



3D culture technologies of cancer stem cells: promising ex vivo tumor models

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Abstract

Cancer stem cells have been shown to be important in tumorigenesis processes, such as tumor growth, metastasis, and recurrence. As such, many three-dimensional models have been developed to establish an *ex vivo* microenvironment that cancer stem cells experience under *in vivo* conditions. Cancer stem cells propagating in three-dimensional culture systems show physiologically related signaling pathway profiles, gene expression, cell–matrix and cell–cell interactions, and drug resistance that reflect at least some of the tumor properties seen *in vivo*. Herein, we discussed the presently available Cancer stem cell three-dimensional culture models that use biomaterials and engineering tools and the biological implications of these models compared to the conventional ones.

Keywords

3D culture; tumor model, cancer stem cells, biomaterials, tumor biology

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Introduction

Recent statistics from the World Health Organization (WHO) report indicates that cancer is the leading cause of death globally, lowering the life expectancy of many populations across the world.¹ Molecular mechanisms of oncogenesis have long been a topic of great interest in a wide range of fields. Cancer stem cells (CSCs) are a small tumor subpopulation of cells that have the potential of self-renewal and multi-differentiation. These aggressive cells are chemo- and radio-resistant, and contribute to the development and progression of malignancy (Figure 1(a)).^{2,3}

Compared with normal stem cells, CSCs show many unique features, including hyper-efficient mechanisms for DNA repair, expression of multidrug resistance-related ATP-binding cassette (ABC) membrane transporters, hypoxic niche resistance, and over-expression of anti-apoptotic proteins.^{4,5} In addition, the difference between CSCs and non-CSCs in the case of cancer may be attributed to epithelial-to-mesenchymal transition (EMT).^{6,7} EMT can be defined as a process whereby epithelial cells turn into mesenchymal cells, which are involved in the progression of malignant tumors. During EMT, cancer cells lose their round, cobblestone-like morphology to become elongated cells with fibroblast-like morphology, resulting in increased

migration and invasion ability (Figure 1(b)).^{8,9} CSCs are considered a key treatment strategy for human cancers and represent new therapeutic targets.¹⁰ However, it has been

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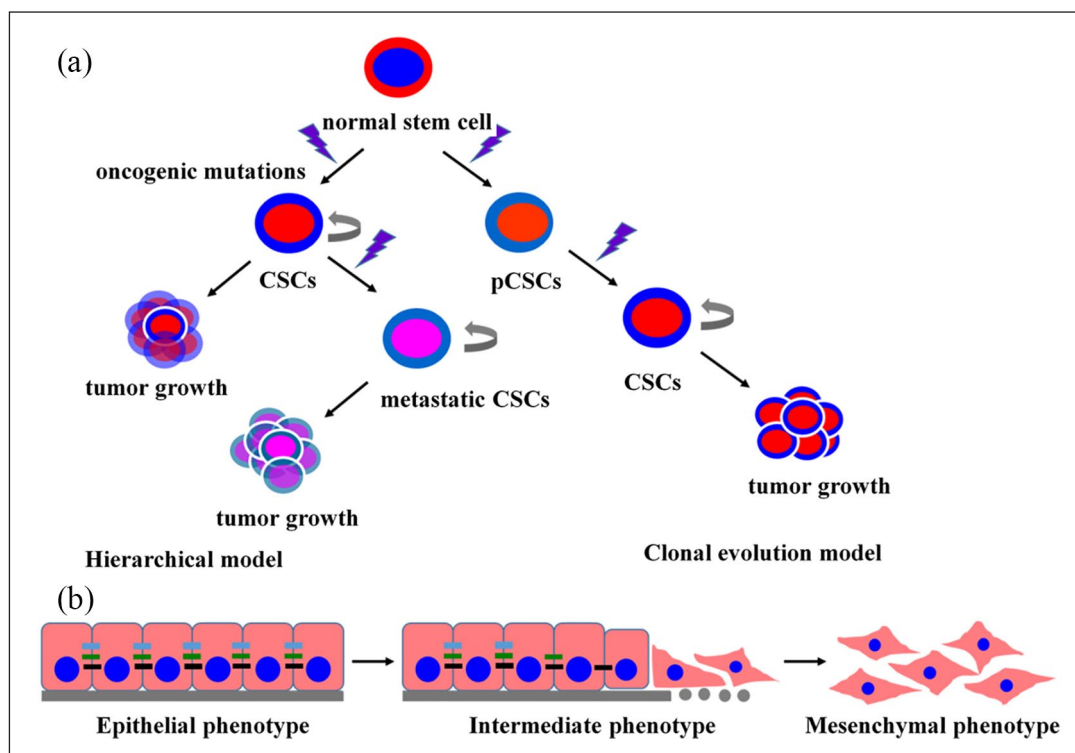


Figure 1. Schematic illustration of cancer stem cell (CSC) models of tumorigenesis and the general features of EMT. (a) CSC models, including the hierarchical and clonal evolution prototypes. CSC subgroups showing self-regeneration properties as well as capacity to differentiate at the apex of tumorigenesis hierarchy. (b) Schematic of EMT process: cancer cells lose their round, cobblestone-like morphology to become elongated with fibroblast-like morphology. CSCs, cancer stem cells; pCSC, precancerous stem cells; EMT, epithelial-mesenchymal transformation.

shown that CSCs are difficult to culture *in vitro*, which is the main constraint in the study of CSC biology and drug discovery. Previous studies have shown that CSCs depend on a niche that regulates their proliferation and differentiation, analogous to normal stem cells.¹¹

Considering the important part that CSCs play during tumorigenesis, including tumor growth and radioresistance, it is essential to create tumor models similar to the *in vivo* condition. With the development of biotechnology methodologies, three-dimensional (3D) cell cultures are widely accepted as one of the most effective ways to elucidate the molecular mechanisms of CSCs. In the 3D culture systems, cells are grown to encourage cell–matrix and cell–cell interactions mimicking tumor microenvironment.¹² Compared to two-dimensional (2D) cultures, the 3D culture systems allow cells to present more appropriate tissue physiology, anatomy, and structure.^{13–16} Signaling pathways in 3D culture also show different profiles in terms of cell migration, morphology, proliferation, and viability.^{15–18} Exemplar 3D culture models of CSCs are the serum-free culture suspension system and culture with a basement membrane scaffold.^{19,20}

Many recent studies have reported various 3D culture models of CSCs using biomaterials and advanced technological tools. Herein, we summarize the developed

3D culture models of CSCs and discuss the biological implications of the models in terms of cell–cell and cell–matrix interactions, gene expression and signaling pathway profiles, and drug resistance. CSC studies in 3D models could contribute to further our understanding of tumorigenesis, tumor growth, metastasis, and recurrence behaviors occurring *in vivo* and assist in potential drug development for tumor therapy.²¹

3D culture models of CSCs

Numerous studies have demonstrated that certain cancer cells could re-acquire CSC traits via intrinsic stem-associated gene expression and extrinsic tumor microenvironment.^{22–26} Over the past few decades, many 3D culture methods of CSCs in the form of spheroids have been developed. Typical methods for forming spheroids include scaffold-free methods such as the ultra-low attachment plate method, hanging drop method, suspension culture method, and scaffold-based techniques (Figure 2).²⁷ Tumorspheres are primarily generated by a suspension of single cells in serum-free conditions.^{28,29} A subgroup of tumor cells that can survive in a serum-free culture is identified and isolated from a group of tumor cells, then used to form tumorsphere. These cells can proliferate and expand clonally

