Recent advances in lung cancer organoid (tumoroid) research (Review)

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Received July 13, 2023; Accepted February 1, 2024

DOI: 10.3892/etm.2024.12672

Abstract. Lung cancer is the most critical type of malignant tumor that threatens human health. Traditional preclinical models have certain defects; for example, they cannot accurately reflect the characteristics of lung cancer and their development is costly and time-consuming. Through self-organization, cancer stem cells (CSCs) generate cancer organoids that have a structure similar to that of lung cancer tissues, overcoming to some extent the aforementioned challenges, thus enabling them to have broader application prospects. Lung cancer organoid (LCO) development methods can be divided into three broad categories based on the source of cells, which include cell lines, patient-derived xenografts and patient tumor tissue/pleural effusion. There are 17 different methods that have been described for the development of LCOs. These methods can be further merged into six categories based on the source of cells, the pre-treatment method used, the composition of the medium and the culture scaffold. These categories are: i) CSCs induced by defined transcription factors; ii) suspension culture; iii) relative optimal culture medium; iv) suboptimal culture medium; v) mechanical digestion and suboptimal culture medium; and vi) hydrogel scaffold. In the current review, the advantages and disadvantages of each of the aforementioned methods are summarized, and references for supporting studies are cited.

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Abbreviations: 2D, two-dimensional; 3D, three-dimensional; AO, airway organoid; CSCs, cancer stem cells; DMEM, Dulbecco's modified Eagle's medium; F12, Ham's F 12 nutrient medium; FBS, fetal bovine serum; IL-6, interleukin-6; KLF4, Kruppel-like factor 4; LCO, lung cancer organoid; LUAD, lung adenocarcinoma; N-hydap, N-hydroxyapiosporamide; NSCLC, non-small cell lung cancer; OCT3/4, octamer-binding protein 3/4; OSK, OCT3/4, SOX2 and KLF4; PDX, patient-derived xenograft; PE, pleural effusion; PGE2, prostaglandin E 2; RORs, retinoic acid receptor-related orphan receptors; SCLC, small cell lung cancer; SOX2, SRY-box transcription factor 2; TME, tumor microenvironment; Wnt, wingless and int-1

Key words: lung cancer, organoids, establishment methods, advantages, disadvantages

Contents

1. Introduction

Lung cancer constitutes 11.4% of all newly diagnosed cases of cancer, and is the leading cause of cancer-associated mortality worldwide (1). Lung cancer is a heterogenous disease that is divided into two major categories based on histopathology: Small cell lung cancer (SCLC) and non-SCLC (NSCLC). NSCLC accounts for 85% of lung cancer cases, and has several subtypes, including lung adenocarcinoma (LUAD), lung squamous cell carcinoma and large cell lung carcinoma (2). SCLC is a type of neuroendocrine tumor that is classified into two subtypes: SCLC and combined SCLC (3,4). Importantly, in addition to different histopathological subtypes, the heterogeneity of lung cancer also refers to differences between patients with the same subtype, or differences among cells in the same tumor tissue (5-10). The heterogeneity of lung cancer affects clinical treatment, since patients with the same pathological type may have diverse reactions to the same treatment (11-14). To effectively treat lung cancer, it is necessary to explore the source of lung cancer heterogeneity, identify specific antitumor drugs and achieve personalized treatment for patients.

Conventional two-dimensional (2D) culture and patient-derived xenograft (PDX) models are useful tools that assist in understanding the mechanisms underlying the occurrence, development and heterogeneity of lung cancer. However, these tools have certain limitations. Culture methods, passage numbers and other unexpected factors may cause tumor cell lines to lose the phenotype and genotype of a primary tumor in a 2D culture model (15-17). Moreover, as 2D culture models lack extracellular matrix, stromal

cells and immune cells, they cannot accurately simulate the tumor microenvironment (TME) and hence the conditions affecting cancers *in vivo* (18-21). Moreover, the defects of 2D culture models frequently cause antitumor drugs to show efficacies and toxicities *in vivo* that are different from those obtained *in vitro* during drug screening, which causes rapid drug screening to be challenging (22,23). Compared with 2D culture models, PDX models in which researchers transplant surgically resected tumor tissues into immunodeficient mice, are more accurate in representing the phenotype, genotype and TME of the parental tumor (24-28). However, the following issues can be observed: i) The proportion of transplantations that are successful in the establishment of PDX models is sometimes too low (29-31); ii) the process of successfully developing PDX models for drug screening is time consuming (32,33), and the condition of the patient often deteriorates during this period; iii) murine stromal cells gradually replace patient stromal cells, which changes the TME of the PDX model (34,35); and iv) the mechanism of interaction between tumor cells and immune cells in a PDX model is challenging to investigate because general PDX models lack immune cells (36,37).

