

Three-dimensional cell culture: A powerful tool in tumor research and drug discovery (Review)

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Abstract. In previous years, three-dimensional (3D) cell culture technology has become a focus of research in tumor cell biology, using a variety of methods and materials to mimic the *in vivo* microenvironment of cultured tumor cells *ex vivo*. These 3D tumor cells have demonstrated numerous different characteristics compared with traditional two-dimensional (2D) culture. 3D cell culture provides a useful platform for further identifying the biological characteristics of tumor cells, particularly in the drug sensitivity area of the key points of translational medicine. It promises to be a bridge between traditional 2D culture and animal experiments, and is of great importance for further research in the field of tumor biology. In the present review, previous 3D cell culture applications, focusing on anti-tumor drug susceptibility testing, are summarized.

Contents

1. Introduction
2. Current preclinical tumor models
3. The methods of 3D cell culture
4. Future trends and conclusions

1. Introduction

As one of the basic techniques utilized to study tumor cell biology, the continual development of tumor cell culture techniques is vital. Traditional cell culture methods use a two-dimensional (2D) monolayer. With continuous improvements being made, this method has become a standard technology in life sciences at present. However, due to the inherent flaws of traditional 2D culture, it fails to correctly

imitate the architecture and microenvironments of *in vivo*, which makes 2D-cultured cells different from cells growing *in vivo* in terms of morphology, proliferation, cell-cell and cell-matrix inter-connections, signal transduction, differentiation and other aspects (1,2). In order to improve these simulations of cell microenvironments *in vivo*, 3D culture has become the next frontier of cell biology research.

As the intersection between tumor cell biology and tissue engineering, 3D *in vitro* tumor models simulate the *in vivo* physiological microenvironment, and may be useful at the pre-clinical development stage to identify potentially successful prototypes and eliminate failures at an early stage. This means that it has potential to bridge the gap between traditional monolayer cell culture and tumor cytology experiments *in vivo*. Therefore, an increasing number of tumor biologists have begun to emphasize the importance of 3D tumor cell culture (3). Subsequently, the advent of 3D culture has seen rapid advancement in the past few decades, as evidenced by the increasing number of studies in this area (4,5), including preclinical drug screening, cancer stem cell maintenance and differentiation, signal abnormal transduction and other aspects (6-8). For example, compared with 2D monolayer cultures, cells in 3D culture generally exhibit a reduced sensitivity to certain chemotherapeutic agents (9). These results and the exponentially increasing number of studies surrounding this topic convey the importance of 3D tumor cell culture, one of the primary reasons for the present review.

Thus, in the present review, the current preclinical models in cancer research are briefly described in order to promote understanding of the necessity of novel cell culture system. In addition to this, the advantages and challenges of 3D *in vitro* tumor models are discussed in terms of a mechanistic understanding of tumor cell physiology and in therapeutic evaluations of anti-tumor drug discovery.

2. Current preclinical tumor models

At present, *in vitro* anti-tumor drugs tests are mainly performed by detecting the reaction of tumor cells in a 2D monolayer culture dish. It is estimated that >80% of cancer biologists still rely on 2D culture techniques to obtain results prior to *in vivo* testing because of its convenience (10). However, in clinical trials, it has become apparent that effective drugs in *in vitro* experiments have no or weak efficacy in real patients with tumors (11). This phenomenon, at least

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partially, has been attributed to the fact that cells grown in 2D culture lack the complex 3D tissue architecture and cell-cell or cell-extracellular matrix (ECM) interactions which exist in the body (12). These models therefore fail to fully reflect the pathophysiology of tumor cells, and the real level of resistance to radiation or drugs in the *in vivo* niche (6,13). For example, hepatic cancer cells in 3D culture may tolerate drug treatment, similar to the resistance characteristics of solid tumors *in vivo* (14). Breast cancer MCF-7 cells in 3D scaffolds demonstrated a stronger resistance to tamoxifen in endocrine therapy than those in monolayer culture (7). Additionally, drug resistance induced by cancer stem cells (CSCs) has received substantial attention in previous decades. However, traditional 2D culture fails to provide an adequate CSC enrichment culture (6). These studies suggest that the microenvironment of tumor cells may notably alter the susceptibility of cancer cell drugs. Traditional 2D culture should, therefore, be subject to further development.