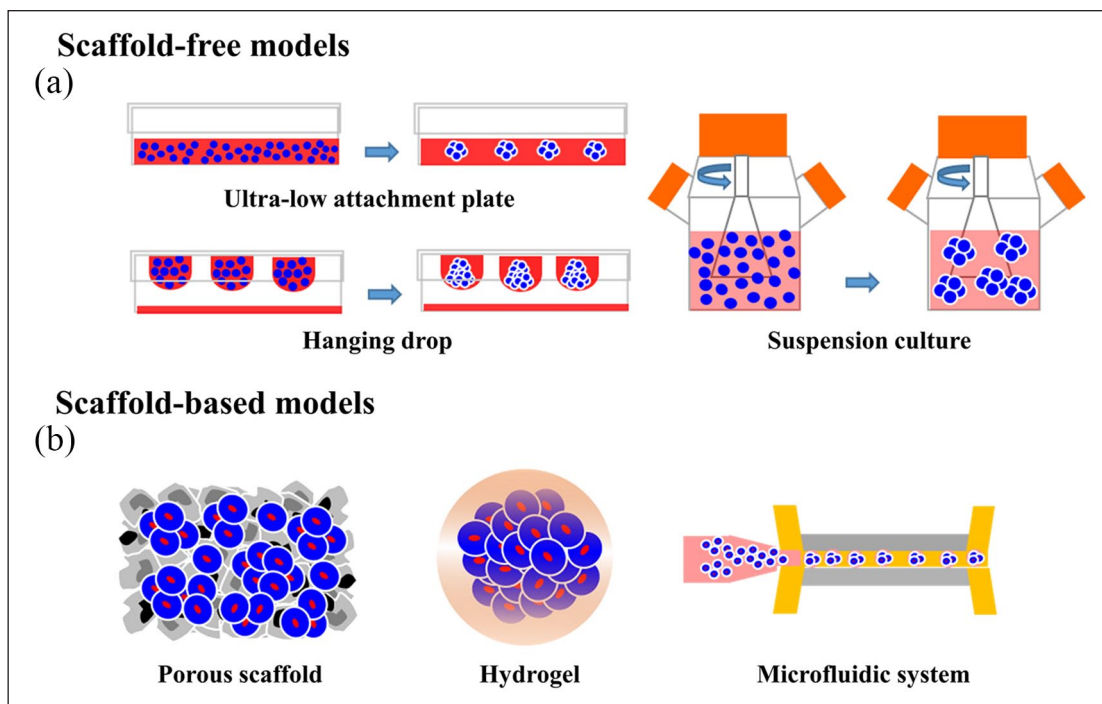


Figure 2. Tumorspheres are 3D cancer stem cell (CSC) models generated by different methods. (a) Scaffold-free methods such as hanging drop, ultra-low attachment plate, and suspension culture. (b) Scaffold-based techniques, such as porous scaffolds, hydrogels, and microfluidic systems.

devoid of serum supplements, suggesting that they may have stem cell-like features. However, recent evidence shows that tumorsphere culture-mediated enrichment of stem cell markers depends on the cell line and therefore, the resulting cells may not exhibit stemness.³⁰ Tumorsphere formation has been achieved with many different tumor cells derived from prostate, skin, breast, and colon.

Scaffold-free 3D CSC culture models

Scaffold-free 3D CSC culture models mainly refers to the use of various physical methods to minimize cell attachment, which suspend the cancer cells onto the medium, and then promote cell aggregation into a spheroid. These methods contain ultra-low attachment plates, hanging drop and suspension culture.

Ultra-low attachment plates. The surface of the ultra-low attachment plate is coated with an inert substance, such as agarose or poly-hydroxyethyl methacrylate,³¹ which can minimize cell attachment.³² This method can isolate CSCs/tumor-initiating cells according to their distinctive anoikis-resistant capacity or anchorage-independent growth.^{33,34} For example, Gao and co-workers³⁵ successfully isolated CSCs from multiple tumor cell lines by a non-adherent culture method, which has significant advantages over other methods. Im et al.³⁶ developed CSC-like cells using A172 glioblastoma cells under conditions of non-adherent

culture with serum deprivation. Krishnamurthy et al.³⁷ applied ultra-low attachment plates to generate head and neck CSC for therapeutic strategies in head and neck cancer studies.

Advantages of this technique include the fact that it is a convenient procedure and multiple cell types (co-culture) can be incorporated.^{16,38} However, the major drawback of CSC spheroids formed with ultra-low attachment plates is that they vary in size. In addition, the mixture of attached cells and spheroids overwhelms assay chemistry.^{15,39}

Hanging drop. The principle behind the hanging drop method is to use the surface tension of a droplet of cells and gravity to suspend the droplet of cells onto the underside of a lid, which could promote cell aggregation into a spheroid. Phosphate-buffered saline is routinely used to suspend the cells to prevent volatilization of the droplets.^{15,16,40,41} Raghavan et al.⁴² formed primary ovarian CSCs using a 3D hanging drop suspension platform to study CSC biology. Rodríguez et al.⁴³ successfully constructed breast CSC using this approach to study the relevance of breast CSC number and human epidermal growth factor receptor 2 regulation. The hanging drop method has been shown to produce uniformly sized spheroids and is applicable to different cells.^{44–46}

However, there are several major drawbacks concerning this method. First, this method is time-consuming to isolate and culture CSCs compared with the other methods.

Second, the osmolarity of the droplet will be elevated owing to evaporation of media, which is not favorable for cell viability and long-term cultivation.^{15,16,47}

Suspension culture. Suspension culture methods can achieve large-scale production of tumor spheroids using bioreactors, such as spinner flask and rotating flasks.^{31,48,49} These methods can decrease the effect of gravity by use of bioreactors and allow rapid production of large quantities of tumor spheroids.⁵⁰ In the suspension culture method, the cell culture medium is stirred using a stirrer or rotating culture flask to prevent cell adhesion, thereby generating tumor spheroids. Appropriate control of stirring or rotating speed is critical for tumor spheroid generation; otherwise, these cells would be damaged by sheer force. Chang et al.⁵¹ successfully cultured hepatoma cell spheroids using rotating wall vessel bioreactors. In their study, spheroids of up to 1 mm in diameter could be obtained. Many tumor-spheres have also been generated from the liver,⁵¹ neuroblastoma,⁵² breast,⁵³ and melanoma⁵⁴ using suspension culture methods.

A major benefit of this method is that a large number of tumor spheroids are formed. Besides, oxygen and nutrients could be distributed evenly around the tumor spheroids. However, mechanical forces and shear stress generated by stirring may damage the cells. Meanwhile, it is difficult to obtain tumor spheroids that are uniform in size and shape with this method.

Biomaterial-based 3D CSC culture models

Biomaterials with different physical structures, such as porous foams and hydrogels, have been used to create 3D CSC culture systems.^{55–57} Furthermore, biomaterials combined with microfabrication technology have been demonstrated to generate more precisely controlled 3D CSC models. Biomaterial-based 3D CSC culture models can contribute not only to the understanding of biological behaviors of CSCs and the mechanisms underlying these events but also to the modeling of various tumors and screening of anti-cancer therapeutics.

