Video Article

Three Dimensional Cultures: A Tool To Study Normal Acinar Architecture vs. Malignant Transformation Of Breast Cells

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Abstract

Invasive breast carcinomas are a group of malignant epithelial tumors characterized by the invasion of adjacent tissues and propensity to metastasize. The interplay of signals between cancer cells and their microenvironment exerts a powerful influence on breast cancer growth and biological behavior[1]. However, most of these signals from the extracellular matrix are lost or their relevance is understudied when cells are grown in two dimensional culture (2D) as a monolayer. In recent years, three dimensional (3D) culture on a reconstituted basement membrane has emerged as a method of choice to recapitulate the tissue architecture of benign and malignant breast cells. Cells grown in 3D retain the important cues from the extracellular matrix and provide a physiologically relevant *ex vivo* system[2,3]. Of note, there is growing evidence suggesting that cells behave differently when grown in 3D as compared to 2D[4]. 3D culture can be effectively used as a means to differentiate the malignant phenotype from the benign breast phenotype and for underpinning the cellular and molecular signaling involved[3]. One of the distinguishing characteristics of benign epithelial cells is that they are polarized so that the apical cytoplasm is towards the lumen and the basal cytoplasm rests on the basement membrane. This apico-basal polarity is lost in invasive breast carcinomas, which are characterized by cellular disorganization and formation of anastomosing and branching tubules that haphazardly infiltrates the surrounding stroma. These histopathological differences between benign gland and invasive carcinoma can be reproduced in 3D[6,7]. Using the appropriate read-outs like the quantitation of single round acinar structures, or differential expression of validated molecular markers for cell proliferation, polarity and apoptosis in combination with other molecular and cell biology techniques, 3D culture can provide an important tool to better understand the cellular changes during malignant transformation and for delineating the responsible signaling.

Video Link

The video component of this article can be found at https://www.jove.com/video/51311/

Introduction

Three dimensional culture (3D) of breast epithelial cells on reconstituted basement membrane is an important model system to study the complex phenotype and associated signaling of normal breast and breast cancer[2,3]. The functional unit of breast is the terminal duct lobular unit (TDLU) which consists of a small duct which branches into acini. The acini are highly organized structures, composed of two cell layers, epithelial and myoepithelial cells, which are surrounded by a basement membrane[5]. The most distinguishing features of normal acini are cellular polarization, attachment to the underlying basement membrane and specialized cell-cell contacts. This intricate organization is disrupted in invasive carcinomas. The widely used 2D cultures, where cells are grown as monolayers, do not allow for the formation of acini. Thus, the monolayer culture is lacking in providing a system to capture the intricate relationship between epithelial cells in normal breast and their deregulation in breast cancer. Functional monotypic epithelial cultures of breast cells have been developed in several laboratories[2,3]. 3D culture involves the growth of breast cells on Matrigel, which is a solubilized extract derived from Engelbreth-Holm-Swarm mouse sarcoma cells and is available commercially. Other 3D substrata like collagen I are also used. The breast cells can be cultured in 3D by 3D embedded assay, where cells are cultured embedded in Matrigel[2]. Alternatively, cells can be cultured by 3D on top assay, also called 3D overlay assay, which is cost effective as it requires lesser volume of Matrigel, and facilitates time lapse monitoring of colony formation by phase contrast imaging and is ideal for *in situ* imaging[2-3]. The 3D on top assay has been used to define the correlation between the acinar phenotype and gene expression profile[7].

3D culture can be effectively used to distinguish normal and benign cells from invasive carcinoma. Normal and benign breast cells form growth arrested polarized structures with a well-defined lumen in the center on Matrigel whereas invasive carcinoma cells grow prolifically and haphazardly with no clearing of the lumen. E6/E7 immortalized human mammary epithelial cells and MCF10A cell line, which is a spontaneously immortalized cell line isolated from a 36 year old patient with fibrocystic changes, have been successfully used to recapture the benign breast phenotype in 3D[3,8] and have served as a model system to study the oncogenic and tumor suppressor function of several molecules[8,9]. These benign cells can be engineered to manipulate gene(s) of interest by the introduction of lentivirus/retrovirus. If the gene(s) of interest is a tumor

suppressor, shRNA mediated knockdown will lead to a malignant transformation whereas if the gene of interest is an oncogene overexpression in benign cells should show a malignant phenotype. In both cases the acinar structures formed in 3D can capture the malignant transformation.