The narrow limitations of conventional tumor cell lines and PDX models have driven researchers to investigate improved preclinical tumor models that preserve the characteristics of primary tumors to the largest extent. Such models are designed to be built in a short amount of time for rapid antitumor drug screening and expanded for the long-term investigation of cancer mechanisms and modification of treatment plans. Tumor organoids, also known as tumoroids (38), have thus emerged. Through self-organization, stem cells generate organoids that retain almost all the features of parental tissues (39,40). Numerous studies have demonstrated that tumor organoids have broad applications in different types of cancer (41-47). Researchers are using organoids to investigate lung cancer, with different studies describing various methods for the establishment of lung cancer organoids (LCOs) (48-80). These methods can be divided into three broad categories based on the source of cells used, namely cancer cell line-based LCOs, PDX-derived LCOs and patient-derived LCOs (Fig. 1). At present, 17 different methods for building LCOs have been described in the literature (Tables I and SI). These methods can be merged into six categories based on the source of cells, the pre-treatment method used, the composition of the medium and/or the culture scaffold: i) Cancer stem cells (CSCs) induced by defined transcription factors (48); ii) suspension culture method (52,66); iii) relative optimal culture medium with serum-free additive, amino acids, growth factor, stemness-related signaling pathway activators and apoptosis signaling pathway inhibitors (49-51,53,55,57,68,76,79,80); iv) suboptimal culture medium (relative optimal culture medium without stemness-associated signaling pathway activators) (71); v) mechanical digestion and suboptimal culture medium (70); and vi) hydrogel scaffold (69,78) (Fig. 1; Tables I, II, SI and SII). The current review presents the advantages and drawbacks of these methods, and cites references for relevant studies. The three broad categories of LCOs are discussed, along with the different methods used to establish LCOs.

2. Cancer cell line-based LCOs

Stem cells are indispensable for organoid generation (81). The ideal methodology for medical research is the use of CSCs from patient tissues to create lung tumoroids. However, ethical issues, success rates and the scarcity of specimens require consideration when using patient tissues. These factors limit the repeatability of studies to a certain extent, and may result in researchers being forced to seek alternative options for the transformation of cancer cell lines into CSCs. Researchers have used transformed CSCs to generate tumoroids, and various methods have been used to transform cancer cell lines into CSCs (82,83). In addition, researchers may be able to expand the CSC population by three-dimensional (3D) spheroid culture to acquire a sufficient number of CSCs for the establishment of tumoroids (84,85). Oshima *et al* (86) showed that colon cancer line cells can be successfully induced to form CSCs by the transfection of stem cell transcription factors, namely a combination of octamer-binding protein 3/4 (OCT3/4), SRY-box transcription factor 2 (SOX2) and Kruppel-like factor 4 (KLF4), known as OSK, into colon cancer cells (87). Subsequently, the authors attempted to induce lung cancer A549 cells into CSCs using this method, and colonies of OSK-A549 cells with chemoresistance, a delayed cell cycle, enhanced sphere formation ability and tumorigenicity were successfully obtained (48). Lung tumoroids were subsequently established by co-culturing the OSK-A549-colony cells with human umbilical vein endothelial cells and human mesenchymal stem cells (Fig. 1) (48). The results of hematoxylin and eosin staining and immunostaining showed that these tumoroids comprised distinctive cohesive cell nests that were analogous to lung cancer tissues (48). Gene expression analysis was conducted to explore changes in the gene expression profile during stem cell transformation (48). The results revealed that interleukin (IL)-6 was expressed at high levels in OSK-A549-colony cells compared with control cells, which increased the resistance of the OSK-A549-colony organoids to chemotherapy and facilitated the ability to construct lung tumoroids from them (48). IL-6 was also found to be expressed at high levels in the majority of patient tissue samples, independently from their gene mutation status and tumor staging (48). These findings suggest that IL-6 has the potential to become a novel therapeutic target for LUAD. This method is a feasible strategy for the generation of lung tumoroids and exploration of the mechanism of lung cancer development by driving lung CSC transformation. The use of cancer cell lines to generate LCOs has the following advantages: i) Cell lines are readily available; ii) there are few ethical issues concerning the use of cell lines to conduct medical research; and iii) repeat tests with the same cell lines can be carried out. However, there are also some disadvantages: i) Random gene mutations may be generated during long-term cell line 2D culture, leading to cell line diversity in terms of the gene profile in different laboratories; and ii) stromal, immune and nerve cells, and capillaries are absent from the tumoroid system, and consequently the interaction of cancer cells with the TME cannot be studied using this type of model. However, this is a common issue in all types of tumoroid models.

Despite their disadvantages, cancer cell line-based LCOs have certain practical utility. There are, however, some aspects of such LCOs that require further investigation: i) Whether