Porous scaffolds. Numerous published articles have shown that owing to better mimicking the *in vivo* environment, porous scaffolds may provide a more favorable environment for tumor cells.^{58,59} Accumulating reports have demonstrated the critical role of porosity, pore shape, and size of the scaffold in cell functions, including growth, division, and migration.⁶⁰ Various approaches have been used to prepare porous scaffolds including particle leaching, phase separation, and emulsification/freeze-drying, among others.^{60–65} Commonly used materials for constructing scaffolds are bioactive ceramics (hydroxyapatite, bioactive glasses), synthetic polymers (such as polyglycolic and polylactic acids), and natural polymers

(including silk, collagen, chitosan-alginate (CA), and hyaluronic acid (HA)).^{66–69}

Polonio-Alcalá et al.⁷⁰ found that polylactic acid (PLA) scaffolds can promote CSC proliferation and enrichment in breast cancer cells. Florczyk and coworkers⁷¹ showed that 3D CA scaffolds promoted enrichment of the CSC population, including the cells of prostate carcinoma, breast cancer, hepatocellular carcinoma (HCC), and glioblastoma cells. HA is a natural polymer that is widely chosen as a 3D tumor model material, as it is abundant in the extracellular matrix (ECM).⁷² Martínez-Ramos and Lebourg.⁷³ reported that U87 astrocytoma cells cultured with HA showed significantly elevated expression of CSC-related proteins, suggesting that the 3D-HA scaffold is a valuable model for developing drugs targeted at CSC. Lee et al.⁷⁴ reported a method using HA-based multilayer films to form, as well as culture pancreatic CSC colonies, which exhibited a dormant, slow-cycling phenotype, and increased expression of CSC-associated genes (OCT4, CXCR4, and CD44v6). One mechanism underlying CSC enrichment in porous scaffolds is that the scaffolds can isolate cancer cells by a unique pore structure that mimics the *in vivo* tumor niche (Figure 3(a) and (b)).^{55,55} Collectively, porous scaffolds are simple and inexpensive to cultivate CSCs, thereby contributing to CSC research.

Porous scaffolds provide support for cell adhesion, growth, proliferation, metabolism and the formation of tumor spheroids. However, there are some limitations, such as low tensile strain strength and inadequate extensibility. In addition, the low usage efficiency of cancer cell due to their poor adhesion on synthetic polymers is a complication in 3D culture models of CSCs.

Hydrogels. Hydrogels are hydrophilic polymers and have advantages of biocompatibility and biodegradability.^{57,76} Because hydrogels are useful for cell growth and exchange of substances between cells, they have been widely applied in tumor models.⁷⁷ The interconnected pores enable the transport of oxygen, nutrients, and metabolites.¹⁵ Hydrogels used for 3D culture are either natural or synthetic polymers.⁷⁸ Rao et al.⁷⁵ wrapped up human prostate cancer cells using alginate hydrogel to enrich CSCs for cancer research and therapy development (Figure 3(c) and (d)). Li et al.⁷⁹ developed glioblastoma tumor-initiating cells (TICs) using a 3D thermo-reversible hydrogel, which showed sufficient, affordable glioblastoma TICs for drug discovery.

Pal et al.⁸⁰ prepared scaffolds by impregnating hydrogels into electrospun scaffolds to study anti-cancer therapeutics against metastasis. The results showed that hydrogel-rich electrospun scaffolds could induce cancer cells to undergo EMT and drive non-CSC to CSC transformation, facilitating the enrichment of CSC phenotypes. Dai et al.⁸¹ prepared GAF hydrogel scaffolds using gelatin, fibrinogen, and alginate as raw materials, and the influence

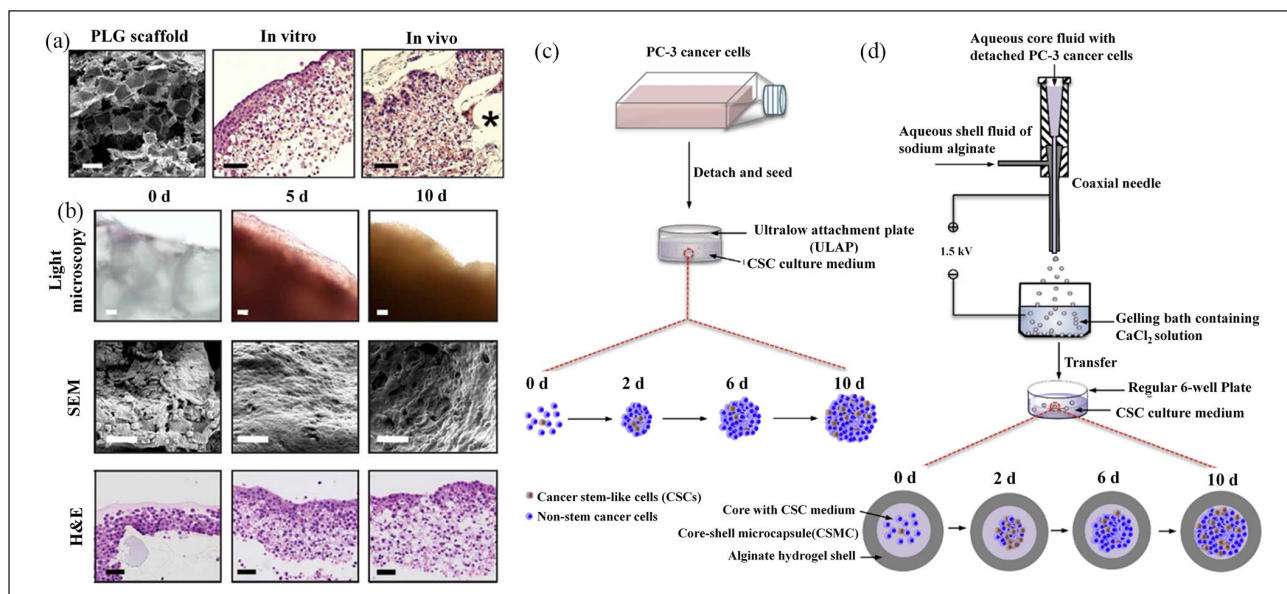


Figure 3. 3D cancer stem cell (CSC) models developed with porous scaffolds (a) and (b) and hydrogels (c) and (d). Characterization of tumor model (a) and (b): (a) Microstructure of cells loaded onto 3D PLG scaffolds visualized under scanning electron microscopy (SEM), and tumor-like tissue hematoxylin and eosin (H&E) stained specimens photomicrographs. Asterisk symbol (*) represent fragments of polymeric scaffolds. (b) Light microscopy, microstructure under SEM, and H&E-stained microphotographs of tumor-like tissues cultured in 3D PLG at different time points. Reproduced with permission from Fischbach et al.⁵⁶ (2007, *Nat Methods*). Enrichment of PC-3 human prostate CSCs using conventional bulk suspension culture versus miniaturized 3D culture (c) and (d): (c) Schematic illustration of conventional bulk suspension culture in ultralow attachment plate. (d) Schematic illustration of miniaturized 3D culture in regular 6-well plates. The core of core-shell microcapsules (CSMCs) containing PC-3 prostate cells was prepared through coaxial electrospay by a coaxial needle. CSMCs were then cultured in regular 6-well plates for at least 10 days. Reproduced with permission from Rao et al.⁷⁵ (2014, *Biomaterials*).

on survival rate as well as inherent characteristics of glioma stem cells with the scaffolds was investigated. In their study, glioma stem cells attained over 86% survival rate, and Nestin glioma stem cell markers showed high levels of expression with the GAF hydrogel scaffolds. Yang et al.⁸² encapsulated breast cancer cells within polyethylene glycol diacrylate (PEGDA) gel conjugated with CD44 binding peptide (CD44BP) to culture breast CSC. The flow cytometry results demonstrated that CD44BP conjugated to PEGDA gel could improve CD44⁺/CD24⁻ percentage, suggesting that this technique can maintain the stemness of breast CSC. Jabbari et al.⁸³ also constructed a PEGDA hydrogel to generate breast CSCs, colorectal CSCs, and gastric CSCs.

