Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) 2024 25(9):736-755
www.jzus.zju.edu.cn; www.springer.com/journal/11585
E-mail: jzus_b@zju.edu.cn

Review

https://doi.org/10.1631/jzus.B2300840

Three-dimensional breast cancer tumor models based on natural hydrogels: a review

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Abstract: Breast cancer is the most common cancer in women and one of the deadliest cancers worldwide. According to the distribution of tumor tissue, breast cancer can be divided into invasive and non-invasive forms. The cancer cells in invasive breast cancer pass through the breast and through the immune system or systemic circulation to different parts of the body, forming metastatic breast cancer. Drug resistance and distant metastasis are the main causes of death from breast cancer. Research on breast cancer has attracted extensive attention from researchers. In vitro construction of tumor models by tissue engineering methods is a common tool for studying cancer mechanisms and anticancer drug screening. The tumor microenvironment consists of cancer cells and various types of stromal cells, including fibroblasts, endothelial cells, mesenchymal cells, and immune cells embedded in the extracellular matrix. The extracellular matrix contains fibrin proteins (such as types I, II, III, IV, VI, and X collagen and elastin) and glycoproteins (such as proteoglycan, laminin, and fibronectin), which are involved in cell signaling and binding of growth factors. The current traditional two-dimensional (2D) tumor models are limited by the growth environment and often cannot accurately reproduce the heterogeneity and complexity of tumor tissues in vivo. Therefore, in recent years, research on three-dimensional (3D) tumor models has gradually increased, especially 3D bioprinting models with high precision and repeatability. Compared with a 2D model, the 3D environment can better simulate the complex extracellular matrix components and structures in the tumor microenvironment. Three-dimensional models are often used as a bridge between 2D cellular level experiments and animal experiments. Acellular matrix, gelatin, sodium alginate, and other natural materials are widely used in the construction of tumor models because of their excellent biocompatibility and non-immune rejection. Here, we review various natural scaffold materials and construction methods involved in 3D tissue-engineered tumor models, as a reference for research in the field of breast cancer.

Key words: Breast cancer; Tumor microenvironment; 3D tumor model; Decellularized extracellular matrix; Natural scaffold materials

1 Introduction

Breast cancer is one of the most important diseases threatening women's health. By 2020, breast cancer had become the most common malignant tumor in the world, accounting for 11.7% of all cancer types and 24.5% of female malignant tumors (Sung et al., 2021). Although the incidence of breast cancer in China is low, the number of new cases accounted for 18.4% of the global incidence of breast cancer, and the incidence in all age groups is increasing rapidly (Lei et al., 2021). Therefore, research on the mechanism of occurrence and progression of breast cancer and its treatment methods has become a research hotspot in recent years (Fig. 1). The in vitro construction of tumor models provides a platform for the study of tumor pathogenesis and drug screening. Tumor models are divided into

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Received Dec. 6, 2023; Revision accepted Feb. 20, 2024; Crosschecked Sept. 2, 2024

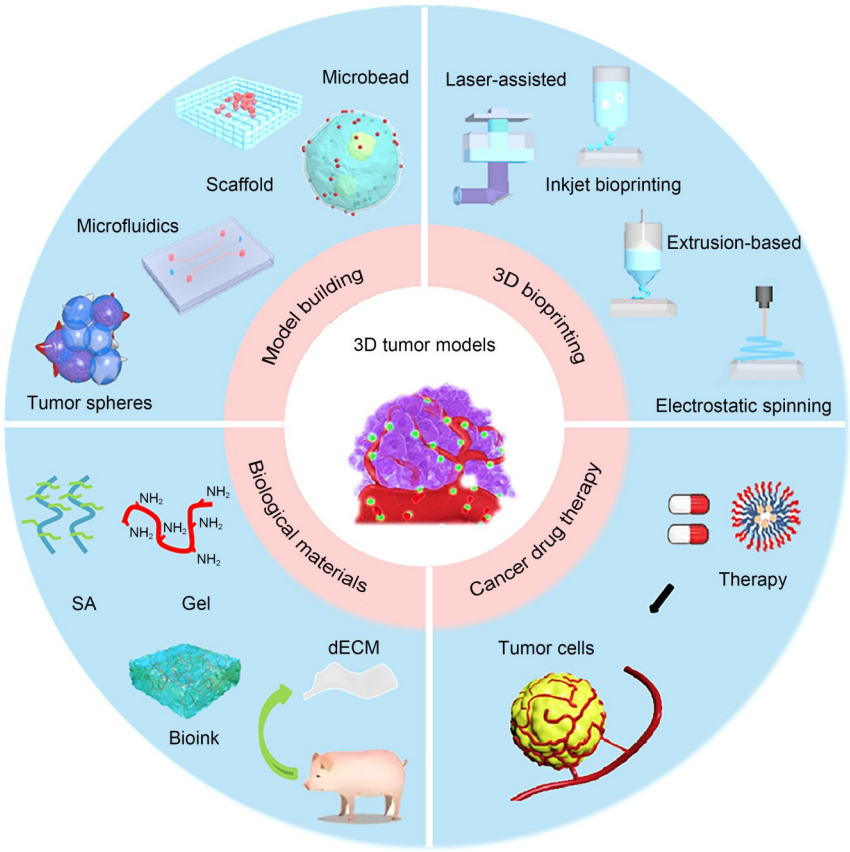

Fig. 1 Research status of breast cancer tumor models. SA: sodium alginate; Gel: gelatin; dECM: decellularized extracellular matrix.

two-dimensional (2D) and three-dimensional (3D) models. Two-dimensional models have the advantages of being fast, simple, cheap, and easy to copy. However, due to their lack of the third dimension of the tumor microenvironment (TME), simulation is not accurate, the ability to predict the body's reaction is low, and in long-term culture cancer cell-specific heterogeneity gradually disappears through genetic and transcriptional evolution. Three-dimensional models have spatial relevance that recapitulates cell–cell and cell–matrix interactions facilitating more accurate simulation of tumor tissue formation and progression in vivo. Three-dimensional tumor models serve as an effective bridge between traditional 2D models and patient-derived xenograft (PDX) or animal experiments.

2 Breast cancer and metastatic breast cancer

The mammary gland structure includes multiple layers of endothelial tissue and a large amount of adipose tissue surrounded by a basement membrane, and contains blood vessels, lymphatic vessels, and various stromal cells (Stingl et al., 2006). In a normal breast, the stratified endothelial cells are composed of two different cell populations: muscle endothelial cells and endothelial cells. Breast cancer usually begins with ductal hyperplasia and develops into a tumor under the constant stimulation of various carcinogenic factors, which may lead to invasion and metastasis. Factors such as extracellular matrix (ECM) and macrophages in the TME play an important role in the generation and progression of breast cancer (Sun et al., 2017). In breast diseases, tissues often show heterogeneity, which is related to the development of the primary stage of a normal breast. In the continuous proliferation and differentiation of cells, changes in genetic information and phenotype lead to the generation of heterogeneity (Almendro and Fuster, 2011).

Normal somatic cells are subjected to strict growth promotion and regulation, which helps to maintain the normal structure and function of tissues. However, due

to the mutation of tumor suppressor genes, cancer cells can proliferate indefinitely for a long time without any external stimulation because telomerase prevents chromosome shortening and allows cells to replicate widely. There are two hypotheses about the initiation and development of breast cancer: cancer stem cell theory and stochastic theory. The cancer stem cell theory holds that all tumor subtypes come from the same stem cells or progenitor cells, and their acquired genetic and epigenetic mutations will lead to different tumor phenotypes (Polyak, 2007). According to the stochastic theory, each tumor subtype originates from a single cell type (stem cells, progenitor cells, or differentiated cells), and stochastic mutations can gradually accumulate in any breast cell. When enough mutations are accumulated, they will be transformed into tumor cells (Sgroi, 2010; Cleary et al., 2014). Although these two theories are supported by a large amount of data, neither can fully explain the origin of human breast cancer. Therefore, it is important to study the tumorigenesis mechanism of normal breast stem cells and progenitor cells to understand the factors driving breast tumorigenesis.

Exposure to estrogen may cause DNA damage and genetic changes, leading to the occurrence of breast cancer (Cavalieri et al., 2006). Under normal circumstances, the immune system can recognize cancer cells and DNA-damaged cells and kill them. The occurrence of breast cancer may be caused by mutation of genes involved in these protective pathways and the blockage of signal pathways under the stimulation of the external environment. This may result in the failure of this effective immune defense and surveillance function, and failure to identify and kill cancer cells in time, allowing cancer cells to proliferate continuously and finally develop into cancers and tumors (Goff and Danforth, 2021).

Malignant tumors are metastatic, which is an important cause of death in 90% of cancer patients (Chaffer and Weinberg, 2011). In the process of tumor metastasis, the cells lose adhesion, gain motility, escape from the original tumor tissue, penetrate through the basement membrane and infiltrate into the capillaries and lymphatic vessels, then enter the systemic circulation, and finally infiltrate into the surrounding tissues and form secondary tumors (Gupta and Massagué, 2006) (Fig. 2). In addition to local recurrence, breast cancer tends to metastasize distally to bone, brain, liver, lung, and distal lymph nodes (Yates et al., 2017). Bone metastasis is the most common, being found in 70% of metastatic breast cancer patients (Weilbaecher et al., 2011), followed by the liver (about 30% (Hess et al., 2006)) and the brain (about 10%–30% (Bachmann et al., 2015)). Different breast cancer subtypes are associated with significantly different overall survival rates and a tendency to metastasize to specific organs (The Cancer Genome Atlas Network, 2012).

