Video Article

Non-enzymatic, Serum-free Tissue Culture of Pre-invasive Breast Lesions for Spontaneous Generation of Mammospheres

Virginia Espina¹, Kirsten H. Edmiston², Lance A. Liotta¹

¹Center for Applied Proteomics and Molecular Medicine, George Mason University

²Virginia Surgery Associates

Correspondence to: Virginia Espina at vespina@gmu.edu

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Abstract

Breast ductal carcinoma *in situ* (DCIS), by definition, is proliferation of neoplastic epithelial cells within the confines of the breast duct, without breaching the collagenous basement membrane. While DCIS is a non-obligate precursor to invasive breast cancers, the molecular mechanisms and cell populations that permit progression to invasive cancer are not fully known. To determine if progenitor cells capable of invasion existed within the DCIS cell population, we developed a methodology for collecting and culturing sterile human breast tissue at the time of surgery, without enzymatic disruption of tissue.

Sterile breast tissue containing ductal segments is harvested from surgically excised breast tissue following routine pathological examination. Tissue containing DCIS is placed in nutrient rich, antibiotic-containing, serum free medium, and transported to the tissue culture laboratory. The breast tissue is further dissected to isolate the calcified areas. Multiple breast tissue pieces (organoids) are placed in a minimal volume of serum free medium in a flask with a removable lid and cultured in a humidified CO₂ incubator. Epithelial and fibroblast cell populations emerge from the organoid after 10 - 14 days. Mammospheres spontaneously form on and around the epithelial cell monolayer. Specific cell populations can be harvested directly from the flask without disrupting neighboring cells. Our non-enzymatic tissue culture system reliably reveals cytogenetically abnormal, invasive progenitor cells from fresh human DCIS lesions.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51926/>

Introduction

Proliferation of epithelial cells within the confines of breast ducts and alveoli (ductal carcinoma *in situ*) is recognized as an obligate precursor to invasive ductal and lobular breast carcinoma. Nevertheless, the molecular mechanisms and cell population dynamics that permit progression to invasive cancer are poorly understood. Elucidating the survival mechanisms used by pre-invasive breast carcinoma cells, or any pre-invasive tumor, may reveal therapeutic strategies for killing, or even preventing, pre-invasive neoplasms¹. However, simple low-cost methods for functionally studying human pre-invasive lesions have been lacking. Although *in vitro* monolayer culture of transformed cell lines is an established laboratory method, the phenotype and genotype of these immortalized cell lines fails to recapitulate the molecular status of primary human tumor cells². Furthermore, even the non-tumorigenic MCF-10A cell line, which recapitulates 3-D mammary gland architecture, fails to adequately represent the functional phenotype and molecular characteristics of an individual patient's pre-invasive breast lesion^{3,4}.

To determine if stem-like neoplastic cells capable of invasion existed within the ductal carcinoma *in situ* (DCIS) cell population, we developed a methodology for collecting and culturing sterile human breast tissue at the time of surgery (**Figure 1**)⁵. Our *ex vivo* breast organoid culture system does not rely on enzymatic tissue disruption, basement membrane extract matrix, or fibroblast depletion, for isolating and propagating mammosphere-forming cells from fresh human breast ductal carcinoma tissue⁶⁻⁸. Our new system is based on the principle of cell streaming/migration⁵. The discernible breast ducts, and surrounding stroma are submerged in a minimum volume of serum-free nutrient medium (just enough to cover the duct fragments) to maximize gas exchange, with the cut surface of the duct exposed to the culture medium, but in no specific orientation in the flask (**Figure 1E-F**). This culture system allows cells to migrate out of the duct and into/onto the autologous stroma and culture flask. The nutrient medium, supplemented only with Epidermal Growth Factor (EGF), insulin, and antibiotics, supports growth of mixed cell populations emanating from the organoid. The tissue culture flask has a removable, re-sealable lid that allows the organoids and/or cells to be harvested without disrupting the entire flask or neighboring organoids, while maintaining a sterile humidified environment.