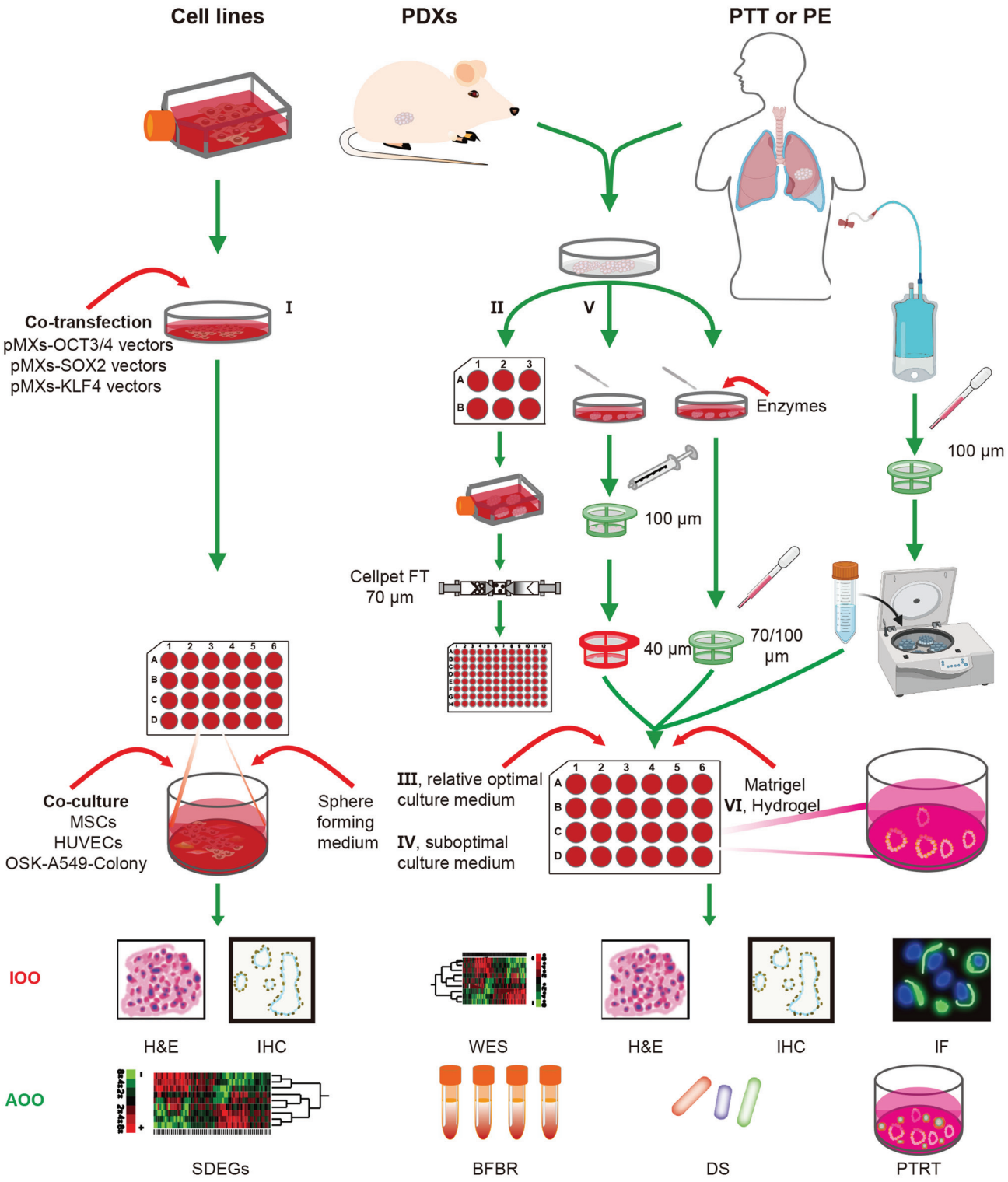

Figure 1. Schematic diagram of lung cancer organoid culture and application. Processes I-VI are shown. AOO, application of organoids; BFBR, biobank for basic research; DS, drug screening; H&E, hematoxylin-eosin staining; HUVECs, human umbilical vein endothelial cells; IF, immunofluorescence; IHC, immunohistochemistry; IOO, identification of organoids; KLF4, Kruppel-like factor 4; MSCs, mesenchymal stem cells; OCT3/4, octamer-binding protein 3/4; PDXs, patient-derived xenografts; PE, pleural effusion; PTRT, production of tumor-reactive T cells; PTT, patient tumor tissue; SDEGs, screening for differentially expressed genes; SOX2, SRY-box transcription factor 2; WES, whole exome sequencing. Some parts of the figure were made using Biorender (https://biorender.com) and ScienceSlides (http://www.scienceslides.com).

lung cancer cells can be transformed into lung CSCs by transfection with other combinations of stem cell transcription factors such as OCT3/4, SOX2, KLF4, cellular myelocytomatosis oncogene and/or Nanog homeobox; ii) differences among stem cells induced by transfection with different combinations of stem cell transcription factors; iii) whether other lung cancer cell lines, especially SCLC cell lines, can also be transformed into lung CSCs and used to successfully establish tumoroids using this method,; and iv) whether this approach is suitable for use in drug screening. In addition, as

Table I. Characteristics of methods for the establishment of lung cancer organoids.

First author/s, year/s	Type of cancer	Stage	Cell source	Success rate of organoid establishment	Success rate of pure LCO establishment, %	Success rate of recovery	Definition of long-term culture, passages	Number of studies	(Refs.)
Ogawa *et al*, 2017	NSCLC (LUAD)	-	A549 cell line	-	-	-		1	(48)
Li *et al*, 2020	NSCLC	I-III	PTT	71.43% (10/14)	-	100% (10/10)	-	1	(49)
Han *et al*, 2022	NSCLC	-	PTT	-	-	-	-	1	(50)
Wang *et al*, 2019	NSCLC (LUAD)	-	PTT	-	-	-	-	1	(51)
Zhang *et al*, 2021	NSCLC (LUAD)	I	PTT	-	-	-	-	1	(52)
Li *et al*, 2020 and 2021	NSCLC (LUAD)	I-III	PTT	80% (12/15)	-	-	-	2	(53,54)
Shi *et al*, 2020; Liu *et al*, 2022	NSCLC (LUSC, LUAD)	III	PTT/PDX	88% (57/65)	-	-	>10	2	(55,56)
Sachs *et al*, 2019; Dijkstra *et al*, 2018; Cattaneo *et al*, 2020; Dijkstra *et al*, 2020; Bie *et al*, 2021; Sándor *et al*, 2021; Kim *et al*, 2021; Padmanabhan *et al*, 2021; Yokota *et al*, 2021	NSCLC	-	PTT/PE	41-88%	7-92.7	-	>10	8	(57-65)
Tamura *et al*, 2018; Takahashi *et al*, 2019	NSCLC (LUSC, LASC)	-	PTT/PE					2	(66-67)
Ma *et al*, 2021	NSCLC (LUAD)	-	PTT/PE	-	-	-	-	1	(68)
Mazzocchi *et al*, 2022	NSCLC (LUAD)	IV	PE	-	-	-	-	1	(69)
Hu *et al*, 2021	NSCLC (LUAD, LUSC), SCLC	III-IV	PTT	79% (81/103)	-	-	-	1	(70)
Liu *et al*, 2022; Kim *et al*, 2019; Jung *et al*, 2019; Chen *et al*, 2020; Chen *et al*, 2022; Peng *et al*, 2022	NSCLC (LUAD, LCLC, LUSC, LASC), SCLC	-	PTT	58-87%	71	70% (39/56)	>10		(56,71-75)
Choi *et al*, 2021	SCLC	-	PTT	80% (8/10)	-	-	7-12	2	(76,77)

