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Chapter

In vitro Approaches to Model Breast Tumor Complexity

Heizel Rosado-Galindo, Lyanne Suarez and Maribella Domenech

Abstract

Cell culture technologies have provided biomedical researchers with fast and accessible tools to probe the breast tumor microenvironment. Exponential progress in fabrication methods combined with multiparametric approaches have enabled the development of cell culture model systems with enhanced biological complexity to identify key aspects that regulate breast cancer (BC) progression and therapeutic response. Yet, the culture parameters and conditions employed influence the behavior of tumor cells, thereby affecting its tissue biomimetic capabilities. In this chapter we review the wide range of culture platforms employed for the generation of breast tumor models and summarize their biomimetic capabilities, advantages, disadvantages and specific applications.

Keywords: culture platforms, microfluidics, organoids, 3D bioprinting, tumor microenvironment, co-culture

1. Introduction

Cell culture is an integral tool in biomedical research. It refers to the removal of cells from tissues or organs, into an artificial *in vitro* environment. The cells may be directly removed from the tissue before culturing, or they may be derived from a previously established cell line [1, 2]. Among their many applications, *in vitro* cell culture models allow for the evaluation of the physiology and biochemistry of cells; the study of mutagenesis and carcinogenesis; and drug research and development [1–3]. Furthermore, *in vitro* models provide a faster and more cost-effective alternative to *in vivo* animal models, while also allowing researchers to control and alter the cellular microenvironment.

Breast tumors are complex systems, composed of different cell subpopulations with distinct tumorigenic capabilities within the tumor. *In vitro* cell culture models have been one of the basic techniques utilized in BC research. Despite the many advances in the field, there is still a need for suitable tumor models that can accurately mimic the disease. Two-dimensional (2D) culture models have been commonly used in BC studies over the years. These have provided valuable insight about the molecular mechanisms involved in the pathology of the disease, yet 2D models are not able to properly model BC complexities [4]. Similarly, animal models require specialized animal facilities, are expensive, laborious, along with the consideration of pharmaco-and toxicokinetic differences between animal and humans which

can make results unreliable [5]. Hence, the development of tumor models that can mimic to some extent the complexity present in the tumor microenvironment (TME) is imperative.

The TME is heterogeneous and plays a significant role in tumor development, progression and metastasis [6]. It is composed of multiple cell types such as fibroblasts, myoepithelial and endothelial cells, infiltrated immune cells (e.g., T cells, macrophages), adipocytes and mesenchymal stem cells (MSC), along with the extracellular matrix (ECM) and soluble factors [7, 8]. These cell types are important for modeling the disease as it has been shown that tumor prognosis is not solely based on the tumorigenic cells, but also on how those cells communicate with their environment [9]. For example, cancer associated fibroblasts (CAFs) have been demonstrated to promote cancer cell aggressiveness and survival by the secretion of growth factors and cytokines and the creation of a "protective niche" against drugs [8, 10, 11]. Similarly, immune cells promote angiogenesis [12], immunosuppression, invasion and metastasis [13, 14]. Furthermore, adipocytes and MSCs have been shown to be involved in the secretion of factors related to matrix remodeling, invasion and survival of the tumor [15, 16]. Thereby, models that include multiple cell types are likely to be more mimetic of the pathology and predictive of responses in tissues. As such, custom microscale platforms have been developed to accommodate multiple cell types in spatially defined patterns and locations to enable examination of multi-cell type interactions. Such models include those related to angiogenesis and metastatic processes [17–19], and due to the lack of spatial control it would have been difficult to recreate such interactions in traditional culture platforms highlighting the applicability of custom platforms for multi-cell type interactions.

The identification of relevant parameters from the tumor microenvironment is imperative for proper assessment and predictability of efficacy of experimental therapies. For this reason, 3D cell culture systems have become more popular due to its potential to better mimic the complexity of the TME and thereby increase the physiological relevance of the study [20, 21]. This modality incorporates scaffolds and 3D cell constructs that have been shown to impact cell proliferation, morphology, signaling and drug resistance in a more physiologically relevant manner [22–25].

Mimicking BC complexity is challenging, however, progress in microfabrication techniques, tissue engineering and cancer biology have paved the way to more sophisticated models with enhanced biomimetic capabilities that will help to elucidate the intricate nature of BC. In this chapter, we discuss the wide range of culture platforms employed for the generation of breast tumor models and summarize their biomimetic capabilities, advantages, disadvantages and specific applications.

