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Advantages and research progress of three-dimensional culture systems for lung cancer drug screening (Review)

YIHE TIAN[1,2*], HAI LIU[1,3*], MINHONG ZHANG[1], AIPING CUI[1,2], HAO CHEN[1,4], LEI SHI[1], JUNYAO ZHU[1], MINGCHUN LI[5], BIAO WANG[1], HAO HUANG[3,4] and JIANQIONG YANG[1,6,7]

[1]The Clinical Medicine Research Center of The First Clinical Medical College, Gannan Medical University, Ganzhou, Jiangxi 341000, P.R. China; [2]School of Rehabilitation Medicine, Gannan Medical University, Ganzhou, Jiangxi 341000, P.R. China; [3]Jiangxi Province Key Laboratory of Pharmacology of Traditional Chinese Medicine, Gannan Medical University, Ganzhou, Jiangxi 341000, P.R. China; [4]College of Pharmacy, Gannan Medical University, Ganzhou, Jiangxi 341000, P.R. China; [5]Department of Oncology, First Clinical Medical College, Gannan Medical University, Ganzhou, Jiangxi 341000, P.R. China; [6]Ganzhou Key Laboratory of Antitumor Effects of Natural Products, Gannan Medical University, Ganzhou, Jiangxi 341000, P.R. China; [7]Ganzhou Key Laboratory of Osteoporosis Research, Gannan Medical University, Ganzhou, Jiangxi 341000, P.R. China

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Abstract. Conventional two-dimensional cell cultures predominantly grow as monolayers, lacking the complex spatial architecture of *in vivo* tumors. However, three-dimensional (3D) tumor culture systems can overcome these limitations by reconstituting cell-cell and cell-matrix interactions, thereby recapitulating the essential hallmarks of solid tumors, including spatial gradients of oxygen, growth factors and metabolites. 3D tumor models are essential preclinical tools in lung cancer research, providing valuable resources for studying cancer biology and developing novel anticancer drugs. The present review examines different 3D culture methods, highlighting the benefits and applications of multicellular tumor spheroids and organoid models in the screening process for anti-lung cancer drugs. The present review aims to provide a novel perspective on tumor biology and *in vitro* drug screening, and a theoretical basis for developing and applying 3D culture models.

Correspondence to: Professor Jianqiong Yang, The Clinical Medicine Research Center of The First Clinical Medical College, Gannan Medical University, 23 Qingnian Road, Zhanggong, Ganzhou, Jiangxi 341000, P.R. China
E-mail: yangjianqiong2010@163.com

[*]Contributed equally

Abbreviations: 3D, three-dimensional; MCTS, multicellular tumor spheroid; NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; LUAD, lung adenocarcinoma; TME, tumor microenvironment; ECM, extracellular matrix; MSC, mesenchymal stem cell; CAR, chimeric antigen receptor; PTX, paclitaxel; nCmP, nanocomposite microparticle; LCO, lung cancer organoid

Key words: 3D culture, lung cancer, MCTS, LCO, drug screening

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1. Introduction

Lung cancer is one of the most common causes of cancer-related mortality worldwide, accounting for 18.7% of all cancer fatalities (1). The incidence of lung cancer in China has shown a persistent upward trend since 2016 (2). Despite advancements in early detection and standardized treatment protocols for patients with lung cancer, the majority of cases are still diagnosed at advanced stages with poor prognosis (3,4). Histologically, lung cancer is categorized into two main types: Non-small cell lung cancer (NSCLC; 85%) and small cell lung cancer (15%) (1). One study found that among patients with epidermal growth factor receptor (EGFR)-mutated NSCLC treated with EGFR tyrosine kinase inhibitors, after stratifying patients into risk groups based on a scoring system incorporating age, performance status, metastatic sites (liver, brain, bone, pleura), and disease control rate, the 5-year survival rate in the low-risk group was 23.4% (5). NSCLC is divided into two principal histological subtypes: Lung squamous cell carcinoma and lung adenocarcinoma (LUAD) (6). Currently, the mechanisms of drug action against NSCLC include promoting apoptosis (7) and inducing cellular autophagy in tumor cells (8). However, early-stage NSCLC is typically asymptomatic and often diagnosed at advanced stages, resulting in more challenging treatment processes with a median survival period of <1 year (9). Therefore, establishing *in vitro* biological models that are rapid and efficient, relatively low-cost, operationally simple, and

capable of accurately replicating both the genetic characteristics of *in vivo* tumors and their responses to therapeutic agents holds notable importance for oncology research.

Three-dimensional (3D) culture is recognized as the most promising *in vitro* model for oncological drug testing, as it can recapitulate key *in vivo* tumor features, including the cellular heterogeneity, intercellular signaling, structural architecture, extracellular microenvironment, growth kinetics, gene expression profiles and drug resistance mechanisms (10,11). Spheroids represent one of the simplest and most effective 3D culture models, and are formed through the self-assembly of multiple cell types (e.g., tumor cells, stromal fibroblasts and immune cells) (12). The complex niche environment surrounding neoplastic cells, referred to as the tumor microenvironment (TME), comprises diverse cellular components (such as tumor endothelial cells, cancer-associated fibroblasts and immune cells) and extracellular constituents (such as cytokines, growth factors, hormones and exosomes) (13). Tumor-stroma interactions dynamically regulate multiple biological characteristics of tumor cells, including proliferation, migration and drug resistance (14). The TME governs fundamental processes of tumorigenesis, progression and metastasis, and profoundly impacts pharmacological therapeutic efficacy (15). Due to their enhanced controllability of microenvironmental variables, 3D cell culture systems are increasingly being employed in biomedical research domains such as developmental biology, regenerative medicine, tissue engineering and oncology (16,17). Therefore, the present review aims to summarize the different 3D culture methods and the research progress on their use for drug screening in lung cancer.

The present review is a narrative overview of advances in 3D culture systems for lung cancer drug screening. The literature search was performed in three electronic databases, including PubMed (https://pubmed.ncbi.nlm.nih.gov), Web of Science (https://www.webofscience.com) and ScienceDirect (https://www.sciencedirect.com). The search period covered publications from June 1997 to April 2026. The main key words used were '3D culture', 'lung cancer', 'MCTS', 'LCO' and 'drug screening'. Articles were initially screened by reviewing the titles and abstracts, after which 115 relevant publications were selected for detailed full-text examination. All of these were ultimately included in the present review.

2. Research models

Two-dimensional (2D) culture models. The current mainstream method for *in vitro* cell culture is 2D culture. Cells grown in 2D monolayers have equal access to nutrients and growth factors present in the culture medium, resulting in uniform growth and proliferation (18). Although the preferred *in vitro* 2D culture model is standardized and simple, it is a monolayer. The cells cultured using this method exhibit notable differences in both morphology and architecture compared with their *in vivo* counterparts under physiological conditions. The 2D culture model cannot reproduce the TME to mimic the real cellular environment of tumors in the human body (19). In addition, it cannot mimic cell-cell and cell-extracellular matrix (ECM) interactions, nor be used to study the complex spatial organization of cells and cell interactions (20). Cells cultured in 2D systems experience homogeneous access to nutrients, oxygen and pharmaceutical agents within the culture medium (21). Consequently, when utilizing monolayer-cultured tumor cells as *in vitro* models for anticancer drug testing, substantial discrepancies emerge compared with *in vivo* models. These differences compromise the efficiency of drug screening processes, potentially leading to miscalculations of therapeutic dosages and subsequent impairment of clinical efficacy (19).

