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Three-Dimensional Culture of Human Breast Epithelial Cells: The How and the Why

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Abstract

Organs are made of the organized assembly of different cell types that contribute to the architecture necessary for functional differentiation. In those with exocrine function, such as the breast, cell–cell and cell–extracellular matrix (ECM) interactions establish mechanistic constraints and a complex biochemical signaling network essential for differentiation and homeostasis of the glandular epithelium. Such knowledge has been elegantly acquired for the mammary gland by placing epithelial cells under three-dimensional (3D) culture conditions.

Three-dimensional cell culture aims at recapitulating normal and pathological tissue architectures, hence providing physiologically relevant models to study normal development and disease. The specific architecture of the breast epithelium consists of glandular structures (acini) connected to a branched ductal system. A single layer of basoapically polarized luminal cells delineates ductal or acinar lumena at the apical pole. Luminal cells make contact with myoepithelial cells and, in certain areas at the basal pole, also with basement membrane (BM) components. In this chapter, we describe how this exquisite organization as well as stages of disorganization pertaining to cancer progression can be reproduced in 3D cultures. Advantages and limitations of different culture settings are discussed. Technical designs for induction of phenotypic modulations, biochemical analyses, and state-of-the-art imaging are presented. We also explain how signaling is regulated differently in 3D cultures compared to traditional two-dimensional (2D) cultures. We believe that using 3D cultures is an indispensable method to unravel the intricacies of human mammary functions and would best serve the fight against breast cancer.

Keywords

3D culture; Breast epithelium; Basoapical polarity; Cancer progression; Tissue architecture

Introduction

1.1. Structure and Function of the Breast Epithelium

The mammary gland is composed of a series of branched ducts that connect the functional glandular units (acini) to the nipple (see Fig. 1). As early as 1840, Sir Astley Paston Cooper published his observation of a branched organization of the mammary gland with distinct lobes or ductal systems, which each opens at the nipple (1). The existence of multiple ductal systems, the number of which varies between individuals, is now well established (2, 3).

The branching ducts, as well as acini located at the extremities of each ductal system, are composed of two cell layers: an inner layer of secretory luminal epithelial cells, with apical microvilli, surrounded by contractile myoepithelial cells. The luminal cells of acini are arranged radially with tight junctions between cells located at the narrow width of the cells,

near the central lumen (see Fig. 1). The primary function of the acinus is milk secretion. Milk flow into the ducts is powered by the contractions of the myoepithelial cells.

The breast epithelium has a unique behavior compared to other tissues in the human body since it continues to develop after birth. It undergoes extended remodeling with cycles of branching, acini formation, and dissolution of epithelial structures during puberty, pregnancy, lactation, and involution. Moreover, there are less extensive and repeated modifications during menstrual cycles (4). The BM, a specialized form of ECM linking epithelial and connective tissues, and the adjacent stroma that traps an abundance of soluble factors constitute the microenvironment of the epithelium. Myoepithelial cells and luminal cells are in contact with the BM (5, 6), the composition of which also undergoes modifications according to the physiological status of the mammary gland (7, 8). The BM is constituted of a polymeric network of collagen IV and laminins, notably laminins-111 and -332. The laminins and collagen IV are interconnected by nidogen and perlecan (9). Many of the BM components are involved in crucial signaling events that regulate tissue-specificity and function. The framework for such signaling was proposed already in the early 1980s (10), and the first proof in the mammary gland was provided for the expression of the milk protein β-casein which is controlled by laminin-111/β1 integrin signaling (11). The BM is also a repository for growth factors and cytokines that upon binding to their receptors trigger specific intracellular signals (12, 13).

A key feature of all luminal epithelia is the basoapical polarity axis. Transmembrane integrins at the basal side of cells serve as anchorage points and receptors for BM components. They trigger intracellular signaling and participate in the perception of the cells' microenvironment. They cooperate with growth factor receptors to control essential cellular processes such as survival, proliferation, and differentiation (14–16). Among the cell–BM contacts, basal polarity is specifically determined by the interaction between laminin-332 and α6/β4 integrin dimers that form hemidesmosomes (15). Lateral cell–cell contacts are mediated by apical tight junctions, adherens junctions, and in some instances desmosomes (17). The location of tight junctions, the uppermost apical cell–cell adhesion complex, is paramount as it permits to separate cell membrane components and receptors between the apical and basolateral cell membranes and thus, strictly defines apical polarity. The tight seal generated by tight junctions prevents milk leakage in-between cells during lactation. The apical junctional complex formed by tight and adherens junctions also organizes the cytoskeleton and associated signaling pathways, which ultimately impinges on nuclear functions. Thus, the basoapical polarity axis permits unidirectional secretion of milk components in the lumen, as well as structured integration of hormonal and mechanical signals exerted by the microenvironment.

Characterizing the mechanisms underlying normal cell behavior in the context of an organized ductal system is critical to understanding which alterations are necessary for breast cancer to progress. This is particularly important for prevention research related to breast cancer that aims at reducing the burden of this important public health concern.

1.2. Architectural Alterations During Breast Cancer Development and Progression

Tissue architecture (i.e., the organized arrangement of cells into specific multicellular structures) has been shown to be critical for the maintenance of functional differentiation and cell survival (18). It comes as no surprise that alterations in tissue architecture are needed to permit tumor formation and that tissue and cellular organization is commonly used by pathologists to precisely diagnose breast cancer. It has been proposed that the loss of apical polarity is a critical event necessary for tumor development (19). Indeed, 3D culture models of breast acini show that only cells with disrupted apical polarity can be pushed into the cell cycle (19). This hypothesis is supported by the observations reported by

several laboratories that tight junction proteins can influence cell proliferation and act as tumor suppressors (17). The impact of basal polarity on the maintenance of apical polarity is not clearly determined. Our recent results suggest that collagen IV and hemidesmosomes both influence the integrity of tight junction organization (20), indicating that microenvironmental alterations might be sufficient to perturb apical polarity. The steps that follow initial apical polarity alterations and ultimately lead to the multilayering of epithelial cells characteristic of preinvasive neoplastic stages (hyperplasia and carcinoma in situ) remain to be uncovered. Among the internal cellular changes associated with apical polarity loss is the relocation of the cell nucleus away from the basal side, as observed in cells of preinvasive neoplastic stages (21).

Breast cancer progression toward invasive stages is accompanied by the breakage of the BM (22) which allows cells to invade the underlying ECM and move into the surrounding tissue. Invasive breast tumors in vivo have been characterized by altered expression and localization of BM proteins (23) and receptors, indicating that both the organization of the microenvironment and receptor–ligand interactions are profoundly altered (24, 25). Changes in intracellular organization have also been observed during tumor progression. Notably the nucleus of invasive tumor cells displays striking changes in the distribution of splicing factor speckle components, heterochromatin and euchromatin domains, and the nuclear mitotic apparatus (NuMA) protein compared to phenotypically normal acinar cells in culture (refs. 19, 26, 27 and unpublished results from the Lelièvre laboratory). Interestingly, the distribution patterns of the chromatin-associated NuMA protein observed in 3D cultures that mimic phenotypically normal and cancerous tissues have been observed also in vivo using archival biopsy samples (Lelièvre and Knowles, unpublished data), suggesting that architectural changes observed in 3D cultures are good predictors of what could be seen in real tissues.

The pioneering work of Mintz and Illmesee (28) with teratocarcinomas brought the paradigm shifting concept that tissue architecture can override genetic and genomic changes (29). The subsequent demonstration that it is possible to induce acinus-like structures from cancer cells with profoundly altered genomes by simply modulating signaling pathways (see Subheading 3.3) has unambiguously revealed the critical role played by epithelial architecture in controlling cell fate (30).

