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Modeling Breast Acini in Tissue Culture for Detection of Malignant Phenotype Reversion to Non-Malignant Phenotype

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ABSTRACT

Backgrounds: Evidence is accumulating to support disruption of tissue architecture as a powerful event in tumor formation. For the past four decades, intensive cancer research with the premise of "cancer as a cell based-disease" focused on finding oncogenes or tumor suppressor genes. However, the role of the tissue architecture was neglected. Three dimensional (3D) cell cultures which can recapitulate major aspects of the microenvironment are appropriate models for exploring cancer. For the first time in Iran, we have launched Matrigel based non-malignant, tumorigenic and reverted breast 3D cell cultures. **Methods:** Non-tumorigenic MCF-10A and tumorigenic MCF-7 breast cell lines were cultured on plastic and Matrigel. MCF-7 cell lines were reverted to normal phenotype via AIIB2 and LY 294002 inhibitors against β1 integrin and class I phosphatidylinositol 3-kinase, respectively. **Results:** MCF-10A acini were distinguishably different from MCF-7 on Matrigel. MCF-10A formed organized hollow spherical structures which were in stark contrast to the MCF-7 disorganized cluster of cells. Matrigel allowed visual monitoring of MCF-7 cells treated with inhibitors. After treatment of MCF-7 cells, we observed reversion of MCF-7 phenotype toward normal, comparable to MCF-10A acini. **Conclusion:** The 3D culture provides a microenvironment which allows malignant and non-malignant cells to demonstrate near physiological behavior and this can distinguish non-malignant from malignant cells. The 3D culture also allows visual monitoring of malignant phenotype reversion to organized spheres. *Iran. Biomed. J. 13 (4): 191-198, 2009*

Keywords: Tissue architecture, Three dimensional (3D) culture, Modeling, Human mammary epithelial cells, Matrigel

INTRODUCTION

Genetic mutation and disruption of tissue architecture are thought to contribute to cancer initiation and progression [1]. For the past four decades, intensive cancer research has been conducted with the premise of "cancer as a cell based-disease" that focused on finding oncogenes and tumor suppressor genes which are thought to have a causal role in cancer initiation and progression [2]. An increasing number of researchers who explored molecular mechanism of cancer with the premise of "cancer as a tissue/organ based-disease," have focused on the role of the tissue architecture and epithelial-stoma interaction on cancer progression [3, 4]. Investigators conducting cancer research under this premise need to work across diverse hierarchical levels of complexity [5].

Although the bulk of our knowledge about cancer is gained through work with cell cultures in two dimensional (2D) *in vitro* models, the 2D culture system does not recapitulate true cellular organization reflective of true histological characteristics and disruption of this histology in cancer [2, 6]. Three dimensional (3D) cell cultures, modeling histological features of glandular epithelium, such as the mammary gland, include polarized morphology, specialized cell-cell contacts, and attachment to an underlying basement membrane; provide the possibility of exploring cancer at tissue level of biological organization [7].

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In addition, loss of polarity and disruption of tissue architecture are hallmarks of cancer [1]. The 3D cell cultures provide a physiologically relevant 3D environment to emulate tumor architecture and behavior [8].

We wanted to see if we could launch 3D cell cultures described by pioneering work of Mina J. Bissell's lab for malignant and non-malignant mammary epithelial cells described more extensively by Joan S. Brugge's lab [7, 9, 10]. We cultured malignant and non-malignant human mammary epithelial cells on commercial basement membrane gel, Matrigel. Normal and malignant cells in this more physiologically relevant environment undergo differential organization and a few days after culture generate either normal polarized or disorganized acini, respectively. We also treated malignant cells with inhibitory agents and cultured the cells on Matrigel which led to formation of reverted acini morphologically comparable to normal acini.

MATERIALS AND METHODS

Standard, 2D cell cultures. MCF-10A cells (non-malignant mammary epithelial cell line derived from a 36-year-old Caucasian female) and MCF-7 cells (epithelial cell line derived from the pleural effusion of a 69-year-old Caucasian female with adenocarcinoma of breast were obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA) and the Pasteur Institute of Iran, respectively. MCF-10A cells were cultured in DMEM/F12 (Invitrogen, USA) supplemented with 5% donor horse serum, 20 ng/ml epidermal growth factor (EGF), 10 µg/ml insulin, 100 µg/ml hydrocortisone, 1 ng/ml cholera toxin, 50 U/ml penicillin, and 50 mg/ml streptomycin [7]. MCF-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 mg/ml streptomycin.