Animal models are an important tool for tumor research. Animal model testing is primarily conducted to monitor drug bioavailability, therapeutic efficacy and dose-limiting toxicity (15). Any novel drugs must undergo preclinical testing in animal models prior to human clinical trials. However there remain a number of issues with these models, including higher costs, species differences, limited availability and feasibility (16). Furthermore, ethical issues in relation to the use of animals in tumor research are highly controversial. The first guideline of the animal model is that animals should be replaced with other methods wherever possible (17). Therefore, novel *in vitro* cell culture models are encouraged by the majority of funding agencies (4), in order to reduce the number of animals used in tumor research and drug evaluation.

To resolve these issues, 3D tumor cell culture methods have been developed where the culture environment takes into account the spatial organization and ECM of the cell. The common goal for these methods is to restructure a biomimetic 3D multicellular tumor model, which may bridge the gap between the conventional 2D *in vitro* and the animal testing models. Tumor cells in 3D models have physiological properties similar to those *in vivo* (5). Thus, 3D culture may be a powerful tool in tumor and relevant drug research. Marked advances have been made in the basic development of 3D tumor models so far, and prominent studies using 3D tumor cell models to simulate the tumor microenvironment or assess drug delivery in the past 5 years are summarized in Table I (6,8,18-35). Considering the advantages of 3D tumor models, the present review provides an overview of the methods and techniques successfully devised for practicing 3D tumor cell culture.

3. The methods of 3D cell culture

Multicellular tumor spheroids (MCTS). MCTS are aggregates of cancer cells grown in suspension or embedded in gels using 3D culture methods. This model partly recapitulates *in vivo* tumor microenvironments (36). For example, larger MCTS (critical size, 400 μm) sustain oxygen and nutrient gradients that often result in the formation of a necrotic core similar to those in poorly vascularized tumors (37). There are several different methods used to create MCTS models (Fig. 1), each with their distinct advantages and disadvantages.

Suspension culture. The suspension culture method was invented to isolate and culture neural stem cells from rat striatal cells in 1970s (38). Subsequently it became a widely used 3D cell culture method. The main features of this method are a serum-free and artificially low adhesion cell growth microenvironment (Fig. 1A). Specifically, the culture medium has no serum but contains a high concentration of growth factors, which are differ slightly between cell lines. For example, glioma suspension MCTS medium contains Dulbecco's modified Eagle's medium, Nutrient Mixture F12, B27, recombinant human epidermal growth factor and basic fibroblast growth factor (39). Modifying cell culture surfaces by using 1.5% agar-coated plates (40) promotes spheroid culture formation by preventing cells attaching to their surface. According to the above conditions, the majority of tumor cells may grow and aggregate into a sphere with a diameter ranging from 20 μm -1 mm (Fig. 1H). These suspension MCTS demonstrate not only numerous characteristics of solid tumor cells, but also tend to exhibit more drug-resistance to either conventional chemotherapy or monoclonal antibody drugs (41,42). In addition, the suspension MCTS culture method is widely used for enriching CSC subpopulations. Under a serum-free environment, highly differentiated cells gradually die and cells with stemness potential proliferate and survive. Following several passages, CSC subpopulations may be enriched and purified (43). The enrichment of CSCs leads to increased drug resistance, accounting for the traditional view that drug penetration into the tumor spheroids may be poor. As the suspension culture model is simple and easy, it may be generated from a wide range of tumor cell types and is easily accessible for relevant experimentation, making this method compatible with high-throughput drug screening (44). However, the cells in suspension culture have no migratory movement. Furthermore, the success rate of long-term passages is low (45) and real *in vivo* tumor cells are not inaccessible with serum. Another important problem with drug testing is lack of defined endpoints and an accurate means for testing cell viability in 3D culture. There are no accurate cell viability assays to assess drug response throughout MCTS so far (46).