Benign single cells plated in 3D start to proliferate and form round spherical structures. By day 5-8 this spherical aggregate of cells will differentiate into a surrounding polarized layer of cells that is in contact with the Matrigel, and an inner mass of less polarized cells, which lack contact with the Matrigel. In the next 10-15 days the inner cells will start to die by apoptosis forming a clear lumen. The malignant cells will grow haphazardly losing their polarity. Highly malignant cells usually form branching structures. These differences in phenotype can be captured by time lapse phase contrast imaging and by *in situ* immunostaining with relevant molecular markers. The most commonly used markers are alpha 6-integrin, a basal polarity marker, in combination with the proliferation marker phospho-histone 3 and the apoptotic marker cleaved caspase-3. The latter is important to determine whether there is lumen formation in the center of the acini, a feature of normal and benign breast cells. Other polarity and cell-cell contact markers include GM130, laminin, ERM, E-cadherin. Alternately the breast cancer cell lines can be engineered to manipulate the gene of interest. In this case reversion to a more growth arrested benign phenotype can be used as a read out to distinguish from the cancer phenotype.

3D culture can also be carefully used to delineate the signaling pathways involved in malignant transformation[10-11]. Using the above mentioned read-outs, pharmacological inhibitors, blocking antibodies or recombinant proteins can be used be pinpoint at the molecular signaling leading to malignant transformation. With continued efforts from various laboratories it is possible to extract the cells from 3D to isolate RNA and also prepare lysates for immunoblotting and immunoprecipitation. These strategies are described in a stepwise and detailed manner in the protocol.

Protocol

1. Preparation of Materials and Culture Media

1. Thaw growth factor reduced (GFR) Matrigel at 4 °C O/N.
2. Warm up complete media for required cell line at 37 °C. MCF10A- ATCC specified media; HME- Ham's F-12 medium supplemented with 5% fetal bovine serum, 1 µg/ml hydrocortisone, 5 µg/ml insulin, 10 ng/ml epidermal growth factor, and 100 ng/ml cholera toxin; SUM149- Ham's F-12 media supplemented with 5% FBS, 2 µg/ml hydrocortisone and 5 µg/ml insulin; and MDA-MB-231-10% FBS-DMEM
3. Precool an 8-well chamber slide on ice.
4. Prepare tissue culture hood and supplies.

2. Three Dimensional Culture in 8-well Chamber Slide

1. Coat each well of the 8-well slide with 40 µl of GFR Matrigel. Add the Matrigel in the center of the slide and then spread using a P200 pipette tip. Try to spread as evenly as possible, avoiding bubbles and overspreading which can lead to meniscus formation.
2. Incubate the slides at 37 °C under 5% CO_2. While the Matrigel is solidifying, trypsinize the cells, collect and spin at 150 x g in tissue culture centrifuge for 4 min.
3. Count the cells and dilute to 12,500 cells/ml in complete media of the respective cell line. Add GFR Matrigel to 2% and add 400 µl to each well of a Matrigel precoated chamber slide.
4. Incubate the slides in the CO_2 incubator. Give fresh complete media changes every 4th day until 15 days.
5. *Treatment with pharmacologic inhibitors:* For inhibitor studies add small molecule inhibitors to the complete media at the time of plating followed by fresh media changes supplemented with the inhibitors every three days while growing the cells on GFR Matrigel.
6. *Treatment with purified protein:*
    1. Plate the cells following steps 2.1-2.3. Allow the cultures to grow for 4 days followed by serum starvation for 16 hr.
    2. At day 5, treat the cells with appropriate concentration of purified protein in 0.1% FBS supplemented media to the serum starved cells. Give fresh media change with purified protein after 2 days.
    3. At day 10 replace the conditioned media with complete HME media. Allow the cultures to grow for another 5 days.
7. *Immunofluorescence staining of acinar structures grown on Matrigel:*
    1. Prepare 2% paraformaldehyde, 0.5% Triton X-100 and 100 mM glycine in 1x PBS, pH 7.4. Prepare immunofluorescence buffer (IF buffer) that comprises of 1x PBS containing 0.1% bovine serum albumin, 0.2% triton X-100, 0.05% Tween-20.
    2. Use a P200 pipette tip to carefully aspirate the conditioned media.
    3. Fix the acinar structures with 2% freshly prepared paraformaldehyde at room temperature (RT) for 20 min.
    4. Permeabilize the cells with 0.5% Triton X-100 for 10 min at 4 °C.
    5. Wash the cultures three times with 100 mM glycine, 10-15 min for each wash.
    6. Block the cells with 10% goat serum in IF buffer, called primary block, for 1 hr at RT.
    7. Incubate the cultures with a 20 µg/ml goat anti mouse F(ab´)$_2$ fragment in primary block buffer for 30 min. This step is important to block the immunoreactive mouse IgG species in the Matrigel.
    8. Incubate with primary antibody in the second blocking solution (Primary block+20 µg/ml goat anti mouse F(ab´)2 fragment) O/N at 4 °C.
    9. Wash three times with IF buffer for 10-15 min each with gentle rocking.
    10. Incubate with fluorescent conjugated secondary antibodies in IF buffer for 45 min.
    11. Wash 3x for 10 min each with IF buffer with gentle rocking.
    12. Mount the slides with anti-fade reagent containing DAPI to visualize the nuclei.
    13. Allow the slides to dry O/N at RT.
    14. Image acquisition: Acquire confocal images at the colony midsection of cells grown on top of Matrigel. For more information acquire a series of optical sections throughout the entire length of the acinar structure by Z stacking.

