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Research Article

Modelling and Validating Three-Dimensional Human Breast and Cancerous Human Breast Tissues In Vitro

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ABSTRACT

In this study three dimensional (3-D) *in vitro* models of normal breast and breast cancer tissues were developed to mimic closely the *in vivo* tissue microenvironment and therefore providing reliable models for *in vitro* studies as well as testing of novel cancer therapies. Normal and cancerous human breast cell lines were used to construct 3-D artificial tissues, where de-epidermalised dermis (DED) was used as a scaffold for both models. Morphological analyses were conducted using haematoxylin and eosin staining. Biomarkers including keratin 5 and 19 as well as α smooth muscle actin and mucin 1 were used to confirm and validate the reliability of the proposed models using immunohistochemical techniques. Findings suggest that the 3-D *in vitro* models described in this work can serve as functional models of both human normal and cancerous breast tissues. Multiple structures similar to ducts and lobules of human breast *in vivo* were observed in 3-D *in vitro* models by the use of H&E, some breast cancer colonies seen in the cancerous 3-D model were similar to the ducto-lobular structures observed in normal 3-D model of the breast but the former cells were more loosely connected, irregular and largely disorganized. The established 3-D *in vitro* model of normal breast showed the development of ducto-lobular structures composed of an inner cell layer which was stained positive with α mucin 1 antibody, a biomarker that is characteristic for luminal cells; and also an outer basal layer of cells that was stained positive for α smooth muscle actin, a biomarker of myoepithelial cells.. Keratin staining in 3-D *in vitro* models also resembled the pattern observed *in vivo* where keratin 5 was detected in both luminal and myoepithelial cells of normal breast model (NTERT cells), whereas keratin 19 was present in breast cancer model (C2321 cells). These 3-D models successfully recapitulate both normal and pathological tissue architecture of breast tissue and has the potential for various applications in the evaluation of breast cancer progression and treatment.

Introduction

Culturing cells in conventional 2-D conditions is routinely used as an initial model system to evaluate the safety and effectiveness of various potentially therapeutic compounds. Studies that involve culturing cells in flat monolayers, in two dimensional cell cultures often do not reflect the *in vivo* situation observed in tissues due to the altered geometric and mechanical restrictions enforced on cells [1]. Tissue architecture has been shown to be essential for the maintenance of cell differentiation [2]. In cancer research, the tumour microenvironment and the architecture of extracellular matrix (ECM) are the major factors that determine cancer development and responses to treatment. ECM is a crucial element that promote normal epithelial polarity and differentiation [1]. Moreover, ECM was reported to dictate the phenotype of mammary epithelial cells [3, 4]. Therefore, reliable and robust three-dimensional *in vitro* tumour models will play an important role in cancer research by providing information on tissue organization and cellular differentiation.

Although current progress in the 3-D culture systems development is very dynamic and advances in tissue engineering have enabled the elaboration of various *in vitro* models of normal breast and breast cancer, further research is still needed [5]. In particular, difficulties in mimicking

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breast cancer development and progression in a laboratory setting indicate that there is still a need to develop *in vitro* human breast models which can more accurately represent breast diseases, such as breast cancers [6].

In this study, we created and described a novel 3-D *in vitro* models for both normal and cancerous mammary tissues. We have mimicked the ECM characteristics and tissue microenvironment by using ECM components of human DED. By the use of hematoxylin and eosin (H&E) staining, the morphology of normal and cancerous cell arrangements that formed multicellular structures was compared in these 3-D *in vitro* models. To verify the reliability of 3-D *in vitro* model of the breast, keratin 5, keratin 19, α-smooth muscle actin (α actin) and MUC 1 biomarkers were used and analysed using immunohistochemical techniques.

Methodology

I Cell Lines

All cell lines were purchased from the American Type Culture Collection (UK). Breast cancer cell lines were grown in RPMI (Gibco Invitrogen, UK) media supplemented with 10% Fetal Bovine Serum – FBS (BioSera, UK), 1% antibiotic (Sigma, UK), supplemented with 1mM of sodium pyruvate (Gibco Invitrogen, UK). Human keratinocytes (NTERT) were grown in Keratinocyte-SFM medium with L-Glutamine, without Calcium Chloride (Gibco Invitrogen, UK) supplemented with Keratinocyte-SFM Supplement Human recombinant Epidermal Growth Factor (2.5µg/L) and Bovine Pituitary Extract (BPE) (25mg/L) (Gibco Invitrogen, UK). All cells were grown at 37ºC in a humidified atmosphere of 5% CO_2 with the media being changed regularly depending on the growth rates of different cell lines.

