids differentiate better in vivo than in vitro. The authors compared the efficiency of differentiation of HLOs grown in vitro and HLOs seeded onto a bioartificial microporous poly(lactide-co-glycolide) (PLG) scaffold, cultured for 5–7 days in vitro and transplanted into epididymal fat pads of mice (Dye et al. 2016). The use of PLG scaffold with HLOs demonstrated that 8 weeks after transplantation 100% of the recovered constructs possessed huMITO + and NKX2.1 + mature airway-like structures, while in the control group (HLOs in a Matrigel plug), no tissue recovery was observed. In addition, 8 weeks after transplantation, multiple epithelial structures per cross section were observed. The identified airway-like structures were densely surrounded by mesenchymal cells and there were pockets of organized cartilage throughout the transplants, while HLOs seeded on the scaffold and grown in vitro for 4–8 weeks did not represent epithelial structure. Therefore, the authors concluded that the scaffold provides critical support for the engraftment and survival of the lung epithelium and the combination of the scaffold and the in vivo environment ensures the growth and maturation of the HLO epithelium. Later, in a 2019 study, the authors showed that the rate of scaffold degradation affects the HLO maturation—rapidly degrading polymers lead to an increase in the size of airway structures (Dye et al. 2020). The use of scaffolds in research is advancing the field of regenerative medicine, where matrices play an important role and should provide a stable and supportive vehicle to deliver cells to the desired location in vivo (Aisenbrey et al. 2020). Matrigel, due to its animal origin, makes it difficult to use it in the future in human transplantation in clinic due to potential immunogenicity (Schneeberger et al. 2017; Kozlowski et al. 2021). therefore, the creation of suitable matrices is an important direction for future research.

It was also shown that the stage of transplanted HLO culture did not affect the survival of the HLO lung epithelium (Dye et al. 2016). However, in (Chen et al. 2018)

transplanted HLOs at different stages of cultivation into the subcapsular pocket of the kidney of immunodeficient B-NSG mice and showed that it is better to use 41-day HLOs to obtain mature AT1-like cells and AT2 cells after long-term transplantation, since at this time the expression of the genes of distal cells of the alveolar epithelium reaches maximum. Earlier, on days 21 and 31 of culturing HLOs in vitro, there is a peak in the expression of the genes specific for lung progenitors and stem cells (NKX2.1, SOX9, SOX2, and P63) and transplanted HLOs can differentiate into bipotent progenitor cells after long-term engraftment (Chen et al. 2018). The authors also showed that 100–120 days after transplantation HLOs possessed a vascular network (ACTA2 +) and a neuronal network (PGP9.5 +), resembling the human lung.

The issue of vascularization is important in transplantation, since it is critical to ensure the distribution of oxygen and nutrients in large organoids in vivo and to provide the integration of airway organoid grafts with the host tissue (Tan et al. 2017; Vargas-Valderrama et al. 2020). In (Tan et al. 2017) obtained multicellular airway organoids containing vascular structures by combining human primary adult bronchial epithelial cells with human primary adult microvascular lung endothelial cells and human primary adult lung fibroblasts at a ratio of 10:7:2; they were transplanted under the kidney capsule of NSG mice (Tan et al. 2017). One week after implantation, organoids were visible in the kidney capsule, with human specific CD31 + endothelial cells within airway organoids. However, staining with specific anti-human antibodies demonstrated that the vast majority of proliferating cells was not within airway organoids, but rather within host tissue. Six weeks after implantation, the organoids regressed in size, abundant host vasculature invaded the organoid area where proximal secretory airway cells (CC10 +) and distal alveolar cells (AQP5 + and SPC +) were observed. Thus, the authors showed that bronchial epithelial, mesenchymal, and vascular cells in the airway organoids can survive and undergo significant maturation after engraftment in vivo; however, ectopic transplantation, as mentioned earlier, prompted a shift towards cell lineage commitment and differentiation towards mature, non-proliferating states, limiting the regenerative potential of the current system.