3. Three Dimensional Culture in 2-well Chamber Slides for Immunoblotting

1. Precool a 2-well chamber slide on ice. Follow steps 2.1-2.4 scaling up the reagents for the 2-well chamber slide *e.g.* coat with 160 µl of Matrigel and add 1.6 ml of cell/Matrigel mix to each well of the 2-well slide.
2. Harvest the acinar structures from the Matrigel using the commercially available 3D culture cell harvesting kit following manufacturer's instructions.
3. Dilute the cell wash and cell harvesting buffers to 1x and precool to 4 °C. Washing and dislodging of Matrigel should be carried out on ice.
4. Wash the acinar structures 3x using the cell wash buffer.
5. Collect the acini into 15 ml centrifuge tubes using cell harvesting buffer followed by 30 min incubation on a rocker at 4 °C. Mix suspension well with a 1 ml pipette. This will break the Matrigel in small pieces and release most of the cells in the cell harvesting buffer.
6. Pellet the cells at 200 x g in a culture centrifuge. Remove the floating layer of hydrogel from the pelleted cells with a P200 pipette tip.
7. Resuspend the lysates in SDS sample buffer provided with the kit. RIPA lysis buffer can also be used.

4. Quantification of Malignant vs. Benign Breast Phenotype

1. Grow cells in triplicates for each treatment set following steps 2.1-2.4.
2. Take phase contrast images of acinar structures from each well at 5X or 10X resolution using a phase contrast microscope attached to a slide shifter and a computer. Take the images by moving the slide from one frame to another and thus covering the entire well of the chambered slide.
3. Count the total number of acinar structures, the number of single round flat acini and the number of multiacinar structures or haphazardly growing structures lacking organization and with branching processes emanating from it.
4. Calculate the percentage of single round flat acinar structures vs. malignant structures and plot as column graph. Calculate the significance by Student's t-test.

Representative Results

3D culture can be effectively used to differentiate malignant phenotype of human breast epithelial cells from the benign breast. Single benign cells plated on Matrigel grow as organized spherical structures that can easily be imaged as single round flat acini in one plane using phase contrast imaging. The single malignant cells plated on Matrigel grow haphazardly, show very high refractive index and are difficult to image in one plane. As shown in **Figure 1**, the benign HME and MCF10A cells form spherical looking acini whereas highly metastatic breast cell lines SUM149 and MDA-MB-231 grow as branching tubular structures with processes emanating from them. Such a deviation from the benign phenotype can be used as a read out to confirm the malignant transformation and can be used to define the function of a molecule as a tumor suppressor or as a tumor promoter. As shown in **Figure 2**, the knockdown (KD) of tumor suppressor gene CCN6 triggers a malignant transformation. HME cells engineered to have down regulated levels of CCN6 by lentivirus mediated transfection lose their organization from very early on. At day 5 the benign control cells were growing as perfect spherical structures whereas the CCN6 KD cells show a disorganized phenotype. By day 15 the benign control cells become growth arrested and show a polarized layer of cells surrounding a well-defined central lumen whereas the KD cells grow prolifically to form disorganized large tubular structures which continue to grow if allowed (**Figure 2**).

