# Three-dimensional models to study breast cancer (Review)

MAIRA HUERTA-REYES[1] and ARTURO AGUILAR-ROJAS[2]

[1]Medical Research Unit in Nephrological Diseases, Mexican Institute of Social Security, National Medical Center Century XXI, Specialty Hospital, Mexico City 06720;
[2]Medical Research Unit in Reproductive Medicine, Mexican Institute of Social Security, Obstetric and Gynecological Hospital, Mexico City 01090, Mexico

Received October 5, 2020; Accepted January 8, 2021

DOI: 10.3892/ijo.2021.5176

**Abstract.** Breast cancer (BC) is the most commonly occurring cancer and primary cause of cancer-related mortality in women worldwide. Investigations into BC have been conducted in *in vitro* and *in vivo* models. Of these models, the cultivation of tumor cell lines in two-dimensional models is the most widely employed *in vitro* model to study tumor physiology. However, this approach does not accurately model all aspects observed in tumors. To address these limitations, three-dimensional (3D) *in vitro* models have been developed. In these, it is possible to reproduce the interaction between tumor cells and the extracellular matrix, as well as the interrelationship between tumor cells and stromal cells, in order to replicate the interactions observed within the 3D environment of *in vivo* tumors. The present review summarizes the most common 3D *in vitro* models used to study BC, including spheroid models, organ-on-a-chip models, hydrogel models and bio-printed models, with a discussion of their particular advantages and limitations.

**Contents**



*Correspondence to:* Dr Arturo Aguilar-Rojas, Medical Research Unit in Reproductive Medicine, Mexican Institute of Social Security, Obstetric and Gynecological Hospital, 289 Río de la Magdalena, Col. Tizapán San Ángel, Álvaro Obregón, Mexico City 01090, Mexico
E-mail: a_aguilar@unam.mx

*Key words:* breast cancer, three-dimensional model, spheroids models, mammosphere, organ-on-a-chip models, hydrogel models, bio-printing models

## 1. Introduction

There has been substantial improvement during previous decades concerning understanding of the genetics and molecular pathways involved in the development of breast cancer (BC). However, BC remains the most commonly occurring cancer and primary cause of cancer-related death in women worldwide (1). For example, data reported by the World Health Organization estimate that there will be >20 million new cases of BC in the world by 2025 (2). The most frequent cause of mortality in cases of BC is due to tumor metastasis and disease recurrence, reflected by a <22% 5-year survival rate in patients with metastasis or recurrence (3).

BC tumors exhibit a high degree of complexity resulting from interactions between various cell types, including proliferating tumor cells and non-cancerous cells in the stroma, such as cancer-associated fibroblasts, endothelial cells, infiltrating inflammatory cells, adipocytes and immune cells (4). Another important component is the extracellular matrix (ECM) (5). In breast tumors, the ECM is primarily comprised of collagens, as well as fibronectin, laminins, glycoproteins, polysaccharides and lipids from adipose tissue (6,7). Of note, the ECM progressively changes during the course of BC, becoming denser and richer in collagen; along with the appearance of new blood vessels, both of these are events are associated with the onset of metastasis (8-10). In the tumor microenvironment, there are frequently progressive changes in the expression of certain receptors in tumor cells, including estrogen receptor (ER), progesterone receptor and/or human epidermal growth factor receptor 2 (HER2), as well as the gene expression patterns of specific genes, some of which are used to perform molecular categorization of breast tumors (11). The sum of all of these properties results in a highly heterogeneous environment that influences tumor proliferation and cellular metastasis. Due the high incidence of BC and its importance as a public health problem, understanding the role of each element in BC tumors comprises a great challenge for research groups to develop new and more effective treatments.

In order to study the progression of BC, *in vitro* and *in vivo* models have been utilized to recapitulate the main aspects of human tumors. The cultivation of tumor cell lines in two-dimensional models (2D) is the most widely employed *in vitro* model used to study tumor physiology (12,13). This technique has the advantage of providing an inexpensive

and standardized high-throughput system. Additionally, characterization of a specific cell response under a specific condition is possible. However, in this model, it is not possible to replicate all aspects observed physiologically in tumors, such as the diversity in cellular population, ECM compounds and interactions in a three-dimensional (3D) environment (14). Therefore, information obtained from these 2D models should be interpreted with caution and extensively confirmed. In order to overcome these limitations, animal models have been used (14). In these, certain deficiencies that arise from 2D culture of cell lines are resolved; however, other points must be considered, such as the low or absent expression of specific biomarkers observed in human BC, that mean that they are cannot fully recapitulate the human disease.

Although the tumor niche is highly complex, its recapitulation in new models may improve understanding of the roles that its components serve in leading to tumor progression and metastasis. *In vitro* 3D models are proposed to address some of these issues, such as the 3D conditions and interactions with ECM, thus developing cellular conditions closer to those observed *in vivo* (15). In addition, under 3D conditions, stromal cells can be added in physiologically relevant proportions to the culture to mimic the cellular composition observed *in vivo* (15). The advantages of these approaches for displaying tumor characteristics have been demonstrated. For example, tumor cells in 3D models exhibit greater resistance to drugs and invasive ability compared with those cultured under 2D conditions (16,17). The present review explores the application of these 3D models to study BC, discussing spheroid models, organ-on-a-chip models, hydrogel models and bio-printed models in particular. To easily consult the applications of each model, a brief summary of them and their advantages and disadvantages is presented in Table I.

## 2. Spheroid models

Spheroids or mammospheres, depending on whether they derive from BC tumor cells or BC cell lines, are micro-scale 3D models characterized by their ability to form spherical aggregates of cells by auto-assembly (radii, 100-600 $\mu$m) (18-20). Spheroids display several features observed in tumors, such as a central necrotic core surrounded by quiescent viable cells and, in the outer layer, a coat of actively proliferating cells (Fig. 1A) (18). In these models, other tumoral features have been observed, such as gradients of pH, oxygen and metabolic compounds (Fig. 1A) (21), and, in some cases, micrometastases (22). Nevertheless, the major inconvenience that these models involve is that they are expensive, and their production is time-consuming.

At present, there are several methods for producing mammospheres in order to allow for cellular growth in suspension or embedded in a 3D matrix, including the hanging drop method (23). In this method, drops of media containing cells are seeded on the lids of culture plate. To increase the thickness of the media, a viscous component is usually added. Then, the cells are cultured in an upside-down position to promote cell aggregation and the formation of spheroids by gravity (21). Although this method is simple and inexpensive, a rigorous standardization process is necessary (24). Using this method, it was demonstrated that, in spheroids developed from MCF-7, BT474 and trastuzumab-resistant BT474 cells, hypoxia regulates resistance to trastuzumab through an increase in BC stem cells and the expression of HER2 (25). This approach has also been adapted for high-throughput screening using 3D printing methodology (26). An alternative method for producing mammospheres involves cell culture in spinner flasks. In this method, a cell suspension is cultured under continuous agitation to prevent adhesion to the flask, allowing cell-cell attachment and the formation of spheroids (19,20). The advantage of this method lies in the production of mammospheres on a large scale, but specialized equipment is required, and the experimental conditions need to be standardized to avoid cell damage, and variation in spheroid size and shape (21). Another approach is the liquid overlay technique (23). In this method, cells are seeded in round-bottomed well plates pre-coated with agarose or polyethylene glycol (PEG) to create a non-adherent substrate. These conditions encourage cellular aggregation, avoiding their attachment to the surface of the plate (21,23,27). Currently, this is one of the most widely used methods to develop mammospheres. Using this approach, the important role of cellular communication during sphere growth, differentiation and collective invasion was demonstrated (28). Additionally, the impact of tumor stiffness on metastasis was recently demonstrated (29); in spheroids from MDA-MB-231 cells seeded in high-stiffness scaffolds, a low invasive capability was observed, but cells with high amounts of actin-enriched protrusions and high levels of Mena protein were also observed. These elevated levels of Mena were in turn associated with remodeling of the fibronectin matrix to promote cell migration (29).

Different stages of BC invasion have been studied using spheroid models. The process of breaching the basement membrane by BC cells and subsequent invasion of the ECM were studied in spheroids from non-tumor MCF10A cells and malignant MCF10A-HRas cells surrounded with basement membrane proteins, seeded within a collagen matrix intended to replicate the ECM (30). Under these conditions, it was shown that the basement membrane prevents the invasion of non-tumor cells; conversely, tumor cells were able to breach this membrane and invade the surrounding matrix. Furthermore, in malignant cells, although invasive abilities were independent of matrix-degrading enzymes, the breaching of the basement membrane was a completely enzyme-dependent process (30). Additionally, the responses to chemotherapeutic agents under 3D conditions have been evaluated. In non-proliferative SUM1315 spheroids and in proliferative MDA-MB-231 spheroids, a less sensitive response was observed to cisplatin, docetaxel and epirubicin compared with 2D cell cultures (31). An important point to be considered during spheroid implementation is that sphere size affects the nutrient distribution in the model core, consequently diminishing cellular growth and response (32). In order to address this, several studies have been conducted. For example, comparison of several procedures to develop spheroids revealed that the liquid overlay technique is an efficient tool to generate uniform mammospheres from the commonly used BC cell lines, MCF-7 and MDA-MB-213 (19); additionally, this methodology permitted the development of co-cultures with human fibroblasts (33). The major disadvantages with this approach concern the implementation of the proper parameters required during cultivation and the acquisition of a sufficient skill level to ensure a regular size and shape in the spheroids (34).