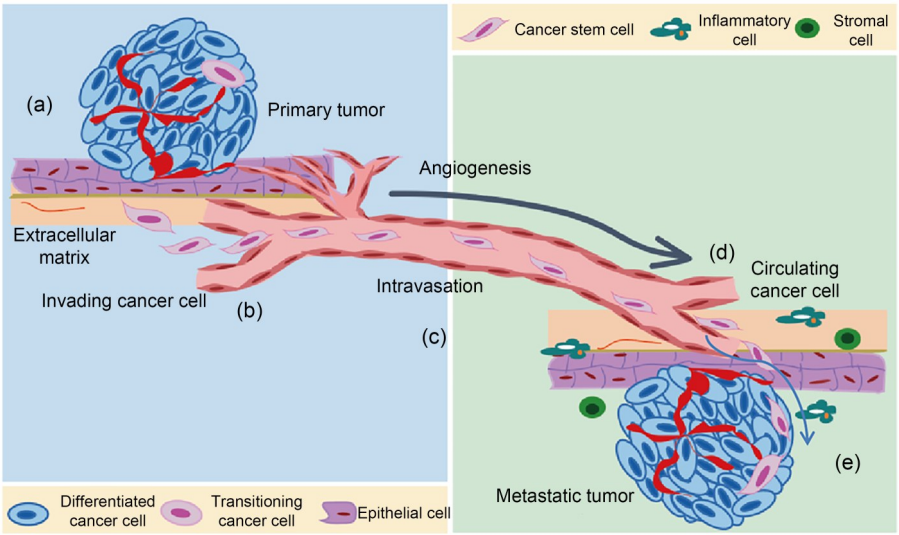

Fig. 2 Metastatic process. (a) Cancer cells within the primary tumor acquire an invasive phenotype. (b) Cancer cells invade into the surrounding matrix and move toward blood vessels, where they intravasate to enter the circulation. (c) Circulating tumor cells (CTCs) travel through the circulation to distant organs. (d) CTCs exit the circulation and invade the microenvironment of foreign tissues. (e) Single cancer cells adapt to the microenvironment, survive, and start to proliferate.

To separate individual or small groups of cancer cells from the primary tumor and start the metastasis process, these cells must acquire the ability to migrate and invade. These properties enable cells to degrade and move to blood vessels and lymphatic vessels through the ECM of surrounding tissues, which in turn provide them with access to distant secondary sites (Chen et al., 2018). Under normal conditions, the cells forming the endothelial layer in the breast tissue are tightly bound to the adjacent cells and the basement membrane through adhesion junctions, tight junctions, desmosomes, and half desmosomes, effectively fixing the cells in the endothelial layer. This strict physiological restriction not only inhibits the invasion of normal epithelial cells, but also hinders the invasion of many benign cancer endothelial cells to the outside. However, with the development of tumors, cancer cells are no longer controlled by intercellular interactions, first dissolving the underlying basement membrane, then invading the adjacent interstitial cavity, and penetrating other organs through the blood. This process is achieved through a molecular mechanism similar to epithelial–mesenchymal transition. Cancer-associated fibroblasts and immune cells, including macrophages and regulatory T cells, promote this process by immune-editing and providing soluble growth factors.

Through physical obstruction, complementary adhesive contact, and binding to chemokine gradients, tumor spheres are attracted to capillaries at secondary sites and adhere to the vascular bed (Azevedo et al., 2015). Tumor cells resident here may grow into intravascular metastases (a metastatic tumor starting from intravasation of a secondary tissue), and may also reactivate proteolytic and migration mechanisms to promote extravascular metastasis of the secondary tissue. The tissue microenvironment of the secondary site is different from that of the primary site, and most cells die due to incompatibility of molecular adhesion and growth interactions (Chambers et al., 2002). Tumor cells can remain dormant in tissues in the form of single cells or clinically undetectable micrometastases, and only a small proportion of tumor cells develop into obvious metastases. It has been suggested that tumor cells undergo reverse transformation from mesenchymal cells to epithelial morphological cells and re-establish the proliferation mechanism. The long incubation period from initial treatment to the onset of metastatic disease is a major feature of estrogen receptor positive breast cancer. This indicates that tumor cells can remain dormant for many years, so it is difficult to detect breast cancer in the early stage. How and when dormant cells regain their growth potential is still unclear, but this stage has more potential for therapeutic intervention (Eckhardt et al., 2012).

There is heterogeneity within tumor tissues, which is caused by the inherent instability of cancer-related genomes. DNA mutation, chromosome rearrangement, and epigenetic changes in the process of cell proliferation can lead to heterogeneity. High metastatic proliferation from different tumor cell populations has a higher gene mutation rate than non-metastatic proliferation from the same tumor, which is an early link between cancer metastasis and genetic instability (Xin et al., 2019). Therefore, it is very important to study metastatic breast cancer.

3 Tumor microenvironment and 3D in vitro tumor models

With the continuous deepening of research on the internal structural pole of a tumor and its physiological mechanism, it is generally believed that a tumor is a 3D network formed by dynamic ECM, immune cells, and endothelial cells (Fig. 3), in which there are very complex cell–cell and cell–ECM interactions. The basement membrane, stromal cells and their released growth factors, ECM degradation proteases, chemokines, and cytokines also have important effects on tumor progression and invasion (Hartl, 1995).

PDX and animal models are the most effective ways to study the mechanism of tumor progression and to screen and evaluate anti-cancer drugs. Such models can reveal the roles of the TME, immune system, and angiogenesis in the tumor treatment response. However, due to their high cost, long time course, ethical problems, and lack of quantitative data collection, they have certain limitations in practical application (Fong et al., 2016). At present, the research and development of tumors rely mainly on traditional 2D cell culture systems. Two-dimensional monolayer culture has been widely used because of its advantages such as speed, simplicity, low cost, and ease of replication. Information about cancer mechanisms, signaling pathways, and targeted therapies was originally obtained through a 2D cell culture system. However, due to the spatial complexity and anisotropy of tumor tissue in vivo, the lack of the third dimension in 2D culture leads to

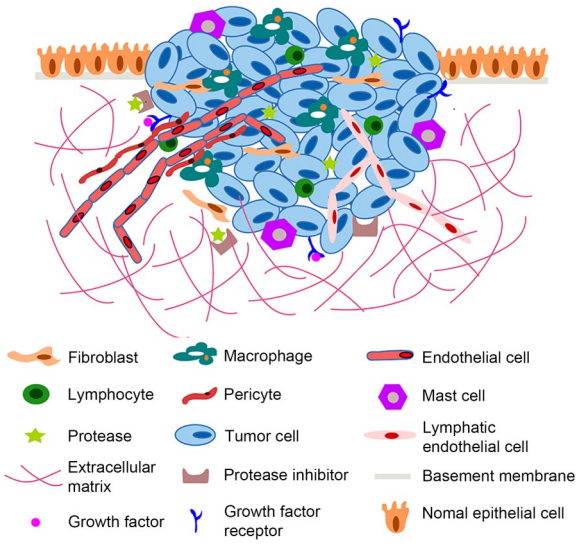

Fig. 3 Schematic view of the tumor microenvironment. The tumor microenvironment consists of cancer cells and various types of stromal cells, including fibroblasts, endothelial, mesenchymal stem cells, and immune cells, embedded in the extracellular matrix. Adapted from Xu et al. (2014), Copyright 2014, with permission from Elsevier.

inaccurate simulation of the TME and a low ability to predict the response in vivo. At the same time, the experimental observations are blurred to a certain extent, resulting in misleading and contradictory results (Huang and Gao, 2018). In long-term culture, the specific heterogeneity of cancer cells gradually disappears through genetic and transcriptional evolution. Therefore, it is difficult to provide accurate information on the efficacy of drug therapy and does not represent truly complex tumor structures. In recent years, 3D models have attracted increasing attention because they have spatial correlation, can reproduce the interactions between cells and between cells and the matrix, and can more accurately simulate the formation and progression of tumor tissues in vivo. Compared with 2D models, 3D models are less sensitive to some antitumor drugs and clinical treatments due to hypoxia and slower circulating cell responses, so they have more value in drug screening and clinical treatment (Zhang et al., 2023). Three-dimensional tumor models have become an effective bridge between traditional 2D models and PDX or animal experiments (Nath and Devi, 2016).

With the deepening of research, there are now many different 3D tumor models and construction methods. Three-dimensional tumor models can be divided into scaffold-free multicellular tumor spheres and biomaterial-based tumor models. The preparation methods include suspension culture, suspension methods, hydrogels, porous scaffolds, microencapsulation methods, and microfluidic technology (Zhang et al., 2020). Compared with traditional 2D culture, artificial 3D culture provides a better platform for cell proliferation and interaction.