Morphogenesis (on-top) assay. Confluent MCF-10A cells were treated with 0.05% trypsin + 0.53 mM EDTA and then resuspended in DMEM/F12 medium supplemented with 20% donor horse serum. The cells were spun down and again resuspended in assay medium (DMEM/F12 supplemented with 2% donor horse serum, 10 µg/ml insulin, 1 ng/ml cholera toxin, 100 µg/ml hydrocortisone, 50 U/ml penicillin and 50 µg/ml streptomycin) at a concentration of 4.8×10^4 cells per 4.0 ml. Eight-well chambered slides (Nunc, Rochester, NY, USA) were coated with 50µl Matrigel per well and left to solidify for 15 min. Matrigel is a basement membrane extract derived from Engelbreth-Holm-Swarm (EHS) tumor (chondrosarcoma) that forms a 3D gel at 37°C. It is also known as EHS extract, reconstituted basement membrane, and Cultrex. Matrigel (BD Bioscience, No. 354230) provides a closer simulation of basal lamina on which epithelial cells rest *in vivo* [11]. The cells suspended in assay media were mixed 1:1 with assay medium containing 4% Matrigel and 10 ng/ml EGF, and also 400 µl assay medium containing Matrigel. Then, the cells were added to each chamber of the Matrigel-coated eight-well chamber slide. Assay medium containing 5 ng/ml EGF was replaced every 4 days [7]. MCF-7 cells were treated with trypsin and first resuspended in DMEM medium supplemented with 10% fetal bovine serum. The cells were spun down and again resuspended in DMEM medium supplemented with 5% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin at a concentration of 6×10^4 cells per 4.0 ml. Eight-well chamber slides (Nunc, Rochester, NY, USA) were coated with 50 µl Matrigel per well and left to solidify for 15 min. The cells were mixed 1:1 with DMEM medium containing 5% FBS and 4% Matrigel and were added to each chamber of the Matrigel-coated eight-well chamber slide. For further observation of malignant acini development along a time course, assay medium (DMEM, 5% FBS containing 4% Matrigel) was replaced every 4 days (data not shown).

Antibody purification and reversion assay. AIIB2 hybridoma cells were obtained from DSHB/ University of Iowa, USA. Hybridoma cells were cultured in DMEM medium supplemented with 15% fetal bovine serum. Supernatant was collected, spun down and filtered. The antibody (rat monoclonal IgG1) recognizes human, dog, sheep, and bovine β1 integrin. Our objective was to block MCF-7 β1 integrin. Antibody was purified via the protein G column. MCF-7 cells were suspended in DMEM containing 5% FBS. The cell suspension was diluted 1:1 with DMEM containing 5% FBS, 4% Matrigel, and 0.16 mg/ml AIIB2 antibody. Subsequently, 400 µl of 1:1 dilusion of cells was added to each well of the chamber slide. The same procedure was used with LY 294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one, MW = 307.4 (Biomol ST420, USA).

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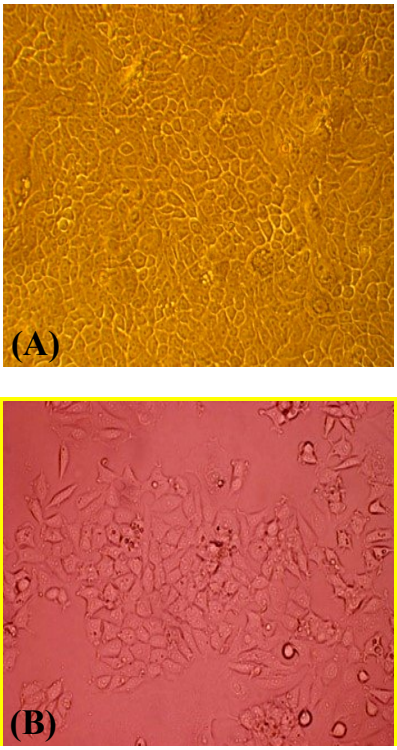

Fig. 1. Phase contrast micrograph of **(A)** confluent MCF-10A and **(B)** subconfluent MCF-7 cells cultured on plastic (two dimensional) for 5 and 3 days (20×), respectively. These adherent growing cells are morphologically indistinguishable from each other.

