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Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures

Jayanta Debnath,[a,b] Senthil K. Muthuswamy,[a,c] and Joan S. Brugge[a,*]

[a] *Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA*
[b] *Department of Pathology, Brigham and Women's Hospital, Boston, MA 02115, USA*
[c] *Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11274, USA*

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Abstract

The three-dimensional culture of MCF-10A mammary epithelial cells on a reconstituted basement membrane results in formation of polarized, growth-arrested acini-like spheroids that recapitulate several aspects of glandular architecture in vivo. Oncogenes introduced into MCF-10A cells disrupt this morphogenetic process, and elicit distinct morphological phenotypes. Recent studies analyzing the mechanistic basis for phenotypic heterogeneity observed among different oncogenes (e.g., ErbB2, cyclin D1) have illustrated the utility of this three-dimensional culture system in modeling the biological activities of cancer genes, particularly with regard to their ability to disrupt epithelial architecture during the early aspects of carcinoma formation. Here we provide a collection of protocols to culture MCF-10A cells, to establish stable pools expressing a gene of interest via retroviral infection, as well as to grow and analyze MCF-10A cells in three-dimensional basement membrane culture.

Keywords: Three-dimensional culture; MCF-10A; Mammary acini; Oncogenes; Luminal apoptosis; Glandular architecture

1. Introduction

Glandular epithelial cells, such as those in the mammary gland, have several distinguishing histological features including a polarized morphology, specialized cell–cell contacts, and attachment to an underlying basement membrane (Fig. 1A). The development and maintenance of this polarized structure are critical for the form and function of epithelial cells [1]. Moreover, the pathogenesis of epithelial tumors, termed carcinomas, requires the disruption of this intact, well-ordered architecture.

Studies of primary human tumor tissue and mouse models of epithelial tumors have provided information on the genetic events involved in carcinoma formation. Although these approaches have been critical for understanding epithelial tumors, they are relatively intractable for studying the biochemical and cell biological pathways involved in tumor formation, especially the mechanisms responsible for early oncogenesis. In contrast, the molecular underpinnings of the signaling machinery involved in oncogenic transformation have been studied primarily in fibroblasts and, to a lesser extent, epithelial cells cultured as monolayers on tissue culture plastic. However, because monolayer cells do not recapitulate the glandular structure of epithelium in vivo, they also do not provide the optimal system for fully understanding how the regulation of proliferation, cell death, and differentiation influence the form and function of glandular epithelium, both in the normal state and during early tumor formation [1,2].

Two imminent questions that remain unanswered by traditional methods are: (1) What are the mechanisms and signaling pathways involved in organizing individual cells into a well-ordered polarized glandular architecture? (2) Does disruption of these pathways influence the architectural progression of cancer? Undoubtedly, these questions require unique approaches that can deal with the limitations imposed by monolayer cell culture and mouse modeling or human tissue studies.

* Corresponding author. Fax: 1-617-432-3969.
E-mail addresses: Jayanta_debnath@hms.harward.edu (J. Debnath), muthuswa@cshl.edu (S.K. Muthuswamy), joan_brugge@hms.harvard.edu (J.S. Brugge).

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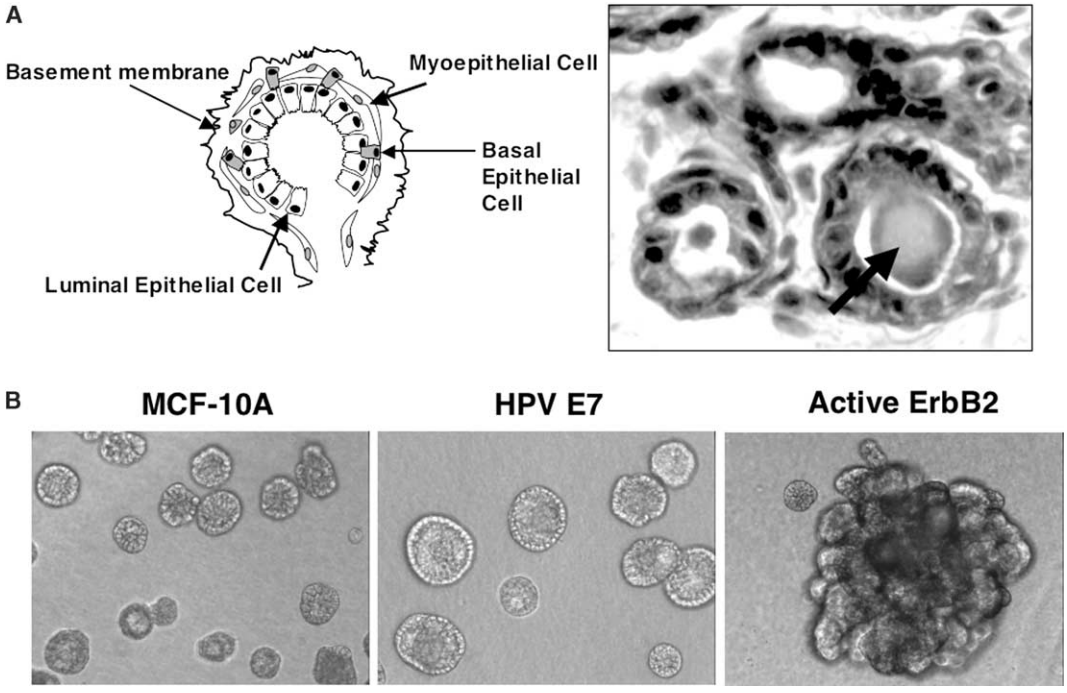

Fig. 1. Morphological architecture of mammary gland in vivo and MCF-10A acini in vitro. (A) Schematic (left) of a lobule from human mammary gland; mammary epithelium possesses a polarized architecture surrounding a hollow lumen, which is surrounded by an inner layer of luminal epithelial cells and an outer layer of myoepithelial and basal epithelial cells. A hematoxylin- and eosin-stained tissue section (right) of acini within human mammary tissue; the lumens of mammary acini in vivo often contain proteinaceous secretory material (black arrow). (B) Phase-contrast micrographs of normal and oncogene-expressing MCF-10A acini cultured on basement membrane for 20 days. Normal MCF-10A (left) acini possess a spherical architecture similar to that observed in vivo. Acini expressing HPV E7 (center) are 30% larger in size, but retain a hollow, spherical architecture, whereas activation of ErbB2 (right) during morphogenesis results in complex multiacinar structures. For further details, see [8,9].

One such experimental strategy involves the three-dimensional (3D) culture of epithelial cells. The value of studying the morphogenesis of glandular epithelium in vitro using three-dimensional culture systems has been recognized for many years [2–4]. Unlike monolayer cultures, mammary epithelial cells grown in three dimensions recapitulate numerous features of breast epithelium in vivo, including the formation of acini-like spheroids with a hollow lumen, apicobasal polarization of cells making up these acini, the basal deposition of basement membrane components (collagen IV and laminin V), and, in some cases, the production of milk proteins [5–7]. Thus, 3D epithelial culture provides the appropriate structural and functional context fundamental for examining the biological activities of cancer genes.