Transplantation of lung organoids for repair of lung injury

Model of lung injury in mice can be created by direct damage to the bronchoalveolar epithelium and capillary endothelium due to intratracheal administration of acid or bleomycin, prolonged hyperoxia, prolonged mechanical ventilation at high tidal volume, or intravenous injection of oleic acid or endotoxin (lipopolysaccharide) (Aeffner et al. 2015). Ischemia/reperfusion models, sepsis models, influenza virus models, and secondary peritonitis models can also be used. 'Smoking mouse' models accurately reflect the pathophysiology of COPD, since cigarette smoke is the main cause of this disease (Vlahos et al. 2014). There are several works on transplantation of lung organoids into the area of lung injury (Nikolić et al. 2017a; Miller et al. 2018; Weiner et al. 2019). In 2015, sublethal whole-body irradiation of the recipient before the transplantation of cells was developed (Rosen et al. 2015). Sublethal irradiation was performed 48 h after naphthalene treatment to clear the precursor niches in the lungs, reduce the competition of stem cells and improve engraftment of infused donor cells. It was shown that exposure to irradiation before treatment with naphthalene did not lead to effective destruction of the endogenous pool of precursors. Although the authors transplanted human fetal lung tissue and not organoids, we consider this study to be a key publication on the long-term engraftment of transplanted lung cells and organoids. The need for irradiation to engraft the lungs with transplanted cells is the most significant obstacle for the clinical development and implementation of this approach. The search for other ways to eliminate recipient's own stem cells in the appropriate niches is necessary for the development of organoid transplantation (Table 2). Although numerous studies show that irradiation is not necessary, the efficiency of cell engraftment after irradiation is significantly improved.

In addition to the competition of stem cells, there is the problem of immune rejection in allotransplantation, which is usually solved by chronic suppression of the immune system. Hillel-Karniel et al. developed a protocol that allowed efficient transplantation across major genetic barriers by co-infusion of T cell-depleted hematopoietic progenitor cells together with lung cells and treatment with cyclophosphamide after transplantation (Hillel-Karniel et al. 2020). On the other hand, obtaining organoids from donor's own iPSCs eliminates the problem of immune rejection, which is a great advantage.

An analysis of the literature shows that human organoids can be transplanted into mice, and after a few days or weeks human cells can be detected. In 2018, transplantation of bud tip progenitor organoids derived from hPSCs into the airways of injured mouse lungs was performed (Miller et al. 2018). A short-term engraftment experiment showed that 79% of the cells expressed the markers SOX2 and SOX9, half of the cells expressed the club cell marker SCGB1A1 and a quarter of the cells expressed the goblet cell marker MUC5AC; no other cell markers were observed. Three groups of mice were involved in the long-term engraftment experiment: (a) received an injury but no injection of cells; (b) received an injury and injection of undifferentiated hPSCs; and (c) received an injury and injection of bud tip organoid cells. Lungs in animals of all experimental

Table 2 Current research on organoid transplantation in vivo

Organoid types	Cell sources	Cell types in the organoids	Transplantation sites/method of introduction	Pre-transplantation processing	Duration	Outcomes	Number of objects	References
Mouse AT2 organoids	Mouse CD45 − EPCAM + β4 − AT2 cells	Cells expressing SPC and Lamp3 markers	Nostrils of influenza-injured recipient mice 11 days post infection	Dissociation and transplantation of 20×10^3—150×10^3 cells	13 days	AT2 organoids either maintain AT2 lineage or express markers of dysplastic regeneration. Recovery of mice with AT2 organoids did not differ from the control	3	Weiner et al. (2019)
Multi-lineage human airway organoids	Human bronchial epithelial cells and human microvascular lung endothelial cells	Bronchial epithelial cells, microvascular lung endothelial cells, and lung fibroblasts	Kidney capsule of NSG mice	-	1 week	Visible organoids. Presence of human CD31 + cells	4	Tan et al. (2017)
					6 weeks	Regression of organoids in size. Presence of host vasculature in the organoid area. expression of proximal secretory airway and distal alveolar epithelial markers		
Embryonic mouse lung organoids	Mouse fetal lung cells	Proximal and distal epithelial cells	Renal capsules of GFP-expressing syngeneic C57/BL6 mice	Transplantation of organoids	5 days	Formation of pulmonary-like tissue constructs. Almost complete absence of transplanted cells in vessels	N/A	Mondrinos et al. (2014)
Multi-lineage mouse lung organoids	Mouse embryonic lung cells	Lung cells	Renal capsules of immunodeficient mice	Dissociation and transplantation	6 days	Formation of structures resembling saccules and composed of interconnected airspaces lined by flattened epithelial cells	N/A	Chapman et al. (2011)
Human lung organoids mixed with E13.5 mouse lungs	Human embryonic lung cells and mouse embryonic lung cells	Cells expressing SOX2 and SOX9 markers	Kidney capsule of NSG mice	Transplantation of cell pellet	3 weeks	Human cells expressed SOX9, SOX2 and assembled into columnar epithelial. Mouse parts of the graft contained either squamous epithelium or columnar epithelium	3 replicates with 4 samples each	Nikolić et al. (2017a)
					7–12 weeks	Human cells expressed low levels of SOX9. Human cell population included goblet cells. Rare patches of differentiated airway cells were identified in 2/3 of the organoids lined with basal, goblet, and ciliated cells		
Human embryonic lung organoids	Human embryonic lung cells	Cells expressing SOX2 and SOX9 markers	Intraperitoneal injection into NSG mouse after exposure to bleomycin	Dissociation and transplantation of 6×10^5 cells	8 days	Human cells expressed SOX2, SOX9, FOXA2, KRT5, TRP63, and MUC5AC markers	8	

