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Three-dimensional culture of tumor cells (Review)

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Abstract. While conventional two-dimensional (2D) cell cultures and animal models have been cornerstone technologies in cancer research, they possess limitations in replicating human tumor pathophysiology. Notably, 2D models fail to capture key tissue-level architecture and cell-cell interactions, whereas animal models are often constrained by species-specific discrepancies and high costs, which limits their applicability in investigating precise human tumor mechanisms. To overcome these shortcomings, three-dimensional (3D) tumor models have emerged as a powerful complementary platform. The present review comprehensively explores the unique capabilities of 3D models in maintaining tumor heterogeneity, simulating the dynamic tumor microenvironment and accelerating high-throughput drug screening. The present review also highlights the transformative potential of 3D models in personalized medicine and in deciphering the mechanisms underlying metastasis. Finally, the present review proposes a visionary roadmap for *in vitro* 3D model innovation, with the goal of guiding their effective translation from foundational research to clinical decision-making in the future.

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1. Introduction

In vitro pharmacological and pathological models serve as indispensable tools in antitumor drug discovery and development. *In vitro* tumor cell culture not only enables the investigation of specific tumor cell behaviors such as growth state, metastasis, differentiation, signal transduction, gene expression and other key processes (1) but also offers the advantages of high efficiency and cost-effectiveness. In comparison, tumor animal models have limitations that restrict their application, including high costs, lengthy experimental duration, low controllability, high vulnerability to internal and external environmental variables, species-specific differences that compromise accurate prediction of human drug responses and the inability to construct all human tumor models (2). Collectively, these attributes establish the *in vitro* method as a foundational platform for antitumor drug development and screening (3). Cell culture models can generally be categorized into two main categories: Two-dimensional (2D) and three-dimensional (3D) cell models. As research on cell culture models progresses, several limitations of 2D cell models have been revealed, including low cell viability, susceptibility to cell morphology damage and the absence of actual tissue structure (4). This structural deficiency restricts cell-cell and cell-extracellular matrix (ECM) interactions, thus failing to mimic the *in vivo* tumor microenvironment. Therefore, 2D models prove inadequate for accurately replicating the actual growth characteristics of tumors *in vivo* (5). By supporting cell proliferation and interactions within a 3D space, 3D cell models facilitate the 3D structural formation of cell populations (6). This system better simulates and replicates *in vivo* cell biology, resulting in enhanced culture efficiency, increased yields of cytokines, antibodies and other biomolecules, and improved overall cell proliferation (7). Current 3D models for studying tumor cells can be classified into two principal categories based on scaffold utilization: Scaffold and scaffold-free models. To systematically evaluate these culture modalities, Table I provides a comprehensive framework that delineates their respective technical boundaries and translational potential, thereby guiding researchers in selecting the most appropriate model for specific research objectives. Among these, scaffold-based models have emerged as a central platform in cutting-edge translational applications such as 3D bioprinting and organ-on-a-chip technologies, due to the marked diversity and tunability of their biomaterial components (8-10). Therefore, the present review first outlines fundamental research progress on scaffold-based models,

followed by an examination of the core mechanisms underlying scaffold-free systems. Building upon this foundation, the present review integrates and discusses the potential of various *in vitro* 3D models for drug-screening and clinical applications. Lastly, the present review offers a forward-looking perspective on bridging the gap between model systems and clinical practice in the future.

2. Scaffold models

Scaffolds are the most extensively used materials in 3D cell culture, acting as an artificial matrix that replicates the complex 3D structure and key characteristics of living tissues. The primary function of scaffolds encompasses providing structural support for cells and serving as a medium for the diffusion of soluble factors, thereby facilitating key cellular processes such as adhesion, migration, proliferation, differentiation and long-term survival, which collectively lead to the formation of tissues and organs (11). The various types of scaffolds utilized in 3D cell culture include solid porous scaffolds, hydrogel scaffolds, non-hydrogel scaffolds, fibrous scaffolds, microfluidic chips, microsphere scaffolds and 3D printing techniques. Advances in enabling technologies, such as 3D printing and microfluidic chips, are rapidly expanding the applications of scaffold-based 3D culture systems, making them invaluable tools for cancer research, high-throughput drug screening and clinical translation.

Solid porous scaffolds. Solid porous scaffolds can be prepared using simple processes (12) with materials exhibiting good permeability and uniform density (13). Furthermore, high-performance solid composite materials exist that are suitable in manufacturing scaffolds that meet the requirements of diverse cell culture systems.

Alafnan *et al* (14) fabricated microneedle patches using a polyethylene glycol diacrylate diphenyl phosphine oxide polymer, which were then coated with gemcitabine and sodium carboxymethyl cellulose to establish an *in vitro* drug delivery system for inflammatory breast cancer treatment. In a separate study, Bai *et al* (15) created a stimulus-responsive scaffold by incorporating graphene oxide (GO) into a copolymer of polyacrylium-g-polylactic acid. When integrated with polycaprolactone (PCL) and gambogic acid (GA), this composite scaffold demonstrated a selective response to tumors and exhibited notable accumulation of GO/GA in breast tumor cells under acidic conditions *in vitro*, with only minor effects on normal cells at physiological pH. This previous study suggested that pH-responsive photothermal combination therapy is more effective in inhibiting tumor growth compared with independent treatments. Dettin *et al* (16) engineered a novel 3D culture scaffold through the conjugation of hyaluronic acid with ion-complementary self-assembling peptides, which effectively promoted the proliferation of HCC1569 and MDA-MB-231 human breast cancer cell lines. This platform was successfully implemented for evaluating electroporation efficacy, demonstrating that the 3D scaffold culture system may advance brain tumor electroporation research compared with conventional 2D models, providing a reliable platform for the validation of novel electroporation-based drug delivery protocols (17).

Hydrogel scaffolds. Hydrogel scaffolds are porous 3D structures that support cell adhesion, proliferation and migration. As shown in Fig. 1, the process of hydrogel scaffold-based 3D cell culture involves mixing the cell suspension with hydrogel followed by incubation for *in vitro* 3D cultivation. In tumor cell culture, naturally derived hydrogel scaffolds sourced from the ECM have demonstrated notable support, plasticity and biocompatibility (18,19), whereas hydrogels derived from artificial tissue culture have also been extensively implemented (18). The selection of appropriate scaffold materials is key to 3D culture and materials optimal for cell viability, proliferation, observation, detection and other pertinent aspects should be used (20). Prompted by these considerations, researchers have developed a series of 3D hydrogel scaffold materials through systematic comparison of diverse culture methodologies, which are now extensively employed in 3D cultivation of tumor cells (11,21). Hydrogels exhibit physical properties resembling human tissues, including softness, elasticity and permeability, which enable them to provide structural support for cells, facilitate nutrient diffusion and assist in metabolite transport. However, hydrogel scaffolds have certain limitations; for example, variations in material composition may impede nutrient influx and distribution, adversely affecting cell culture (22). Commonly used gel-forming materials include fibrin, agarose, polyethylene and ethylene glycol. Fu *et al* (23) developed a fiber-hydrogel composite scaffold loaded with platelet-rich plasma, which combined efficient material transport with enhanced cell adhesion and exhibited an elastic modulus similar to that of breast tumor tissue, thereby promoting cell aggregation and reducing resistance to chemotherapeutic agents. Therefore, this composite scaffold provides a valuable platform for *in vitro* oncology research and antitumor drug efficacy prediction. Quazi *et al* (24) constructed a polypeptide drug delivery carrier using stepwise functionalization of a nano-DNA hydrogel scaffold, which was loaded with a cell-penetrating anticancer peptide via electrostatic adsorption to achieve light-triggered drug delivery. This delivery system demonstrated safety, specificity and high efficiency, establishing an ideal platform for anticancer peptide administration and offering a promising strategy for future cancer treatment. By integrating a microfluidic system with a soft hydrogel, Jiang *et al* (25) established a 3D *in vitro* model mimicking the *in vivo* lung gland microenvironment. Analysis of cancer cell morphology, proliferation and invasion within this model provided key insights into the invasive mechanisms of *in vivo* lung cancer cells.

Engineering of PCL into nano-porous PCL (NP-PCL) yields an emerging biomaterial characterized by an extensive nanoporous network structure, which provides chondrocytes with an improved growth environment and markedly enlarged adsorption surface area. Furthermore, NP-PCL exhibits notable mechanical properties and closely mimics the structural characteristics of native bone, thereby markedly enhancing chondrocyte adhesion and proliferation (26).

Non-hydrogel scaffolds. Natural scaffolds, fabricated from biological substances including collagen, chitosan, polysaccharides, seaweed salts, ECM components and other materials (27), exhibit high biocompatibility and biodegradability. However, their structural strength is typically inferior to that of synthetic

Table I. Scaffold-based vs. scaffold-free culture methods.

A, Scaffold model

First author, year	Subtype	Description	Advantages	Limitations	Applications/examples	(Refs.)
Alafnan, 2022	Solid porous scaffolds	Polymers (for example, PEGDA) with porous structures for cell support and drug delivery	Simple fabrication, high permeability and uniform density	Limited dynamic microenvironment simulation	Gemcitabine-loaded microneedles for inflammatory breast cancer	(14)
Fu, 2024	Hydrogel scaffolds	Natural/artificial hydrogels (for example, fibrin and agarose) mimicking ECM properties	High biocompatibility, nutrient diffusion and structural flexibility	Variable nutrient transport depending on material composition	Breast tumor drug resistance studies	(23)
Kim, 2019	Non-hydrogel scaffolds	Natural materials (collagen and chitosan) for controlled drug release	Biodegradable and supports sustained/controlled drug delivery	Lower mechanical strength compared with synthetic scaffolds	Glioblastoma drug resistance screening	(33)
Liu, 2021	Fibrous scaffolds	Silk/chitosan-based scaffolds with web-like structures for cell adhesion	High surface area and promotes gas/nutrient exchange	Complex fabrication and high cost	Photothermal therapy for post-surgical tumor ablation	(34)
Xu, 2023	Microfluidic chips	Nano/microchannels for cell culture and analysis	Precise fluid control, mimics vascular dynamics and high-throughput screening	High manufacturing complexity	CTC isolation with 90% purity	(40)
Hen and Jv, 2023	Microsphere scaffolds	Spherical carriers (for example, alginate) loaded with cytokines/nutrients	Adjustable porosity and enhances mass transfer	Challenges in uniformity control	HepG2 spheroid formation for drug testing	(44)
Liu, 2023	3D bioprinting	Customized scaffolds using biocompatible materials	High precision and tailored mechanical properties	High cost and technical complexity	3D-printed microfluidics concentration gradient chip and a paper chip	(46)

B, Scaffold-free models

First author, year	Subtype	Description	Advantages	Limitations	Applications/examples	(Refs.)
Safari, 2021	Hanging drop culture	Cells aggregate in liquid droplets via surface tension	Simple and limited equipment is required	Limited droplet volume (<50 μl) and inappropriate for large-scale use	Prostate cancer co-culture model	(55)
Wang, 2019	Spheroid culture	Cells self-assemble into 3D spheres in non-adherent conditions	Retains stemness and mimics tumor heterogeneity	Variable sphere-forming efficiency across cell types	Pancreatic CSC enrichment	(61)

Table I. Continued.