Recently, our laboratory has used the immortalized, nontransformed mammary epithelial cell line MCF-10A to characterize events associated with morphogenesis in vitro and to analyze the phenotypic effects of oncogenes on the morphogenesis of glandular structures. For example, overexpressing cyclin D1 or inactivating the retinoblastoma (Rb) protein with human papillomavirus E7 within mammary epithelial acini results in excessive proliferative activity within acinar structures cultured on basement membrane gels. However, despite this enhanced proliferation, the structures retain a hollow morphology because the proliferating cells lacking contact with the basement membrane undergo apoptosis [8]. In contrast, activation of the ErbB2 oncoprotein during mammary epithelial morphogenesis elicits a multiacinar phenotype, which is notable for excess proliferation, and filling of the luminal space; apparently, both protection from apoptosis and changes in apicobasal polarization contribute to this complex phenotype (Fig. 1B) [8,9].

These studies have illustrated that three-dimensional culture systems provide an important tool to interrogate the how cancer genes influence glandular architecture as well as model early events involved in carcinoma formation. Hence, these culture systems may serve as a valuable addition to the technical repertoire used by cancer cell biologists. This article provides a detailed primer of the methods used in our laboratory to culture MCF-10A cells, establish stable pools expressing a gene of interest, as well as grow and analyze MCF-10A cells in three-dimensional basement membrane culture.

2. Use of MCF-10A mammary epithelial cells in acinar morphogenesis

Several methods have been developed to culture epithelial cells as three-dimensional spheroids. Certain

tumor-derived cell lines, such as LIM 1863 colon carcinoma cells and DU4475 mammary carcinoma cells, spontaneously form gland-like organoids when grown in suspension culture [10,11]. Although spheroids derived from tumors may be useful for studies of tissue organization, cell–cell adhesion, and, in some cases, apicobasal polarization, the structures derived from these cell lines do not exhibit normal growth control properties, which limits their utility for assaying the biological activities of proliferative oncogenes.

Madin–Darby canine kidney (MDCK) cells, a commonly used nontranformed epithelial cell line, form polarized cysts with a hollow lumen when embedded as single cells within collagen gels [12,13]. Recent studies in MDCK cells have delineated the importance of assembling a laminin-rich basement for the establishment of apicobasal polarity during cyst formation [14]. Various nontransformed mammary epithelial cell lines can also be induced to undergo morphogenesis into acinus-like structures, including S2 cells (from the HMT progression series) and MCF-10A cells, which are human in origin, as well as mouse mammary epithelial cell lines, such as TAC-2 and EpH4 [5,15–18]. Primary mouse and human mammary epithelial cells also form polarized structures with a hollow lumen when cultured in basement membrane gels [5,19]. To form acini, mammary cells are usually cultured in a matrix of reconstituted basement membrane derived from Engelbreth–Holm–Swarm (EHS) tumor (commercially available as Matrigel); EHS tumor basement membrane is composed primarily of the matrix components laminin, collagen IV, and entactin; recent studies have indicated that the exogenously provided laminin in the EHS tumor-derived matrix is responsible for driving the morphogenetic process when mammary epithelial cells are cultured three-dimensionally [6].

Our laboratory has used primarily the MCF-10A cell line for three-dimensional assays. MCF-10A is a spontaneously immortalized, but nontransformed human mammary epithelial cell line derived from the breast tissue of a 36-year-old patient with fibrocystic changes [20]. These cells exhibit numerous features of normal breast epithelium, including lack of tumorigenicity in nude mice, lack of anchorage-independent growth, and dependence on growth factors and hormones for proliferation and survival [20]. Importantly, after 15 days in 3D culture, MCF-10A cells form acinar structures that retain an important characteristic found in glandular epithelium in vivo; they exhibit low levels of proliferation and remain stable with respect to both acinar size and cell number [8,9]. This suppression of proliferation observed within MCF-10A acini is of extreme experimental value, because it represents an important parameter to interrogate in analyzing the effects of oncogenes on MCF-10A morphogenesis.

Although MCF-10A cells are commonly recognized as a "normal" breast epithelial cell line, certain caveats should be recognized. First, although the karyotype is stable and near-diploid, these cells are nonetheless cytogenetically abnormal [20]. Specifically, these cells harbor genetic abnormalities commonly associated with the in vitro culture of mammary epithelial cells, including deletion of the locus containing *p16* and *p14ARF*, as well as amplification of *MYC* [K. Chin and J. Gray, personal communication; 21,22]. These changes may phenotypically cooperate with the introduction of additional oncogenes into acini. Notably, MCF-10A cells express wild-type *p53* [23].

The second limitation is epigenetic in nature and is associated with the loss of architectural cues of mammary cells during immortalization and long-term culture in vitro. The ducts and lobules that make up the mammary gland are distinguished by the presence of a central hollow lumen enclosed by an epithelium composed of multiple cell types, including luminal secretory cells, and the surrounding myoepithelial cells and basal cells (Fig. 1). Recent evidence indicates that the immortalization of primary human mammary epithelial cells (HMECs) in culture results in the outgrowth of basal epithelial cells [24]. Accordingly, MCF-10A cells are negative for estrogen receptor (ER) and express markers commonly associated with a basal epithelial phenotype, including high-molecular-weight cytokeratins and the p53 family member, ΔNp63α [20,24,25]. Although luminal epithelia are widely believed to give rise to the majority of human breast cancers, recent evidence suggests that a subset of breast cancers exhibit a basal phenotype and may have arisen from basal epithelia [26,27].

Despite the presence of these genetic and epigenetic changes, the morphogenesis of MCF-10A cells on reconstituted basement membrane results in growth-arrested structures, similar to those obtained with other normal human mammary epithelial cells [5]. Furthermore, the ability to disrupt morphogenetic events by introducing various oncogenes underscores the value of this system in evaluating biological properties of cancer genes that are not assayable in monolayer cultures.

MCF-10A cells are grown on standard tissue culture plastic in a 5% CO_2 humidified incubator at 37 °C. The growth medium contains several additives and is composed of DMEM/F12 supplemented with 5% donor horse serum, 20 ng/ml epidermal growth factor (EGF), 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, and antibiotics [20]. Recipes for the various cell culture media and stock solutions for the additives are provided in Table 1.