3.1 Natural scaffold materials

Scaffolds are 3D porous, fibrous, or permeable biomaterials that allow the transport of liquids and gases and promote interaction between cells. The material of the tumor tissue-engineered scaffold determines its final performance, so the selection of an appropriate material is crucial. The main scaffold materials commonly used in tissue engineering research include natural materials and synthetic materials. Natural materials refer to macromolecular substances that originally exist in nature and can be directly used with little or no processing, including decellularized ECM (dECM), gelatin, and sodium alginate (SA). Natural materials generally do not cause immune rejection of receptors. Generally, cell surface receptors have specific recognition sites that can regulate and induce cell proliferation and differentiation. Synthetic materials are macromolecular polymers that can accurately adjust physical and chemical properties and biomechanical characteristics by chemical methods. They include polyvinyl alcohol, polylactic acid, and polycaprolactone, and come from a wide range of raw materials. Accurate synthesis allows control of their advantages. Natural materials have good biocompatibility and degradability, but their mechanical properties are not good. Synthetic materials have excellent mechanical properties, but their biocompatibility is insufficient, which often provokes an immune rejection reaction. Therefore, synthetic materials are generally combined with natural materials in a certain way, so as to combine the advantages and functions of each material to build a composite scaffold with suitable hardness and mechanical properties. Table 1 summarizes recent studies using dECM, gelatin, and SA scaffolds.

3.1.1 Decellularized extracellular matrix

Inspired by research on organ regeneration for orthotopic transplantation in tissue engineering, it is generally believed that the ECM (fibrin proteins (such as types I, II, III, IV, VI, and X collagen and elastin)

Table 1 Examples of scaffolds based on natural materials

Support material	Fabrication method	Cell type	Inoculated cell number	Outcome	Crosslinking mode	Reference
dECM	Liquid cover	MDA-MB231, MCF-7	3×10^4 cells/hole	Self-assembly of dECM-3D tumor matrix sphere with adjustable size		Ferreira et al., 2021
	Freeze-drying	MDA-MB-231	1×10^5 cells/scaffold	Prepared 3D dECM scaffolds with different hardness		Lv YG et al., 2021
		MCF-7, 4T1	5×10^5 cells/mL	3D tissue-enginnered tumor model can better simulate tumors in vivo	Chemical crosslinking	Li et al., 2019
Gel	Hydrogel	HCC1806	1×10^6 cells/mL	The primary mammary gland like organ tissue transplanted in mice was successfully cultivated	Photo-crosslinking	Casey et al., 2017
	Encapsulation	MDA-MB-231	1×10^6 cells/mL	Provide a platform to more accurately simulate the internal conditions	Enzyme-crosslinking	Fang et al., 2014
	Mould	3T3-L1, HCC1806, MDA-MB-231	1×10^7 cells/mL	Interstitial carcinoma interaction highly depends on ECM stiffness	Photo-crosslinking	Yue et al., 2018
	Photocuring	MCF-7, HUVECs	4×10^5 cells/mL	Created tumor on chip with endothelial barrier	Photo-crosslinking	Aung et al., 2016
	Freeze-drying	MCF-7	1×10^5 cells/scaffold	Gelatin provides an improved environment for MCF-7 cell proliferation	Physical/chemical crosslinking	Redmond et al., 2022
SA	Microextrusion printing	MDA-MB-231	1×10^6 cells/mL	It can be used to maintain and expand patient-derived cancer spheroid	Physical crosslinking	Flores-Torres et al., 2021
	Encapsulation	MCF-7, MC3T3-E1	2×10^4 cells/microbead	Saffron can promote apoptosis of breast cancer cells	Physical crosslinking	Zhu et al., 2021
		MCF-7, MDA-MB-231, EA.hy926	5×10^5 cells/mL	Tumor like microcapsules become a good candidate for clinical and drug level uses	Physical crosslinking	Ertekin et al., 2022

dECM: decellularized extracellular matrix; Gel: gelatin; SA: sodium alginate; HUVECs: human umbilical vein endothelial cells.

and glycoproteins (such as proteoglycan, laminin, and fibronectin), which regulate protein complexes, participate in cell signaling, and promote growth factor binding and cell adhesion) and the vascular system preserved in the matrix after cell removal may provide a natural environment for the growth of cancer cells (Li et al., 2019). Therefore, dECM has also been gradually applied to simulate the microenvironment of tumor tissue in vitro. Since different tissue sources and decellularization methods affect the structure and chemical composition of dECM, it is very important to select an appropriate decellularization method according to the cell and lipid content, density, and thickness of the target tissue, to minimize the adverse effects on the ECM.

dECM has been widely used in various fields of tissue engineering due to its unique advantages. Its materials come from a wide range of sources including pig, rabbit, cattle, sheep, and patient tumor tissues. A variety of in vitro tumor models can be prepared

based on dECM materials. Porcine-derived breast tissue has been used for decellularization processing in combination with ultra-low adhesion surfaces to construct organotypic 3D metastatic breast cancer fibroblast models enriched in dECM microfilament fragments, overcoming the morphological variability associated with cell-containing dECM models (Yang et al., 2022) (Fig. 4a). Similarly, pig kidney cellular matrix can be used as raw material to prepare dECM scaffolds (Fig. 4b). A chemical crosslinking method is used to improve the mechanical properties and physical characteristics of cellular scaffolds derived from pig kidney, and human breast cancer cell line (MCF-7) is used to construct tumor models (Ferreira et al., 2021).

In addition to the acellular matrix of normal tissue, a tumor-derived acellular matrix can be used to prepare 3D scaffolds with different stiffness to simulate the human breast TME, and can be used for preclinical drug screening to a certain extent (Lv YG et al., 2021) (Fig. 4c). In addition, dECM models can be combined with tumor chip technology to provide an attractive and high-performance platform for in vitro TME simulation, enabling combination of therapeutic strategies and a diversity of biophysical and biochemical model parameters (Tamayo-Angorrilla et al., 2022) (Fig. 4d).

dECM can enhance the migration of normal cells and the invasion of cancer cells, and regulates cell behavior to maintain tissue integrity. Among many animal tissues used for acellular research, the liver is the largest organ of the body and the center of metabolism. The liver ECM contains collagen, fibronectin, laminin, glycosaminoglycan, proteoglycan, and a variety of non-soluble growth factors, which can provide a natural growth environment for cells (Lang et al., 2011). In addition, due to the metastatic nature of malignant tumors, it is of great significance to construct tumor models using liver dECM to study the characteristics and mechanisms of metastatic tumors.

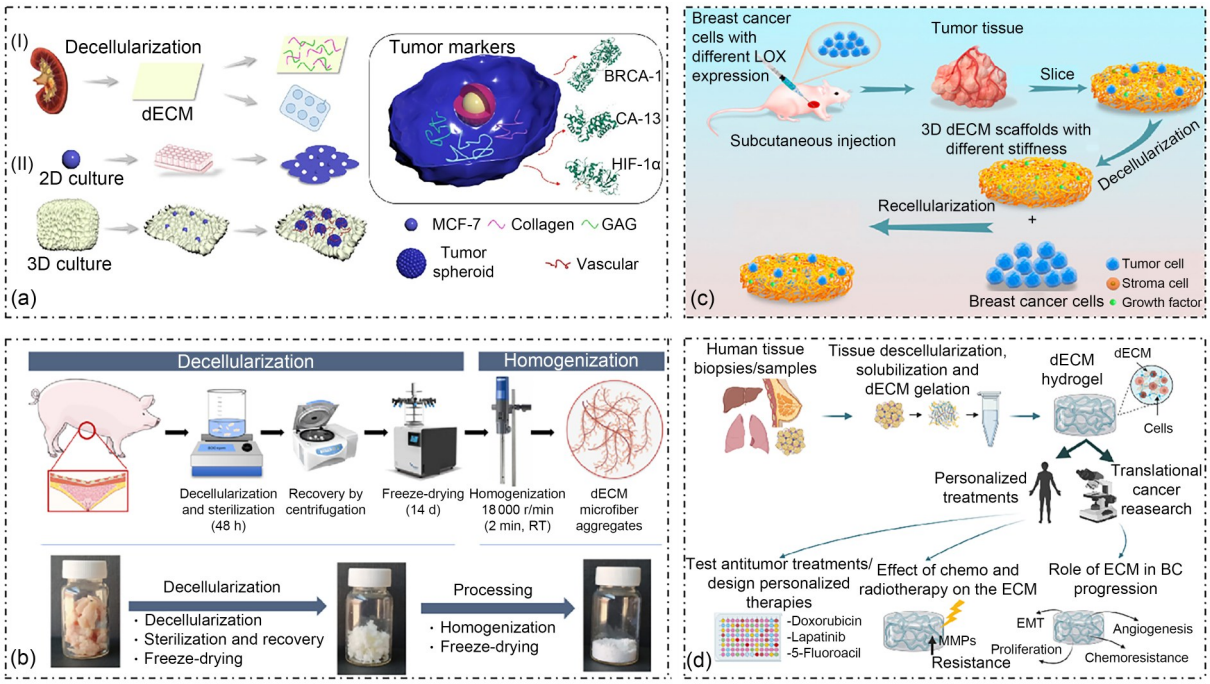

Fig. 4 Three-dimensional (3D) in vitro tumor model studies based on an acellular matrix. (a) Improving the performance of the derived scaffolds using porcine kidney decellularized extracellular matrix (dECM) combined with a chemical crosslinking method; tumor model construction using human breast cancer cell line (MCF-7) (Yang et al., 2022). (b) An organotypic 3D metastatic breast cancer model rich in dECM-derived breast cancer fibroblasts constructed using dECM combined with an ultra-low adhesion surface. Reprinted from Ferreira et al. (2021), Copyright 2021, with permission from Elsevier. (c) Preparation of 3D scaffold based on tumor-derived dECM to simulate the microenvironment of human breast tumor tissue (Lv YG et al., 2021). (d) Using a dECM model combined with tumor microarray technology provides an attractive and high-performance platform for in vitro tumor microenvironment (TME) simulation (Tamayo-Angorrilla et al., 2022). GAG: glycosaminoglycan; BRCA1: breast cancer susceptibility protein 1; CA-13: cytogenetic abnormalities of chromosome 13; HIF-1α: hypoxia-inducible factor-1α; RT: room temperature; LOX: lysyl oxidase; BC: breast cancer; EMT: epithelial–mesenchymal transformation; MMPs: matrix metalloproteinases.