2. Cell culture modalities

2.1 Two-dimensional and three-dimensional culture

The traditional cell culture methods for studying breast cancer employ two-dimensional monolayer cultures, where cells grow flat on a surface. Two-dimensional culture is still widely used, but with advances in microfabrication now surfaces can be modified with nanostructure topographies and different levels of stiffness to mimic to some extent the physical properties of the matrix surface. These topographies (e.g., roughness, surface geometry) have the capability of providing biomimetic surfaces that have been shown to modify the morphology, proliferation and signaling, among others, of cells [26]. Similarly, changes in the mechanical properties of the ECM (e.g., stiffness) are related to increasing malignant phenotype [27], cancer progression, signaling [28–30] and

drug sensitivity [31]. Despite these technological advances in 2D cultures, multiple studies have shown that cell cultures in 2D felt short to mimic cell phenotypes associated with disease progress such as cell invasion, cell function and expression of pathological markers [4, 23, 32]. In some cases, utilizing 2D culture systems has resulted in the loss of essential cell signaling pathways, hence limiting the ability to fully evaluate cell–cell and cell-ECM interactions [33]. Evidence has also shown that there are inconsistencies when comparing cell morphology, receptor expression, and polarity between cells grown in 2D and the *in vivo* setting [34].

In order to bridge this gap in biological complexity, multiple methods employing 3D cell culture systems have emerged and continue to be steadily improving, aiming to produce the most *in vivo*-like structures. Essentially, 3D models can be divided into two groups: cell aggregates (spheroids) and biomaterial constructs [35]. The most basic 3D culture models use scaffolds of synthetic (e.g., polydimethylsiloxane-PDMS, polylactic acid-PLA) and natural (e.g., collagen, Matrigel®, hydrogels) biomaterials to investigate the effect of ECM properties on cancer behavior. Spheroids have been used mostly for drug screening applications since it has been demonstrated they more closely resemble the *in vivo* environment [36]. Growing BC cells in 3D has also revealed a more realistic drug response [21, 37], cell proliferation and morphology [38], and better representation of tumor heterotypic phenotype and TME [39, 40]. For example, single-cell RNA sequencing of breast cancer spheroids have uncovered cell clusters with specific functions (e.g., proliferation, invasion) that provide evidence of the heterotypic nature and complexity of breast tumors [41]. **Figure 1** below depicts the main *in vitro* 2D and 3D culture modalities along with the most predominant co-culture models (discussed in the next subsection) to study cell crosstalk.

2.2 Co-culture

Cancer is a heterogeneous disease and even though there have been various advances in cell culture modalities, thorough comprehension of the crosstalk between cancer and non-cancer cells is still not fully understood [42]. Co-culture and multi-culture models have been long established as appropriate tools for