B, Scaffold-free models

First author, year	Subtype	Description	Advantages	Limitations	Applications/examples	(Refs.)
Kumar, 2024	Rotary cell culture	Simulates microgravity via rotational motion	Low shear stress and promotes tissue-like structures	Equipment-dependent and parameter optimization required	Pancreatic cancer-β cell interaction studies	(64)
Du, 2022	Ultra-low adsorption	Cells form spheroids on non-adhesive surfaces	Easy operation and no specialized tools	High cost of culture plates	Effect of umbilical cord mesenchymal stem cell supernatant on esophageal squamous cell carcinoma spheres	(69)
Calamak, 2020	Bioreactor culture	Controlled environment (temperature and fluid dynamics) for large-scale culture	High reproducibility and scalable	Complex setup and maintenance	HCT-116 colorectal cancer cells in a peristaltic continuous flow bioreactor to simulate physiological hemodynamics	(71)
Jaganathan, 2014	Magnetic suspension	Magnetic nanoparticles guide 3D cell assembly	Precise spatial control and scaffold-free	Requires magnetic particle integration	Magnetic suspension coculture system for breast cancer cells and fibroblasts	(78)
Zhang, 2020	Agarose coating	Agarose gel prevents cell adhesion and promotes spheroid formation	Non-toxic and simple protocol	Lacks cell-matrix interaction	HepG2 spheroid-based drug screening	(80)

3D, three-dimensional; PEGDA, polyethylene glycol diacrylate; ECM, extracellular matrix; CTC, circulating tumor cells; CSC, cancer stem cells.

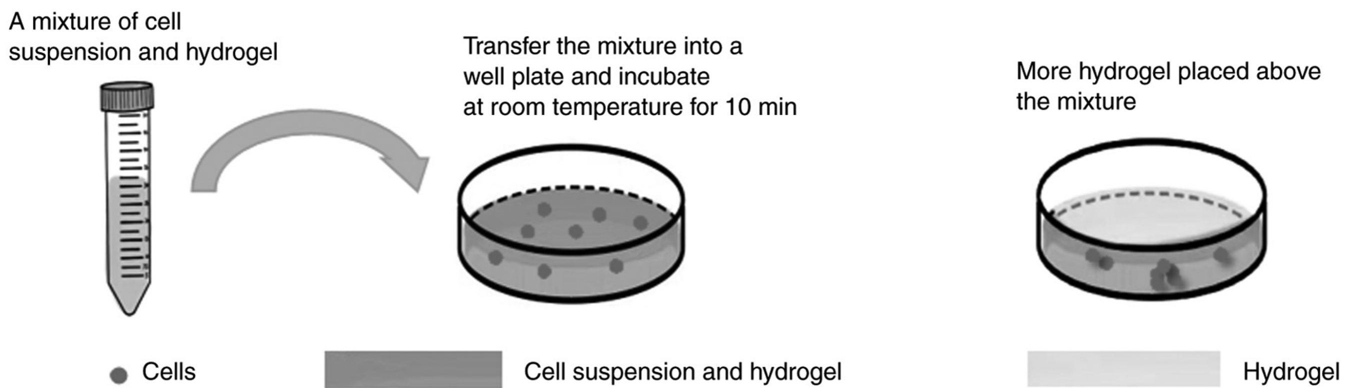

Figure 1. Schematic illustration of 3D cell culture using a hydrogel scaffold. 3D, three-dimensional.

alternatives. The development of novel drug delivery systems has facilitated the engineering of chitosan-based targeted formulations for sustained and controlled release, and targeted delivery, which offer notable advantages for enhanced drug absorption, improved bioavailability and reduced adverse effects. A key example is folic acid-modified nanoparticles, synthesized by conjugating folic acid onto chitosan nanoparticles (28). Upon loading with chemotherapeutic agents, such as paclitaxel and fluorouracil (29), they can be internalized into tumor cells via endocytosis, thereby enabling the recognition and elimination of an extensive range of cancer cells (30). Paclitaxel demonstrates marked efficacy in the treatment of colon cancer. It exerts its therapeutic effect by interfering with microtubule dynamics, which are essential for cancer cell division, thereby inhibiting cell proliferation and ultimately inducing apoptosis (31).

Chaicharoenaudomrung *et al* (32) demonstrated that human glioblastoma cells cultured in 3D calcium-alginate scaffolds exhibited reduced proliferation, enhanced tumor sphere formation, upregulation of stemness-associated genes (CD133, Sox2, Nestin and Musashi-1) and increased expression of differentiation-related markers (glial fibrillary acidic protein and β-tubulin III) compared with those under 2D conditions. Further investigation into the cellular response to anticancer agents, including doxorubicin and cordycepin, revealed that cells in the 3D scaffold system developed markedly stronger drug resistance. These findings support the utility of 3D calcium-alginate cultures as a valuable platform for anticancer drug-screening and resistance mechanism analysis. In a related study, Kim *et al* (33) employed 3D matrix scaffolds to culture two bladder cancer cell lines, SBT31A and T24, both expressing cyclin D1b mRNA, and evaluated the antitumor effects of cyclin D1b knockdown via small interfering RNA. Their results indicated that suppression of cyclin D1b expression promoted apoptosis, attenuated cancer stemness and epithelial-mesenchymal transition (EMT) and therefore, suppressed malignant phenotypes in bladder cancer cells.

Fibrous scaffolds. Fibrous scaffold materials offer notable advantages due to their fibrillar architecture, which provides extensive surface area for cell attachment, proliferation and differentiation. These scaffolds feature a dense, spiderweb-like pore network within a pliable 3D structure, facilitating nutrient exchange and gas transport. They are primarily categorized into natural fiber scaffolds (for example, silk fibroin and chitosan) and synthetic fiber scaffolds (for example, PCL).

Liu *et al* (34) constructed a core-shell fibrous scaffold by uniformly coating 3D-printed alginate-gelatin scaffolds first with PCL and subsequently with polydopamine to impart a pronounced photothermal effect, thereby achieving on-demand drug release triggered by near-infrared irradiation. The released doxorubicin, in combination with photothermal therapy, demonstrated effective liver cancer suppression and ablation both *in vitro* and *in vivo*. This scaffold presents a promising strategy for localized tumor therapy and tissue regeneration, particularly in patients with cancer post-surgery. It can be implanted at the resection site to eliminate residual or recurrent cancer cells while promoting the repair of surgically induced tissue defects.

Microfluidic chips. Microfluidic chips are technological platforms fabricated by etching microscale to even nanoscale channels and analytical detection units on substrates such as glass and silicon (35). Functioning as a miniature technological system, these devices enable diverse applications including microscale sample analysis, cell culture and signal transduction (36). Their internal architecture resembles a miniaturized chemical laboratory, incorporating fluidic channels, reaction platforms, analytical detection modules and separation components, with dimensional characteristics comparable to those of human capillaries (37). This technology demonstrates multiple advantages, including precise fluid flow control, minimized reagent consumption, enhanced detection efficiency and reduced processing times, while simultaneously enabling comprehensive simulation of *in vivo* microenvironments and supporting 3D tumor cell culture (38).

Microfluidics technology demonstrates unique advantages in modulating the physicochemical properties that govern drug release behavior. A previous study by Matsuura-Sawada *et al* (39) revealed that both lipid concentration and flow rate ratio markedly influenced liposome structure and drug release profiles. When the lipid concentration was maintained at ≥50 mmol/l with a flow rate ratio of 3, multilamellar liposomes were predominantly formed. The barrier effect conferred by their bilayer lipid membranes markedly reduced the drug release rate, resulting in a cumulative release of <58% over 72 h. By contrast, under conditions of low lipid

concentration (10 mmol/l) or a high flow rate ratio (flow rate ratio=9), unilamellar liposomes became the dominant structure, leading to a comparatively faster drug release. By flexibly adjusting these two key parameters, liposomes with varying structures can be engineered and their proportions controlled, thereby enabling precise regulation of drug release kinetics. Xu *et al* (40) developed an integrated microfluidics chip for online labeling, separation and enrichment of rare circulating tumor cells (CTCs) from blood samples, followed by analysis via inductively coupled plasma mass spectrometry (ICP-MS). Using HepG2 cells as a model, the team combined single-cell ICP-MS to quantitatively analyze asialoglycoprotein receptors on individual cells. Lanthanide-labeled anti-asialoglycoprotein monoclonal antibodies and anti-epithelial cell adhesion molecule-modified magnetic beads were prepared as signal probes and magnetic probes, respectively, enabling specific cellular recognition. Using the application of targeted magnetic separation techniques for aggregation and sorting within a designated separation zone, both the average cell recovery rate and purity of HepG2 cells were observed to be notably high. This methodology was thus established as a viable strategy for the absolute quantification of asialoglycoprotein on individual liver cancer cells, providing an efficient analytical platform to investigate targeted drug delivery in cancer therapeutics.