3.1.2 Gelatin

Gelatin is a hydrolyzate of collagen. It is composed of 20 amino acids, of which glycine accounts for one-third and hydroxyproline and proline together for one-third, and has good immunogenicity, hydrophilicity, and temperature sensitivity. When gelatin is dissolved in water, a solution with high viscosity can be formed, and a hydrogel can be created after the temperature is reduced. Therefore, scaffolds with high resolution can be prepared by 3D printing (Bahcecioglu et al., 2020). Because of its good biocompatibility and easy gelation, gelatin has become one of the materials widely used in tissue-engineered scaffolds.

Gelatin can reduce the viscosity and shear stress of biological ink. Thus, it can improve the printability of the ink, especially for the printing of cells carrying biological ink, which can reduce cell damage. For example, Redmond et al. (2022) developed a gelatin-based scaffold that can support the attachment and proliferation of breast cancer cells as a 3D culture model. The addition of gelatin is conducive to the preparation of scaffolds with suitable porosity, pore size, and mechanical properties to accurately replicate the stiffness of thin ECM in human breast cancer. It provides a platform for the evaluation of gene expression profiles and therapeutic agents for breast cancer (Fig. 5a). Based on gelatin material, the gelatin polymer is covalently crosslinked with polyethylene glycol crosslinking agent, and the layered protein-derived Tyr-Ile-Gly-Ser-Arg (YIGSR) polypeptide or vascular endothelial growth factor (VEGF) pseudo QK polypeptide is coupled with gelatin to form a hydrogel that can be used to regulate cell binding and growth factor signal transduction (Su et al., 2019). Results showed that the concentrations of peptide and crosslinking agent affected the swelling of the hydrogel, and interaction with the gelatin would affect the formation of a hydrogel network structure (Fig. 5b). Shah et al. (2022) studied the proliferation and morphology of two separate breast cancer cell lines (MCF-7 and MDA-A) and stem cell populations in gelatin in controlled normal and MB 231 tissues. Hard and dense hydrogel (10 kPa) and acidic pH (6.5) increased the expression of epithelial cancer stem cell (E-CSC) and mesenchymal cancer stem cell (M-CSC) markers (Fig. 5c).

Gelatin has stable properties and low cytotoxicity, which can promote the adhesion and growth of cells. When used in the construction of tissue-engineered models, it can show high scaffold formability and cell activity. Its good hydrophilic property is also conducive to the exchange of nutrients and metabolic wastes between cells and media in the system. However, the mechanical stability of gelatin is low and it deforms easily under external action. This can be improved by modifying some groups of gelatin or mixing with other materials (Xu et al., 2021).

3.1.3 Sodium alginate

SA is a readily available natural polymer extracted from natural brown algae and composed of β-D-mannuronic acid (M sugar) and α-L-guluronic acid (G sugar). It is biocompatible and can be crosslinked with divalent cations under mild conditions to form hydrogels. Combining it with other biopolymers can significantly change the properties of the gel and make it suitable for different applications (Piras and Smith, 2020). In tissue-engineered models, it can be used as a substitute for glycosaminoglycan and is therefore widely used to immobilize proteins and cells (Tang et al., 2017). SA has become one of the commonly used scaffold materials for constructing tumor models in vitro.

$CaCl_2$ is usually used as crosslinking agent to construct tumor models in vitro with SA. The concentrations of $CaCl_2$ and SA affect the hardness and mechanical properties of the scaffold. Liu et al. (2018) used collagen and alginate as biomaterials to prepare a hydrogel with adjustable mechanical properties for use in the in vitro construction of a metastatic breast cancer model (Fig. 6a). The hardness, mechanical properties, and pore structure of the scaffold can be adjusted by changing the concentration of Ca^{2+} in the crosslinking agent, but the concentration of SA has no significant effect on the growth or proliferation of cells on the scaffold (Liu et al., 2018). The gelatin/SA composite biomaterial hydrogel was prepared by 3D printing technology and the effects of the SA concentration on the mechanical properties of the scaffold, cell adhesion rate, and tumor sphere size were studied (Jiang et al., 2020) (Fig. 6b). The results showed that the SA concentration affects the printing quality of the scaffold: the higher the SA concentration, the higher its elasticity, and the slower the scaffold collapses. The adaptability of the scaffold to yield stress is enhanced, so the roughness is reduced. However, SA has no obvious effect on the biological performance of the

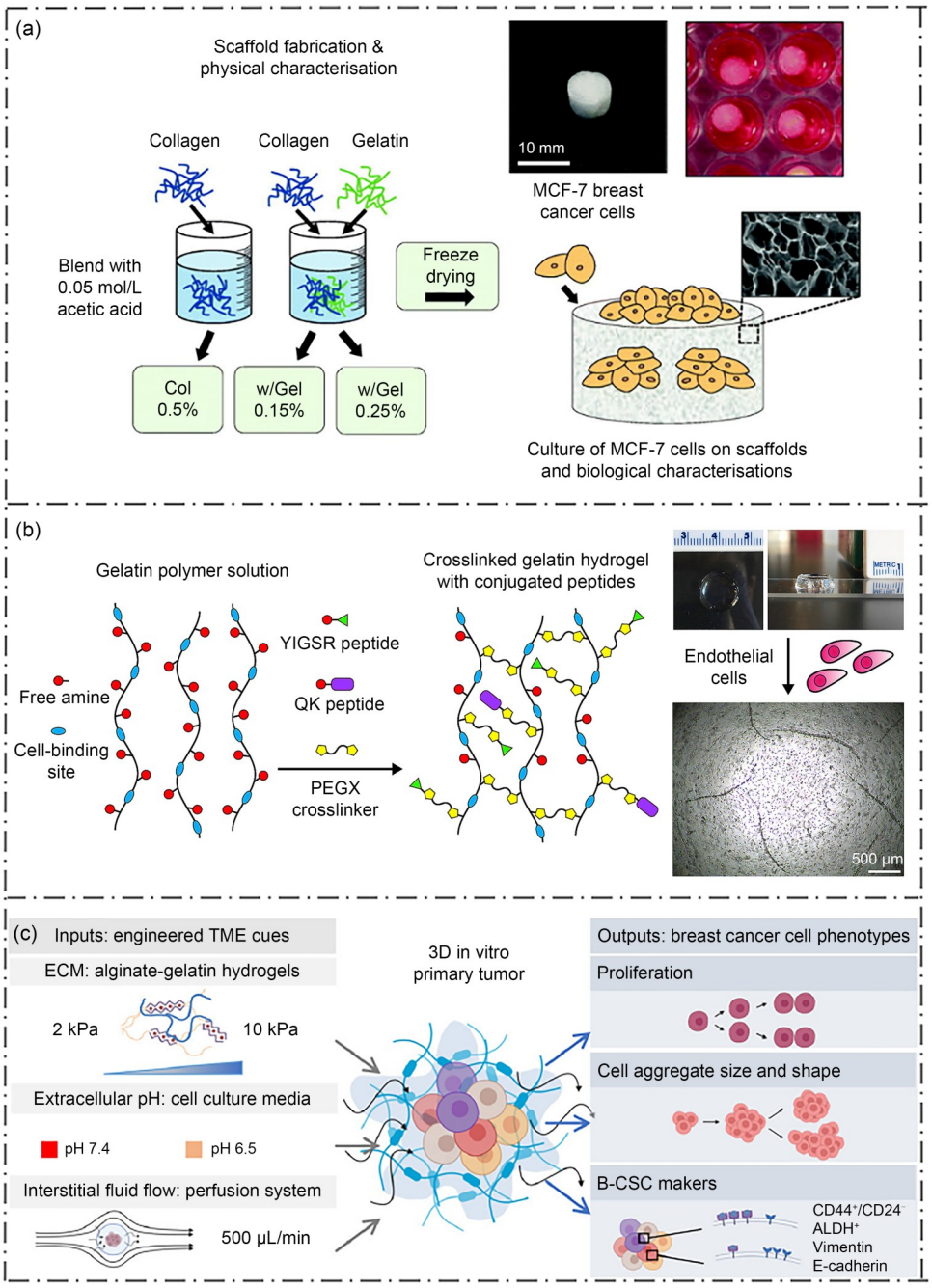

Fig. 5 Gelatin-based 3D in vitro tumor model studies. (a) Hydrogel tumor model with the extracellular matrix (ECM) stiffness of human breast cancer constructed based on gelatin material (Redmond et al., 2022). (b) Preparation of polyethylene glycol-crosslinked gelatin gel and polypeptide coupling. Reprinted from Su et al. (2019), Copyright 2019, with permission from Elsevier. (c) 3D in vitro models with controlled physicochemical properties simulating normal and tumor breast tissues based on gelatin (Shah et al., 2022). Col 0.5%: collagen 0.5% (0.005 g/mL); w/Gel 0.15%: Col 0.5% with the addition of 0.15% (0.0015 g/mL) gelatin; w/Gel 0.25%: Col 0.5% with the addition of 0.25% (0.0025 g/mL) gelatin; YIGSR: Tyr-Ile-Gly-Ser-Arg; PEGX: a poly(ethylene glycol) (PEG) crosslinker; TME: tumour microenvironment; ECM: extracellular matrix; B-CSC: breast cancer stem cell; CD: cluster of differentiation; ALDH: aldehyde dehydrogenase.

scaffold. This shows that SA has good biocompatibility, and increasing the concentration of SA has no effect on cell growth or proliferation.