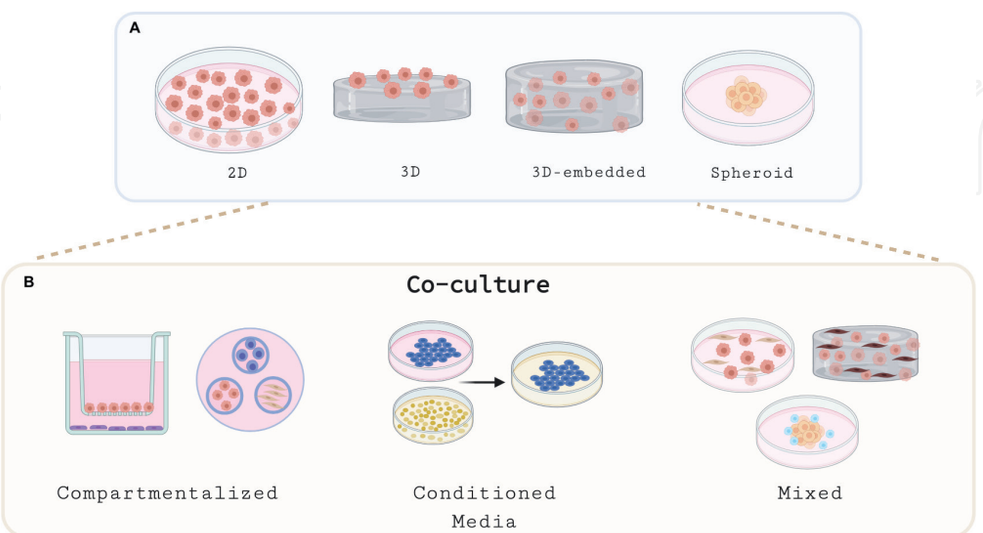

Figure 1.
In vitro *culture modalities. A) Cells can be cultured* in vitro *as 2D monolayers, over a 3D scaffold (synthetic or natural material), embedded into a scaffold material or as spheroid constructs. B) Yet, co-culture and multi-culture models are implemented in order to better understand tumor-stroma interactions and cross-talk. The three main co-culture modalities used are compartmentalized, conditioned media and mixed, which incorporate cells cultured in 2D monolayers, 3D scaffolds or spheroids. Created with BioRender.com*

evaluating breast cancer heterotypic interactions *in vitro* [6]. Co-culture refers to the culturing of two different cell lines, while multi-culture models involve three or more different cells. Historically, co-culture models have been the predominant approach in research. However, despite their ability to identify factors mediating cancer and stromal interactions, co-culture models are deficient in incorporating microenvironment structure, dimensionality, and functional response [42]. With the hopes of bridging the gap between *in vitro* and *in vivo* studies, new research has been moving away from the study of only two cell types, to studying multi-cell type systems. This type of model permits researchers to control and evaluate the influence of each cell culture component. It also allows the study of important cell–cell heterotypic signals, which would be impossible to study with a 2-cell type model [43].

There have been an increasing number of studies looking to compare tri-culture models with the more traditional mono-culture or co-culture methods. With the intention of better understanding the bone microenvironment, Pagani *et al.* compared a tri-culture model of osteoblasts, osteoclasts, and endothelial cells; to single and co-cultures. The results demonstrated that the behavior of the three cell types cultured together was very different from the single or the co-culture model, in terms of proliferation, activity, and viability. These results correlate with previously established data regarding their behavior *in vivo* [44]. Regier *et al.* evaluated how increased model complexity would affect gene expression. The results demonstrated that gene expression changes based on the type of model utilized; suggesting how tumor and stromal cells would respond to microenvironments of increased complexity *in vivo* [42]. Loy *et al*. investigated the effect a tri-culture model would have on angiogenesis and compared it to simpler models. The results showed that the tri-culture model promoted cell-matrix remodeling and early expression of elastic fiber-related proteins. It also reiterated the significance of multi-culture methods since culturing with fibroblasts, endothelial cells, and smooth muscle cells was required to obtain tissues with appropriate physiological-like properties [45]. All three of these studies highlight the increasing need and importance of more complex heterotypic cultures.

Co-culture models involve a cell growing arrangement, where two or more different cells are cultured with some amount of contact between them [46]. The communication between the cells may be bi-directional or multi-dimensional, and it can happen at the macro-scale or at the micro-scale [47]. The method of choice should be dependent on what is the focus of each individual study and can be grouped in: compartmentalized, conditioned media and mixed culture.

2.2.1 Compartmentalized

The segregated or compartmentalized model consists of two or more physically separated cells, cultured in a shared environment [6]. This type of culture is preferred when studying paracrine interactions of cells that are not located in close proximity in tissues. Also, this method is useful to identify target cells based on soluble factor signaling since the cells individual response can be examined, facilitating the identification of factors that may play a role in tumor growth and advancement. In compartmentalized co-cultures, one cell population is seeded in the bottom of the standard well, and the other is seeded on a top insert or in an adjacent compartment. By doing this, the cell types remain separated, while still being able to exchange soluble signals in their shared environment [48]. Indirect cell culture eliminates heterotypic interactions mediated by contact between the cell types, which can be seen in direct cell culture. It also allows for cell type specific readouts, which are unachievable in direct cell culture [6]. Such method has

provided evidence on genes involved behind stromal invasiveness and metastasis, and the crucial role of fibroblasts in proliferation of estrogen-dependent human breast carcinomas [6, 49, 50]. Gonzalez *et al.* utilized a 2D indirect co-culture method with human BC cells and human umbilical vein endothelial cells to evaluate the process behind angiogenesis; concluding that melatonin may be an alternative for preventing tumor angiogenesis [51]. While Chiovaro *et al.* analyzed the role of ECM proteins in bone metastasis, showing that tenascin-W promotes cancer cell migration and proliferation [52].