Many authors have classified hydrogels into two categories based on their differences of raw materials: natural hydrogels (collagen hydrogels, fibrin hydrogels) and synthetic hydrogels (PEGDA hydrogels). Each type of these hydrogel systems have distinct advantages and disadvantages for specific 3D cell cultures, in terms of ranges of controllable properties and long-term cell viability. For example, the chemical structures of natural hydrogels similar to glycosaminoglycans and therefore provide natural hydrogels with controlled permeabilities. While natural polymers also have several disadvantageous features, such

as poor mechanical properties. There are increasing efforts to modify gel-forming polymers or crosslinking molecules with multifunctional groups and also cross-link multipolymer systems, in order to further improve the controllability of the microstructures and properties of hydrogels.

Microfluidic devices. Microfluidics is a technique developed based on the advances in biology, physics, materials science, and engineering. In this technology, numerous experimental steps, such as sample preparation, reaction, separation, and assay, can be integrated into a microfluidic chip with diameters of 1 mm, which makes the detection process more miniaturized and intelligent. Owing to its high efficiency, excellent sensitivity, and exact controllability, this technique has been widely used in medical research.⁸⁴ In recent years, microfluidics has been applied to the generation, isolation, and characterization of CSCs. Use of microfluidics can generate a large number of spheroids with uniform size and shape for high-throughput screening (HTS) (Figure 4).^{85,86} For example, microfluidic devices composed of microwells (250–450 μm) with vasculature-mimicking microfluidic channel connections could support growth of CSCs. Here, the glass plate was coated with a 3D growth matrix (hydrogel and a porous membrane) to promote cell aggregation and spheroid

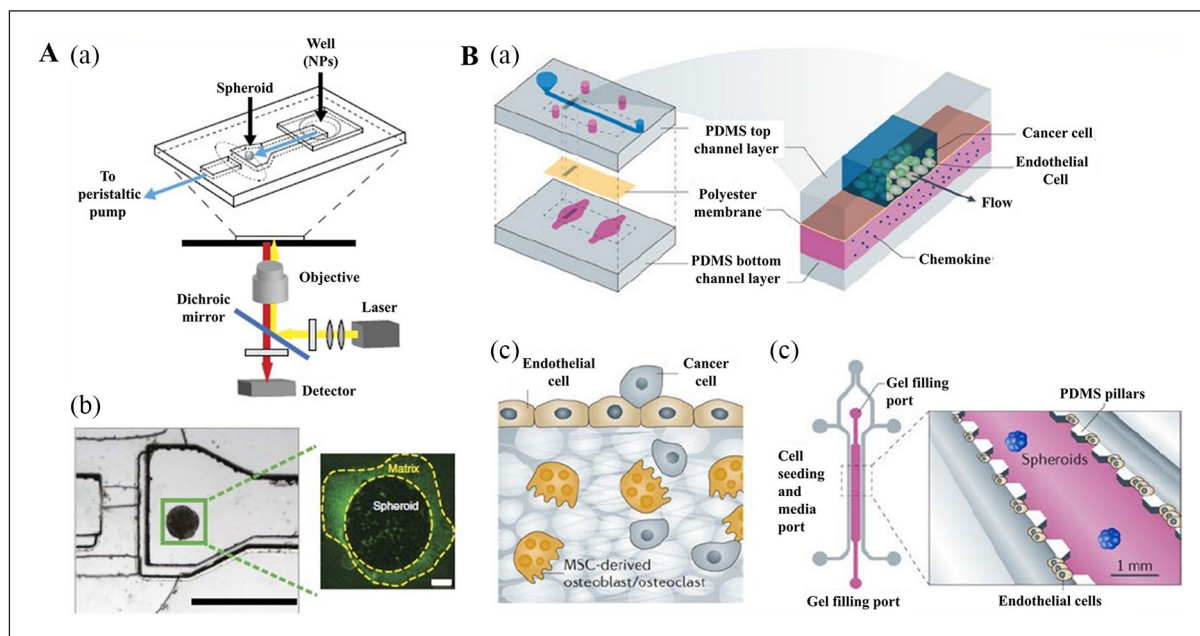


Figure 4. 3D cancer stem cell (CSC) models developed using microfluidic devices. (A, a, b) Schematic of the tumor-on-a-chip. (A, a) Schematic of the polydimethylsiloxane microfluidic device at the microscope stage. (A, b) The microfluidic device (left) showing the channel width of 600 μm at the inlet, which extends to 1200 μm in the imaging chamber where the spheroid is immobilized. The height of the channel is 250 μm and decreases to 25 μm at the end of the imaging chamber, forming a dam. A spheroid (right) stained for 10 min with anti-Laminin-FITC, then flushed for 5 min with imaging media. (B, a-c) Schematic of the organ-on-a-chip model. Reproduced with permission from Albanese et al.⁸⁵ (2013, *Nat Commun*). (B, a) An endothelium-on-a-chip microvascular established in a segmented microfluidic channel allowed endothelial cells cultured on a chemokine supplemented permeable membrane to undergo activation and basal stimulation during the investigation of attached circulating cancer cells in breast tumor metastasis. The impacts of chemokines, including tumor necrosis factor, were examined via incorporating the chemokines in the bottom channel. The endothelium pre-treated with tumor necrosis factor attracted more tumor cells compared to the untreated endothelium. (B, b) Migration of breast tumor cells to the bone was examined in microfluidic device with endothelial cell culture from human umbilical vein next to bone cells derived from mesenchymal stem cells of the human bone marrow held a 3D collagen gel. Movement of cancerous cell to the bone was detected. (B, c) To assess the epithelial-mesenchymal transition (EMT) during malignancy, spheroids of lung cancer were fixed in micropatterned 3D matrices connected closely to endothelial cells lining on the microchannel. EMT examination was performed with microfluorometry to identify cancer spheroids distribution. Reproduced with permission from Esch et al.⁸⁶ (2015, *Nat Rev Drug Discov*).

formation.¹⁵ Moreover, a microfluidic device was designed to control the flow rate to keep the cells in suspension, which may allow the formation of spheroids. Zhang et al.⁸⁷ manufactured a mechanical separation chip via microfluidic technology to isolate and screen breast cancer cell lines. In their experiments, breast cancer cells with high flexibility and metastasis could easily pass through the mechanical separation chip, and these cells were identified to possess stem cell properties and the ability to form tumorspheres. Zhao et al.⁸⁸ developed microfluidic devices that were prepared from polydimethylsiloxane by standard soft lithography and replica molding to investigate CSCs. The microfluidic devices consisted of 4 functional channels: main channels, endothelial cell channels, symmetric chambers, and fluidic channels. Many smaller horizontal bridge microposts connected all the chambers and parallel channels. Hexagonal columns formed numerous gaps between them to hold the matrigel. The polydimethylsiloxane was glued to a glass coverslip. The microfluidic device

provided both 2D and 3D culture, as well as co-culture environments with no effect on cell viability.

High-throughput biosensor technologies as the basis of new-generation cell-based HTS techniques provide analytical information by the recognition of real time biological events employing a physical transducer. And this technique also has the advantage of generating a large number of spheroids with uniform size and shape for HTS.⁴² However, the main drawback of microfluidic devices is the complexity of its design and manufacture. Moreover, spheroids generated by microfluidic devices are difficult to collect for subsequent analysis.