SA can provide a TME that resembles the body more closely for cell growth. Fig. 6c shows a 3D platform of a shell core structure based on micron alginate,

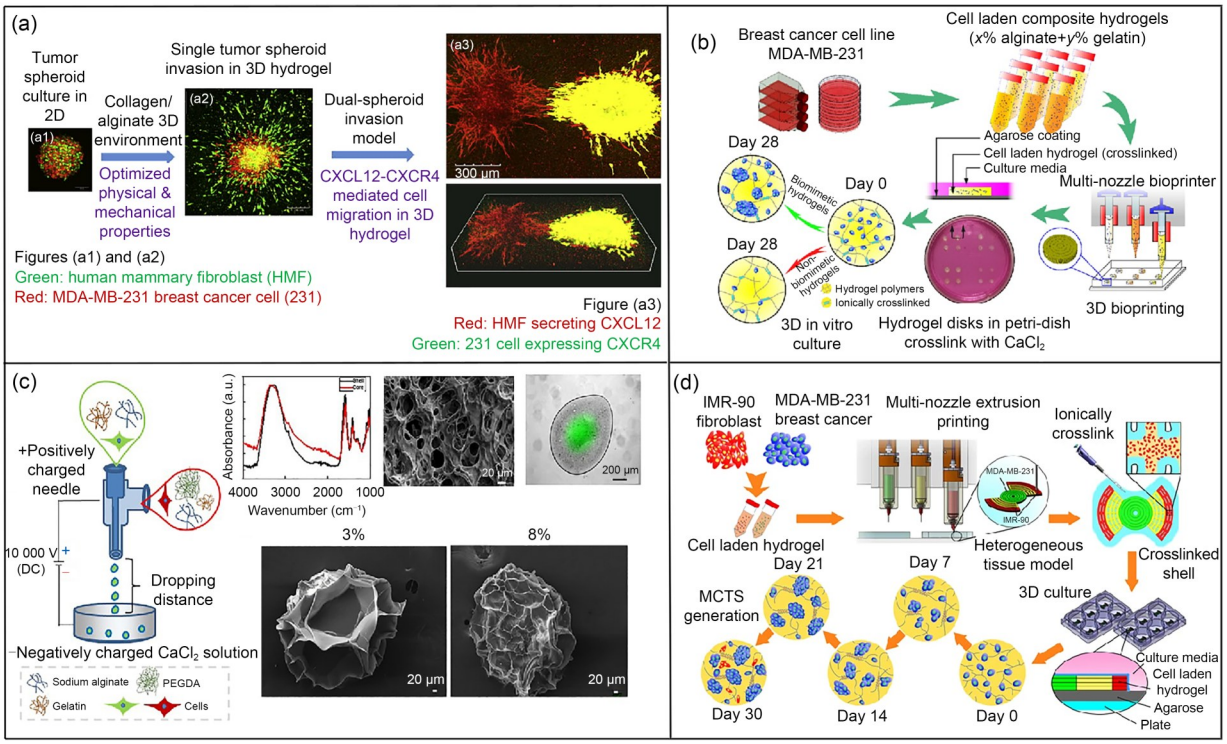

Fig. 6 Sodium alginate (SA)-based 3D in vitro tumor model studies. (a) A hydrogel with adjustable mechanical properties prepared based on collagen and alginate and used for the construction of a metastatic breast cancer model. Reprinted from Liu et al. (2018), Copyright 2018 Acta Materialia Inc., with permission from Elsevier. (b) A gelatin/SA composite biomaterial hydrogel prepared by 3D printing used to study the effects of SA concentration on the mechanical properties of scaffolds, cell adhesion rate, and tumor sphere size. Reprinted from Jiang et al. (2020), Copyright 2019, with permission from IOP Publishing Ltd. (c) A 3D platform of putamen structure based on micron scale alginate simulates cell–cell interactions in the tumor microenvironment in vivo. 3%=0.03 g/mL; 8%=0.08 g/mL. Reprinted from De et al. (2021), Copyright 2021, with permission from Elsevier. (d) A combined heterogeneous tumor model of MDA-MB-231 triple-negative breast cancer cells and IMR-90 fibroblasts based on sodium alginate preparation (Jiang et al., 2017). CXCL12: C-X-C motif chemokine 12; CXCR4: C-X-C chemokine receptor type 4; DC: direct current; PEGDA: poly(ethylene glycol) diacrylate; MCTS: multicellular tumour spheroid; a.u.: arbitrary unit.

which divides different cells by dividing the space in the system to simulate the intercellular interaction in the TME in vivo (De et al., 2021). Compared with the traditional 2D environment, the levels of stem cell markers and proteins in this system increase, indicating that SA provides an environment that more closely resembles tumor tissue for cell growth in vivo.

SA can be combined with other biopolymers to significantly change the properties of gelatin for different purposes. For example, hydrogel scaffolds were prepared based on gelatin/SA material, where SA improved the viscosity of ink during the printing process, and its mechanical properties could be enhanced by ion cross-linking. Co-culture of MDA-MB-231 triple-negative breast cancer cells and IMR-90 fibroblasts embedded in specific locations in the matrix formed multicellular tumor spheroids (Fig. 6d) (Jiang et al., 2017).

Some studies have shown that SA can be degraded by sodium citrate, so after SA-based scaffolds improve cell growth, cells can be recovered with sodium citrate (Castiaux et al., 2019). Nevertheless, due to the lack of interaction with integrins, SA lacks cell adhesion, and cells cannot grow and proliferate on pure SA materials. Therefore, SA is often not used alone in the construction of tissue-engineered models, but modified by adding gelatin, hyaluronic acid, and other materials (Hutmacher, 2010).

3.2 Model construction methods

In recent years, 3D tumor models have received increasing attention, and researchers have used a variety of methods to prepare different types of 3D tumor models in vitro, such as microbeads, tumor spheres, organoids, microfluidics, and 3D bioprinted scaffolds.

3.2.1 Microgel beads

A microgel bead is a kind of biological microsphere with a particle size of 100–3000 μm and is used to coat cells or microorganisms (Jiang et al., 2017). Through this method, cells can be embedded in an environment with soft texture, good stress protection and biocompatibility to achieve the objectives of cell proliferation, induced differentiation, immune isolation, gene delivery, and slow drug release. Therefore, they are widely used in cancer treatment, drug delivery, environmental repair, and the food industry (Luo et al., 2018).

Fig. 7a shows a spherical microbead with uniform size, which is used to wrap different subtypes of breast cancer cells with flow-focusing microfluidic technology. Research showed that although the number of cells and the size of globules increased exponentially, the overall size of the microgel did not change, which indicated that the cells in the micro gel could reorganize the surrounding polymer matrix to adapt to the increase in the number of cells (Lee and Cha, 2020). The effect of the structure and composition of a 3D tumor model on its drug resistance was studied using a double lotion technology to prepare porous microbeads (Brancato et al., 2018). Compared with a tumor ball formed by single culture, the diffusion coefficient of doxorubicin in the co-culture model of fibroblasts and tumor cells was higher and the cell activity was lower (Fig. 7b). This shows that the pores in the microgel bead model are interconnected and cells can diffuse and grow in the scaffold. The mechanical properties of the bead can be adjusted in a large range by changing the concentration of the polymer, and the structure and composition of the model have a great impact on its drug resistance.

In general, the complex microstructure inside the cell microgel beads enables oxygen, nutrients, growth factors, and other substances to diffuse inward and metabolic wastes to diffuse outward, thus providing a good external environment for cell growth and proliferation. Microgel beads have strong mechanical properties and can adjust the distribution of cells in their interior by controlling their volume. The coated cells are not easy to exude.

3.2.2 Three-dimensional bioprinting

Three-dimensional bioprinting is an emerging additive manufacturing technology. The required materials can be obtained through computer-aided design of 3D geometry and layer-by-layer deposition of biological ink materials. It has accurate reproducibility, low cost, and high throughput and efficiency, and so is increasingly being applied in the field of tissue engineering (Mao et al., 2020). Currently, 3D bioprinting can be divided mainly into inkjet bioprinting, extrusion-based bioprinting, laser-assisted bioprinting, and electrospinning according to the type of process (Fig. 8). Table 2 shows an example of tissue engineering using a 3D-printed composite scaffold.