Microsphere scaffolds. Microsphere scaffolds, characterized by spherical structures with particle sizes ranging between 10 and 100 μm, contain cytokines and nutrients key to stimulating cell proliferation and division (41). By adjusting preparation parameters or incorporating bioactive components such as hepatic decellularized matrix (42), the mass transfer efficiency of these scaffolds can be markedly enhanced, thereby further mimicking the *in vivo* microenvironment, and promoting the proliferation and invasion of tumor cells.

Qiu *et al* (43) achieved efficient separation of CTC-like particles using an acoustic fluidic chip coupling system, utilizing carboxylic acid-functionalized polystyrene microspheres encapsulating aminated mesoporous acoustic particles to simulate CTCs. Hen *et al* (44) prepared uniformly sized methacrylated alginate microspheres via microfluidic technology combined with online UV-induced crosslinking and subsequently fabricated large-pore microspheres using freeze-drying techniques. Evaluation using HepG2 cells as a model system demonstrated that these microspheres supported cell aggregation into spheroids with viability >85% and promoted cellular proliferation, indicating their suitability as 3D cell culture scaffolds. The results confirmed that these scaffolds sustained anticancer drug release and effectively suppressed cancer cell proliferation *in vitro*, suggesting their potential applications in cancer research and drug screening in the future.

3D bioprinting. 3D bioprinting is an advanced manufacturing technique that fabricates solid structures through the integration of computer-aided design, systematic analysis of target scaffold characteristics (including material properties, geometry and dimensions) and programmed processing procedures. Fig. 2 illustrates the control system components for 3D bioprinting, including the pump, printhead, actuator, bioink, nozzle, environmental control system and high-speed camera, along with the resulting honeycomb-like scaffold model produced after printing. The integration of 3D bioprinting with alternative scaffold materials for tumor cell culture and viability assessment offers distinct advantages, particularly in achieving enhanced mechanical strength and toughness along with demonstrated non-cytotoxic properties. However, this special culture method imposes strict requirements on both computers and printers, and its high cost combined with these equipment demands currently limits its application (45).

Liu *et al* (46) developed an integrated platform comprising a 3D-printed microfluidics concentration gradient chip and a paper chip to investigate the effects of hydrogen sulfide on tumor cells and intracellular signaling molecules, demonstrating that sustained exposure to low concentrations effectively induced apoptosis and suppressed proliferation in malignant cells. Jin *et al* (47) constructed a concentric cylindrical tetra-culture model containing gallbladder carcinoma (GBC) and endothelial cells, fibroblasts and macrophages using 3D bioprinting technology, and validated the model characteristics by combining hematoxylin and eosin staining, immunofluorescence labeling and single-cell RNA sequencing. Using comparative transcriptomics analysis, this model was reported to effectively recapitulate key features of the tumor microenvironment and heterogeneity, induce more aggressive tumor cell phenotypes and provide a high-fidelity platform for GBC biological studies and antitumor drug development.

3. Scaffold-free culture

Scaffold-free cell culture approaches fundamentally promote the self-assembly of tumor cells into 3D spheroid-like aggregates through autonomous cellular processes (48). Established methodologies encompass hanging drop cultures, spheroid formation techniques, the rotary cell culture system (RCCS), ultra-low adsorption cell culture, bioreactor cultures, magnetic suspension culture and agarose coating protocols (49). Cellular models derived from these platforms demonstrate advantages of reduced experimental costs, operational simplicity and suitability for industrial production (50), rendering them particularly suitable for high-throughput drug-screening applications. However, its extensive implementation faces challenges including prolonged culture durations, high equipment investment and limited imaging penetration, which makes it difficult to ensure the uniformity of experimental results (51).

Hanging drop culture. The hanging drop method involves depositing cell suspension onto the bottom surface of a culture dish or plate, forming hanging drops via liquid surface tension. The vessel is then inverted, enabling cells to aggregate at the bottom of the drops under gravity through intercellular adhesion, thereby forming 3D structures (52). While this technique offers notable advantages in generating homogeneous spheres with controllable size and shape (53), it presents technical challenges for subsequent drug treatment procedures and morphological analysis, thereby limiting its practical applicability for large-scale cultivation.

Rodoplu *et al* (54) demonstrated a notable increase in both the percentage area and total length of tumor angiogenesis-associated blood vessels following a 6-day co-culture of embryonic bodies and tumor spheroids on a microfluidic hanging-drop

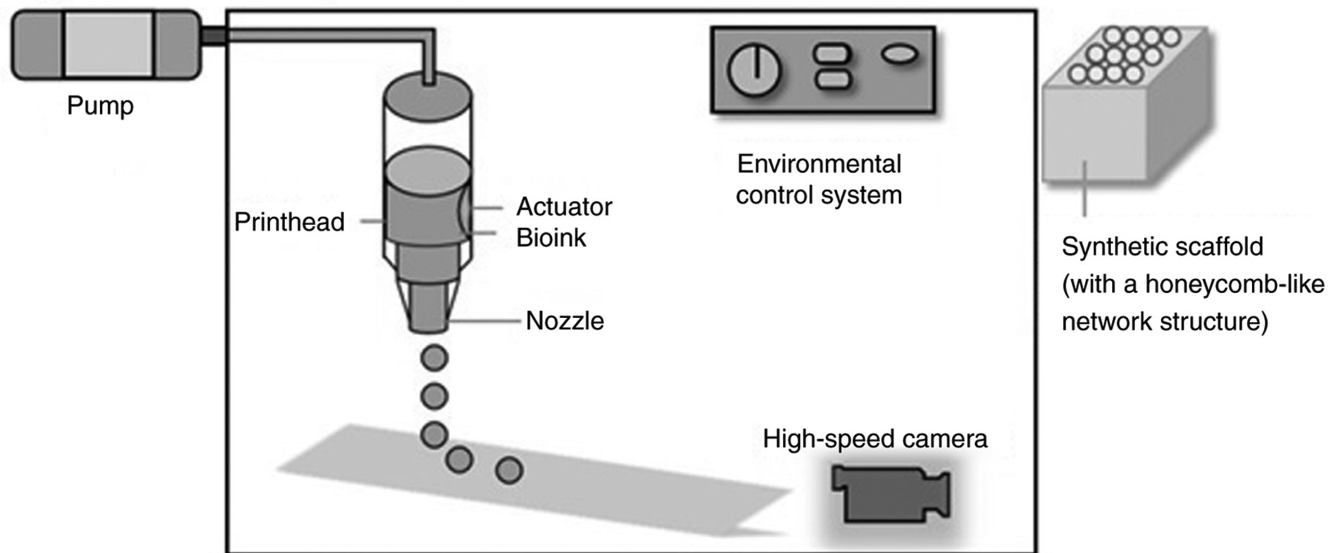

Figure 2. 3D printing system with the synthesized scaffold. 3D, three-dimensional.

platform, which establishes this methodology as a straightforward and efficient approach for generating co-cultured cell spheroids. Safari *et al* (55) employed the hanging drop technique to establish a 3D co-culture model of prostate cancer cells, revealing that conditioned medium derived from human amniotic mesenchymal stromal cells exhibited potent anticancer activity, a finding that provides compelling evidence supporting the therapeutic potential of stem cell-based strategies in suppressing prostate cancer progression.

Spheroid culture. The spheroid culture method refers to a technique wherein cells aggregate to form clump-like cellular assemblages that subsequently develop into spherical structures through 3D cultivation (56). Fig. 3 illustrates the process of forming 3D cell spheroids: 2D cultured cells are first grown to the logarithmic phase, then centrifuged. The harvested cells are diluted to create a cell suspension, which is uniformly dispensed into multi-well plates. Under gravity, the cells aggregate to form 3D spheroids. For cancer stem cells (CSCs), their sphere-forming capacity serves as a key diagnostic parameter in evaluating the self-renewal potential of individual cells under specific growth conditions (57). While tumor cells such as glioma and breast carcinoma cells demonstrate relatively high sphere-forming rates, epithelial-derived tumor cells including hepatocellular and colorectal carcinoma cells tend to exhibit comparatively lower sphere-forming rates (58). Therefore, when investigating the growth potential of these tumor cell types, culture conditions should prevent adherent growth while allowing tumor cells to form spherical aggregates in specialized media. While this method is relatively low-cost, the thickness of the formed spheroids and their inherent light scattering/absorption properties markedly affect imaging quality, thus requiring more sophisticated optical instruments and advanced analytical algorithms to achieve accurate data interpretation.

Xue *et al* (59) cultivated spheroids in specialized culture plates and demonstrated that silica stimulation induced an anti-apoptotic phenotype in myofibroblasts through activation of the nuclear factor erythroid 2-related factor 2/Bax pathway. Sun *et al* (60) conducted comparative assessment of 22 liver injury-positive and 5 liver injury-negative compounds using lung cancer cells cultured in spheroid or 2D systems, revealing that the spheroid culture markedly enhanced model sensitivity in detecting compound cytotoxicity compared with conventional 2D culture. Wang *et al* (61) demonstrated that pancreatic CSCs enriched via spheroid culture methods exhibited higher co-expression levels of stemness-related genes CD24 and CD44 compared with conventional 2D cell culture systems, thus establishing spheroid culture as a suitable platform in maintaining pancreatic CSCs under *in vitro* conditions. Raggi *et al* (62) employed spheroid culture to enrich stem-like subpopulations in human intrahepatic cholangiocarcinoma and via extracellular flux analysis using Seahorse technology coupled with high-resolution respiratory measurements, the study established that the respiratory phenotype of cholangiocarcinoma cells in the spheroid culture was markedly more efficient compared with that in the monolayer culture. The spheroid culture platform enabled direct microscopic visualization of morphological characteristics and growth status within individual wells, while simultaneously facilitating relatively straightforward environmental control during experimental manipulations, such as reagent addition and medium replacement, thereby offering particular advantages for implementation in small-scale laboratory settings.

RCCS. The RCCS simulates microgravity conditions by inducing rotational motion of cells, tissues and culture medium in a state approximating free fall (63), which effectively promotes cellular proliferation and differentiation while facilitating intercellular signaling transduction. Operating without propellers or mechanical agitators, the system generates minimal shear stress that poses negligible cellular damage, with additionally adjustable rotation speeds enabling controlled reduction of sedimentation rates as cellular aggregates develop.