1.3. The Third Dimension Factor in Cell Culture

Monolayer cultures (2D cultures) have been very useful models for gene discovery and early work on viral transformation but are a far cry from physiologically relevant models. Flattened cell morphologies and the spatial plane of cell–cell contacts obtained in 2D cultures are strikingly different from those observed in tissues. Cell shape is known to influence cell behavior including growth and nutrient uptake (31, 32) and gene expression (29) (see Subheading 1.7), which may partially explain the decreased expression of tissue-specific genes often observed in 2D cultures.

Organ cultures are physiologically relevant model systems but are often technically challenging and typically short-lived due to necrosis in tissue explants. Culturing cells in 3D is a more flexible alternative to organ culture. One important goal is to enable the formation of tissue structures that have precise geometrical and functional signatures. This can be achieved by providing cells with proper mechanical and chemical signals from both specific types of architectural components of the ECM and soluble molecules. Serum varies in composition and contains high levels of growth factors and hormones in unpredictable concentrations. It is known to disrupt the ability of cells to express their tissue-specific functions (33) and its use should be avoided if at all possible.

Although most cells in culture synthesize ECM components, the establishment of 3D structures from single cells usually requires the use of hydrogels (viscoelastic meshworks consisting of two or more components, one of which is water) containing exogenous ECM components that provide the structural and biochemical signaling necessary for the formation of the correct architecture and differentiation status. Originally, 3D cultures were performed in floating collagen gels, as demonstrated with murine cells (34). Using this method, morphological characteristics of differentiation, including basoapical polarity, was maintained in culture. Cells at the surface of the gel formed monolayers and some cells below the gel surface rearranged themselves to form acinus-like structures. Nowadays, Engelbreth-Holm-Swarm (EHS) extracts are commonly used as hydrogels (see Subheading 1.5). While a large number of human breast cancer cell lines can be cultured in 3D to mimic tumor development (35), the recapitulation of phenotypically normal acinar phenotypes is more challenging and usually requires the presence of BM components in the exogenous hydrogel (20, 36). A few non-neoplastic breast epithelial cell lines have been used in 3D cultures including MCF-10A (37) and HMT-3522S1 (38) cells (for a comparison of the MCF-10A and S1 models please refer to ref. 20). This chapter mainly focuses on the 3D culture of the HMT-3522 series derived from S1 cells that provides a well-studied model of breast cancer progression. The use of primary cells as complements to immortalized cell lines is also discussed.

1.4. The Example of the HMT-3522 Series: A Progression of Breast Neoplastic Phenotypes in 3D Culture

The HMT-3522 progression series is derived from a benign mammary fibrocystic lesion (38). HMT-3522 cells became spontaneously immortalized in culture, giving rise to the non-neoplatic S1 cell line. In the presence of the appropriate substratum (i.e., BM components and a specific mechanical environment) (20), S1 cells differentiate into basoapically polarized acinus-like structures of approximately 30 μm in diameter containing 25–35 cells (36) (see Fig. 2). Basoapical polarity is a critical feature of normal breast epithelia. The polarity axis is evidenced by the presence of basal BM components (laminin-332 and collagen IV) deposited by the cells, basally localized α6/β4 integrins—the bona fide laminin receptor dimer in breast epithelia—lateral cell–cell adherens junctions (with Ecadherin and β-catenin used as markers), and lateroapical tight junctions (with ZO-1 and ZO-2 core plaque proteins used as markers). The presence of a tiny lumen (often less than the size of a single cell) in S1 acini further indicates close resemblance to acini in the resting mammary gland (20). S1 cells express the luminal marker cytokeratin 18 (30). They have a number of genetic alterations (39) probably linked to their immortalized cell line status. A sub-population of S1 cells carries a mutation in p53 (His to Asp at codon 179). This mutation may confer a slight growth advantage, since its frequency in the cell population progressively increases with passage numbers (40). This phenomenon requires that the use of the S1 cells be restricted to passages below 60, to avoid the drift of the cell population. Acini formed by HMT-3522S1 cells are typically composed of one layer of luminal epithelial cells. In contrast, acini in the mammary gland are constituted by a layer of luminal cells surrounded by contractile myoepithelial cells (see Subheading 1.1 and Fig. 1). Therefore, in 3D monoculture models, luminal cell–ECM contacts are prevalent, whereas in mammary glands in vivo, luminal cells are mostly in contact with myoepithelial cells and make only punctual contacts with the BM (5, 6). Myoepithelial cells are largely responsible for making BM components (41). Surprisingly, although S1 cells are luminal in their behavior and main characteristics, the basal portion of these cells displays myoepithelial characteristics (e.g., presence of vinculin). We believe that these myoepithelial characteristics permit the formation of the appropriate endogenous BM necessary for acinar differentiation.

Malignant transformation of the S1 cells was achieved in vitro by altering the composition of the culture medium (39, 42). The HMT-3522S2 subline, growing independently of EGF and bearing characteristics of preinvasive carcinoma (43–45), was isolated. These cells are actually sensitized to EGF and would not thrive in its presence. They also require coating the flasks with collagen I for 2D culture. S2 structures in 3D culture are heterogeneous in size. Homogeneous S3 sublines (HMT-3522S3-A, S3-B, and S3-C) have been derived from S2 3D cultures by selecting for colony size (45). S3 cell lines display progressive loss of polarity, genomic anomalies, and gene expression changes characteristic of preinvasive to invasive transition (45).

S2 cells that had reached passage 238 were found to produce tumors in nude mice. The T4-2 subline was obtained from a tumor after two rounds of in vitro–in vivo mouse passage. T4-2 cells are highly tumorigenic and have a triple-negative phenotype (i.e., no expression of estrogen, progesterone, and ErbB2 receptors) (45). In 3D culture, T4-2 cells develop structures reminiscent of invasive breast tumors (19, 30). The nodules reach approximately 200 μm in diameter after 10 days in culture (see Fig. 2). Thereafter, the size of the nodules remains relatively stable due to a balance between cell division and apoptosis.

Compared to widespread models of aggressive and metastatic cancers that give little insights into the early events of cancer progression, the HMT-3522 cancer progression series offers unique opportunities to study early phases of tumorigenesis. The series allows for direct comparisons between nonmalignant acini models (S1), preinvasive nodules (S2), and invasive tumors (T4-2). There exists other interesting breast cancer progression series. Non-neoplastic MCF-10A parent cell lines were reported to form acinus-like structures devoid of apical polarity as their acini usually lack apical tight junctions (20, 46, 47). These cells can undergo malignant transformation upon exogenous expression of chimeric ErbB2 receptors (48). This receptor consists of the ErbB2 intracellular domain fused to the synthetic ligand-binding domain of the FK506-binding protein (FKBP) and to the extracellular and transmembrane domains of the p75 nerve growth factor receptor. Homodimerization of chimeric p75-ErbB2 can be induced with a FKBP ligand, leading to the activation of ErbB2 signaling without interfering with endogenous ErbB2 receptors. Activation of p75-ErbB2 in MCF-10A acini produced in the presence of EHS extracts induces cell proliferation and multilayering. The resulting structures retain epithelial properties and are not invasive; thus, they may represent a model of early stage mammary cancer in vitro (48). MCF-10A cells have also been transformed using T24 Ha-ras, giving rise to the MCF10-AneoT cell line (49). The MCF10-AT cell lines, derived from xenograft-passaged MCF10-AneoT, represent premalignant stages. However, a subset of MCF10-AT xenographs developed into preinvasive and invasive carcinomas in immunodeficient mice. One such tumor was isolated and cultured in vitro, leading to the malignant MCF10CA1 cell line (50, 51).