The proper care and passage of MCF-10A cells in monolayer culture is of utmost importance. MCF-10A cells should not be allowed to grow for more than 3 to 4 days without passage. Furthermore, omitting any of

Table 1
Medium recipes for MCF-10A cells

Component	Growth Medium[a]	Resuspension Medium[a]	Assay Medium[a] (without EGF)
DMEM/F12 (Invitrogen No. 11965-118)	500.0 ml	400.0 ml	500.0 ml
Horse serum (Invitrogen No. 16050-122)	25.0 ml (5% final)	100.0 ml (20% final)	10.00 ml (2% final)
EGF (100 μg/ml stock)[b]	100 μl (20 ng/ml final)	—	—
Hydrocortisone (1 mg/ml)[c]	250 μl (0.5 μg/ml final)	—	250 μl (0.5 μg/ml final)
Cholera toxin (1 mg/ml stock)[d]	50 μl (100 ng/ml final)	—	50 μl (100 ng/ml final)
Insulin (10 mg/ml stock)[e]	500 μl (10 μg/ml final)	—	500 μl (10 μg/ml final)
Pen/Strep (100× solution, Invitrogen No. 15070-063)	5.0 ml	5.0 ml	5.0 ml

[a] For each medium type, premix all of the appropriate additives, sterile filter through a 0.2-μm filer, and add to DMEM/F12 medium bottle.
[b] EGF (Peprotech, 1 mg): Resuspend at 100 μg/ml in sterile dH_2O. Store aliquots at −20 °C.
[c] Hydrocortisone: (Sigma No. H-0888, 1-g bottles) Resuspend at 1 mg/ml in 200-proof ethanol and store aliquots at −20 °C.
[d] Cholera toxin: (Sigma No. C-8052, 2-mg vials) Resuspend at 1 mg/ml in sterile dH_2O and allow to reconstitute for about 10 min. Store aliquots at 4 °C.
[e] Insulin (Sigma No. I-1882, 100-mg vials) Resuspend at 10 mg/ml in sterile dH_2O containing 1% glacial acetic acid. Shake solution and allow 10–15 min to reconstitute. Store aliquots at −20 °C.

these additives is detrimental to the growth of MCF-10A cells and can be easily recognized by morphological changes in the cells when grown as a monolayer on tissue culture plastic. Finally, careful attention should always be paid to the monolayer appearance of the cells. When confluent, MCF-10A cells adopt a cobblestone morphology (Fig. 2B), which is typical of mammary epithelial cells. Any deviation from this morphology should be cause for concern; if the cells appear fibroblastic or spindle cell-like, it is unlikely that they will properly form acinar structures in 3D culture. A protocol for passaging cells grown on 10-cm^2 dishes follows.

2.1. Protocol 1: care and passage of MCF-10A cells in monolayer culture

1. Aspirate the Growth Medium (Table 1), and rinse with 10.0 ml of phosphate-buffered saline (PBS).
2. Aspirate the PBS and add 2.0 ml of 1× trypsin solution (0.05% trypsin:0.53 mM EDTA, Cellgro No. 25-052-C1). It is not advisable to use high-concentration trypsin solutions when passaging cells.
3. Aspirate the trypsin leaving a thin film behind. The cells tend to clump if the excess trypsin is not removed, making it difficult to obtain a single-cell suspension.

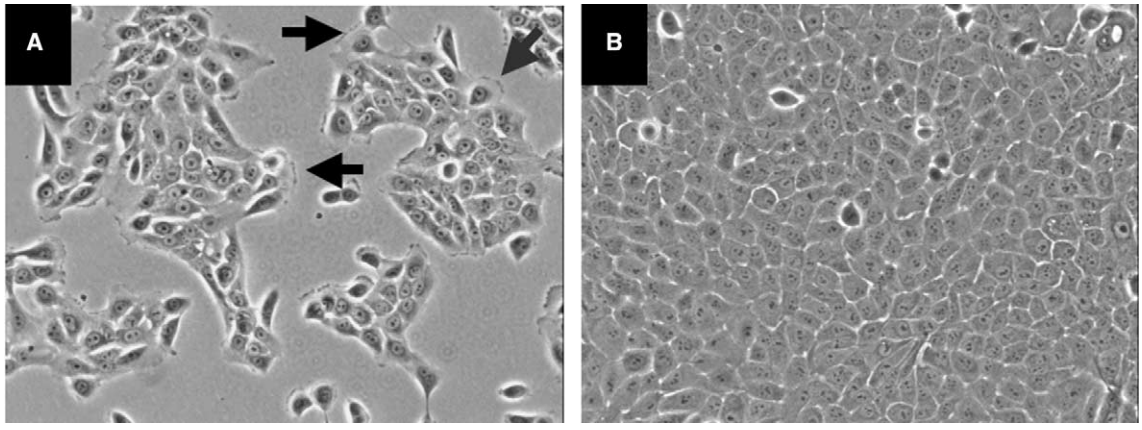

Fig. 2. Phase-contrast morphology of MCF-10A cells grown as a monolayer. (A) Subconfluent MCF-10A cells (15 h following trypsinization and passage) grow as clusters; lamellipodia are often observed at the edges of these clusters (black arrows). (B) At confluence, MCF-10A cells assume a cobblestone morphology, typical of epithelial cell lines.

4. Incubate in a 5% CO_2 humidified incubator at 37 °C for 15–25 min. Check the extent of trypsinization after 10 min, gently tapping the plate to dislodge the cells. Continue every few minutes. The cells should be completely dissociated from the plate to avoid clonal selection of adherent cells.
5. Once cells are dislodged, add 1.0–2.0 ml of Resuspension Medium (Table 1) and pipet to break up cell clumps.
6. Transfer the cells to a 15-ml conical tube and rinse the plate with another 1.0 ml of Resuspension Medium. Add an additional 1.0 ml of Resuspension Medium to the conical tube. Ultimately the cells are resuspended in 3–4 ml. If you are dealing with multiple plates, it is very important to process only one or two plates at a time. The cells will reattach if they are not resuspended in a timely manner after the serum is added.
7. Spin down the cells at 150*g* in a tissue culture centrifuge for 3–5 min.
8. Aspirate the medium and resuspend the cells in 1.0 ml of MCF-10A Growth Medium. Add 4.0–5.0 ml to the tube, mix the cells, and plate 1.0 ml cells per 10-cm dish in a total of 10 ml of MCF-10A Growth Medium (1:5 to 1:6 dilution). A 1:5 passage ratio becomes confluent in 2.5 to 3 days, and a 1:6 passage ratio, in 3.5 to 4 days.
9. Keep track of the passage number; cells may start behaving aberrantly in 3D morphogenesis assays starting at passage 35.