The hanging drop method is a special type of suspension culture (47). It uses a small quantity of cell suspension dropped onto a culture plate, and then the plate is inverted to create droplets, as the micro-liquid adhesion with the substrate surface is greater than its own weight. Cells will aggregate, proliferate and grow into a spheroid at the liquid-air interface of the medium drop tip (Fig. 1B). This method is relatively simple, and it has a ~90% reproducibility rate for producing 1 MCTS per droplet for numerous tumor cell lines (45). At present, dedicated commercial 384-well droplet suspension plates have emerged (48). But, similar to the suspension culture, its limitations are apparent. For example, to prevent the droplet falling, the recommended drop volume is only 10-20 μl . In this case, the general number of cells in MCTS is not >500. In addition, once the cell culture is initiated, the medium cannot be replaced and it is difficult to add the drug in the middle of culture. These inherent limitations confine the widespread use of this technique in drug discovery.

Device-assisted culture. Device-assisted suspension culture is an improvement of the static suspension culture, depending

Table I. Previous 3D cell culture models to simulate the tumor microenvironment and assess drug. Arranged according to tumor type.

Author, year	Model	Cells	3D model	Results	(Refs.)
Talukdar and Kundu, 2012	Breast cancer	MDA-MB-231	Non-mulberry silk fibroin protein scaffolds	Notably higher drug concentrations are required to achieve a comparable reduction in cell viability and invasive potential in 3D cultures than 2D cultures	(18)
Chen <i>et al.</i> , 2012		MCF-7	Collagen scaffolds	High-level expression of CSC-associated properties of MCF-7 cells cultured in 3D were confirmed	(6)
Dunne <i>et al.</i> , 2014		MCF-7, BT474, SKBR3	Scaffolds	Breast cancer cells grown in 3D on the decellularized scaffolds demonstrated reduced sensitivity to doxorubicin relative to 2D cell culture	(19)
Maguire <i>et al.</i> , 2016		MCF-10	Multicellular tumor spheroids	Genetic dependencies can be uncovered when cells are grown in 3D conditions similar to <i>in vivo</i>	(20)
Sha <i>et al.</i> , 2015	Gastric cancer	BGC-823	Multicellular tumor spheroids	Anti-EGFR-IRGD may improve anticancer drugs, such as doxorubicin, bevacizumab, nanoparticle permeability and efficacy throughout the tumor mass	(21)
Kundu <i>et al.</i> , 2013	Hepatocarcinoma	HepG2	Tasar silk fibroin scaffolds	3D multicellular model demonstrates insight into hepatocarcinoma progression and offers a prediction of cellular response to transfection efficacy, drug treatment and therapeutic intervention	(22)
Xu <i>et al.</i> , 2013	Lung cancer	Primary lung cancer cells, SPCA-1	Spheroids in device-assisted culture	Developed a high-throughput model for assessing drug sensitivities <i>in vitro</i> . There was a large discrepancy between drug sensitivity levels in 2D versus 3D.	(23)
Simon <i>et al.</i> , 2016		A549	Hydrogels	3D model can help regulate the exposure of oxygen to subpopulations of cells in a tissue-like construct either prior to or following therapy	(24)
Stratmann <i>et al.</i> , 2014		HCC827, A549	Decellularized scaffolds	Quantitative read-outs for proliferation, apoptosis and invasion were established in the complex 3D tumor model	(25)

Table I. Continued.