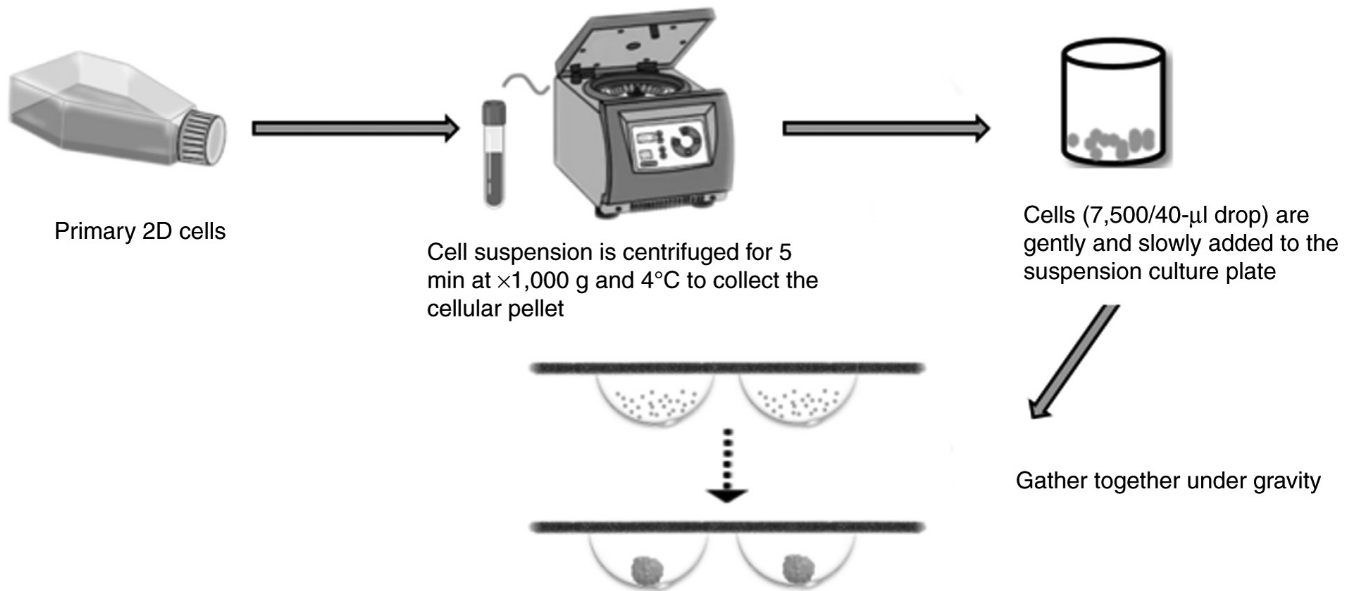

Figure 3. Schematic diagram of forming 3D cell spheroids from 2D cultures using the spheroid culture method. 3D, three-dimensional; 2D, two-dimensional.

Utilizing a 785 nm semiconductor laser for cellular stimulation and Raman spectra acquisition, Kumar *et al* (64) established a 3D RCCS to co-culture multiple pancreatic ductal adenocarcinoma (PDAC) cell lines with MIN6 pancreatic β-cells, while systematically investigating morphological characteristics and viability using integrated time-lapse imaging, confocal and scanning electron microscopy, and immunohistochemical analysis. This methodology successfully generated a co-culture platform capable of forming 3D PDAC spheroids and β-cell aggregates (pseudo-islets), with cellular surface morphology and growth patterns closely recapitulating the *in vivo* microenvironment, while further demonstrating the propensity of PDAC cells to surround and invade the pseudo-islet structures. Belloni *et al* (65) cultured bone marrow stromal cells with tumor cells in a RCCS, revealing that this 3D culture model effectively recapitulated tumor-mesenchymal transition processes and provided a physiologically relevant platform for drug-screening applications.

Ultra-low adsorption cell culture. The ultra-low adsorption cell culture method offers operational simplicity without requiring additional equipment, employing only round-bottomed vessels coated with inert, non-adhesive surfaces to prevent cellular attachment to vessel walls, thereby promoting cell aggregation and adhesion into 3D spheroids. However, the high cost of these specialized culture dishes or plates restricts their widespread implementation (66).

Using ultra-low adsorption plates, Malhão *et al* (67) generated multicellular aggregates from four breast cell lines and characterized them using morphometric analysis, qualitative cytology and quantitative immunohistochemistry, revealing that while each cell line formed homogeneous multicellular aggregates, distinct structural heterogeneity existed between different lines. In another experimental study (68), the team employed this ultra-low adsorption approach to cultivate 3D spheroids, observing compact structural integrity, robust cellular viability and straightforward procedural implementation. In a separate study, Du *et al* (69) demonstrated that culture supernatants from umbilical cord mesenchymal stem cells markedly enhanced spheroid formation in esophageal squamous cell carcinoma cultures prepared using ultra-low adsorption plates.

Bioreactor culture. Bioreactors represent specialized cultivation systems engineered to accommodate specific culture methodologies, with key parameters, including temperature, humidity, pressure, nutrient supply, CO_2 concentration, and physical or chemical stimuli, mimicking *in vivo* conditions with high fidelity. This configuration enables more precise regulation and enhanced controllability compared with alternative culture environments while facilitating streamlined tumor cell cultivation, thereby finding extensive applications in biomedical research (70).

Calamak *et al* (71) cultured HCT-116 colorectal cancer cells in a peristaltic continuous flow bioreactor to simulate physiological hemodynamics, and demonstrated that this system induces reprogramming of cancer cells toward a mesenchymal niche while accurately replicating circulatory conditions. These findings established that hemodynamic forces alter membrane composition and morphological characteristics in malignant cells, providing notable insights in the development of novel cancer therapeutics. Huo *et al* (72) investigated a 3D perfusion bioreactor that maintained neuroblastoma tissue architecture and cellular matrix integrity for 7 days, enabling continuous drug response monitoring using isothermal microcalorimetry. This platform additionally incorporated 56 metabolic assessment methods with rapid detection and high sensitivity to advance personalized treatment for neuroblastoma. Bober *et al* (73) designed a bioreactor-based 3D culture system combining non-invasive proton magnetic resonance (MR) relaxation time measurements at 1.5 Tesla with immunohistochemical analysis to evaluate trastuzumab delivery efficiency in breast cancer cells (CRL2314) vs. normal controls (HTB-125). The results revealed notably reduced relaxation

times in both treated and untreated CRL2314 cells compared with HTB-125 cells, validating MR relaxation time analysis as an effective approach in assessing drug responses and cellular viability in 3D culture models.

Magnetic suspension culture. Magnetic suspension culture employs a hydrogel medium composed of bacteriophages and magnetic iron oxide particles to establish 3D cultures, enabling spatial control over cellular aggregates through magnetic manipulation to achieve multicellular organization in coculture systems (74). Nevertheless, this technique presents several limitations: The magnetic beads are costly, potentially cytotoxic at elevated concentrations and enable limited aggregate yield (75). By integrating quantitative mass spectrometry-based proteomics with magnetic suspension culture, Vu *et al* (76) characterized proteomic alterations in squamous cell carcinoma cells, demonstrating that the absence of xenogenic protein scaffolds permits integrated analysis of cells with their endogenous ECM. Magnetic suspension has thus been demonstrated as a valuable methodology in elucidating proteomic dynamics underlying 3D tissue architecture. Qin *et al* (77) identified circadian rhythms in tumor cells maintained in suspension culture, advancing drug delivery strategies through stage-specific pharmacological interventions. Jaganathan *et al* (78) developed a magnetic suspension coculture system for breast cancer cells and fibroblasts, quantifying tumor size and cellular density while comparing phenotypic characteristics with *in vivo* tumors and examining matrix protein composition. Their results confirmed that this approach can facilitate precise control over tumor cell composition and density, rapidly generating large-scale breast tumor models within 24 h that markedly recapitulate the *in vivo* tumor microenvironment for antitumor drug evaluation.

Agarose coating. Agarose, a polysaccharide extracted from marine algae, dissolves in boiling aqueous solution and solidifies into non-cytotoxic gels upon cooling. These resultant gels inherently lack cell adhesion motifs, thereby effectively preventing the attachment of human and animal cells (79). Fig. 4 illustrates the following procedure: A quantified agarose solution is dispensed into a 96-well plate, cooled to room temperature and subsequently supplemented with a quantified cell suspension for culture. Ultimately, the formation of clustered cell spheroids is observed.

Zhang *et al* (80) established a 3D HepG2 cell model using 96-well flat-bottom plates combined with agarose gel, observing that well-defined cellular spheroids formed on the agarose surface when seeding densities were maintained between $1.975\text{x}10^3$ and $1\text{x}10^4$ cells/well. With increasing cell inoculation numbers, spheroid volumes expanded progressively, demonstrating a strong linear association within the density range of $1.975\text{-}6.667\text{x}10^3$ cells per well. Chen *et al* (81) systematically investigated the effects of primary components of tea polyphenols on breast CSCs cultured via the agarose-coating method. Using integrated experimental approaches including cell migration assays, scratch tests and cellular repair assessments among other techniques, the research team established that transcriptional downregulation of key EMT genes effectively suppressed invasive phenotypes in breast CSCs, diminished transcriptional activation of breast cancer marker genes, thus preventing manifestation of self-renewal characteristics. These findings markedly expand the potential pharmacological applications of primary tea polyphenol components in anticancer therapeutic development. Capitalizing on the unique thermally-responsive properties of agarose that undergo physical cross-linking at 35-40˚C, Gong *et al* (82) employed agarose as a gelatin substitute for 3D bioprinting at both 10 and 24˚C. Comparative evaluation revealed that structures fabricated using agarose pre-gelation methods exhibited notably enhanced dimensional precision, structural stability and mechanical rigidity compared with those produced using gelatin pre-gelation approaches.

4. Application of 3D culture technique for tumor cells

3D cell culture models have demonstrated marked potential in both assessing drug safety and efficacy, and modeling diverse pathological conditions (83). This 3D culture technology serves as a versatile platform for culturing tumor cells *in vitro*, enabling systematic investigation of oncogenesis, metastatic progression, invasive behavior, recurrence patterns and therapeutic strategies, while simultaneously facilitating drug discovery and screening applications (84).

