Review Article

Promising preclinical models for lung cancer research—lung cancer organoids: a narrative review

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Background and Objective: Traditional cell line models are the commonly used preclinical models for lung cancer research. However, cell lines cannot recapitulate the complex tumor heterogeneity and cannot mimic the microenvironment of human cancer. Recently, 3D multicellular *in vitro* self-assembled models called "organoids" have been developed at a fast pace in the field of research, which can mimic the actual primary tumor. At present, several studies have reported on protocols of lung cancer organoids (LCOs) generation, and using LCOs can provide novel insight into the basic and translational research of lung cancer. However, the establishment of the LCO models remains challenging due to the complexity of lung cancer and the immaturity of organoid technology, so it is necessary to understand the influences of different methodologies on LCO generation and review the applications and limitations of LCO models.

Methods: In this review, we searched the literature in the recent ten years in the field of LCOs.

Key Content and Findings: We summarized the methodology, the problems, and the solutions in the LCOs generation, its application and limitations, as well as proposing future challenges and perspectives.

Conclusions: Currently, LCOs are successfully generated via exploring the methodology by the researchers. Though there are still challenges in clinical application, LCOs are applied in some cancer studies including investigation of anti-cancer treatment response *in vitro*, modeling tumor immune microenvironment, and construction of organ chips, which are forging a promising path towards precision medicine.

Keywords: Lung cancer; organoids; culture medium; preclinical models

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Introduction

Major improvements have been made in lung cancer treatment with the emergence of molecular targeted therapies and immunotherapies (1-3). The presence of an oncogenic driver mutation, such as epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK), renders a tumor sensitivity to target tyrosine kinase inhibition (4,5). In addition, with the emergence of immune checkpoint inhibitors (ICIs), significant survival benefits have been produced in the treatment of some advanced lung cancer and cancer therapy has been greatly enriched (6,7). Despite the huge improvement, identifying new therapeutic targets and overcoming drug resistance remain

Table 1 The search strategy summary

Items	Specification
Date of search	May, 2023
Databases and other sources searched	PubMed
Search terms used	Lung cancer, organoid
Timeframe	From 2013 until 2023
Inclusion criteria	Literature in English were all included
Selection process	Studies were initially screened by X.C. and L.Y., reviewed by Y.H. and obtained consensus via discussion among all authors

a big challenge. Therefore, it is crucial to undertake cancer biology and translational research via *in vitro* and *in vivo* model systems that can accurately recapitulate patients' tumors.

Conventional 2D cell lines are easy to obtain and culture, and they represent the widely used *in vitro* preclinical model which is used for basic research and anti-tumor drug testing. Up to now, more than 300 lung cancer cell lines have been established *in vitro* via a culture system (8). Yet, a large number of studies using cell line models with dramatic success eventually failed in clinical trials. The reason might be related to the inherent genetic drift during the process of passage, and clonal selection is usually performed in fast-growing tumor populations (9). Therefore, tumor heterogeneity and actual drug response of parental tumor might not be able to be reflected. By contrast, *in vivo* patient-derived xenograft (PDX) models generated from patient tumor tissue implantation in immunodeficient or humanized mice can better retain tumor heterogeneity and mimic tumor microenvironment (TME). However, the establishment of PDX models is more time-consuming and costly (10,11).

To address these problems, 3D biomimetic *in vitro* models have been sequentially developed, with which patient-derived tumor spheroids have been the first studied. Through 3D cell culture *in vitro*, cancer cells can aggregate into microsized spherical structures, mimicking histological structures and growth kinetics of solid tumors to a certain extent. On this basis, patient-derived organoid models have been developed to further maintain the characteristics of the primary tumor. Through using specific animal-derived proteinaceous extracellular matrix (ECM) gels and growth factors cocktails, self-organization and self-assembly of the organoids can be guided. Currently, 3D organoid culture technologies have emerged as a relatively low-cost and representative platform to model cancer heterogeneity (12-14). Though several studies have reported the successful experience of lung cancer organoids (LCOs) generation, the culturing methods and conditions remain immature. In this review, we mainly focused on the culturing method of LCOs, including tissue collection, culture medium, and procession, all of these are the key factors to the success. Besides, we also discussed the big challenges and application of LCOs. We present this article in accordance with the Narrative Review reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-341/rc).

