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The need for complex 3D culture models to unravel novel pathways and identify accurate biomarkers in breast cancer

Britta Weigelt[1], **Cyrus M Ghajar**[2], and **Mina J Bissell**[3]

[1]Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA

[2]Public Health Sciences Division/Translational Research Program, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA

[3]Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

Abstract

The recent cataloging of the genomic aberrations in breast cancer has revealed the diversity and complexity of the disease at the genetic level. To unravel the functional consequences of specific repertoires of mutations and copy number changes on signaling pathways in breast cancer, it is crucial to develop model systems that truly recapitulate the disease. Here we discuss the three-dimensional culture models currently being used or recently developed for the study of normal mammary epithelial cells and breast cancer, including primary tumors and dormancy. We discuss the insights gained from these models in regards to cell signaling and potential therapeutic strategies, and the challenges that need to be met for the generation of heterotypic breast cancer model systems that are amenable for high-throughput approaches.

Keywords

Three-dimensional cell culture; co-culture; microenvironment; tissue architecture; signaling pathway; breast cancer

1. INTRODUCTION

Breast cancer is a heterogeneous disease, encompassing multiple entities with distinct biological and clinical features [1]. The massively parallel sequencing endeavors performed by The Cancer Genome Atlas (TCGA; www.cancergenome.nih.gov), the International Cancer Genome Consortium (ICGC [2]) and individual investigators have provided a comprehensive characterization of breast cancer mainly at the genomic, but also the

Correspondence to: Mina J Bissell, Life Sciences Division, Lawrence Berkeley National Laboratory, One Cyclotron Road, MS:977, Berkeley, CA 94720, USA. mjbissell@lbl.gov; Phone: 510-486-4365; Fax: 510-486-5586. Britta Weigelt, Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA. weigelt@mskcc.org, Phone: 212-639-2332; Fax: 212-639-2502.

7. CONFLICT OF INTEREST

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transcriptomic and epigenomic level. The use of this technology has demonstrated that breast cancers harbor heterogeneous constellations of somatic mutations and only few highly recurrently mutated driver genes [3–6]. In fact, at base-pair resolution each breast cancer appears to be unique in its repertoire of genetic aberrations [6]. Despite this genetic heterogeneity seen between breast cancers, it is important to note that the number of specific signaling pathways activated in each molecular subtype of the disease seems to be limited [5]. In addition, massively parallel sequencing analyses of breast cancers have revealed intra-tumor genetic heterogeneity in a substantial proportion of cases [4,7,8]. In fact, it is currently accepted that at least a subset of breast cancers are composed of mosaics of tumor cell clones, which in addition to the founder genetic events present in all cells, also display additional genomic alterations.

It has been posited that the microenvironment exerts selective pressures on cancer cells, such as during the metastatic process or when the environment is changed due to an external selective pressure, such as drug treatment. Indeed, there is burgeoning evidence stemming from sequencing endeavors to demonstrate that primary breast cancers and their matched distant metastases are distinct in their mutational landscapes, and enrichment of populations of cancer cells harboring specific genetic alterations in the primary *versus* the metastatic site and *vice versa* has been observed [9,10]. Also drug treatment has been reported to result in the selection of subclones, present in varying frequencies in the primary tumors, harboring mutations conferring resistance to the therapeutic agent [11–14].

The impact of environmental cues on cancer is not restricted to biological or exogenous bottlenecks as exemplified above. In fact, it is plausible that throughout tumorigenesis and tumor progression, the microenvironment plays a pivotal role, as cancer cells are exposed to local selective pressures stemming from the structural and cellular microenvironment. In fact, a tumor cell is not an island [15]; instead, breast cancer cells interact with each other and with their surrounding non-malignant cells, hormones, secreted factors and the extracellular matrix (ECM). These complex microenvironmental interactions and forces contribute profoundly to the behavior, phenotype and evolution of cancer cells. For example, in estrogen receptor-negative breast cancer, increased expression levels of immune response pathway genes or increased presence of lymphocytic infiltration have been shown by independent investigators and studies to be the strongest predictor of outcome and, potentially, of chemotherapy benefit [16–19].

Given the genomic complexity of breast cancer, understanding the epistatic interactions between mutations, as well as their effects on tissue function and endocrine, paracrine and autocrine signaling is germane for the development and validation of prognostic and predictive strategies. Most studies investigating the effect of genetic/epigenetic aberrations *in vitro* on specific aspects of cellular processes such as transformation, proliferation or signaling have been performed in oversimplified model systems, not taking alterations in tissue architecture, cell-cell interactions, or cell-microenvironment interactions into account. The understanding of the functional consequences of specific repertoires of genomic aberrations on signaling and pathway dependencies within and between the cancers cells but also with their surrounding microenvironment require model systems that truly recapitulate the disease. To date, the vast majority of functional studies using cancer cell lines are

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performed in traditional monolayer cultures, however, and culture systems that fully mirror human breast cancer, primary and metastatic, and its diverse cellular microenvironment have yet to be developed further.

Here, we provide an overview of the three-dimensional (3D) cell culture models currently being employed for the study of breast cancer, including co-culture systems. In addition, we discuss how these models can be used for the dissection of cell-cell and cell-stroma interactions and of the role of specific genetic aberrations or signaling pathways in normal and malignant mammary epithelial cells.