Author, year	Model	Cells	3D model	Results	(Refs.)
Lee <i>et al.</i> , 2013	Ovarian cancer	1847, A2780, CaOV3, COV644, EFO27, ES-2, FUOV1, HEY, IGROV1	Multicellular tumor spheroids	3D cell culture is an improved reflection of the histological, biological and molecular features of tumors compared with primary cultures in 2D. In terms of chemosensitivity, 7 out of 11 cell lines were more resistant in 3D models. 7 out of 11 cell lines also demonstrated increased resistance to paclitaxel	(26)
Shin <i>et al.</i> , 2013		HEY	Multicellular tumor spheroids	Taxol-induced apoptosis was detected in monolayer conditions but not in spheroid cultures.	(27)
Loessner <i>et al.</i> , 2013		OV-MZ-6	Multicellular tumor spheroids	Combinatorial approaches of paclitaxel and KLK/MAPK inhibition may be more efficient than chemotherapeutics alone	(28)
Yang and Zhao, 2011		A2780, A2780/DDP, SK-OV-3	Nanofiber scaffolds	Ovarian cancer cells demonstrated between two and five-fold higher drug resistance (flourouracil, paclitaxel and curcumin) relative to 2D culture	(29)
Fitzgerald <i>et al.</i> , 2015	Prostate cancer	PC3, LNCaP	Collagen-based scaffold	The two cell lines in 3D demonstrated higher resistance to docetaxel. Non-viral delivery vectors, lipofectamine and a modified cyclodextrin, mediated gene knockdown in this 3D model	(30)
Xu <i>et al.</i> , 2014		LNCaP	HA-based gels	The hydrogel serves as a diffusion barrier for nanoparticles. Cells cultured in 3D were more resistant to docetaxel treatment relative to 2D	(31)
Lv <i>et al.</i> , 2016	Glioma	Primary cells, U87	Collagen scaffolds	3D-cultured cells also demonstrated enhanced resistance to chemotherapeutic alkylating agents, with a much higher proportion of glioma stem cells and upregulation of MGMT	(32)

Table I. Continued.

Author, year	Model	Cells	3D model	Results	(Refs.)
Ma <i>et al.</i> , 2016		U251	Polystyrene scaffolds coated with laminin	The results indicate the influence of 3D context on integrin expression, specifically, the upregulation of the laminin-binding integrin's alpha 6 and beta 4	(33)
Pedron <i>et al.</i> , 2013		U87	HA gels	Clustering of glioma cells was observed exclusively in HA gels and expression of malignant genes was revealed to vary bi-phasically with incorporated HA content.	(34)
Munson <i>et al.</i> , 2013		RT2, U87, C6, 9L	HA-collagen gels	Interstitial flow in this 3D model increases glioma invasion by a CXCR4-dependent mechanism	(35)
Fong <i>et al.</i> , 2013	Ewing sarcoma	TC-71	Electrospun poly(ϵ -caprolactone) scaffolds	Ewing sarcoma cells cultured in 3D were more resistant to traditional cytotoxic drugs, and exhibited remarkable differences in the expression pattern of the IGF-IR/mammalian target of rapamycin pathway	(8)

2D, two-dimensional; 3D, three-dimensional; CSC, cancer stem cell; KLK, kallikrein-related peptidases; MAPK, mitogen-activated protein kinases; HA, hyaluronic acid; MGMT, O-6-methyltransferase; CXCR4, C-X-C motif chemokine receptor 4; IGF-IR, insulin-like growth factor receptor.

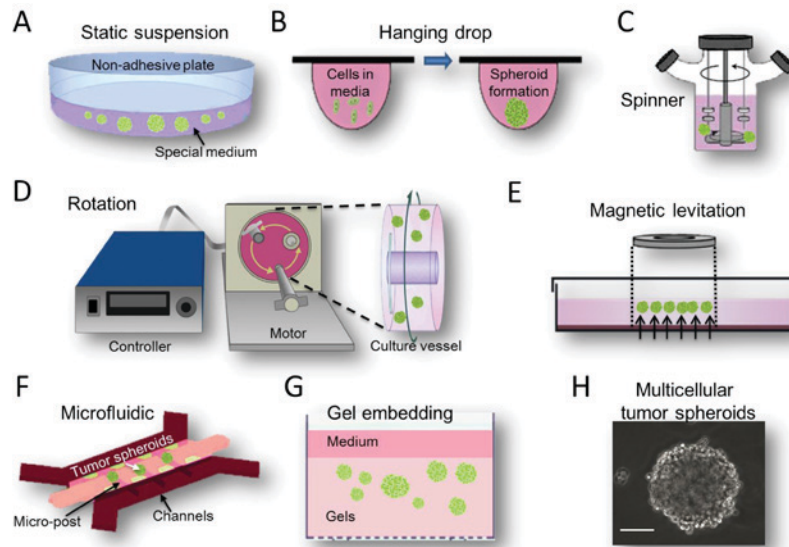


Figure 1. Methods available for MCTS formation. These methods include (A) static suspension; (B) hanging drop methods; (C) spinner bioreactor; (D) rotational bioreactor; (E) magnetic levitation; (F) microfluidic system; and (G) gel embedding. (H) A classic MCTS was observed by inverted phase contrast microscope (scale bar, 100 μm). MCTS, multicellular tumor spheroids.

on several biological devices, including magnetic levitation, spinner or rotational bioreactors and microfluidic devices. The main feature of these bioreactors is to prevent tumor cell adherence or to suspend movement, so that they may grow into MCTS. Additionally, microcarriers or microcapsules are often used in combination to increase the efficiency of cell growth and enhance the protection of the moving cells (49).