Biological implications of 3D CSC models

The tumor microenvironment is different from that in a 2D culture. However, in a 3D culture, CSCs display different types of tumor biology, including sustained angiogenesis,

tissue invasion, metastasis drug resistance, tumor-immune cell interactions, and EMT, which are much closer to the reality in humans.^{19,48,89,90}

Hypoxia and metabolism in 3D CSC models

In general, CSCs requires a specific microenvironment to maintain self-renewal and asymmetric divisions where hypoxia is the predominant feature.^{91,92} When tumorspheres are enlarged beyond several hundred microns in diameter, the cells grown on the outermost layer of the tumorspheres could consume a lot of oxygen and nutrients, leading to preferential growth of cells located in the marginal zone. Meanwhile, the cells located in the innermost layers of tumorspheres undergo growth arrest or even necrosis due to an insufficient supply of oxygen and nutrients. Several studies have demonstrated that hypoxia could result in altered gene expression, promoting tumor angiogenesis, and metabolic shift in cancer cells.^{93,94} In addition to a condensed structure resulting from a tightly aligned cell layer, 3D cancer models reproduce unique features of tumor hypoxia and necrosis in the innermost layer,⁹⁵ offering an efficient system to investigate hypoxic mimic biology of cancers and thus develop new anti-cancer therapies.^{96–98}

Maintenance of stemness of CSCs is closely associated with the hypoxic environment.^{99,100} Hypoxia is obligatory for the formation of a CSC niche.²² This hypothesis was supported by studies that demonstrated that primitive hematopoietic stem cells inhabit areas with low oxygen pressure, given that they are likely to occupy regions that are far from the vessels.^{101,102} Emerging evidence also suggests that under a hypoxic environment, the stemness of breast cancer cells can be enhanced to increase malignancy and therapeutic resistance.¹⁰⁰

The hypoxic tumor microenvironment could also activate CSC-related signaling pathways. The CSCs properties are regulated by a network of complex interacting signaling pathways. Some of them are critical for maintaining the stemness of CSCs, including the Wnt, Notch, and Hedgehog pathways. Studies in breast cancer cells have found that stem cell characteristics are largely mediated through the activation of Wnt/ β -catenin and Notch signaling pathways under the hypoxic tumor microenvironment.¹⁰³ This was proved by a study that hypoxia could enhance the stemness properties of glioblastoma and colorectal cancer by Notch, Hedgehog, and Wnt signaling pathways.¹⁰⁴

Hypoxia is regarded as the main characteristic of the tumor microenvironment, which could contribute to the maintenance of stemness and promote tumor progression.¹⁰⁵ There is mounting evidence that hypoxia induces stemness in differentiated progenitor and non-CSCs via stem gene activation and dedifferentiation.^{106–108} Hypoxia-inducible factors (HIFs) is important in maintaining

stemness and EMT process under anoxia, as hypoxia induces stemness characteristics of CSCs via activating HIFs.^{92,109,110} The HIFs comprise a constitutively expressed subunit and an oxygen-associated α subunit (HIF-1 α -3 α).¹¹¹ Many studies have reported that HIFs play a critical function in the regulation of the CSC phenotype.^{112–117}

The cells are cultured in the 2D culture system uniform contact with oxygen, which cannot create a continuous oxygen concentration gradient. Thus, the 2D cell culture system does not mimic a hypoxic environment similar to *in vivo* conditions.¹¹⁸ Therefore, hypoxia level and hypoxia-controlled expression of genes are different between cells cultivated in 3D and 2D culture models. For example, Stankevicius et al.¹¹⁹ investigated the changes in gene expression associated with the maintenance of CSCs, such as genes involved in hypoxia, multipotency, CSC marker, and EMT in human colorectal carcinoma cell lines HT29 and DLD1 cultured in 2D vs 3D cell culture conditions. The authors selected hypoxia-related genes of GLUT1, CAIX, and VEGFA. The results showed that the HT29 and DLD1 cells cultured in a 3D lamin-rich-ECM environment showed higher levels of GLUT1, VEGFA, and CAIX gene expression relative to traditional 2D monolayer cell cultures. Klimkiewicz et al.¹²⁰ built a 3D micro-melanoma tumor using a 3D system perfecta to study and compare melanoma cell monolayers and melanoma spheroids. The results of enzyme-linked immunosorbent assay showed that there was constant amounts of HIF-1 α in melanoma spheroids cultured in the 3D system, while HIF-1 α amounts rose and subsequently fell in 2D cell monolayers. In another study, clinical samples demonstrated a positive correlation between HIF-1 α and estrogen receptor alpha (ER α) expression. DelNero et al.¹²¹ established a 3D culture alginate system with controlled oxygen, and examined expressed gene patterns of cancer cells grown in 2D and 3D in the same normoxic or hypoxic conditions using microarray assay. Microarray gene expression analysis of tumor cells grown in 2D vs 3D under hypoxic or ambient conditions showed a remarkable association of culture dimension and hypoxia reaction, indicating that response to hypoxia mainly depends on the condition where cell are grown either 2D or 3D environments (Figure 5). Whitman et al.¹²² observed a dramatic reduction in ER α in 2D cultures and the stabilization of ER α in 3D cultures.

Angiogenesis in 3D CSC models

Angiogenesis in cancer provides oxygen and nutrients, which favors cancer cell growth and represents a prerequisite and biological underpinning of metastasis for tumor growth, invasion, progression, and metastasis.^{123,124} Recent findings suggest that CSCs are involved in promoting angiogenesis, thereby promoting cancer metastasis.¹²⁵ Therefore, inhibiting cancer angiogenesis is considered an