2. THREE-DIMENSIONAL CELL CULTURE MODELS

The acini (also called alveoli in breast) and ducts of the normal mammary gland are highly organized structures, with a central lumen lined by polarized luminal epithelial cells and surrounded by an outer layer of myoepithelial cells. The epithelium is separated from the surrounding stromal ECM and stromal cells by a basement membrane (BM) (reviewed in [20]). In contrast, in invasive breast cancer, the neoplastic epithelial cells are in direct contact with the stroma [20] comprised of stromal ECM, adipose tissue, blood vessels, lymphatics as well as lymphocytes, macrophages, and fibroblasts, amongst other cell types. It has been observed that in invasive breast cancers the myoepithelial cells are generally lost, whereas there is an increase in myofibroblasts and immune cells in the stroma and enhanced vascularization [20–24].

In the 1970s it was shown that collagen gels, once floated in the cell culture medium, could allow epithelial cells of different tissues and origins to maintain much of their tissue structure and some of their differentiated functions [25–27]. The mechanisms by which the collagen gel could allow partial functional tissue-specific differentiation was not at all clear, in particular because on similar floating gels, mammary cells would produce milk proteins whereas liver cells would produce albumin [28]. Using patterns of C14-labeled glucose metabolites, we showed initially that patterns of functional differentiation indeed were cell- and tissue-specific (for review see [15]). We also showed that the milk protein (beta-casein) detected was synthesized *de novo* [29,30]. Subsequently, our studies have also demonstrated that there is a temporal and direct relation between the endogenous production of BM by the cells in culture and the subsequent expression of tissue-specific genes [31,32]. Thus we suggested then, and proved later in our acini model, that what allows tissue specific functions to be expressed on floating collagen gels is not the collagen gel itself, but the ability to lay down an endogenously made BM upon floatation to which the cells respond (Fig. 1).

Based on these observations we proposed that the ECM in general [33] and BM in particular, which is composed of laminins, collagens, tenascin, and proteoglycans [34], are more than simple scaffolds. Instead, we postulated a “dynamic reciprocity” between the ECM and the nucleus of a cell where the ECM provides signaling cues via transmembrane receptors and the cytoskeleton to the nuclear matrix and chromatin to maintain tissue integrity [35]. This would explain why normal epithelial cells when grown as monolayers on tissue culture plastic lose morphological organization and almost all of their tissue-specific

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functions and resemble cancer cells [15]. To address these problems, we developed 3D culture systems using a BM gel derived from Engelbreth-Holm-Swarm (EHS) tumors [36] (see below) to study tissue-specific functions of normal mouse mammary cells [37–39], and later normal and malignant human breast cells [40]. The basement membrane-like gels, which we call laminin-rich ECM (lrECM) gels, restore more of functional and morphological differentiation than do floating collagen gels. In fact, these 3D lrECM models allow cells to organize into structures that mimic their *in vivo* architecture (i.e. acini) and to investigate gene functions and signaling pathways in a more physiologically relevant context than two-dimensional (2D) monolayers (for a historical and more recent review see [41]).

The most commonly used lrECMs for the study of non-malignant and malignant breast cells and cell lines are Matrigel™ (BD Biosciences) and Cultrex® BME (Trevigen), which are BM preparations extracted from the EHS mouse sarcoma. This tumor is rich in ECM proteins, in particular laminin but also collagen IV, heparan sulfate proteoglycans, and contains several growth factors [42,43]. LrECM gels could be made also from a mixture of laminin 111 and collagen 1 from rat tail collagen [44]. For 3D cell culture assays, cells are either completely embedded in lrECM [40] or seeded on top of a thinner lrECM gel overlaid with a dilute solution of lrECM ([45]; for protocols see [46,47]) (Fig. 2). Contrary to monolayer cultures, where non-malignant and malignant breast epithelial cells often exhibit similar morphologies and doubling times, 3D culture assays allow phenotypic discrimination between non-malignant and malignant breast epithelial cells: non-malignant cells form polarized, growth-arrested acinus-like colonies when grown in lrECM, whereas breast cancer cells form disorganized, non-polarized and proliferative colonies [15,40,47–50].

The study of non-malignant cells in 3D culture has led to the characterization of factors playing a role in the establishment and maintenance of the polarized epithelial architecture and in oncogene-induced alterations of this structure (see section 4). As mentioned above, the BM is generally lost in invasive breast cancers, and given that these cells are unable to form an endogenous BM due to destruction by MMPs [51], tumors *in vivo* are surrounded by different ECM/ECM-peptides; for the study of breast cancer cells, lrECM therefore may not be entirely reflective. In addition, we know that ECM stiffness is an important regulator of the cellular response, and that tumors are stiffer than normal tissue [52–55](Fig. 3). The elastic modulus (measured in Pascals) of normal mouse mammary gland has been reported to be similar to that of lrECM, whereas that of established tumors from MMTV-*Her2/neu*, *Myc*, and *Ras* transgenic mice is significantly higher (>20x) [55]. Interestingly, mammary epithelial cells cultured in low concentration 3D collagen gels organize into polarized acinar and ductal structures, whereas these cells lose this polarized morphogenesis and have an invasive phenotype in high concentration collagen gels [56]. One should keep in mind that EHS-derived matrices are biological products from mouse tumors; thus the exact composition and protein content may vary between lots, and therefore would require testing and adjustment of each lot before use (i.e. dilution or addition of high concentration collagen) to achieve the correct softness/stiffness representative of either normal or malignant mammary tissue.