The magnetic levitation method is a novel suspension culture technology invented by n3D Bioscience (Fig. 1E). In this method are magnetized with NanoShuttle-PL during an overnight incubation and cultured on magnetic nanoparticles in dedicated plates, where they are levitated off the bottom by a magnet placed above the plate. This method does not require any artificial substrate or specialized media or equipment. Additionally, 3D cultured cells may also be picked up and transferred using magnetic tools, including the MagPen (50). Using this approach, Su *et al.* (51) studied the niches and conducted a pharmaceutical synergism analysis of myeloma stem cells.

The spinner bioreactor culture method was derived from the study of tumor cells in terms of radiotherapy resistance by Durand and Sutherland (52) in 1970s. This system includes a container to hold the cells culture and an impeller or paddle stirring continuously to ensure the cells suspended and medium mixed (Fig. 1C). The liquid flow not only prevents cell adhesion, but also ensures the uniform distribution of various nutrients and oxygen. It is conducive to cellular sphere formation and metabolism. A disadvantage of this method is that the foam and fluid shear force generated by the agitation process may cause cell damage, but if the stirring speed is too low, the cells would drop and adhere easily. The rotational bioreactor culture method was adapted from the former method by the National Aeronautics and Space Administration in 1992. It is designed to use the culture container's self-rotating ability to generate microgravity (Fig. 1D), and to be used for the purpose of researching the biological characteristics of the cells in this

case (53). The system is now widely used in the 3D culture of tumor cells. It may reduce damage more effectively by the fluid shear force than the former device, but it still fails to avoid mechanical damage by collision between the cells and the bioreactor wall. These bioreactor systems may produce a large number of uniform cellular spheres. Certain researchers have reported that using these two types of bioreactors may improve amplification efficiency by 8-30x greater than that of the 2D method on the culture of stem cells (54,55). Various tumor models, including hepatocellular carcinoma (56), neuroblastoma (57), breast adenocarcinoma (58) and melanoma (59) have been successfully engineered using spinner/rotational bioreactors.

Microfluidic devices, which allow spatial control over fluids in micrometer-sized channels, have become a valuable tool to further increase the physiological relevance of 3D tumor models (Fig. 1F). These devices process or manipulate micro-liquid, using microchannels with dimensions of 1-1,000 μm (60). MCTS are generated within microfluidic channels. Spheroid formation may be controlled precisely in the microfluidic device. Continuous perfusion under physiological conditions during spheroid formation allows for faster formation and increased uniformly in size (61). Microfluidic platforms also allow the formation, maintenance, and testing of spheroids within a single device.

Gel embedding culture. MCTS in suspension culture lose their complicated ECM microenvironments. The solid tumor cells *in vivo* should not form suspended spheroids, they associate with other cells and the ECM (62). As the ECM affects cellular organization and cell function, novel 3D culture methods that incorporate ECM arguably mimic *in vivo* situations more accurately, as they allow for cell-ECM interactions. Gel is used as a substrate for 3D cell culture. It is a type of highly hydrophilic polymer with a soft tissue-like stiffness which aims to mimic the ECM. Tumor cells grown in the gel usually form a

spheroid-like structure spontaneously (Fig. 1G). This structure contains not only cell-cell adhesions, but also supports contact between the cells and artificial ECM. When tumor cells are allowed to grow in gels, there is architectural support and important signaling motifs to aid in 3D cancer growth, which often leads to higher resistance to chemotherapy. For example, when human epithelial ovarian cancer cells were cultured on 3D hydrogel system as MCTS, a higher survival rate was observed following exposure to paclitaxel (63). A number of commonly used gel methods are described below.