Methods

This review consists of relevant data of LCOs searched in the PubMed database, of which "lung cancer" and "organoid" were used as the search terms. In addition, the literatures we reviewed were published in English in the recent ten years and the process for identifying the literature search is listed in *Table 1*.

Methodology of LCOs

The LCOs derived from cancer tissues are considered to be able to represent the genetic and epigenetic characteristics of cancer. Currently, one of the most commonly used approaches for organoid generation is the ECM scaffold method (15,16). Briefly, the tumor samples were collected from the clinical sites and minced into pieces (1–2 mm^3), followed by incubation with digestive enzymes. Then the dissociated cells and cell clusters were resuspended with cold Matrigel before being seeded in plates, and the Matrigel drops were left solidified and overlaid with tumor-type specific culture medium. After verification by histology and sequencing, further experiments can be performed

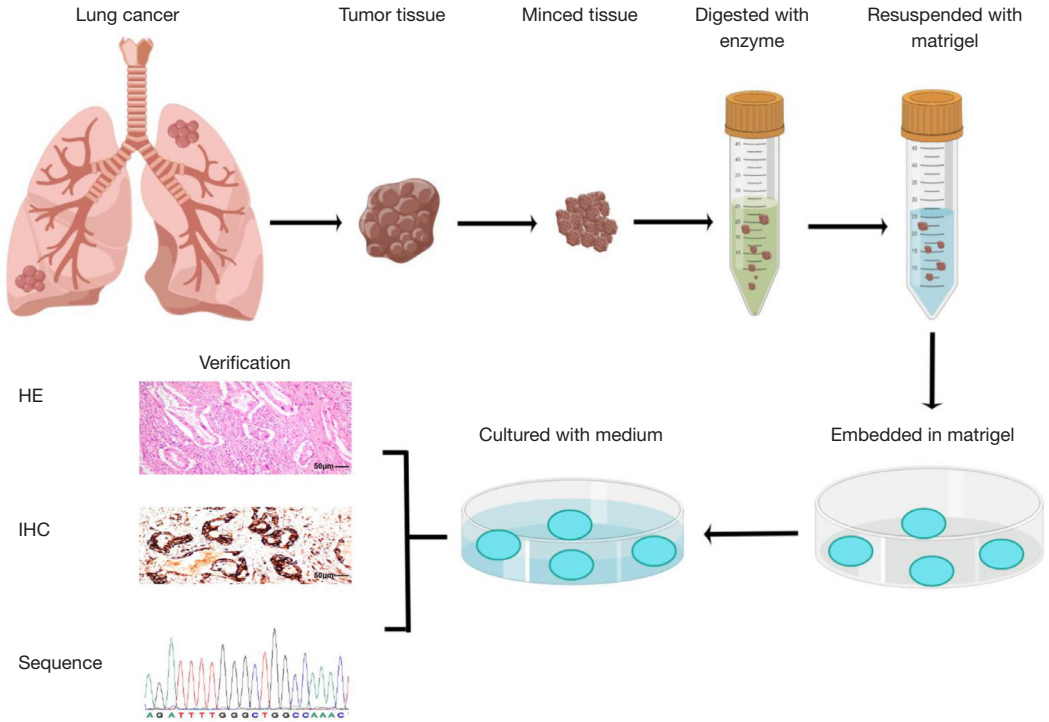

Figure 1 Workflows for establishment and identification of lung cancer. HE, hematoxylin and eosin; IHC, immunohistochemistry.

smoothly (*Figure 1*).

Patient specimen collection

The sufficient number of active cancer cells is the key factor for the success of organoids culture. Most studies used surgically resected samples with 1 to 4 cm^3 for harvesting more cancer cells to generate organoids, and the success rate reported previously varied widely, from 40.7% to 100% (17-19). Biopsy specimens with the size of 0.2 to 0.4 cm^3 have also been used in some studies, of which the success rate was reported to be only 23% to 28% (20,21). Besides, lymph node metastases and malignant pleural effusions have also been used to generate organoids (22-24).