Three-dimensional cell culture systems other than lrECM models are used in the study of cancer aiming to improve the accuracy of cell line-based research (Fig. 2). A simple 3D

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approach is the spheroid culture without exogenous ECM, which takes advantage of the fact that many cell types spontaneously aggregate [57]. These spheroids can be generated by culturing cells using low-attachment or coated plates, and also hanging-drop methodologies, rotary cell culture systems and other mono- or multicellular spheroids techniques are used (for review see [58]). Considerable progress has been made over the past years in the engineering of materials for 3D *in vitro* culture systems ranging from naturally and synthetic polymers to macroporous scaffolds to support the formation of 3D structures (for reviews see [59–62]) (Fig. 2). These tissue-engineered models undoubtedly may be more advanced and reproducible than conventional spheroid or lrECM based 3D cell culture models, although the context of different tissues and organs may require different assays and techniques. Current 3D cell-laden matrix designs using selective “click” or “clip” chemistries can couple or remove functionalities from 3D culture systems in an attempt to recapitulate tissue function not only in space but also in time (4D cell culture technology; reviewed in [63]). Although in theory this sounds exciting, in practice, all gel systems including lrECM, change their endogenous make-up of ECM molecules and structure as a function of time, which is why cells in 3D lrECM can make a functional BM. It should be noted, however, that for 3D cell cultures to be employed as a standard technique in research laboratories, the ease of use and applicability for specific read-outs as well as costs are crucial.

Most cancer biology studies performed to date, both in 2D and 3D model systems, have employed cell lines established decades ago. These cell lines have been maintained on tissue culture plastic. Given the known genetic and epigenetic heterogeneity of human cancers, it is likely that these cell lines are preferentially composed of clones that are fittest to grow on tissue culture plastic [64]. In recent years, with the development of approaches that have resulted in higher rates of success to establish patient-derived primary breast cancer mouse xenografts, which are likely more representative of the human disease than single cancer cell line models, novel opportunities have emerged (reviewed in Williams et al. [64] and Tentler et al. [65]). Cell lines obtained from these patient-derived xenografts can be established without being subjected to 2D tissue culture plastic. In addition, the establishment of primary organoids from human tumors directly on 3D lrECM has been described [66,67]. It should be noted, however, that in the case of breast cancer, not all subtypes of the disease are equally amenable to propagation as patient-derived xenografts or cell lines; for instance, the success rate for the establishment of such models of estrogen receptor-negative cancers is much greater than that of estrogen receptor-positive tumors [68,69]. In addition, these approaches seem not to overcome the challenges posed by intra-tumor genetic heterogeneity, as xenografts have been shown to be derived from specific subclones of the primary tumor [9,70]; however, as these approaches more closely recapitulate the human disease, this selection may not be as strong as that caused by culturing the cells directly on plastic without any microenvironmental cues.

With the wealth of genomic data at our hands, high-throughput applications of 3D culture models, in particular those using cancer cell models that closely recapitulate the human disease, are pivotal for the modeling and understanding of the epistatic interactions between mutations found in breast cancer to date, and many laboratories and biotechnology

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companies are currently developing high-throughput 3D culture applications. A consortium (PREDECT; www.predect.eu) partnering academia, enterprises and pharmaceutical companies is now focusing on the generation of *in vitro* and *in vivo* models for breast, prostate and lung cancers, including 2D/3D organotypic (co-)cultures, to provide more appropriate *in vitro* platforms for target validation and drug discovery.

3. HETEROTYPIC THREE-DIMENSIONAL CELL CULTURE MODELS

The most common 3D cell culture systems discussed above use monocultures, i.e. only one cell type. To fully recapitulate the histological complexity of the normal breast and invasive breast cancers, not only extracellular matrices and scaffolds are required but also stromal cells [71], which interact physically or via paracrine signaling with the epithelial cells [72].

Introduction of myoepithelial cells to the 3D collagen cultures of luminal epithelial cells leads to bilayered acinar structures in a way akin to those found in the mammary gland [73], although cells from different tissues of an organ such as luminal and myoepithelial cells of the mammary gland preferentially adhere to their own kind in such models. In invasive breast cancer, the cross-talk between cancer-associated fibroblasts (CAFs) and neoplastic cells [74–76] and between immune and malignant cells [77,78] has been shown to play a role in the malignant progression and behavior. Although the importance of these heterotypic cell-cell interactions are long appreciated, the implementation of cultures including multiple cell types has proven challenging. Access to primary human breast tumor-derived stromal cells is limited for routine in culture experimentation, although decent human fibroblast cell lines from different organs do exist. Whether these immortalized cell lines faithfully represent the distinct types of stromal cells, however, is not well studied. In addition, optimal culture conditions that allow each cell type to grow and maintain a differentiated state in combination with the other cell types for longer periods of time have yet to be fully established. Other challenges to be overcome include the analysis of the experiments performed, including the isolation of the individual cell types or the identification of the origin of secreted factors [79,80]. Due to these technical obstacles, the heterotypic cell models described in the literature to date mostly employ co-cultures of a single stromal cell type with normal and malignant mammary epithelial cells (but see below, [81]).