Table I. Application of 3D models to study BC.

| Model type | Description | Advantages | Disadvantages | Applications | (Refs.)[a] |
|---|---|---|---|---|---|
| Spheroid | Micro-scale 3D model that reproduces early tumor stages. They have a spheroidal shape, with a central necrotic core, surrounded by quiescent cells and an outer layer with proliferating cells | Gradients of pH, oxygen, and metabolic compounds, as in *in vivo* conditions | Their production is expensive and time-consuming, and some methods require a rigorous standardization process | Reproduction of tumor features: Spheroids can be generated using single cell lines or via co-culture of several cell lines, with well-regulated communication between cells to enable successful formation, differentiation, and invasion | (19,28,33,40,41) |
| | | | | Chemosensitivity: Spheroids are less sensitive to cisplatin, docetaxel and epirubicin compared with 2D cell cultures, potentially due to an increase in BC stem cell number in 3D conditions | (25,31) |
| | | | | Metastasis and invasion: Spheroids embedded in high-stiffness scaffolds exhibit tumor cells with reduced invasiveness compensated by actin protrusions that remodel the fibronectin matrix and promote cell invasion; additionally, it has been demonstrated that tumor cells are able to breach basement membranes | (29,30) |
| | | | | High-throughput screening: Novel methodologies have been developed to produce organoids of a standardized size to be used in the high-throughput screening of chemotherapeutics and molecular inhibitors | (49,50,58-60) |
| Organ-on-a-chip | Micropatterned surfaces capable of supporting the correct spatial arrangement of cells. Microfluidic applications can mimic the specific stages of tumor cell distribution, as well as the gradients of biomolecules | Due to their small size, smaller sample sizes and reagent volumes are employed | Qualified personnel are required for its design and use | Study of tumor microenvironment: Close communication between BC cells, macrophages, stromal cells and fibroblasts has been investigated, as well as the effects of microenvironmental signals on BC progression | |

Table I. Continued.

| Model type | Description | Advantages | Disadvantages | Applications | (Refs.)[a] |
|---|---|---|---|---|---|
| | | Their manufacturing characteristics enable high reproducibility and large-scale production | PDMS, the material commonly used for their construction, absorbs small hydrophobic molecules, interfering with some drugs-screening studies | Metastasis and invasion: The culture of spheroids in microfluidic devices enables the study of early invasion processes, as well as the important role of cell adhesion molecules on motility and the importance of cell interactions between tumoral, endothelial and immunological cells in invasion; additionally intravasation processes can be studied | (57,63,65,67, 68,70) |
| Hydrogel | Scaffolds that reproduce a simplified tumor microenvironment to replicate specific stages of tumor behavior | Low toxicity and good biocompatibility | Mechanical weakness under some culture conditions | Study of tumor microenvironment: The use of synthetic hydrogels has supported their application in 3D models due their capability to reproduce natural matrixes; those models have been studied to investigate the effects of hypoxic conditions on gene expression and the impact of 3D conditions on cancer stem cell populations | (77,78,94-96) |
| | | Promoting cell attachment and proliferation, and the presentation of tumor cues for cell migration | | Study of interactions between BC cells and stroma: Scaffolds models have been used to study interactions between tumor cells, fibroblasts and osteoblasts, highlighting the participation of soluble factors in promoting tumoral progression | (79-81) |
| | | | | Metastasis and invasion: With this approach, the importance of the ECM for cell migration has been demonstrated | (82) |
| Bio-printing | Bio-printing employs printable hydrogels encapsulating living cells in order to sequentially form a 3D scaffold | Good performance for recreating the complex tumor environment in high-resolution under 3D conditions | Challenges include improvements in cell viability, resolution and print fidelity | Study of the interactions between BC cells and stroma: Successful co-culture of BC cells and mesenchymal stem cells using a bioink comprised of alginate and highly hydrated cellulose has been reported | (119,120) |

Table I. Continued.

| Model type | Description | Advantages | Disadvantages | Applications | (Refs.)[a] |
|---|---|---|---|---|---|
| | | | The type and concentration of the selected bioink can greatly affect the scaffold properties | Implementation of bio-printing to develop organoids: Using bio-printing principles, it has been possible to obtain organoids with a standardized size on a large scale | |

[a]References apply to the applications of models described in each corresponding row. BC, breast cancer; 2D, two-dimensional; 3D, three-dimensional; ECM, extracellular matrix; PDMS, polydimethylsiloxane.

As previously mentioned, the main obstacle in the implementation of spheroid technology lies in the difficulty to make the size and shape of the spheres consistently reproducible. Previous studies showed that small spheroids (diameter, 100-300 $\mu$m) lose oxygen, nutrient and biochemical compound gradients; as a result, they also lose the hypoxic core (35,36). Conversely, large spheroids (diameter, 500-1,000 $\mu$m) frequently exhibits extensive necrotic cores due to the lack of nutrients and stimuli, rendering their biological relevance questionable (33,37). Additionally, the analysis of these large spheroids using conventional microscopy tools is complicated (37). Spheroids of medium size (diameter, 300-500 $\mu$m) exhibit an equilibrium between the biological signatures of tumors, and their study using conventional tools is also viable (38). Therefore, methods have been developed that attempt to generate spheroids of optimal size in a more consistent, efficient and scalable manner. Among these methods, the magnetic levitation method inserts magnetic nanoparticles into the cells for assembling 3D models (39). These models allow the formation of spheroids from single-tumor BC cell lines such as MDA-MB-231, Hs785bst and Hs371.t, or their co-cultivation with human pulmonary fibroblast cell lines such as SUM159 (40) and, cells from patient-derived xenografts (PDXs) (41). In all these models, preservation of tissue architecture and the expression profile of key biomarkers from the original tissue was observed (40,41). A major advantage of this approach that it is important to emphasize is its capability to develop spheroids in a short period of time; however, in terms of challenges concerning its implementation, it is important to mention that specialized instrumentation and particular laboratory skills are required (42).

3D printing is another approach used to develop mammospheres (43), involving the incorporation of living cells within biomaterials via the use of robotic manufacturing to provide control over cell distribution (44). For example, using the laser-direct writing bio-printing approach, spheroids were constructed of MDA-MB-231 cells (45); this approach enabled control of the size, spatial placement and overall geometry of the aggregate. Another example is the use of 96-well magnetic bio-printed plates (41). In this method, PDX-derived cells were coated with a nanoparticle assembly of iron oxide and iron nanoparticles cross-linked with poly-L-lysine; then, the formation of spheroids was conducted via cell seeding under a magnetic field in the 96-well magnetic bio-printed plates, a method that has been successfully employed as preclinical platform for high-throughput drug screening (41). Finally, another methodology to develop mammospheres involves the use of an aqueous two-phase system in which two hydrophilic solutions are mixed to entrap the cells into drops of a more hydrophilic phase. The usefulness of this method to develop spheroids was showed in MDA-MB-157 cells (46). Likewise, to form spheroids, MDA-MB-231 cells were absorbed into drops of aqueous dextran; subsequently, these drops were immersed in PEG and culture medium to ensure free diffusion of nutrients and the removal of waste between both phases. These spheroids displayed a standardized size and the typical features of solid tumors, such as compact morphology, hypoxia and ECM deposition (47,48). In addition, they were also successfully used as models in the high-throughput screening of chemotherapeutics and molecular inhibitors (49,50).

In conclusion, the features observed in organoids, such as a necrotic core surrounded by quiescent cells and a coat of actively proliferating cells, enable study of the early stages of tumor progression. The presence of gradients of pH, oxygen and metabolic compounds, and micrometastases provide the possibility for the high-throughput screening of chemotherapeutics and molecular inhibitors. However, for this, it is necessary to resolve technical challenges, such as the development of approaches to produce organoids on a large scale and consistently. At present, there are several systems to produce these and the application of novel techniques could allow, within a few years, the production of these models on a large scale.

### 3. Organ-on-a-chip models

A combination of microfabrication approaches, such as soft lithography, molding and micromachining techniques, has been biologically adapted to develop microfluidic 3D devices (51,52). These models are constructed primarily with optically clear plastic, glass or flexible polymers such as polydimethylsiloxane (PDMS) (53). As a result of developing micropatterned surfaces, tumor configuration and microenvironmental cues are reproduced in the 3D conditions, supporting the accurate arrangement of cells. The major advantage of these chips over static models is through the manipulation of microfluidic amounts of fluids and living cells via channels with dimensions of hundreds of $\mu$m that mimic tumor cell distributions, as well as the gradients of nutrients and factors (54,55). Despite the great advantages shown by microfluidic chips, there are certain limitations that should be considered in terms of their implementation. First, their standardization requires qualified personnel for their design and use. Second, PDMS, which is the material most commonly utilized for chip construction, is able to absorb small hydrophobic molecules, resulting in interference in certain studies on drug screening (56). Finally, the use of spheroids-on-chips results in difficulties in providing long-term cultures (24).