Tissue digestion

The final number of active cells mainly depends on the samples after disaggregation. Enzymatic digestion is the most common way in several studies, and few studies chose mechanical processing. To obtain more active cells, the digestive enzyme cocktail and digestion time are particularly important. Collagenase, neutral proteases, and elastase hyaluronidase were the most commonly used enzymes for tissues (25). By using digestive enzymes, the attachments between cells and ECM can be cleaved, and there is no need to pursue a state of excessive digestion. Cattaneo *et al.* reported that the ideal digestion condition was a mix of 70% single cells and 30% clusters of 7–10 cells (26). The choice of digestive enzyme used for detaching cells mainly includes trypsin and trypLE (an analogue of trypsin). Although trypsin with a stronger enzymolysis effect has limited adverse effects on cell characteristics, the use of trypLE was more common in organoid digestion, because its milder effect can avoid overtrypsinization (27,28). The total digestion time mainly depends on the sample size and tumor type, and generally, it may vary from 15 to 30 minutes for biopsies and 45 to 60 minutes for surgical resections. We should avoid overtrypsinization as much as possible, and check the status of the digestion by using a microscope to evaluate whether the tissue has dissociated into single cells.

Culture system

To mimic the tissue conditions the original tumor growing in, the culture system of LCOs commonly consists of ECM and culture medium containing various growth factor

cocktails. The obtained single tumor cells or cell clusters are plated in the 3D ECM hydrogel such as basement membrane extract (BME), which was used as an artificial lamina propria in organoid models inspired by an approach pioneered by Lee *et al.* (29). Matrigel or other BME both are commonly used ECM protein mix, a complex mixture of different ECM proteins including laminin, fibronectin, collagen and heparin sulfate proteoglycans, which can maintain necessary cell-ECM interactions (30). As for culture medium, it usually consists of various supplements, such as B27, *N*-acetylcysteine, nicotinamide, noggin, WNT/R-spondins, epidermal growth factor (EGF), and fibroblast growth factor (FGF), all of which are associated with cell survival and stemness/differentiation balance (21,31). Therefore, understanding the function of these supplements is essential for LCO generation (*Table 2*) (21).

Verification and characterization of LCOs

Once organoids are established, the next important thing is to identify the organoids for further experiments. Evaluating the similarity between LCOs and the primary tumor is critical for the value of LCOs. Several approaches for organoid validations have been proposed to verify the characteristics of LCOs. These include histomorphology, immunohistochemistry, and genetic profiles. Firstly, hematoxylin-and-eosin (H&E) stains can be used to assess the morphological features which consist of the major structure of LCOs and the morphology of the cells. The study by Dijkstra *et al.* demonstrated that normal lung airway organoids (LAOs) grew as relatively well-organized cystic structures, while the LCOs grew mostly as irregular solid structures. However, they found that a few LCOs can be both cystic or solid (18). Therefore, it is not enough to evaluate the LCOs via histomorphological features alone. Immunohistochemistry (IHC) is another useful method to further aid in the development of classification. Thyroid transcription factor 1 (TTF-1), cytokeratin 7 (CK7), tumor protein 63 (p63), and CK5/6 were the most commonly used markers for IHC (32). In the study of Dijkstra *et al.*, the authors further suggested that the loss of polarized p63 staining pattern can be used to differentiate LAOs from LCOs (18). In addition, in clinical practice, these markers can be used to distinguish the subtypes of lung cancer, especially for lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC), where LUAD usually shows positive TTF-1 and CK7, and LUSC shows positive p63 and CK5/6.

Genetic profiles help to further describe the characteristics of established LCOs compared with primary tumors. Dijkstra and colleagues first generated copy number (CN) profiling to distinguish normal LAOs and LCOs (18). The result showed that CN was normal in LAOs, while most of the primary non-small cell lung cancer (NSCLC) samples showed multiple gains and losses. Besides, whole exome sequencing (WES), whole genome sequencing (WGS), and targeted gene panel sequencing might provide additional molecular information, which can differentiate between tumor and normal organoid as well as confirming the parental accordance and clonal heterogeneity of the LCOs (33). Among them, WGS is considered the most accurate method for identification of the patient-derived organoids due to the access to the whole genome information, but considering the high cost, many researchers have chosen WES as the more cost-effective and less labor-intensive alternative (34). Similarly, detection of commonly mutated genes in lung cancer using a small panel such as *EGFR*, *TP53*, *KRAS*, and so on can save the cost, but some potential discordances between organoid and the original tumor may not be found, given that the tumors may not necessarily carry these mutations. RNA sequencing was another way to identify the gene expression profiles in LCOs (35). We, therefore, recommend a complete verification and characterization of LCOs should include the morphological and IHC assessment, combined with a genetic profile analysis by CN, WES, or RNA sequencing.