1.5. Challenges and Limitations of 3D Cultures

One of the major challenges of 3D culture is to reproduce a microenvironment close enough to the in vivo situation to permit proper differentiation into phenotypically normal tissues, and to accurately mimic different tumor stages. During the development of 3D models of specific tissues, it is important to test for compliance with the architecture and physiology of the in vivo counterparts. An ECM-like hydrogel substratum and an appropriate medium need to be carefully chosen, in order to solve the problem under study using conditions relevant to the organ in vivo. Ideally, well-characterized hydrogels with defined ECM components should be used. However, with the exception of collagen I, pure ECM components are very costly and difficult to formulate as hydrogels. As an alternative, an ECM mixture isolated from Engelbreth-Holm-Swarm mouse sarcoma cells (52) provides an acceptable BM approximation in terms of components and organization, especially when studying noninvasive breast tissue structures and tumors. Commercially available EHS

extracts contain laminin-111, type IV collagen, proteoglycans, and entactin. The composition of commercially available EHS extracts is not fully elucidated and varies between lots; this implies that lots need to be tested for a particular application (see Note 1). Engineered ECM-like hydrogel substrata are being developed as alternatives to EHS extracts, but, to our knowledge and to this date, there is no report that they allow non-neoplastic breast epithelial cells to recapitulate acinar differentiation.

In the mammary glands, acini are connected to ducts (see Fig. 1); whereas human acinar models in 3D cultures are isolated sphere-like entities that rarely branch out. The 3D culture protocols aim at keeping the growth conditions as constant as possible using chemically defined cell culture media and carefully tested lots of EHS-derived hydrogels. Despite these efforts, variations inherent to the biological origin of the additives used in the cell culture media and components of the hydrogel are difficult to control, as is the heterogeneity of primary cultures or even established cell lines. Moreover, the physiological changes found in vivo (e.g., estrous cycles and functional changes associated with puberty, pregnancy and menopause) are yet to be readily explored using 3D culture models.

Ultimately, results obtained using 3D models need to be validated in vivo. This is a challenge when working with human cells. Available in vivo alternatives are murine models bearing human cells (e.g., cells cultured in cleared fat pad) and human tissues from biopsies and reduction mammoplasties. Experiments with xeno-graft models can be influenced by the animals' physiology and the context into which the cells are injected, and human biological samples provide only frozen moments in time. Nevertheless, there have been several compelling examples showing that what was discovered in 3D cultures indeed illuminated important phenomena in vivo (see Subheading 1.7).

1.6. Primary Cells Versus Cell Lines

Primary cells are nonimmortalized cells obtained directly from tissues. Breast tumor cells can be obtained from breast cancer patients undergoing surgical treatment whereas non-neoplastic mammary cells are typically derived from reduction mammoplasty or from milk. Primary cells have proven to be useful to study breast phenotypes (53). They are heterogeneous and hence truly represent their tissue of origin. Primary cells do not carry genetic (polyploidy, mutations) and phenotypic (e.g., rapid growth) alterations linked to immortalization. However, their use in culture is limited due to the small number of divisions achievable in vitro, which hampers long-term studies. Variability within primary cell populations also represents a challenge since it may reduce experimental reproducibility. Finally, compared to immortalized cell lines, the access to primary cells is limited.

1.7. Examples of Applications of 3D Lumen Breast Epithelial Cell Cultures

This section is a nonexhaustive description of the use of 3D human breast epithelial cultures in a number of research areas (54). Due to space limitations, we have focused only on a few studies presenting compelling and original demonstrations of the usefulness of 3D cultures to study different aspects of breast tissue biology.

Three-dimensional tissue models have provided unique information pertaining to cell signaling, notably that there exist crosstalks between signaling pathways that are only

[1]Matrigel™ is only good for 2 years when stored at –80°C. Due to variability inherent to biological materials, new gel lots need to be tested in order to ensure reproducibility between experiments using different lots. A typical test procedure consists of culturing side-by-side S1 cells using new as well as currently used gel lots. After 10 days in culture, morphological characteristics (diameter and smoothness of the multicellular structures) are evaluated and basoapical polarity and cell proliferation are scored after immunostaining for specific markers (see Note 4). T4-2 tumor phenotypes also need to be validated when changing the gel lot.

established under certain architectural conditions (i.e., when an acinus is formed). The crosstalk between integrin and growth factor receptors signaling in the glandular epithelium is now well established (16). Originally, it was shown that epidermal growth factor receptor (EGFR) and β1 integrin signal transduction pathways were coupled in breast acini obtained in 3D culture, but not in 2D cultures of the same breast epithelial cell line (55). The predominance of tissue architecture in establishing specific signaling networks was confirmed by the restoration of the crosstalk in cancer cells induced to form basally polarized acinus-like structures. This example illustrates the large impact of tissue architecture on signaling from the microenvironment (29, 56).

Three-dimensional cultures are being used extensively also to study how basoapical polarity is established, maintained, and compromised in mammary epithelia. In particular, laminin-111 was shown to be essential for proper polarization. Luminal epithelial cells cultured on collagen I substratum adopted an inverted polarity; but proper polarity could be rescued by laminin-111 (23). Blocking ECM signaling through β4 integrins prevented the phenotypic reversion of the malignant T4-2 HMT3522 cells (30, 57), and blocking the same signaling pathway in S1 acini compromised apical polarity (20), suggesting that the establishment of basal polarity is needed to maintain apical tight junctions. The underlying mechanisms remain to be discovered.

Intracellular organization in 3D cultures often closely resembles the organization in vivo. In particular, the nuclear structure of acini produced in 3D culture and that observed on sections of acini in resting mammary glands are strikingly similar. This observation is illustrated by the distributions of the nuclear mitotic apparatus (NuMA) protein (ref. 58, Knowles and Lelièvre, unpublished), and certain markers of higher order chromatin organization (59). A tremendous advantage of 3D culture models over fixed human tissues is that they can be used for functional experiments with physiological relevance. Studies conducted with 3D acini and 3D tumor models have revealed that nuclear organization actively modulates cell and tissue phenotypes (60). A clear demonstration of the impact of nuclear organization on cell behavior in epithelial cells came from studies performed with mammary acini in 3D culture (26). Alterations induced in the distribution of the nuclear protein NuMA using function blocking antibodies, expression of dominant negative truncated forms of the protein, and siRNAs were shown to impair acinar differentiation, alter BM integrity and lead to proliferation and cell death (19, 26, 59). This “dynamic reciprocity” between NuMA and the cell phenotype was not observed in 2D cultures. More particularly, the work performed in 3D culture demonstrated that the protein NuMA was influencing the higher order organization of chromatin (59) normally achieved upon acinar differentiation (19). Recurring findings that changes in cell shape during differentiation are accompanied by the remodeling of nuclear organization and alterations in gene expression profiles (60) strengthen the importance of studying biological processes in tissue contexts.