Collagen is a widely used material in gel embedding culture (64). As a key ECM component, collagen has excellent biocompatibility for the vast majority of tumor cells. The earliest application of collagen is in tumor tissue culture. Certain tumor tissues are embedded in collagen gel for culture following cutting (diameter ~1 mm), and researchers have demonstrated that the tumor tissue structure and cell viability are maintained (65). Subsequently, certain researchers selected collagen for tumor tissue culture and drug sensitivity testing (66). Tumor cell culture with collagen gel was originally applied in breast cancer research. It has been revealed that the breast cancer cells in collagen gel embedding culture may form tube-like structures, which are similar to typical breast cancers *in vivo* (67). In CSC research, a novel colorectal cancer stem cell-enriched cell line was established by 3D culture in Collagen I (68). For drug sensitivity testing of hepatocellular carcinoma lines, cancer cells in collagen gel presented with increased drug-resistance compared with those in monolayer culture (69). With the improvement of gel technologies, complex ECM mimetic materials consisting of collagen and hyaluronic acid (HA) have been developed. These are suitable for investigating the behaviors and drug testing of glioblastoma cells (70).

Alginate is a natural polymer derived from brown seaweed. It gels in the presence of calcium ions, and is often used for the encapsulation of various types of cells. The main advantage of alginate gel culture is that gelation may be accomplished at room temperature following addition of the cells to the polymer. It allows the cells to mix into the gel-liquid uniformly and grow nondestructively through the process of gelation. The hepatocytes in alginate gel may grow and maintain the function of albumin synthesis (71). Thus, this culture gel substrate may maintain hepatocytes function well. Breast cancer cells grown in alginate gel 3D culture form MCTS, which have stronger chemotherapy resistance compared with breast cancer cells grown in 2D culture (72). In addition, a previous study successfully enriched multiple CSCs by culturing them in alginate gel beads (73), which suggested that alginate gel is a useful biomaterial for enriched CSCs culture.

Matrigel derives from Engelbreth-Holm-Swarm mouse tumor cell-derived basement membrane proteins which include collagen IV, laminin, entactin, perlecan, multiple cytokines and growth factors (74). It has wider commercial applications in numerous tumor cellular experiments including 3D culture and tumor invasion models. 3D cultured breast cancer cells in Matrigel exhibit a bidirectional cross-modulation of β 1-integrin and epidermal growth factor receptor signaling via the mitogen-activated protein kinase signaling pathway, but this reciprocal modulation does not occur in monolayer

culture (75). These different signal pathways included transforming growth factor β family (76) and phosphatase and tensin homolog/platelet-derived growth factor signaling network (77), which are relevant with chemotherapy and radiotherapy resistance. CSC research using Matrigel has also been performed. For example, CD271+ uveal melanoma stem cells may undergo vasculogenic mimicry in 3D Matrigel culture (78). Another previous study revealed that nicastrin, a novel type I transmembrane glycoprotein, is associated with breast cancer stem cell properties using Matrigel culture (79). The defects of Matrigel are that it is expensive, has complex compositions and uncertain proportions between different batches of ingredients (80).

To date, novel gel techniques emerge constantly. Other new commercial gel materials include PathClear Grade Basement Membrane gel (AMS Biotechnology, Ltd., Abingdon, UK), ECM gel (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), ECL Cell Attachment Matrix (Merck KGaA) and Geltrex (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA). In the future, corresponding ECM components may be extracted from different tissues to prepare the gel according to tumor type, in order to improve mimicry of the tumor microenvironments *in vivo*. The potential drawback of the gel embedding culture approach is that gel lacks the cross-linked network system for the mechanical support of tumor cell growth, meaning that it is difficult to grow tumor cells diffusely.

Scaffold culture. 3D scaffolding is a product of tissue engineering developments. It may act as a surrogate for the missing ECM, representing the available space of tumor tissue, providing the physical support for cell growth, adhesion and proliferation, and causing the cells to form an appropriate spatial distribution and cell-cell or cell-ECM interaction. There are a number of differences between 2D and 3D scaffold culture. A notable comparison is that tumor cells cultured in 3D scaffolds exhibit morphological similarities to tumor cells cultured in human tumor tissues (Fig. 2). For example, glioblastoma U87 cells are fusiform, flat and epithelioid in 2D culture, but grew as small, round or ovoid cells and formed complex structures with cilia or microvilli on their surface in 3D scaffold culture (32).