Protocol

Human breast tissue was collected from patients enrolled in a research study, with written informed consent, following Department of Defense, George Mason University, and Inova Health System Institutional Review Board's approved protocols.

1. Prepare Nutrient Rich Medium with Growth Factors and Antibiotics

1. Prepare stock solutions of insulin, epidermal growth factor (EGF), streptomycin sulfate and gentamicin sulfate.
 1. Reconstitute insulin in sterile filtered water to a final concentration of 10 mg/ml. Aspirate 10 ml of Type 1 reagent grade water in a sterile 10 ml disposable syringe. Attach a 0.22 μ m polyethersulfone filter to the syringe. Dispense the sterile filtered water into a sterile 15 ml conical tube.
 2. Add 10 ml of sterile filtered water to a 100 mg vial of insulin. Mix the contents briefly on a vortex mixer. Keep the insulin vial on ice. Dispense 450 μ l of the insulin stock solution into labeled, sterile microcentrifuge tubes and store at -20 °C. Insulin stock solution is stable until the expiration date on the vial.
 3. Prepare stock solution of epidermal growth factor (EGF): Add 500 μ l sterile water to 500 μ g EGF (stock 1 μ g/ μ l). Mix the contents briefly on a vortex mixer. Keep the EGF vial on ice.
 1. Prepare a working stock solution of EGF: Add 100 μ l of the stock EGF solution to 900 μ l nutrient medium (working stock will be 0.1 μ g/ μ l). Mix the contents briefly on a vortex mixer. Dispense 50 μ l of the EGF working stock solution into labeled, sterile microcentrifuge tubes and store at -80 °C.
NOTE: EGF stock solution (1 μ g/ μ l) is stable for 1 year at -80 °C; working stock solution (0.1 μ g/ μ l) is stable for 2 months at -80 °C.
 4. Weigh 40 mg of streptomycin sulfate on an analytical balance. Place the streptomycin sulfate into a sterile 15 ml conical tube. Add 4 ml of nutrient medium. Mix the contents briefly on a vortex mixer. The solution should be slightly pink. Store at 4 °C protected from light. Streptomycin sulfate solution is stable for 7 days.
 5. Weigh 20 mg of gentamicin sulfate on an analytical balance. Place the gentamicin sulfate into a sterile 15 ml conical tube. Add 2 ml of nutrient medium. Mix the contents briefly on a vortex mixer. The solution should be yellow. Store at 4 °C protected from light. Gentamicin sulfate solution is stable for 7 days.
2. Prepare two 200 ml batches of nutrient medium supplemented with human recombinant EGF (10 ng/ml final conc.), insulin (10 μ g/ml final conc.), streptomycin sulfate (100 μ g/ml final conc.) and gentamicin sulfate (20 μ g/ml final conc.).
 1. Add 200 ml nutrient medium to a vacuum filter flask equipped with a 0.2 μ m polyethersulfone filter flask and a 250 ml receiving bottle. Add 20 μ l working stock EGF, 200 μ l stock insulin, 2 ml stock streptomycin sulfate, and 400 μ l stock gentamicin sulfate to the 200 ml of nutrient medium. Attach the filter flask to a vacuum. Filter the medium. Discard the filter and label the flask as "nutrient rich medium". Store at 4 °C for up to 14 days.