Several studies have reported on the co-culture of breast cancer cells with fibroblasts [74,82–86], with immune cells such as macrophages [87,88] or with adipocytes [89–92] generally using lrECM, collagen or spheroid models and demonstrated their reciprocal communication and the effect of stromal cells on the behavior and signaling of breast cancer cells (see below). It should be noted, however that the effect of stromal cell lines on gene expression or drug response has also been examined using transwell assays or direct co-culture of stromal and breast cell lines in conventional 2D monolayers [93–95]. In addition, also multicellular-culture systems have been reported, including breast tumor cells with a mix of fibroblasts, vascular smooth muscle cells and pericytes in 3D collagen gels [74], or MCF10A, human fibroblasts and adipocytes in a lrECM/collagen mixture on porous silk protein scaffolds [96]. Recently, a tunable 3D breast cancer tissue test system combining heterocellular tumor spheroids, polymeric microcarriers and adipocytes has been developed

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to investigate the behavior of breast cancer cells in response to different environmental stimuli [97].

For the study of disseminated breast tumor cell dormancy, we have recently engineered organotypic models of lung- and bone marrow-microvascular niches using three different cell types. For this, microvascular niches derived from the culture of human umbilical vein endothelial cells (HUVECs) were seeded on top of either lung fibroblasts or bone marrow mesenchymal stem cells and breast cancer cells were seeded on top of the formed microvasculature [81] (Fig. 4). These organotypic 3D models recapitulated what was observed *in vivo* in mice and zebrafish, where the stable trunk of the microvasculature allowed breast cancer cell lines to become dormant but where the vessels were sprouting, the tumor cells 'woke' up and grew, revealing that a stable microvasculature induces sustained quiescence of breast cancer cells [81].

Finally, in a way akin to the synthetic 3D scaffolds, also in the field of co-culture systems, tissue engineering now focuses on the production of patterned culture strategies supporting the study of heterotypic cell-cell contacts [98] with the aim of developing artificial organs and tissues. The design of such multicellular normal or cancerous breast cell *in vitro* models will then allow the dissection of the heterotypic interactions between mammary epithelial or breast cancer cells and normal *versus* tumor-associated stromal cells, as well as the effects on therapy response.

4. SIGNALING PATHWAYS AND COMPLEX BIOLOGICAL MODELS

Signaling pathways that function in parallel in cells growing on cell culture plastic become reciprocally integrated or reprogrammed when cultured in lrECM or with stromal cells. In our laboratory, in addition to the usual breast cancer cell lines and, we have utilized the HMT3522 breast tumor progression series with the nonmalignant (S1), pre-malignant (S2, S3) and malignant (T4-2) human breast epithelial cell lines derived from the reduction mammoplasty of a woman [99,100]. When cultured in 3D lrECM, this series can be used as a model for studying acquisition, maintenance, disruption, and reacquisition of tissue polarity. As described above, the non-tumorigenic S1 cells form growth-arrested, polar colonies with hollow lumen (resembling breast 'acini'), whereas the tumorigenic T4-2 cells forms disorganized colonies that continue to proliferate [40]. By studying these cells in 3D lrECM we observed that the T4-2 tumorigenic phenotype could be reverted by interfering with a number of signaling pathways [101]. The inhibition of β1-integrin or EGFR, of different components of MAPK and PI3K signaling pathways, or of Raf-induced MMP9 in the T4-2 tumor-like colonies leads to a functional and morphological reversion of these tumorigenic cells to growth-arrested acinar-like structures [51,100,102–104]. In fact, the attenuation of specific aberrant signaling pathways in cancer cells grown in 3D lrECM leads to normalization of key signaling pathways to the level of those present in normal cells, a feedback inhibition that does not occur in 2D cultures [102,105]. For example, the β1-integrin and EGFR signaling pathways were shown to be coupled and bidirectional when cells are propagated in a 3D lrECM environment but not in 2D [102].