At present, different aspects of the behavior of BC tumors have been evaluated using organ-on-a-chip models. For example, the early stages of BC tumors have been explored. Using these chips, co-culture of breast tumor spheroids with human mammary ductal epithelial cells and mammary fibroblasts in a compartmentalized 3D microfluidic device was successfully implemented (Fig. 1B) (57). This design consists of upper and lower microchannels separated by a thin ECM-derived membrane (Fig. 1B). On the upper side of the membrane, mammary epithelial cells and spheroids were seeded. On the lower side of the membrane, a stromal layer was added that contained mammary fibroblasts. Both chambers were permanently infused with culture medium (Fig. 1B). This chip was able to recapitulate the general aspects of the mammary duct and of ductal mammary carcinoma, and also allowed the study of anticancer drugs and their effects on proliferation and invasion (57).

Additionally, the impact of the microenvironment has been studied in terms of growth and progression in other models. For example, the co-culture of mammary fibroblasts and T47D human BC cells with different ECM proteins (collagen, fibronectin and laminin) showed that fibroblasts and high amounts of fibronectin stimulate the growth of tumor cells (58). In addition, in a cell panel of BC cells, it was determined that microenvironmental signals promote the ligand-independent activation of ER$\alpha$ (59). Furthermore, by means of a multi-culture device, BC cells, macrophages and stromal cells were co-cultured, and it was determined that cellular interactions altered gene expression in all of the evaluated cells (60). Another important aspect studied using micro-chip models is the epithelial-mesenchymal transition of BC cells. This is a cellular process normally observed during embryogenesis; however, during tumor progression, this process leads to the loss of cell-cell and cell-ECM interactions following the activation of specific genes, promoting a mesenchymal phenotype with high metastatic potential and resistance to therapeutic drugs (61). To investigate this process, a chip with two microfluidic channels separated by a thick matrix layer was employed (Fig. 1C) (62,63). In this device, MDA-MB-231 were seeded into the upper channel. Then, they were attracted toward the matrix layer to the lower channel (Fig. 1C). By using this microfluidic system, it was determined that cells not expressing E-cadherin (MDA-MB-231) invaded mostly as single cells, whilst cells with normal E-cadherin expression (MCF-7) collectively invaded (63).

In addition, migration and invasion processes have also been studied using organ-on-a-chip tools (64). Through the use of a multifluidic platform, a physical interaction was demonstrated between metastatic BC cells and an endothelial monolayer that facilitated tumor invasion through a collagen type I gel (65). In two parallel microfluidic channels, a co-culture was evaluated comprising human umbilical vein endothelial cells (HUVECs) as an endothelial layer and MCF-10A, MCF-7 or MDA-MB-231 cells as epithelial cells (66). In this system, a higher migration profile was observed for MDA-MB-231 cells compared with MCF-10A and MCF-7 cells, with a lack of movement from HUVEC cells. The system also was proposed as a tool to study the interaction between endothelial and epithelial layers and their effects during cell migration and invasion. Additionally, the interaction between tumor cells and immunological cells was evaluated using a microfluidic 3D migration assay (67), which reported that macrophages promoted an increase in the speed and persistence of cancer cell migration.

The establishment of models to study the intravasation of BC cells into blood capillaries has been challenging. To address this, microfluidic approaches have been used. For example, in a microfluidic system consisting of concentric layers containing BC cells and vascular networks formed by HUVECs, it was demonstrated that only the highly metastatic MDA-MB-231 cell line, and not the less invasive MCF-7 cell line, was able to either enhance invasion or intravasation, or to increase vascular permeability (Fig. 1D) (68). Similar results were observed in the co-culture of primary human BC cells and the vascular network; the permeability of the vessels was significantly increased in response to tumor cells or tumor cell-conditioned medium (69). Furthermore, these models have been utilized to study BC cell responses against anticancer therapies (70).

In brief, the greatest advantage of these models is their small size, allowing the use of smaller sample sizes and reagent volumes. As a result, they have high reproducibility and capacity for large-scale production. Additionally, their

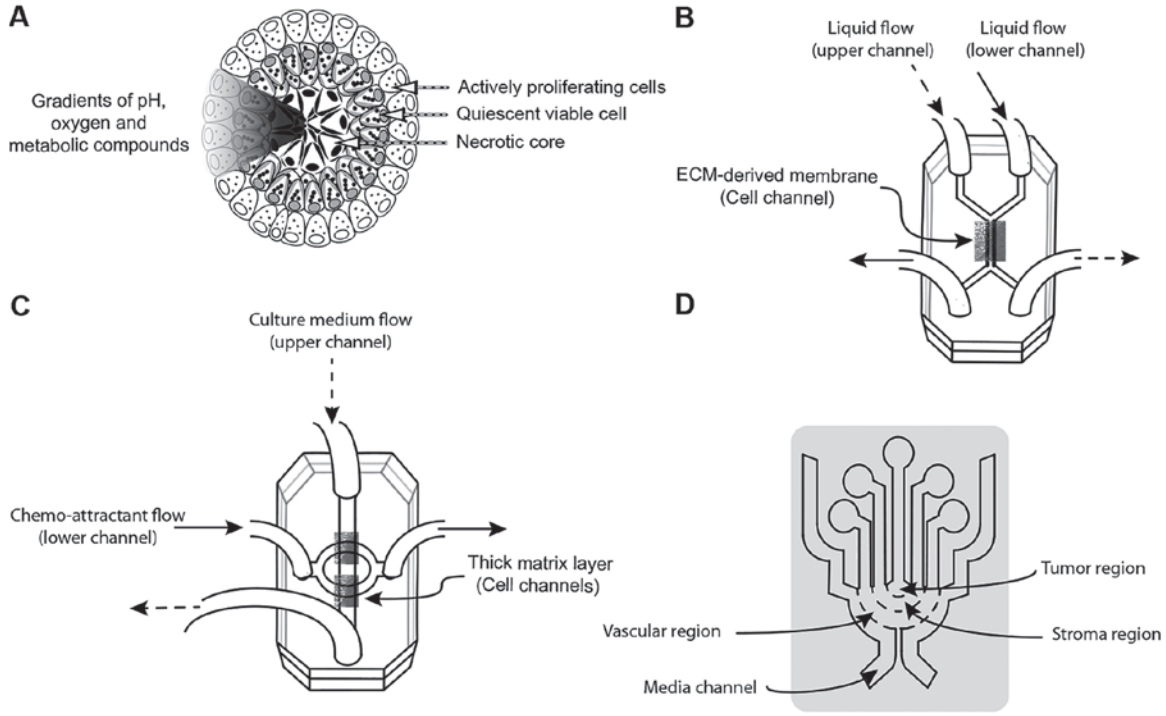


Figure 1. Schematic representation of 3D models employed to study BC. (A) Schematic of spheroid models, spherical aggregates of cells with tumor features, such as a central necrotic core surrounded by quiescent viable cells and a coat of actively proliferating cells. Additionally, as in tumors, gradients of pH, oxygen and metabolic compounds are observed. These models are used to study early-stage breast cancer. (B) Schematic of an organ-on-a-chip model: A 3D compartmentalized microfluidic device with an upper channel and a lower channel, separated by an ECM-derived membrane. Above this membrane, mammary epithelial cells and spheroids are cultivated. A stromal layer and mammary fibroblasts are added in the lower channel. This chip is able to recapitulate the general aspects of mammary ducts and ductal carcinoma. (C) 3D microfluidic device compartmentalized into two microfluidic channels separated by two thick matrix layers. In the upper channel, BC cells such as MDA-MB-231 or MCF-7 cells are seeded. In the lower channel, chemo-attractants are added. This model is employed to study epithelial-mesenchymal transition. (D) Microfluidic system consisting of concentric layers containing tumor cells, a stromal region and vascular cells, used to study the intravasation of BC cells into the blood capillaries. Liquid flow into the upper channel is represented by dashed arrowheads. Liquid flow into the lower channel is represented by solid arrowheads. BC, breast cancer; ECM, extracellular matrix; 3D, three-dimensional.

design permits the study of specific stages of BC development, employing single cells or the co-culture of cell lines under controlled conditions. By the use of these models, the study of BC cell interactions with vascular networks, endothelial and epithelial cells has been possible, along with the study of ECM components during tumor metastasis, invasion or angiogenesis.

## 4. Hydrogel models

Hydrogel models are scaffolds that reproduce a simplified tumor microenvironment to understand tumor behavior (71). These platforms are fabricated with a naturally derived hydrogel, such as collagen (72,73), fibrin (74) or reconstituted basement membrane (Matrigel) (75,76). The advantages of hydrogel models include low toxicity, biocompatibility, the promotion of cell attachment and proliferation, and the display of tumor cues for cell migration (71). However, as in all of the models, they demonstrate limitations, such as mechanical weakness under certain culture conditions.