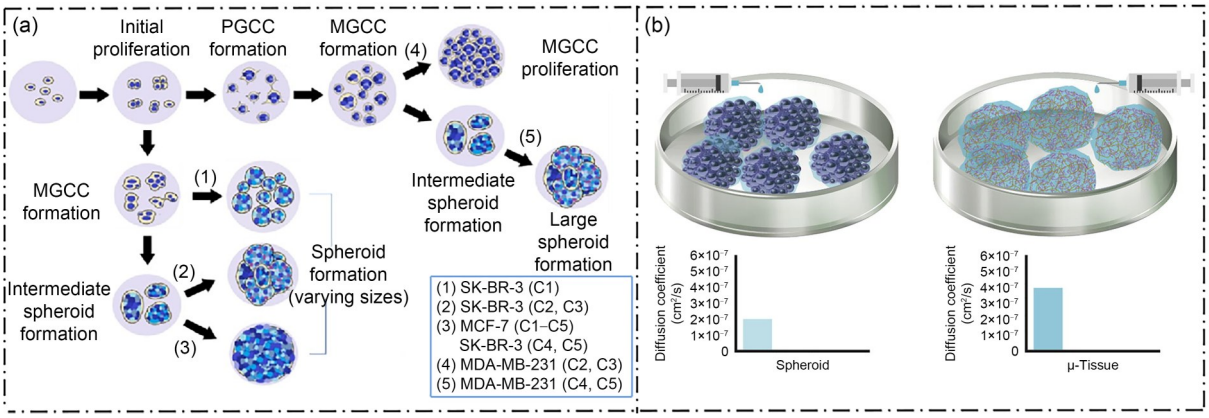

Fig. 7 Research methods and applications of microgel bead tumor models. (a) Spherical microgel beads with uniform size were prepared based on flow-focusing microfluidic technology to encapsulate different subtypes of breast tumor cells. Reprinted from Lee and Cha (2020), Copyright 2020, with permission from Elsevier. (b) Based on gelatin materials, porous microgel beads prepared by double lotion technology were used to study the effects of structure and composition of a 3D cancer model on its drug resistance. Reprinted from Brancato et al. (2018), Copyright 2018 Acta Materialia Inc., with permission from Elsevier. MGCC: multinucleated giant cancer cell; PGCC: polyploid giant cancer cell.

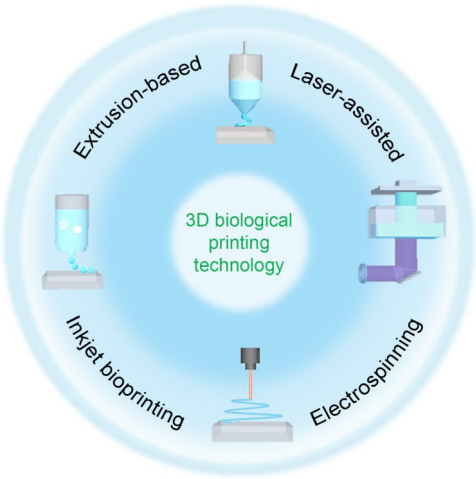

Fig. 8 Three-dimensional (3D) bioprinting divided into four types according to the processes involved, including inkjet bioprinting, extrusion-based bioprinting, laser-assisted bioprinting, and electrospinning.

Inkjet bioprinting was the first bioprinting technology (Tuan et al., 2002) and is very similar to traditional 2D inkjet printing. It has the characteristics of fast printing speed, low cost, and a relatively high cell survival rate, but cannot deposit sticky materials. Laser-assisted bioprinting originated from laser direct writing and laser-induced transmission technology. The precise structure is manufactured by layer-by-layer technology to obtain a high print resolution scaffold. Compared with inkjet printing, laser-assisted printing can avoid direct contact between the dispenser and biological ink, and will not cause mechanical stress on cells, so the cell viability after printing is high (usually more than 95%) (de Pieri et al., 2020). Extrusion-based bioprinting is an improvement of inkjet printing. Bio-ink is extruded from the nozzle (a coaxial or multi-nozzle type) layer by layer through mechanical pressure. It has high flexibility and can print sticky materials that cannot be deposited by an inkjet printer (Murphy and Atala, 2014). Electrospinning bioprinting uses a variety of materials to print microfiber and nanofiber structural supports. The materials are spun in the form of fibers, while the solvent evaporates between the spinneret and the collector. A scaffold printed by this method has the characteristics of a high specific surface area, high porosity, and easy processing, but the range of materials used by this method is limited (Santschi et al., 2019).

To build a highly vascularized tumor model in vitro, researchers have used sacrificial ink to print microchannel scaffolds. For example, petroleum jelly and liquid paraffin are used as sacrificial materials to embed printing in a matrix of bacterial cellulose to form a perfusion microchannel (Cheng et al., 2019). A vascular system was obtained by implanting human umbilical vein endothelial cells (HUVECs) into the microchannel, and MCF-7 cells were seeded onto the surrounding matrix to build a 3D vascularized breast tumor model (Fig. 9a). This sacrificial printing method provides a new strategy for building a simple and low-cost tissue model in vitro.

A 3D printing scaffold can gather cancer cells with multiple cell types in a spatially related manner, which can simulate some characteristics of solid tumor tissue, such as tumor morphology, gradient distribution of chemical and biological factors, and interaction between the tumor and its matrix. Breast cancer cells and stromal cells encapsulated with pig breast tissue bio-ink have been 3D-printed to create artificial tumors (Blanco-Fernandez et al., 2022) (Fig. 9b).

Compared with the traditional hydrogel preparation method, a 3D-bioprinted tissue-engineered model can accurately control the material structure, physical properties, and cell distribution according to requirements, increase the specific surface area, improve the mechanical properties, reduce the dissolution risk, and be more conducive to cell adhesion (Lv KN et al., 2021). In addition, 3D printing can directly coat biological materials such as cells and growth factors in biological ink, eliminating the inoculation step and making the operation easier.

3.2.3 Microfluidic technology

Microfluidic technology is a new technology for precise control and operation of nanometer-level fluids by using microtubules (Whitesides, 2006). The physical principles at the micro-scale differ from those at the macro-scale. For example, at the micro-scale, laminar flow and diffusion are better than convection. As these physical parameters also play a regulatory role in the biochemical processes in the tissue TME, microfluidic technology has received extensive attention in studies of microfluidic technology and cell biology. The advantage of microfluidic technology is that it can realize the precise control and design of the space-time distribution and co-culture of materials, cells, and biomolecules, and can better reduce the invasion of tumors in organisms (Dhiman et al., 2021).

Table 2 Examples of 3D bioprinting composite scaffolds for breast tissue engineering

Fabrication	Biomaterial composition	Cell type	Inoculated cell number	Outcome	Cell inoculation mode	Reference
Laser-assisted bioprinting	GelMA	MDA-MB-231, MCF7, MCF10A	6×10^6 cells/mL	It can independently decouple the embedded regions of different cells in the tumor model and adjust their stiffness	Online printing	Peela et al., 2016
Electrospinning	Gel, SA, curcumin	MCF-7		Provide a new type of implantable therapy	Inoculation	Chen et al., 2021
Inkjet bioprinting	Polylactic acid glycolic acid, Gel, chitosan	MDA-MB-231, NIH3T3, HUVEC	4×10^6 cells/scaffold	Multifunctional smart scaffold is an excellent treatment after breast cancer resection	Inoculation	Shi et al., 2020
	GelMA	MDA-MB-231s	2×10^6 cells/mL	Propose a new 3D tumor array chip drug-screening system with "layer cake" structure	Online printing	Xie et al., 2020
Extrusion-based bioprinting	GelMA, SA, type I collagen, dECM	hAMSC, MCF-7	1.5×10^6 cells/mL	Reproduce the complex composition of breast tumors	Online printing	Blanco-Fernandez et al., 2022
	GelMA, Pt(IV)	4T1	2.5×10^5 cells/scaffold	A novel photopolymerization 3D scaffold with Pt(IV) prodrug initiator	Inoculation	Zhang et al., 2022
	Gel, SA	MDA-MB-231	1.0×10^6 cells/mL	Gel preparation depends on the rate and frequency of self-assembly into multicellular tumor spheres	Online printing	Jiang et al., 2020
	Gel, SA	MDA-MB-231, IMR-90	1×10^6 cells/mL	Reconstruct in vitro physiological simulated tissue model with cell heterogeneity	Online printing	Jiang et al., 2017
	Hydroxyethyl, cellulose, SA, Gel	MCF-7	1×10^7 cells/mL	3D bioprinting technology was first used to study the structure activity relationship	Online printing	Li et al., 2020
	Gel, SA	MCF-7	3×10^6 cells/mL	Quantitative evaluation of the efficacy of anticancer drugs	Online printing	Hong and Song, 2022

Gel: gelatin; GelMA: methacrylated Gel; SA: sodium alginate; dECM: decellularized extracellular matrix; HUVEC: human umbilical vein endothelial cell; hAMSC: human adipose-derived mesenchymal stem cell.