If multiple cells need to be examined, co-culture platforms, such as transwells, are not useful since they are limited to only two compartments. Hence, the use of customizable culture systems such as microscale devices, is warranted [6]. Our group developed compartmentalized microwell culture platforms, in which we show the contribution of multiple cell types to the sensitivity to heat therapy in tumor cells [43]. The data shown indicates that the presence of macrophages and fibroblasts had a significant protective effect against heat stress in BC cells, thus, perturbing the effectiveness of heat therapy. Others have employed multi-cell type cultures to deconvolute cell communication of metastatic breast tumors. Regier *et al.* developed a compartmentalized multi-culture method, utilizing BC epithelial cells, bone marrow cells, and human monocytes. The platform allowed the creation of a substantial dataset made up of cell specific gene expression patterns. This was possible by collecting data from an individual cell type, while communicating through paracrine interactions in a heterotypic culture. The study also compared tri-culture to mono-culture and co-culture, which led to the demonstration of how stromal and tumor cells respond differently based on the complexity of the micro-environment [42]. This reiterates the importance of utilizing multi-cultures versus the more traditional co-cultures. A drawback with this method is that physical contact between cells cannot be completely prevented in the long term [47]. In addition, because cell-seeding sometimes requires more than one step, the process may be considered somewhat complicated and time-consuming [6].

2.2.2 Conditioned media

Conditioned media transfer utilizes two separately cultured cell populations, where one culture medium is utilized to nourish the other [48]. This type of method is simple and allows one-way signaling from effector to responder [6]. The advantage of utilizing this method is that conditioned media can be profiled for the identification of secreted soluble factor-related effects is possible [47]. Consequently, the role of signaling molecules could be tested in a specific response [6]. Also, this method is useful when the cells of interest cannot be cultured together such as studies involving tumor cells and microbes [53]. However, when employing multiple cell types, the method becomes a bit more complex since identification of the secretor and recipient cells can be complicated. Additionally, when this type of method is utilized, there is no cross-communication within the cells and it is not possible to study bi-directional signals [48]. For this reason, this type of method would not be ideal if the goal is to study multi-cell type interactions that naturally occur in the *in vivo* tumor environment.

2.2.3 Mixed co-culture

In mixed cell culture, different types of cells are cultured together. Just as with conditioned media transfer, this type of method is accessible and simple. It can be done in 2D or 3D using traditional well plates [6]. If the cells are cultured together in a standard plate, the method is referred to as direct or mixed cell culture.

However, if a transwell insert or adjacent compartments are utilized, the method is denoted as indirect or compartmentalized cell culture. Unlike the conditioned media method, mixed co-culture does allow for bi-directional paracrine and juxtacrine signaling, which is of great importance when studying multi-cell type interactions in breast cancer [6]. Because of the cellular arrangement, this method is also ideal for studying how cell–cell contact affects cell behavior [54]. When performing multi-cell type studies, the direct method simply requires the inclusion of the additional cell lines mixed.

Mixed co-culture experiments shed light on distinct microenvironment features based on cancer subtype; and potential mechanisms behind invasive phenotypes [55, 56]. Camp *et al*. compared the interaction of fibroblasts with the basal-like subtype versus the luminal subtype. The results were increased migration and expression of interleukins in the basal-like BC cell lines, which reiterates the important role of the TME in cancer progression [10]. Buess *et al.* also looked into evaluating the role of aspects of the TME by studying tumor-endothelial interactions and determining gene expression changes [56]. Multiple other studies have been done utilizing these culture modalities and have provided insight into further understanding the disease [6]. Yet, a disadvantage of this method is the lack of control of the spatial location of cells which can be important when examining and quantifying changes in some tumor cell behaviors such as cell migration and invasion. Also, single cell studies will require multiple cell separation steps that will make this method more time consuming and increase the number of cells needed for analysis due to cell loss during sample handling.

3. Culture platforms for enhanced biomimetic capabilities

Despite the development and application of the aforementioned cell culture methods, thorough understanding of cancer development and progression continues to be a challenge. As shown in **Figure 2**, *in vitro* cell models are mainly categorized in 2D and 3D (as discussed before) and thus, these models become more complex as research continues to be centered on creating experimental models that can mimic cell evolution on the bench with the goal of understanding the biology of the disease and identifying key therapeutic targets. Despite the advances that came with the implementation of 3D multi-culture systems, there still remains a scarcity of models that can recreate the biological complexity of the tumor microenvironment. Biomimetics can be defined as technology that utilizes or emulates tissue function with the intention of improving human lives [57]. Effective biomimetic models need to contribute a 3D environment permissive of cell phenotypic stages while enabling multi-cell type interactions [58]. As cell culture methods continue to evolve, innovative approaches are being created with the hopes of overcoming the limitations of the more traditional methods. **Table 1** summarizes the advantages, disadvantages and applications of advanced biomimetic *in vitro* 3D culture technologies.