Animal models. Animal models provide a more physiologically relevant system, since the tumor develops within a complex and dynamic microenvironment (22). Animal models recapitulate the *in vivo* context, including the complex TME. Common examples include genetically engineered mice (23), zebrafish (24), xenografts (25) and allografts (26). Animal models are indispensable for lung cancer research, providing critical insights into the biology and progression of the disease, and potential therapeutic strategies. By utilizing these models, researchers can shed light on the intricate interactions between cancer cells and the TME, which is vital for understanding tumor dynamics and treatment efficacy (27). While animal models can simulate physiological complexity at the whole-organism level, they present insurmountable challenges in terms of external validity due to poor applicability to clinical settings and inherent interspecies differences between animals and humans (28). Systematic studies on the predictive value of animal models have revealed weak associations between animal-derived data and human clinical outcomes (29,30). The reliability of animal models for drug screening is further compromised by variables such as animal sex, sample size, age and stress levels in experimental animals (30). Furthermore, clinical trial failures often stem from patient heterogeneity, a critical factor that conventional methodologies and animal models fail to assess adequately (31). These limitations have driven the development of precision medicine and corresponding advanced drug screening approaches (32).

3D culture models. Current mainstream 3D cancer models primarily include multicellular tumor spheroids (MCTSs), organotypic multicellular spheroids, tumor-derived organoids and tumor spheroids (33). As illustrated in Fig. 1, these lung cancer models can be generated from different cellular sources-such as cancer cell lines, patient tissues or circulating tumor cells - and can be further co-cultured with other cell types to increase complexity, ultimately serving as powerful platforms for biomedical applications including drug screening and personalized medicine. Organoids and tumor spheroids are 3D culture models that provide more physiologically relevant insights into tumor biology than traditional 2D cultures (34). However, organoids and tumor spheroids differ notably in their culture methods, structural characteristics and applications. Spheroids generally form through the self-assembly of single-cell suspensions and do not require exogenous scaffolding. By contrast, organoids represent more complex 3D structures that typically rely on scaffold-based culture systems (35). Organoids closely mimic the architecture and function of native tumors and often incorporate multiple cell types along with elements of the TME (36). Organotypic culture is a culture method in which undamaged organs in the living body are isolated and cultured *in vitro*, so that they can

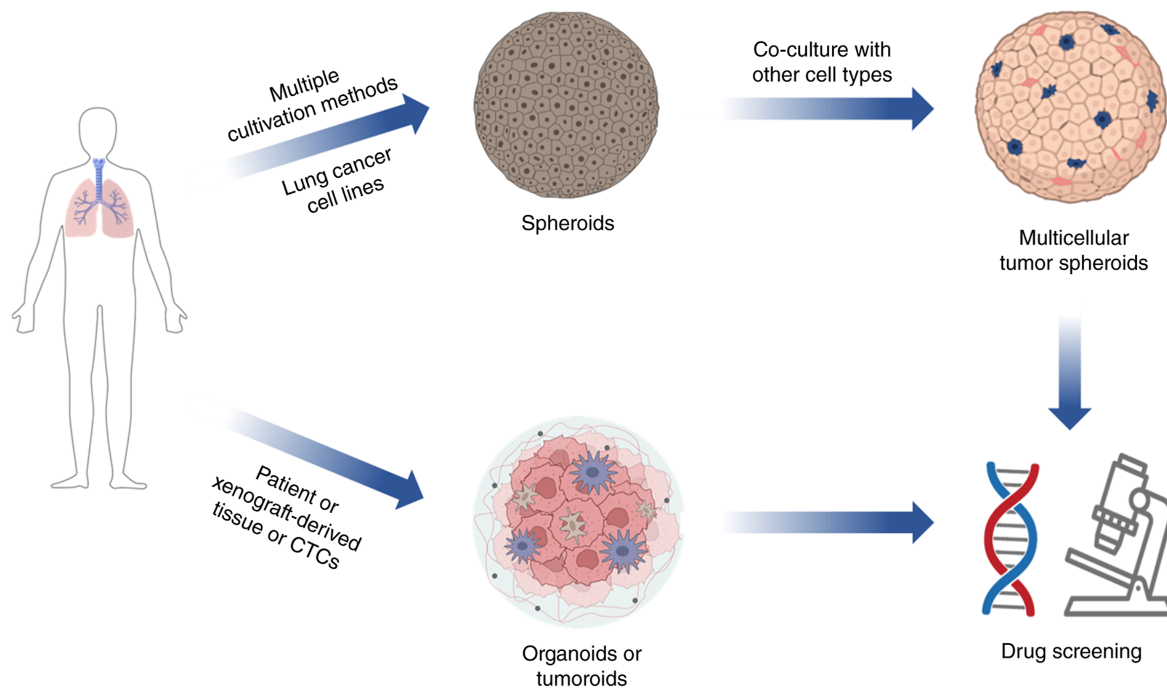

Figure 1. Schematic diagram of constructing 3D tumor models from different cellular sources for drug screening. Primary pathways are illustrated for developing physiologically relevant 3D tumor models. Lung cancer cell lines serve as a reproducible source for generating spheroids using multiple cultivation methods. The complexity and relevance of these spheroids can be enhanced through co-culture with other cell types (such as fibroblasts and immune cells) to form advanced multicellular tumor spheroids. Alternatively, clinically relevant models with preserved tumor heterogeneity can be established directly from patient or xenograft-derived tissue or CTCs, yielding organoids or tumoroids. Both types of advanced 3D models constitute powerful platforms for a broad spectrum of biomedical applications, including drug discovery, personalized medicine and fundamental cancer research. 3D, three-dimensional; CTC, circulating tumor cell.

maintain 3D growth *in vitro* and maintain the original tissue structure. Organotypic culture can successfully preserve the intact TME (37). However, organ culture has inherent limitations: First, obtaining organ tissues is severely restricted by their limited availability. Second, once the organ is isolated and cultured, the proliferative activity of its cells decreases notably and the proliferative vigor is challenging to maintain (38). To solve the aforementioned complications, a 3D culture model in which tumor cells grow and proliferate *in vitro* to form tumor spheroids has emerged. The method is usually applied to cell lines from solid tumor spheroids. Notably, such cell lines from solid tumor spheroids survive well in avascular conditions (39). *In vitro*-constructed MCTSs are of great value in cancer research, with applications including, but not limited to, radiobiological studies (40) and novel chemotherapeutic drug development (41).

3D culture technology represents an advancement beyond conventional 2D culture systems. Compared with 2D systems, 3D culture establishes more physiologically relevant gradients of oxygen, nutrients, metabolic wastes and signaling molecules. 3D culture facilitates comprehensive cell adhesion, signal transduction and differentiation, while promoting cell-cell and cell-matrix interactions, creating a microenvironment that is an improved simulation of *in vivo* conditions. This enables cultured tumor cells to exhibit characteristics closely resembling those of solid tumors *in vivo* (16). As shown in Fig. 2, these characteristics include spatial gradients of oxygen, nutrients and drugs from the periphery to the core, as well as the coexistence of proliferating, quiescent and necrotic cell populations (42). Consequently, *in vitro* 3D cell culture models are being progressively developed and implemented for chemotherapeutic drug screening applications. Compared with animal studies, 3D cell culture systems offer distinct advantages. Firstly, they circumvent ethical concerns associated with human experimentation while addressing the critical discordance observed between animal models and clinical outcomes (with ~90% of *in vivo* animal experiment results failing to align with clinical trial findings) (43). Furthermore, 3D cultures substantially reduce drug screening costs, shorten the screening timeline, and enable the incorporation of diverse cell types and enhanced physiological complexity. Additionally, this approach overcomes the limitations inherent to animal models, including prolonged experimental cycles, high costs and incompatibility with high-throughput screening approaches (44).