3. Establishment of stable pools in MCF-10A cells by retroviral infection

Retroviral expression vectors can be used for the stable expression of genes in MCF-10A cells. We have found that vectors that use the long terminal repeat regions of Moloney leukemia virus are very effective for stable expression in MCF-10A cells (e.g., pBABE, pMSCV, pLNCX, pLXSN). Because MCF-10A cells are of human origin, it is necessary to use virus that either possesses an amphotropic envelope or is pseudotyped with the envelope of vesicular stomatitis virus (VSV). Established retroviral producer lines have been developed for virus production with either of these envelopes, including VSV-GPG cells and Phoenix cells [28–30]. Readers should refer to the Nolan laboratory web site (http://www.uib.no/mbi/nolan/NL-Homepage. html) or the original references for details of transfection and virus production. Notably, the aforementioned lines are derived from HEK293 cells, making them easily transfectable using calcium phosphate- or lipid-based methods. Virus-containing supernatant can be collected and used to directly infect MCF-10A cells using the protocol below. When establishing stable pools of MCF-10A cells, it is desirable to infect with a multiplicity of infection (MOI) of 3 to 5, which ensures that each host cell integrates at least a single copy of the retrovirus. Furthermore, because MCF-10A cells, like all epithelial cell lines, exhibit nonspecific clonal variability, working with a diverse pooled population of stable expressors is desirable to ensure that the observed phenotypes can be attributed to the overexpressed gene product of interest. If clones are derived, multiple independent lines should be examined prior to drawing any conclusions about phenotypes that may arise during morphogenesis. Although the retroviruses produced by VSV-GPG and Phoenix cells are replication incompetent, strict BL2 safety guidelines should be employed when working with these reagents.

3.1. Protocol 2: retroviral infection of MCF-10A cells and selection of stable pools

1. The night prior to infection, plate early-passage MCF-10A cells at 500,000 cells per 10-cm^2 dish. If titer is being determined, several plates should be made to test several dilutions of the viral supernatant obtained from a producer line. In addition, if a drug selection scheme will be used, extra plates (that will not be infected) should be prepared to serve as positive controls for drug-induced killing. Cells will be 15–20% confluent on the day of infection.
2. On the day of infection, virus-containing supernatant from the producer line is collected and filtered through a 0.45-μm filter to remove cell debris. Virus can be stored as aliquots at −80 °C until use; if a frozen aliquot is being used for infection, it should be thawed quickly in a 37 °C water bath. Repeated freezing and thawing should be avoided.
3. To titer viral preparations, dilute the stock into 4 ml of MCF-10A Growth Medium. A range of dilutions from 1:4 to 1:4000 is a good starting point; once the titer is determined, the appropriate dilution to obtain a MOI of 3 to 5 can be used in subsequent infections to generate pools of cells containing a single copy of the vector.
4. Add polybrene to the "virus incubation cocktail" from step 3 at a final concentration of 8 μg/ml. A 1000× stock solution (8 mg/ml) of polybrene (Sigma) is prepared by dissolving the solid in sterile H_2O and sterile filtering this solution with a 0.2-μm filter. Aliquots stored at −20 °C can be repeatedly frozen and thawed.
5. Aspirate the medium from the cells and add the virus incubation cocktail. Return to a 5% CO_2 humidified incubator at 37 °C.
6. Five hours postinfection, feed the cells with an additional 6 ml of MCF-10A Growth Medium.

7. Replace the medium on the following day (18 h post-infection).
8. If the retroviral vector includes a drug selection marker, begin drug selection at 36 to 48 h postinfection. Concentration ranges for commonly used drugs include: 0.5–2.0 μg/ml puromycin, 250–300 μg/ml active G418, and 250–500 μg/ml hygromycin. Drug selection should be continued until the uninfected drug-treated controls have completely died; for puromycin or hygromycin, this will occur by 4 to 5 days of treatment, and for G418, 9 to 10 days of treatment. If cells become confluent, they should be split (1:3 passage) to maintain the efficacy of the selection agent.
9. Following selection, the cells should be passaged once to allow recovery from drug treatment. Thereafter, the stable pools can be used for the 3D morphogenesis assays discussed below as well as other applications. It is optimal to use pools of cells shortly after infection since continued passage of cells can lead to loss of the vector if expression of the transduced gene has any negative effects on cell proliferation. Infection of fresh cells avoids complications due to unpredictable negative effects.

4. Three-dimensional culture of MCF-10A cells on reconstituted basement membrane

In most previous studies, mammary cells have been cultured in three dimensions by totally embedding in a gel of reconstituted basement membrane. Although MCF-10A cells can be cultured using a total embedment method, our laboratory has used primarily an overlay method in which single cells are seeded on a solidified layer of Growth Factor Reduced Matrigel measuring approximately 1–2 mm in thickness (Fig. 3). The cells are grown in an Assay Medium (see Table 1) containing 5 ng/ml epidermal growth factor (EGF) and 2% Matrigel; these conditions are sufficient to drive the development of these cells into individual polarized acinar units with a hollow lumen. Notably, the basic recipe for Assay Medium in Table 1 lacks EGF, allowing for addition of the desirable concentrations of that growth factor in an assay; obviously, the concentration of any of the additives used to culture MCF-10A cells can be manipulated in a similar manner depending on the experimental question being asked.

The overlay method has several beneficial features. First, the acini that develop are larger than those obtained with total embedment, making it easier to discern the hollow lumen as well as the presence of luminal apoptosis. Second, the acini can easily be stained in situ, eliminating the need for cryosectioning in the majority of cases; the subcellular location of numerous markers can be analyzed by confocal microscopy (Table 2). Third, proteins are easier to extract for immunoblotting analysis. Finally, smaller amounts of Matrigel, an expensive reagent, are used than in total embedment protocols.

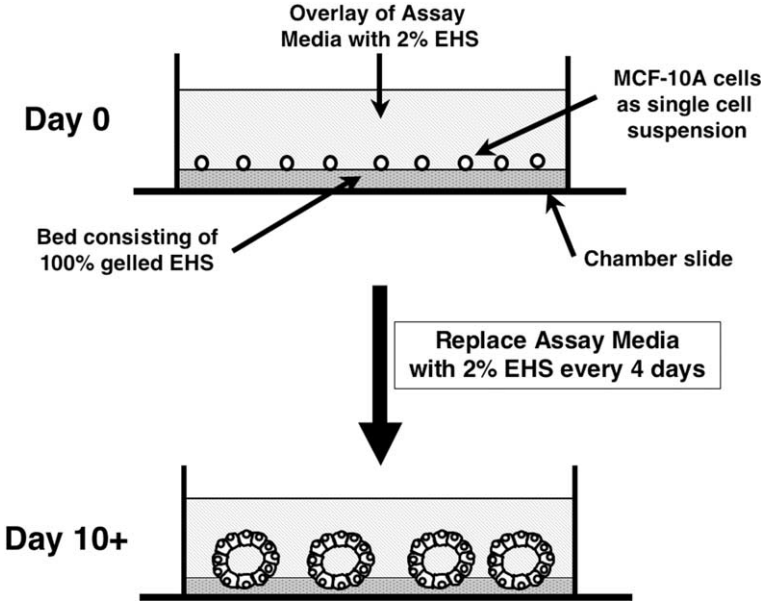

Fig. 3. Schematic of overlay method for 3D culture of MCF-10A cells on matrigel. The well of a chamber slide is initially coated with 100% Matrigel and allowed to solidify, forming a gelled bed of basement membrane measuring approximately 1 mm in thickness. MCF-10A cells are seeded onto this bed as a single-cell suspension in an Assay Medium containing growth factors, hormones, and 2% Matrigel. The Assay Medium is replaced every 4 days. Cells proliferate and form clusters after 5–6 days in 3D culture, and subsequently form acini. Experimental details are provided in Section 4.1.