LY 294002 is a potent and specific cell-permeable inhibitor of PI3K. In *in vitro* assays using purified P13K inhibitor, LY 294002 displays IC50 of 1.4 µM and inhibits all isoforms equally at final concentration of 10 µM. For further observation of malignant acini development along a time course, assay medium (DMEM, 5% FBS) containing 4% Matrigel plus an inhibitory agent was replaced every 4 days as previously mentioned [12].

Cryosectioning and immunostaining. After medium aspiration, 18% and 30% sucrose in PBS were added to acinar structures for 15 minutes each. After removing all sucrose, Tissue-Tek OCT compound (Miles Laboratories, USA) was added. The slides were placed on dry ice mixed with ethanol and sections (5-8 µm) were obtained. The slides were then incubated for 10 min with 5% normal goat serum appropriately diluted in TBS to block non-specific bindings. To stain for human collagen IV, slides were incubated in 1:50 dilution of mouse anti-human antibody (DAKO, Glostrup, Denmark) followed directly by incubation with serially diluted biotinylated anti-mouse antibody, and FITC-conjugated streptavidin for 45-60 min. Nuclei were counterstained with diamino-phenylindole (DAPI, Sigma, Taufkirchen, Germany and the cells were visualized using a Nikon Diapot 200 microscope. All immunofluorescent images were recorded at 40× magnification.

Nuclear staining. Assay media were removed from wells and acini were washed once with PBS. Acini were incubated with PBS containing 0.5 ng/ml DAPI (Sigma, Taufkirchen, Germany) or 0.5 ng/ml propidium iodide (Sigma, Taufkirchen, Germany) at room temperature for 15 min and then rinsed once with PBS at room temperature for 5 min. Immunofluorescent images were captured by a Nikon TE300 microscope equipped with a mercury lamp and CCD camera. Confocal analysis was performed with Nikon PCM2000 CLSM confocal microscopy system.

RESULTS

MCF-10A and MCF-7 in 2D culture. Immortalized, non-transformed human mammary epithelial cell line, MCF-10A, and malignant human mammary cell line, MCF-7 were cultured on plastic as standard (2D) cell culture (Fig. 1). As previously described MCF-10A and MCF-7 cell lines adhere to the surface of the culture dish and begin to grow on plastic as monolayer, morphologically indistinguish-able from each other.

MCF-10A and MCF-7 in three dimension. In spite of their 2D growth and organization on a plastic dish, normal and malignant glandular epithelial cells grow and organize three dimensionally *in vivo* [13]. Normal epithelial cells such as those in the mammary gland also have several distinguishing histological features including a polarized morphology, specialized cell-cell contacts, and attachment to an underlying basement membrane. We asked whether non-malignant mammary epithelial cell line, MCF-10A, can generate organized acinar structures with approximate reconstruction of the basement membrane using Matrigel in tissue culture. Confluent MCF-10A cells were harvested, resuspended in assay medium and seeded as single cells on top of Matrigel. These cells begin to proliferate on reconstituted basement membrane (rBM) and after a few days make well-organized 3D spheroid architectures (Fig. 2A). Morphologically,

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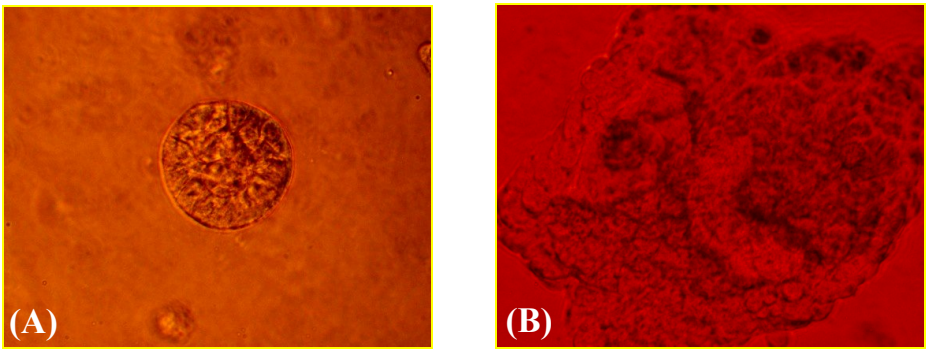