3.1 Microfluidics

Microfluidic platforms can be utilized to scale down the traditional culture modalities, yet they enable to customize the culture environments to examine more complex interactions [64]. This technology employs microsystems that allow the manipulation of small fluid volumes and control over the spatial location of cell clusters [70]. Its application to improve 3D cell culture models has been increasing since 2012, particularly in BC research [71]. In comparison to macroscopic culture,

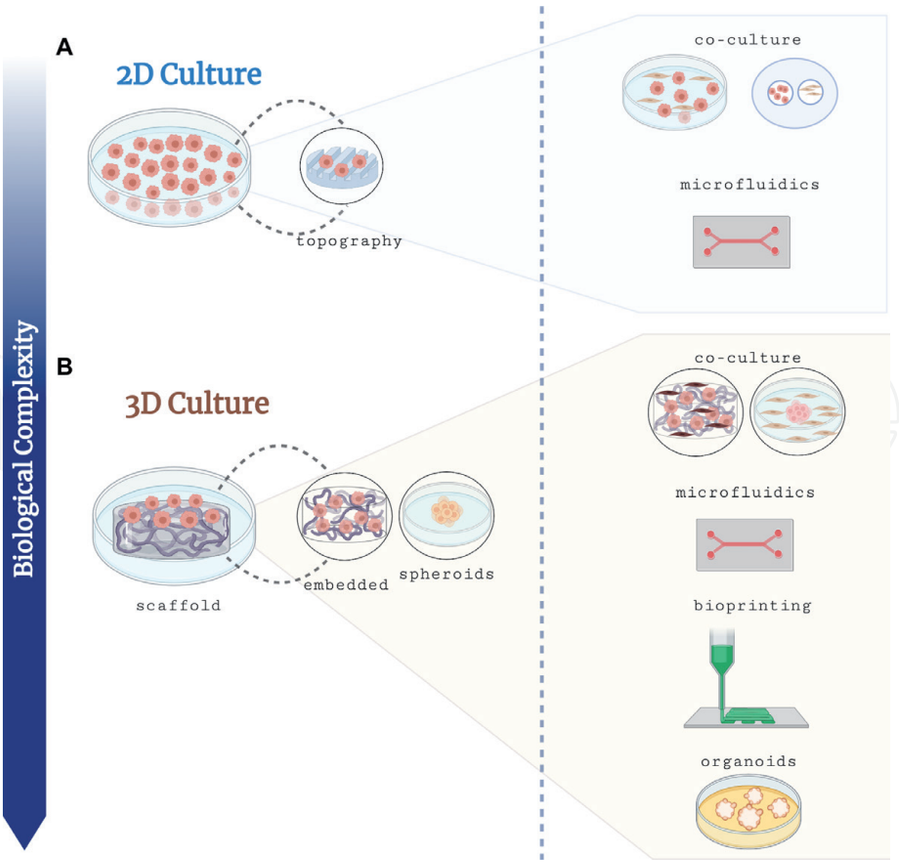

Figure 2.
Culture platforms employed in breast cancer models. A) Simple 2D platforms consist of cells cultured in flat, nano- or micro- structured substrates (left) that mimic to some extent tissue topography; or they can combine co-culture and microfluidic devices (right) to increase the complexity of the model and better resemble tumor-stroma interactions. B) In three-dimensional models, cells are culture in scaffolds and constructs that further imitate the architecture of the tumor (left). Co-culture and advanced 3D models such as microfluidics, bioprinting and organoids are capable of duplicating the TME and provide physiologically relevant insights about the disease (right). Created with BioRender.com

microfluidic cell culture models have several significant advantages that, when employed, lead towards better biomimetic models. Firstly, cells may be cultured in a spatially controlled environment by controlling fluid patterns and proximity across culture compartments [72–77]. This technology permits the combination of multiple cell types and to control cell patterning, to recapitulate to some extent tissue observations. For example, microfluidic devices permit the study of angiogenesis while also allowing the study of endothelial migration and evaluation of cell response in co-culture [71, 78]. Also, microfluidics can implement continuous perfusion conditions, and controlled gradients, which are both characteristics that also resemble the cancerous *in vivo* environment more closely. Gradients are found in angiogenesis, invasion, and migration whereas perfusion is crucial in vasculature and cell extravasation as well for nutrient replenishment. Finally, microfluidic systems enable high-throughput arrays and pose lower contamination risk and reagent consumption which make them very appealing for studies with limited cell samples such as those that employ patient-derived tissues [70, 71].