Table 2
Useful antibodies and fluorescent reagents for the analysis of MCF-10A mammary acini

Antibody or stain	Purpose	Normal subcellular distribution in acini	Source	Species (clone if monoclonal)	Fixation[a]
Active (cleaved) caspase 3	Apoptosis marker	Dying cells in luminal space	Cell Signaling	Rabbit	Formalin, PFA
Active (cleaved) caspase 9	Apoptosis marker	Dying cells in luminal space	Cell Signaling	Rabbit	Formalin, PFA
α6 integrin	Basolateral polarity	Strong basal with weaker lateral staining	Chemicon	Rat (GoH3)	Formalin, PFA, M/A
α-Tubulin	Cytoskeleton	Microtubule cytoskeleton	Sigma	Mouse (DM1α)	−20 °C methanol only
β-Catenin	Cell–cell junctions	Lateral	BD	Mouse (14)	Formalin, PFA, M/A
Collagen IV, human specific	Basement membrane	Basal	DAKO	Mouse (CIV 22)	Formalin, PFA
DAPI	Nuclear counterstain	Nuclei (basally located)	Sigma	N/A	Formalin, PFA, M/A
Discs large (hDlg)	Basolateral polarity	Strong basal with weaker lateral staining	Santa Cruz	Mouse (1D11)	Formalin
E-cadherin	Cell–cell junctions,	Lateral	BD	Mouse (15)	Formalin, PFA
E-cadherin	Cell–cell junctions,	Lateral	Sigma	Mouse (DECMA)	Formalin, PFA
Ethidium bromide	Cell death marker	Selective stains dying cells in luminal space of *unfixed* cultures	Sigma	N/A	No fixation
GM130	Apical polarity	Golgi (apically located)	BD	Mouse (35)	Formalin, PFA
Laminin V, human specific	Basement membrane	Basal	Chemicon	Mouse (D4B5)	Formalin, PFA
LAMP-1	Lysosomes	Punctate, cytoplasmic	BD	Mouse (25)	Formalin, PFA
Ki-67	Proliferation marker	Nuclei of proliferating cells	Zymed	Rabbit	Formalin, PFA, M/A
Phospho-Akt (Ser 473)	Akt kinase activation	Matrix attached cells	Cell Signaling	Rabbit (IHC Specific)	Formalin, PFA, +Inhibitors
Phospho-Akt substrate	Akt kinase activation	Matrix attached cells, primarily membrane localized	Cell Signaling	Rabbit	Formalin, PFA, +Inhibitors
Phospho-ERM (Ezrin/Radixin/ Moesin)	Cell size/shape; actin-rich structures (e.g., microvilli)	Plasma membrane, with enrichment on apical surface	Cell Signaling	Rabbit	Formalin, PFA, +Inhibitors
Phosphotyrosine	Phosphotyrosine	Matrix attached cells, cytoplasmic distribution	Santa Cruz	Mouse (PY20)	Formalin, PFA, +Inhibitors
Phalloidin	Cytoskeleton	Actin cytoskeleton	Molecular Probes	N/A (Alexa Fluor conjugated)	PFA only
Propidium Iodide	Cell death marker	Selectively stains dying cells in luminal space of *unfixed* cultures	Sigma	N/A	No fixation

[a] *Details of fixation method.* Formalin: Room-temperature 2% formalin in phosphate-buffered saline PBS, pH 7.4 (20 min fixation). PFA: Room-temperature 2% freshly prepared paraformaldehyde in (PBS), pH 7.4 (20 min fixation). M/A: −20 °C 1:1 methanol:acetone (10–12 min fixation). +Inhibitors: Add phosphatase inhibitors *during fixation and permeabilization* at the following final concentrations: 1 mM sodium orthovanadate (freshly prepared), 10 mM sodium fluoride, and 10 mM β-glycerophosphate.

4.1. Protocol 3: overlay three-dimensional culture of MCF-10A cells on matrigel

1. Handling Matrigel: We obtain Growth Factor Reduced Matrigel from BD Biosciences (BD No. 354230). Thaw on ice overnight at 4 °C. Matrigel remains liquid on ice but solidifies rapidly when warmed, so it should be handled on ice at all times. Once thawed, the Matrigel can be stored as 1.0-ml aliquots at −20 °C. Because there is lot-to-lot variability in Growth Factor Reduced Matrigel, our laboratory tests individual lots, prior to purchasing large quantities for experiments. In determining the appropriateness of a lot for 3D assays, we prefer those with protein concentrations ranging from 10 to 12 mg/ml and an endotoxin level less than 2 units/ml. For any interesting experimental observation, it is advisable to repeat the assay with two or more independent lots of Matrigel to confirm the generalizability of the result.
2. Add 40 μl of Growth Factor Reduced Matrigel to each well of an eight-well glass chamber slide and spread evenly in the well using the tip of a P-200 pipetman. Take care not to generate air bubbles or overspread; this will form a high meniscus on the border. If you are not experienced in handling Ma-

trigel, the chamber slide can be precooled on a tray of ice, which provides extra time to spread the EHS. Place the slides in a cell culture incubator to allow the basement membrane to solidify for at least 15 min. The coated chamber slides must be promptly placed in the cell culture incubator promptly to avoid dehydration.

3. While the Matrigel is solidifying, trypsinize a confluent plate of cells (see Section 2.1 for details) and resuspend in 2.0 ml Resuspension Medium. Use an additional 3 ml of this medium to rinse the plate and combine in a 15-ml conical tube.
4. Spin the cells at 150*g* in a tissue culture centrifuge for 3 min.
5. Resuspend the cell pellet in 2 ml of Assay Medium (see Table 1 for recipe) lacking EGF. Gently triturate cells 10 to 25 times with a P1000 pipetman to ensure that a single-cell suspension has been obtained. Then, add additional Assay Medium to achieve a final volume of 8 to 10 ml.
6. Count cells and dilute in Assay Medium to achieve a final concentration of 25,000 cells/ml.
7. Prepare a stock of Assay Medium plus 4% Matrigel. Also add EGF or other growth factors, pharmacological agents, etc., to this medium at twice the final concentration that is desired. For standard assays in our laboratory, this corresponds to an EGF concentration of 10 ng/ml. It is necessary to make 200 μl of this stock for each well of the chamber slide; furthermore it is recommended that enough stock solution for "$n + 1$" assays be prepared to account for volumetric errors in pipetting.
8. Mix the cells from part 6 (25,000 cells/ml) with the Matrigel-containing medium from part 7 in a 1:1 ratio.
9. Plate 400 μl of this mixture per well on top of the solidified Matrigel in each well of the chamber slide from part 1. This corresponds to a final overlay solution of 5000 cells/well in medium containing 2% Matrigel and 5 ng/ml EGF.
10. Allow the cells to grow in a 5% CO_2 humidified incubator at 37 °C. The cells should be refed with Assay Medium containing 2% Matrigel and 5 ng/ml EGF every 4 days. (The day that the assay is set up corresponds to Day 0; thus, feed on Days 4, 8, 12, 16, etc.). The cells should form clusters by Day 5 or 6 of 3D cultures and subsequently start forming a hollow lumen.