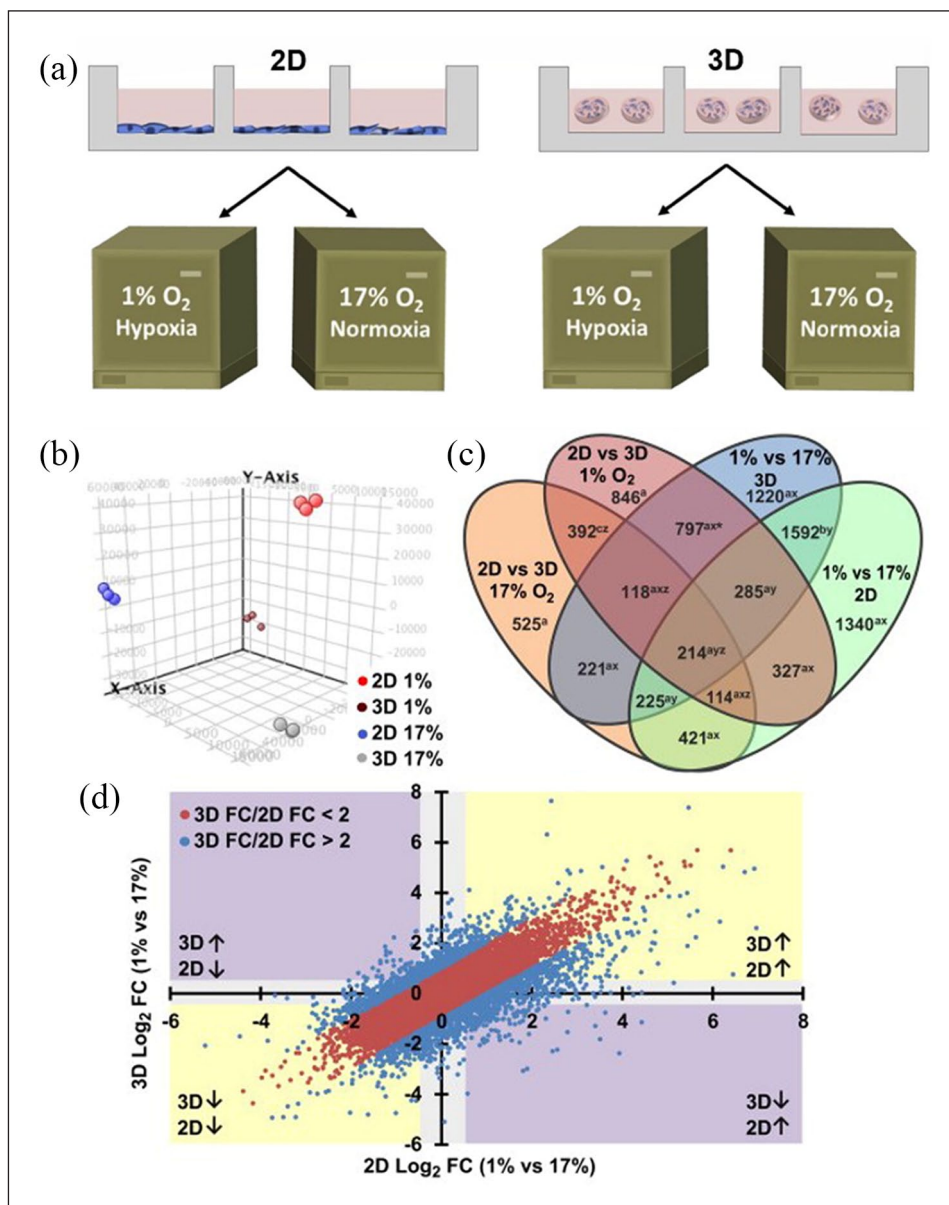


Figure 5. Schematic diagram of cell culture model and overall changes in gene expression under different conditions. (a) OSCC-3 cells were cultured in traditional 2D monolayer cell cultures or microfabricated alginate disks and incubated in normoxia (17% O₂) or hypoxia (1% O₂) condition for a week, respectively. (b) GeneSpring GX 12.6.1 software was used to in principal component analysis (PCA) of microarray results. Each substrate assembling and oxygen concentration demonstrated the dependability of every treatment to produce autonomous and self-reliable gene expressed patterns. (c) Genes were related to dimensionality as well as oxygen level variations according to Venn diagram. (d) The 2D hypoxia vs normoxia (x-axis) and 3D hypoxia vs normoxia (y-axis) scatterplots indicate the degree (FC: fold change) and trend (↑ and ↓: up- and down-regulation, respectively) of gene expression variations. Reproduced with permission from DelNero et al.¹²¹ (2015, *Biomaterials*).

efficient therapeutic strategy. Growing CSCs in 3D can mimic tumor angiogenesis, which allows the evaluation of drug effects on angiogenesis.

Numerous studies have shown that CSCs can elevate the expression of angiogenic factors in hypoxic environments, suggesting that CSCs play pivotal roles in tumor progression and angiogenesis.^{120,125,126} CSCs may switch tumor neovascularization, resulting in promoting tumor development. Three pathways are known for tumor

neovascularization by CSCs, namely production of proangiogenic factors, transdifferentiation, and formation of vasculogenic mimicry (VM).¹²⁷ CSCs have been reported to contribute to the formation of VM, a unique pattern of blood supply, induced by endothelial cells and vascular smooth muscle-like cells, through non-endothelium lining channels.^{128–132}

3D tumor models with CSCs have demonstrated some unique patterns of angiogenesis. For example, Bray et al.¹³³

mimicked tumor angiogenesis by a hydrogel culture system built on glycosaminoglycan, which could recreate prostate and breast tumor vascularization. This microenvironment model often recreates tumor vascularization breast and prostate. The different types of cells grown within this model were more tolerant of chemotherapy than those in 2D cultures and exhibited a tumor inhibition profile as that seen *in vivo*. In another study, Chiew et al.¹³⁴ developed a classical system to analyze cancer development and angiogenesis using ECs and HepG2 HCC cells. This model could resemble tumor angiogenesis under the HCC microenvironment, which enables investigation of the cellular signaling pathways involved in HCC progression. The results revealed that the 3D model exhibited similar levels of protein expression relative to HCC xenograft. Also, the 3D model showed increased expression of vital signaling proteins, including Akt/mTor and p70s6k, which was not observed in the 2D model. This could be attributed to strong association among liver cancer and ECs, thus facilitating the EC maturation, protein synthesis as well as the cancer cells development in 3D co-culture. Also, the levels of VEGF expression was higher in the 3D co-culture, indicating a higher secreted VEGF in 3D than 2D co-culture by HepG2-DsRed cells. Miller et al.¹³⁵ reported the culture of primary human clear cell renal cell carcinoma (ccRCC) cells using 3D culture system (“ccRCC-on-a-chip”) to study tumor angiogenesis. Based on their findings, expression of key angiogenic factors, including ANGPTL4, PGF, and VEGFA, in primary human ccRCC cells is enhanced in 3D cultures compared to that in 2D monolayers. In study by Agarwal et al.,¹³⁶ established “bottom-up” strategy for designing 3D vascularized human tumor which showed the ability to form complex 3D vascular networks that is controllable by incorporating cancerous cells into hydrogel-shelled microcapsules for reduced 3D culturing. In this study, the results showed that expression of vasculogenesis and angiogenesis-associated genes (e.g. VEGF) can indeed be discharged to the surrounding via the alginate shell and the secreted VEGF in the microcapsule confined 3D tumors was considerably higher than those released by 2D cultures. Also, the typical blood vessels dimension of the engineered derived tumors was higher compared to those formed from the 2D grown cells (Figure 6).

EMT in 3D CSC models

EMT has great significance for embryogenesis and maintains the integrity of the embryo.⁶ Recent studies have linked EMT with both metastatic progression of cancer and acquisition of stem cell characteristics, leading to treatment resistance, progression, and metastasis of malignant tumors.^{137–141} The activation of EMT processes in embryogenesis and tumor progression may induce changes in the physiological function of cells. Epithelial cells lose their round, cobblestone-like morphology and become

elongated cells with fibroblast-like morphology, which results in a change in cell regulatory factors, enhancing cell motility.¹⁴²

Considering the importance of EMT in drug resistance and tumor metastasis, it is necessary to establish a 3D culture design to mimic *in vivo* environments to be able to evaluate the reversibility of EMT and its role in tumorigenesis. Essentially, EMT is examined in 3D as well as 2D culture models. In 2D cultures, the cell form is confined to a “flat” plain, whereas in 3D models metastatic CSCs cells *in vivo* found at the edge of tumors forming aggregates, spherical forms, and colonies.^{143,144} Some studies showed that EMT activation is associated with the characteristics of stem cell traits for both neoplastic and normal cells.^{141,145} EMT is a crucial factor for CSC formation,^{141,146} and can result into transformation of epithelial to mesenchymal characteristics in cells, including high invasion and motility, bestowing them with stem cell-like features.¹⁴⁷ EMT can lead to loss of polarity and phenotype in epithelial cells, and this includes their connection with the basement membrane. This can make them gain high invasion and migration, anti-apoptotic, and ECM degradation capacities, leading to drug resistance and metastasis of malignant tumors.