Several molecular markers can be used to advantage to differentiate between the benign vs. malignant breast phenotype. The benign breast shows a differentiated growth arrested phenotype by day 15 in 3D culture. The acini have a deposition of basement membrane that can be visualized by immunostaining with laminin V as is clearly shown in control cells in **Figure 3**. These cells are well polarized and shows the presence of alpha-6 integrin basally and GM130 apically. E-cadherin is present at the cell-cell junctions indicating an intact cell-cell contact in benign cells. The malignant cells like CCN6 KD show disruption of basement membrane indicated by laminin V staining. These cells lose their polar organization and as such can show loss of alpha-6 expression as in case of CCN6 KD cells or haphazard expression of alpha 6 integrin and GM130. CCN6 KD and MDA-MB-231 cells which undergo epithelial to mesenchymal transition show loss of E-cadherin. However, malignant cells can still express E-cadherin but localization might not be restricted to the cell-cell junctions (**Figure 3**).

The signaling pathways involved in the malignant transformation can be delineated using a combination of pharmacological inhibitors and purified recombinant human (rh) proteins. As shown in **Figure 4** the malignant phenotype of CCN6 KD HME cells can be reverted to single round acinar structures by exogenous treatment with rhCCN6 protein. Confocal imaging at the mid-section of the DAPI stained acinar structures clearly show polarized acini with central lumen in CCN6 KD cells treated with rhCCN6. Similarly the invasive SUM149 and MDA-MB-231 cells treated with P38 MAP kinase inhibitor shows the reduction in the malignant phenotype (**Figure 5A**). The reversion to a benign like phenotype can be quantified by calculating the percentage of single round acinar structures as compared to the malignant structures. The specificity of the inhibitors can be appreciated by the fact that AKT inhibitors fail to show any effect on CCN6 KD cells whereas treatment with the MAP kinase inhibitor PD98059 shows a remarkable reversion to single round acinar structures (**Figure 5B**).

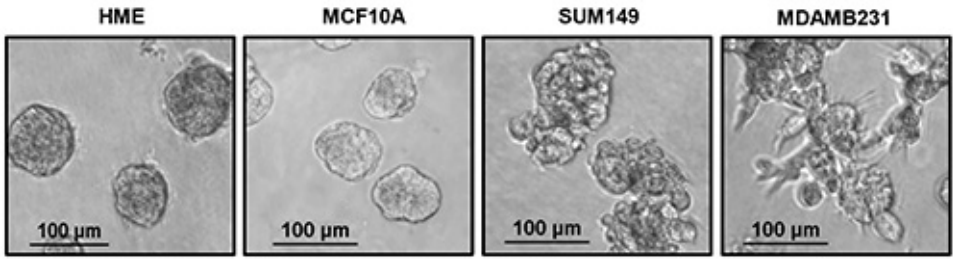

Figure 1. Phase contrast images of benign vs. malignant breast cells grown on Matrigel for 10 days. The benign immortalized human mammary epithelial cells (HME) and spontaneously immortalized MCF10A cells grow as single round flat acini devoid of any protrusion. Whereas the malignant human breast cell lines SUM149 and MDA-MB-231 grow as highly disorganized structures. Scale bar represents 100 µm. Click here to view larger image.