5. Analysis of morphogenetic parameters within 3D cultures by fluorescent and immunofluorescent techniques

The utility of MCF-10A mammary acini grown in three-dimensional culture as an experimental tool is due largely to the ability to analyze the spatial and temporal aspects of key biological processes (e.g., proliferation, apoptosis) and signal transduction events during morphogenesis. Recently, we have delineated a temporal progression of biological processes that contribute to the development and maintenance of a hollow glandular architecture within MCF-10A acini, which is schematically portrayed in Fig. 4. First, single cells seeded on a basement membrane gel must proliferate to form acini, and remarkably, several events contributing to the formation of a hollow architecture precede the suppression of proliferation within MCF-10A acini. During the early stages of morphogenesis, evidence of apicobasal polarization becomes evident within cell clusters prior to evidence of bona fide lumen formation. At Days 5 through

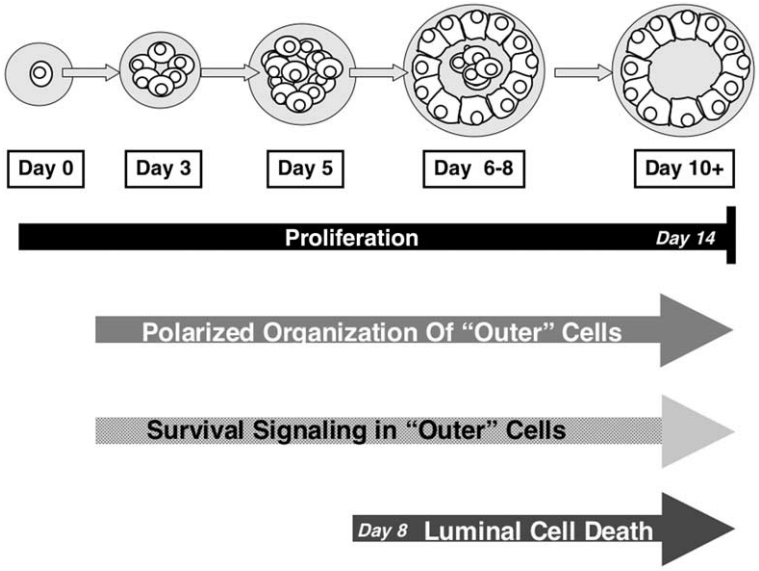

Fig. 4. Schematic of biological events during MCF-10A acinar morphogenesis. Details are provided in Section 5 of the text.

8 in culture, two populations of cells within each acinus become evident: a well-polarized outer layer of cells that is in direct contact with matrix, and an inner subset of cells that are poorly polarized, lacking contact with the matrix. Notably, there is also a dichotomy in the activity of Akt, a serine–threonine kinase with well-known survival functions, between these two populations of cells. By Day 8 of culture, the centrally located, nonpolarized cells began to die by apoptosis, which coincides with the formation of a hollow lumen [8].

Importantly, by establishing this time course of morphogenetic events, we have provided a framework to guide the further examination of various cancer genes on modulating the development of a hollow, polarized acinar architecture. Undoubtedly, analysis of the mechanisms by which a gene may modulate acinar morphogenesis requires effective imaging methodologies to monitor the aforementioned parameters. In Table 2 are listed useful reagents for analyzing these parameters, including cell–cell adhesion, basement membrane deposition, and polarity. Representative immunostains are shown in Fig. 5. A basic indirect immunofluorescence staining technique for 3D cultures is provided in Section 5.1; this protocol should be viewed as a starting point that requires optimization on a case-by-case basis. In most instances, but not all, antibodies and staining conditions that work for indirect immunofluorescence of MCF-10A cells grown as monolayers on glass coverslips also work in 3D cultures; thus, the optimization of untested antibodies can be carried out in this manner.