The 3D model has been widely used to investigate molecular events in EMT.¹²³ To reflect CSC and EMT properties in a 3D context, Liu et al.¹⁴⁸ constructed a collagen scaffold to research adenoid cystic carcinoma (ACC) cell biological function in 3D culture system. Here, ACC-83 cells seeded in collagen scaffold were compared to ACC-83 cells in 2D culture. The EMT and angiogenesis associated genes expressions were considerably increased in 3D culture. Moreover, the collagen scaffold could improve ACC-83 cell migration, invasion and resistance to chemotherapeutic agents. From this, they concluded that the collagen scaffolds provide a new platform for CSC research in diseases. Also, to explain the effect of composition and biophysical features of ECM on pancreatic cancer EMT, Puls et al.¹⁴⁹ also cultured pancreatic ductal adenocarcinoma in a type I collagen oligomer 3D matrices, suggesting that classic EMT changes could also be observed in 3D models of the mammary gland acinus. On the contrary, the frequently used 2D *in vitro* cell culture system does not model the conditions of *in vivo* cancer EMT. Huang and Hsu¹⁵⁰ used chitosan-coated and HA as the material to produce chitosan-hyaluronic acid (CH) membranes. Human non-small cell lung cancer (NSCLC) cells were seeded onto CH membranes to promote generation of tumor spheroid. In this study, they examined the biological function of tumor spheroid by comparing NSCLC cells on CH membranes (3D culture) or tissue culture polystyrene (conventional 2D culture). The result showed that compared with the conventional 2D culture, the expression levels of EMT- and stemness-associated genes were considerably increased in 3D culture. Besides, the NSCLC seeded on the CH membranes displayed more

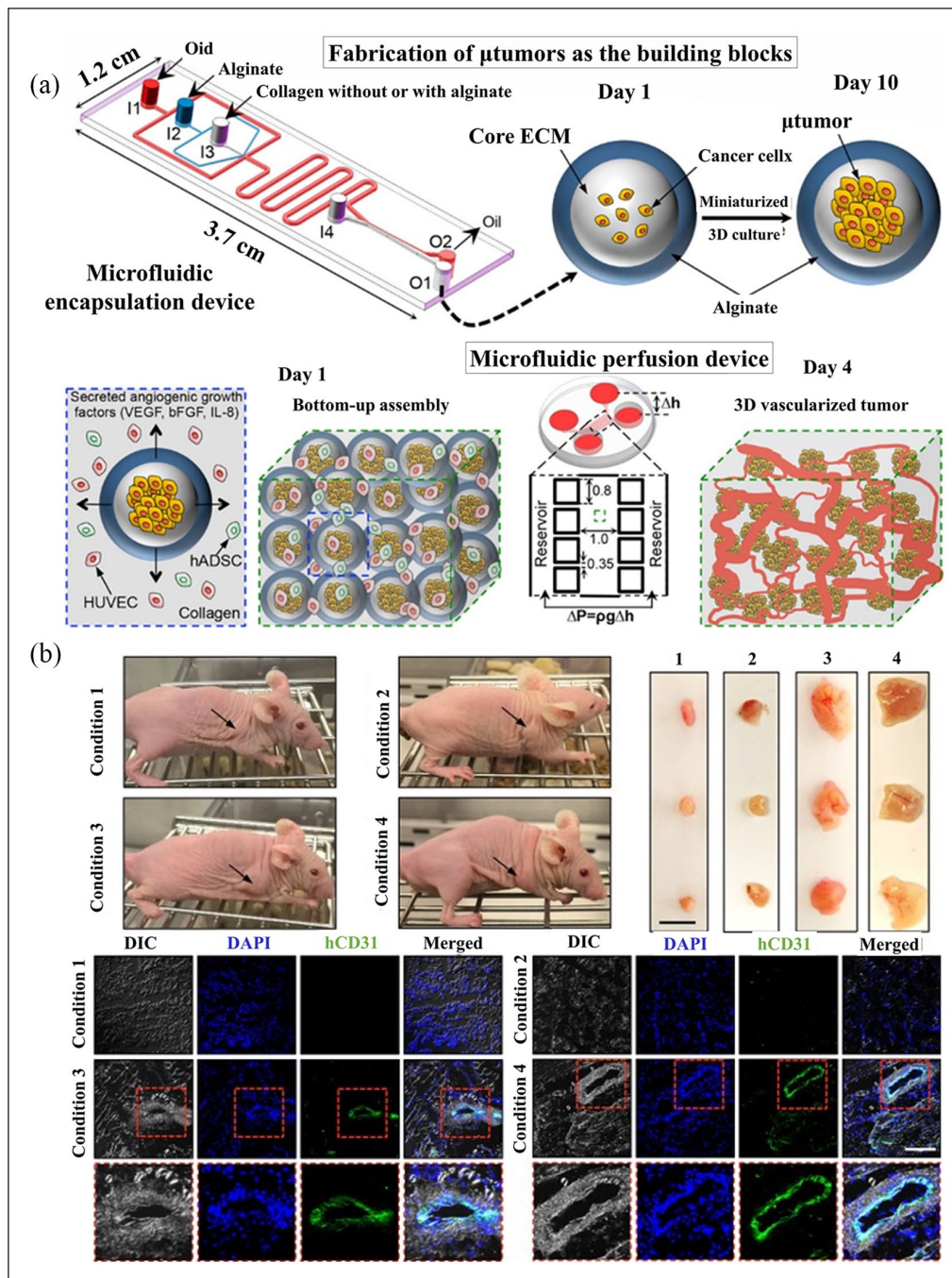


Figure 6. (a) A scheme of the bottom-up approach of generating 3D vascularized human tumor. (b) In vivo tumorigenicity of the 3D-engineered system of encapsulated microtumors, HUVECs, and hADSCs in collagen. Reproduced with permission from Agarwal et al. (2017, *ACS Nano*).¹³⁶

aggressive characteristics and resistant to antineoplastic drugs. These results illustrated that CH was valuable for CSC research and antineoplastic drugs development. (Figure 7).

Drug resistance in 3D CSC models

Multidrug resistance (MDR) refers to the fact that when cancer cells are exposed to a type of chemotherapeutics for

long-term, they could acquire resistance to that type of chemotherapeutics, and also develop cross-resistance to other chemotherapeutics.^{151,152} MDR remains a tough clinical problem for researchers and a difficult issue in the treatment of cancer, leading to tumor recurrence and progression.¹⁵³

Studies have indicated that CSCs play crucial role in the emergence of MDR, which is the main cause of tumor treatment failure.^{152,154} Resistance to these treatments can

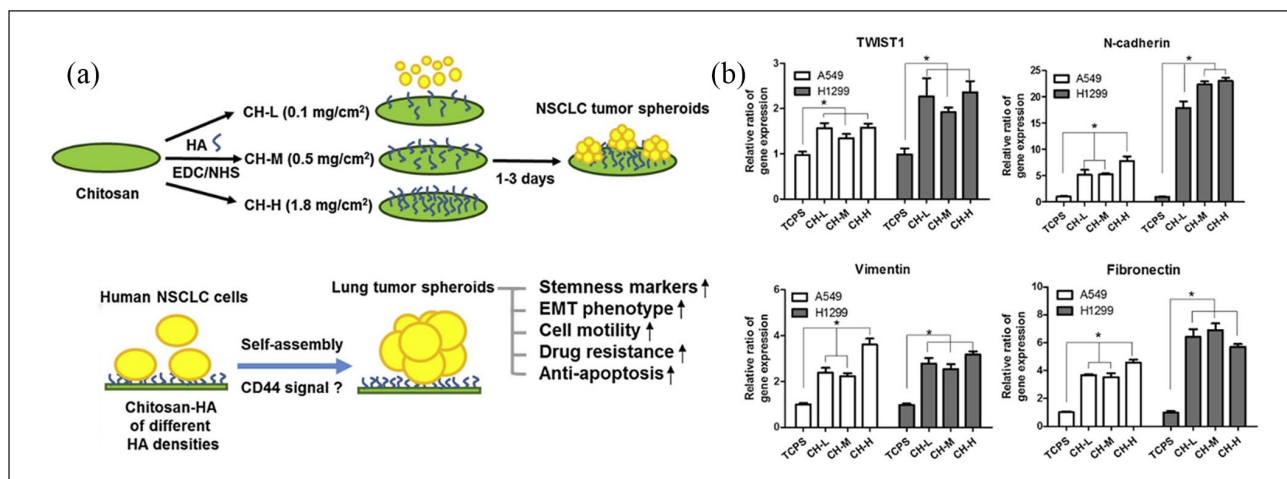


Figure 7. (a) Synthesis of chitosan-hyaluronic acid (CH) matrices under diverse hyaluronic acid (HA) concentrations and formation of tumor spheroids from non-small cell lung cancer (NSCLC) cells via CD44 signaling modulation. (b) Features of EMT from qRT-PCR, denoted by expressed TWIST1, N-cadherin, vimentin, and fibronectin mRNA in a week. Reproduced with permission from Huang et al.¹⁵⁰ (2014, *Biomaterials*).