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For all reversion studies the inhibitors used were added to the culture at the single cell stage [41,51,100,102–104]. To make the assay more relevant to the clinic, we developed two additional modifications of our 1992 assay for testing drugs. We allowed the non-malignant S1, pre-malignant S2 or other tumor cell lines to form their respective non-malignant and malignant phenotypes *before* inhibitors were added, either by removing individual colonies from inside lrECM gels and placing them onto agarose or methyl cellulose without disturbing the 3D structures they had formed [106], or allowed the cells to form their respective structures on top of the lrECM and tested these directly [107]. In the first series of studies, we tested six different apoptotic agents used in the clinic and showed that resistance to cell killing directly correlated with the colony ('tissue') polarity rather than with the malignant phenotype or the rate of growth [106]. The second series of studies demonstrated further that treatment with the β1-integrin inhibitory antibody AIIB2 leads to decreased proliferation and increased apoptosis of several breast cancer cell lines, but not of the nonmalignant cell line S1 [107]. Furthermore, we observed that inhibition of β1-integrin enhanced the efficacy of radiotherapy human breast cancer xenografts [108], providing evidence to suggest that β1-integrin could be a therapeutic target for breast and other types of cancer [109]. Three-dimensional lrECM culture models have been adopted in screens for the discovery of novel targets in breast cancer [110] (Fig. 5). As mentioned above, EGFR tyrosine kinase inhibitor treatment leads to phenotypic reversion of malignant T4-2 cells into growth-arrested, polarized structures resembling non-malignant cells. Using T4-2 cells transduced with a cDNA library, we identified *FAM83A* as a candidate gene conferring resistance of T4-2 cells to reversion and death in 3D lrECM when treated with an EGFR tyrosine kinase inhibitor [110].

In a way akin to S1 cells, the 3D culture of the non-malignant MCF10A mammary epithelial cells, and its progression series, leads to formation of growth-arrested acinus-like structures that recapitulate some aspects of glandular architecture [40,46]. The introduction of oncogenes or activated growth factor receptors into HMT3522 or MCF10A cells disrupts their morphogenesis and elicits distinct morphological phenotypes [111,112]. Expression of signaling pathway effectors such as a conditionally active variant of AKT in MCF10A cells have been shown to elicit large, misshapen structures as a result of enhanced proliferation and increased cell size [113], whereas hyperactivation of transmembrane receptors such as HER2 or CSF-1R has been shown to disrupt cell polarity, and leads to repopulation of the luminal cavity, and/or the formation of multiacinar structures [111,114]. In fact, the multi-acinar phenotype induced by HER2 in MCF10A cells propagated in 3D lrECM shows great similarities with human *HER2* amplified DCIS lesions [111]. Also *DEAR1*, a gene encoding a member of the TRIM subfamily of RING finger proteins, has been reported to be a dominant regulator of acinar morphogenesis in normal mammary gland cells and to be mutated/ deleted in breast cancer [115]. The MCF10A model has also been used to unravel signaling pathways in breast cells. For example, p57 has been identified as an effector of crosstalk between the ERK and Akt pathways in MCF10 cells, which played a key role in determining the proliferative response of these cells to the growth factors EGF, IGF-1 or also insulin [116]. In addition, it was shown that the cell culture context (i.e. 2D vs 3D) led to differential IL-6/STAT3 signaling and dependency for migration, invasion and tumorigenesis in MCF10A-Ras cells [117]. The 3D lrECM models of non-malignant

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mammary epithelial cells, while few, have great value in studying the effects of loss-of-function or gain-of-function of specific genes on cell signaling and tumorigenesis.

The 3D models not only can distinguish between normal and malignant cell phenotypes, but can also be utilized to learn details of differences between the tumor cell lines. When grown in lrECM, breast cancer cell lines form tumor-like structures with characteristic morphologies, which have been categorized into four classes, referred to as Round, Mass, Stellate and Grape-like [49]. These 3D morphologies are in part reflective of underlying genetic aberrations or gene expression patterns as breast cancer cell lines of 'Stellate' 3D morphology lack E-cadherin, whereas those with 'Grape-like' morphology have elevated HER2 expression levels [49]. Furthermore, there is evidence to suggest that cell-cell and cell-ECM interactions provided by 3D models alter signaling pathways in breast cancer cells when compared to monolayer cultures. The activation of signaling pathways downstream of HER2 has been observed to switch between PI3K and MAPK pathways in *HER2* amplified breast cancer cell lines either grown in spheroids [118] or 3D lrECM [119] compared to 2D culture plastic, which affects the response of these cell lines to HER2-targeting agents. In fact, the ability to activate parallel signaling pathways regulating proliferation and survival depending on the environmental cues highlights the cross-talk between pathways, which may adapt to different microenvironments and selective pressures such as targeted therapies.

5. BIOMARKERS AND COMPLEX BIOLOGICAL MODELS

Cancer cell lines grown in conventional monolayer cultures have been used to link pharmacological data with genomic information and helped identify rare genotypes associated with targeted therapy response [120–122]. Some of these genomic response predictors identified *in vitro* have been or are in the process of being translated into the clinic (e.g. [123–125]). These data suggest that monolayer cultures are powerful tools identifying those subsets of cancers whose proliferation is reliant on the activation of a specific oncogenic driver, most of which are kinases.

In contrast, a large number of studies using standard *in vitro* culture systems have reported also on the identification of biomarkers for prognosis, markers for metastatic progression or potential drug targets, claiming great potential for their use in human breast cancer. Regrettably, only few, if any, of these biomarkers, have been translated into clinical practice. To improve the success rate of the implementation of biomarkers and drug targets and/or the development of candidate drugs identified *in vitro*, more reliable and representative human breast cancer tissue surrogates may bridge the gap between cell line and *in vivo*/clinical studies. To date, biomarkers studies in breast cancer using 3D model systems are limited to those employing lrECM cultures, and represent proof of principles that by allowing tissue organization and ECM signaling, specific aspects important for the behavior of cells *in vivo* are recapitulated also *in vitro*. For example, a microarray-based "3D-signature" was developed based on the genes differentially expressed between the initially disorganized proliferating non-malignant mammary epithelial cells when seeded in 3D lrECM and the final organized polarized growth-arrested cells [126]. This 3D-signature, which mainly captured cell cycle-related genes, was shown to predict outcome in three human breast cancer microarray datasets [127].