Problems and solutions in the LCOs generation

Currently, although researchers are struggling for optimizing the LCO generation procedures, there are a number of problems for these models. Due to the complexity of lung cancer and the immaturity of organoid technology, it is worth thinking during establishment of LCO models about how to determine the composition of conditioned medium, maintain the tumor heterogeneity and shorten the time for organoid preparation.

The choice of conditioned medium for LCO generation

The biggest problem for organoids generation is that normal lung epithelial cells in the lung organoids often grow faster than the lung tumoroids, leading to the failures of LCOs establishment (13,18). To overcome this obstacle, some studies on tumor purifying have been reported. Sachs *et al.* reported the use of nutlin-3a, an MDM2

Table 2 Supplements applied in lung cancer organoid cultures

Supplements	Signaling pathway	Function
B27 supplement	A serum-free culture supplement	Increase survival rate of tumorspheres and prevention of adherences
		Improve the sphere-forming efficiency and sustain the propagation of tumor spheres
N2 supplement	A serum-free culture supplement	Maintain an undifferentiated state of organoids
N-acetylcysteine	Antioxidant	Protect cells from severe levels of stress
Nicotinamide	Co-enzyme precursor	Increase the efficiency of organoids differentiation
SB202190	p38 MAPK signaling	Protect cells from environmental stress-induced apoptosis
Y-27632	Inhibition of ROCK signaling	Prevents anoikis-induced cell death
A83-01	Inhibition of TGF-β/Smad signaling	Promote the proliferation of stem cells
		Maintain the undifferentiated state of stem cells
Noggin	Inhibition of TGF-β/Smad signaling	Promote the proliferation of stem cells
		Maintain the undifferentiated state of stem cells
R-spondin-1	Wnt/β-catenin signaling	Maintain the stemness and promote the self-renewal of stem cells
Wnt 3A	Wnt/β-catenin signaling	Maintain the stemness and promote the self-renewal of stem cells
FGF-2	FGFR1c, 3c signaling	Keep the survival of organoids
FGF-4	FGFR1c, 2c signaling	Keep the survival of organoids
		Maintain the stemness of stem cells
FGF-7	FGFR2b signaling	Induce organoid branching
FGF-10	FGFR2b signaling	Promote differentiation of lung stem cells
		Inducer of lung organoid formation and branching
EGF	PI3K, ERK1/2, JAK/STAT, β-catenin, and calcium signaling	Drive proliferation of organoids
IGF-1	IGF-1R signaling	Keep the survival of organoids
Nutlin-3a	Inhibition of P53/MDM2 signaling	Select TP53 mutated cancer organoids
Smoothened agonist	Hedgehog signaling	Promote differentiation of stem cells
CHIR 99021	Inhibition of GSK-3β signaling	Promote self-renewal of stem cells
Prostaglandin E2	E series prostaglandin receptors signaling	Keep the survival of organoids
Dexamethasone	Corticosteroid receptor signaling	Induces alveolar maturation and increases the versatility of lung organoids.
Forskolin	cAMP signaling	Promote cell proliferation

FGF, fibroblast growth factor; EGF, epidermal growth factor.

inhibitor, to lead airway organoids with *TP53* wild-type into apoptosis, and at the same time, to allow the outgrowth of tumoroids with mutant *TP53* (20). Moreover, it has been confirmed that deprivation of specific growth factor based on the culture medium for normal airway can avoid the normal cell contamination, and subsequently increase the survival rate of organoids generation (36). Yokota *et al.* have compared the growth of lung tumoroids in the LAO medium developed by the Clevers laboratory with those in other LCO culture media reported by the other three laboratories (37). Ultimately, the author found that LAO medium with nutlin-3a was superior to other different

media. However, only approximately 50–60% of lung cancer patients harbor the *TP53* mutation (38), of which might not be suitable for the generation of LCOs with wild TP53. To avoid eradication of the LCOs without *TP53* mutation, Ebisudani *et al.* prepared a second cancer-selective condition based on nutlin-3a, where they halted ERBB signaling by removing EGF/insulin growth factor-1 (IGF-1)/FGF-2 and adding a pan-ERBB inhibitor to restrict normal airway and alveolar organoids growing and obtain pure LCOs (39). Through the investigation of genotype-phenotype correlations, they found that LCOs of different histologic subtypes exhibited different EGFR pathway dependency and drug sensitivity. All in all, it remains unknown whether all growth factors would be required for each LCO and the culture medium formulations from different laboratories are still immature, due to the complexity of histology and genetic background of lung cancer. With the deepening understanding of organoid culture technology, the improvements and adjustments for culture systems are increasing, and the different LCO systems published are compared in *Table 3*.