Table I. Continued.

First author/s, year/s	Type of cancer	Stage	Cell source	Success rate of organoid establishment	Success rate of pure LCO establishment, %	Success rate of recovery	Definition of long-term culture, passages	Number of studies	(Refs.)
Gmeiner *et al*, 2020	SCLC	-	PDX	-	-	-	-	1	(78)
Chen *et al*, 2022	SCLC	-	PDX	-	-	-	-	1	(79)
Redin *et al*, 2022	SCLC	-	PDX	-	-	-	-	1	(80)

LASC, lung adenosquamous carcinoma; LCO, lung cancer organoid; LCLC, large cell lung carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; NSCLC, non-small cell lung cancer; PDX, patient-derived xenograft; PE, pleural effusion; PTT, patient tumor tissue; SCLC, small cell lung cancer.

some studies have reported that pyroptosis plays an important role in the occurrence and development of lung cancer (88-90), it is not yet known if cancer cell line-based LCOs can be used for researching the mechanism of drug-induced lung cancer cell pyroptosis. To broaden the application scope of cancer cell line-based LCOs, these unknown factors require elucidation.

3. PDX-derived LCOs

As PDXs effectively maintain the characteristics of primary tumors, including the phenotype, genetic profile and TME, they are widely used as preclinical models to explore the mechanisms of tumorigenesis and development, screen anti-tumor drugs and discover novel therapeutic methods (91-94). However, the high cost and time-consuming process of developing PDXs limit their usage (95,96). To use the full advantages of PDXs while avoiding their disadvantages, investigators have developed PDX-derived organoid models that have already been applied to multiple tumor types, and shown to preserve the genomic and transcriptomic profiles, protein markers and drug response of primary PDXs (94,97-99). These models have been used to study the pathogenesis of lung cancer and screen antitumor drugs (Fig. 1). A total of four methods to create PDX-derived LCOs are presented in the current review (Table I) (55,78-80). Only one of these was used to establish PDX-derived organoids of NSCLC (55), while the other three were used to generate PDX-derived organoids of SCLC (78-80).

Shi *et al* (55) reported a method for the creation of PDX-derived organoids of NSCLC using relatively optimal culture medium, with short- and long-term PDX-derived LCO cultures. The study found that the PDX-derived LCOs reflected the histological and cell lineage characteristics, and drug sensitivity of the parental PDXs to a large extent, in both short- and long-term culture. Even following the prolonged culture of LCOs, the mutations, copy number landscape and gene expression profiles of organoids and primary PDXs were comparable. In addition, short-term PDX-derived LCO culture was able to establish tumoroid models rapidly, and the drug sensitivity of the LCOs was consistent with that of the parental PDXs. These findings indicate that these LCOs have certain application prospects in drug screening.

Delayed diagnosis, high aggressiveness, susceptibility to relapse and poor prognosis are the basic characteristics of SCLC (4,100-102). Although SCLC is divided into four subtypes based on expression of the transcription factors achaete-scute homolog 1, neuronal differentiation 1, POU class 2 homeobox 3 and Yes1 associated transcriptional regulator (103), there is no specific and effective treatment for each subtype (104). Traditional preclinical models perform poorly in the exploration of novel markers and treatment methods for SCLC (105). Therefore, it is urgently necessary to develop more efficient preclinical models. Tumor organoids have unique advantages in that regard, and researchers have been attempting to establish SCLC organoids (71,72,77-80). Gmeiner *et al* (78), Chen *et al* (79) and Redin *et al* (80) have each reported methods for the culture of SCLC PDX-derived organoids to explore drug resistance mechanisms and screen specific antitumor drugs. The study by Gmeiner *et al* (78) indicated that overexpression of the transcription factor

Table II. Characteristics of the culture medium in different methods for the establishment of lung cancer organoids.