Specific recognition of the influence of tissue architecture on tumor development has come from the demonstration that cancer cell phenotype can be reverted by selectively modulating cell communication with the microenvironment (18). Relatively simple experiments had already indicated that the arrangement of tumor cells could impact their behavior. Indeed tumor cells were found to change their sensitivity to cancer chemotherapeutic drugs when placed on soft agarose (61). The behavior displayed by cells organized into a tumor was in fact similar to that of xenografts, indicating that the formation of a tumor nodule (instead of a flat monolayer of cells) mimicked the in vivo situation concerning the response to treatments. Later on, it was demonstrated that in breast cancer cells placed in 3D culture, drug sensitivity was influenced by basal polarity, and notably that hemidesmosome-directed signaling was conferring resistance to treatments aimed at killing cancer cells (57). These findings have important implications for research on DNA repair (Rizki, Jasin, and Bissell,

unpublished) and apoptosis, and for the development of chemotherapeutics. They clearly indicate that 2D cultures, although easily amenable to high-content assays, are poor predictors of the effect of drugs in vivo.

Finally, 3D cultures in hydrogels have revealed the importance of mechanical stimuli for tissue differentiation. Manipulation of the mammary gland's microenvironment stiffness (using glutaraldehyde fixation of collagen I gels or by mixing collagen I to laminin-111 gels) was found to affect β-casein expression (24). Interestingly, increased ECM stiffness characteristic of malignant tumors resulted in increased cell growth and altered organization of non-neoplastic mammary epithelial cells (62).

In summary, virtually all cellular processes studied so far appear to be influenced by the architectural and microenvironmental contexts. Hence, 3D culture models provide invaluable tools to elucidate fundamental biological questions under physiologically relevant conditions.

2. Materials

2.1. Cell Culture Medium for HMT-3522 Cells

Unless indicated otherwise, additives are dissolved in Milli-Q water, filter-sterilized (0.22 μm), aliquoted, fast-frozen in liquid nitrogen and stored at –80°C.

1. Dulbecco's Modified Eagle's Medium (DMEM/F12, Life Technologies™), stored at 4°C.
2. Prolactin (Sigma-Aldrich®): Diluted to 1 mg/mL (30.3 IU/mL) in 26 mM (2.22 mg/mL) sodium bicarbonate. Stable for 1 year at –80°C and 1 month at 4°C.
3. Insulin (Sigma-Aldrich®): Dissolved as a 2 mg/mL concentrated stock in 5 mM HCl. The stock solution is filter-sterilized and diluted to 100 μg/mL in sterile Milli-Q water. Stable for 6 months at –80°C and 1 month at 4°C.
4. Hydrocortisone (Sigma-Aldrich®): Dissolved as a 5 mg/mL concentrated stock solution in 95% (v/v) ethanol. The stock is further diluted to 0.5 mg/mL (1.4×10^{-3} M) in ethanol and kept at –80°C. Stable for 1 year at –80°C and 1 month at 4°C.
5. β-Estradiol (Sigma-Aldrich®): Dissolved as an 8 mg/mL concentrated stock solution in 95% (v/v) ethanol. The stock solution is then serial-diluted in ethanol to 2.67×10^{-5} mg/mL (10^{-7} M). β-Estradiol should be protected from light. Aliquots can be stored for 6 months at –80°C and 1 month at 4°C.
6. Sodium selenite (BD Biosciences): Dissolved as a 20 mg/mL concentrated stock and serial-diluted to 2.6 μg/mL. Stable for 1 year at –80°C and 1 week at 4°C.
7. Transferrin (Sigma-Aldrich®): Diluted to 20 mg/mL. Stable for 3 months at –80°C and 1 month at 4°C.
8. Epidermal growth factor (EGF, BD Biosciences): Dissolved to 20 μg/mL. Stable for 3 months at –80°C and 1 week at 4°C.

2.2. Propagation of HMT-3522 Cells in Monolayer (2D) Cultures

1. Cell culture flasks (T-75).
2. Solution of trypsin (0.25%) and ethylenediamine tetraacetic acid (EDTA, 1 mM) (Life Technologies™).

3. Soybean trypsin inhibitor (SBTI, Sigma-Aldrich®): Prepared as a 10 mg/mL dilution in sterile Milli-Q water, filter-sterilized, aliquoted, fast-frozen, and stored at –80°C. SBTI solution is stable for 6 months at –80°C and 2 weeks at 4°C.
4. Type I collagen solution (PureCol®, Advanced Biomatrix) and sterile PBS, used for coating plastic surfaces for the culture of HMT-3522S2, S3, and T4-2 cells: 9 mL of a (1:44) PureCol® :PBS dilution is added to a 75 cm^2 flask for a minimum of 24 h at 4°C. Before use, the PureCol® dilution is aspirated and the flask is rinsed with 5 mL DMEM/F12.

2.3. Culture of HMT-3522 Cells in 3D

1. Four-well chamber slides (Lab-Tek™, Nunc) are used for immunostaining experiments and are amenable for direct imaging using the drip or HTP 3D culture methods (see Subheading 3.2).
2. Four-well plates (Nunc) are used for immunostaining experiments on frozen sections of 3D cultures obtained from embedded 3D culture methods (see Subheadings 3.2 and 3.6).
3. Tissue culture dishes (Falcon, 35 mm in diameter) are used for biochemical assays (protein, RNA, and DNA extracts).
4. Basement membrane matrix from EHS extracts (Matrigel™, BD Biosciences; other sources may be appropriate). Matrigel™ is kept at –80°C and thawed on ice 24 h before use. If rigorously kept at 0°C, Matrigel™ is stable for 1 month after thawing; it can only be thawed twice. Thus, usually a 10 mL bottle is thawed to prepare 1–2 mL aliquots that will be frozen and kept at –80°C until use. For the optimal culture of human breast cell lines currently available, it was observed that Matrigel™ should have low levels of endotoxins (<4 U/mL) and a protein concentration below 13 mg/mL. In addition, certain research teams prefer to use growth-factor depleted Matrigel™. Due to the variability between lots, Matrigel™ lots need to be tested before use (see Note 1).
5. Collagen I gel (PureCol®), used to induce the formation of nonpolarized nodules from S1 cells of sizes similar to that of acini obtained with Matrigel™ (see Subheading 3.3).

2.4. Retrieval of Cells and Tissue Structures from the Extracellular Matrix

1. Dispase (50 U/mL, BD Biosciences).
2. Collagenase type IV (250 U/mg, Life Technologies™).

2.5. Introduction of Small Peptides and Antibodies into Live Cells in 3D Culture

1. Permeabilization buffer: 25 mM HEPES buffer (pH 7.2), 78 mM potassium acetate, 3 mM magnesium acetate, 1 mM ethylene glycol-bis(2-aminoethylether)- *N, N, N′, N′*-tetraacetic acid (EGTA), 300 mM sucrose, 1.0% (w/v) bovine serum albumin. Filter-sterilize and store aliquots at –20°C. Permeabilization buffer can be stored at 4°C for up to 1 month.
2. Digitonin (caution, toxic material): Dissolved as a 0.5% solution in Milli-Q water, filter-sterilized and kept at 4°C. Heating (90°C, up to 4 h) is necessary for solubilization when making the stock solution.
3. Peptides and function-blocking antibodies (this is application-specific and normally used to modify the action of an endogenous protein of interest).

2.6. Cryosections from Embedded 3D Cultures

1. 18% (w/v) sucrose (ACS grade) in PBS.
2. 30% (w/v) sucrose in PBS.
3. Tissue-Tek® optimum cutting temperature (O.C.T.).
4. Dry ice.
5. Cryostat.
6. DAPI (Life Technologies™).
7. Superfrost glass slides.