3. Spheroid culture methods

MCTSs are commonly referred to as 3D *in vitro* models that can mimic the tissue microenvironment. MCTSs are spherical structures of a specific diameter with a dense core, composed of a population of tumor cells. The central region of an MCTS typically becomes hypoxic due to the limited access of cells to oxygen and nutrients. By contrast, the cells at the edge of the spheroid can grow normally (45). By combining advantages such as ease of construction, high reproducibility and compatibility with high-throughput drug screening, MCTSs have become a commonly used model for preclinical antitumor drug evaluation (46-49). The formation of an MCTS is roughly divided into the following three steps

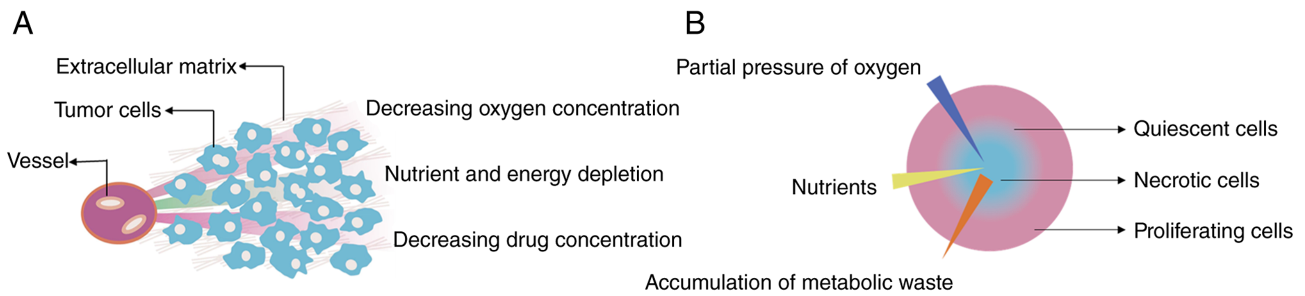

Figure 2. Physiological characteristics of solid tumors *in vivo* and basic structure of three-dimensional tumor spheroids. (A) Schematic representation of the *in vivo* solid tumor microenvironment, illustrating gradients of oxygen concentration (decreasing partial pressure of oxygen), nutrients/energy depletion and decreasing drug concentration from blood vessels toward the tumor interior. (B) Corresponding cellular heterogeneity and physicochemical gradients within the tumor, showing proliferating cells near vessels, quiescent cells in intermediate regions, necrotic cells in the core and accumulation of metabolic waste.

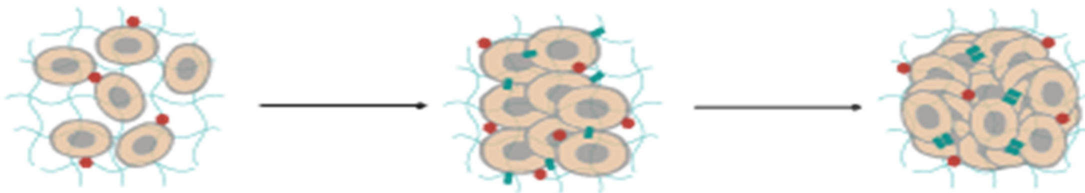

Figure 3. Formation process of MCTSs. This figure illustrates the sequential stages and underlying cellular mechanisms involved in the formation of a MCTS. Firstly, a suspension of individual cells begins to aggregate and gradually form loosely adherent tumor spheroids. In this step, interactions between cell surface integrins and extracellular matrix components facilitate the initial aggregation of individual cells. Second, E-calmodulin enhances the strong adhesion of the initial cell aggregates by binding to peripheral cellular calmodulin, promoting further aggregation of loosely adherent spheroids. In addition, the β-catenin complex promotes cell signaling, and actin also affects aggregation and stemness by facilitating contact between neighboring cells, resulting in the formation of MCTSs with strong adhesion. MCTS, multicellular tumor spheroid.

(Fig. 3) (50,51). First, cells form loose aggregates through integrin-ECM interactions. Then, cadherin expression is upregulated, initiating cell compaction. Finally, homophilic cadherin-cadherin interactions mediate the formation of discrete, dense spheroids that continue to grow. Current construction methods include the spinner culture, pellet culture and hanging-drop method. The advantages and disadvantages of different MCTS construction methods are summarized in Table I (52-59).

Lung cancer organoids (LCOs) are generated by culturing lung cancer stem cells derived from patient tissue samples in an ECM such as Matrigel, with the addition of growth factors to promote their proliferation and differentiation (60). As the same malignant tumor shows different genetic characteristics and phenotypic heterogeneity among different patients, and tumor tissues in different parts of the body of the same patient, personalized precision medicine has become a novel trend in tumor treatment, and the emergence of tumor organoids provides an opportunity for precision medicine (61). Individualized therapy is the latest concept in lung cancer treatment, which refers to the development of an individualized treatment plan with the best efficacy and the least toxicity according to the biological characteristics of the tumor, in order to maximize the clinical benefit to the patient (62,63).

As early as 2009, small intestinal organoids with intestinal structure were successfully cultured using adult stem cells derived from the mouse intestine, which led to an increase in organoid research (64). Novel 3D cell models have been used for drug screening, predicting patient response to therapy and providing guidance for personalized medication, which has been considered a breakthrough in tumor stem cell technology in the last decade (65). Organoid models constructed from tissue samples from patients with lung cancer can reproduce the histomorphology and biomarker characterization of tumors in patients, maintain tumor heterogeneity and exhibit drug sensitivity similar to that of their lung cancer tissues of origin. As illustrated in Fig. 4A, tumor organoids derived from patient tumor tissues or biopsy samples can be used for high-throughput drug screening, genetic testing, discovery of new therapeutic targets and the establishment of a tumor organoid biobank, thereby supporting basic research and drug development (66). In parallel, Fig. 4B shows the clinical application pathway: Patient-derived tumor organoids are subjected to drug sensitivity screening to guide individualized treatment decisions (67). Together, these organoid models serve a key role in individualized drug administration and the prognosis of lung cancer, and have the potential to be used as reliable preclinical models for lung cancer research (68). Organoid production methods are generally divided into three

Table I. Technical methods, principle, advantages and disadvantages of multicellular tumor spheroid construction methods.

First author/s, year	Technical methods	Principle	Advantages	Disadvantages	(Refs.)
Baral *et al*, 2026	Hanging-drop	Uses surface tension and gravitational force	Easy, convenient operation and low cost	Only suitable for short-term spherical culture, the medium is easy to evaporate, and the medium cannot be changed frequently	(52)
Ryu *et al*, 2019	Pellet culture	Uses centrifugal force to concentrate cells	Generates uniformly sized and shaped spheres in a short period of time, enabling precise control of the cell culture environment	Unable to be mass-produced	(53)
Pang *et al*, 2025	Microfluidic chip	Cell aggregation in microwells via microfluidic flow	Ability to rapidly form large, more homogeneous spheres of cells compared with conventional methods (e.g., the hanging-drop or liquid overlay method)	Higher costs	(54)
Zhang *et al*, 2024	3D bioprinting	Building of cell spheres with 3D printers.	Ability to accurately control the size of the sphere.	Higher costs	(55)
Moghe *et al*, 2026	Rotating wall vessel	Uses constant circular rotation of the vessel to create a low-shear, microgravity environment that promotes cell aggregation into spheroids	Ability to promote cell proliferation and differentiation, as well as mass production of cell spheres	Self-assembly of cell spheres cannot be observed in real time	(56)
Depresle *et al*, 2025	Liquid overlay	Overlay uses non-adhesive materials to inhibit cell attachment	Automated production of spheres with uniform morphology and size, and easy dosing or medium change	Experimental instruments are costly and the culture plates (or the non-adherent coating) are not reusable, requiring a lot of labor and material resources	(57)
Dauphin *et al*, 2025	Spinner culture	Use continuous stirring to keep cells in suspension and promote cell-cell aggregation	Mass production is possible	Shear stress-induced cell damage; unsuitable for shear-sensitive/anchorage-dependent cells	(58)

Table I. Continued.

First author/s, year	Technical methods	Principle	Advantages	Disadvantages	(Refs.)
Haisler *et al*, 2013	External force	Use of external forces to levitate cells	Suitable for long-term culture, high versatility, easy to operate, low cost, short incubation time	Susceptible to external electric fields	(59)

3D, three-dimensional.