		Culture medium									
						Stemness-related signaling pathway activators		Apoptosis signaling pathway inhibitors			
First author/s, year	Method	Medium	SFA	Amino acid	Growth factor	Wnt signaling	BMP signaling	TGF-β signaling	Rho-ROCK signaling	p38 MAPK signaling	(Refs.)
Ogawa *et al*, 2017	I	DMEM	-	-	FGF2, insulin, transferrin	-	-	-	-	-	(48)[a]
Zhang *et al*, 2021	II	DMEM/F12	N2/B27	-	EGF, FGF10, FGF2, HGF	-	-	-	-	-	(52)[b]
Tamura *et al*, 2018	II	FBIM002[c] (suspension culture)	-	-	-	-	-	-	-	-	(66)
Li *et al*, 2020	III	AdDMEM/F12	N2/B27	GM, Gln, NAM, NAC	EGF, FGF10, FGF2, PGE2, gastrin 1	Wnt3A, RSPO1	NOG	A83-01	-	SB202190	(49)[a]
Han *et al*, 2022	III	AdDMEM	B27	GM, NAM, NAC	EGF, FGF10, FGF2, PGE2	RSPO	NOG	A83-01	Y27632	SB202190	(50)
Wang *et al*, 2019	III	AdMEM/F12	B27	GM, NAC, NAM	EGF, FGF10, FGF2, DHT	RSPO	NOG	A83-01	Y27632	SB202190	(51)
Li *et al*, 2020	III	AdDMEM/F12	B27	GM, NAM, NAC	FGF10, FGF7	RSPO1	NOG	A83-01	Y27632	SB202190	(53)
Shi *et al*, 2020	III	AdDMEM/F12	B27	GM, NAC	EGF, FGF10, FGF4	CHIR99021	NOG	A83-01	Y27632	-	(55)
Sachs *et al*, 2019	III	AO medium; AdDMEM/F12	B27	GM, NAM, NAC	FGF10, FGF7	RSPO1	NOG	A83-01	Y27632	SB202190	(57)
Ma *et al*, 2021	III	LUAD organoid\culture medium (OmaStem®)[d]	-	-	-	-	-	-	-	-	(68)
Choi *et al*, 2021	III	AdDMEM/F12	N2/B27	-	FGF2, EGF	Wnt3A, RSPO1	NOG	A83-01	Y27632	-	(76)

Table II. Continued.

		Culture medium									
						Stemness-related signaling pathway activators		Apoptosis signaling pathway inhibitors			
First author/s, year	Method	Medium	SFA	Amino acid	Growth factor	Wnt signaling	BMP signaling	TGF-β signaling	Rho-ROCK signaling	p38 MAPK signaling	(Refs.)
Chen *et al*, 2022	III	DMEM/F12 (PRF)	B27	Glutamine, NAC, NAM	FGF10, EGF, FGF7	RSPO3	-	A83-01	Y27632	SB202190	(79)
Redin *et al*, 2022	III	DMEM/F12	B27	NAC, NAM	EGF, FGF, gastrin 1	RSPO	NOG	A83-01	Y27632	-	(80)[e]
Kim *et al*, 2019	IV	DMEM/F12	N2/B27	-	FGF2, EGF	-	-	-	Y27632	-	(71)
Hu *et al*, 2021	V	DMEM/F12	N2/B27	GM, NAC, NAM	EGF	-	-	A83-01	Y27632	SB202190	(70)[f]
Mazzocchi *et al*, 2022	VI	RPMI-5	-	-	-	-	-	-	-	-	(69)
Gmeiner *et al*, 2020	VI	DMEM	-	-	-	-	-	-	-	-	(78)

[a]Culture includes bovine serum albumin; [b]culture includes inactivated fetal bovine serum; [c]FBIM002 medium from the Fukushima Translational Research Project (Fukushima/Japan); [d]OmaStem® Lung Cancer Medium (Human), OM14 (Guangzhou/China); [e]culture includes HyClone serum; [f]culture includes forskolin and dexamethasone. adDMEM, advanced Dulbecco's modified Eagle's medium; AO, airway organoid; B27, B-27 supplement; BMP, bone morphogenetic protein; DHT, dihydrotestosterone; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; F12, Ham's F 12 nutrient medium; FGF, fibroblast growth factor; Gln, L-glutamine; GM, Glutamax; HGF, hepatocyte growth factor; LUAD, lung adenocarcinoma; MAPK, mitogen-activated protein kinase; N2, N-2 supplement; NAC, N-acetyl-l-cysteine; NAM, nicotinamide; NOG, Noggin; PGE2, prostaglandin E 2; PRF, phenol red-free; Rho, Ras homology; ROCK, Rho-associated kinase; RPMI-5, RPMI 1640 medium with 5% fetal bovine serum; RSPO, R-spondin; SFA, serum free additive; TGF-β, transforming growth factor-β; Wnt, wingless and int-1.

E2 promoter binding factor 1-3 caused the upregulation of thymidylate synthase and the increased malignancy of SCLC. To test the efficacy of thymidylate synthase inhibitors in the treatment of SCLC, the authors created SCLC PDX-derived organoid models and used them to demonstrate that SCLC is sensitive to CF10, a novel fluoropyrimidine polymer. Retinoic acid receptor-related orphan receptors (RORs), including RORα, RORβ and RORγ, participate in a variety of physiological and pathological reactions through ligand-dependent interactions with co-regulators (106). Chen *et al* (79) found that the high expression of RORγ improved SCLC cell growth and inhibited apoptosis, while the RORγ antagonists XY018 and GSK805 eliminated this effect in H446 and H1048 cells. These results were verified in SCLC PDX-derived organoids. Chemical library screening, and cellular thermal shift and surface plasmon resonance assays were used to identify N-hydroxyapiosporamide (N-hydap), as a potent and selective RORγ antagonist. N-hydap was more efficient at suppressing the growth and survival of cancer cells than GSK805 and XY018, which was confirmed using SCLC PDX-derived organoid models. The study by Chen *et al* (79) provides a new approach for the screening of targeted antitumor drugs. Redin *et al* (80) described another method for the generation of SCLC PDX-derived organoids, which they used to verify the curative effect of the YES1-specific inhibitor CH6953755 on SCLC. All three methods involve the use of newly established, not passaged, PDX-derived organoids to explore the mechanism of lung cancer development and screen antitumor drugs. The study findings indicate that short-term culture PDX-derived LCOs are reliable in the preclinical research of lung cancer.