3. Methods

3.1. Propagation of Non-Neoplastic and Neoplastic Breast Epithelial Cells

Culture cell lines from the HMT-3522 breast cancer progression series (39) in the absence of serum in chemically defined H14 medium (38, 63) (see Note 2 and Subheading 3.1, step 1). The same culture medium is used for nonmalignant S1, preinvasive S2/S3 cells, and malignant T4-2 cells, except that EGF is omitted from S2, S3, and T4-2 cultures. HMT-3522 cell lines are propagated in 75 cm^2 cell culture flasks. For S2, S3, and T4-2 cultures, PureCol®-coated flasks are used (see Subheading 2.2, step 4). Rigorous attention should be paid to seeding density to avoid changes in phenotypes (see Note 3).

Splitting cultures of HMT-3522S1, S2, and T4-2 cells:

1. Prepare fresh H14 medium (volumes of additive stocks, see Subheading 2.1, per 10 mL medium are indicated in brackets): DMEM/F12, 5 μg/mL (0.15 IU/mL) prolactin (50 μL), 250 ng/mL insulin (25 μL), 1.4 μM hydrocortisone (10 μL), 0.1 nM β-estradiol (10 μL), 2.6 ng/mL sodium selenite (10 μL), 10 μg/mL transferrin (5 μL), 5 ng/mL EGF (2.5 μL).
2. Remove the medium, rinse briefly with 1 mL trypsin/EDTA, aspirate the solution, and incubate for 7–12 min in 1 mL trypsin/EDTA solution at 37°C.
3. Suspend the dislodged cells in 10 mL DMEM/F12 supplemented with 180 μL of 10 mg/mL SBTI for trypsin inactivation and count cells using a hemocytometer.
4. Centrifuge (115 × *g*, 5 min, room temperature) volumes of cell suspension corresponding to 1.75×10^6 cells (S1, 2.3×10^4 cells/cm^2) or 875,000 cells (S2 and

[2]A major reason for not using serum for the culture of HMT-3522 cells is to have a defined medium to monitor tissue phenotypes and signaling. It is also desirable to prevent exposure to concentrations of growth factors and cytokines that the cells do not generally experience in vivo.

[3]In order to avoid phenotype drifts (i.e., selection of cells with more or less aggressive behavior or less differentiation capabilities), cells are only used within defined passage numbers (passages 52–60 for S1 cells; T4-2 cells can be split usually ten times without seeing a shift in aggressiveness compared to the first passage used in 3D culture). In addition, S1 cell cultures are split no sooner than day 8 and no later than day 12. These cells need time to deposit BM components on the cell culture surface. Passages performed before day 8 would select for rapidly adhering cells that do not differentiate well. Waiting longer than 12 days may lead to difficulties in detaching cells with trypsin. T4-2 cell cultures are split when 70–80% confluence is reached (typically after 4–5 days in culture) as waiting longer would select for cells with less aggressive phenotype in 3D culture. Finally, the culture medium is replaced no earlier than 48 h after plating the cells in order to avoid selection for fast-adhering (more aggressive) S1 cells and to allow cells to benefit from the production of paracrine and autocrine factors during the initial phase of the culture. In 2D culture, HMT-3522S1 cells organize into patches (islands) delineated by a layer of cells that display a morphology typical of epithelial cells, with nuclei basally located against the inside of the island and cytoplasm extending toward the outside of the island (see Fig. 2). The proliferation rate of S1 and T4-2 cells in seed cultures needs to be monitored since aberrant growth rates often reflect changes in cellular metabolism that have profound consequences on the phenotypes obtained in 3D cultures.

T4-2, 1.15×10^4 cells/cm^2). Resuspend cell pellets in 10 mL H14 medium and seed in 75 cm^2 flasks.

5. Change the culture medium every 2–3 days. A medium change before 2 days would deplete cells of important ECM components, whereas a medium change after 3 days might be damaging as nutrients will become too scarce. Use strictly 5% CO_2 and 37°C for the cell incubator environment as deviating from these settings would lead to profound changes in cell behavior, and possibly death, in 3D culture.

3.2. 3D Culture Systems: Variations on a Theme

Cells are cultured in 3D in the presence of the hydrogel. Several techniques have been developed (refs. 20, 64, see Fig. 3) for this type of culture. Irrespective of the culture method used, normal and malignant phenotypes obtained in 3D culture need to be validated (see Note 4). The embedded method was initially used for HMT-3522 cultures (36). In this setting, individual cells are included within EHS-derived gels covered by culture medium. The technique offers the advantage of providing a homogeneous microenvironment to the cells. It is useful to study the invasive phenotype of tumors rather than the invasive capabilities of individual cells measured in Boyden chambers (65). However, the thick gel layer renders a number of applications challenging, in particular direct fluorescence imaging. Embedded cultures can be cryo fixed and sectioned for immunostaining experiments (see Subheading 3.6).

3.2.1. Embedded Culture

1. Prepare H14 medium (see Subheading 3.1).
2. Apply a thin coat of EHS-derived gel to the tissue culture surface (10 μL/cm^2). Dispense the EHS-derived gel quickly by spreading the drop deposited in the center of the culture vessel using a pipette tip and let solidify by incubating 5 min at 37°C in the cell culture incubator. The EHS-derived gel aliquot used to prepare 3D cultures should be kept on ice at all times to avoid solidification and degradation.
3. Detach cells with trypsin and count cells (see Subheading 3.1).
4. Centrifuge a cell suspension volume corresponding to the desired amount of cells per individual culture device (if several wells or culture dishes are used, cell amounts cannot be pooled due to the nature of the embedding technique as described below). For S1 cells, use 90,000 cells/cm^2 of culture device. For S2, S3, or T4-2 cultures, use 45,000 cells/cm^2 of culture device.
5. Resuspend the cell pellet in a volume of DMEM/F12 that corresponds to 10% of the total EHS-derived gel volume that will be needed. The total EHS-derived gel volume is typically 0.15 mL/cm^2.

[4]Quality control is essential to confirm that tissue structures obtained in 3D culture indeed mimic physiologically relevant tissue structures in vivo. The first step in the validation process is the morphological comparison between structures obtained in 3D culture and their counterpart in vivo. Under optimal conditions, S1 cells in 3D culture should differentiate into smooth spherical structures of ~30 μm in diameter, whereas T4-2 cells should produce irregular structures with a broader size distribution (50–200 μm range). Differentiated mammary acini are quiescent (i.e., cells have exited the cell cycle) and basoapically polarized (see Figs. 1 and 2). In a typical S1 acini culture, close to 100% of the acini display correctly distributed basal markers and approximately 75% of the acini display correctly distributed apical markers under optimal culture conditions. The percentage of apically polarized acini may drop to 50% or 60% from one experiment to another since apical polarity seems very sensitive to microenvironmental conditions (20). In contrast, T4-2 tumor-like structures contain proliferating cells and, although certain basal and apical polarity markers are expressed, these makers typically show highly disorganized distributions. The percentage of quiescent cells can be monitored by Ki67 immunostaining. Basal polarity markers include type IV collagen, laminin-332, and α6 and β4 integrins. Tight junction protein ZO-1 is used as a robust apical polarity marker.

6. Mix EHS-derived gel (0.15 mL/cm^2 of tissue culture surface) to the cell suspension by pipetting up and down briefly (usually once or twice). Avoid forming bubbles. Transfer immediately to the tissue culture container, adding the cell suspension evenly across the surface precoated with EHS-derived gel.
7. Place at 37°C in the cell culture incubator for 30 min to let the EHS-derived gel solidify.
8. Add H14 medium (volume = volume of EHS-derived gel × 1.5) slowly to the side of the culture vessel to avoid disturbing the gel.
9. Place 3D cultures back in the cell culture incubator. Replace H14 medium every 2–3 days. For S1 cultures, it might be necessary to use H14 without EGF starting day 7 of culture to optimize acinar differentiation. For S2, S3, and T4-2 cultures, always use H14 without EGF.