The biological relevance of 3D-based scaffold models made with natural hydrogels has been investigated. Comparisons between 2D and 3D conditions reveal that, although ERα protein was expressed in a 3D collagen-scaffold model maintained in hypoxia, these molecules were downregulated in 2D models under the same hypoxic conditions (77). Additionally, scaffolds comprised of polycaprolactone (PCL), a biocompatible polymer, exhibit an increase in cancer stem cell populations under 3D conditions (78). These data demonstrate the effects of culture environment on cellular response and indicate that 3D models mimic *in vivo* conditions. Naturally-derived hydrogels have been employed to evaluate novel behaviors of BC cells. For example, in order to study the complex interactions between BC cells and stroma, human fibroblasts were seeded on a Petri dish pre-coated with collagen. Above this layer, a mix of Matrigel + MDA-MB-231 cells was added (Fig. 2A) (79). This collagen-Matrigel scaffold demonstrated that MDA-MB-231 cells and human fibroblasts interact by the release of soluble factors secreted by tumor cells (79), which promotes alterations in fibroblast shape, motility and gene expression. In a reciprocal manner, fibroblasts release soluble factors that accelerate BC cell aggregation (Fig. 2A) (79). These data emphasize the important role of the stroma on tumor growth.

Another study evaluated the interaction between MC3T3-E1 pre-osteoblasts and MDA-MB-231 tumor cells in a dense 3D collagenous environment (cellularized scaffold from rat tail collagen) (80). Under these co-culture conditions, BC cells promote MC3T3-E1 differentiation into osteoblasts, reducing the mineralization of the osteoblast-mediated media, which is hypothesized to result in promoted bone resorption. Similarly, in a 3D scaffold model developed by a collagen-glycosaminoglycan combination, it was demonstrated

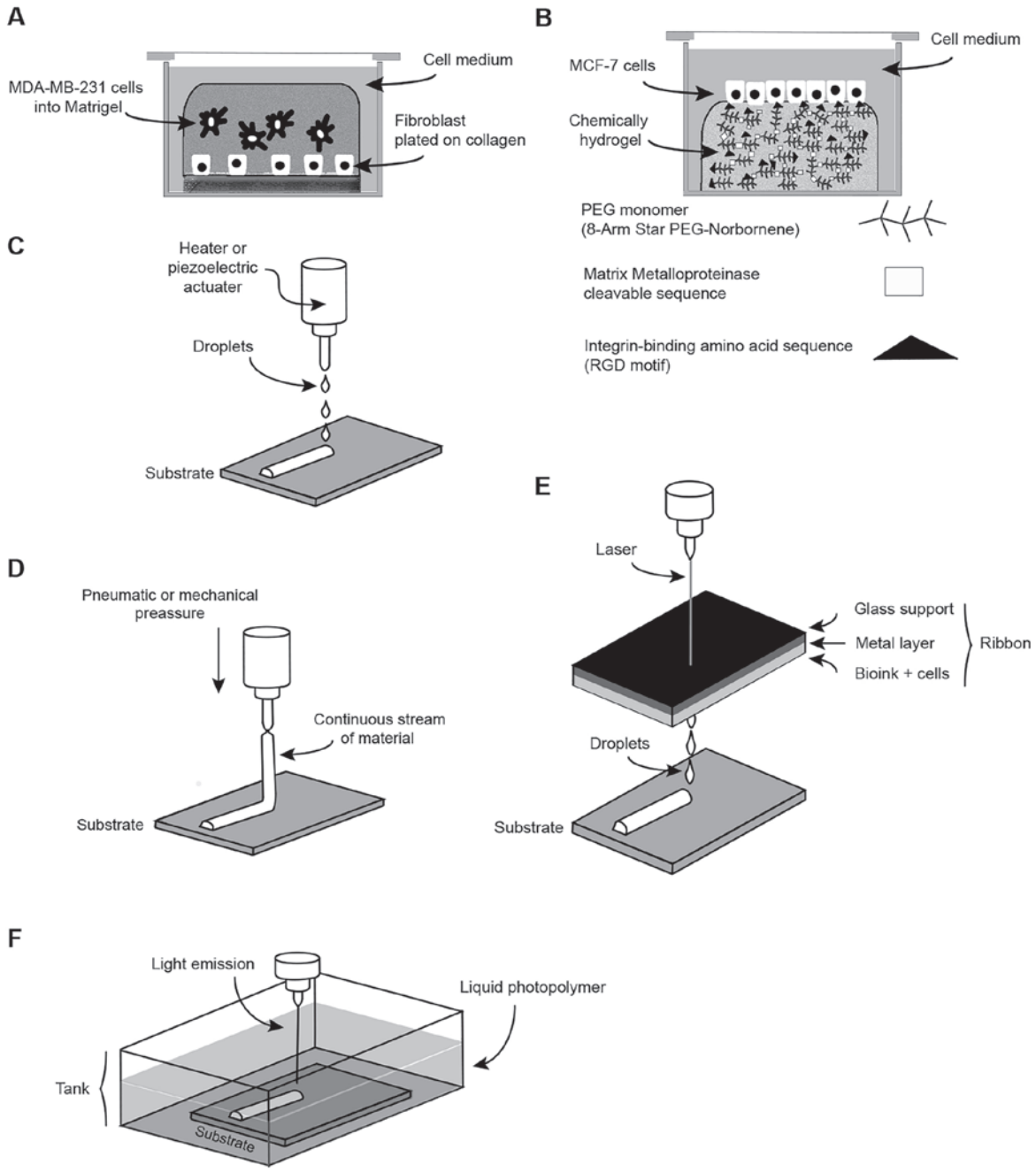


Figure 2. Schematic representation of additional 3D models employed to study BC. (A) 3D-based scaffold fabricated with naturally derived hydrogel (collagen + Matrigel). Human fibroblasts are seeded above the collagen layer; above these, a mix of Matrigel and BC cells are added. The interaction between tumor cells and fibroblasts has been studied using this system. (B) 3D-based scaffold fabricated with a synthetic hydrogel. In this case, the matrix contains PEG monomers, linked with matrix metalloproteinase-cleavable sequences. To improve cell adhesion, proliferation and migration, the integrin-binding amino acid sequence is added. This model has been utilized to demonstrate the biological relevance of the synthetic hydrogel. (C) Inkjet bio-printing method promotes the formation of droplets of bioink via thermal or piezoelectric processes. The resulting microbubbles are deposited onto a substrate to print the desired pattern. (D) Micro-extrusion bio-printing is a pressure-driven technology to produce a continuous stream of material directly deposited onto the substrate. (E) Laser-assisted bio-printing is a method that uses laser energy to vaporize a thin layer of metal and eject the biomaterial in droplets. (F) Stereolithographic bio-printing involves the photopolymerization of liquid photopolymers via irradiation with ultraviolet, infrared or visible light. BC, breast cancer; 3D, three-dimensional; PEG, polyethylene glycol.

that the murine mammary adenocarcinoma 4T1 cell line was able to mineralize under appropriate conditions (80). These findings support the hypothesis that BC cells possess the ability to osteomimitize in order to promote metastasis into the bone microenvironment (81) and alter the bone microenvironment for survival. Additionally, the importance of collagen fibers, independent of matrix stiffness during tumor cell invasion, was revealed using collagen scaffolds and MDA-MB-231 cells (82). Although this observation was previously postulated following observation of human samples (83,84), it was verified in a 3D model. Furthermore, the importance of substrate fibers in promoting cell migration was shown. This was studied in 3D scaffolds comprising Matrigel, collagen type I and porcine ECM-derived tissue matrix gel (TMG). Comparison of these

hydrogels revealed that in collagen and TMG matrix, in which there were high amounts of fibers, MDA-MB-231 cells exhibited more cellular protrusions, which could be associated with their invasive properties (82).

As mentioned above, the use of natural hydrogels has certain limitations. To counteract this, chemical hydrogels have been developed (85). Among them, the most used in tumor research are PEG, poly(lactic acid), poly(glycolic acid) and poly(lactic-co-glycolic acid) (PLGA) (86). One important characteristic displayed by these models is their ability to reproduce 3D tumor environment properties, such as adhesiveness, degradable components and the diffusion of chemoattractant molecules. Thus, they can be designed as modular settings containing peptide sequences or protein domains in the hydrogel backbone; for example, the PEG monomer, (8-arm star PEG-norbornene), incorporates a cross-linker sequence and cell-adhesion domains (Fig. 2B) (87). Adhesion domains utilize biological molecules such as the integrin-binding amino acid sequence (RGD motif), which more effectively reproduce tumor aspects such as cellular proliferation, adhesion and migration (Fig. 2B) (88,89). Other cell adhesion molecules that are utilized include fibronectin/vitronectin (RGDS motif), collagen (GFOGER motif) and laminin (IKVAV motif) combined with PEG to develop scaffolds with different matrix densities (90). The successful proliferation of the MDA-MB-231 and T47D cell lines was observed using these molecules; the cell lines responded distinctly according to the synthetic matrix employed (90). Finally, cross-linker sequences such as the matrix metalloproteinase-cleavable sequences (KCGGPQGIAGQGCK-NH2) can be added to permit cell invasion (Fig. 2B) (87). Recently, the biological relevance of chemical hydrogels such as PEG in terms of reproducing the effects of natural hydrogels such as collagen and Matrigel was evaluated (87). It was shown using the MCF-7 cell line that PEG hydrogels exhibit similar performance compared with collagen and Matrigel scaffolds, rendering them suitable for use in cell culture experiments (87). Additionally, MDA-MB-231 and MCF-7 cells recently exhibited their ability to grow on both natural hydrogels, such as a 1% alginate scaffold, and synthetic matrices, such as a thiol-modified hyaluronan (HA-SH cross-linked with PEGDA) device (91).