The aforementioned four methods can be divided into two categories, those using relative optimal culture medium and those using a hydrogel scaffold. The methods developed by Shi *et al* (55), Chen *et al* (79) and Redin *et al* (80) are in the former category, while that developed by Gmeiner *et al* (78) is in the latter category. The details of these methods are presented in Table II.

These studies used four different digestion methods to dissociate tissues into single cells. Digestive strategies using the combination of Liberase TM, which comprises a combination of collagenase I, II and thermolysin, with TrypLE involve lower concentrations of enzymes with higher digestive efficiency compared with those that use a combination of collagenase II and TrypLE. Digestive methods using collagenase IV alone also have a higher digestive efficiency compared with those that using a combination of collagenase II and TrypLE. This may be due to collagenases I and IV having higher activity than collagenase II. Notably, treatment with TrypLE, a recombinant trypsin-like protease (107) used in two different digestive enzyme combinations, has been shown to result in a significantly higher cell viability compared with trypsin (108). Any pure collagenase is not able to effectively dissociate tissues into single cells (109). The combination of any collagenase and TrypLE may have a greater ability to generate single cells from tissues than either enzyme used alone.

Cell-Titer Glo reagents manufactured by Promega Corporation were used to measure the viability of organoids in the method reported by Chen *et al* (79). To avoid interference with the detection of fluorescence, phenol red-free Dulbecco's modified Eagle's medium/Ham's F 12 nutrient medium (DMEM/F12) was used.

Chen *et al* (79) added glutamine to the culture medium. Glutamine serves as a nitrogen source for the biosynthesis of a number of important substances, including nucleotides, nicotinamide adenine dinucleotide, the protein glycosylation precursor glucosamine-6-phosphate, and asparagine (110-112). Although mammalian cells have the ability to synthesize glutamine *de novo*, numerous types of cancers cannot grow and proliferate in an environment lacking exogenous glutamine (113). As DMEM/F12 contains glutamine, most methods that use DMEM/F12 to culture cells do not involve the addition of extra glutamine to the culture medium (52,71,80). More importantly, glutamine naturally breaks down to form ammonia (114), which is toxic for mammalian cell cultures (115). Therefore, excessive glutamine may be disadvantageous for cells. In one study, the authors replaced the glutamine in DMEM/F12 with L-alanyl-L-glutamine, known as Glutamax, and found it to be a suitable substitute because of its improved solubility and stability during cell culture (116).

CHIR99021 was added to the culture medium in the procedure described by Shi *et al* (55); this LCO culture method is the only approach discussed in the present review to include CHIR99021 in the culture medium. CHIR99021 activates the wingless and int-1 (Wnt) signaling pathway via the inhibition of glycogen synthase kinase 3β (117,118). Activation of the Wnt signaling pathway maintains the stemness of stem cells and organoid formation (119,120). CHIR99021 has been used to generate organoids from other types of cancer tissue, such as bladder cancer tissue (121). Shi *et al* (55) reported that their method of establishing LCOs has a higher success rate (88%) than other methods, which may be due to the addition of CHIR99021. However, this hypothesis requires verification by additional experiments.

Gmeiner *et al* (78) used HyStem-HP Hydrogel as a 3D scaffold for organoid culture. The type of 3D scaffold has numerous desirable features, including high transparency and cellular affinity, and being easy to standardize. These suggest it has application prospects in the field of organoid culture.

Redin *et al* (80) added 10% HyClone serum to the LCO culture medium. This is the only method covered in the present review that involves supplementation of the LCO culture medium with serum. Although some studies have demonstrated that the proper addition of fetal bovine serum (FBS) enhances organoid formation (122-124), FBS contains unknown components that might cause the organoid culture to fail (125). Therefore, most methods of organoid culture do not include FBS. Nonetheless, the role of FBS in organoid culture is worthy of exploration.

Redin *et al* (80) added gastrin I to their culture medium. Gastrin is an important growth factor for digestive system tumors (126), which can prolong the survival time of digestive system tumoroids (127-134), and is often added to the medium used to culture them. However, it is not clear whether gastrin addition is beneficial to lung tumoroid culture.

PDX-derived LCOs have certain advantages compared with cancer cell line-based or patient-derived LCOs: i) The phenotype, genetic profiles and heterogeneity of parental tumors are more effectively preserved in PDX-derived LCOs than in lung cancer cell line-based LCOs; ii) compared with patient

samples, PDXs are easier to obtain and can undergo long-term expansion by passaging, which ensures the repeatability and sustainability of experiments; iii) the establishment of PDX-derived LCOs has lower requirements for ethical approval compared with patient-derived LCOs; and iv) PDX-derived LCOs are easier to develop than patient-derived LCOs (55). However, there is a clear disadvantage of PDX-derived LCOs, in addition to the defects common to all LCOs: PDX-derived LCOs may be contaminated by mouse cells during short-term culture, which is likely to affect the phenotypic identification of tumors, genotype analysis or antitumor drug screening. These factors may restrict their application.