Microfluidic chips can be used to construct tumor models in vitro and test the therapeutic effect of anticancer drugs because of their real-time regulation and dynamic culture. On the microfluidic chip OrganoPlate®, a dynamic tumor chip was constructed by perfusion culture medium and used to test the therapeutic effect of anticancer drugs on triple negative breast cancer. The chip showed stronger drug resistance than 2D culture (Lanz et al., 2017) (Fig. 10a). Using a micro-array column in the microfluidic channel to fix the tumor cells, a

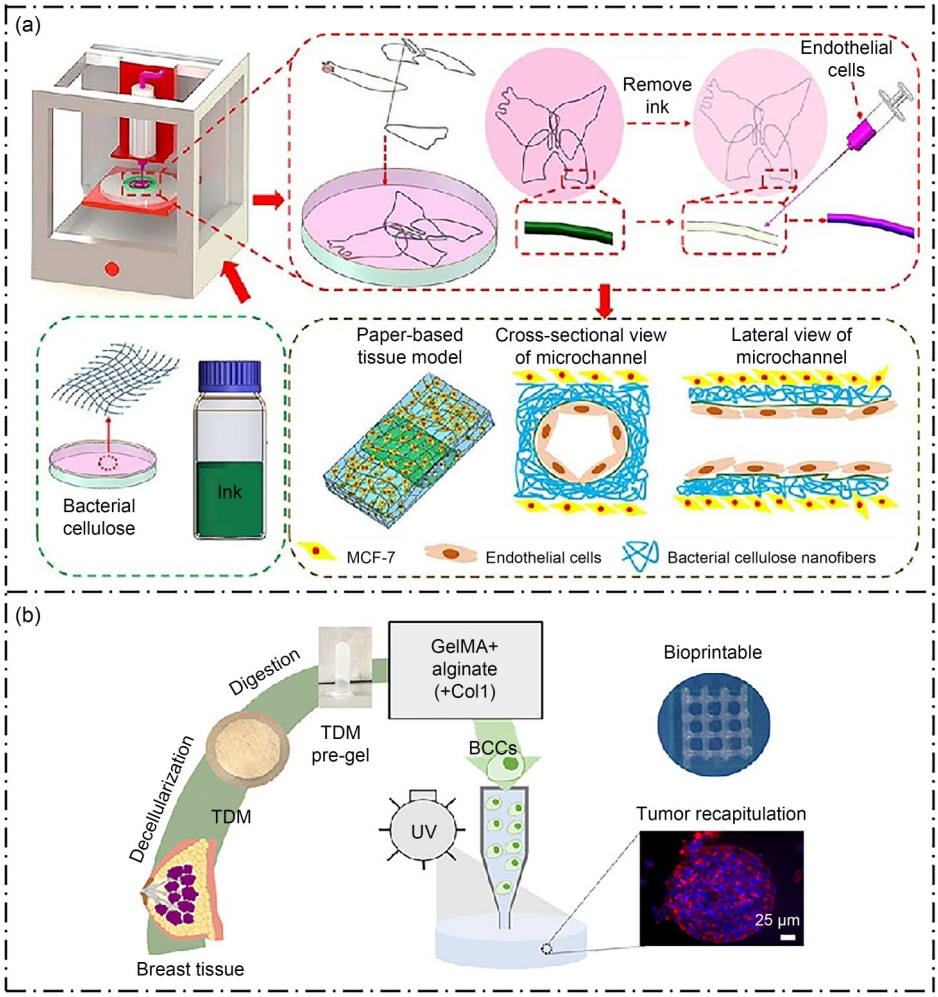

Fig. 9 Research methods and applications of 3D-printed tumor models. (a) Preparation method of a flexible microfluidic paper device based on embedded printing of sacrificial materials. Reprinted with the permission from Cheng et al. (2019), Copyright 2019 American Chemical Society. (b) Artificial tumor production based on 3D printing of pig breast tissue bio-ink (Blanco-Fernandez et al., 2022). TDM: tissues and organ-derived matrix; GelMA: gelatin methacrylamide; Col1: collagen type 1; UV: ultraviolet; BCCs: breast cancer cells.

3D collagen barrier is formed around the 3D cancer cell cluster through the aggregation process of a polyelectrolyte complex to build a microfluidic cancer cell migration model. The model allows real-time observation of the migration and invasion process of metastatic breast cancer cells (MX-1) from the 3D cell aggregates across the collagen barrier (Toh et al., 2018) (Fig. 10b). The real-time monitoring function of the microfluidic model is outstanding. As the growth and movement patterns of tumor cells in the system can be observed in real time, this model will facilitate further research on the processes of cell invasion, different movement patterns, and ECM degradation.

In order to restore biological processes of cells in the TME in vitro, researchers have improved the original microfluidic chip, and its shape is no longer limited to a column. For example, Gioiella et al., (2016) built a breast cancer model with a microfluidic chip. Two compartments were added to the microfluidic chip to accommodate stromal tissue and epithelial tumor tissue, respectively, to achieve regional division and interaction between stroma and tumor tissue, and restore upper skin mesenchymal interaction in the TME (Fig. 10c). Microfluidic chips could also be combined with bioprinting to directly bioprint perfusion and microvascular tumor environment models into customized fluidic chips (Nothdurfter et al., 2022) (Fig. 10d). A platform made in this way is suitable for the future research on tumor angiogenesis and metastasis in precise medical methods.

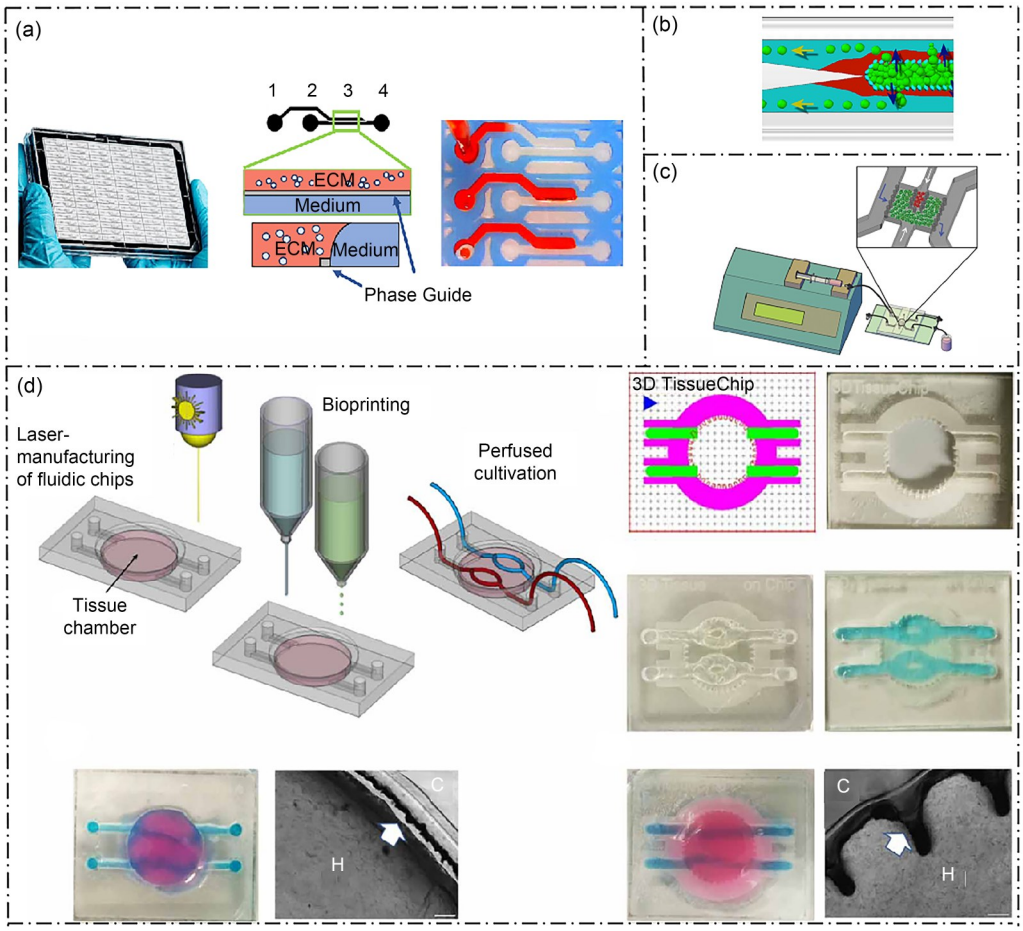

Fig. 10 Research methods and applications of microfluidic tumor models. (a) Dynamic microfluidic tumor chip for 3D breast cancer treatment response testing (Lanz et al., 2017). ECM: extracellular matrix. (b) A microfluidic cancer cell migration model constructed by the coagulation of polyelectrolyte complexes (Toh et al., 2018). (c) Breast cancer model constructed with a microfluidic chip to reduce epithelial–mesenchymal interaction in the tumor microenvironment. Reproduced from Gioiella et al. (2016) by permission of John Wiley & Sons. (d) A tumor model constructed by combining a microfluidic chip with biological printing (Nothdurfter et al., 2022). Arrow: detachment from walls; H: hydrogel; C: chip.

Compared with traditional tumor model construction methods, microfluidic technology can develop dynamic and real-time in vitro models, which more closely represent the TME in vivo. This technology can not only accurately control the flow and distribution of media and cells, but also realize the real-time monitoring and observation of cell activities and medium exchange status in the model by combining with other equipment, which is very helpful for the study of tumor infiltration and migration.

3.2.4 Scaffold-free suspension culture

In addition to tumor models with stroma, scaffold-free multicellular tumor spheres are commonly used in the construction of tumor models in vitro. The spheres have the advantages of a simple structure and easy replication (Hutmacher, 2010), and a large number of tumor spheres with high activity can be produced in a short time. However, due to the lack of ECM, they are unable to reduce the cell–cell and cell–ECM interactions in the TME in vivo, so there are certain limitations in the accuracy of simulation.