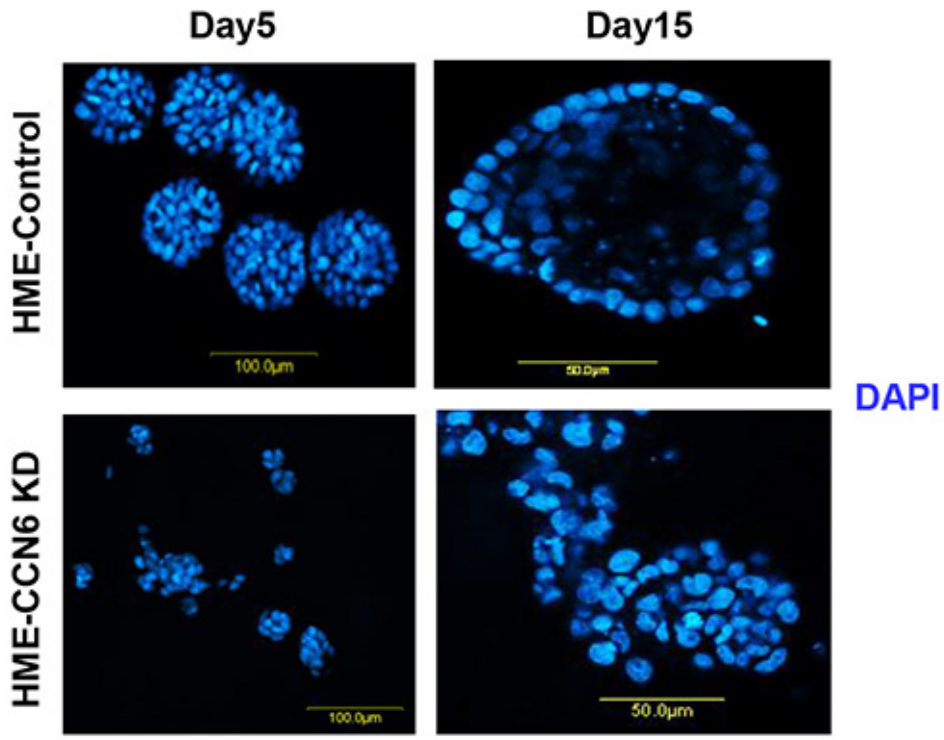

Figure 2. Confocal imaging of HME-control and CCN6 KD cells grown on Matrigel for 15 days. Blue depicts DAPI for nuclear staining. The KD of CCN6 in HME cells triggers a malignant phenotype. The malignant CCN6 KD cells grow in a disorganized manner from the beginning. At day 5 in 3D culture KD cells lack organization whereas the benign control cells grow as single round acini that become growth arrested and differentiated acinar structures with well-defined lumen by day 15 of growth on Matrigel. On the other hand the KD cells present a very invasive, branching and tubular phenotype. Scale bar represents 50 µm. Click here to view larger image.