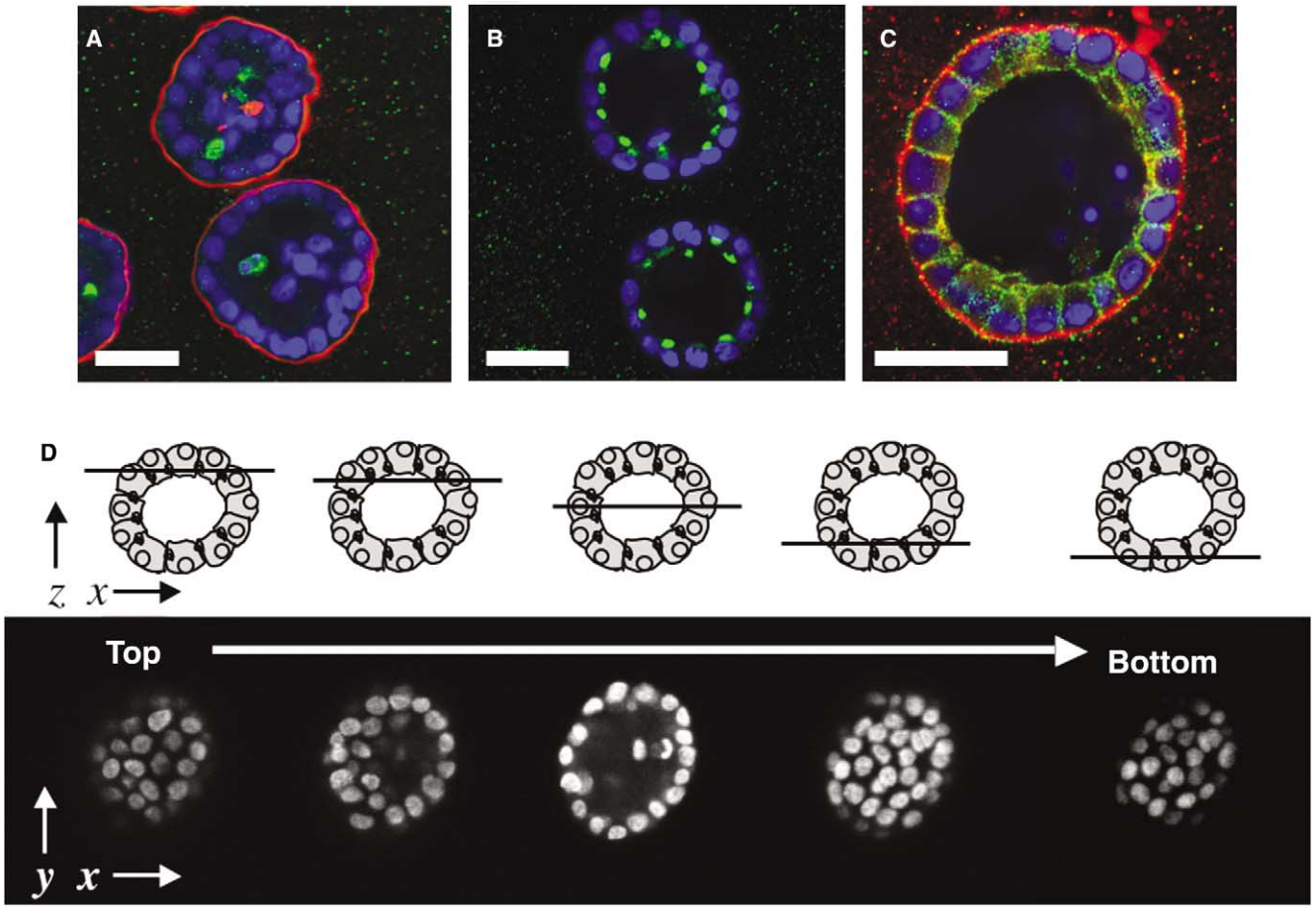

Fig. 5. Representative confocal microscopic imaging of MCF-10A acini. (A) MCF-10A cells were cultured on basement membrane for 8 days and immunostained with antiserum to activated caspase 3 (green) to detect apoptosis, and anstiserum to laminin V (red), to delineate the secretion of basement membrane. DAPI-stained (blue) equatorial cross sections show apoptotic cells in the center of developing acini, whereas cells in direct contact with basement membrane are viable. Bar = 25 μm. (B) Day 20 MCF-10A acini were immunostained with antibodies to the Golgi protein, GM130 (green), which illustrated the apical orientation of the Golgi apparatus toward the hollow lumen of acini. Bar = 25 μm. (C) Day 20 MCF-10A acini were immnuostained with antibodies toward phospho-ERM (ezrin/radixin/moesin) (green) and hDlg (human disk large) (red). DAPI-stained equatorial cross sections illustrate that phosphorylated ERM proteins and hDlg are both immediately subjacent to the plasma membrane within acini; phospho-ERM is distributed over the entire cell membrane whereas hDlg is basolateral in location. Bar = 25 μm. (D) Serial confocal cross sections (*x*–*y* axis) through a Day 15 MCF-10A acinus. The schematic diagrams overlying each section illustrate the relative position of the optical section with respect to the *z* axis. The floor and the roof of the spherical structure appear as a sheet of cells because the optical section cuts *en face* across the surface contour of the sphere; in contrast, the equatorial and adjoining cross sections appear as a ring because the section cuts through the hollow lumen.

Two important parameters that warrant specific discussion are the analysis of proliferation and apoptosis. We have monitored proliferation by immunostaining with an antibody toward Ki-67, a nuclear protein that is expressed only during late G_1 through M phase of the cell cycle and is commonly employed by pathologists to detect proliferating cells [31,32]. We have had limited success with other assays for proliferation within MCF-10A acini, notably bromodeoxyuridine (BrDU) labeling; however, other laboratories have effectively used this technique in 3D mammary epithelial culture systems [15].

Two techniques appear to be very useful for monitoring cell death in MCF-10A acini: (1) in situ staining with ethidium bromide, and (2) immunostaining for the cleavage product of activated caspase 3. The first technique uses a DNA-intercalating dye that cannot enter living cells but can readily label dying cells whose membrane integrity has been compromised; it is a simple and rapid assay to examine and quantify cell death (see Section 5.3) in a culture of interest. The latter technique is more specific for the involvement of a "type I" apoptotic process because it detects the activation of an executioner caspase [33]. Both techniques have indicated that cell death is confined to cells occupying the luminal space within both normal and oncogene-expressing MCF-10A structures [8].

5.1. Protocol 4: indirect immunofluorescence staining of MCF-10A acini cultured in matrigel

1. Fixation: Aspirate the medium from each well of the chamber slide, and immediately fix acini with 2% formalin (Sigma) or 2% paraformaldehyde (2% PFA in PBS, pH 7.4, freshly prepared) for 20 min at room temperature. Acini can also be fixed using a 1:1 mixture of methanol:acetone at −20 °C for 10–12 min. For eight-well chamber slides, 500-μl volumes are appropriate for the fixation, permeabilization, and all subsequent washing steps in this protocol.
2. Permeabilization: If formalin or paraformaldehyde is used for fixation, permeabilize with PBS containing 0.5% Triton X-100 for 10 min at 4 °C. Depending on the antibody used for immunostaining, the detergent concentration or duration of permeabilization may require modification.
3. Glycine rinse: Rinse three times with PBS/glycine (130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 100 mM glycine), 10–15 min per wash at room temperature.
4. Primary block: Incubate with 200 μl/well IF Buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 7.7 mM NaN_3, 0.1% bovine serum albumin, 0.2% Triton X-100, 0.05% Tween-20) + 10% goat serum for 1–1.5 h at room temperature.
5. Secondary block: Aspirate the primary block and incubate with 100 μl/well secondary block (IF Buffer + 10% goat serum + 20 μg/ml goat anti-mouse $F(ab')_2$ fragment (Jackson ImmunoResearch No. 115-006-006) for 30–40 min.
6. Primary antibody: Incubate with primary antibody in the secondary block solution (see step 5) overnight (15–18 h) at 4 °C. Table 2 provides a list of useful antibodies and staining reagents our laboratory has used. Although optimal antibody concentrations should be determined empirically on a case-by-case basis, 1:100 to 1:200 dilutions of the primary antibody are a good starting point.

Occasionally, overnight incubation at 4 °C elicits liquefaction of the basement membrane and extensive lifting of the acini during subsequent washing steps. This varies with each lot of Matrigel used for the morphogenesis assay and, unfortunately, cannot easily be predicted. If this problem arises, it is advisable to perform the primary antibody incubations overnight at room temperature rather than 4 °C. If room temperature incubations are attempted, the primary and secondary blocking solutions (steps 5 and 6) should be sterile filtered to avoid the chance of bacterial contamination.

7. Rinse three times (20 min each) with IF Buffer at room temperature with gentle rocking.
8. Secondary antibody: Incubate with fluorescent conjugated secondary antibody in IF Buffer + 10% goat serum for 40–50 min at room temperature.

We recommend Alexa conjugated, highly cross-absorbed secondary antibodies from Molecular Probes used at 1:200 dilution; in our experience, these secondary reagents exhibit low levels of background and minimal cross-reactivity between species, making them useful for double immunostaining procedures.