In general, the procedures for preparing the scaffolds fall into one of two major categories: 1, natural polymers derived from natural polymer materials, including collagen, chitosan, glycosaminoglycans (mainly hyaluronic acid), fibroin, agarose, alginate, and starch (mainly used as additives); and 2, synthetic polymers, containing polyglycolic acid, polylactic acid, polyorthoester and their copolymers or blends, as well as the aliphatic polyester polycaprolactone (81). Natural polymers have improved biocompatibility and lower toxicity, while the artificial synthetic polymers have higher versatility, reproducibility, enhanced workability, and in a majority of cases may be processed more easily than the former, but are not bioactive (82). The processing techniques of scaffolding preparation are much more complicated than gel embedding. For providing a more well-defined architecture for tumor cell growth and improved repeatability, the porosity, mechanical strength, structural stability and degradation kinetics of the scaffold needs to be controlled. A wide range of techniques are used in generating different scaffolds,

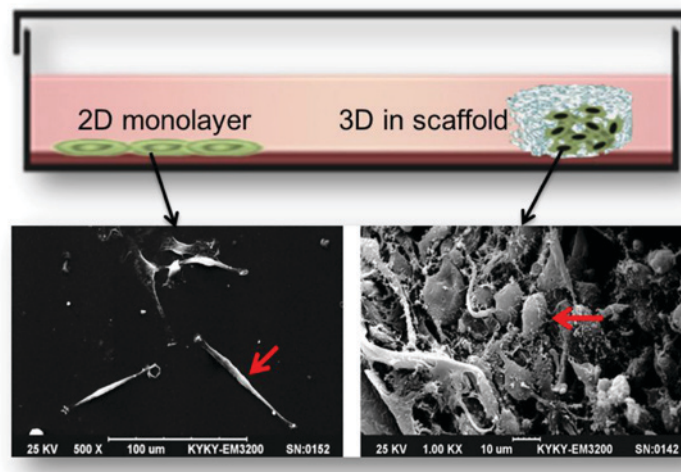


Figure 2. Comparison of tumor cells morphology between 2D and 3D scaffold culture. U87 cells in 2D (left) and 3D scaffold (right) in a scanning electron microscope image (scale bars, 100 and 10 μm). 2D, two dimensional; 3D, three dimensional.

including solvent casting/particulate leaching, freeze-drying, phase inversion, electrospinning, stereolithography, selective laser sintering, shape deposition manufacturing, 3D printing, robotic microassembly and fused deposition modeling (81). Among these techniques, solvent casting, freeze drying, phase inversion and fiber electrospinning are used a majority of the time. But the latter three techniques may become the main methods of preparing precise micro-scaffolds in the future.

A number of highly influential studies regarding 3D scaffolding culture in drug research and CSCs have been reported. Dhiman *et al* (7) studied the growth of breast cancer MCF-7 cells on porous chitosan scaffolds, and revealed that the response of cancer cells to tamoxifen was a 10-fold increase in drug resistance compared with those in monolayer culture. Fong *et al* (8) established an *in vitro* 3D Ewing sarcoma model with porous 3D polycaprolactone (PCL) scaffolds. The results revealed that the tumor cells on scaffolds were not only more resistant to traditional cytotoxic drugs but also exhibited remarkable differences in the expression pattern of the insulin-like growth factor-1 receptor/mammalian target of rapamycin pathway. Furthermore, Chen *et al* (6) studied breast cancer cell growth on a porous collagen scaffold, and revealed that the porous scaffolds not only induced the diversification of cell morphologies but also extended cell proliferation and notably increased the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinases. Additionally, 3D collagen scaffolding upregulated a subpopulation of breast cancer stem cells, and xenografts with 3D cells formed larger tumors. These results indicate that 3D collagen scaffolds may provide a useful platform for anti-cancer therapeutics and CSC research. Furthermore, breast cancer stem cell expansion in PCL scaffolds (83), ovarian cancer stem cell behavior and drug resistance investigated in 3D basement membrane extract scaffolds (84) and glioma stem cells proliferating in 3D chitosan-alginate scaffolds (85) were observed in these studies, sequentially.