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Biomarker studies using more complex 3D cell culture models are yet to be performed. In particular, for high-throughput drug screening approaches more complex breast cancer models have great potential and, as the tumor microenvironment has been shown to play a role in drug resistance and tumor dormancy [81,128], may provide new leads for the development of biomarkers and treatment approaches. In addition, given the differences in signaling pathway activation between cells grown in 2D and 3D culture systems, novel approaches for 3D culture systems (e.g. based on hydrogel-based scaffolds, nanoparticles or meshes [129–136]) may provide novel avenues for high-content [137–139], RNA interference [140], cDNA [110] and chemical screens [141,142] that may help address fundamental questions emerging from the recent massively parallel sequencing studies of human cancers. In fact, novel technologies and approaches for 3D tissue culture systems that are scalable and could possibly be employed in high-throughput experiments have been developed (Table 1), and one could envisage the implementation of these approaches to define which genetic aberrations found in cancer cells are actual drivers of the disease. The use of 3D culture systems is likely to be even more important in the case of loss of function mutations, given that this approach would more accurately define which pathways would be in synthetic lethal interactions with the mutations found if the microenvironmental cues are present, which cannot be recapitulated in 2D models.

6. CONCLUSION

The use of 3D lrECM culture systems to model normal mammary epithelial morphogenesis, dissect pathways involved in breast cancer progression and assess the effects of potential oncogenes or tumor suppressor genes on the polarized acinar-like structures has proven extremely valuable. In fact, the matrix stiffness of commercially available lrECM seems to closely mirror that of normal breast tissue [55], and mouse mammary epithelial cells cultured in 3D lrECM gels were shown to respond to lactogenic hormones by producing and secreting milk proteins [29,39,105]. For breast cancer, in particular for understanding of the epistatic interactions between genetic aberrations, the structural and stromal microenvironment and drug response, 3D heterotypic cell culture models more representative of the different types of primary breast tumors and the metastatic setting are required. Such model systems should be amenable to high-throughput screening approaches and high-content read-outs. One may hypothesize that for the design of truly representative 3D multicellular breast cancer cell culture systems that can be effectively implemented as standard *in vitro* models in research laboratories, multidisciplinary tissue engineering and translational breast cancer research efforts will be required.

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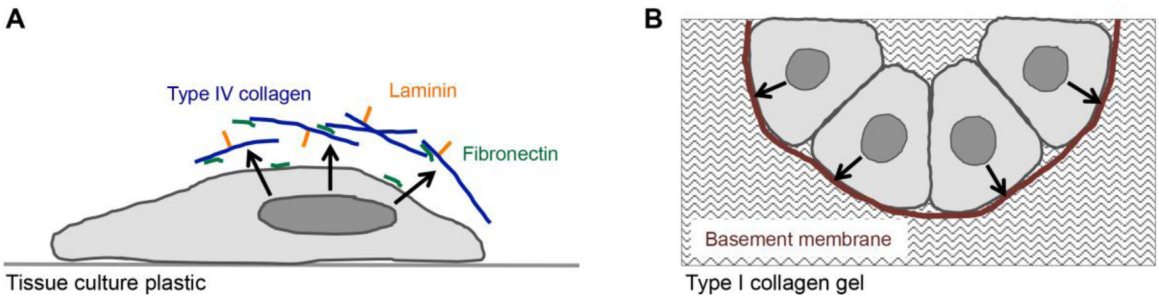

Fig. 1. *De novo* synthesis of extracellular matrix components of cultured normal mammary epithelial cells is dependent on the substratum

(A) Normal mammary epithelial cells cultured on tissue culture plastic secrete laminin and other ECM proteins such as fibronectin and type IV collagen but fail to organize, whereas (B) normal mammary epithelial cells grown cultured on type I collagen gels deposit an endogenously synthesized basement membrane and recapitulate their *in vivo* phenotype [31,32].