3.2.2. Drip Culture—The drip method is an alternative to the embedded method which offers greater flexibility in the experimental design, especially for high-resolution imaging applications.

1. Coat the tissue culture surface with EHS-derived gel (see Subheading 3.2.1, step 2), but use 50 μL of gel per cm^2. Let solidify by incubating 30 min at 37°C in the cell culture incubator.
2. Prepare H14 medium (see Subheading 3.1).
3. Detach cells with trypsin and count cells (see Subheading 3.1).
4. Centrifuge a cell suspension volume corresponding to the desired amount of cells. For S1 cells, use 42,000 cells/cm^2 (e.g., 400,000 cells per 35 mm dish or 50,000 cells per well in 4-well chamber slides). For S2, S3, or T4-2 cultures, use 21,000 cells/cm^2.
5. Resuspend the cell pellet in H14 medium in half of the final volume of H14 culture medium (e.g., 650 μL for a 35 mm dish or 200 μL per well for 4-well chamber slides) and add the cell solution drop by drop evenly over the cell culture surface.
6. Let the cells settle down for 5 min.
7. Drip a volume equivalent to that used for the cell suspension of 10% (v/v) Matrigel™ in H14 onto the cells (evenly over the entire culture surface) and return the vessels to the incubator. The resulting final EHS-derived gel concentration of 5% in the culture medium appears to be optimal to obtain apical polarity with non-neoplastic HMT-3522S1 cells (20).
8. Change the culture medium (H14) every 2–3 days (see Subheading 3.2.1). Tissue structures are typically analyzed between day 10 and day 12 of culture, unless specific treatments of tissue structures need to be tested (see Subheading 3.3). After the day of plating, the gel drip does not need to be repeated when changing the medium.

The drip method has also been used for 3D culture of other non-neoplastic (e.g., MCF-10A) and malignant breast cell lines including MCF-7, MDA-MB-231, BT20, and BT-474 (35).

3.2.3. "High Throughput" Culture—A new technique has been developed for HMT-3522S1 acini culture (ref. 20, see Fig. 3). This technique, referred to as "high throughput" (HTP), can be used to obtain S1 acini with basoapical polarity and morphological characteristics indistinguishable from acini in "drip" or "embedded" cultures.

The HTP technique circumvents the need to coat cell culture surfaces with EHS-derived gel, hence reducing handling time and permitting the production of large quantities of acini for high-content screening methods and biochemical analyses. The absence of a gel coat on the culture surface greatly reduces background signals in live cell and immunostaining experiments.

1. Follow steps 2–8 from Subheading 3.2.2. Deposit S1 cell suspensions directly on the glass or plastic substratum.
2. Change the culture medium every 2–3 days, removing EGF on day 6. Cells are used after 8 days for imaging or biochemical experiments. Cells in 3D culture are in general more sensitive to alterations in their environment compared to 2D culture (see Note 5), irrespective of the method used for 3D culture.

3.3. Reversion of Tumor Cells and Manipulation of Nonmalignant Phenotypes

The phenotype of malignant cells can be “reverted” to mimic phenotypically normal acinus-like spheroids or partial acinar differentiation (see Fig. 2). This approach is particularly useful to determine if a difference in phenotype or behavior between non-neoplastic and neoplastic cells results from differences in tissue architecture, cell cycle status, or the genetic background. Moreover, models of non-neoplastic multicellular structures like the acini can be manipulated, notably to affect the basoapical polarity status. Established protocols for tumor cell reversion and for the manipulation of non-malignant phenotypes in 3D culture are referenced in Table 1. A powerful method is to add function-blocking antibodies targeting integrins to the culture medium starting the day of cell plating. 1 mL of cell suspension is first incubated for 30 min with 15 μg/mL antibodies at 37°C; then cells are plated in 3D culture in the presence of 15 μg/mL antibodies. This technique has been used to obtain reverted T4-2 structures resembling S1 acini but lacking apical polarity.

The same approach was applied to block the establishment of basoapical polarity (57) in S1 cultures. It is also possible to treat with antibodies or small molecules after the establishment of non-malignant acinar structures and tumor-like colonies, hence mimicking clinically relevant situations to assess specifically the effect of potential anticancer drugs on their tumor targets and/or the side effects on normal tissue (66). An example of the application of this method is illustrated by the suppression of β4-integrin signaling in S1 acini (20). Preformed acini are released from the drip culture with dispase (see Subheading 3.5) and incubated with function blocking antibodies (30 min at 37°C), before replating in the presence of antibodies using the drip method (see Subheading 3.2.2).

3.4. Design of Functional Experiments in a 3D Culture Setting: Mechanical and Biochemical Aspects

Cells in 3D culture usually have a morphology that does not seem amenable to the use of classical transfection reagents. Moreover, non-neoplastic breast epithelial cells usually become quiescent after a few days in the presence of EHS-derived or collagen 1 gels. To circumvent these limitations, cells can be transiently transfected in 2D culture, just before plating them under 3D culture conditions. This approach can be applied to silence the expression of genes coding for proteins with relatively slow turnover using RNA interference (59). Stable transfection of cells in 2D culture prior to use in 3D culture is also possible (57, 59), but clonal selection should be avoided since some of the clonal

[5]In our experience, cells are much more resilient to alterations in their environment when cultured in 2D compared to 3D. For example, subtle variations in the cell culture medium—such as altered water quality or variations in additive concentrations—may have no visible effect on cells cultured in 2D while profoundly altering cell behavior (differentiation, proliferation, survival) in 3D culture. Moreover, treatments with certain drugs or dyes that may be well tolerated by cells in 2D cultures might drastically change phenotypes in 3D culture and even lead to cell death.

populations might lack/lose differentiation capability. Other possibilities include viral infection followed by selection of stably transduced cells in 2D culture prior to placing them in 3D cultures (67). If the transgene should only be expressed following differentiation in 3D culture, conditional expression should be used (68).

Function-blocking antibodies or peptides can be introduced inside cells in 2D and 3D cultures to disrupt protein functions or to manipulate signaling pathways. This method requires plasma membrane permeabilization (58, 59):

1. Remove the cell culture medium and cover the cells with 0.01% (w/v) digitonin in permeabilization buffer (see Subheadings 2.5, step 1 and 2.5, step 2).
2. Incubate for exactly 1 min (30 s in 2D culture) at room temperature.
3. Aspirate digitonin solution promptly and wash twice 5 min at 37°C with permeabilization buffer.
4. Remove the permeabilization buffer and add interfering peptides or function-blocking antibodies (generally at 15 μg/mL) in H14 medium. Proper controls should also be used (e.g., corresponding nonspecific immunoglobulin for function-blocking antibody experiments).
5. Incubate at 37°C. Incubation times need to be empirically determined, but they typically range between 30 min and 5 h. Longer incubations are not useful as the cell membrane pores created by digitonin will close. The cells can be kept in culture for several days. Normally, cells should get rid of the antibodies after a couple of days, but if the antibodies were trapped in a specific cellular compartment, they might stay until the protein that they target undergoes turnover.
6. Proceed with the analysis. Antibodies introduced upon cell permeabilization can be detected in immunostaining experiments by adding only the fluorescently labeled secondary antibodies.

Although technically challenging, microinjection can be applied to 3D tissue-like structures (20) and could be used for nucleic acid delivery.