Recent studies in microfluidic systems have highlighted their capability to recreate and profile some of the biological complexity of the tumor microenvironment. Such studies have revealed important information regarding the processes involved in metastasis and how the tumor microenvironment contributes. For example, single cell RNA sequencing using microfluidic devices have revealed the diversity of the breast epithelium, which sheds light about early tumorigenesis and tumor progression [79, 80]. In addition, microfluidic devices pose as an advantage

Model	Advantages	Disadvantages	Application	Ref.
3D Microfluidics	Small size samples, spatial and temporal control, reduced reagent volumes, controlled gradients, high-throughput	Mechanical stress, complicated set-ups, material fabrication	Invasion, metastasis, vasculature, modeling TME	[20, 37, 59, 60]
Bioreactors	Long term culture, effective nutrient distribution, large scale	Contamination risk, expensive, specialized equipment, low throughput, limited spatial resolution, high cell numbers needed	Metastasis, drug discovery	[61–63]
3D bioprinting	Controlled spatial arrangement of cells and matrix, biomolecular gradients, high-throughput	Lower cell viability, material challenges, lack of standardized methods, high cell numbers needed	Migration, angiogenesis, drug discovery, modeling TME	[64–66]
Organoids	Small size samples, retain parental tumor phenotype, can be preserved as biobanks, mimetic of tissue function	Lack of standardized methods, heterogeneous cell samples, high variability across replicates	Drug discovery, invasion, metastasis	[67–69]

Table 1.
Comparison of in vitro *3D BC models.*

to personalized medicine by aiding in the selection of appropriate pharmacologic agents. In this regard, Lanz *et al.* developed a 3D microfluidic device, OrganoPlate®, to be utilized for therapy selection. They showed that MDA-MB-231 (cell line isolated at MD Anderson from a pleural effusion of a 51-year old Caucasian woman) cells embedded in Matrigel® became more sensitive to the drug, thus confirming along with previous studies that drug response is tuned by the ECM. The results were promising and even though further validation is warranted, it appears to be a fine tool for pharmacologic selection and response prediction [37]. Similarly, Yildiz-Ozturk *et al.* studied the cytotoxicity of carnosic acid and doxorubicin on MCF-7 and MDA-MB-231 BC cell lines and demonstrated the importance of biomimicry in *in vitro* platforms [20]. A breast metastatic microfluidic model was developed by Kong *et al.* to mimic the metastasis of circulating breast cancer cells (CBCCs) to the lung and other organs. Their microfluidic device allowed the flow of CBCCs over primary cell culture chambers, which would have been impossible with static conditions. They demonstrated that the metastatic potential of these cell lines was in concordance with animal models, providing a cost-effective and time-saving alternative [81]. Bersini *et al.* also developed a microfluidic co-culture model made up of metastatic BC cells, and collagen gel-embedded bone marrow-derived stem cells (hBM-MSC) lined with endothelial cells to create an osteo-conditioned microenvironment and access extravasation and micrometastases to bone tissue [59]. They found that BC receptors CXCR2 and bone-secreted chemokine CXCL5 play major roles in the extravasation process. However, due to the complexity of the design, their platform is not high throughput compatible, which adds many challenges, particularly to obtain multiple replicates in a short time. Also, in general it is important to notice that most of the organ on chip microfluidic platforms focus on the metastatic stage of the disease, leaving an evident need for research focusing

on the early stages of breast cancer. Yet, some efforts are being done to overcome this gap. As an example, Choi *et al.* developed a compartmentalized microfluidic device that enabled co-culture of tumor spheroids and normal mammary epithelial cells in close proximity to fibroblasts, with the goal of providing a model that allows researchers to closely examine the mechanistic progression of early-stage breast ductal carcinoma *in situ* (DCIS) [82].