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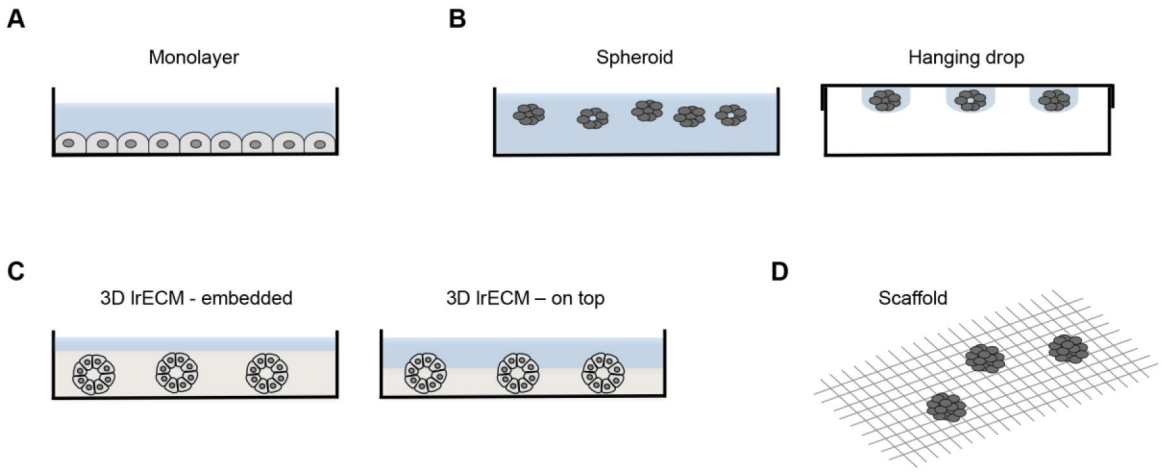

Fig. 2. Three-dimensional cell culture systems

For the study of breast cancer, (A) Conventional monolayer cell cultures [120,121,146]; (B) Spheroid cultures, where cells spontaneously aggregate either growing in low attachment plates (left) or hanging drops (right) [80,118,138,147]; (C) 3D lrECM cultures, where cells are either embedded (left) or growing on top of the ECM overlaid with a dilute solution of lrECM (right) [39,40,49,111]; and (D) Natural or synthetic polymers and macroporous scaffolds support the formation of 3D structures of cells [52,97], have been employed.

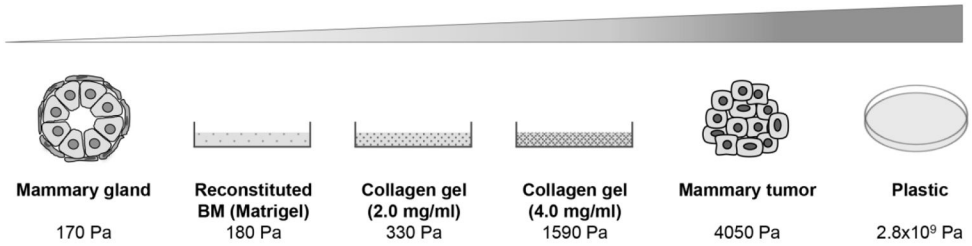

Fig. 3. Stiffness of tissues and culture model systems

Stiffness (or elastic modulus), measured in Pascals (Pa), of normal mouse mammary gland, mouse tumors, and matrices and plastic used in cell culture models. Data from [55,128]. BM, basement membrane.

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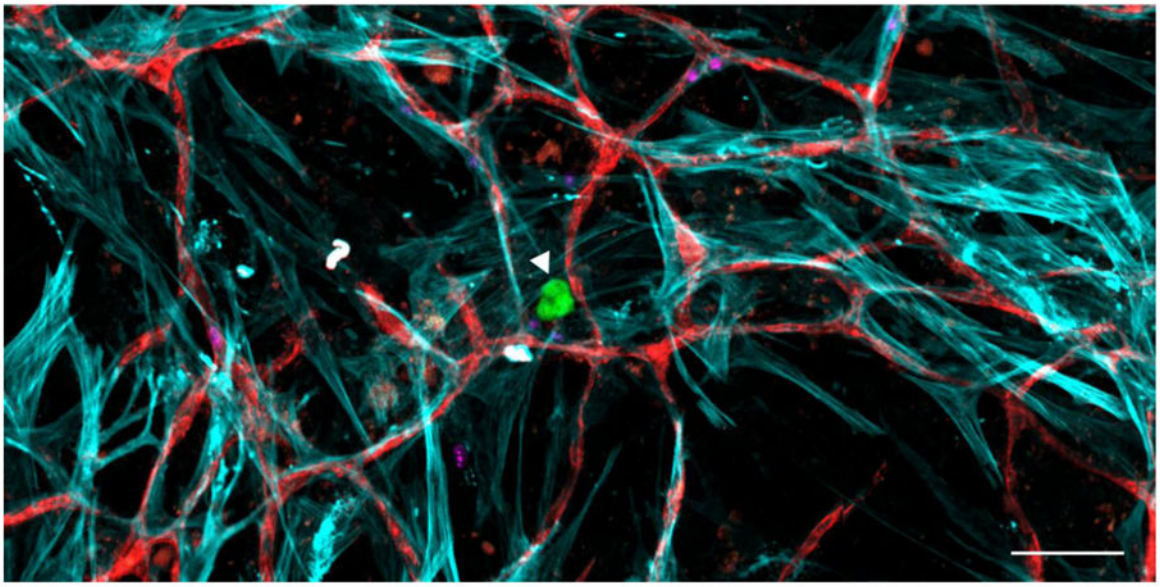

Fig. 4. Complex three-dimensional cell culture model: microvascular niche

Lung-like microvascular niches were created by co-culture of lung fibroblasts and human umbilical vein endothelial cells (see [81]). This assay was used to demonstrate that the microvascular endothelium (denoted by CD31 immunostaining, red) steers malignant, genotypically aberrant breast epithelial cells (T4-2 cells, green, as indicated by white arrowhead) into a quiescent state characterized by a lack of Ki-67 immunostaining (magenta). In this assay, a subset of lung fibroblasts express alpha-smooth muscle actin (cyan) and associate intimately with the microvasulature, behaving as pericytes. Scale bar=100 μM. (C.M. Ghajar & M.J. Bissell, unpublished data [81]).