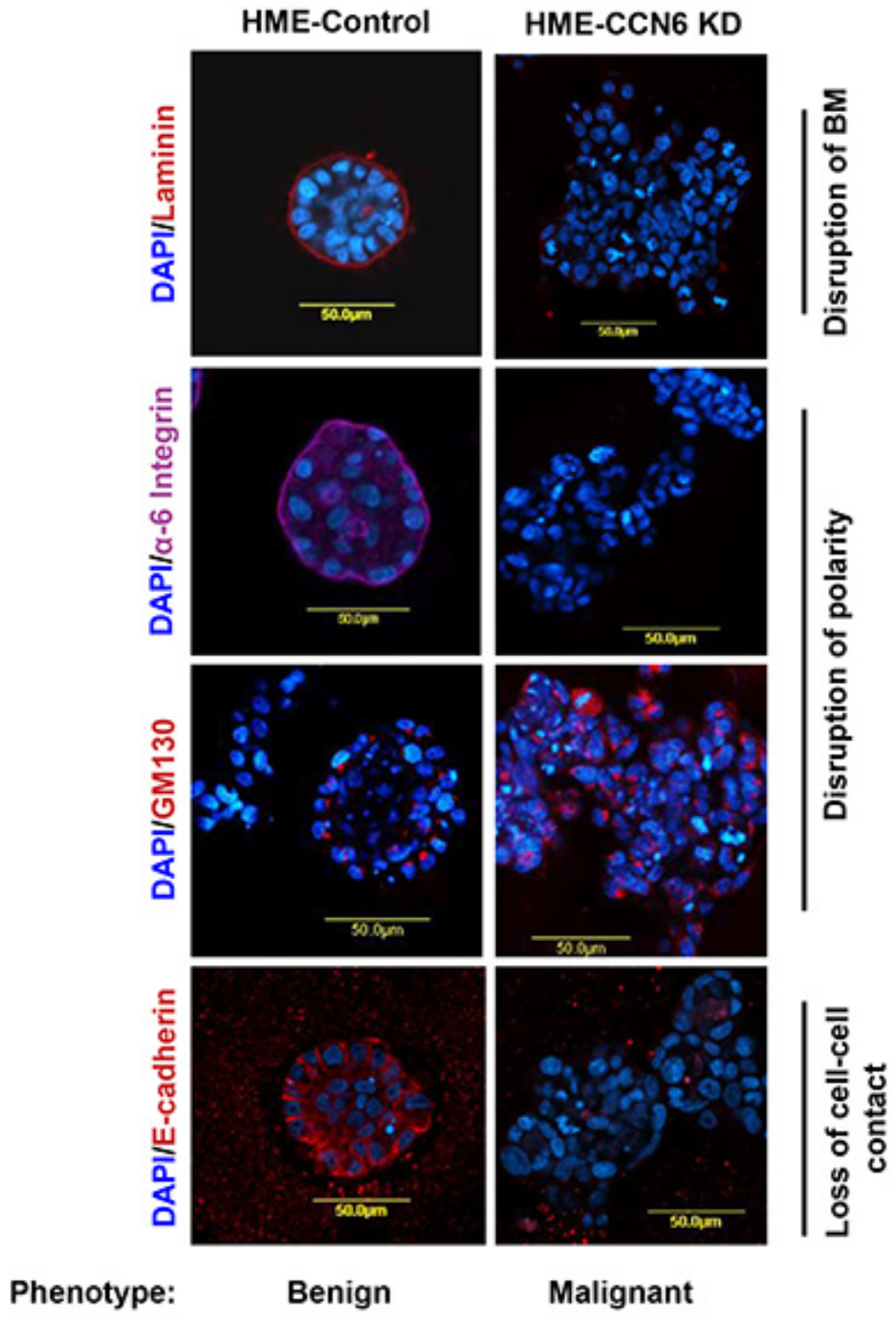

Figure 3. Confocal imaging shows differential expression of molecular markers in benign vs. malignant breast cells. CCN6 KD HME cells show loss of laminin V, representing the disruption of basement membrane, loss of basal polarity marker alpha-6-integrin, indicating loss of polarity and loss of cell-cell contact marker E-cadherin. However the control cells show deposition of basement membrane (an intact laminin layer around the acini), basal expression of alpha 6 integrin, apical expression of GM130 and presence of E-cadherin at cell-cell junction. Scale bar represents 50 μm. Click here to view larger image.