be subcategorized into intrinsic and acquired. MDR of tumors can be categorized into intrinsic resistance and acquired drug resistance. Intrinsic resistance is a preexisting factor before the start of chemotherapy, thus inducing certain treatments useless. Acquired drug resistance occurs gradually during the course of chemotherapy, and seems to be the main reason for tumor treatment failure.¹⁵⁴

3D CSC culture models could simulate the *in vivo* situation to study therapy resistance because they can reflect real drug responses. Studies have indicated that tumor-spheres cultured in 3D models may show increased treatment resistance compared with cancer cells cultured in traditional 2D culture, reflecting the real resistance level against an anti-cancer drug.^{39,123,155,156} Many mechanisms of CSC resistance have been explored. First is the overexpression of ABC transporters. These are complex molecular pumps which mainly act as catalyst in active transport, by hydrolysing ATP. In a clinical setting, these may involve pumping out of the drug by ABC transporters, directing drugs and removal via exocytosis in vesicles, as well as low drug uptake.¹⁵⁷ Second is the overactivation of the DNA damage response (DDR). Radiotherapy is a local tumor treatment used to kill tumor cells, while CSCs over-activate DDR, which produces drug-tolerant states.^{158,159} Third is cell-cycle promotion and/or cell metabolic alterations. Most CSCs are in the state of cell-cycle quiescence, except the state of self-renewal and cell division, which reduces damage from anticancer drugs. This is because some anticancer drugs are cell-cycle-specific agents, which only act on cancer cells in the proliferating phase.^{152,160} Finally, apoptosis evasion and activation of pro-survival pathways is another resistance mechanism, as one of the key mechanisms of chemotherapy treatment is inducing cancer cell apoptosis.¹⁵¹ When cancer cell apoptosis is disrupted, cancer cells could show resistance to

chemotherapy treatment. In addition, the tumor microenvironment, activation of aldehyde dehydrogenase, and developmental pathways also play an important role in CSC resistance.^{161–163} In conclusion, CSCs play a key role in tumorigenesis and promote MDR phenotype via multiple mechanisms.

Rija and Li¹⁶⁴ introduced a fabricated reconstructable tissue matrix scaffold system native tissue from ECM tissue-like structure and pliability to test effectiveness of two anti-cancer medications, taxol and 4-hydroxytamoxifen (HT), in 2D and 3D cultures. The BT474 and T47D cells were seeded onto 2D and 3D surface scaffolds and evaluated with Live/Dead Cell assay and CCK-8 reagent post the anti-cancer drugs administering. According to the results, BT474 and T47D cells exhibited a faster proliferation with distinct growth tendency in the 3D scaffolds among the scaffold sets, and higher robust growth was observed in 2D models. The administered medication suppressed the proliferation of cells in the 3D and 2D groups in a time-dependent manner, but the impact of drug suppression in 3D cultures was lower than in 2D. Thus, indicating that 3D cell cultures have better biological and clinical feasibility than 2D cultures (Figure 8).

Concluding remarks

In this communication, we discussed about different 3D models of cancer for CSCs enrichment, putting an emphasis on the biomaterials- and engineering-based approaches and designs. Development of 3D culture models similar to the conditions of *in vivo* tumorigenesis enables better understanding the key events including tumorigenesis, tumor growth, metastasis, and recurrence in the *in vitro* conditions that resemble tumor microenvironment in

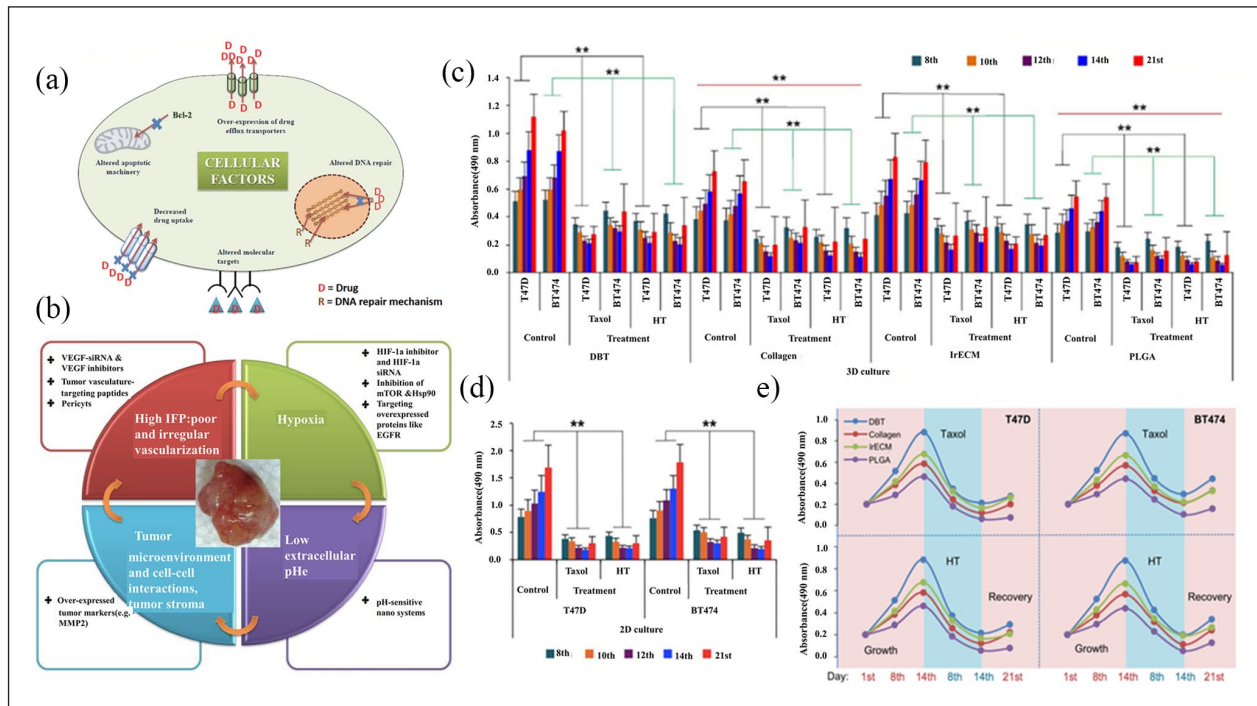


Figure 8. Drug resistance in cancer cells (a) Mechanisms of intrinsic multidrug resistance in cancer cells. (b) Intrinsic relationship between multidrug resistance and physiological factors. Reproduced with permission from Patel et al.¹⁶⁵ (2013, *Adv Drug Deliv Rev*). The susceptibilities of T47D and BT474 cells to 4-hydroxytamoxifen and paclitaxel from different cultivation methods. (c) and (d). (c) The histogram shows absorbance values of T47D and BT474 cells treated with 4-hydroxytamoxifen and paclitaxel from four different scaffolds. (d) Proliferation of T47D and BT474 cells treated with 4-hydroxytamoxifen and paclitaxel cultured in 2D culture were assessed by measuring the absorbance. (e) Proliferation/inhibition curve plots. Reproduced with permission from Rija et al.¹⁶⁴ (2017, *Sci Adv*).

humans. Even with the active studies in this area, current 3D models of cancer for CSC enrichment are limited in terms of variability in cell size and homogeneity, protocol standardization and mass production. Tackling these issues with advanced 3D CSC models may facilitate tumor modeling and drug development in the near future.



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