Maintenance of the tumor heterogeneity

It has been shown that organoids can capture tumor heterogeneity and serve as an *ex vivo* platform for personalized treatment, though this model cannot fully reflect the disease *in vivo* (42). According to the results of genomic profiling in LCO research, the discordance between the organoid and the original tumor is usually obvious but acceptable for recapitulating primary tumors. Roerink *et al.* have reported that the colorectal cancer (CRC) organoids generated from different tumor clones of the same tumor exhibited obvious genetic diversification and different responsiveness to therapy (43), indicating that intra-tumor diversification might be a reason for the discordance. Moreover, the ongoing normal tumor evolution and the heterogeneous organoid cultures for tumor selection might also lead to the discordance, so the use of low passage cultures can minimize the effect of potential *in vitro* selection by media or accumulation of *in vitro* mutations. To confirm the obtained organoid with maintenance of the original tumor heterogeneity, genetic characterization is necessary.

Long time for LCO generation

Currently, the lengthy time in generating LCOs hampers the application of organoids in clinical practice, especially for drug sensitivity tests to predict the drug response, which requires prolonged *in vitro* expansion to generate enough quantities of LCOs and obtain quick responses for drugs. However, a few weeks or even months are required for the current drug test. Recently, Hu *et al.* by employing an integrated superhydrophobic microwell array chip (InSMARchip), demonstrated that LCOs, even at passage 0, can be used to perform one-week drug tests, which holds great promise for clinical drug sensitivity screening *in vitro* (36). For future studies, more efforts should be made to improve the culture technique to shorten the time.

Application and limitation of LCOs

Drug sensitive screening

Currently, common preclinical models used for drug screening in lung cancer therapy include 2D cell culture, spheroids, organoids, and xenografts. Patient-derived tumor cell lines grown in 2D cultures are ideal for studying single cancer cells in response to drugs via rapid and long-term expansion with low costs and high reproducibility, but they cannot fully recapitulate the biological *in vivo* interaction within the entire human body and the genetic background of parental tumors. As for the 3D tumor spheroids model, it can further maintain parental characteristics and predict treatment response compared to cell lines model, attributed to its high number of cell-cell interactions, cell–ECM interactions, and the gradients of gases, nutrients, and pH from inner to outer. Organoids model as a special 3D model with higher thresholds and costs for generation is capable of recapitulating primary tumors more accurately, which is similar to the PDX model. As for the PDX model with the transplanted patient-derived tumor tissue growing in the biological system of model animals, it contains more complete TME, which may give the treatment response closer to human body compared to the organoids model. However, the PDX model is unsuitable for large-scale drug screening due to their time-consuming nature and high costs (44,45). In conclusion, the high similarity to their parental tumors in respect of phenotypes and genotypes as well as the possibility of rapid and long-term expansion, make LCOs more suitable for the high-throughput drug screening.

To date, only a few studies have reported on the use of organoids from primary NSCLC or metastatic sites for drug screening. Sachs *et al.* demonstrated that LCOs are