Table 2 (continued)

Organoid types	Cell sources	Cell types in the organoids	Transplantation sites/method of introduction	Pre-transplantation processing	Duration	Outcomes	Number of objects	References
Human lung spheroids	Human lung and transbronchial cells	Cells expressing ProSPC, AQP5, CCSP, CD90, and CD105 markers	Intravenous injection into NSG mice	Dissociation and injection of 5×10^6 cells	1, 4, 7, 11, and 20 days	Donor cells were found in lungs and liver. In lungs, cells expressed alveolar, secretory, and epithelial markers	10	Dinh et al. (2017)
Human lung organoids	Human embryonic stem cells	Basal cells, immature ciliated cells, smooth muscle myofibroblasts, and alveolar-like cells	Subcapsular pocket of the kidney of NSG mice	Transplantation of 35-day HLOs using forceps	4–6 weeks	The stage of HLO culture and the site of transplantation did not affect the graft efficiency	3–6	Dye et al. (2016)
			Greater omentum of NSG mice	Suturing of 65-day HLOs using non-absorbable suture	12 weeks		13	
HLOs seeded on a PLG			Epididymal fat pads of NSG mice	Immersion in Matrigel with 500 ng/mL FGF10	4–15 weeks	100% of the recovered constructs possessed airway-like structures	23	
Human lung organoids	Human embryonic stem cells	Proximal or/and immature distal airway epithelial cells	Subcapsular pocket of the kidney of B-NSG mice	Transplantation of 10–20 organoids (1–2 mm^3) per mice	14 days	21-day HLOs expressed markers of lung progenitor cells	3 per group	Chen et al. (2018)
					100–120 days	21-day HLOs expressed markers of AT1 and AT2 cells 41-day HLOs could differentiate into mature AT1-like and AT2 cells HLOs possessed vascular and neuronal networks		
Human bud tip organoids	Human induced pluripotent stem cells	Enriched for SOX2 and SOX9 co-expressing cells	Trachea of NSG male mice 24 h after intraperitoneal injection of naphthalene	Dissociation and transplantation of 0.5×10^6—0.6×10^6 cells	7 days	Cells engrafted and retained expression of SOX2 and SOX9	15	Miller et al. (2018)
					6 weeks	8 out of 15 surviving animals had cell engraftment. Lungs successfully recovered from the injury. All engrafted cells were SOX2+, had a mucus-producing phenotype and expressed ciliated and neuroendocrine cell markers. Alveolar cell-specific markers were not detected		

Table 2 (continued)

Organoid types	Cell sources	Cell types in the organoids	Transplantation sites/method of introduction	Pre-transplantation processing	Duration	Outcomes	Number of objects	References
Human lung bud organoids (LBOs)	Human pluripotent stem cells	Cells expressing lung (NKX2.1), epithelial progenitor (p63), mesodermal (PDGFRA) and endodermal (SHH) markers	Kidney capsule of NSG mice	Mixing of 20–25-day LBO cells with Matrigel and transplantation of 1×10^6 cells	1.5 months	Organoids containing tubular structures lined by epithelium and surrounded by mesenchymal tissue	5 – 7 per time point	Chen et al. (2017b)
					5 months	Branching structures appeared. Branch tips expressed markers of AT2 cells, while stalks and central tubules expressed markers of proximal cells		
					7 months	The presence of dome-shaped groups of cells in the airway-like structures and areas of growth containing AT1 and AT2 cells		