Biological behavior of tumors. 3D tumor cell culture technology enables more accurate simulation of the authentic *in vivo* growth environment under *in vitro* conditions (85), demonstrating unique advantages and profound implications for investigating diverse tumor biological behaviors, therapeutic interventions and drug-screening processes (86). For example, $CD271^+$ uveal melanoma stem cells may undergo vasculogenic mimicry in 3D Matrigel culture (87). Another previous study revealed that nicastrin, a novel type I transmembrane glycoprotein, is associated with breast cancer stem cell properties, as determined using Matrigel culture (88).

Tumor angiogenesis. Vascular endothelial growth factor serves as a key regulator of pathological angiogenesis in tumors, activating endothelial cells to migrate, develop tip cells and ultimately anastomose into nascent blood vessels (89). To access the circulatory system, malignant cells must reside in proximity to the vasculature, due to their fundamental dependence on oxygen and nutrient supply for survival and proliferation. This complex process necessitates coordinated interactions among cells, extracellular matrices and signaling networks. 3D tumor cell culture systems can generate neovascular networks that closely mirror native vascular architecture, thereby establishing an advanced platform for investigating tumor migration and invasion mechanisms *in vitro* (90). Methods to vascularize various tissue/organ types using several synthetic and naturally occurring biomaterials have already been established. For example, Lazzari *et al* (91) constructed a poly-HEMA-based 3D tumor model by co-culturing PANC-1, MRC-5 and human umbilical vein endothelial cells to synthesize vascularized tumor spheroids of pancreatic cancer cells.

Tumor microenvironment. The tumor microenvironment comprises multiple cellular components, including endothelial cells, fibroblasts, pericytes, adipocytes and immune cells,

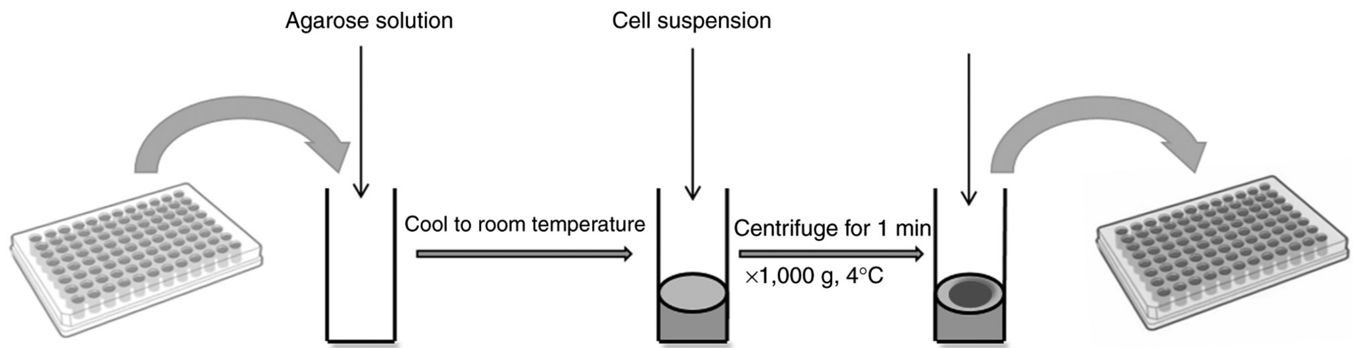

Figure 4. Schematic diagram of the agarose scaffold method for culturing cell spheroids in a 96-well plate.

which collectively contribute to tumor initiation, progression and metastasis (92). Through dynamic intercellular interactions, signal transduction and cellular communication, it provides key support for tumor survival and growth, while simultaneously modulating key processes such as immune responses, angiogenesis and drug resistance (93). Furthermore, the tumor microenvironment facilitates proto-oncogene expression and tumor-promoting protein production while impairing immune cell functionality. Due to the bidirectional regulatory interplay between tumors and their microenvironment, elucidating these complex mechanisms carries notable importance in understanding tumor biology, and advancing diagnostic and therapeutic strategies (94). Amaral *et al* (95) employed two different 3D cell culture techniques, the hanging drop and the forced floating with ultra-low attachment plates, to form human bladder cancer RT4 spheroids. These models are gaining popularity, given their ability to reproduce key aspects of the tumor microenvironment, concerning the 3D tumor architecture, as well as the interactions of tumor cells with the extracellular matrix and surrounding non-tumor cells (86).

CSCs. CSCs, which are characterized by self-renewal capacity, differentiation potential, high tumorigenicity and enhanced drug resistance, can withstand non-specific treatments including radiotherapy and chemotherapy, while serving key roles in tumor initiation, metastatic progression, drug resistance development and disease recurrence (96). The self-renewal capability and unlimited proliferative potential of these cells represent fundamental mechanisms sustaining tumor cell population viability, and their migratory activity may initiate tumor cell dissemination (97). When maintained in prolonged dormant states, CSCs harbor diverse drug-resistant molecules and demonstrate reduced sensitivity to exogenous physicochemical agents that typically eliminate tumor cells, such as anthracyclines, taxanes, anti-metabolites and alkylating agents (98) In recent years, the identification of novel targets specifically present in CSCs has emerged as a promising direction in the development of innovative antitumor therapeutics (99-101). CSCs grown in 3D model assays offer marked potential in understanding therapy response rates. Such cells have already been successfully isolated from a 3D model of human osteosarcoma treated with epirubicin. In this manner, 3D CSC models have provided novel insights into tumor drug resistance (102).

Development and screening of antitumor drugs. The field of anticancer drug development continues to grow with increasing demand for specifically targeted therapeutics (103). However, conventional 2D culture-based drug-screening platforms exhibit notable limitations, as antineoplastic efficacy observed *in vitro* frequently fails to translate to clinical settings (104). This translational gap primarily originates from the inherent inability of 2D systems to replicate key features of the native tumor microenvironment (105), which serves a key role in driving tumor progression, metastatic dissemination and drug resistance mechanisms. Therefore, developing 3D culture models that more accurately mimic tumor cell interactions with ECM components is of key importance. Such advanced models provide vital platforms for precisely evaluating chemotherapeutic performance and cytotoxic responses, thereby markedly enhancing the predictive capacity of high-throughput screening for anticancer compounds.

To address this challenge, researchers have developed a 3D *ex vivo* tumor model system utilizing the AXTEX-4D™ platform (106). This system is characterized by its capacity to generate physiologically relevant microenvironments through self-assembly of endogenously secreted matrix components without requiring exogenous scaffolding materials, thereby spontaneously establishing biochemical gradients that better approximate *in vivo* conditions. In immuno-oncology applications, the platform has demonstrated efficacy in evaluating therapeutic outcomes through monitoring key parameters, including immune cell proliferation, migration, infiltration, cytokine secretion and tumor-specific cytotoxicity (107). As a highly integrated physiomimetic system, it serves as a robust platform for high-throughput screening of immunotherapeutic agents, markedly enhancing the predictive accuracy and translational relevance of preclinical drug evaluation (105).

Rosendahl *et al* (108) established 3D coculture models of MCF7 and MDA-MB-231 human breast cancer cell lines within 2,2,6,6-tetramethylpiperidine 1-oxyl cellulose nanofibril (TEMPO-CNF) scaffolds, observing multilayered tumor growth with distinct morphological patterns, while demonstrating that these TEMPO-CNF scaffolds upregulated the expression of stem cell marker CD44 and migration markers Vimentin/SNAI1 in MCF7 cells compared with 2D culture systems, thereby establishing TEMPO-CNF as a promising biomaterial in developing 3D culture platforms applicable to anticancer drug screening.

5. Significance and prospects

Technical challenges requiring resolution. Notwithstanding their enhanced physiological relevance, the broad implementation of 3D models faces constraints from several key technical hurdles.

First, operational expenses markedly exceed those of conventional 2D systems, primarily due to dependence on specialized and costly components such as commercial basement membrane extracts and sophisticated bioreactor systems designed for extended culture maintenance and perfusion requirements. Furthermore, high-resolution imaging of 3D specimens typically necessitates advanced instrumentation including confocal or light sheet microscopy, representing considerable additional investment. These cumulative costs restrict accessibility for resource-constrained laboratories and thereby reduce implementation scalability in high-throughput screening campaigns.

Second, reproducibility and standardization issues present notable obstacles. Batch-to-batch variations in scaffold materials introduce considerable experimental variables that compromise result reliability (109), while manual production methods in scaffold-free models frequently generate spheroids with inadequate size uniformity that consequently demonstrate elevated data variability (110). The field currently lacks unified culture protocols, standardized analytical criteria and clearly defined efficacy endpoints, thus preventing direct comparison and integration of experimental data across different laboratories.

Furthermore, the intrinsic 3D architecture of these models creates notable challenges for imaging procedures and subsequent data interpretation. From an optical perspective, light scattering and absorption phenomena restrict both imaging depth and resolution, particularly in specimens >200 μm in thickness, often necessitating specialized methodologies such as tissue clearing methods or light sheet fluorescence microscopy (111). From an analytical standpoint, extracting quantitative parameters from 3D images, including cellular viability, morphological characteristics and spatial heterogeneity in protein expression, proves markedly more complex compared with corresponding 2D analyses (112). This process requires sophisticated computational algorithms and artificial intelligence capabilities to execute tasks involving 3D cell segmentation, structural reconstruction and phenotypic characterization (113), thereby demanding enhanced computational resources and specialized technical expertise.

Future directions and innovation. To address these challenges and facilitate clinical translation of 3D tumor models, future investigations should concentrate on the following key domains.