Fig. 2. Phase contrast micrograph of **(A)** well-organized MCF-10A acini and **(B)** disorganized MCF-7 malignant acini cultured on basement membrane gel for 10 days (20×). These three dimensional (3D) structures were in sharp contrast to both MCF-10A and MCF-7 growing on plastic. Although MCF-10A and MCF-7 cells growing on plastic were morphologically indistinguishable (Fig. 1), 3D malignant and normal acinar structures were visibly distinguishable.

these 3D acini formed in 3D environment had different structures than adherent MCF-10A cells growing on plastic.

MCF-10A Demonstrate Evidence of Polarity. Previous studies have shown acini producing milk *in vivo* and 3D spheroid architectures which are formed on basement membrane *in vitro* have polarized architecture surrounding a hollow lumen [6]. In addition, acinar structures formed on Matrigel can produce components of basement membrane *de novo,* and due to polarization of these structures, secrete basement membrane components to the basal side of the acini a few days subsequent to seeding [9]. To examine whether our acini have these properties, cryosection of MCF-10A acinar structures at day 14 was prepared and immuno-stained against human specific Collagen IV, one of the components of *de novo* synthesized basement membrane. Our acini also had synthesized *de novo* Collagen IV and had secreted it out of these apico-basal polarized acini. Concomitant nuclear counterstaining via DAPI also revealed these acini had a hollow lumen (Fig. 3). Counterstaining of MCF-10A acinar structures, at day 11, with Propidium iodide and examination with confocal microscopy further corroborated three dimensionality of the acinar structures (Fig. 4). During the process of oncogenesis, disruption of tissue architecture is one of the earliest powerful events on the road to tumor formation [14]. We asked whether malignant MCF-7 cells create disorganized acinar structures like malignant acini just beginning to form *in vivo.* Subconfluent MCF-7 cells were harvested and seeded as single cells on top of Matrigel. After seeding, malignant cells start to proliferate on Matrigel and after a few days they construct large disorganized acini relative to MCF-10A acinar structures (Fig. 2B). Disorganized malignant acini formed in a more physiologically relevant environment were 3D structures which were in sharp contrast to the malignant adherent MCF-7 growing on plastic. Although MCF-10A and MCF-7 cells growing on plastic were morphologically

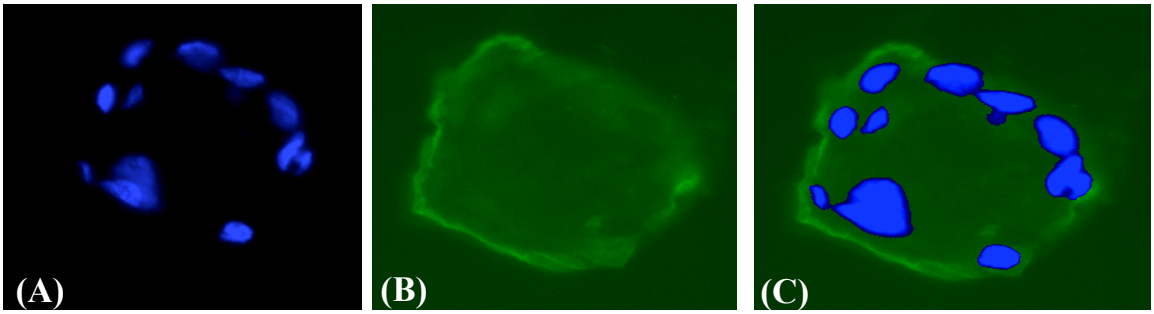

Fig. 3. Cryosection of MCF-10A acinar structures, at day 14, was prepared and **(A)** counterstained with diaminophenylindole (DAPI) and **(B)** immunostained against human specific collagen IV, one of the components of synthesized *de novo* basement membrane (BM). **(C)** Superimposition of (A) and (B). Basal secretion of synthesized *de novo* collagen IV indicates polarization of MCF-10A acini. Concomitant nuclear counterstaining via DAPI also revealed hollow lumen of these acini.