2. Tissue Acquisition and Grossing

1. In the operating suite, maintain sterile technique after procuring the breast tissue. Place the breast tissue in a sterile tray and cover the tray with sterile plastic wrap.
 1. Transport the tissue in the covered tray to Radiology/Pathology as required by your institution. Do not open the tray. Transport times vary within institutions. Tissue can remain viable in this covered tray at RT for up to 45 min post excision. However, prompt processing of the tissue into nutrient rich medium provides optimal conditions for subsequent organoid culture.
2. Gross tissue dissection to identify areas of DCIS within the breast tissue: Use sterile gloves, blades, scalpels, tissue marking dyes, and vinegar during tissue dissection to maintain tissue sterility.
 1. Clean the work area with 70% ethanol or 1% bleach. Open the sterile gloves and place the glove wrapper on the work surface. Place the sterile interior of the glove wrapper face up. Put on the sterile gloves.
 1. Clean the surface of the specimen container with 70% ethanol. Remove the plastic wrap from the tissue specimen container and place the specimen on the glove wrapper.
 2. Dip two cotton tipped swabs into the tissue marking dye. Apply the dye to the surface of the tissue by rolling the swabs across the tissue surface.
NOTE: Each pathology department has a standardized dye color/tissue orientation protocol. Tissue is labeled with dye to orient the tissue in relation to its position in the patient. The dye is blotted or painted onto the tissue. Do not pour the dye over the tissue. Pouring the dye can cause the dye to run/drip into tissue crevices thus confusing the orientation of surgical margins. Tissue orientation provides the surgeon and pathologist with anatomical landmarks to a) describe the tissue specimen, and b) determine the location of the surgical margins in relation to the tumor.
 3. Pour distilled white vinegar (5% acetic acid) onto sterile gauze pads. Blot the dyed tissue with the vinegar soaked gauze. Discard the gauze. Vinegar is used to fix the tissue marking dyes.
 4. Cut the breast tissue into vertical slices approximately 5 mm thick. Do not cut all the way through the tissue (**Figure 2**). Observe/ palpate the tissue for areas of calcification. Identify DCIS lesions by their characteristic firm, pale appearance surrounded by reddish, rubbery borders that feel gritty (due to calcium spicules).
NOTE: In some cases, comedo (pimple-like) areas can be seen as white dots, representing necrotic material from large diameter DCIS lesions.

5. Cut out areas of DCIS/calcified breast tissue, including a small amount of surrounding breast tissue. Place the breast tissue into a sterile 50 ml tube containing 20-30 ml of nutrient rich medium prepared in step 1.
 1. Mix the tissue and medium by gently inverting the tube several times. Discard the medium and add fresh medium. Place the tube containing the tissue and medium into an insulated container and transport the tissue to the tissue culture lab.

3. Tissue Culture

1. Working in a biological safety cabinet, pour the tissue and a small amount of the nutrient medium into a sterile Petri dish. Using a sterile scalpel cut away and discard the fibrous tissue. Cut the breast tissue into pieces (organoids) approximately 3 mm² (**Figure 1C-D**). Try to cut the tissue so that each organoid contains at least one discernable duct segment with surrounding stroma.
2. Open the lid of the tissue culture flask. Using sterile tweezers or forceps, place the organoids into the flask. Close the lid. Discard the petri dish.
3. Add 11 ml serum-free nutrient rich medium (prepared in step 1) to the tissue culture flask. Close the flask and swirl the flask so the organoids and medium are evenly distributed across the flask surface (**Figure 1E-F**).
4. Incubate the flask at 37 °C in a humidified 5.0% CO₂ atmosphere for 2 days. Do not move the flask during this time.
5. On day 2 post incubation, remove the flask from the incubator to check for potential bacterial/fungal contamination. Avoid sudden, sharp movements, or swirling of the flask. Place the flask on an inverted microscope stage.
 1. Observe the medium for bacteria, yeast, and/or fungus. If no contamination is noted, return the flask to the incubator for an additional day. If contamination is noted, discard the flask and contents in an appropriate container.
6. On day 3 post incubation, replace the conditioned medium with fresh medium.
 1. Place 11 ml of medium in a sterile tube at 37 °C for 20-30 min. Remove the tissue culture flask from the incubator. Without disturbing the organoids, remove and discard the medium in the flask using a sterile serologic pipette.
 2. Using a new sterile serologic pipette, add 11 ml of the pre-warmed fresh medium. Very gently rotate the flask to distribute the medium across the flask surface.
 3. Incubate the flask at 37 °C in a humidified 5.0% CO₂ atmosphere.