Additionally, 3D porous scaffolds from synthetic polymers have demonstrated their utility for mimicking tumor conditions (92,93). For example, in a 3D porous PCL scaffold model, it was demonstrated that MDA-MB-231 cells exhibit enhanced proliferation and a significant increase in the expression of genes associated with BC metastasis, tissue remodeling and cancer inflammation, indicating the recreation of *in vivo* conditions in the 3D model (94). Similarly, in 3D scaffolds made from PCL, it has been observed that the 3D microenvironment promotes the cell dormancy of chemoresistant BC cells compared with cells cultured under 2D conditions (95). Finally, it was reported that MDA-MB-231 cells seeded in PLGA and PCL porous scaffolds exhibited increased expression of ECM receptors and reduced sensitivity to 4-hydroxytamoxifen treatment compared with cells cultured under 2D conditions (96).

In conclusion, these hydrogel models have opened the possibility to study the cellular response of single tumor cells and stroma cells to specific environments. Additionally, they have been used to evaluate how this cellular communication promotes tumor growth, invasion, metastasis and changes in the microenvironment in order for them to colonize and survive in new tissues.

## 5. Bio-printing models

In bio-printing models, 3D bio-printing technologies are used to create complex structures (97). In them, 2D layers of biomaterials or bioinks, which are printable hydrogels with living cell encapsulation, are sequentially printed to form a 3D scaffold (98). Currently, the most common methods of bio-printing employed in Biology include inkjet printing (99,100), micro-extrusion printing (101), laser-assisted printing (102) and stereolithographic printing (103). For all of these, their principal advantage is their ability to reproduce high-resolution 3D structures that recreate the complex tumor environment. However, at present, there are specific challenges that should be resolved for each method, such as cell viability, resolution and print fidelity; in addition, the bioink selected and its concentration are also important points that impact the printing characteristics. The specific properties, advantages and disadvantages of each method are covered below.

In the inkjet printing method, the biomaterial that contains the cells is vaporized into microbubbles via a thermal process (a heated element is used to form droplets) or a piezoelectric process (acoustic waves are used to eject droplets), and they are deposited to print the desired pattern (Fig. 2C) (97,104,105). This method has several advantages, such as high resolution (~50 $\mu$m), the capacity to replicate complex biological structures and high cell viability (97). Its principal disadvantage is its inability to print large-scale scaffolds; in addition, the use of viscous bioinks represents a challenge (106).

Micro-extrusion printing is a pressure-driven technology that is connected with nozzles or needles to cartridges loaded with bioink (Fig. 2D). In this method, the biomaterial is driven by pneumatic or mechanical pressure to produce a continuous stream of material that is directly deposited onto the substrate (Fig. 2D) (107). Some advantages include the ability of this method to print high cell densities in viscous biomaterials and the possibility of using multiple cartridges to print heterogenous structures with several types of cells (101,107). Notable drawbacks include a reduced resolution in comparison with inkjet printing (~100 $\mu$m) (97), the possible distortion of cell structures and the loss of cellular viability due to the force used to expel the biomaterial (107,108).

In the laser-assisted approach, laser energy is used to vaporize a thin layer of metal and eject the biomaterial in droplets. In brief, the procedure consists of three steps. In the first of these, a laser source is focused on a laser-absorbing support called the ribbon. In this ribbon, there are three layers: A transparent glass support, a thin layer of metal and the layer of bioink containing cells (Fig. 2E) (97). In the second step, the metal layer is vaporized by laser pulses to release bioink droplets. In the third step, the free droplets containing the cells are printed on the receiving slide (Fig. 2E) (106). The major advantages shown for this methodology are a high cellular viability (97) and a high resolution for printing 3D models (10-50 $\mu$m) (109). However, there are some limitations to consider for its implementation. For example, during the fabrication process, rigorous standardization is necessary to

minimize the possibility of over-drying of the sample by the laser power, and to adjust the distance between the ribbon and receiving slide (102). Another important point to bear in mind is the high cost of the required equipment (104).

Stereolithographic printing is the oldest technique of bio-printing that exists (110). In this approach, a liquid photopolymer or photoinitiator is irradiated with ultraviolet (UV), infrared or visible light to promote its photopolymerization (Fig. 2F). The 3D scaffold is formed by stacking all of the solidified layers via a layer-by layer process (layer heights ranging from 25-200 $\mu$m) (Fig. 2F) (106,111) The resolution for this method ranges from 5-300 $\mu$m and is dictated by the light source (112). In terms of advantages, the limitations observed using other printing methods resulting from the bioink viscosity are avoided. Additionally, the fabrication speed of 3D scaffolds using this method is fast, which can provide high-resolution molds, and eliminate the distortion of cell structures and the loss of cellular viability observed using micro-extrusion printing. However, there are some disadvantages, such as the difficulty in fabricating using multimaterial structures (112). Another point to consider is the illumination source; when the modeling of the 3D structures employs UV or laser light, it can affect the cells and introduce mutations in them. To eliminate the harmful effects of this radiation, it is possible to employ this method using visible light, allowing the manufacturing of 3D scaffolds containing cells throughout the polymer resin (113). Also, the selection of the concentration of the photoinitiator is a critical point, as the amount of this element defines the stiffness and matrix density of the scaffold; however, a high concentration may induce cytotoxic effects (97).

Biomaterials or bioinks employed in bio-printing are hydrogels that protect the cells during the printing process and also reproduce the ECM environment to support cellular functions such as cell viability, proliferation and morphology. The type of bioink selected will exert an important effect on the 3D scaffold structure, and certain properties, such as viscosity, gelation and cross-linking capabilities, must be considered prior to its selection (97). In general, two types of biomaterials are utilized to produce 3D platforms: Natural polymers and synthetic polymers (110). Among the natural substrates used are alginate (114), collagen (115), fibrin (116), gelatin and hyaluronic acid (117). Some examples of synthetic polymers include PCL, PEG, PLGA and Poloxamer 407 (110). Of note, natural polymers are the most frequently used bioink due to their biological compatibility (118).

The implementation of micro-extrusion printing has allowed for the successful co-culture of mesenchymal stem cells and MDA-MB-231 cells in bioinks comprised of alginate and highly hydrated cellulose in order to explore the communication between these cells (119). Additionally, through the implementation of bio-printing, organoids from MCF-7 and MDA-MB-468 tumor cells, as well as MCF-12A non-tumor cells, have been generated using a novel human-derived breast hydrogel (120).

These systems also permit the study of microenvironmental effects on tumor progression; however, to our knowledge, there are few works at present that employ this method to study BC. Nevertheless, it is predicted that their implementation in upcoming years will provide new data concerning the progression of BC, facilitating the development of new and more effective therapies.

## 6. Conclusions

Present understanding of the development of BC derives from studies conducted in patients and animal models, or from the culture of cell lines under 2D conditions. Although the information obtained from these studies has been very important, each method and model exhibits limitations. To generate solutions for these challenges, novel 3D models have been developed. In these, various aspects observed under *in vivo* conditions have been recapitulated, such as interactions between tumor cells and the stroma, as well as the presence of the ECM and 3D environment. These particularities have rendered possible the study of novel aspects of BC progression under standardized conditions. However, their regular implementation requires the resolution of some important points. For example, in spheroid models, the lack of vascularization limits their use as a model of the genesis of tumors, although not the later stages of tumor development. Concerning organ-on-a-chip and hydrogel models, these have a well-organized spatial distribution of tumor cells and ECM components, but their implementation reproduces only one specific point in tumor progression. Finally, the implementation of bio-printing methods depends upon resolving several technical challenges, such as improvements in bioink materials and cell-seeding conditions.

Advances concerning present knowledge of BC progression require the use of 3D models and improved laboratory skills. Additionally, it is necessary to improve these models to allow their regular use, as well as to improve their ability to reflect the complexity of the tumor microenvironment and increase knowledge of cancer biology. To resolve these limitations, interdisciplinary work between various areas of science will be necessary. These improvements without doubt will be an important step for the development of more efficient therapeutic strategies. Based on current understanding of 3D models, it is predicted that in the near future, it will be possible, for example, to combine two or more conventional 3D tumor models, such as organoids contained in microfluidic devices or bio-printed scaffolds, raising the possibility of even more complex models that will be able to recapitulate complex cell-ECM interactions and tumor compartmentalization and further understanding BC.

## Acknowledgments

Not applicable.

## Funding

This study was supported by the Health Research Fund of the Mexican Institute of Social Security (IMSS; grant nos. FIS/IMSS/PROT/EMER18/1848 and FIS/IMSS/PROT/G15/1472). Funding was also received courtesy of Research Career Development Awards from the IMSS Foundation.