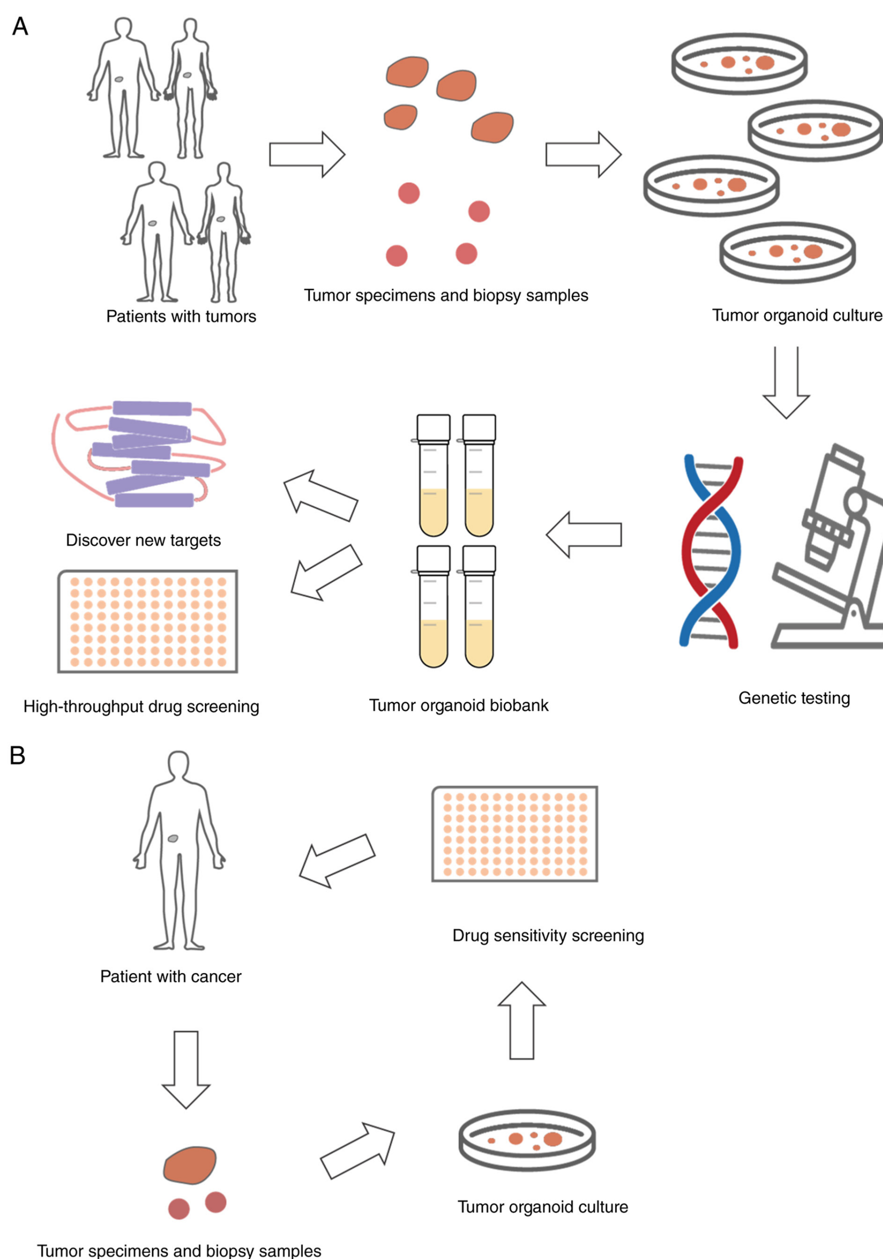

Figure 4. Application pathways of tumor organoids in basic research and clinical translation. The figure is primarily divided into two directions: (A) Application in personalized precision medicine and drug development. Tumor tissue or biopsy samples are obtained from patients with cancer for tumor organoid culture. Successfully cultured organoids can be used for high-throughput drug screening to guide personalized clinical treatment or for genetic testing to discover novel therapeutic targets, thereby advancing targeted drug development. (B) Personalized clinical application. Tumor specimens and biopsy samples from an individual patient with cancer are used to generate patient-derived tumor organoids, which are then subjected to drug sensitivity screening to guide clinical treatment decisions.

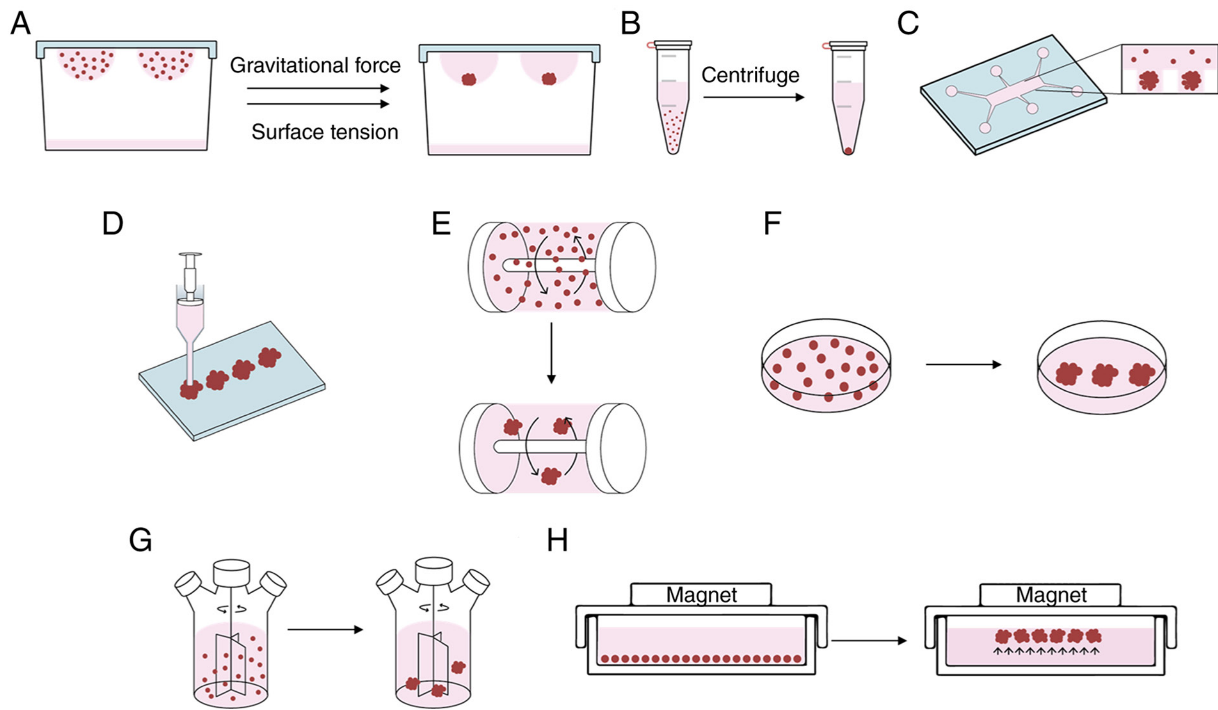

Figure 5. Spheroid culture methods. (A) Hanging-drop method. (B) Pellet culture. (C) Microfluidic chip method. (D) Three-dimensional bioprinting. (E) Rotating wall vessel method. (F) Liquid overlay method. (G) Spinner culture. (H) External force method.

categories: Conventional 3D culture, organoid-on-a-chip and 3D bioprinting (69).