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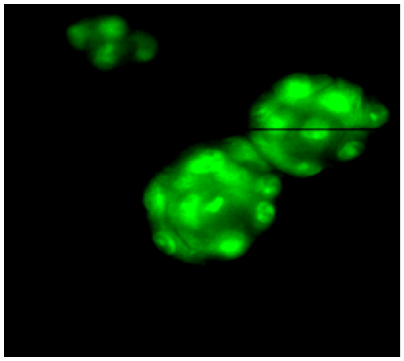

Fig. 4. Nuclear staining of MCF-10A acinar structures, at day 11, with propidium iodide and visualization with confocal microscopy corroborated the three dimensionality of acinar structures.

indistinguishable, 3D malignant and non-malignant acinar structures were morphologically very different.

Visual monitoring of malignant phenotype reversion to organized spheres. Previous studies have shown that MCF-7 cells overexpress β1 integrin protein and deregulation of β1 integrin and its downstream signaling component, phosphatidylinositol 3-kinase (PI3K) may play a causal role in the expression of malignancy in human mammary epithelial cells [10, 15]. AIIB2 inhibitory monoclonal antibody can block the function of β1 integrin on plastic and 3D cultures [10]. We questioned whether malignant 3D acinar structures can demonstrate the inhibitory potential of therapeutic agents visually in tissue culture via a simple light microscope. Subconfluent MCF-7 cells harvested and treated with AIIB2 inhibitory monoclonal antibody against β1 integrin were seeded as single cells on top of the basement membrane gel. After seeding, malignant cells start to proliferate on Matrigel and after a few days they generate morphologically reverted organized spheroid acini comparable to MCF-10A acini (Fig. 5). Similar results were obtained by using LY 294002 against PI3K, a downstream component of β1 integrin signaling pathway (Fig. 5D). For further analysis of normal, malignant and reverted acini, nuclei were stained with DAPI and were examined with fluorescent microscopy. The 3D organization of reverted acini was markedly different from malignant acinar structures but morphologically comparable to non-malignant acini (Fig. 6).