Even though microfluidic devices have given the opportunity to better replicate the tumor environment, there are still some caveats to its use. Silicone-based devices have been shown to sequester small hydrophobic molecules, which can compromise the results of some studies [70], yet researchers have been addressing this by modifying the material to make it more hydrophilic and reduce molecule sequestration [60]. Also, microfluidic devices in some cases can induce mechanical stress to the cells [83], which can modulate cell responses in an unpredictable manner, and are often limited by complicated set-ups [70], which limits their broad adoption by the scientific and clinical community. As such, simpler fabrication methods and commercial availability of customizable microscale platforms is desirable to overcome such limitations.

3.2 Bioreactors

Despite the numerous advantages of the aforementioned 3D culture methods, the duration of culture and nutrient availability can be a limitation in static cultures particularly to enable observations that occur in cells over periods of several weeks. In this case, perfusive systems, such as bioreactors, are more appropriate. A bioreactor is a canister that allows the 3D culture of cell clusters for extended periods of time. It is coupled to sensors and actuator components allowing for the controlled delivery of oxygen, nutrients and other parameters [84]. Goliwas *et al.* developed a perfused 3D BC surrogate model utilizing a bioreactor system that incorporated breast carcinoma epithelial cells and stromal fibroblasts into an extracellular matrix. The study demonstrated that using a bioreactor allowed for analysis of longer growth periods and a greater degree of growth when compared to solid models [85]. Bioreactors have also been utilized to study metastatic progression of breast cancer, and as potential drug development platforms for cancer treatment. Krishnan *et al.* utilized a compartmentalized bioreactor model, with osteoblasts and metastatic BC cells, to study the colonization of osteoblastic tissue. In their design, cultured osteoblasts were monitored over longer periods and exhibited more *in vivo*-like characteristics, compared to 2D cell cultures [86]. Marshall *et al.* developed a physiologically relevant bioreactor system that could be potentially used for pharmacologic development. Their construct was capable of supporting and perfusing larger volume, which poses as an advantage to lab-on-a-chip systems [62]. Other studies have also used bioreactors to assess drug response of BC tissue [63, 87]. Despite bioreactors being an ideal option for cultures that require long-term analysis, there are some factors that might damper their use. Membrane bioreactors may become contaminated and multilayer cell growth may cause transfer limitations [88]. Also, its complex composition and dimensionality limits their implementation in convectional labs and limits the number of experimental replicates [89].

3.3 Three-dimensional (3D) bioprinting

Another technology that has emerged in recent years and that is being applied to 3D culture technology is 3D bioprinting. Its development has been possible thanks to advances in 3D printing technology, biomaterials and tissue engineering

methods. Three-dimensional (3D) bioprinting consists of printing cells together with ECM components, biomaterials and bioactive factors [90]. It has been shown that bioprinting techniques can be used to generate 3D tumor models that can better resemble the TME [90, 91]. This has been achieved as bioprinting provides the ability of controlling the spatial arrangement of cells, creating biomolecular gradients and well-organized vessel-like structures (vasculature) within a micron scale resolution [92, 93]. Therefore, bioprinted tumor models are used for angiogenesis, migration and drug development and screening studies as well as TME models [65, 94]. Although 3D bioprinting is widely used in tumor research, very few studies use bioprinted models for BC. Yet, most of these studies are focused on BC metastasis and drug resistance. A study performed by Zhou *et al.* evaluated the interaction between triple negative breast cancer cells (TNBC) and osteoblasts to assess metastatic progression in bone. They found that osteoblasts increased VEGF secretion and therefore, enhanced the proliferation of BC cells, while osteoblast proliferation was inhibited [58]. Bioprinted BC models have also been used for drug resistance studies. Swaminathan *et al.* bioprinted pre-formed MDA-MB-231 spheroids along with breast epithelial cells and vascular endothelial cells and evaluated plaxitacel chemoresistance in mono and co-culture. They demonstrated that bioprinted spheroids are more resistant to plaxitacel as it has been shown before in other studies. Yet, this resistance was decreased in co-culture with vascular endothelial cells highlighting the importance of replicating the TME complexities *in vitro* [95]. Another study by Duan *et al.* examined drug resistance using 3D bioprinted constructs of BC cells and adipose-derived mesenchymal stem cells (ADMSC). They found increased chemoresistance in BC cells cultured with ADMSC in comparison to monoculture and, thus provided a model to better understand the role of ADMSC in BC progression [66]. Likewise, Campbell et al. bioprinted MCF-7 cancer cells and showed higher resistance to Tamoxifen compared to monolayer culture, providing a more biological-like behavior [66, 96]. Despite the flexibility of 3D bioprinting systems, there are some challenges that need to be overcome to ease its application. Maintaining high viability and original phenotype is an issue in some bioprinting techniques due to exposure of cells to shear stress. Therefore, close control of bioink viscosities, extrusion rates, among other parameters, is imperative [97]. Also, lack of process standardization and guidelines pose another challenge for study comparison and reproducibility.