4. Maintenance of Established Organoid/Epithelial Cell Colonies

1. Prepare fresh medium every 2 weeks and replace the media in the tissue culture flask 3 times per week.
 1. Place 11 ml of medium in a sterile container at 37 °C for 20-30 min. Remove the flask from the incubator. Using a sterile serologic pipette, remove and discard the conditioned medium from the flask, being careful not to disturb the organoids.
 2. Using a new sterile serologic pipette, add 11 ml of the pre-warmed fresh medium. Very gently rotate the flask to distribute the medium across the flask surface. Incubate the flask at 37 °C in a humidified 5.0% CO₂ atmosphere.
2. After 10 - 14 days in culture, remove any pieces of tissue in the culture flask that are not adherent.
 1. Periodically harvest cells and/or organoids from the flask for propagation into new culture flasks, for xenograft transplantation, or for phenotypic and/or molecular analysis.
 2. Remove the tissue culture flask from the incubator. Spray the flask with 70% ethanol. Wipe off excess ethanol on the flask with a clean paper towel that has been sprayed with 70% ethanol.
 3. Organoid propagation:
 1. Open the lid of the flask and place the lid face-up in the biological safety cabinet. Under microscopic visualization, locate the organoid(s) to be harvested. Use sterile tweezers or forceps to pick-up an organoid.
 2. To propagate the organoid in a new culture flask, place the organoid in the new flask. Add 11 ml fresh, warm medium. Incubate at 37 °C, in a humidified 5% CO₂ atmosphere as described in steps 3.3 – 3.6.3. Replace the cell culture medium in the tissue culture flask three times per week.
4. Harvesting cells:
 1. Under direct microscopic visualization, gently scrape and aspirate cells and mammospheres using a 1,000 µl pipette with sterile disposable pipette tips. Aspirate the cells and surrounding medium. Dispense the cells/medium into a sterile microcentrifuge tube.
5. Spin the cells briefly in a mini-centrifuge at 12,100 x g for 5 sec. Remove and discard the medium.
 1. For DNA analysis, immediately freeze the cell pellet on dry ice, in a small volume of medium (10 µl), with long term storage at -80 °C.
 2. For proteomic analysis, lyse the cells in 10 µl of 8 M urea for mass spectrometry or 2x SDS tris-glycine buffer for western blotting/reverse phase protein microarrays. Alternatively, spin cells in a cytocentrifuge to make cell smears for immunohistochemical analysis.
6. After harvesting cells from a flask, remove and discard the remaining medium. Add 11 ml of warm, fresh nutrient rich medium. Incubate the flask at 37 °C, in a humidified 5% CO₂ atmosphere.

Representative Results

Workflow for procuring and culturing sterile breast ductal carcinoma *in situ* tissue

Breast tissue sterility is maintained from the operating room to the cell culture lab via minor changes in typical hospital pathology workflow (**Figure 1**). Tissue is transported in a sterile container with a plastic film cover which allows radiologic assessment while maintaining tissue sterility. Gross tissue processing of a breast lumpectomy or mastectomy sample is performed with sterile gloves, blades, and tissue marking dyes. Gross morphologic appearance of breast ductal carcinoma *in situ* resembles pale, slightly raised areas with a gritty/firm texture, surrounded by reddish/yellow rubbery tissue. Areas of ductal hyperplasia and DCIS may feel "gritty" and firm due to calcifications. These areas of breast tissue often appear tan or a slightly different color than the surrounding breast tissue. However, ADH can only be distinguished after tissue collection and pathologic review of the stained tissue sections. Breast tissue remains viable by immersing the tissue and/or ductal organoids in serum-free nutrient medium supplemented with human recombinant EGF (10 ng/ml), insulin (10 µg/ml), streptomycin sulfate (100 µg/ml) and gentamicin sulfate (20 µg/ml)⁵. Cell culture flasks with removable/re-sealable lids permit periodic harvesting of cells/organoids (**Figure 1E-F**). This model successfully propagated human breast pre-invasive lesions from more than 20 patients diagnosed with atypical ductal hyperplasia (n = 2) and ductal carcinoma *in situ* (n = 18).