Hanging-drop method. The hanging-drop method is a commonly used method for 3D culture of tumor spheroids (70,71). After flipping the lid of a Petri dish with attached cell suspension droplets, the cell suspension will hang on the lid due to surface tension. The tumor cells within the droplets will spontaneously gather at the tip of the droplets due to gravity, which promotes the coalescence of the cell droplets at the air-liquid interface to form a spheroid (72). An appropriate amount of PBS must be added to the petri dish to prevent evaporation of the liquid within the droplets. As only one tumor spheroid is formed within each suspension droplet, the application of this method allows for the accurate control of the size and homogeneity of the tumor spheroids (Fig. 5A) (73). In addition to the adjustable size of the spheroids, the suspension droplet culture system also has other advantages. Since it is a small-scale experiment, it does not require expensive or specialized equipment to form spheroids; a multichannel pipette can easily generate numerous spheroids (74,75). Notably, mesenchymal stem cells (MSCs) cultured using the hanging-drop method can secrete large amounts of anti-inflammatory and antitumor factors, which are important in disease treatment (76). However, the traditional hanging-drop method also has notable limitations. These include the inability to change the medium level frequently and the likely evaporation of the culture solution within the suspension droplet, which causes inconvenience in the subsequent experimental work, and thus, this method is only suitable for short-term culture (77).

The 96-well spheroid plate minimizes manual handling and transfer of spheroids, and prevents their fusion. This shift in cultivation methodology enhances the spheroid formation throughput, improves spheroid uniformity and reduces the skill required to generate consistent spheroids (78). For applications demanding a large number of spheroids (ranging from 100 to 1,000) or substantial biomaterial, alternative methods utilizing agarose micromolds or agarose stamps may be preferable. These methods offer several key advantages: i) High throughput and scalability; ii) production of highly uniform spheroids; iii) cost-effectiveness and reusability of the molds; and iv) easy integration with standard multi-well plates for downstream analyses (78,79). Conventional 3D culture methods often require tedious liquid handling steps to separate chimeric antigen receptor (CAR)-T cells and tumor cells killed by CAR-T cells from the spheroids when performing cytotoxicity assays (80). The advent of the 3D hanging spheroid plate has addressed this challenge by facilitating both spheroid formation and the subsequent separation of unbound and dead cells from the spheroids during the assay (81). The 3D hanging spheroid plate features a unique design where each well comprises a hanging dripper, spheroid wells and waste wells (82). The process begins in the hanging-drop reservoir, where tumor spheroids are formed and subsequently co-cultured with CAR-T cells. For the cytotoxicity readout, the droplet containing the spheroid is deposited into the spheroid separation well (81). A key innovation of this system is that by tilting the plate beyond an angle of 20˚, unbound CAR-T cells and dead tumor cells are efficiently transferred into the adjacent waste well through a connecting channel, leaving the intact spheroid isolated for further analysis (82). The traditional 3D culture method, whilst simple and easy to use, offers limited precise control and low reproducibility (83).

Pellet culture. The pellet culture method is a simpler approach for tumor spheroid formation compared with the hanging-drop

method. In this method, cells are incubated on a shaker for 1 h to maximize cell-cell contact and interaction, promoting tumor spheroid formation. Subsequently, the cell suspension is centrifuged at a low speed (28 x g for 5 min), concentrating the cells at the bottom of a conical-shaped test tube via centrifugal force (Fig. 5B). Afterwards, the supernatant is removed to harvest the cell pellet, and the pellet is resuspended in a spheroid-forming culture medium. After estimating the cell count, the cells in the medium are dispensed into 96-well U-bottom plates with a cell-rejecting surface (74,84). Pellet culture can be used to induce MSC differentiation. This system is particularly suitable for stem cell differentiation through chondrogenesis, as the cell-cell interactions within the pellet microenvironment mimic pre-chondrogenic cell cohesion during embryogenesis. MSCs can change their morphology in pellet culture from fibroblasts to polygonal shapes similar to chondrocytes. Thus, pellet culture can be used to study cartilage formation signaling pathways and assess stem cell chondrogenic potential (85,86). Although the pellet culture method has the advantage of generating uniformly sized and shaped spheroids in a short period, it requires a large number of cell culture flasks to fill the porous plate, making it difficult to generate spheroids on a large scale and unsuitable for high-throughput screening (53).

Microfluidic chip method. Microfluidic chip technology, as a novel culture method, has attracted increasing attention for its prospects in the pharmaceutical industry (87,88). Integrating the 3D culture of tumor cells onto a microfluidic chip provides a novel platform for high-throughput screening of antitumor drugs (89). To facilitate cell aggregation, the microfluidic microwells are first functionalized with a layer of cell-ECM material. Subsequently, a controlled flow rate is applied to generate hydrodynamic forces within the device. This induces a rotational motion that keeps the cells in suspension, thereby promoting their organization into aggregates within the coated microwells. The appropriate concentration of the cell suspension is infused through the microchannels into microwells, and then the cells are cultured on the chip to form the tumor spheroids (Fig. 5C) (90). The advantages of this method compared with conventional spheroid culture methods (e.g., the hanging-drop or liquid overlay method) are that more homogeneous tumor spheroids can be formed quickly, the cell culture environment can be precisely controlled, and the tumor spheroids can be observed and imaged in real time by biosensors (91). The disadvantage is that the design and processing of the chip are more complicated, and it is difficult to remove the formed tumor spheroids for further experimental analysis (92,93).

Organ-on-a-chip models refer to microfluidic-based platforms that mimic the microarchitecture and functionality of human organs, recreating various physiological milieus (94,95). These platforms are also termed 'microphysiological systems' (96). The cells utilized in these models can be derived from primary cells, cell lines or stem cells. Specifically, 'organoid-on-a-chip' denotes the integration of organoids into microfluidic devices. This hybrid approach not only preserves the biological complexity inherent to organoids but also leverages the controlled microenvironments of the chip to enhance the maturity and functionality of the organoids (97). Therefore, microfluidics, a technology characterized by sub-millimeter fluid engineering operations, is important as an ideal technological platform for *in vitro* cellular research, and has future applications in disease diagnosis and treatment and clinical biology research (98).

3D bioprinting. 3D bioprinting, an additive manufacturing technique, is an emerging technology that converts computer aided designs into physical objects (Fig. 5D) (99). The advancement of 3D bioprinting technology and its integration with biomaterials have provided unprecedented opportunities for constructing *in vitro* tumor models (100). 3D bioprinting uses bioinks (mainly consisting of biomaterials, cells and growth factors) to construct 3D tumor models layer by layer and obtain functional organoids through subsequent *in vitro* culture (101,102). Inkjet-based 3D printing is suitable for encapsulating organoids within bioinks to form stable, isolated niches that facilitate the self-assembly of organoids. This technique enables high-precision droplet deposition, making it applicable in scenarios demanding meticulous placement of cells and biomaterials (103). The advantages of this method are the ability to form tumor spheroids of the desired size and shape that can be easily controlled in a short period of time, and that the technology can be used for high-throughput rapid screening due to its precision, controllability, versatility, flexibility and rapidity (104). Furthermore, the method shows great potential for geometrical and spatial control because 3D bioprinting allows the precise layer-by-layer deposition of bioinks, enabling the fabrication of tumor spheroids with customized sizes, shapes and internal architectures that mimic the complex spatial organization of native tumors. The disadvantages are the complexity of the technique and the high cost of the instrumentation. 3D bioprinting technology has led to the emergence of a novel era of biomedical engineering, and its multiple advantages have become a major driver for the rapid development of drug screening methods and drug delivery systems (105).

Rotating wall vessel method. In the rotating wall vessel method, the culture system is rotated horizontally around the x-axis to create a microgravity environment, which promotes the aggregation of cells into spheroids (Fig. 5E) (106). Studies have shown that this microgravity affects gene expression in MSCs (107,108). Under microgravity conditions, chondrogenic and osteogenic gene expression is reduced in stem cells, while adipogenic gene expression is elevated (109). The literature suggests that initially the rotational speed is ~15 rpm, and as the tumor spheroids gather into progressively larger clusters, the rotational speed is increased to 25 rpm to keep the cell clusters in suspension (110). Compared with conventional static culture, the culture environment of the rotating microgravity bioreactor can enhance the promotion of cell proliferation and differentiation, providing an effective platform for studying disease pathogenesis and exploring novel therapeutic strategies (111). The method can be applied to a variety of cell lines and is capable of generating a large number of usable tumor spheroids in an environment characterized by low shear stress, high dissolved oxygen levels and low nutrient concentration, with the disadvantage that the self-assembly of tumor spheroids cannot be observed in real time (112).