Table 3 Different patient-derived LCO systems

Reference	Sample	Histology	Medium/ECM	Supplements	Success rate	Applications
Pauli *et al.* 2017 (33)	Resection/biopsy	LUAD	DMEM/Matrigel	B27, N-acetylcysteine, R-spondin-1, noggin, FGF-10, FGF-2, EGF, A83-01, Y-27632, SB202190, nicotinamide, prostaglandin E2	50% (1/2)	High throughput drug screening
Dijkstra *et al.* 2018 (19)	Resection/biopsy	LUAD/LUSC/other	DMEM/F12/ Geltrex	B27, N-acetylcysteine, R-spondin-1, noggin, FGF-10, FGF-7, A83-01, Y-27632, SB202190, nicotinamide, nutlin-3a	100% (6/6)	Induce and analyze tumor-specific T cell responses via organoids co-cultured with PBMC
Sachs *et al.* 2019 (20)	Resection/biopsy	NSCLC	DMEM/F12/ BME	B27, N-acetylcysteine, R-spondin-1, noggin, FGF-10, FGF-7, A83-01, Y-27632, SB202190, nicotinamide, nutlin-3a	• Resections of primary NSCLC: 88% (14/16) • Biopsies of metastatic NSCLC: 28% (5/18)	Long-term expansion of LCOs, validation, and drug testing
Kim *et al.* 2019 (40)	Resection	LUAD/LUSC/ SCLC/LCNEC	DMEM/F12/ Matrigel	B2, N2, FGF-2, EGF, ROCK inhibitor	87% (20/23)	Established a living biobank of 80 lung cancer organoids
Dijkstra *et al.* 2020 (18)	Resection/biopsy	LUAD/LUSC/ SCLC/LCNEC/ others	DMEM/F12/ Geltrex	B27, N-acetylcysteine, R-spondin-1, noggin, FGF-10, FGF-7, A83-01, Y-27632, SB202190, nicotinamide	24/59 (40.7%)	Evaluation of methods to determine tumor purity of organoids of lung cancer organoids
Li *et al.* 2020 (17)	Resection	LUAD	DMEM/F12/ Matrigel	B27, N-acetylcysteine, R-spondin-1, noggin, FGF-10, FGF-7, A83-01, Y-27632, SB202190, nicotinamide	12/15 (80%)	Established a living biobank of lung cancer organoids and undertaken high throughput drug screening
Shi *et al.* 2020 (35)	Resection/PDX	LUAD/LUSC	DMEM/F12/ Matrigel	B27 supplement, N-acetylcysteine, noggin, FGF-10, FGF-4, EGF, A83-01, Y-27632, CHIR-99021, SAG	• Organoids maintained in short-term culture: 72% (47/65) • Organoids maintained in long-term culture: 15% (10/65)	Established a protocol for the development of NSCLC organoids and may be useful for future drug screening
Kim *et al.* 2021 (41)	Resection/pleural effusion	LUAD	DMEM/F12/ Matrigel	B27, R-spondi-1, FGF-7, FGF-10, noggin, A83-01, SB202190, Y- 27632	83% (83/100)	Examined the ability of lung cancer organoids to predict clinical responses
Hu *et al.* 2021 (36)	Resection/biopsy	LUAD/LUSC/ SCLC/others	DMEM/F12/ Matrigel	B27 N2, nicotinamide, N-acetylcysteine, Y-27632, EGF, SB202190, A83-01, Forskolin, Dexamethasone	78.6% (81/103)	Establishing Lung cancer organoids analyzed on microwell arrays predict drug responses
Yokota *et al.* 2021 (37)	Resection/biopsy	LUAD/LUSC/ SCLC/others	DMEM/F12/ Matrigel	B27, N-acetylcysteine, R-spondin-1, noggin, FGF-10, FGF-7, A83-01, Y-27632, SB202190, nicotinamide, nutlin-3a	• Organoid/tumoroid formations: 82.9% (34/41) • Tumoroid lines for long-term culture: 7% (3/41)	Comparing different culture mediums to determine which tumoroid culture system is most efficient
Wang *et al.* 2023 (24)	Malignant pleural effusion/ resection/biopsy	LUAD/LUSC/ SCLC/ASC/PSC	DMEM/F12/ Matrigel	B27, N2, EGF, FGF, Y-27632	74.8% (160/214)	Prediction of locally advanced or metastatic lung cancer tumor in response to chemotherapy and targeted therapy
Ebisudani *et al.* 2023 (39)	Malignant pleural effusion/ resection/biopsy/ circulating tumor cells/ sputum	LUAD/LUSC/ SCLC/LCNEC	DMEM/F12/ Matrigel	B27, N-acetylcysteine, EGF, IGF-1, FGF-2, R-spondin-1, noggin, A83-01, Wnt 3A, A83-01, Y-27632 LCOs enrichment: nutlin-3a, pan-ErbB inhibitor, remove of EIF (EGF, IGF-1 and FGF-2)	Malignant pleural effusion/resection/biopsy: 17.8% (35/197) Circulating tumor cells: 8.3% (3/36) Sputum: 20% (5/25)	Established a biobank of lung cancer organoids for investigation of genotype-phenotype mapping