Scaffold-less multicellular spheres are widely used because they can be formed in a short time and are easily prepared. Researchers use stirring, aggregation, and other methods to form tumor spheres to build 3D tumor cell models. Chen et al. (2022) proposed a 24-hole 3D hanging spheroid plate (3DHSP), with each hole containing a suspended emitter, sphere hole, and waste hole. In the emitter, a tumor sphere is formed and mixed with chimeric antigen receptor (CAR) T cells. The size of the sphere can be determined optically

without using any fluorescent label (Fig. 11a). A scaffold-free 3D tumor sphere model is established in the stirred culture system that can adjust and control physical and chemical parameters in real time (Santo et al., 2016). Its dynamic culture medium can also better realize the material exchange between cells and media inside the tumor ball. Through this method, a compact, stable, and repeatable tumor ball can be obtained (Fig. 11b).

To alleviate the problem that simple tumor spheres cannot reduce the cell–cell and cell–ECM interactions in the TME in vivo due to the lack of ECM, and given the diversification of formation platforms of tumor spheres, researchers combined hydrogels to form tumor spheres for building tumor models. Liu et al. (2022) developed a programmable multi-functional 3D cancer cell invasion microbuckets-hydrogel (Mb-H) platform by integrating various functional variable

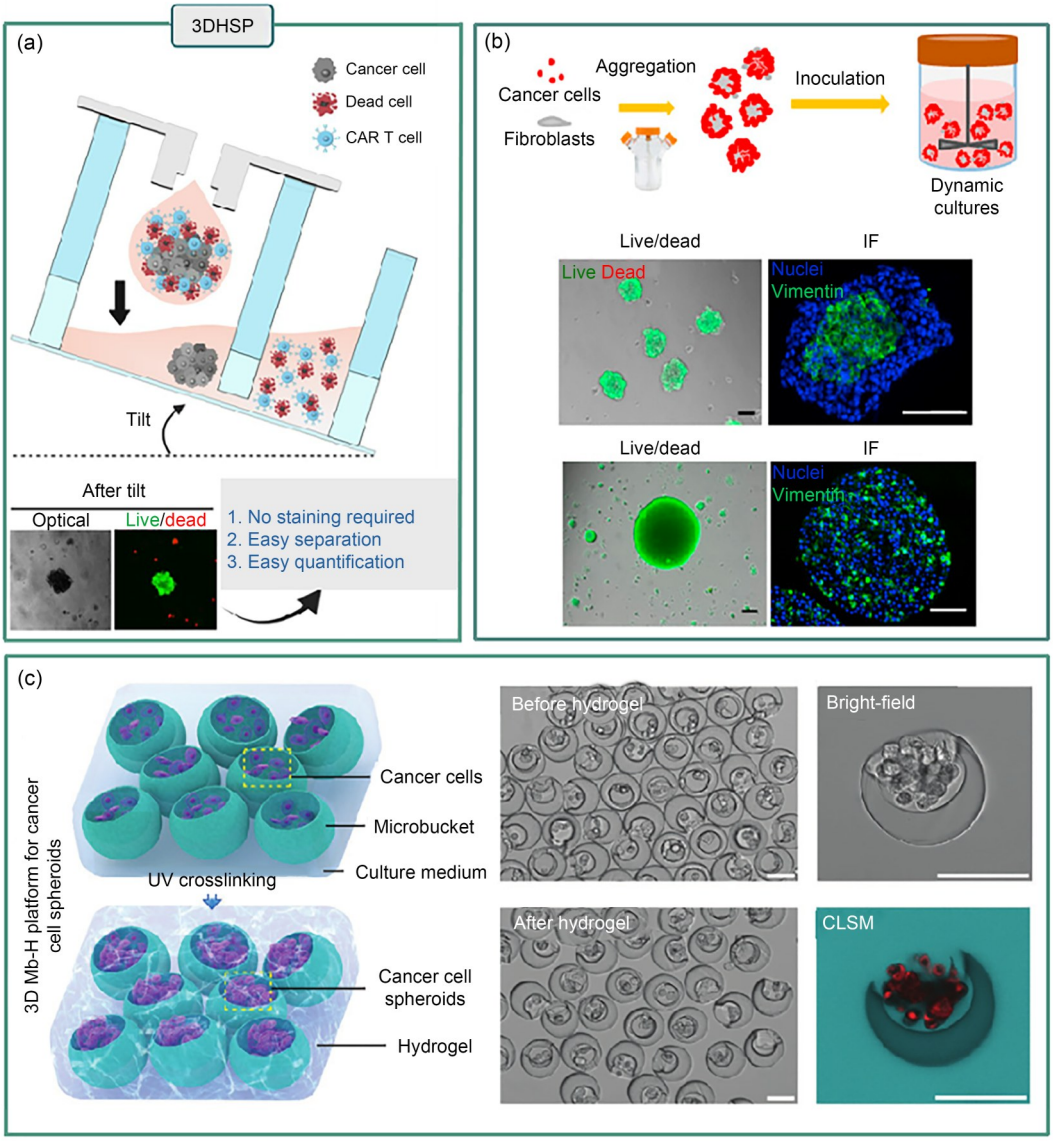

Fig. 11 Research methods and applications of scaffold-free tumor sphere models. (a) Tumor spheres formed based on 24-well plate 3D hanging spheroid plate (3DHSP) can be determined by optics (Chen et al., 2022). (b) Scaffold-free 3D tumor spheres were established in a stirred culture system. Reprinted from Santo et al. (2016), Copyright 2016, with permission from Elsevier. (c) Three-dimensional invasive migration of cancer cell spheroids was studied by integrating various functional variable microbubbles and extracellular matrix-like hydrogels (Liu et al., 2022). Scale bar=100 μm. CAR: chimeric antigen receptor; IF: immunofluorescence; Mb-H: microbuckets-hydrogel; UV: ultraviolet; CLSM: confocal laser scanning microscope.

microbubbles and ECM-like hydrogels. This enables cancer cells to gather to form cancer cell spheres, and shows the relationship between single cell migration and cell collective migration in the epithelial–mesenchymal transformation (EMT) process of cancer cell invasion (Fig. 11c) (Liu et al., 2022).

The main advantage of scaffold-free suspension culture is that it can construct stable, uniform, and reproducible tumor spheres on a large scale, and allows the existence of a dynamic culture environment. However, due to the lack of ECM, this method is not suitable for further tumor-related research.

4 Conclusions and prospects

Drug resistance and distant metastasis are the main causes of death from breast cancer, so it is crucial to study the mechanism and drug responses of metastatic breast cancer. Approved drugs for cancer treatment are still lacking. It takes a long time to develop a new anticancer drug, and the probability of obtaining approval of candidate drugs that eventually enter clinical trials is extremely low. In addition, due to the lack of sufficient preclinical models to test the efficacy and toxicity of new drugs, the failure rate of drugs is very high. The TME includes peripheral immune cells, blood vessels, ECM, lymphocytes, fibroblasts, and signal molecules. The interaction between malignant cells and non-malignant cells produces the TME, which affects the development and progression of cancer. Therefore, it is necessary to reconstruct 3D tissue-engineered tumor models in vitro by combining the mechanical properties and chemical characteristics of the target tissue, as well as the correct transport of nutrients, oxygen, metabolites, and signal molecules.

The 3D tissue-engineered tumor models provide a more realistic and controllable environment for the combination of factors such as specific cells, cytokines, cell–cell and cell–ECM interactions, nutrients, and oxygen concentrations, and are conducive to the simulation of the natural TME of tumor growth, invasion, and metastasis. Therefore, they are of great significance for studying the mechanism of cancer and screening cancer drugs in vitro.

Although great success has been achieved in creating in vitro 3D breast tumor models to improve the prediction of preclinical studies, the existing models still have major challenges and limitations. The current in vitro tumor models cannot replicate the full complexity of human tumor function. First, most of the current 3D in vitro tumor models are relatively simple. Only their morphology is similar to that of in vivo tumors, and they fail to reflect the natural TME. Second, the construction of a tumor vascularization system in vitro still needs to be solved, because these vessels not only affect cancer progression, but also greatly affect drug delivery in tumor tissues. Therefore, standardization and validation tests are still needed to develop a highly heterogeneous, cost-effective, stable, and reproducible representative 3D in vitro tumor model for cancer mechanism research and drug discovery and screening. The development of tumor models will facilitate drug research and further clinical testing of more therapeutic methods, and accelerate the transformation of new cancer therapies into clinical practice.

Data availability statement

Data will be made available on request.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (No. 31670978), the Fok Ying Tung Education Foundation (No. 132027), the State Key Laboratory of Fine Chemicals (No. KF1111), the Fundamental Research Funds for the Central Universities (Nos. DUT22YG213 and DUT22YG116), and the Basic and Applied Basic Research Major Program of Guangdong Province (No. 2020B1515120001), China.

Author contributions

Yan SHU and Bing LI were involved in conceptualization, visualization, writing – original draft, and writing – review & editing. Hailin MA and Jiaqi LIU were involved in visualization and writing – original draft. Yuen Yee CHENG was involved in writing – review & editing. Xiangqin LI, Tianqing LIU, Chuwei YANG, and Xiao MA were involved in supervision and writing – review & editing. Kedong SONG was involved in conceptualization, project administration, supervision, visualization, and writing – review & editing. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Yan SHU, Bing LI, Hailin MA, Jiaqi LIU, Yuen Yee CHENG, Xiangqin LI, Tianqing LIU, Chuwei YANG, Xiao MA, and Kedong SONG declare that they have no conflict of interest.

This review does not contain any studies with human or animal subjects performed by any of the authors.

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