9. Rinse three times (20 min each) with IF Buffer at room temperature with gentle rocking.
10. To counterstain nuclei, incubate with PBS containing 5 μM TOPRO-3 (Molecular Probes) and/or 0.5 ng/ml 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 15 min at room temperature.
11. Rinse once with PBS for 5 min at room temperature.
12. Mount with freshly prepared Prolong Antifade Reagent (Molecular Probes) and allow to dry overnight at room temperature. Once dry, slides can be stored at 4 °C for up to 1 week or at −20 °C for up to 2 months.

5.2. Guidelines for image acquisition

The microscopic imaging of structures grown in three-dimensional matrices involves a unique set of challenges compared with imaging of cells grown as monolayers on glass coverslips. First, the thickness of structures, which ranges from 50 to 100 μm, must be

recognized during image acquisition. Consequently, confocal microscopy is required to examine most of the immunofluorescent markers. Second, because an acinus grown in basement membrane culture is three-dimensional in nature, the entire series of optical sections throughout each individual structure should be viewed to appreciate any local changes in a particular marker. Serial DAPI-stained cross sections though a normal acinus are shown in Fig. 5D. In normal structures, the equatorial cross section is easily found and, ostensibly, provides the most poignant illustration of the polarized hollow glandular architecture. However, when the morphogenetic process is disrupted by an oncogene, such as the activation of ErbB2, the complex architecture is best appreciated by taking a series of optical cross sections through the structure [9].

The primary determinant in acquiring high-quality images is the optimization of the immunostaining conditions; as discussed above, establishing these conditions is highly empirical in nature. However, even when this has been accomplished, a common problem during confocal analysis is the ''graininess'' or ''noisiness'' of the image, due to the interference of out-of-focus light in these thick specimens. Certain parameters during microscopy can be adjusted to improve image quality. The most useful are increasing the acquisition time and either line-averaging or frame-averaging while acquiring the image. Reducing the pinhole size can also be helpful; however, this is usually accompanied by an unacceptable decrease in the signal. In our experience, the pinhole size should be adjusted to provide an optical section thickness (determined as full width half-maximum) of 0.7 to 0.9 μm. For further information concerning image acquisition and confocal microscopy, an extensive, well-catalogued collection of useful Internet links dealing with numerous practical and theoretical issues in microscopy, including confocal analysis, is available from the University of Arizona Center for Toxicology Experimental Pathology Core (http://swehsc.pharmacy.arizona.edu/exppath/micro/index.html).

5.3. Protocol 5: ethidium bromide staining for analysis of cell death during 3D culture

1. Remove medium from the 3D cultures to be analyzed and carefully wash each well with 500 μl PBS.
2. Prepare a mixture of 1 μM ethidium bromide (Sigma) in PBS and add this solution to acini. Incubate for 15–30 min at 37 °C. If desired, 5 μM calcein AM (Molecular Probes) can also be added to counterstain viable cells.
3. Analyze using indirect immunofluorescence on a microscope equipped with a mercury lamp. Ethidium bromide-positive cells are readily detected on the rhodamine channel available on standard filter sets. If a high autofluorescent background interferes with proper microscopic examination, the staining solution can be replaced with PBS.
4. For a quantitative measure of cell death within a specific culture, the culture is stained with ethidium bromide (calcein AM should not be added in these assays) for exactly 15 min and the acini containing one or more ethidium bromide-positive cells within a culture are counted; at least 200 structures should be counted for such quantification.

6. Guidelines for preparation of protein lysates from 3D cultures

In addition to indirect immunofluorescence, three-dimensional cultures can be examined by immunoblotting to assess protein expression levels, the presence of protein complexes, as well as phosphorylation events. Protein lysates for immunoblot analysis can be prepared directly from three-dimensional cultures using detergent based-lysis buffer such as RIPA buffer (1% NonidetP-40, 0.2% SDS, 0.5% sodium deoxycholate, 150 mM sodium chloride, 50 mM Tris–HCl, pH 7.4, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 5 μg/ml apoprotinin, 5 μg/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride). In certain cases, particularly when determining the expression of an intracellular protein, the cells can be isolated from the Matrigel culture prior to cell lysis using trypsin (0.25% trypsin, 0.1% EDTA) or PBS/2.5 mM EDTA at 37 °C; the isolation of single-cell suspensions from 3D cultures usually takes 30–60 min. Fortunately, with this method, the cells are separated away from the EHS matrix, allowing for the enumeration of cell number and protein concentration prior to lysis.

However, because these extraction methods require the disruption of cell–cell and cell–matrix interactions and prolonged treatment times, they are not recommended for the detection of cell surface proteins, protein complexes, and phosphorylation events; instead, cultures should be directly lysed with a detergent-containing buffer for these studies. In this case, the quantification of protein concentration of the lysates is not possible due to the abundance of protein in the Matrigel. However, colorimetric assays used to measure the activity of stable cytosolic enzymes, such as lactose dehydrogenase (LDH), can be used to normalize protein lysates from 3D cultures; these assays are readily available as kits from various commercial sources (e.g., Promega No. G1780). Moreover, after assessing the expression levels of a protein, immunoblots should be stripped and reprobed for a structural protein (e.g., actin or tubulin) to evaluate equal loading of lysates.

Because the numbers of cells obtained from 3D cultures are usually much lower than that commonly used

to make lysates from monolayer cells, detection by immunoblot analysis can be difficult, particularly for low-abundance molecules. Hence, if appropriate reagents are available, the immunoprecipitation of proteins from 3D lysates, followed by immunoblotting of the electrophoretically resolved immune complexes (IP-Western), can be extremely valuable for the detection of low-abundance proteins.

7. Concluding remarks

The disruption of an intact glandular structure, including loss of apicobasal polarity and filling of the luminal space, is a hallmark of epithelial cancer, even at its earliest stages; yet, very little is known about the mechanisms that elicit these changes during carcinoma formation. The morphogenesis of MCF-10A mammary acini in three-dimensional basement membrane culture provides a biologically rich system and the appropriate structural context in which to assay the effects of oncogenes on glandular architecture. Notably, previous studies in our laboratories have concentrated on gain-of-function approaches via the overexpression of known oncogenes. However, recent advances in gene-specific silencing in mammalian cultured cells using the retroviral delivery to stably express small hairpin RNAs broach the exciting possibility of employing loss-of-function approaches in MCF-10A cells and, hence, facilitate the analysis of tumor suppressors on glandular architecture [34,35]. Finally, although the protocols we have provided focus on the use of a single mammary epithelial cell line, we believe that they can be readily adapted to model cancers in other glandular tissues, especially as epithelial cell lines from various organs become more commonly available. Overall, the development of such in vitro models should provide new opportunities to gain biological insight into the mechanisms that promote the disruption of glandular structure during carcinoma progression and help create a mechanistic framework in the search for new diagnostic markers and therapeutic targets in epithelial cancers.

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