LCO, lung cancer organoid; ECM, extracellular matrix; LUAD, lung adenocarcinoma; DMEM, Dulbecco's modified eagle medium; FGF, fibroblast growth factor; EGF, epidermal growth factor; LUSC, lung squamous cell carcinoma; PBMC, peripheral blood mononuclear cell; NSCLC, non-small cell lung cancer; BME, basement membrane extract; SCLC, small cell lung cancer; LCNEC, large cell neuroendocrine carcinoma; PDX, patient-derived xenograft; SAG, Smoothened agonist; ASC, adenosquamous carcinoma; PSC, pulmonary sarcomatoid carcinoma; IGF, insulin growth factor.

amenable to drug screening, and differential responses of NSCLC organoids to conventional chemotherapeutics such as cisplatin or paclitaxel, but also showed sensitivity towards the tyrosine kinase inhibitors (TKIs) such as erlotinib and gefitinib in *ERBB2*-mutant organoids (20). In the same year, another study by Kim *et al.* established a biobank of 80 LCOs and found a *BRCA2*-mutant NSCLC responded to olaparib, an *EGFR*-mutant LCOs responded to erlotinib and an *EGFR*-mutant/*MET*-amplified LCOs responded to crizotinib, which provided a prospect for drug testing in LCOs (40). Shi *et al.* also verified that *KRAS* mutant organoids were much more sensitive to the MEK inhibitor (35). Yokota *et al.* demonstrated that the combination of Bcl-2 inhibitor (navitoclax) and survivin inhibitor (YM-155) might be effective for EGFR-TKI-resistant NSCLC organoids (37). Recently, high-throughput drug screening is considered to be a more efficient way to utilize the advantages of organoids. Li and coworkers established 12 human LUAD derived organoids from 15 patients with different subtypes of LUAD and showed the ability to identify potential biomarkers and to perform high-throughput drug screening with 24 anti-cancer drugs including standard chemotherapy and targeted agents (17).

LCOs for modeling TME

Patient-derived tumor organoids have emerged as a promising model that can maintain the heterogeneity of primary cancers and recapitulate the TME. The TME comprises an immune cellular network, mesenchymal-derived cells (pericytes and fibroblasts), and endothelial cells, which greatly fosters tumor progression and the efficacy of therapeutic responses. As an important means of lung cancer treatment, immunotherapy needs the support of immune cells for its curative effect, pushing the development of corresponding co-culture models mimicking tumor immune microenvironment (TIME). Previously, 2D cell line models usually served as a common approach for exploring the mechanisms of cancer immunology by co-culture with exogenously immune cells, such as those from autologous peripheral blood mononuclear cells (PBMCs) (46-48). However, this approach was mainly based on monolayer cancer cell structures, which is far from the actual situation of TIME. The advent of 3D organoids represented a more ideal *in vitro* model for studying tumor immunobiology and developing novel approaches for personalized medicine in lung cancer.

Currently, there are two approaches that can be used to add immune cells to the organoid culture system to generate TIME in organoids. One is reconstituted models, in which immune cells, such as those from autologous PBMCs, are isolated and subsequently co-cultured with grown organoids. Dijkstra *et al.* demonstrated that co-cultures of LCOs and PBMCs could enrich the tumor-reactive T cells from patients with mismatch repair deficient (MRD) CRC and NSCLC, where LCOs were generated from six NSCLC patients and autologous tumor-reactive CD8+ T cells were obtained in two out of six cases (19). Recently, another study reported by Li *et al.* pointed out that CAR-T cells targeting B7-H3 (B7-H3.CAR) effectively exhibit antitumor activity *in vitro* against LCOs, and achieved T-cell-mediated targeting of NSCLC brain metastases (49). Another is holistic native TIME models, which preserve primary tumor cells and endogenous immune and non-immune stromal cells. Neal *et al.* reported an air-liquid interface (ALI) co-culture system that can be used to propagate LCOs (50). In this system, large regions of tumors could be grown in their native state and thus also faithfully preserve a diversity of native syngeneic tumor-reactive tumor-infiltrating lymphocytes, including T cells, B cells, NK cells, and macrophages, while the immune components would decline over time (<30 days), and did not persist beyond 2 months with IL-2 supplementation. Notably, although these LCO platforms have been confirmed with capacities of predicting the responsiveness to immune-checkpoint inhibitors and considered with huge potential for clinical translation, they still need more clinical data to support it.