4. Patient-derived LCOs

Patient-derived LCOs are ideal for researching the mechanism of initiation, development and drug resistance of lung cancer, and for exploring new biomarkers, antitumor drugs and treatment protocols. The samples used to create patient-derived LCOs mainly originate from cancer tissue, including that obtained during surgery or biopsy, or from the exfoliative tumor cells present in pleural effusion (PE) (Fig. 1). In the current review, 13 methods used to generate LCOs from patient samples are presented (Tables I, II, SI and SII) (49-53,55,57,66 ,68-71,76). These involve all six categories of methods used to establish organoids. The success rates of organoid and pure LCO establishment using these methods are variable (57,60), which might influence subsequent mechanistic research and drug screening. Therefore, it is necessary to compare and analyze the details of the methods investigated in these studies to select the ideal method for the culture of LCOs.

The relative optimal culture medium method is the most popular, and the most representative method among all those reported is that described by Sachs *et al* (57). Although different laboratories generated LCOs via similar methods, the success rate of organoid establishment ranged from 41 to 88%. The success rate range of pure LCO establishment was also diverse, ranging from 7 to 92.7% (60,63). Dijkstra *et al* (60) reported lower success rates of organoid establishment (41%) and LCO establishment (17%) compared with other studies (57,63). Patients with stage IV adenocarcinoma accounted for 78% of all patients in the study by Dijkstra *et al* suggesting that the low success rates might be due to the degree of tumor malignancy. Kim *et al* (63) used 77 malignant effusion samples, three brain metastasis samples, a single bone metastasis sample and two primary lung tumor samples from patients with advanced LUAD to successfully generate LCOs, and the success rates of organoid and pure LCO establishment were 83 and 92.7%, respectively. According to these results, it can be concluded that samples from malignant effusions or metastatic foci easily form pure cancer organoids. This may be due to the airway organoid (AO) culture medium being more suitable for normal airway epithelial cell growth and samples from malignant effusions or metastasis foci being less easily contaminated by normal epithelial cells than those from primary lung tissues. If normal epithelial cells are not removed during the pretreatment process, cancer cells are rapidly overtaken by normal epithelial cells during organoid culture, leading to failure of the cancer organoid culture (135,136). However, surgically resected tumor tissues are a prominent source of material for LCO culture. To make full use of these tissues, a number of researchers have sought to devise improved culture methods. Kim *et al* (71) developed a new method using LCO suboptimal medium free of Wnt3a, Noggin and A83-01 to culture lung tumoroids, which improved the growth of cancer organoids and inhibited that of normal epithelial organoids. When surgically resected tumor tissues were used to establish organoids, the success rates were 58-87% (71,73), which were comparable with the 41-88% success rates of the method described by Sachs *et al* (57). Moreover, a success rate of pure LCO establishment of 71% was observed (73), which is higher than the 17% reported by Dijkstra *et al* (60). Therefore, it is speculated that LCO suboptimal medium may be superior to AO medium in cancer organoid culture. It is noteworthy that that the pretreatment method used by Kim *et al* (71) differed from that used by Sachs *et al* (57). Sachs *et al* (57) used only collagenase to digest tissue. By contrast, Kim *et al* (71) used DNase and collagenase/dispase to isolate single lung cancer cells. Gohi *et al* (137) reported that digestion using a combination of collagenase and DNase is conducive to the maintenance of cell surface antigen integrity and cell activity. These findings are meaningful for subsequent cancer organoid culture. Therefore, the pretreatment method reported by Kim *et al* (71) may partially contribute to the high success rates of organoid establishment and pure LCO establishment that were obtained. The importance of pretreatment methods was supported by Hu *et al* (70), who found mechanical processing to be more beneficial for tumor organoid formation than enzymatic digestion, with the latter being beneficial for normal organoid formation. Moreover, their study revealed that a medium without R-spondin and Noggin is conducive to the establishment of pure LCOs. Overall, it may be easier to generate higher purity LCOs with an acceptable success rate by the use of mechanical digestion and the suboptimal culture medium method (Tables I; SI).

In addition to the success rates of organoid establishment and pure LCO establishment, researchers have evaluated the sustainability of LCOs, which includes the expansion and efficient reconstitution of cryopreserved LCOs. Short-term organoid culture is sufficient to perform drug screening for patients whose cancer tissue has been used to generate LCOs. However, the long-term expansion of tumor organoids and efficient reconstitution of cryopreserved organoids are necessary to provide sufficient tumor organoids for the establishment of LCO biobanks for use in long-term studies, such as those for antitumor drug discovery, the elucidation of drug resistance mechanisms and improvement of treatment protocols. Four methods of LCO long-term culture are covered in the present review (55,57,71,76). In terms of time, different definitions of tumoroid long-term culture have been proposed. However, as regards passage number, the definitions of tumoroid long-term culture are similar (>10 passages). The results of the study by Yokota *et al* (65) showed that the AO medium is a more robust tumor organoid culture medium than the media used by Kim *et al* (71) and Shi *et al* (55). While AO medium is suitable for the growth of all lung epithelial cells, some LCOs with particular mutations may also be long-term expanded in AO medium (65). The study of Yokota *et al* (65) revealed that activation of the Wnt/β-catenin pathway is a prerequisite for