4. Future trends and conclusions

Different approaches offer different advantages and disadvantages, as summarized in Table II. Due to the inherent

differences in complexity and functionality, the choice of model is usually dependent on the application. Additionally, advances in tumor cell biology, tissue engineering, biomaterials, micro-fabrication and microfluidics have enabled the rapid development of 3D tumor cell culture. Future prospects include ensuring that the main features of culture substrate are controllable and adjustable with more advanced materials and processing technologies. Depending on the different tumor types and research purposes, different biomaterials are of varying suitability for 3D culture in each specific study. For example, biomedical polymer scaffolds with low degradation rates should be manufactured for the research of bone cancer metastases. Additionally, scaffolds with added growth factors including VEGF may potentially promote the study of vascular mimicry. In addition, combining with different types of bioreactors, for example gel embedding or scaffolds used in conjunction with a micro-fluid bioreactor, may resolve the problem of cell metabolites or drugs diffusing limitedly. Furthermore, a co-culture system with two or more types of cells established under the 3D culture platform, including the co-culture of cancer cells, immune cells and endothelial cells, may mimic real tumor niches more effectively and help to clarify the effect of interactions among cancer cells, ECM and other neighboring cells (86). Automated quantification of 3D cultures is required in order to achieve high throughput drug screening programs (87). In addition, CSCs are novel therapeutic targets (88), and 3D tumor cell culture ought to be a useful technique in this area due to its CSC-enriching function. Greater focus on exploring appropriate ECM components in a 3D model is also critically important for monitoring tumor cell responses to exogenous cues, including growth factor activation or chemotherapy (32,33).

In summary, the biological influence of the 3D microenvironment on tumor cell differentiation, progression, metastasis and chemotherapy-resistance has gained increased recognition. 3D culture, which ranges from the simple cell spheroid model to complex tissue-engineered constructs, serves an increasingly important function in tumor cell biology research. Gel embedding and scaffolds have a number of advantages in simulating the 3D structure of tumor cells *in vivo* compared

Table II. Comparison of various methods of 3D tumor cell culture.

Method	Advantages	Disadvantages
Static suspension culture	Relatively simple, low cost, spheroid production is easily accessible, reproducible	Continuous passage culture difficult, relatively labor intensive, only autocrine ECM
Hanging drop	Control of spheroid size, low cost if using standard 96-well plate, uniform spheroid size, homogenous spheroids, suitable for high-throughput testing	Expensive if using specialized plates, long-term culture difficult, small culture volume, medium exchange difficult
Magnetic levitation	Relatively simple tool, efficient, does not require any specialized media	Colors the 3D culture brown and limit certain applications, numerous cells also adhere to the bottom of the plate
Spinner/rotational based approaches	Mass production, long-time culture, relatively simple	Require specialized equipment, not possible to control uniformity of spheroid (size, composition), exposed to high shear force
Microfluidic device	Suitable for control of spheroid (size, parameters), continuous perfusion for faster spheroid formation	Difficulty collecting cells for analysis, require expensive and complicated equipment
Gel embedding	Resemblance to the <i>in vivo</i> conditions, gel provides rich network of ECM signals	Poor mechanical properties, certain components of natural gel are variable and undefined, lacks vasculature
Scaffolds	Maximum resemblance to the <i>in vivo</i> conditions, composition and it is possible to precisely control ECM presentation, possible to combine with preferred growth factors	Expensive for large-scale production, trouble in cells dissociation from scaffold, complex operation

3D, three dimensional; ECM, extracellular matrix.

with diverse suspension culture methods, because they are more accurate at modelling the full range of microenvironmental cues, including 3D cell-cell and cell-ECM interactions. In multiple cancer research areas, particularly in the direction of drug discovery and CSC enrichment, 3D culture has unique advantages. An optimized 3D tumor model for the research of drug discovery *in vitro* will require cancer biologists and tissue engineers to increase efforts in this field.

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