3.5. Dissolution of Hydrogels for Biochemical Assays

Classical biochemical analyses of RNAs and proteins are possible using 3D cultures. Although some of these experiments can be performed by extracting the cellular material directly from 3D cultures containing the hydrogel, it is often better to first isolate the tissue structures from the exogenous substratum, typically by solubilization of the ECM using proteases (collagenase for collagen I gels or dispase for EHS-derived gel). Cell suspensions are then processed using standard protocols. Below is a protocol used for the isolation of HMT-3522S1 acini and T4-2 tumor nodules:

1. Remove the cell culture medium and add dispase solution (0.75 mL/mL of EHS-derived gel).
2. Incubate for 30 min at 37°C.
3. Wash cells three times in DMEM/F12 medium and once in a buffer compatible with downstream applications (e.g., PBS supplemented with protease and phosphatase inhibitors). Each wash is followed by centrifugation (450 × *g*, 5 min, room temperature). *Note* : A similar method is used to release tissue structures to be treated with function-blocking antibodies against BM receptors before replating the structures in the presence of EHS-derived gel. In the latter case, all four washes

are in DMEM/F12 medium. Do not reduce the number of washes; otherwise, cells will be injured by leftover proteases.

4. Cell pellets are further processed for RNA or protein analysis, or subcellular fractionation using standard protocols.

In contrast to trypsin/EDTA treatments, dispase (or collagenase) treatment does not affect cell–cell contacts. This is important since disruption of cell–cell junctions with EDTA would result in profound changes in cellular organization and gene expression that may influence the outcome of the experiments. The recently developed HTP culture for HMT-3522S1 acini (ref. 20, see Subheading 3.2.3) is an alternative for larger-scale production of acinus-like structures. With the HTP culture, dispase treatments with shorter incubation time (10 min) can be used to release the acini.

3.6. High-Resolution Imaging

Immunostaining can be performed on tissue structures obtained with different 3D culture techniques (see Subheading 3.2). Embedded cultures are too thick for direct immunolabeling and imaging. Therefore, cryosections have to be prepared (20, 30):

1. Remove the cell culture medium from 3D embedded cultures in four-well plates.
2. Incubate for 15 min in 18% sucrose at room temperature, rocking slowly.
3. Remove the solution and incubate for 15 min in 30% sucrose at room temperature, rocking slowly.
4. Remove the sucrose solution as much as possible and add Tissue-Tek® O.C.T. freezing solution to the cells.
5. Freeze 3D cultures by placing the 4-well plate on dry ice for 30 min.
6. Gently remove 3D culture blocks using a tweezer and store at –80°C. When ready to use, make frozen sections with a cryostat using a thickness setting between 4 and 20 μM. Check that sections contain cells by staining with DAPI and viewing under a fluorescence microscope every few slices.
7. Deposit the cryosection on superfrost glass slides and maintain slides at –20°C in the cryostat chamber until use or storage.
8. Store sections at –80°C until immunofluorescence labeling. Immunostaining can also be performed directly on 3D cultures (“drip” and “HTP” methods). These types of 3D cultures offer the advantage of avoiding artifacts caused by sectioning and permit volume imaging. Direct immunostaining often results in high fluorescence background due to the EHS-derived gel, in particular when using the drip method. To control background issues, blocking and washing steps are doubled compared to standard immunostaining protocols. S1 acini range between 25 and 35 μm in diameter, whereas T4-2 nodules are typically 50–200 μm in diameter after 10 days of 3D culture (see Fig. 2). These intact structures can be easily imaged using confocal laser scanning microscopy.

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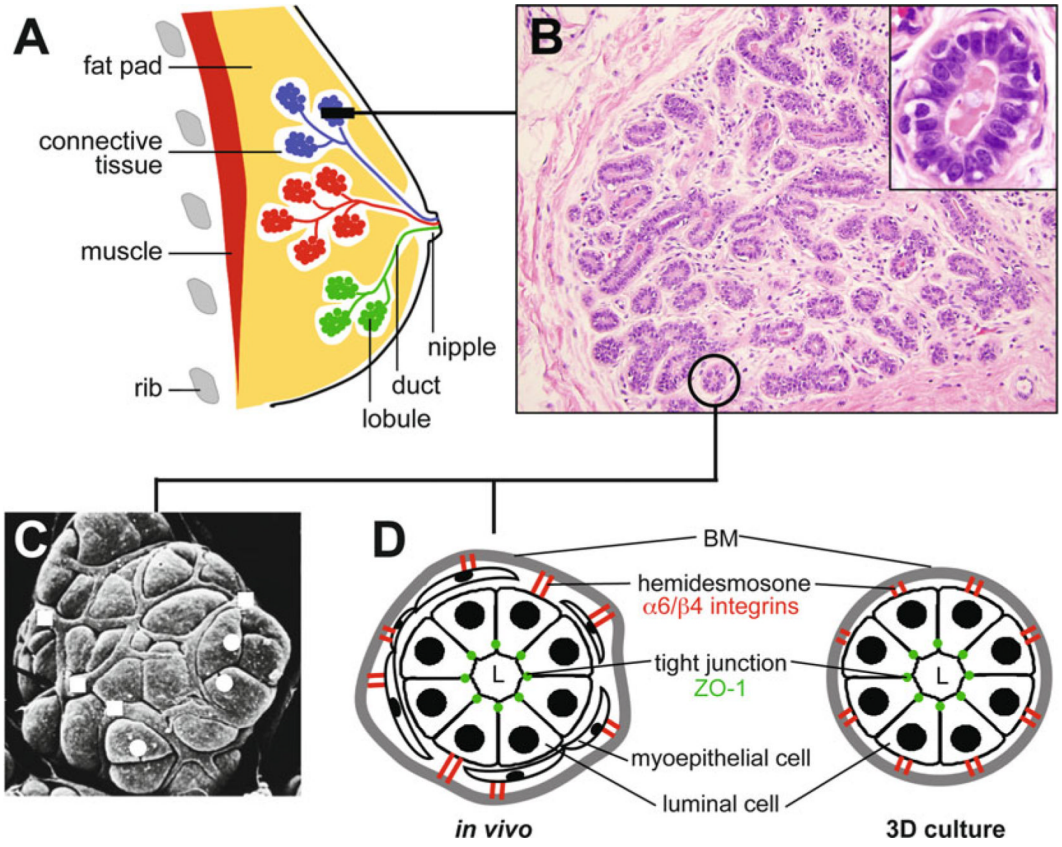

Fig. 1.

Anatomy of the mammary gland. (**A**) Schematic representation of the breast. Distinct ductal systems are represented with different colors. (**B**) Cross section of a breast lobule stained with hematoxylin and eosin (nuclei appear in *blue*). A higher magnification view of the smallest structural and functional mammary gland unit (acinus) is shown in the inset. (**C**) Scanning electron micrograph from an acinus. Luminal cells (*white circles*) and myoepithelial cells (*white squares*) are visible. Reproduced from Rosen's Breast Pathology (Third Edition, 2009) with permission from Wolters Kluwer. (**D**) Schematic of cross sections through an acinus in vivo (*left*) and in 3D culture (*right*). Basoapical polarity is evidenced by cell–cell tight junctions (*green dots*) at the apical pole and cell–BM junctions (hemidesmosomes, *red*) at the basal side. Polarity markers are indicated. In 3D monoculture lacking myoepithelial cells, cell–BM contacts are exclusively mediated by luminal cells. Nuclei are shown in *black*. *L* lumen.