the maintenance of certain LCOs (*TPM3-ROS1; TP53*^{K120Sfs*3}) in long-term culture. This was verified by Choi *et al* (76), who found that Wnt3A and R-spondin1 do not promote SCLC tumoroid formation but play important roles in the long-term culture of SCLC tumoroids. Therefore, if the specific mutations of lung cancer tissues are unknown, AO medium appears to be a suitable choice for lung tumoroid long-term culture. Nevertheless, the removal of activators of the Wnt/β-catenin pathway did not influence other lung tumoroids in long-term culture, for example LCOs (*BRAF*^{G469A}*; TP53*^{T155P}) (65). These results are consistent with those of Kim *et al* (71), who found that most LCOs could be long-term expanded in LCO suboptimal medium. In general, the efficient reconstitution of cryopreserved LCOs is essential for the establishment of LCO biobanks. The cell viability of LCOs before cryopreservation and the methods of cryopreservation used determine the success or failure of recovery. Although LCO suboptimal medium contains fewer reagents than relative optimal culture medium, Kim *et al* (71) reported that tumoroids cultured in the former medium had a high recovery success rate (70%) after freezing. This indicates that LCO suboptimal medium can effectively sustain cell viability and provide high recovery success rates. In a study by Shi *et al* (55), long-term culture was achieved for 15% of NSCLC organoids, and these organoids had good cell viability, with all of them being recoverable after >1 year of cryopreservation and continued passaging. Hu *et al* (70) chose tumoroids with fast growth rates and short generation intervals for cryopreservation. Of the five lung tumoroids used, four were successfully cryopreserved and thawed. These results showed that cell viability is a critical factor in the efficient reconstitution of cryopreserved LCOs. Therefore, it is necessary to screen for high-viability LCOs before cryopreservation.

Jung *et al* (72) developed a one-stop microfluidic device for use in tumoroid culture and drug screening. The microphysiological system allows the quantity of lung tumoroids and concentration of drugs to be controlled, which facilitates the standardization of drug screening. Tamura *et al* (66) reported a suspension culture method which they used to generate patient-derived tumor organoids for the Fukushima Translational Research Project (Fukushima-PDOs). However, a long culture time of 3-6 months was required to successfully establish Fukushima-PDOs, which is unsuitable for the rapid screening of drugs for patients who have provided cancer tissues. The Fukushima-PDOs were found to be able to expand long term, which may contribute to the establishment of LCO biobanks for use in long-term studies. As uniform cell sizes are important for high-throughput screening, the authors used CellPet FT to mince the tumoroids and obtain organoids of similar sizes. The study by Dijkstra *et al* (58) described how tumor-reactive T cells can be produced through the coculture of peripheral blood lymphocytes and tumor organoids. These T cells specifically kill tumoroids, and the method enables T-cell-based therapies and interactions between T cells and tumor cells to be researched *in vitro*.

In general, patient-derived LCOs are improved preclinical models is comparison with other traditional models for the following reasons: i) Patient-derived LCOs have excellent fidelity because they are directly structured with patient tumor tissue or PE; ii) patient-derived LCOs can be generated in weeks or even days, and short-term cultured organoids are able to predict the responses of patients to antitumor drugs. The integrated superhydrophobic microwell array chip (InSMAR-chip) shortens the time for drug screening to 1 week, thereby saving precious time for patients requiring treatment (70); and iii) the combination of patient-derived LCOs and microfluidic devices can standardize drug screening to help clinicians in the formulation of appropriate medication plans. Patient-derived LCOs also have certain limitations: i) As samples originate from patients with lung cancer, they are of high value and if the establishment of patient-derived LCO fails, it is challenging to compensate for the loss; ii) although patient-derived LCOs can be long-term expanded, immortality of patient-derived LCOs *in vitro* has not yet been achieved.

A number of issues remain to be resolved including: i) How the optimization of patient-derived LCO culture methods can be achieved to eliminate normal organic contamination and maintain long-term lung tumoroid culture and even achieve immortality; ii) how the standardization of patient-derived LCO culture methods can be accomplished; iii) how the creation of a co-culture system of patient-derived LCOs and the microenvironment can be realized; and iv) how the success rate of LCO establishment from patient biopsy samples can be enhanced.

5. Conclusions

LCOs derived from three major resources greatly promote preclinical research, with applications including the exploration of cancer mechanisms, searching for novel tumor biomarkers, screening of antitumor drugs and improving treatment plans. The three major types of lung tumoroids, which are cancer cell line-based LCOs, PDX-derived LCOs and patient-derived LCOs, are complementary, and the results of studies on the three major types of lung tumoroids have provided a more comprehensive understanding of the pathogenesis of lung cancer (48,50,52). Furthermore, there are six different categories of methods that can be used to establish LCOs. Appropriate models or methods can be selected based on the requirements of researchers. However, the present review puts forth several suggestions: i) AO medium is a more robust tumor organoid culture medium than other media used for the long-term culture of LCOs; ii) the mechanical digestion and suboptimal culture medium method is more conducive to the establishment of pure LCOs, while the mechanical dissociation method is beneficial for the passage of organoids; iii) malignant PE samples appear to have a high tendency to establish pure LCOs; and iv) the use of CellPet FT to process organoids is beneficial for standardization in high-throughput screening. The achievement of standardization is important in lung tumoroid culture. The combination of bioengineering technology, including microfluidic devices and the InSMAR-chip, and lung tumoroid culture has accelerated the standardization of lung tumoroid applications to some degree. It is hypothesized that with technical progress, lung tumoroids will have broader application prospects in the future.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

Not applicable.

Authors' contributions

QZ wrote the manuscript and prepared the figures. MZ conceived and designed the study. MZ revised the manuscript critically for important intellectual content. Both authors read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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