Technological integration and automation. i) 3D bioprinting. Bioprinting technology serves as a robust methodology to address standardization challenges by enabling precise and reproducible deposition of cellular components and biomaterials. Through the layered assembly of multiple cell types with gradient biofactors, this approach facilitates the construction of highly biomimetic and structurally controllable tumor microenvironment models (114). These advanced systems prove particularly valuable for investigating complex pathological processes including tumor invasion and metastatic progression (114). Long-term perspectives encompass applications in vascular regeneration and cartilage repair, with potential clinical translation toward surgical reconstruction of blood vessels and cartilage tissues, alongside the development of personalized drug delivery platforms (115).

ii) Organ-on-a-chip platforms. The integration of 3D tumor models with microfluidics technology enables the development of organ-on-a-chip systems. These platforms dynamically simulate physiological parameters including hemodynamic flows, mechanical stresses and inter-tissue interactions, thereby achieving more accurate recapitulation of *in vivo* drug distribution and metabolic processing. Such technological synergy provides an innovative framework for enhancing the physiological relevance of preclinical drug evaluation methodologies (116).

Data integration and intelligent analysis. i) Multi-omics data integration. Integrating phenotypic readouts from 3D models with subsequent multi-omics analyses enables elucidation of underlying molecular mechanisms (117), where this model-to-omics strategy establishes a key bridge connecting observed phenotypic responses with their mechanistic drivers, while facilitating identification of novel therapeutic targets and predictive biomarkers.

ii) Artificial intelligence and machine learning. Targeting artificial intelligence to process complex 3D imaging and multi-omics datasets allows identification of patterns imperceptible through conventional analysis (118), enabling automated high-content phenotypic screening and supporting development of robust predictive models in evaluating therapeutic efficacy and patient prognosis.

Defining clinical translation pathways. Future research endeavors should prioritize systematic validation of 3D models by rigorously establishing associations between their predictive accuracy and clinical outcomes. Advancing standardization of technologies, such as 3D bioprinting and organ-on-a-chip systems, while integrating them comprehensively with multi-omics analyses and artificial intelligence will enable the development of more predictive tumor models. These advancements will not only improve the fundamental understanding of cancer biology but also expedite the development of innovative anticancer therapies, ultimately propelling the field of precision oncology toward novel frontiers.

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SD and HL conceptualized and designed the present review. SD, HW, DX, JY and SM drafted the manuscript, and prepared the tables and figures. Data authentication is not applicable. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

References

1. Madrid MF, Mendoza EN, Padilla AL, Choquenaira-Quispe C, de Jesus Guimarães C, de Melo Pereira JV, Barros-Nepomuceno FWA, Lopes Dos Santos I, Pessoa C, de Moraes Filho MO, *et al*: In vitro models to evaluate multidrug resistance in cancer cells: Biochemical and morphological techniques and pharmacological strategies. J Toxicol Environ Health B Crit Rev 28: 1-27, 2025.
2. Faber MN, Sojan JM, Saraiva M, van West P and Secombes CJ: Development of a 3D spheroid cell culture system from fish cell lines for in vitro infection studies: Evaluation with Saprolegnia parasitica. J Fish Dis 44: 701-710, 2021.
3. Langhans SA: Using 3D in vitro cell culture models in Anti-cancer drug discovery. Expert Opin Drug Discov 16: 841-850, 2021.
4. Lu X, Liu X, Zhong H, Zhang W, Yu S and Guan R: Progress on Three-dimensional cell culture technology and their application. Sheng Wu Yi Xue Gong Cheng Xue Za Zhi 40: 602-608, 2023 (In Chinese).
5. Sugimoto M, Kitagawa Y, Yamada M, Yajima Y, Utoh R and Seki M: Micropassage-embedding composite hydrogel fibers enable quantitative evaluation of cancer cell invasion under 3D coculture conditions. Lab Chip 18: 1378-1387, 2018.
6. Zhang T, Zhang KN and Ke CX: Research progress of three-dimensional cell culture in bladder cancer. J Med Res 50: 16-18, 2021.
7. Zhang C, Yang Z, Dong DL, Jang TS, Knowles JC, Kim HW, Jin GZ and Xuan Y: 3D culture technologies of cancer stem cells: Promising ex vivo tumor models. J Tissue Eng 11: 2041731420933407, 2020.
8. Jovanović Stojanov S, Grozdanić M, Ljujić M, Dragičević S, Dragoj M and Dinić J: Cancer 3D Models: Essential tools for understanding and overcoming drug resistance. Oncol Res 33: 2741-2785, 2025.
9. Lonkwic KM, Zajdel R and Kaczka K: Unlocking the potential of spheroids in personalized medicine: A systematic review of seeding methodologies. Int J Mol Sci 26: 6478, 2025.
10. Kaur N, Savitsky MJ, Scutte A, Mehta R, Dridi N, Sogbesan T, Savannah A, Lopez D, Yang D and Ali J: Navigating cancer treatment: A journey from 2D to 3D cancer models and nanoscale therapies. ACS Pharmacol Transl Sci 8: 3773-3800, 2025.
11. Abuwatfa WH, Pitt WG and Husseini GA: Scaffold-based 3D cell culture models in cancer research. J Biomed Sci 31: 7, 2024.
12. Wang X, Kawazoe N and Chen G: Interaction of immune cells and tumor cells in gold nanorod-gelatin composite porous scaffolds. Nanomaterials (Basel) 9: 1367, 2019.
13. Wang X, Zhang J, Li J, Chen Y, Kawazoe N and Chen G: Bifunctional scaffolds for the photothermal therapy of breast tumor cells and adipose tissue regeneration. J Mater Chem B 6: 7728-7736, 2018.
14. Alafnan A, Seetharam AA, Hussain T, Gupta MS, Rizvi SMD, Moin A, Alamri A, Unnisa A, Awadelkareem AM, Elkhalifa AO, *et al*: Development and characterization of PEGDA microneedles for localized drug delivery of gemcitabine to treat inflammatory breast cancer. Materials (Basel) 15: 7693, 2022.
15. Bai G, Yuan P, Cai B, Qiu X, Jin R, Liu S, Li Y and Chen X: Stimuli-responsive scaffold for breast cancer treatment combining accurate photothermal therapy and adipose tissue regeneration. Adv Funct Mater 29: 1904401, 2019.
16. Dettin M, Sieni E, Zamuner A, Marino R, Sgarbossa P, Lucibello M, Tosi AL, Keller F, Campana LG and Signori E: A Novel 3D scaffold for cell growth to asses electroporation efficacy. Cells 8: 1470, 2019.
17. Fang Z, Chen L, Moser MA, Zhang W, Qin Z and Zhang B: Electroporation-based therapy for brain tumors: A review. J Biomech Eng 143: 100802, 2021.
18. Lou J and Mooney DJ: Chemical strategies to engineer hydrogels for cell culture. Nat Rev Chem 6: 726-744, 2022.
19. Radulescu DM, Neacsu IA, Grumezescu AM and Andronescu E: New insights of scaffolds based on hydrogels in tissue engineering. Polymers (Basel) 14: 799, 2022.
20. Wang C, Ye X, Zhao Y, Bai L, He Z, Tong Q, Xie X, Zhu H, Cai D, Zhou Y, *et al*: Cryogenic 3D printing of porous scaffolds for in situ delivery of 2D black phosphorus nanosheets, doxorubicin hydrochloride and osteogenic peptide for treating tumor resection-induced bone defects. Biofabrication 12: 035004, 2020.
21. Yang X, Wang Y, Mao T, Wang Y, Liu R, Yu L and Ding J: An oxygen-enriched thermosensitive hydrogel for the relief of a hypoxic tumor microenvironment and enhancement of radiotherapy. Biomater Sci 9: 7471-7482, 2021.
22. Yang A, Bai Y, Dong X, Ma T, Zhu D, Mei L and Lv F: Hydrogel/nanoadjuvant-mediated combined cell vaccines for cancer immunotherapy. Acta Biomater 133: 257-267, 2021.
23. Fu SJ, Liu XX, Hu MB, Li CJ ,Wang FJ and Wang L: Preparation and characterization of fiber-hydrogel composite scaffolds for three-dimensional culture of breast tumorcells. J Donghua Univ (Nat Sci) 50: 11-19, 2024.
24. Quazi MZ and Park N: DNA Hydrogel-based nanocomplexes with Cancer-targeted delivery and Light-triggered peptide drug release for Cancer-specific therapeutics. Biomacromolecules 24: 2127-2137, 2023.
25. Jiang R, Huang J, Sun X, Chu X, Wang F, Zhou J, Fan Q and Pang L: Construction of in vitro 3-D model for lung cancer-cell metastasis study. BMC Cancer 22: 438, 2022.
26. Yao J, Li J, Du G and Zhao JM: Effect of nano-microporous polycaprolactone combined with bone marrow mesenchymal stem cells on cartilage injury in rabbits. Guangdong Med J 35: 3307-3309, 2014.
27. Mahendiran B, Muthusamy S, Selvakumar R, Rajeswaran N, Sampath S, Jaisankar SN and Krishnakumar GS: Decellularized natural 3D cellulose scaffold derived from Borassus flabellifer (Linn.) as extracellular matrix for tissue engineering applications. Carbohydr Polym 272: 118494, 2021.
28. Nemati M, Bani F, Sepasi T, Zamiri RE, Rasmi Y, Kahroba H, Rahbarghazi R, Sadeghi MR, Wang Y, Zarebkohan A and Gao H: Unraveling the effect of breast cancer patients' plasma on the targeting ability of folic acid-modified chitosan nanoparticles. Mol Pharm 18: 4341-4353, 2021.
29. Tian XH: Preparation of pH-responsive Chitosan Anticancer Drug Carriers and Their Application. Jinzhou Medical University, 2019.
30. De Araújo JT, Tavares Junior AG, Di Filippo LD, Duarte JL, Ribeiro TD and Chorilli M: Overview of chitosan-based nanosystems for prostate cancer therapy. Eur Polymer J 160: 110812, 2021.
31. Pham DT, Saelim N and Tiyaboonchai W: Paclitaxel loaded EDC-crosslinked fibroin nanoparticles: A potential approach for colon cancer treatment. Drug Deliv Transl Res 10: 413-424, 2020.
32. Chaicharoenaudomrung N, Kunhorm P, Promjantuek W, Heebkaew N, Rujanapun N and Noisa P: Fabrication of 3D calcium-alginate scaffolds for human glioblastoma modeling and anticancer drug response evaluation. J Cell Physiol 234: 20085-20097, 2019.
33. Kim CJ, Terado T, Tambe Y, Mukaisho KI, Sugihara H, Kawauchi A and Inoue H: Anti-oncogenic activities of cyclin D1b siRNA on human bladder cancer cells via induction of apoptosis and suppression of cancer cell stemness and invasiveness. Int J Oncol 52: 231-240, 2018.
34. Liu C, Wang Z, Wei X, Chen B and Luo Y: 3D printed hydrogel/PCL core/shell fiber scaffolds with NIR-triggered drug release for cancer therapy and wound healing. Acta Biomater 131: 314-325, 2021.
35. Niculescu AG, Chircov C, Bîrcă AC and Grumezescu AM: Fabrication and applications of microfluidic devices: A review. Int J Mol Sci 22: 2011, 2021.
36. Modena MM, Chawla K, Misun PM and Hierlemann A: Smart cell culture systems: Integration of sensors and actuators into microphysiological systems. ACS Chem Biol 13: 1767-1784, 2018.