## Availability of data and materials

Not applicable.

### Authors' contributions

MHR and AAR conceived and drafted the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

### References


1. Ahmad A: Breast cancer statistics: Recent trends. Adv Exp Med Biol 1152: 1-7, 2019.
2. Rivera-Franco MM and Leon-Rodriguez E: Delays in breast cancer detection and treatment in developing countries. Breast Cancer (Auckl) 12: 1178223417752677, 2018.
3. Global Burden of Disease Cancer Collaboration; Fitzmaurice C, Dicker D, Pain A, Hamavid H, Moradi-Lakeh M, MacIntyre MF, Allen C, Hansen G, Woodbrook R, *et al*: The global burden of cancer 2013. JAMA Oncol 1: 505-527, 2015.
4. Hanahan D and Coussens LM: Accessories to the crime: Functions of cells recruited to the tumor microenvironment. Cancer Cell 21: 309-322, 2012.
5. Mittal S, Brown NJ and Holen I: The breast tumor microenvironment: Role in cancer development, progression and response to therapy. Expert Rev Mol Diagn 18: 227-243, 2018.
6. Conklin MW and Keely PJ: Why the stroma matters in breast cancer: Insights into breast cancer patient outcomes through the examination of stromal biomarkers. Cell Adh Migr 6: 249-260, 2012.
7. Insua-Rodríguez J and Oskarsson T: The extracellular matrix in breast cancer. Adv Drug Deliv Rev 97: 41-55, 2016.
8. Friedl P and Alexander S: Cancer invasion and the microenvironment: Plasticity and reciprocity. Cell 147: 992-1009, 2011.
9. Kaushik S, Pickup MW and Weaver VM: From transformation to metastasis: Deconstructing the extracellular matrix in breast cancer. Cancer Metastasis Rev 35: 655-667, 2016.
10. Natal RA, Paiva GR, Pelegati VB, Marenco L, Alvarenga CA, Vargas RF, Derchain SF, Sarian LO, Franchet C, Cesar CL, *et al*: Exploring collagen parameters in pure special types of invasive breast cancer. Sci Rep 9: 7715, 2019.
11. Vuong D, Simpson PT, Green B, Cummings MC and Lakhani SR: Molecular classification of breast cancer. Virchows Arch 465: 1-14, 2014.
12. Comşa Ş, Cimpean AM and Raica M: The story of MCF-7 breast cancer cell line: 40 years of experience in research. Anticancer Res 35: 3147-3154, 2015.
13. Ravi M, Sneka MK and Joshipura A: The culture conditions and outputs from breast cancer cell line in vitro experiments. Exp Cell Res 383: 111548, 2019.
14. Kim JB, O'Hare MJ and Stein R: Models of breast cancer: Is merging human and animal models the future? Breast Cancer Res 6: 22-30, 2004.
15. Bersini S, Jeon JS, Dubini G, Arrigoni C, Chung S, Charest JL, Moretti M and Kamm RD: A microfluidic 3D in vitro model for specificity of breast cancer metastasis to bone. Biomaterials 35: 2454-2461, 2014.
16. dit Faute MA, Laurent L, Ploton D, Poupon MF, Jardillier JC and Bobichon H: Distinctive alterations of invasiveness, drug resistance and cell-cell organization in 3D-cultures of MCF-7, a human breast cancer cell line, and its multidrug resistant variant. Clin Exp Metastasis 19: 161-168, 2002.
17. Lovitt CJ, Shelper TB and Avery VM: Evaluation of chemotherapeutics in a three-dimensional breast cancer model. J Cancer Res Clin Oncol 141: 951-959, 2015.
18. Mehta G, Hsiao AY, Ingram M, Luker GD and Takayama S: Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy. J Control Release 164: 192-204, 2012.
19. Froehlich K, Haeger JD, Heger J, Pastuschek J, Photini SM, Yan Y, Lupp A, Pfarrer C, Mrowka R, Schleußner E, *et al*: Generation of multicellular breast cancer tumor spheroids: Comparison of different protocols. J Mammary Gland Biol Neoplasia 21: 89-98, 2016.
20. Bahcecioglu G, Basara G, Ellis BW, Ren X and Zorlutuna P: Breast cancer models: Engineering the tumor microenvironment. Acta Biomater 106: 1-21, 2020.
21. Nagelkerke A, Bussink J, Sweep FC and Span PN: Generation of multicellular tumor spheroids of breast cancer cells: How to go three-dimensional. Anal Biochem 437: 17-19, 2013.
22. Singh M, Mukundan S, Jaramillo M, Oesterreich S and Sant S: Three-dimensional breast cancer models mimic hallmarks of size-induced tumor progression. Cancer Res 76: 3732-3743, 2016.
23. Zhang W, Li C, Baguley BC, Zhou F, Zhou W, Shaw JP, Wang Z, Wu Z and Liu J: Optimization of the formation of embedded multicellular spheroids of MCF-7 cells: How to reliably produce a biomimetic 3D model. Anal Biochem 515: 47-54, 2016.
24. Asghar W, El Assal R, Shafiee H, Pitteri S, Paulmurugan R and Demirci U: Engineering cancer microenvironments for in vitro 3-D tumor models. Mater Today (Kidlington) 18: 539-553, 2015.
25. Rodríguez CE, Berardi DE, Abrigo M, Todaro LB, Bal de Kier Joffé ED and Fiszman GL: Breast cancer stem cells are involved in trastuzumab resistance through the HER2 modulation in 3D culture. J Cell Biochem 119: 1381-1391, 2018.
26. Zhao L, Xiu J, Liu Y, Zhang T, Pan W, Zheng X and Zhang X: A 3D printed hanging drop dripper for tumor spheroids analysis without recovery. Sci Rep 9: 19717, 2019.
27. Metzger W, Sossong D, Bächle A, Pütz N, Wennemuth G, Pohlemann T and Oberringer M: The liquid overlay technique is the key to formation of co-culture spheroids consisting of primary osteoblasts, fibroblasts and endothelial cells. Cytotherapy 13: 1000-1012, 2011.
28. Huang S, Yuan N, Wang G, Wu F, Feng L, Luo M, Li M, Luo A, Zhao X and Zhang L: Cellular communication promotes mammosphere growth and collective invasion through microtubule-like structures and angiogenesis. Oncol Rep 40: 3297-3312, 2018.
29. Berger AJ, Renner CM, Hale I, Yang X, Ponik SM, Weisman PS, Masters KS and Kreeger PK: Scaffold stiffness influences breast cancer cell invasion via EGFR-linked Mena upregulation and matrix remodeling. Matrix Biol 85: 80-93, 2020.
30. Guzman A, Sánchez Alemany V, Nguyen Y, Zhang CR and Kaufman LJ: A novel 3D in vitro metastasis model elucidates differential invasive strategies during and after breaching basement membrane. Biomaterials 115: 19-29, 2017.
31. Dubois C, Dufour R, Daumar P, Aubel C, Szczepaniak C, Blavignac C, Mounetou E, Penault-Llorca F and Bamdad M: Development and cytotoxic response of two proliferative MDA-MB-231 and non-proliferative SUM1315 three-dimensional cell culture models of triple-negative basal-like breast cancer cell lines. Oncotarget 8: 95316, 2017.
32. Smalley KS, Lioni M, Noma K, Haass NK and Herlyn M: In vitro three-dimensional tumor microenvironment models for anticancer drug discovery. Expert Opin Drug Discov 3: 1-10, 2008.
33. Costa EC, Gaspar VM, Coutinho P and Correia IJ: Optimization of liquid overlay technique to formulate heterogenic 3D co-cultures models. Biotechnol Bioeng 111: 1672-1685, 2014.
34. Costa EC, de Melo-Diogo D, Moreira AF, Carvalho MP and Correia IJ: Spheroids formation on non-adhesive surfaces by liquid overlay technique: Considerations and practical approaches. Biotechnol J 13, 2018.
35. Kosaka T, Tsuboi S, Fukaya K, Pu H, Ohno T, Tsuji T, Miyazaki M and Namba M: Spheroid cultures of human hepatoblastoma cells (HuH-6 line) and their application for cytotoxicity assay of alcohols. Acta Medica Okayama 50: 61-66, 1996.
36. Kelm JM, Timmins NE, Brown CJ, Fussenegger M and Nielsen LK: Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. Biotechnol Bioeng 83: 173-180, 2003.
37. Robertson FM, Ogasawara MA, Ye Z, Chu K, Pickei R, Debeb BG, Woodward WA, Hittelman WN, Cristofanilli M and Barsky SH: Imaging and analysis of 3D tumor spheroids enriched for a cancer stem cell phenotype. J Biomol Screen 15: 820-829, 2010.