Liquid overlay method. The liquid overlay method, also referred to as static suspension culture, involves culturing cells on specially designed plates coated with polyhydroxyethyl methacrylate (113) or agarose (114), which results in the formation of spheroids by reducing cell adhesion to the non-adherent culture plate (Fig. 5F). As cell adhesion is inhibited, the liquid overlay method enables the rapid cultivation of reproducible, morphologically uniformly sized and well-defined spheroids in an automated manner (115). This method is more convenient for changing the cell culture solution and drug administration without interfering with tumor spheroid formation compared with the hanging-drop method. In addition, the liquid overlay method can be performed using 96-well ultra-low adsorbent plates, making it ideal for inoculating cells at the desired density. However, the preparation of culture plates used in the liquid overlay method is labor-intensive, requires skilled personnel, is relatively costly and the plates (or their non-adherent coating) are not reusable (112). Despite the non-adhesive properties of agarose, this biomaterial still has drawbacks in culturing cancer cells; agarose has difficulty interacting with tumor cells and is unable to activate specific signaling pathways associated with the response of tumor cells to therapeutic processes because agarose lacks the bioactive adhesive motifs that engage cell surface receptors (e.g., integrins) (116).

Spinner culture. The spinner culture method generates 3D aggregates by using a magnetic stir bar to create a turbulent flow field that mixes air and nutrients-a process driven by magnetic force, which was initially used to study the effects of microgravity on cells and tissues (117). The spinner culture method has been successfully applied for the large-scale cultivation of bacteria and yeast to produce vaccines, recombinant proteins and other metabolites, and in tissue engineering for generating cartilage constructs as well as for expanding mesenchymal stem cells on microcarriers (Fig. 5G) (118,119). During system operation, a constant rotational speed regulation serves as the critical parameter (120). The advantage of this method is that the fluid environment helps exchange substances between the tumor spheroids and allows for the formation of multiple tumor spheroids (121). In addition to reducing the stirring speed, the tumor spheroids were studied by changing the medium to add drugs or growth factors to the spheroids (48). A notable drawback of this method is that the mechanical stirring device in the culture system imposes substantial fluid shear stress on the cells, thereby causing a certain degree of cellular damage. Consequently, this approach is likely unsuitable for cells with low adhesion strength or high sensitivity to shear forces, or those that are anchorage-dependent and prone to anoikis when in suspension (122).

External force method. The external force method uses external forces such as magnetic fields, electric fields and ultrasound to concentrate dispersed cell suspensions into high-density solutions, thereby promoting cell aggregation into spheroids. This method relies on magnetic forces termed magnetic levitation, which improves upon traditional suspension techniques by enabling more rapid and uniform spheroid formation without the need for mechanical agitation, generating higher yields with better reproducibility and allowing co-culture of multiple cell types (Fig. 5H) (123,124). For example, cells are incubated with magnetic cationic liposomes containing Fe_3O_4 magnetic nuclei, and when the magnetic nanoparticles are endocytosed by the cells, the cells become magnetic under the influence of the magnetic nanoparticles. The magnetized cells are then attracted to the center to form a tumor spheroid by the force of an external magnetic field (59). This method allows for high yields and applications for long-term culture of tumor spheroids, but the resulting tumor spheroids are usually not uniform in size (125). The use of magnetic forces to induce the aggregation of cells into spheroids can be used to elucidate the interactions between cells. However, the introduction of foreign materials (e.g., magnetic nanoparticles) may raise safety concerns, which limits their potential for future clinical applications (e.g., *in vivo* cell therapy or tissue implantation), although this method remains valuable for *in vitro* 3D culture (120).

4. Application of 3D culture technology in the field of lung cancer research

Advances in MCTS modeling for lung cancer drug screening. A previous study has analyzed and compared the morphological characteristics, protein expression, ECM distribution and drug resistance of scaffold-free and scaffold-based A549 spheroids, and found that compared with monolayer A549 cell cultures, scaffold-free and scaffold-based A549 spheroids exhibited elevated levels of epithelial-mesenchymal transition (EMT) markers and protein expression levels associated with drug resistance (126). Drug resistance is a leading cause of treatment failure and mortality in patients with cancer. Among patients who succumb to cancer due to metastasis or recurrence, >90% of cases are closely associated with multidrug resistance, a phenomenon commonly observed in both traditional chemotherapy and novel targeted therapies (127).

Notably, 3D culture systems generally exhibit more potent drug resistance properties, with most compounds showing notably reduced efficacy in a 3D environment compared with 2D culture (128,129). Huang and Hsu (130) successfully accelerated the formation of tumor spheroids of NSCLC cells using an easy-to-prepare chitosan-hyaluronic acid composite membrane as a 3D culture platform. By comparing the biological properties of 3D tumor spheroids formed by this composite membrane with those of 2D-cultured cells, it was found that the expression levels of tumor stem cell-related markers (such as CD133 and CD44) and EMT markers were notably upregulated in the 3D culture system, and that cell viability and invasive capacity were enhanced 2- to 4-fold compared with those in 2D culture. Notably, 3D-cultured cells showed a 5-6-fold enhancement of cisplatin resistance and an even greater 16-56-fold increase in methotrexate resistance, confirming that the composite membrane could provide an efficient 3D culture platform for tumor stem cell research and anticancer drug screening.

In addition, Huang *et al* (131) cultured human A549-iRFP NSCLC cells into MCTSs and inoculated them into the lungs of male nude mice. An orthotopic xenograft model of lung cancer was established via intrapulmonary injection of tumor spheroids to simulate the progression of NSCLC in four clinical stages within 1 month. Tumor resection in this

orthotopic xenograft model resulted in a high postoperative survival rate. The establishment of this novel *in situ* xenograft model of NSCLC opens up numerous opportunities for clinical evaluation of potential anticancer drugs for the treatment of different stages of lung cancer. Zhang *et al* (132) established a patient-derived 3D tumor spheroid culture system using NSCLC patient samples. Under long-term culture conditions, these patient-derived tumor spheroids reproduced the cytological features and markers of the primary tumors. The study also demonstrated the utility of this patient-derived spheroid system for drug screening. The results of the study support the establishment of scalable 3D *in vitro* models of NSCLC for drug screening and allow for potential long-term studies, including studies modeling drug resistance. MCTSs are gradually becoming an important method for novel drug testing and personalized anticancer therapy (133,134).

Gupta *et al* (135) developed a novel air-grown MCTS model for simulating tumors *in vivo* to understand lung cancer biology. In the 3D MCTS model constructed from the A549 cell line, tumor-penetrating peptide iRGD peptide co-application notably enhanced the tumor permeability of paclitaxel (PTX). Notably, the cytotoxic effect of PTX was confined to the outer layer of proliferating active cells, suggesting a barrier effect of the 3D structure on drug diffusion. Guzman *et al* (136) developed a dry powder of nanoparticles containing a PTX nanocomposite microparticle (nCmP) aerosol delivered to the lungs via a dry powder inhaler to treat NSCLC. In a liquid 3D tumor spheroids model, PTX nCmP exhibited growth inhibition effects comparable to free PTX, but the nCmP drug delivery system showed a superior cytotoxicity profile. The 3D air-interface culture platform could be used to evaluate PTX nCmP, showing its potential as an *in vitro* model that exhibits improved simulation of the lung environment and facilitating its further application for evaluating other aerosol preparations.