37. Parihar A and Mehta PP: Lab-on-a-chip devices for advanced biomedicines: Laboratory scale engineering to clinical ecosystem. Royal Society of Chemistry, 2024.
38. Hakim M, Khorasheh F, Alemzadeh I and Vossoughi M: A new insight to deformability correlation of circulating tumor cells with metastatic behavior by application of a new deformability-based microfluidic chip. Anal Chim Acta 1186: 339115, 2021.
39. Matsuura-Sawada Y, Maeki M, Uno S, Wada K and Tokeshi M: Controlling lamellarity and physicochemical properties of liposomes prepared using a microfluidic device. Biomater Sci 11: 2419-2426, 2023.
40. Xu Y, Chen B, He M, Cui Z and Hu B: All-in-One microfluidic chip for online labeling, separating, and focusing rare circulating tumor cells from blood samples followed by inductively coupled plasma mass spectrometry detection. Anal Chem 95: 14061-14067, 2023.
41. Zhu YH, Chen S, Zhang CF, Ikoma T, Guo HM, Zhang XY, Li X and Chen W: Novel microsphere-packing synthesis, microstructure, formation mechanism and in vitro biocompatibility of porous gelatin/hydroxyapatite microsphere scaffolds. Ceramics Int 47: 32187-32194, 2021.
42. Allu I, Sahi AK, Koppadi M, Gundu S and Sionkowska A: Decellularization techniques for tissue engineering: Towards replicating native extracellular matrix architecture in liver regeneration. J Funct Biomater 14: 518, 2023.
43. Qiu H, Wang H, Yang X and Huo F: High performance isolation of circulating tumor cells by acoustofluidic chip coupled with ultrasonic concentrated energy transducer. Colloids Surf B Biointerfaces 222: 113138, 2023.
44. Hen SK and Jv XJ: Preparation of open macroporous alginate microspheres for Three-dimensional cell culture. Polymer Materials Sci Engine 39: 126-133, 2023.
45. Song JQ, Chen HL and Yang FW: Research and Application of 3D Bioprinting Technology in Medical Field. Chin Med Equipment 36: 151-165, 2021.
46. Liu P: Construction and Application of 3D-printed microfluidic chip cell analysis platform. Shandong Normal University, 2023.
47. Jin Y, Zhang J, Xing J, Li Y, Yang H, Ouyang L, Fang ZY, Sun LJ, Jin B, *et al*: Multicellular 3D bioprinted human gallbladder carcinoma forin vitromimicry of tumor microenvironment and intratumoral heterogeneity. Biofabrication 16: 045028, 2024.
48. Mai P, Hampl J, Baca M, Brauer D, Singh S, Weise F, Borowiec J, Schmidt A, Küstner JM, Klett M, *et al*: MatriGrid® based biological morphologies: Tools for 3D cell culturing. Bioengineering (Basel) 9: 220, 2022.
49. Jubelin C, Muñoz-Garcia J, Griscom L, Cochonneau D, Ollivier E, Heymann MF, Vallette FM, Oliver L and Heymann D: Three-dimensional in vitro culture models in oncology research. Cell Biosci 12: 155, 2022.
50. Ayvaz I, Sunay D, Sariyar E, Erdal E and Karagonlar ZF: Three-dimensional cell culture models of hepatocellular carcinoma-a review. J Gastrointest Cancer 52: 1294-1308, 2021.
51. De Pieri A, Rochev Y and Zeugolis DI: Scaffold-free cell-based tissue engineering therapies: Advances, shortfalls and forecast. NPJ Regen Med 6: 18, 2021.
52. Czaplinska D, Elingaard-Larsen LO, Rolver MG, Severin M and Pedersen SF: 3D multicellular models to study the regulation and roles of acid-base transporters in breast cancer. Biochem Soc Trans 47: 1689-1700, 2019.
53. Kelm JM, Timmins NE, Brown CJ, Fussenegger M and Nielsen LK: Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. Biotechnol Bioeng 83: 173-180, 2003.
54. Rodoplu D, Matahum JS and Hsu CH: A microfluidic hanging drop-based spheroid co-culture platform for probing tumor angiogenesis. Lab Chip 22: 1275-1285, 2022.
55. Safari F, Shakery T and Sayadamin N: Evaluating the effect of secretome of human amniotic mesenchymal stromal cells on apoptosis induction and epithelial-mesenchymal transition inhibition in LNCaP prostate cancer cells based on 2D and 3D cell culture models. Cell Biochem Funct 39: 813-820, 2021.
56. Heinrich MA, Huynh NT, Heinrich and Prakash J: Understanding glioblastoma stromal barriers against NK cell attack using tri-culture 3D spheroid model. Heliyon 10: e24808, 2024.
57. Bahmad HF, Cheaito K, Chalhoub RM, Hadadeh O, Monzer A, Ballout F, EL-Hajj A, Mukherji D, Liu YN, Daoud G and Abou-Kheir W: Sphere-formation assay: Three-dimensional in vitro culturing of prostate cancer stem/progenitor sphere-forming cells. Front Oncol 8: 347, 2018.
58. Ferreira SM: Effect of normal and tumor extracellular matrix in cancer stem Cell-Like properties. Universidade do Porto (Portugal), 2019.
59. Xue W, Wang J, Hou Y, Wu D, Wang H, Jia Q, Jiang Q, Wang Y, Song C, Wang Y, *et al*: Lung decellularized matrix-derived 3D spheroids: Exploring silicosis through the impact of the Nrf2/Bax pathway on myofibroblast dynamics. Heliyon 10: e33585, 2024.
60. Sun B, Liang Z, Wang Y, Yu Y, Zhou X, Geng X and Li B: A 3D spheroid model of quadruple cell co-culture with improved liver functions for hepatotoxicity prediction. Toxicology 505, 153829, 2024.
61. Wang J, Kong Y and Deng FS: Enrichment of pancreatic cancer stem cells by sphere-forming culture. Anhui Med J 40: 1303-1305, 2019.
62. Raggi C, Taddei ML, Sacco E, Navari N, Correnti M, Piombanti B, Pastore M, Campani C, Pranzini E, Iorio J, *et al*: Mitochondrial oxidative metabolism contributes to a cancer stem cell phenotype in cholangiocarcinoma. J Hepatol 74: 1373-1385, 2021.
63. Topal U and Zamur C: Microgravity, stem cells, and cancer: A new hope for cancer treatment. Stem Cells Int 2021: 5566872, 2021.
64. Kumar V, Sethi B, Staller DW, Shrestha P and Mahato RI: Gemcitabine elaidate and ONC201 combination therapy for inhibiting pancreatic cancer in a KRAS mutated syngeneic mouse model. Cell Death Discov 10: 158, 2024.
65. Belloni D, Ferrarini M, Ferrero E, Guzzeloni V, Barbaglio F, Ghia P and Scielzo C: Protocol for generation of 3D bone marrow surrogate microenvironments in a rotary cell culture system. STAR Protoc 3: 101601, 2022.
66. Naser Al Deen N, Atallah Lanman N, Chittiboyina S, Lelièvre S, Nasr R, Nassar F, Dohna H, AbouHaidar M and Talhouk R: A risk progression breast epithelial 3D culture model reveals Cx43/hsa_circ_0077755/miR-182 as a biomarker axis for heightened risk of breast cancer initiation. Sci Rep 11: 2626, 2021.
67. Malhão F, Macedo AC, Ramos AA and Rocha E: Morphometrical, morphological, and immunocytochemical characterization of a tool for cytotoxicity research: 3D cultures of breast cell lines grown in Ultra-low attachment plates. Toxics 10: 415, 2022.
68. Yang JY, Liu D, Li L, Li DH and Wang HF: Effect of propofol on pyroptosis of lung cancer A549 cells by NLRP3/ASC/caspase-1 pathway. J China Med Univ 53: 132-141, 2024.
69. Du M, Liu ZJ, Zou F, Cai HH, Li JY and Zhou BMJ: Effect of umbilical cord mesenchymal stem cell supernatant on esophageal squamous cell carcinoma spheres. Chin J Gastroenterol Hepatol 31: 634-637, 2022.
70. Liu B, Han S, Modarres-Sadeghi Y and Lynch ME: Multiphysics simulation of a compression-perfusion combined bioreactor to predict the mechanical microenvironment during bone metastatic breast cancer loading experiments. Biotechnol Bioeng 118: 1779-1792, 2021.
71. Calamak S, Ermis M, Sun H, Islam S, Sikora M, Nguyen M, Hasirci V and Steinmetz LM: A circulating bioreactor reprograms cancer cells toward a more mesenchymal niche. Adv Biosyst 4: e1900139, 2020.
72. Huo Z, Bilang R, Supuran CT, von der Weid N, Bruder E, Holland-Cunz S, Martin I, Muraro MG and Gros SJ: Perfusion-based bioreactor culture and isothermal microcalorimetry for preclinical drug testing with the carbonic anhydrase inhibitor SLC-0111 in Patient-derived neuroblastoma. Int J Mol Sci 23: 3128, 2022.
73. Bober Z, Podgórski R, Aebisher D, Cieślar G, Kawczyk-Krupka A and Bartusik-Aebisher D: Cellular 1H MR relaxation times in healthy and cancer Three-dimensional (3D) breast cell culture. Int J Mol Sci 24: 4735, 2023.
74. Anil-Inevi M, Yilmaz E, Sarigil O, Tekin HC and Ozcivici E: Single cell densitometry and weightlessness culture of mesenchymal stem cells using magnetic levitation. Methods Mol Biol 2125: 15-25, 2020.
75. Hoarau-Vechot J, Rafii A, Touboul C and Pasquier J: Halfway between 2D and animal models: Are 3D cultures the ideal tool to study cancer-microenvironment interactions? Int J Mol Sci 19: 181, 2018.
76. Vu B, Souza GR and Dengjel J: Scaffold-free 3D cell culture of primary skin fibroblasts induces profound changes of the matrisome. Matrix Biol Plus 11: 100066, 2021.
77. Qin WJ, Pan MP, Su ZJ, Hou RR, Lu HJ and Le ZC: Biological clock synchronization of cancer cells basedw on suspension culturedand its application in the study of timing drug administration. J Xiamen Univ (Nat Sci): 138-143, 2021.
78. Jaganathan H, Gage J, Leonard F, Srinivasan S, Souza GR, Dave B and Godin B: Three-dimensional in vitro co-culture model of breast tumor using magnetic levitation. Sci Rep 4: 6468, 2014.