38. Lorenzo C, Frongia C, Jorand R, Fehrenbach J, Weiss P, Maandhui A, Gay G, Ducommun B and Lobjois V: Live cell division dynamics monitoring in 3D large spheroid tumor models using light sheet microscopy. Cell Div 6: 22, 2011.
39. Leonard F and Godin B: 3D in vitro model for breast cancer research using magnetic levitation and bioprinting method. Methods Mol Biol 1406: 239-251, 2016.
40. Jaganathan H, Gage J, Leonard F, Srinivasan S, Souza GR, Dave B and Godin B: Three-dimensional in vitro co-culture model of breast tumor using magnetic levitation. Sci Rep 4: 6468, 2014.
41. Eckhardt BL, Gagliardi M, Iles L, Evans K, Ivan C, Liu X, Liu CG, Souza G, Rao A, Meric-Bernstam F, *et al*: Clinically relevant inflammatory breast cancer patient-derived xenograft-derived ex vivo model for evaluation of tumor-specific therapies. PLoS One 13: e0195932, 2018.
42. Lin RZ and Chang HY: Recent advances in three-dimensional multicellular spheroid culture for biomedical research. Biotechnol J 3: 1172-1184, 2008.
43. Swaminathan S, Hamid Q, Sun W and Clyne AM: Bioprinting of 3D breast epithelial spheroids for human cancer models. Biofabrication 11: 025003, 2019.
44. Ong CS, Yesantharao P, Huang CY, Mattson G, Boktor J, Fukunishi T, Zhang H and Hibino N: 3D bioprinting using stem cells. Pediatr Res 83: 223-231, 2018.
45. Kingsley DM, Roberge CL, Rudkouskaya A, Faulkner DE, Barroso M, Intes X and Corr DT: Laser-based 3D bioprinting for spatial and size control of tumor spheroids and embryoid bodies. Acta Biomater 95: 357-370, 2019.
46. Ham SL, Joshi R, Luker GD and Tavana H: Engineered breast cancer cell spheroids reproduce biologic properties of solid tumors. Adv Healthc Mater 5: 2788-2798, 2016.
47. Ham SL, Thakuri PS, Plaster M, Li J, Luker KE, Luker GD and Tavana H: Three-dimensional tumor model mimics stromal-breast cancer cells signaling. Oncotarget 9: 249-267, 2017.
48. Atefi E, Lemmo S, Fyffe D, Luker GD and Tavana H: High throughput, polymeric aqueous two-phase printing of tumor spheroids. Adv Funct Mater 24: 6509-6515, 2014.
49. Lemmo S, Atefi E, Luker GD and Tavana H: Optimization of aqueous biphasic tumor spheroid microtechnology for anti-cancer drug testing in 3D culture. Cell Mol Bioeng 7: 344-354, 2014.
50. Shahi Thakuri P, Ham SL, Luker GD and Tavana H: Multiparametric analysis of oncology drug screening with aqueous two-phase tumor spheroids. Mol Pharm 13: 3724-3735, 2016.
51. Sackmann EK, Fulton AL and Beebe DJ: The present and future role of microfluidics in biomedical research. Nature 507: 181-189, 2014.
52. Sen AK, Raj A, Banerjee U and Iqbal SR: Soft lithography, molding, and micromachining techniques for polymer micro devices. In: Microfluidic Electrophoresis. Methods in Molecular Biology. Dutta D (ed). Vol 1906. Humana Press, Springer, New York, NY, pp13-54, 2019.
53. Halldorsson S, Lucumi E, Gómez-Sjöberg R and Fleming RMT: Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices. Biosens Bioelectron 63: 218-231, 2015.
54. Sontheimer-Phelps A, Hassell BA and Ingber DE: Modelling cancer in microfluidic human organs-on-chips. Nat Rev Cancer 19: 65-81, 2019.
55. Yum K, Hong SG, Healy KE and Lee LP: Physiologically relevant organs on chips. Biotechnol J 9: 16-27, 2014.
56. Wang JD, Douville NJ, Takayama S and ElSayed M: Quantitative analysis of molecular absorption into PDMS microfluidic channels. Ann Biomed Eng 40: 1862-1873, 2012.
57. Choi Y, Hyun E, Seo J, Blundell C, Kim HC, Lee E, Lee SH, Moon A, Moon WK and Huh D: A microengineered pathophysiological model of early-stage breast cancer. Lab Chip 15: 3350-3357, 2015.
58. Montanez-Sauri SI, Sung KE, Berthier E and Beebe DJ: Enabling screening in 3D microenvironments: Probing matrix and stromal effects on the morphology and proliferation of T47D breast carcinoma cells. Integr Biol (Camb) 5: 631-640, 2013.
59. Lang JD, Berry SM, Powers GL, Beebe DJ and Alarid ET: Hormonally responsive breast cancer cells in a microfluidic co-culture model as a sensor of microenvironmental activity. Integr Biol (Camb) 5: 807-816, 2013.
60. Regier MC, Maccoux LJ, Weinberger EM, Regehr KJ, Berry SM, Beebe DJ and Alarid ET: Transitions from mono-to co-to tri-culture uniquely affect gene expression in breast cancer, stromal, and immune compartments. Biomed Microdevices 18: 70, 2016.
61. Dongre A and Weinberg RA: New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. Nat Rev Mol Cell Biol 20: 69-84, 2019.
62. Eslami Amirabadi H, SahebAli S, Frimat JP, Luttge R and den Toonder JMJ: A novel method to understand tumor cell invasion: Integrating extracellular matrix mimicking layers in microfluidic chips by 'selective curing'. Biomed Microdevices 19: 92, 2017.
63. Eslami Amirabadi H, Tuerlings M, Hollestelle A, SahebAli S, Luttge R, van Donkelaar CC, Martens JWM and den Toonder JMJ: Characterizing the invasion of different breast cancer cell lines with distinct E-cadherin status in 3D using a microfluidic system. Biomed Microdevices 21: 101, 2019.
64. Toh YC, Raja A, Yu H and van Noort D: A 3D microfluidic model to recapitulate cancer cell migration and invasion. Bioengineering (Basel) 5: 29, 2018.
65. Blaha L, Zhang C, Cabodi M and Wong JY: A microfluidic platform for modeling metastatic cancer cell matrix invasion. Biofabrication 9: 045001, 2017.
66. Devadas D, Moore TA, Walji N and Young EWK: A microfluidic mammary gland coculture model using parallel 3D lumens for studying epithelial-endothelial migration in breast cancer. Biomicrofluidics 13: 064122, 2019.
67. Li R, Hebert JD, Lee TA, Xing H, Boussommier-Calleja A, Hynes RO, Lauffenburger DA and Kamm RD: Macrophage-secreted TNFα and TGFβ1 influence migration speed and persistence of cancer cells in 3D tissue culture via independent pathways. Cancer Res 77: 279-290, 2017.
68. Nagaraju S, Truong D, Mouneimne G and Nikkhah M: Microfluidic tumor-vascular model to study breast cancer cell invasion and intravasation. Adv Healthc Mater 7: 1701257, 2018.
69. Tang Y, Soroush F, Sheffield JB, Wang B, Prabhakarpandian B and Kiani MF: A biomimetic microfluidic tumor microenvironment platform mimicking the EPR effect for rapid screening of drug delivery systems. Sci Rep 7: 9359, 2017.
70. Lanz HL, Saleh A, Kramer B, Cairns J, Ng CP, Yu J, Trietsch SJ, Hankemeier T, Joore J, Vulto P, *et al*: Therapy response testing of breast cancer in a 3D high-throughput perfused microfluidic platform. BMC Cancer 17: 709, 2017.
71. Bahlmann LC, Smith LJ and Shoichet MS: Designer biomaterials to model cancer cell invasion in vitro: Predictive tools or just pretty pictures. Adv Funct Mater 30: 1909032, 2020.
72. Chen L, Xiao Z, Meng Y, Zhao Y, Han J, Su G, Chen B and Dai J: The enhancement of cancer stem cell properties of MCF-7 cells in 3D collagen scaffolds for modeling of cancer and anti-cancer drugs. Biomaterials 33: 1437-1444, 2012.
73. Antoine EE, Vlachos PP and Rylander MN: Review of collagen I hydrogels for bioengineered tissue microenvironments: Characterization of mechanics, structure, and transport. Tissue Eng Part B Rev 20: 683-696, 2014.
74. Liu J, Tan Y, Zhang H, Zhang Y, Xu P, Chen J, Poh YC, Tang K, Wang N and Huang B: Soft fibrin gels promote selection and growth of tumorigenic cells. Nat Mater 11: 734-741, 2012.
75. Kleinman HK and Martin GR: Matrigel: Basement membrane matrix with biological activity. Semin Cancer Biol 15: 378-386, 2005.
76. Krause S, Maffini MV, Soto AM and Sonnenschein C: The microenvironment determines the breast cancer cells' phenotype: Organization of MCF7 cells in 3D cultures. BMC Cancer 10: 263, 2010.
77. Whitman NA, Lin ZW, Kenney RM, Albertini L and Lockett MR: Hypoxia differentially regulates estrogen receptor alpha in 2D and 3D culture formats. Arch Biochem Biophys 671: 8-17, 2019.
78. Palomeras S, Rabionet M, Ferrer I, Sarrats A, Garcia-Romeu ML, Puig T and Ciurana J: Breast cancer stem cell culture and enrichment using poly(ε-Caprolactone) scaffolds. Molecules 21: 537, 2016.
79. Wessels DJ, Pradhan N, Park YN, Klepitsch MA, Lusche DF, Daniels KJ, Conway KD, Voss ER, Hegde SV, Conway TP and Soll DR: Reciprocal signaling and direct physical interactions between fibroblasts and breast cancer cells in a 3D environment. PLoS One 14: e0218854, 2019.
80. James-Bhasin M, Siegel PM and Nazhat SN: A three-dimensional dense collagen hydrogel to model cancer cell/osteoblast interactions. J Funct Biomater 9: 72, 2018.
81. Cox RF, Jenkinson A, Pohl K, O'Brien FJ and Morgan MP: Osteomimicry of mammary adenocarcinoma cells in vitro; increased expression of bone matrix proteins and proliferation within a 3D collagen environment. PLoS One 7: e41679, 2012.