Spontaneous mammosphere formation *in vitro* and *in vivo*

Mammospheres and 3-D structures arose spontaneously from multiple, independent human DCIS duct tissue fragments from different patients diagnosed with atypical ductal hyperplasia or ductal carcinoma *in situ* (**Figure 3 & 4**)^{5,9}. Enzymatic disruption of the breast tissue was not performed prior to tissue culture, which resulted in a mixed-cell type culture. Neither serum, basement membrane extract, nor gel-like matrices were required for spontaneous mammosphere formation (**Figure 4**). The mammospheres generated mammary xenograft tumors in a NOD/SCID mouse model with the same growth pattern as that of invasive cancer (**Figure 5**)⁵. These results demonstrate that progenitor cells with invasive potential pre-exist within the human breast DCIS duct but are apparently held in check by the ductal niche and can be coaxed to emerge in organoid culture. These cells constitute a new category of breast stem-like cells that exist prior to the overt manifestation of the invasive phenotype^{5,9,10}.

Confirmation of epithelial derived mammospheres and xenografts

The mammospheres and xenografts derived from the mammospheres were confirmed by immunofluorescence as having epithelial origins. Epithelial cell adhesion molecule (EpCAM) is a membrane glycoprotein expressed on epithelial cells¹¹. Immunofluorescence with a mouse monoclonal antibody reactive to human EpCAM (green) and a nuclear stain (DAPI, blue) showed EpCAM positive cells in the mammospheres in culture (**Figure 6A**) and the center of a NOD/SCID xenograft (**Figure 6B**).

Verification of intact basement membrane boundaries

The mammosphere forming, neoplastic epithelial cells in this culture system were derived from pre-invasive breast lesions that were devoid of frank invasion or microinvasion, as verified by independent pathologic analysis under standard of care histopathologic diagnosis. Multiple organoid structures from the same patient generated mammosphere forming colonies that proved to be tumorigenic. In addition, histopathologic examination of the tissue used for organoid culture revealed confluent intraductal lesions with intact basement membrane boundaries (**Figure 7B**)⁵. Thus it can be concluded that the spontaneous mammospheres formed in this culture system are derived only from pre-invasive neoplastic areas and are not a product of rare areas of microinvasion⁵.

Figure 1. Workflow for maintaining tissue sterility during radiological imaging and gross tissue dissection. (A) In the operating suite, breast tissue (lumpectomy sample shown) is placed in a sterile tray and covered with sterile plastic wrap. The tissue can be imaged directly in the plastic tray. (B) Tissue grossing to identify areas of DCIS. Single-use only tissue orientation dyes are painted onto the tissue surface using sterile cotton tipped swabs. Household distilled white vinegar is poured directly onto the tissue and blotted with sterile cotton gauze. (C & D) Breast tissue is transported to the tissue culture lab in nutrient rich medium supplemented with antibiotics. Tissue dissection, to isolate the areas of DCIS, is performed using sterile gloves and blades/scalpels/scissors. The DCIS tissue is cut into multiple organoids for culture. (E & F) *In vitro* culture of breast organoids. Human DCIS tissue is placed directly in tissue culture flasks with removable lids, without prior enzymatic digestion of the tissue. A minimal amount of serum-free culture medium supplemented with epidermal growth factor and insulin supports cellular growth while maintaining an air-liquid interface around the organoid. [Please click here to view a larger version of this figure.](#)

Figure 2. Illustration of gross tissue processing for breast DCIS tissue. The lumpectomy or mastectomy tissue is cut into thin sections by slicing the tissue vertically without cutting all the way through the specimen. This dissection method is often referred to as "bread loaf technique" since the cut tissue resembles a loaf of bread. The area(s) suspected of containing DCIS are cut out and sliced into 2 - 3 mm slices for diagnostic pathology and organoid culture.