3.4 Organoids

The most recent 3D cell culture modality are organoids. These are 3D heterotypic *in vitro* tissue constructs, derived either from primary tissue or stem cells, that have the ability to mimic the *in vivo* organ [98, 99]. Historically, established cancer cell lines have been widely utilized as single cell models of the cancer disease. However, their use has several drawbacks in terms of their capability to mimic the pathology of the patient. Cell lines can undergo genetic changes, losing the genetic heterogeneity of the original tumor [100]. Organoids also possess substantial similarities to cancer cell lines 3D models (spheroids) such as cell–cell and cell-matrix interactions, gradients of nutrients, oxygen and metabolites, and can be replaced from frozen supplies with ease. They are also relatively easy to handle and can be grown in infinite quantities [101]. Yet, the main characteristic of organoids is their capability to closely resemble and retain the pathology of the parental tumor over several rounds of expansion *in vitro* [102, 103]. They also have shown therapeutic predictability for some drugs and can be preserved as biobanks and expanded, which allows extended incubation [98, 99]. Given the number of mutational processes involved in cancer development and progression, being able to study tumorigenesis in depth

is crucial. Organoids allow for organ-specific mutations to be analyzed and their whole genomes to be sequenced. Intratumor heterogeneity can also be analyzed by growing organoids from separate sections of the same tumor [100]. Another area where organoids can play a major role is drug development. Organoids appear to be much better models for identifying and testing anticancer drugs yet in a patient specific manner. For instance, studies on single cell transcriptomics of organoids have detected differences in drug sensitivity, proving that organoids maintain tumor heterogeneity, which is considered a critical aspect of tumor models [104].

Studies with BC organoids are limited, since this modality has just started to be explored. However, they have gained more popularity in the last few years. Cheung *et al.* used breast carcinoma organoids to understand tumor invasiveness and metastasis. They found that the heterotypic interactions between epithelial subgroups are key to collective invasion [105]. Broutier *et al.* was able to demonstrate that liver cancer derived organoids could be utilized for drug screening testing and identification of potential pharmacologic targets [68]. Sachs *et al.* demonstrated the biomimetic nature of organoids by demonstrating the reflecting histopathology of *in vivo* tumors, as well as HER2 and hormone receptor status. Moreover, drug screening tests were consistent with patient response [69]. These promising findings suggest that organoids will be an ideal alternative model for cancer research. Nonetheless, successfully cultivating patient organoids from biopsy specimens is still a challenge mainly due to low cell recovery and heterogeneity of collected samples, and limited availability of standardized methods [103, 105].

4. Concluding remarks

Breast cancer is an evolutionary disease and cell culture modalities should continue to evolve concomitantly. Even though traditional 2D co-culture methods have provided valuable insights on disease development and progression, there is a need for more heterotypic biomimetic models that can replicate the tumor environment more closely. Some of the consequences of limited biomimetic models has been the large number of investigational drugs that never make it past clinical trials and the lack of clear understanding on the foundations of breast cancer malignant transformation. Aside from the need for more biomimetic models, most of the current research has been focused on the metastatic stage of the disease. Even though understanding tumor progression and the role of its microenvironment is of utmost importance, understanding the early and localized stages of breast cancer is also imperative. Not having an explicit grasp on the biological processes behind progression from early stage to invasive to metastasis has hindered the ability to make a predictive diagnosis in patients with early disease that have a greater probability of invasive cancer progression. Hence, designing new targeted pharmacologic agents becomes a challenge. Despite the continuous development of innovative cell culture modalities, there are still many unanswered questions. However, the hope is that with the emergence of the new methods (bioreactors, organoids, etc.), many of these questions can be interrogated in a controlled and user friendly cell culture environment.

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Conflict of interest

The authors declare no conflict of interest.

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