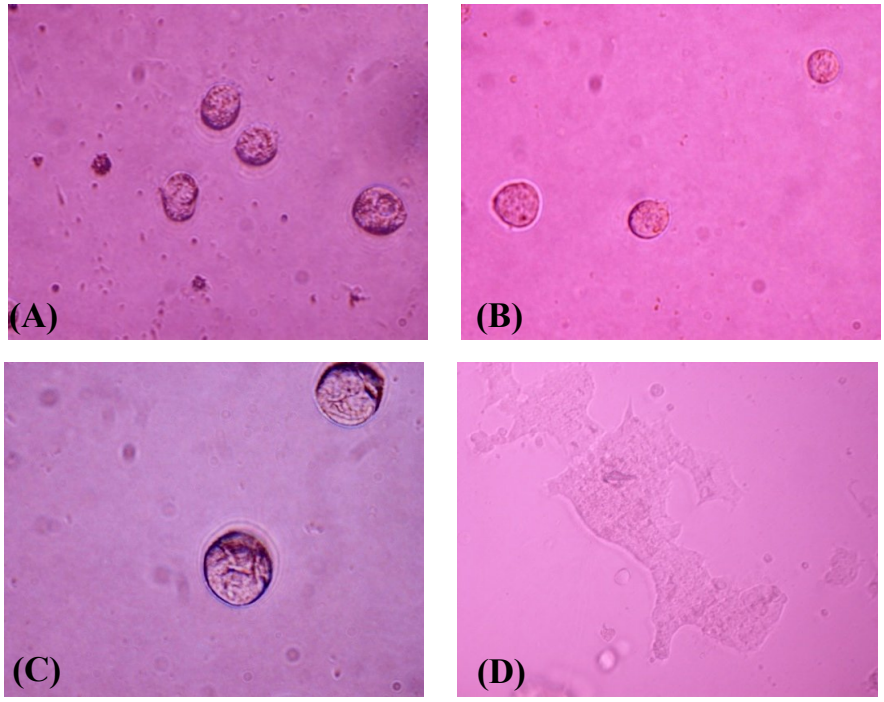

Fig. 5. MCF-7 cells were treated with **(A)** AIIB2 inhibitory monoclonal antibody against β1 integrin and **(B)** LY 294002 against class I phosphatidylinositol 3-kinase cultured three dimensionally and led to formation of reverted acini morphologically comparable to normal MCF-10A acini (**C**) and were visibly different from MCF-7 disorganized cluster of cells (**D**). All figures are at 20× resolution.

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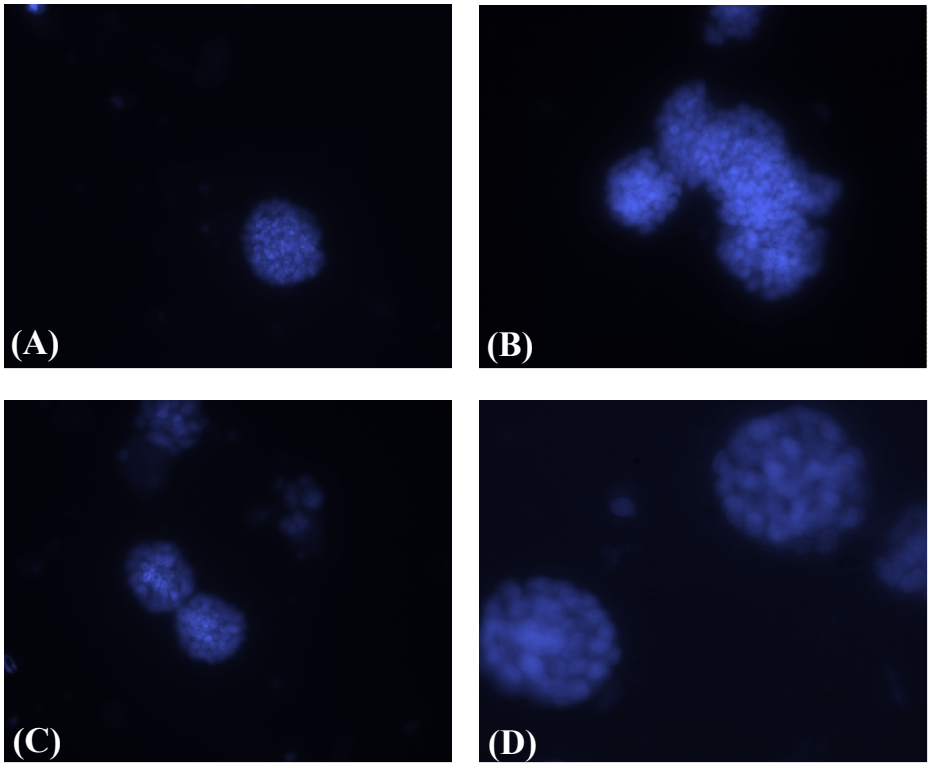

Fig. 6. For further analysis of **(A)** normal (20×), **(B)** malignant (20×) and **(C)** reverted acini by AIIB2 antibody (20×), and (**D)** LY 294002 (40×), nucleoli were stained with diaminophenylindole and examined with fluorescent microscopy. The 3D organization of reverted acini was markedly different from malignant acinar structures.

DISCUSSION

Glandular epithelial cells such as those in the mammary gland rest on and reciprocate dynamically with extracellular matrix (ECM) *in vivo* [16]. ECM provides both biochemical and structural cues for epithelial cells [17]. Consequential biochemical and mechanical signals are not preserved when malignant and normal cells are cultured on 2D plastic dishes [6]. Many of these pivotal messages are retained when cells are three dimensionally cultured on biosynthesized scaffolds, such as the reconstituted basement membrane which we have used [6, 17]. There is increasing corroborative evidence that 3D cell cultures are reliable models for verifying the effects of developing anti-cancer drugs at preclinical trials [13].