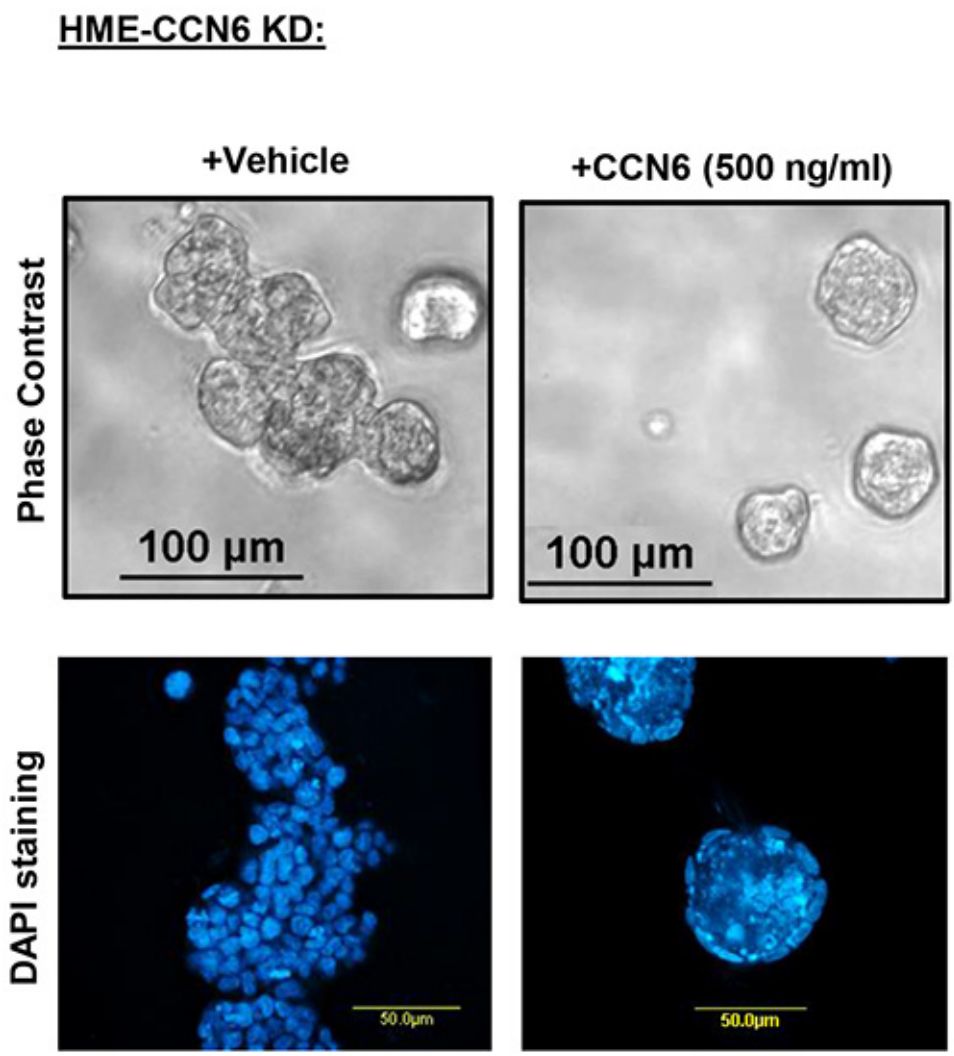

Figure 4. Reversal of the malignant phenotype of CCN6 KD HME cells by exogenous treatment with purified recombinant human CCN6 protein. CCN6 KD cells grow as haphazard, invasive acinar structures in 3D culture. However, CCN6 KD cells treated with 500 ng/ml of rhCCN6 show reversion to single round acinar phenotype with clearing of lumen, indicating a more benign like phenotype. Black scale bar represents 100 µm and yellow scale bar represents 50 µm. Click here to view larger image.