A similar effect on the growth of NSCLC was demonstrated using a multicellular spheroid chip. H1650 NSCLC cell suspensions were incubated in the chip under a pressure range of 145-155 Pa. The chip, which was equipped with a removable cell capture barrier, was capable of forming and extracting 3D tumor spheroids (92). The study provided a simple and effective method for obtaining uniform and small-sized 3D tumor spheroids for the next steps in cell-based biomedical research, including gene expression analysis and tumor spheroid inoculation in animal models. Ganguli *et al* (137) designed a modular and versatile microchip 3D droplet culture platform that allows for reproducible and high-content drug screening. The droplets were designed to form a predetermined geometry, thus enabling manipulation of the geometry of the cultured cell clusters, which can be performed directly on the chip in real time using high-resolution confocal microscopy. In addition, the cells in the micro-platform aggregated into MCTSs notably faster (forming tumor spheroids in 1 day) compared with the conventional hanging-drop culture. Fully automated 3D screening of MCTSs has been demonstrated using a novel angle plate adapter technology that facilitates efficient liquid handling in a 1,536-well format. This approach identified active natural products from the microbial Natural Products Library at UF Scripps (138). Because this method enables high-throughput, automated screening of 3D tumor models in a physiologically relevant microenvironment, thereby improving the predictive accuracy of drug responses and reducing reliance on animal models, it may continue to serve an important role in drug discovery and development for the foreseeable future (139). 3D tumor spheroids are critical for understanding the mechanisms by which the TME contributes to cancer onset and progression, which can lead to metastasis, and can help identify novel molecular therapeutic approaches (140). 3D tumor spheroid models have led to a renewed appreciation of MCTSs due to their ability to mimic the complexity of *in vivo* tumors and serve as a potential bridge between traditional 2D culture and *in vivo* studies (141).

3D *in vitro* co-culture models improve the study of cell-cell and cell-matrix interactions, as well as the role of the microenvironment in cell differentiation, proliferation, apoptosis, gene expression, and tumor cell drug resistance (142). In tumor cell culture for drug screening, co-culture of normal cells with tumor cells could be a potential technique to reconstitute the heterogeneous multicellular environment of solid tumors and to promote tumor migration (53). Due to the multi-component and multi-target action characteristics of Traditional Chinese Medicine, the traditional 2D monolayer cell model is too oversimplified to comprehensively reflect its overall regulatory effects. By contrast, the 3D tumor spheroids model with multi-cell co-culture can more accurately mimic the *in vivo* microenvironment (143). Lamichhane *et al* (144) used the hanging-drop method to establish a tumor spheroid model consisting of epithelial cells, endothelial cells and bone marrow MSCs in lung cancer, and to study the different cell distributions and interactions within the tumor spheroids to investigate the effects of therapeutic drugs on different cells. Tumor spheroids constructed with a mixture of different cells were a good model to study the interactions between cells within the tumor, and it was found that the composition of real tumor tissues could be further simulated by adjusting the ratio of different cells.

MCTSs are an inevitable choice for simulating the TME. However, relatively few cell types and compositions are currently used to construct MCTSs, partly due to the difficulty of co-culturing multiple cells *in vitro* (145). One study found that using fibroblasts, endothelial cells and immune cells in a feeder-layer-like manner as the external microenvironment of tumor spheroids effectively explores stromal cell effects and overcomes co-culture challenges (146). Han and Hsu (147) constructed a co-culture system of MSCs and A549 lung cancer cells on ultra-thin (2 μm) hyaluronan-grafted chitosan, and the cells successfully self-assembled to form 3D tumor co-spheroids with a core-shell structure. To verify the different degrees of tumorigenicity, A549 cells or cells co-cultured with MSCs were transplanted into zebrafish embryos for *in vivo* evaluation, and the tumorigenicity obtained in the zebrafish xenograft model was consistent with that observed *in vitro*. This evidence suggests that a 3D co-culture platform of cancer cells and MSCs based on hyaluronan-grafted chitosan substrates may be a convenient tool for studying cell-cell interactions in tumor-like microenvironments and could potentially be used for *in vitro* cancer drug testing. With the continuous development of precision medicine, MCTSs may be further developed into a reliable platform that can simulate solid tumors to a greater extent than conventional 2D cultures. This platform opens up a valuable pathway for subsequent

high-throughput and high-resolution analyses. Compared with traditional preclinical models, it can more accurately predict clinical outcomes and become an effective tool for real-world drug-based research and individualized tumor treatment.

Research progress on LCOs in personalized precision medicine. Currently, a variety of tumor organoid models have been successfully constructed, including colorectal cancer organoid models (148), gastric cancer organoid models (149), hepatocellular carcinoma organoid models (150), pancreatic cancer organoid models (151), bladder cancer organoid models (152), ovarian cancer organoid models (153), prostate cancer organoid models (154), lung cancer organoid models (155) and breast cancer organoid models (156). These 3D culture systems successfully mimic the biological characteristics of the source tissues in terms of gene expression profiles, tissue structural features and physiological functions, and provide a promising technological platform for developmental biology research, disease modeling, drug screening and cell therapy (157). Several laboratories have successfully established LCO biosample libraries for lung cancer research, including organoid models derived from the tissues of patients with LUAD (158,159). Systematic analyses have shown that such models completely retained the histological architecture (verified by H&E staining), genomic features (confirmed by whole-exome sequencing) and gene expression profiles (analyzed by RNA-sequencing) of the parental tumors (160,161). The models can be used for high-throughput drug screening to further understand the pathophysiology of lung cancer and to facilitate personalized medicine by predicting individual patient responses to anticancer drugs (162).

Lung cancer exhibits notable genetic and phenotypic heterogeneity among individuals, which drives the need for personalized medicine (108). LCOs can be generated from tumor cells isolated from patient tissues (163). The response of LCOs to drugs is based on their genomic alterations. Specifically, olaparib is effective against breast cancer gene 2-mutant-like organoids, erlotinib is effective against EGFR-mutant organoids and crizotinib is effective against EGFR mutation/mesenchymal-epithelial transition factor amplification organoids. LCOs exhibit the functional heterogeneity of tumors that is difficult to capture with traditional techniques (108). One study identified Wnt-dependent and Wnt-independent subtypes in LUAD, regulated by the alveolar factor NK2 homeobox 1 (NKX2-1). Loss of NKX2-1 triggers lineage reprogramming, leading to Wnt dependency and sensitivity to porcupine inhibitors (159). This organoid-based classification model links specific molecular phenotypes (Wnt-dependent vs. Wnt-independent LUAD subtypes defined by NKX2-1 status) to targeted therapies (e.g., porcupine inhibitors for Wnt-dependent tumors), thereby advancing a novel paradigm for precision medicine in lung cancer. Additionally, EGFR-mutated LCOs have been constructed, revealing the mechanisms of resistance to EGFR-tyrosine kinase inhibitors (164). Kim *et al* (165) established LCOs using five cancer tissue subtypes, and surgically resected lung cancer tissues were separated into individual cells or clusters of cells, embedded in Matrigel, and cultured in Wnt3a$^-$ and Noggin-free medium. The organoids of different lung cancer subtypes were obtained in ~4 weeks. H&E staining and immunohistochemical analysis showed that the organoids of the five lung cancer subtypes retained LUAD markers (e.g., napsin-A), as well as the histological features of the cancerous tissues. The lung cancer tissues retained the genetic features of cancer tissues as demonstrated by gene-targeted sequencing, single nucleotide polymorphisms genotyping and mutation concordance analysis. Meanwhile, some related studies have confirmed that patient-derived organoids retained the histological and genetic features of primary tumors, and were reliable models for evaluating drug sensitivity and predicting therapeutic responses in NSCLC, thereby guiding personalized precision medicine (166,167).