AQP5, aquaporin-5; *AT2*, alveolar type 2 cells; *BMP*, bone morphogenic protein; *CC10*, Club cell 10kD protein; *CCSP*, Club cell secretory protein; *CD*, cluster of differentiation; *E14.5*, embryonic day 14.5; *EPCAM*, epithelial cell adhesion molecule; *GFP*, green fluorescent protein; *Lamp3*, lysosome-associated membrane protein 3; *NKX2.1*, NK2 homeobox 1; *NSG mice*, non-obese diabetic (NOD)-severe combined immunodeficient mice; *p63*, transformation-related protein 63; *PDGFRA*, platelet-derived growth factor receptor A; *PLG*, poly(lactic-co-glycolic acid); *proSPC*, prosurfactant protein C; *SHH*, sonic hedgehog; *SOX*, SRY (sex determining region Y)-box; *SPC*, surfactant protein C; *N/A*, not available

groups successfully recovered from the injury. Engraftment of human cells was observed in 8 out of 15 surviving mice that received bud tip organoid injections. Engrafted cells were found in bronchioles, trachea, and primary/secondary bronchi of mice. All engrafted human cells expressed SOX2, with roughly 75% of the cells acquiring a mucus-producing phenotype, ~13% acquiring a ciliated cell profile, and ~0.5% exhibiting a neuroendocrine cell profile. The P63 marker or alveolar cell specific markers were not detected. In 2019, AT2 organoids and primary AT2 cells were transplanted into influenza-injured recipient mice 11 days after infection (Weiner et al. 2019). Mice with cellular transplantation recovered ~ 65% faster than the control group, whereas the transplantation of AT2 organoids did not improve the recovery process. Thirteen days after transplantation, cells from AT2 organoids demonstrated two distinct fates: maintenance of AT2 lineage (SPC +, Lamp3 +) or dysplastic regeneration (Scgb3a2 +, Krt5 +), while primary AT2 cells either retained their AT2 lineage or differentiated into AT1 cells and did not exhibit dysplastic regeneration. The authors concluded that primary AT2 cells which never experienced in vitro conditions may retain a more appropriate lineage restriction upon transplantation.

Another significant problem in the transplantation of organoids derived from embryonic or induced pluripotent stem cells is incomplete differentiation and presence of poorly differentiated cells in the cellular mass that could theoretically form tumors (teratomas, for example) after transplantation. To date, this problem has been insufficiently studied. It is necessary to study the long-term consequences of transplantation of stem cell-derived organoids not only from the point of view of the survival rate of donor cells, but also for the assessment of the risk of tumor formation. In addition, it is necessary to develop protocols for detecting incomplete differentiation of stem cells before transplantation.

Lung organoids are promising instruments in regenerative medicine, since organoids can be obtained from a small amount of donor cells and can provide autologous cells or even tissue for transplantation (Bartfeld et al. 2017). Lung organoids are believed to be grafted onto scaffolds, including a decellularized lung, as it is done today with gastric, hepatic, pancreatic, and small intestinal organoids (Giobbe et al. 2019). Various methods of scaffold grafting with cells and organoids have been reported. For each of these methods, successful growth and maturation of cells were observed, which gives hope for their further development and application to lung organoids.

Organoid technology can be combined with recent advances in genome editing based on CRISPR-Cas9. There are three ways to incorporate CRISPR-Cas9 method in organoid technology: genome modification of cells prior to creation of organoids; genome modification of organoids dissociated into single cells and subsequent re-formation of 3D structures; delivery of Cas9 and sgRNA to the organoids without their dissociation into single cells (Gopal et al. 2020). The first two methods are often used in the studies of lung diseases, including generation of disease-specific lung organoids (Strikoudis et al. 2019), or for the correction of the *CFTR* gene in intestinal organoids (Maule et al. 2020).

Conclusion

Lung organoids are a promising approach in the treatment of pulmonary diseases. The review describes the main protocols for obtaining airway, alveolar, and lung organoids from adult tissue, embryonic progenitors, and induced pluripotent stem cells. Organoids are transplanted into ectopic and orthotopic sites for maturation and formation of vascular and neuronal networks. In most cases, lung organoids are transplanted into an ectopic site, since this opens up the possibility of choosing a site with good access to blood supply and maintenance of vascularization of the grafted tissue; however, the best results of organoid maturation were obtained with orthotopic transplantation. Studies on the transplantation of lung organoids into the area of lung injury showed that organoids engraft, preserve the phenotype, and contribute to the repair of injured tissue. However, there is little research on the fate of transplanted organoids, their vascularization and restoration of tissue architecture over a long period of time.

Since organoids have appeared relatively recently, their use in clinical practice requires their thorough characterization, development of the most effective protocols for obtaining and transplantation, and conductance of preclinical studies. In our opinion, there are still a number of unresolved problems in this area. It is necessary to develop protocols for confirming complete differentiation of stem cells, if they are the source of organoids. Protocols for successful colonization of the lungs with donor cells need to be improved, as irradiation is dangerous for patients. Solving these problems will bring us closer to the clinical use of respiratory organoids for the treatment of various lung diseases.

Funding This work was supported by the Ministry of Science and Higher Education of the Russian Federation.

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