Figure 3. A mixed cell type culture maintains representative *in vivo* cell populations. (A) Phase contrast image of mixed cell culture generated from breast DCIS lesions over 11 weeks (4X magnification). (B & C) *In vitro* organoid cultivation successfully propagated DCIS derived epithelial cells with anchorage independent growth, defined as upward growing and expanding mammospheres, and lobulated, duct-like 3-D formations, in serum free medium supplemented with EGF, insulin, streptomycin and gentamicin (10X magnification). (D) Example mammosphere formed after 11 weeks in culture (40X magnification). [Please click here to view a larger version of this figure.](#)

Figure 4. Spontaneous formation of mammospheres in serum free organoid culture. An example mammosphere formation following 33 days of culture (10X magnification, 20X inset). [Please click here to view a larger version of this figure.](#)

Figure 5. NOD/SCID mouse xenograft model. Xenografts were generated by injecting epithelial cells derived from a patient diagnosed with DCIS (mouse right mammary fat pad) or from a patient diagnosed with invasive DCIS (mouse left mammary fat pad). Xenografts derived from both pure DCIS and invasive DCIS revealed a similar growth pattern and rate. [Please click here to view a larger version of this figure.](#)

Figure 6. Mammospheres are confirmed to be of epithelial origin. Immunofluorescence with anti-EpCAM conjugated to FITC was used to confirm the epithelial origin of mammospheres and mouse xenografts generated from breast ducts containing DCIS. **(A)** EpCAM-FITC positive cells (pseudo-colored green, 488 nm) were only seen in mammospheres of the mixed cell cultures emanating from breast organoids (DAPI (pseudo-colored blue, 408 nm) nuclear stain). **(B)** In formalin fixed paraffin embedded mouse xenograft tissue sections, EpCAM positive cells were only detected in the center of the xenograft tumor section. (20X magnification) [Please click here to view a larger version of this figure.](#)

Figure 7. Collagen IV immunohistochemistry reveals intact basement membranes surrounding ducts. Normal breast ducts (**A**) are surrounded by intact basement membranes enriched in collagen IV (diaminobenzidine = brown staining). Following organoid culture, breast tissue also contains intact basement membranes confirming that the mammospheres are derived from areas of DCIS and not from invasive cancer (collagen IV immunohistochemistry, panel A 4X magnification, panel B 10X). [Please click here to view a larger version of this figure.](#)

Discussion

The culture system described herein constitutes a new model for generating living pre-invasive neoplastic breast cells for basic and translational research studies. In the past, pre-malignant breast cancer progression has typically been studied using three different methods. The first method is histopathologic and genetic analysis of microdissected frozen or fixed human specimens¹²⁻¹⁴. The second method utilizes mouse models that contain hyperplastic alveolar nodules (HAN lesions) that are thought to be similar to human pre-invasive breast lesions¹⁵. The third method uses established breast carcinoma cell lines such as MCF7 sublines (MCF10A) that have a highly differentiated DCIS like morphology^{3,4}. Although these three models have provided molecular clues to breast cancer progression, none of these methods are capable of assessing malignant potential or the molecular phenotype in an individual patient's lesion(s). Histopathologic analysis does not provide information about the functional phenotype of the cells in the pre-invasive lesions. The mouse model of breast cancer progression may not accurately reflect the histomorphology and diversity of human atypical ductal hyperplasia, lobular carcinoma in situ, and ductal carcinoma *in situ*¹⁶⁻¹⁸. Furthermore, the stromal microenvironment and the deposition of extracellular matrix surrounding mouse precursor lesions are markedly different from the human counterpart¹⁶. Spontaneous murine precursor lesions can exhibit a very low level of progression to invasion and metastasis. The third method, cultured cell lines, can provide functional phenotypic information only if transplanted into immune suppressed hosts^{3,4}. In addition the genetic abnormalities of a long passaged cell line may not represent spontaneous breast cancer progression in humans². Finally, it is well established that each patient's neoplasm has a unique combination of genetic and epigenetic abnormalities that drive the rate of growth, differentiated state, and progression to invasion and metastasis^{13,14,17}. Human pre-invasive lesions are multifocal and heterogeneous in cell composition and histomorphology. Furthermore, the biologic malignant potential is unknown for an individual patient's pre-invasive lesion.