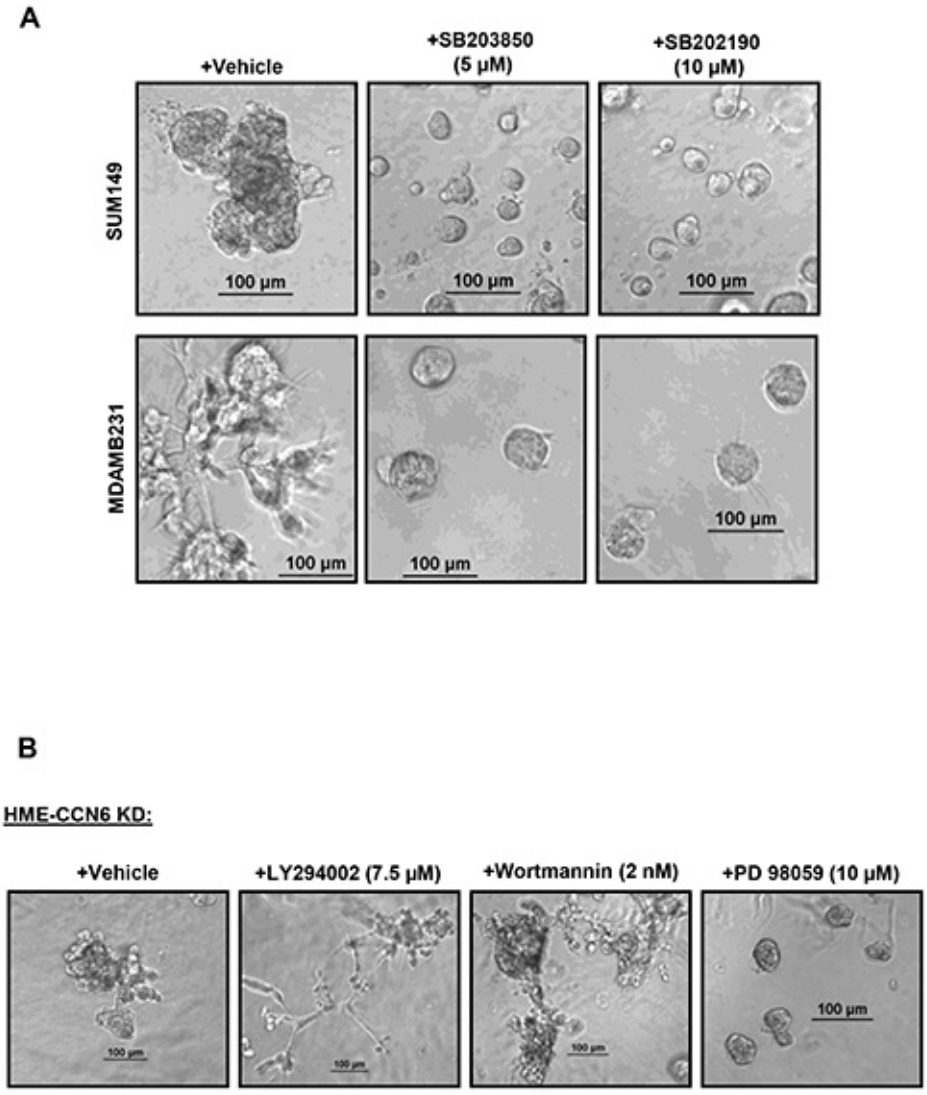

Figure 5. Treatment with small molecule inhibitors revert the malignant phenotype to less malignant or benign like phenotype. A. Treatment of 2 highly metastatic breast cell lines SUM149 and MDA-MB-231 with P38 MAP kinase inhibitors SB203850 (5 μM) and SB202190 (10 μM) reduces their malignant behavior as is reflected in the single round acinar phenotype on 3D. B. CCN6 KD HME cells treated with AKT inhibitors LY294002 (7.5 μM), Wortamannin (2 nM) shows no effect but treatment with MAP kinase inhibitor PD98059 (10 μM) reverts the phenotype to single round organized acinar structures. Click here to view larger image.

Discussion

We have described here the three dimensional culture of breast epithelial cells on a reconstituted basement membrane and the usefulness of this system to differentiate between malignant vs. benign breast phenotype. This system can also be used to culture different cell types from other glandular tissues and has been reported to have been successfully used to culture prostate epithelial, bronchial epithelial, and thyroid epithelial cells, to name a few[12-14]. The importance of 3D culture lies in the fact that it is a physiologically-relevant model. 3D culture provides a means to gain insights into the molecular mechanisms that leads to disruption of the glandular architecture during the malignant transformation. It also provides a working model system that can be helpful in screening novel therapeutic targets for treating cancers. *Many drugs which are found to be effective in 2D culture often have little effect in experimental and clinical applictaions[15]. As such 3D culture can provide an important preclinical tool to predict the effect of compounds in vivo.*

3D culture is a challenging technique and should be performed carefully. GFR Matrigel is the most commonly used substrata for culturing cells in 3D. It can be thawed at 4 °C O/N, aliquoted and frozen at -20 °C for future use. Repeated freeze thawing should be avoided. GFR Matrigel is liquid at 4 °C but solidifies at 37 °C and can be extremely difficult to handle when changing conditioned media with fresh media or while processing the cells for immunocytochemistry. Please do not use a vacuum aspirator to remove the conditioned media as it invariably leads to the loss of entire acinar cultures along with the Matrigel. It is observed that in case of some aggressive cancer cell lines like MDA-MB-231 cells which form very branching network like structures the Matrigel can detach from the surface and is often times floating. Care should be taken to suck media out of these cultures otherwise they will be lost while removing the media.

Equally important is the care and passage of cells growing as monolayer cultures especially for the benign cells like HME and MCF10A. Over confluence or deviance from the prescribed media may affect the acinar formation in 3D.

It is possible to isolate cells from 3D cultures to process for RNA or protein isolation using commercially available 3D culture cell harvesting kit. Alternately three-dimensional cultures can be directly lysed using detergent based-lysis buffer such as RIPA buffer (1% Nonidet P-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 b-glycerophosphate, 5 µg/ml apoprotinin, 5 µg/ml leupeptin, and 100 µM phenylmethylsulfonyl fluoride). However protein quantification is not possible in this case because of protein abundance in the Matrigel. Thus the protein lysates should be normalized with colorimetric assays used to measure the activity of stable cytosolic enzymes, such as lactose dehydrogenase (LDH) and the blots should be reprobed for a housekeeping gene like actin or tubulin or GAPDH [3].

However the cell harvesting from the Matrigel process requires long duration incubation at 4 °C. This can affect the expression profile of the cell surface proteins and as such *in situ* immunostaining is a more appropriate method to analyze the expression of cell surface proteins. For immunostaining antibody incubation on a gentle rocker is helpful in evenly staining the acini but optimal speed and the rocker kind is critical as it can also lead to the dislodging of the Matrigel. The antibody concentration may need optimization but most antibodies were found to be efficient at 1:100 concentration. A more specific list of antibodies is provided in the table. A detailed list of antibodies with their specifications can be found in Debnath *et al.*[2] Simultaneous incubation with more than one primary antibody obtained from different animals like rat, rabbit and mouse can be carried out but should be optimized initially to rule out any cross reactivity.

Disclosures

The authors declare no competing financial interests.

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