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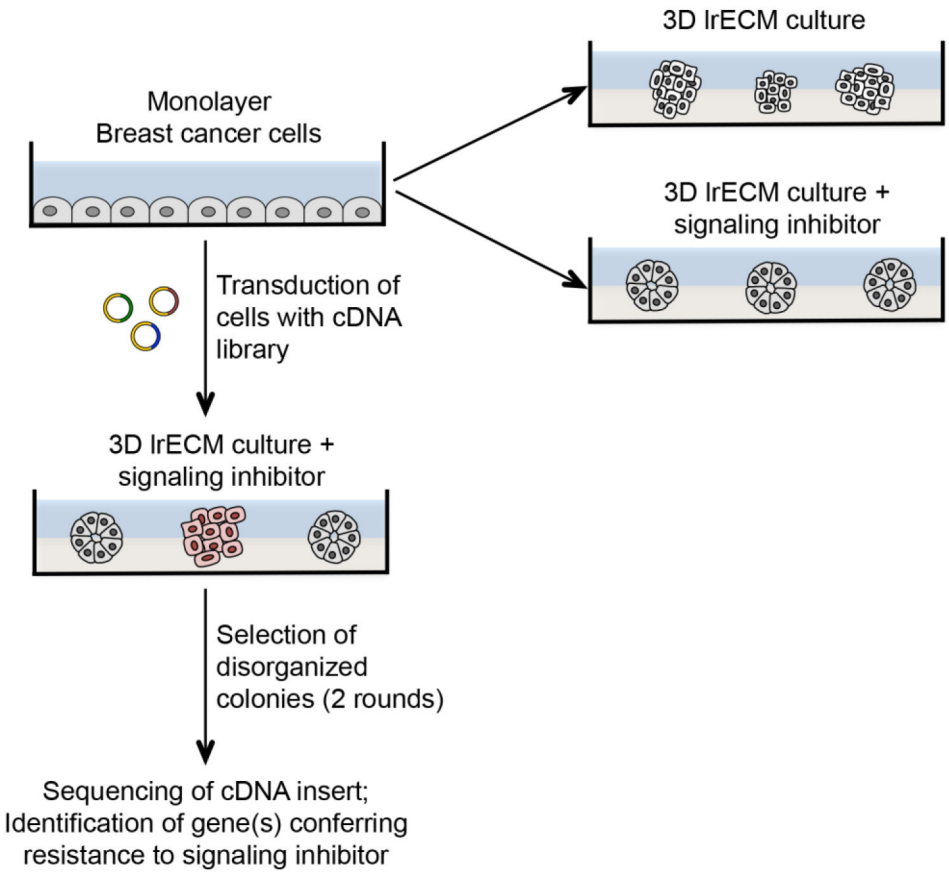

Fig. 5. Screen to identify genes conferring resistance to signaling inhibitors

Breast cancer cells, such as T4-2 cells, form unpolarized tumor-like structures when grown in 3D lrECM cell cultures. Treatment with signaling inhibitors, such as small molecule EGFR tyrosine kinase inhibitors, induces reversion of the tumor-like structures to a polarized acinar phenotype (right). A disorganized unpolarized structure of breast cancer cells transduced with a cDNA library is selected after signaling inhibitor treatment, and the cDNA insert sequenced to identify genes conferring resistance to reversion and signaling inhibition (left) [110].

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Table 1

Examples of scalable 3D culture models for high-throughput screening approaches.

3D Platform	Material	High-throughput endpoints	Instrument required for read-out
Microarray [131,135,143]	Hydrogel (collagen, alginate), lrECM	Viability; metabolite analysis	Microarray scanner; fluorescence microscope; metabolite analyzer
Micro-scaffold array chip [129]	Macroporous gelatin sponge	Viability	Microplate reader; confocal microscope
Microchamber array (spheroids) [144]	Non-attachment honeycomb chambers	Viability (drug response)	Fluorescence microscope
Microtiter plate [130,133,137]	Hydrogel (collagen, alginate)	Viability, morphology; Invasion	Light, fluorescence, confocal microscope; microplate reader
Microtiter plate [132]	Nanoparticles	Cell migration, viability	Mobile-device (iPod); light microscope; microplate reader
Cells-in-Gels- in-Mesh (CiGiM) [134,141]	Stacked sheets of polymer-based mesh supporting cells in lrECM (in 96-well holders)	Viability (drug response); morphology	Fluorescence gel scanner; light, confocal microscope; microplate reader
Microfluidic chip [139]	lrECM in a PDMS device on a glass slide	Viability (drug response)	Fluorescence microscope
Hanging drop microwell plates [138,145]	Microwell plate	Viability (drug response); gene expression	Fluorescence microscope; real-time PCR machine

lrECM, laminin-rich extracellular matrix; PDMS, polydimethylsiloxane.