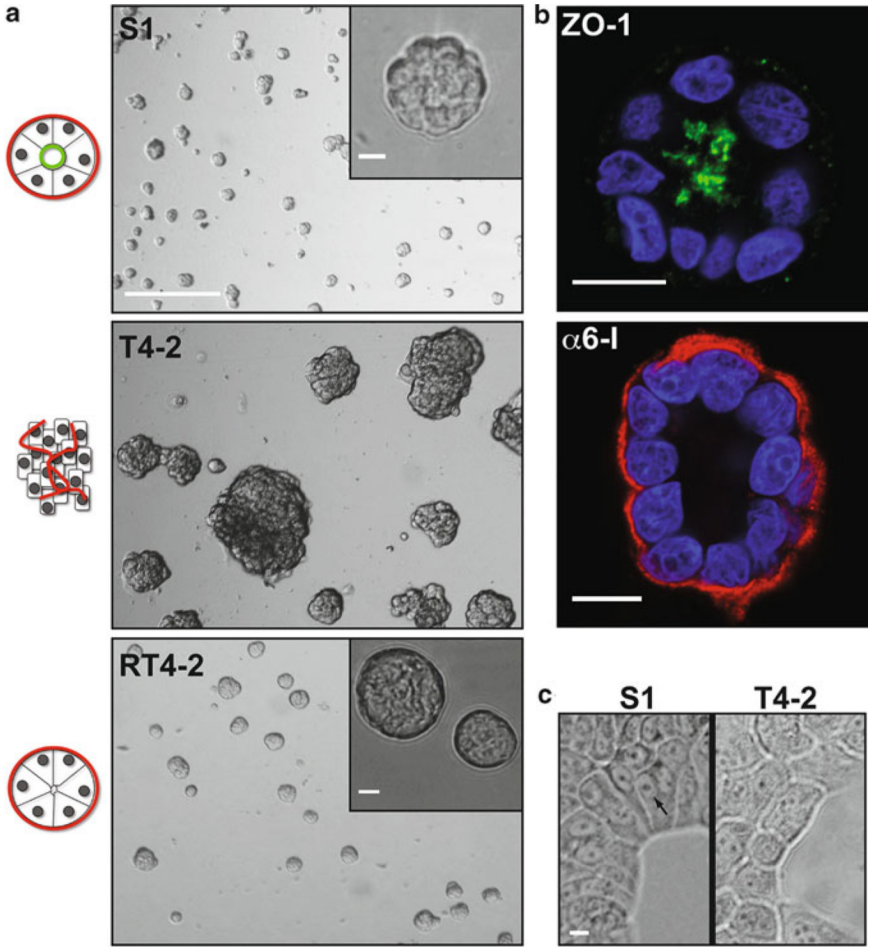

Fig. 2.

Examples of phenotypes of the HMT-3522 progression series. (**a**) Bright field images of HMT-3522 cells in 3D drip cultures. Non-neoplastic HMT-3522S1 cells form structures reminiscent of mammary acini, whereas HMT-3522T4-2 malignant cells develop into disorganized and heterogeneous nodules. T4-2 cell phenotype can be reverted (RT4-2) into spheroid structures similar to S1 acini but lacking apical polarity by blocking specific signaling pathways (see Subheading 3.3). Schematic representation of the tissue structures are shown on the *left*. The distribution of basal and apical polarity markers is highlighted in *red* and *green*, respectively. Size bar, 200 μm (10 μm for insets). (**b**) Immunostaining for ZO-1 (*green*) apical and α6-integrin (*red*) basal polarity markers in S1 acini differentiated in 3D culture. Images were captured by confocal microscopy. Nuclei are stained with DAPI (*blue*). (**c**) Bright field images of 2D cultures on plastic. After an initial proliferation stage, most of the S1 cells exit the cell cycle and organize into “islands.” Cells at the periphery of these islands have a trapezoidal morphology and nuclei are located toward the inside of the island (*arrow*). Size bar, 10 μm (for **b** and **c**).

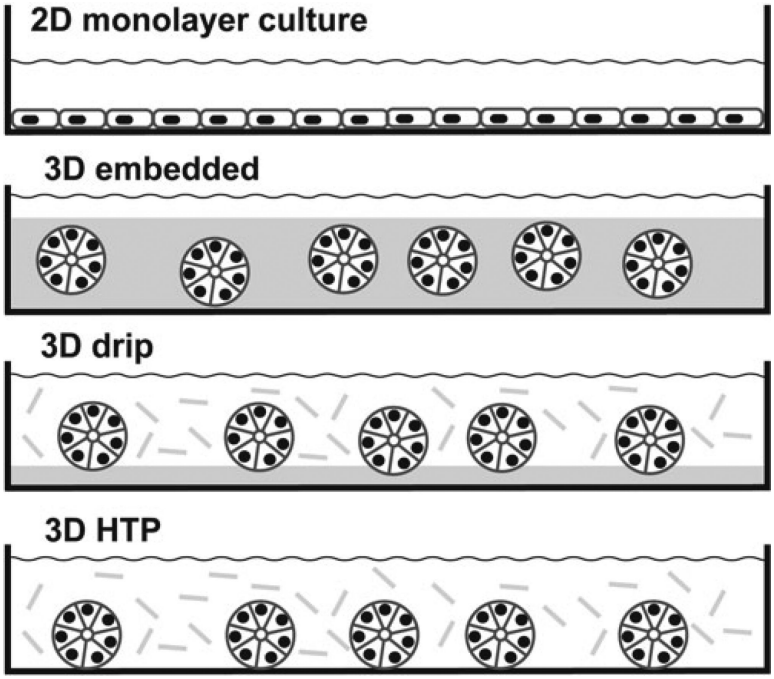

Fig. 3.

Drawings of different cell culture systems. Cells are propagated as monolayer cultures on plastic (2D culture). For 3D assays, cells can be embedded in EHS-derived gel (*gray*, 3D embedded), cultured on top of a thin EHS-derived gel coat with a 5% final gel drip (3D drip), and (for S1 cells) grown directly on glass or tissue culture-treated plastic with a 5% final gel drip (3D HTP). For the 3D drip and HTP methods, the EHS-derived gel is diluted in the culture medium (*gray bars*) and dripped onto the cells at the time of seeding. Drawings represent acini. Cell nuclei are shown in *black.*

Table 1

Tumor cell reversion and manipulation of non-neoplastic phenotypes

Cell line	Treatment	Phenotype	Reference
T4-2	(a)EGFR inhibition using function-blocking antibodies or Tyrphostin (AG1478) (b)β1 integrin blocking (AIIB2 function-blocking antibodies) (c)PI3K inhibition (LY294002)	Spheroid[a]	(30, 55, 69, 70)
MDA-MB-231; MCF-7	(a)β1 integrin blocking + PI3K inhibition (b)β1 integrin blocking + MAPK inhibition (PD98059) (c)E-cadherin expression + β1 integrin blocking (d)E-cadherin expression + PI3K inhibition (e)E-cadherin expression + MAPK inhibition	Spheroid[a]	(69)
MCF-7	CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1) expression	Acinar morphology, “lumen” formation[b]	(71)
S1	Type I collagen	“Reversely polarized” spheroid[c]	(19, 72)
S1	(a)β4 integrin blocking (CD104) (b)α6 integrin blocking (CD49F)	Acini with compromised cell–BM interactions[d]	(20, 57)

[a] Basally polarized spheroid lacking apical polarity

[b] Polarity status not determined

[c] Spheroid with inverted polarity (i.e., apical polarity markers located at the basal side)

[d] Disruption of BM signaling resulting in loss of basal and apical polarity; *EGFR* epidermal growth factor receptor