Our primary tissue culture method overcomes the deficiencies of previous models of human pre-invasive breast cancer and provides the following advantages: 1) The organoid culture system supports growth of neoplastic cell populations within the native tissue microenvironment that spontaneously grow and generate mammospheres that will produce invasive tumors in mouse xenograft models. The cells represent the genotype and phenotype of an individual patient and thereby provide information for personalized therapy, or individual prognosis. 2) The organoid culture system maintains the native cellular subpopulations and provides a means to cultivate non-malignant epithelial cells, stromal cells and immune cells originally present in the primary breast tissue, and carried into culture within the organoid. The low volume of media in the culture system supports oxygen exchange encouraging spontaneous mammosphere formation and differentiated duct and alveoli like structures without the need for an artificial three dimensional scaffolding. 3) The primary cell culture is free from modifications and selection generated by enzymatic dissociation or exogenous genetic modification. Furthermore, the nutrient medium is low-cost and simple to prepare. 4) Molecular and genetic analysis can be conducted on specific cell populations and/or organoids from the culture at different points in time without disrupting the entire culture. 5) The organoid culture system permits the growth, differentiated morphology and cell-cell interactions of the native cell populations which can be studied before and after introduction of therapeutic agents into the culture media.

Although primary tissue culture has certain advantages, it is not without limitations. The organoid culture supports growth of mixed cell cultures, without overgrowth of any one cell type. However, the specific ratio of cell types cannot be controlled and thus may not recapitulate the exact cellular ratios found *in vivo*. Successful organoid cultivation requires sterile tissue collection and processing, both of which are not routine procedures in many community hospital pathology laboratories. Good communication among the clinical researchers, surgical staff, and pathology staff are essential for maintaining sample sterility within the continuum of patient care.

A further limitation of organoid culture is the effect of substratum firmness on cellular phenotype and gene expression^{16,19}. Differentiation of stem cells in culture can be induced by addition of serum-containing medium or may be due to extended time in culture. A stem-like phenotype was maintained after several months in this non-enzymatic, serum-free organoid culture system⁵. However, at some point in time, the cells may differentiate which can be seen morphologically – the cells become smaller, denser, and fail to form mammospheres. To avoid potential issues with changes in cell phenotype over time, molecular experiments, such as transfections or knock down assays, should be performed with young cultures rather than with old cultures (more than 6 months).

The keys to successful organoid culture are using an appropriate volume of medium in the culture flask, and allowing the organoids time to adhere to the tissue culture flask. Excess medium in the flask limits oxygen diffusion, inhibiting mammosphere formation. The first 3 - 7 days of tissue culture are critical for the organoids to attach to the tissue culture flask. In general, if an organoid has not attached by day 14, it likely does not contain any viable DCIS ductal segments and will not become adherent. Organoids that are not adherent by day 14 should be removed from

culture. The lack of viable breast DCIS ducts can be verified following culture by fixing the organoid in 10% formalin and processing the tissue into paraffin blocks for tissue staining and microscopic evaluation.

Our non-enzymatic, serum-free culture system findings support the hypothesis that genetically abnormal neoplastic precursor cells with invasive potential exist within pre-invasive human breast lesions^{5,9,20}. This finding is in keeping with the previous work of Sgroi *et al.*, who conducted genetic analysis of human breast pre-invasive lesions and Damonte *et al.* who studied the mammary intraepithelial neoplasia outgrowth (MINO) murine model of breast cancer progression^{12,14,21}. Taken together with the conclusions of others, our culture model of individual patient pre-invasive lesions supports the concept that the aggressive phenotype of a patient's invasive breast cancer may be largely pre-determined at the pre-invasive stage.

Disclosures

The authors have nothing to disclose.

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