With the advent of precision therapy, *in vitro* drug sensitivity prediction of tumors is an important research direction for individualized treatment (168). Tumor organoid technology has advanced significantly, enabling the development of *in vitro* models that can predict patient responses to anticancer drugs across various cancer types (169). However, in lung cancer, the construction and application of LCO models are more difficult than those in other tumor organoid models due to efficiency and time consumption limitations (170). Hu *et al* (171) used a novel integrated superhydrophobic microtiter array chip, instead of the traditional 96-well plate, for high-throughput 3D cultivation and analysis of LCOs. A week-long drug sensitivity test on LCO microarrays demonstrated great potential in predicting patient treatment response and drug screening. Importantly, the one-week drug test results were cross-validated against three independent benchmarks: i) The drug responses of LCOs correlated with the *in vivo* anti-tumor efficacy observed in patient-derived xenografts; ii) the sensitivity patterns aligned with the specific genetic mutation profiles of the tumors (e.g., EGFR-TKI sensitivity in EGFR-mutant cases); and iii) the test predictions effectively reflected the actual clinical outcomes (e.g., progression-free survival and objective response rates) of the matched patients, establishing the InSMAR-chip platform as a technically feasible means for rapid patient-specific drug response prediction in clinical settings. The cutting-edge technology of combining LCO models with microfluidic chips provides an effective and reliable technological tool for predicting patient-specific drug responses in a clinical setting. A recent study detailed a novel tumor-on-a-chip platform incorporating organoids, which was designed to model the physiological processes of tumor growth and metastasis *in vivo*. This platform enabled practical evaluation of the invasive and proliferative capacities of patient-derived tumor cells, thereby serving as a valuable tool for investigating metastatic mechanisms, and advancing targeted cancer therapies and drug discovery (172).

Although the development of organoids has progressed to some extent, *in vitro* cancer models that can simultaneously reproduce the complexity of the TME and its diverse ECM and genetic properties remain challenging to establish. The emergence of 3D bioprinting technology offers a promising approach to address this limitation, although its full potential has not yet been realized. Dong *et al* (173) utilized 3D bioprinting technology to establish a 3D bioprinting strategy based on a bioink system (sodium alginate, hyaluronic acid and arginine-glycine-aspartic acid peptide) *in vitro*, and successfully constructed an array of LCOs for drug evaluation. Hydrogel, as a support material for cells in 3D models,

is essential for achieving important cell-cell and cell-matrix interactions that induce cells to form tissues and organoids (174). 3D bioprinting allows precise control of the spatial deposition of biomaterials and cells based on predefined positions to create personalized tumor models, enabling the establishment of animal-free and personalized drug-screening platforms (175).

Despite progress in the establishment of LCOs, a large proportion of patients remain unresponsive to current immunotherapies (176). To better recapitulate the living TME, a cell co-culture system can be established by adding stromal cells, adipocytes or lymphocytes to the culture system (177). Dijkstra *et al* (178) demonstrated that the co-culture of autologous tumor-like organs and peripheral blood lymphocytes could be used to enrich tumor-reactive T cells. In addition, the killing efficiency of these T cells against matched tumor organoids could be assessed. A recent study has demonstrated that a gel-liquid interface co-culture model, established between LCOs and matched peripheral blood mononuclear cells, could enhance the interaction between immune cells and tumor organoids (155). This model thereby allows for an improved recapitulation of the systemic antitumor immune response observed *in vivo*.

5. Conclusion and prospects

The present review systematically examines the advantages and research progress of 3D culture systems, including MCTSs and organoids, for lung cancer drug screening, providing a theoretical basis for their application in preclinical drug development. Nevertheless, translating these findings into routine clinical practice remains challenging. To advance this field and enable clinical translation, future research should prioritize the standardization of culture protocols and the integration of emerging analytical technologies. Currently, considerable heterogeneity exists across studies in terms of cell sources, ECM components, spheroid formation methods and culture durations, which limits the comparability and reproducibility of drug response data (179). Furthermore, although 3D models improve the recapitulation of the TME compared with traditional 2D cultures, they still lack key physiological features such as functional vasculature, dynamic immune cell infiltration and complex stromal interactions (180). The majority of existing studies rely on bulk analyses of spheroids or organoids, which may obscure the intrinsic heterogeneity of tumor cell populations and their differential drug responses (181). Integrating patient-derived organoid biobanks with single-cell and spatial multi-omics technologies holds considerable potential for dissecting intratumoral heterogeneity at high resolution and mapping the spatial distribution of drug-resistant subclones (182). Furthermore, combining 3D culture systems with microfluidic organ-on-a-chip platforms and 3D bioprinting will enable the construction of more physiologically relevant models that incorporate multiple cell types and recapitulate tissue perfusion. Addressing these limitations through technological refinement and standardization will accelerate the translation of 3D culture systems from bench to bedside.

The application of spheroids and organoids represents a notable advancement in lung cancer research. They are crucial for studying cancer biology, drug responses and resistance mechanisms. Despite these advancements, challenges remain, including the variability in organoid cultures, the need for enhanced standardization and the difficulty in fully recapitulating the TME. Nevertheless, the continuous refinement of these models holds promise for improving their clinical relevance, facilitating drug development and enhancing the present understanding of cancer progression. These models provide a promising pathway for personalized cancer therapy, reducing reliance on animal models and improving the prediction of human-specific drug toxicity and efficacy, thereby driving innovation in lung cancer research and treatment.

Although both MCTSs and LCOs are superior to 2D culture techniques, they are not mutually exclusive but rather coexist complementarily. MCTSs provide a robust and scalable system for investigating fundamental mechanisms and large-scale compound screening. By contrast, LCOs, although often more resource-intensive and time-consuming to establish, offer high-fidelity patient-specific models for validating personalized treatment strategies. Recently, it has been demonstrated that malignant pleural effusion can be used as a cell source or medium supplement for LCO model establishment; MPE not only improves the success rate of LCO culture by enabling extended passaging and facilitating the initial formation from difficult-to-culture samples, but also supports the rapid generation of LCOs for personalized drug testing (183). Integrating both models through a tiered screening strategy, employing MCTSs for initial high-throughput screening followed by validation with LCOs in a patient-specific context, can effectively optimize the drug development pipeline. In addition, the complementary strengths of tumor spheroids in terms of modeling the TME and organoids in terms of mirroring human tissue specificity, can be leveraged through their integration (184). This synergistic approach bridges the gap between model systems and deepens research insights.

Despite progress, both MCTSs and LCOs face challenges. For MCTSs, these include standardizing size and cellular composition, as well as incorporating complex TME components. Ongoing advances regarding scaffold materials, imaging technologies and culture methodologies may help overcome these limitations in the future. For LCOs, limitations include low culture success rates, difficulty in reconstituting a complete TME (including functional vasculature and immune cells), and reducing the associated time and costs of establishment. One recent study successfully integrated multiple organoid modules on a single chip, enabling high-throughput drug testing using this platform (185). Future directions may involve converging these models with advanced technologies such as microfluidic organ-on-a-chip systems and 3D bioprinting. This integrative strategy will help construct a more physiologically relevant TME, including stromal and immune components, thereby enhancing the predictive power of 3D *in vitro* models.

In summary, the synergistic application of MCTSs and LCOs is driving a paradigm shift in lung cancer research. MCTSs serve as versatile tools for fundamental and applied screening, while LCOs provide a powerful means for personalized oncology. Together, they form a comprehensive and complementary toolkit that enhances the capacity to develop novel anticancer strategies and predicts clinical outcomes

more accurately than conventional 2D cultures or animal models, ultimately advancing the era of precision medicine for patients with lung cancer.

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Authors' contributions

JY and MZ conceived the idea of the study and provided overall supervision for the project. YT and HL collected materials and wrote the manuscript. AC, LS, JZ and HC helped with literature screening and manuscript writing. BW, HH and ML provided constructive guidance and revised the manuscript. Data authentication is not applicable. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

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Competing interests

The authors declare that they have no competing interests.

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