Here, for the first time in Iran, we have launched Matrigel based non-malignant, tumorigenic and reverted breast 3D cell cultures based on pioneering work of our colleagues abroad [7-10]. Malignant and non-malignant cells were cultured in the form of an "On top" assay in which cells were cultured on top of a thin Matrigel overlaid with a dilute solution of Matrigel, as opposed to 3D 'embedded' assay in which cells are cultured embedded in Matrigel [7, 8]. Matrigel also termed Cultrex, laminin-rich extracellular matrix or EHS matrix is a commercial soluble and sterile extract of basement membrane proteins derived from the EHS mouse sarcoma cells [11]. Matrigel provides both biochemical and structural signals that support morphogenesis, differentiation, and growth of malignant and normal mammary epithelial cell lines [11].

The 3D culture allowed phenotypic discrimination between non-malignant and malignant mammary epithelial cells [8]. MCF-10A cells seeded on Matrigel began to proliferate forming a cluster of cells. These clusters of cells dichotomize into two groups of cells at day 5. The first group, the outer layer of cells, is in dynamic reciprocity with Matrigel via receptors such as integrins. Subsequently, the first layer organizes to a well-polarized outer layer of cells which is visible on day 8. The second group which is the poorly organized inner bunch of cells and lacks contact with Matrigel is removed by apoptotic and non-apoptotic processes [7, 16, 18]. This eventually results in growth-

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arrested acinar structures with hollow lumen, as demonstrated by Figure 3. The entire structure obtains apical-basal polarity and cells within the acini express tissue-specific genes such as milk proteins [3]. These acini also deposit basement membrane component basally, such as collagen IV [9]. As demonstrated by Figure 3, we were able to reproduce this finding in our laboratory. On the other hand, 3D culture of malignant cells such as MCF-7 could model several oncogenic phenomena observed in carcinoma such as disorganized architectural morphology, as documented in our laboratory (Fig. 4) [9]. Signature morphology of cells, to some extent, could demonstrate gene expression pattern [19]. Other oncogenic phenomena associated with a tumor, studied in context of an organ system, can be recapitulated by malignant cells in 3D culture. Disrupted apico-basal polarity, failure to generate a hollow lumen, evading proliferative suppression and exhibiting invasive behavior are some of these oncogenic phenomena [14]. The above mentioned differences between malignant and non-malignant primary cultured cells and established cell lines, which are clearly demonstrable in 3D culture, are not achievable with standard 2D culture on plastic [8].

In addition to different biochemical signals, cells cultured three dimensionally receive dissimilar structural messages. It has been shown that cell shape has profound influence on cell fate and gene expression [20]. Cells in acinar form have different geometrical shape from adherent cells and have more condensed chromatin, hypo-acetylated H4 Histone and different gene expression profile [20]. Genes involved in signal transduction are influenced to a greater extent than others [19]. In conclusion, architecture by itself is fate determining [21].

We treated MCF-7 cells with inhibitory agents and cultured them three dimensionally which led to formation of reverted acini morphologically comparable to normal MCF10A acini as demonstrated by Figures 5 and 6. It has been shown that MCF-7 cells overexpress β1 integrin which leads to activation of downstream signaling components via PI3K [10, 15]. The activated signaling cascade divides into two pathways: one pathway results in increased cell proliferation through activation of downstream AKT and another pathway causes decreased cell polarity through activation of Rac1 [15]. The components of the above mentioned pathway seem to be a small section of an entangled network of signaling pathways that coordinately control cell proliferation, polarity, survival and migration. The Bissell lab is intensively deciphering the components of this network [13, 22]. Reversion via each inhibitor had similar effect on acini as judged by morphology and size criteria. Manipulation of one component in this network evidently influences cell proliferation, polarity, survival and migration concomitantly [15].

In conclusion, we have for the first time in Iran established a 3D culture system for non-malignant and malignant mammary epithelial cells on Matrigel. In addition, we used PI3K inhibitor and β1 integrin inhibitor to revert malignant cell structures to acinar-like structures resembling non-malignant acini. Further molecular data on the establishment of this system is under investigation such as documentation of apoptosis inside reverted acini, and suppression of proliferation by Ki67 index.

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