82. Berger AJ, Linsmeier KM, Kreeger PK and Masters KS: Decoupling the effects of stiffness and fiber density on cellular behaviors via an interpenetrating network of gelatin-methacrylate and collagen. Biomaterials 141: 125-135, 2017.
83. Conklin MW, Eickhoff JC, Riching KM, Pehlke CA, Eliceiri KW, Provenzano PP, Friedl A and Keely PJ: Aligned collagen is a prognostic signature for survival in human breast carcinoma. Am J Pathol 178: 1221-1232, 2011.
84. Bredfeldt JS, Liu Y, Conklin MW, Keely PJ, Mackie TR and Eliceiri KW: Automated quantification of aligned collagen for human breast carcinoma prognosis. J Pathol Inform 5: 28, 2014.
85. Wang Y, Mirza S, Wu S, Zeng J, Shi W, Band H, Band V and Duan B: 3D hydrogel breast cancer models for studying the effects of hypoxia on epithelial to mesenchymal transition. Oncotarget 9: 32191, 2018.
86. Peppas NA, Hilt JZ, Khademhosseini A and Langer R: Hydrogels in biology and medicine: From molecular principles to bionanotechnology. Adv Mater 18: 1345-1360, 2006.
87. Livingston MK, Morgan MM, Daly WT, Murphy WL, Johnson BP, Beebe DJ and Virumbrales-Muñoz M: Evaluation of PEG-based hydrogel influence on estrogen receptor driven responses in MCF7 breast cancer cells. ACS Biomater Sci Eng 5: 6089-6098, 2019.
88. Bellis SL: Advantages of RGD peptides for directing cell association with biomaterials. Biomaterials 32: 4205-4210, 2011.
89. Wang F, Li Y, Shen Y, Wang A, Wang S and Xie T: The functions and applications of RGD in tumor therapy and tissue engineering. Int J Mol Sci 14: 13447-13462, 2013.
90. Sawicki LA, Ovadia EM, Pradhan L, Cowart JE, Ross KE, Wu CH and Kloxin AM: Tunable synthetic extracellular matrices to investigate breast cancer response to biophysical and biochemical cues. APL Bioeng 3: 016101, 2019.
91. Schmid R, Schmidt SK, Hazur J, Detsch R, Maurer E, Boccaccini AR, Hauptstein J, Teßmar J, Blunk T, Schrüfer S, *et al*: Comparison of hydrogels for the development of well-defined 3D cancer models of breast cancer and melanoma. Cancers (Basel) 12: 2320, 2020.
92. Feng S, Duan X, Lo PK, Liu S, Liu X, Chen H and Wang Q: Expansion of breast cancer stem cells with fibrous scaffolds. Integr Biol (Camb) 5: 768-777, 2013.
93. Zhang YS, Duchamp M, Oklu R, Ellisen LW, Langer R and Khademhosseini A: Bioprinting the cancer microenvironment. ACS Biomater Sci Eng 2: 1710-1721, 2016.
94. Balachander GM, Balaji SA, Rangarajan A and Chatterjee K: Enhanced metastatic potential in a 3D tissue scaffold toward a comprehensive in vitro model for breast cancer metastasis. ACS Appl Mater Interfaces 7: 27810-27822, 2015.
95. Guiro K, Patel SA, Greco SJ, Rameshwar P and Arinzeh TL: Investigating breast cancer cell behavior using tissue engineering scaffolds. PLoS One 10: e0118724, 2015.
96. Rijal G, Bathula C and Li W: Application of synthetic polymeric scaffolds in breast cancer 3D tissue cultures and animal tumor models. Int J Biomater 2017: 8074890, 2017.
97. Kačarević ŽP, Rider PM, Alkildani S, Retnasingh S, Smeets R, Jung O, Ivanišević Z and Barbeck M: An introduction to 3D bioprinting: Possibilities, challenges and future aspects. Materials (Basel) 11: 2199, 2018.
98. Charbe N, McCarron PA and Tambuwala MM: Three-dimensional bio-printing: A new frontier in oncology research. World J Clin Oncol 8: 21-36, 2017.
99. Derby B: Bioprinting: Inkjet printing proteins and hybrid cell-containing materials and structures. J Mater Chem 18: 5717-5721, 2008.
100. Negro A, Cherbuin T and Lutolf MP: 3D inkjet printing of complex, cell-laden hydrogel structures. Sci Rep 8: 17099, 2018.
101. Panwar A and Tan LP: Current status of bioinks for micro-extrusion-based 3D bioprinting. Molecules 21: 685, 2016.
102. Koch L, Gruene M, Unger C and Chichkov B: Laser assisted cell printing. Curr Pharm Biotechnol 14: 91-97, 2013.
103. Mondschein RJ, Kanitkar A, Williams CB, Verbridge SS and Long TE: Polymer structure-property requirements for stereolithographic 3D printing of soft tissue engineering scaffolds. Biomaterials 140: 170-188, 2017.
104. Cui X, Boland T, D'Lima DD and Lotz MK: Thermal inkjet printing in tissue engineering and regenerative medicine. Recent Pat Drug Deliv Formul 6: 149-155, 2012.
105. Cheng E, Yu H, Ahmadi A and Cheung KC: Investigation of the hydrodynamic response of cells in drop on demand piezoelectric inkjet nozzles. Biofabrication 8: 015008, 2016.
106. Yi HG, Lee H and Cho DW: 3D printing of organs-on-chips. Bioengineering (Basel) 4: 10, 2017.
107. Bishop ES, Mostafa S, Pakvasa M, Luu HH, Lee MJ, Wolf JM, Ameer GA, He TC and Reid RR: 3-D bioprinting technologies in tissue engineering and regenerative medicine: Current and future trends. Genes Dis 4: 185-195, 2017.
108. Chang R, Nam J and Sun W: Effects of dispensing pressure and nozzle diameter on cell survival from solid freeform fabrication-based direct cell writing. Tissue Eng Part A 14: 41-48, 2008.
109. Murphy SV and Atala A: 3D bioprinting of tissues and organs. Nat Biotechnol 32: 773-785, 2014.
110. Park JH, Jang J, Lee JS and Cho DW: Three-dimensional printing of tissue/organ analogues containing living cells. Ann Biomed Eng 45: 180-194, 2017.
111. Sears NA, Seshadri DR, Dhavalikar PS and Cosgriff-Hernandez E: A review of three-dimensional printing in tissue engineering. Tissue Eng Part B Rev 22: 298-310, 2016.
112. Raman R, Bhaduri B, Mir M, Shkumatov A, Lee MK, Popescu G, Kong H and Bashir R: High-resolution projection microstereolithography for patterning of neovasculature. Adv Healthc Mater 5: 610-619, 2016.
113. Lin H, Zhang D, Alexander PG, Yang G, Tan J, Cheng AW and Tuan RS: Application of visible light-based projection stereolithography for live cell-scaffold fabrication with designed architecture. Biomaterials 34: 331-339, 2013.
114. Axpe E and Oyen ML: Applications of alginate-based bioinks in 3D bioprinting. Int J Mol Sci 17: 1976, 2016.
115. Nerger BA, Brun PT and Nelson CM: Microextrusion printing cell-laden networks of type I collagen with patterned fiber alignment and geometry. Soft Matter 15: 5728-5738, 2019.
116. Włodarczyk-Biegun MK and del Campo A: 3D bioprinting of structural proteins. Biomaterials 134: 180-201, 2017.
117. Albritton JL and Miller JS: 3D bioprinting: Improving in vitro models of metastasis with heterogeneous tumor microenvironments. Dis Model Mech 10: 3-14, 2017.
118. Mancha Sánchez E, Gómez-Blanco JC, López Nieto E, Casado JG, Macías-García A, Díaz Díez MA, Carrasco-Amador JP, Torrejón Martín D, Sánchez-Margallo FM and Pagador JB: Hydrogels for bioprinting: A systematic review of hydrogels synthesis, bioprinting parameters, and bioprinted structures behavior. Front Bioeng Biotechnol 8: 776, 2020.
119. Moore CA, Shah NN, Smith CP and Rameshwar P: 3D bioprinting and stem cells. Methods Mol Biol 1842: 93-103, 2018.
120. Mollica PA, Booth-Creech EN, Reid JA, Zamponi M, Sullivan SM, Palmer XL, Sachs PC and Bruno RD: 3D bioprinted mammary organoids and tumoroids in human mammary derived ECM hydrogels. Acta Biomater 95: 201-213, 2019.