79. He YY, Li LJ, Zhang SQ, Li YZ, Yang S and Ji P: Method of constructing cell spheroids based on agarose and polyacrylic molds. Chin J Tissue Eng Res 26: 553-559, 2022 (In Chinese).
80. Zhang J, Fan X, Li M, Dou MJ and Wang HF: Establishment of 3-dimensional culture model of HepG2 cells and viability detection. Central South Pharmacy 18: 1111-1114, 2020.
81. Chen L, Li KM, Ma HJ, Wang F, Zeng SX and Song XQ: The inhibitory effect of EGCG on breast cancer stem cell migration and Self-renewal properties in 3D culture system. J Xinyang Normal Univ Nat Sci Ed 32: 437-442, 2019.
82. Gong C, Kong Z and Wang X: The effect of agarose on 3D bioprinting. Polymers (Basel) 13: 4028, 2021.
83. Wang H, Brown PC, Chow ECY, Ewart L, Ferguson SS, Fitzpatrick S, Freedman BS, Guo GL, Hedrich W, Heyward S, *et al*: 3D cell culture models: Drug pharmacokinetics, safety assessment, and regulatory consideration. Clin Transl Sci 14: 1659-1680, 2021.
84. Zuo DQ and Cai ZD: Progress in three-dimensional cell culture and its application in tumor research. Tumor 38: 502-507, 2023.
85. Rauner G, Gupta PB and Kuperwasser C: From 2D to 3D and beyond: The evolution and impact of in vitro tumor models in cancer research. Nat Methods 22: 1776-1787, 2025.
86. Barbosa MA, Xavier CP, Pereira RF, Petrikaitė V and Vasconcelos MH: 3D cell culture models as recapitulators of the tumor microenvironment for the screening of Anti-cancer drugs. Cancers (Basel) 14: 190, 2021.
87. Valyi-Nagy K, Kormos B, Ali M, Shukla D and Valyi-Nagy T: Stem cell marker CD271 is expressed by vasculogenic mimicry-forming uveal melanoma cells in three-dimensional cultures. Mol Vis 18: 588-592, 2012.
88. Lombardo Y, Filipović A, Molyneux G, Periyasamy M, Giamas G, Hu Y, Trivedi PS, Wang J, Yagüe E, Michel L and Coombes RC: Nicastrin regulates breast cancer stem cell properties and tumor growth in vitro and in vivo. Proc Natl Acad Sci USA 109: 16558-16563, 2012.
89. Eelen G, Treps L, Li X and Carmeliet P: Basic and therapeutic aspects of angiogenesis updated. Circ Res 127: 310-329, 2020.
90. Lugano R, Ramachandran M and Dimberg A: Tumor angiogenesis: Causes, consequences, challenges and opportunities. Cell Mol Life Sci 77: 1745-1770, 2020.
91. Lazzari G, Nicolas V, Matsusaki M, Akashi M, Couvreur P and Mura S: Multicellular spheroid based on a triple co-culture: A novel 3D model to mimic pancreatic tumor complexity. Acta Biomater 78: 296-307, 2018.
92. Kumari S, Advani D, Sharma S, Ambasta RK and Kumar P: Combinatorial therapy in tumor microenvironment: Where do we stand? Biochim Biophys Acta Rev Cancer 1876: 188585, 2021.
93. Gao QD, Duan XF, Li Y, Xu T, Yu YY and Bai GD: Research on the role of resveratrol against breast cancer. China Pharmacy 35: 1408-1412, 2024.
94. Ren X, Zhang L, Zhang Y, Li Z, Siemers N and Zhang Z: Insights gained from Single-Cell analysis of immune cells i91n the tumor microenvironment. Annu Rev Immunol 9: 583-609, 2021.
95. Amaral RLF, Miranda M, Marcato PD and Swiech K: Comparative analysis of 3D bladder tumor spheroids obtained by forced floating and hanging drop methods for drug screening. Front Physiol 8: 605, 2017.
96. Zhang W, Zhao K, Zhang K, Chen X, Hao M and Yang Z: Advances in the biological characteristics and stem maintenance mechanisms of tumor stem cells. Chin J Biotech 40: 391-418, 2024.
97. Li XY, Yang X and Huang M: Advances in cancer therapeutic targets-cancer stem cells. J Logistics Univ PAP (Med Sci) 3: 65-70, 2021.
98. Zhang LS, Shi HY, Zhang T, Wang J, Mao H and Wang XL: Research progress of biomarkers of endometrial cancer stemc cells. Med Recapitulate: 934-939, 2022.
99. Lee H, Kim B, Park J, Park S, Yoo G, Yum S, Kang W, Lee JM, Youn H and Youn B: Cancer stem cells: Landscape, challenges and emerging therapeutic innovations. Signal Transduct Target Ther 10: 248, 2025.
100. Guo S, Zheng S, Liu M and Wang G: Novel anti-cancer stem cell Compounds: A Comprehensive review. Pharmaceutics 16: 1024, 2024.
101. Li YR, Fang Y, Lyu Z, Zhu Y and Yang L: Exploring the dynamic interplay between cancer stem cells and the tumor microenvironment: implications for novel therapeutic strategies. J Transl Med: 21 686, 2023.
102. Kimlin LC, Casagrande G and Virador VM: In vitro three-dimensional (3D) models in cancer research: An update. Mol Carcinog 52: 167-182, 2013.
103. Chunduri V and Maddi S: Role of in vitro two-dimensional (2D) and three-dimensional (3D) cell culture systems for ADME-Tox screening in drug discovery and development: A comprehensive review. ADMET DMPK 11: 1-32, 2023.
104. Crouigneau R, Li YF, Auxillos J, Goncalves-Alves E, Marie R, Sandelin A and Pedersen SF: Mimicking and analyzing the tumor microenvironment. Cell Reports Methods 4: 100866, 2024.
105. Honkala A, Malhotra SV, Kummar S and Junttila MR: Harnessing the predictive power of preclinical models for oncology drug development. Nat Rev Drug Discov 21: 99-114, 2022.
106. Baru A, Mazumder S, Kundu P, Sharma S, Purakayastha BPD, Khan S, Gupta R and Arora NM: Establishment of a three-dimensional triculture model on the novel AXTEX-4D™ platform. Oncol Rep 49: 2, 2023.
107. Goenka A, Khan F, Verma B, Sinha P, Dmello CC, Jogalekar MP, Gangadaran P and Ahn BC: Tumor microenvironment signaling and therapeutics in cancer progression. Cancer Commun (Lond) 43: 525-561, 2023.
108. Rosendahl J, Svanström A, Berglin M, Petronis S, Bogestål Y, Stenlund P, Standoft S, Ståhlberg A, Landberg G, Chinga-Carrasco G and Håkansson J: 3D printed nanocellulose scaffolds as a cancer cell culture model system. Bioengineering (Basel) 8: 97, 2021.
109. Rafieyan S, Ansari E and Vasheghani-Farahani E: A practical machine learning approach for predicting the quality of 3D (bio) printed scaffolds. Biofabrication: 16, 2024 doi: 10.1088/1758-5090/ad6374.
110. Anthon SG and Valente KPL: Vascularization strategies in 3D cell culture models: From scaffold-free models to 3D bioprinting. Int J Mol Sci 23: 14582, 2022.
111. Yoon S, Cheon SY, Park S, Lee D, Lee Y, Han S, Kim M and Koo H: Recent advances in optical imaging through deep tissue: Imaging probes and techniques. Biomater Res 26: 57, 2022.
112. Xu X, Su J, Zhu R, Li K, Zhao X, Fan J and Mao F: From morphology to single-cell molecules: High-resolution 3D histology in biomedicine. Mol Cancer 24: 63, 2025.
113. Ali M, Benfante V, Basirinia G, Alongi P, Sperandeo A, Quattrocchi A, Giannone AG, Cabibi D and Yezzi A: Applications of artificial intelligence, deep learning, and machine learning to support the analysis of microscopic images of cells and tissues. J Imaging 11: 59, 2025.
114. Li Y, Cui H and Cui H: Precision spatial control of Tumor-stroma interactions in cancer models via 3D bioprinting for advanced research and therapy. Adv Funct Materials 35: 2503391, 2025.
115. Hamilton C, Collins V, Butala S, Lee K, Panse N, Pierce A, Borole A, Gupta S, Rahimi S, Truong H and Beckerman W: Current trends and outlook of 3D printing in vascular surgery. JVS-Vascular Insights 2: 100114, 2024.
116. Li Z, Hui J, Yang P and Mao H: Microfluidic organ-on-a-chip system for disease modeling and drug development. Biosensors 12: 370, 2022.
117. Nevedomskaya E and Haendler B: From omics to multi-omics approaches for in-depth analysis of the molecular mechanisms of prostate cancer. Int J Mol Sci 23: 6281, 2022.
118. Yetgin A: Revolutionizing multi-omics analysis with artificial intelligence and data processing. Quantitative Biol 13: e70002, 2025.