Besides immune cells, cancer-associated fibroblasts (CAFs) as an equally important component of TME and responsible for the modification of the ECM proteins and secretion of growth factors, were also included in the study of co-culture with LCOs (51,52). Chen *et al.* (52) reported a 3D coculture system of LUSC organoids with CAFs and ECM that recapitulates the dynamic interactions between LUSC cells and components of the tumor TME. The result showed that CAFs and ECM could override cell intrinsic oncogenic changes in determining the disease phenotype in the context of LUSC and it is a promising model for TME research.

Organ-on-a-chip and organoid-on-a-chip

Human organ chips are microfluidic cell culture devices integrating living human cells with synthetically generated

yet physiologically relevant microenvironments, which may simulate the functions of different organs (53). Compared with traditional 2D culture, organ chips emulate one specific function of an organ which closely resembles the architecture of the human body. Organ-on-a-chip technology aims to develop effective microphysiological models for studying physiological events including pulmonary thrombosis and lung inflammation (54-56). However, most organ-on-a-chip models are based on a single organ without cross-organ communication. Hence, establishing multiorgan-on-a-chip systems can incorporate different organ-on-a-chip into a dynamic circulation system, called a "body-on-a-chip" (56). However, organ-on-a-chip systems lack structural or cellular fidelity compared with organoids. Organoids rely on self-assembly for the establishment of organized tissue structure. However, insufficient nutrients and oxygen might limit the growth of organoids as the size and volume become much bigger than before, and eventually leading to cell death. Therefore, combining the advantages of the two technologies, called "organoid-on-a-chip", can provide a more nutrient and gas exchange to organoids for avoiding cell death and achieving a long-term culture (57,58). Organoid-on-a-chip can serve as a more versatile preclinical model for the broad application of emerging drug discovery processes. Jung *et al.* presented a one-stop microfluidic device enabling both small cell LCOs culturing and drug sensitivity tests directly on a microphysiological system (59).

Future challenges and perspectives

Increasing evidence has shown that LCOs can recapitulate the true genetic, phenotypic, and morphological diversity of distinct neoplastic cell subclones. Despite being widely used, several challenges remain to be addressed for facilitating their future use in clinical practice.

Due to the lack of a standard process for LCO culture, such as tissue processing and medium formulations, current techniques for LCO culture are inherently uncontrolled and irreproducible (60). One goal for LCOs is to obtain a sufficient number of viable cancer cells and to timely establish the organoids. However, the problems of establishing long-term cultured tumoroids and improving the survival rates of pure LCOs are currently limiting factors for the broad clinical application of LCOs. Hence, available protocols will have to undergo additional optimization procedures to further develop and enhance the methodology. In addition, most of studies established organoids by only including cancer cells but without co-culture of mesenchymal-derived cells, endothelial cells, and immune cells. The mainstream approach for studying the association of organoids and the immune microenvironment is co-cultured organoids with immune cells. However, because TIME cells play diverse roles in cancer organoids, progression, and treatment, which are yet to be studied clearly, it remains unknown which kinds of TIME cells should be added to the organoid system.

Currently, researchers have successfully identified the required or dispensable medium components for cancer organoid culture. However, they mainly focus on some interconnected pathways, and some of the medium components are sorely stratified by the mutational status of tumors. Considering that various subtypes of lung cancer have distinct molecular characteristics and evolutionary processes, future efforts should be made to investigate the tumor-specific medium formulations to better suit organoid growth.

Conclusions

Increasing evidence has demonstrated that LCOs are a promising *in vitro* model that is suitable for future basic and translational cancer studies. Though there are high expectations for LCOs, we have to face several challenges so that LCOs can be better applied in clinical practice. Currently, the standard culture protocol remains immature. More experiments are required to compare the different culture protocols on the NSCLC with different histological types and genetic backgrounds, which may help to identify potential genotype-specific supplements that improve the success rate of organoid generation. Besides, LCO as an ideal model for the investigation of anti-cancer treatment response *in vitro*, especially for the immune-checkpoint inhibitors, holds great potential in immunotherapy in lung cancer. Finally, more advanced technology, such as organoid-on-a-chips, which imitates organ development and human diseases, will forge a path toward realizing the considerable promise of therapy response prediction and personalized medicine.

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Footnote

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