II Three-Dimensional *In Vitro* Models

NTERT cells were used to create a control model of normal human breast, whereas breast cancer cells MCF7, C2321 and C2335 were used to produce *in vitro* models of breast cancer. The immortalized human keratinocytes cell line NTERT and the cervical cancer cell lines C33A, C2321 an C2335 were purchased from ATCC (ATCC, UK). All cell lines used were grown on de-epidermalised dermis (DED), immersed in media with the addition of 1 µM estradiol (Sigma, UK). In order to verify the presence of ducto-lobular structures, two antibodies: anti-α actin, that stains myoepithelial cells of the duct and lobule of the breast, and anti-MUC 1, that stains luminal cell in ducts and lobules of the breast, were used. An adjacent normal breast tissue area from breast cancer patient was used as a positive control. Negative controls where primary antibody was omitted and negative isotypic controls were also employed in this study (data not shown).

All cell lines were grown using DED as a scaffold for modelling 3-D *in vitro* systems. Human skin (Euro Skin Bank, Netherlands) was de-epidermalised using method previously described by Zuk *et al*. [7]. The de-epidermalised dermis was cut in small sections (1.5 x 1.5 cm,) washed three times in 1% PBS and incubated at 37ºC in a humidified atmosphere of 5% CO_2 for 24h with culture media. Cells were then seeded on the top of the de-epidermilised dermis inside a metal ring and after 48 hours, after cell attachment, the ring was removed, and cells were further incubated with media supplemented with 1 µM of estradiol for 3-4 weeks with media replenished regularly. The 3D models were then washed in PBS and placed in 4% paraformaldehyde for 48 hours. After fixation models were washed in PBS, dehydrated in 70%, 90% and absolute ethanol respectively and cleared in xylene before being embedded in paraffin wax.

III Staining

The morphology and characteristics of normal and cancerous cell arrangements in the 3-D *in vitro* models created were examined by the use of H&E and immunohistochemistry staining techniques. Paraffin-embedded tissue blocks were cut in 6µm thick sections and stained for both H&E or immunohistochemical staining using methods previously described by Zuk *et al*. [7]. In brief, sections were deparaffinised in xylene and rehydrated through graded alcohols to water. To block endogenous peroxidase sections were incubated in 3% H_2O_2 in methanol for 10 minutes (Sigma-Aldrich Co. Ltd, Poole, UK). Different epitope retrieval techniques were used, according to (Table 1). To block non-specific binding of immunoglobulin sections were incubated in blocking serum (normal horse serum (Sigma, UK) and PBS in 1:1 proportion) for 10 minutes. Sections were then incubated with primary antibody overnight at 4ºC, and after three PBS washes, they were further incubated with biotinylated secondary antibody for 30 minutes at room temperature. Samples were then rinsed in PBS and then incubated with universal avidin biotin complex (ABC) solution (Vector Laboratories Ltd, UK) for 20 minutes at room temperature. Afterward, samples were washed in PBS and sections were developed using 3,3'-Diaminobenzidine (DAB) (Sigma-Aldrich Co. Ltd, UK) substrate solution for up to 5 minutes. Then samples were briefly rinsed in distilled water and counterstained with Gill's hematoxylin solution (Sigma-Aldrich Co. Ltd, UK). Sections were differentiated in 1% acid alcohol for a few seconds, washed in running tap water for 5 minutes and dehydrated through 70%, 90% for 2 minutes and two changes of absolute alcohol for 3 minutes each. Finally, samples were cleared in two changes of xylene, and mounted with xylene based mounting medium before being analysed using a light microscope (Nikon eclipse SOi, UK). Images were captured using Image - Pro Express 6.3 program. A list of primary and secondary antibodies is presented in (Table 1) below.

Table 1: Antibodies used for IHC staining and their characteristics.

1º Ab	Ig type	Concentration	Antigen retrieval	Serum	2 º Ab
Anti α-smooth muscle actin (mouse monoclonal) (Abcam)	IgG2a	2 µg/mL	0.1% Triton-100, 20 min	goat	Goat anti-mouse IgG H&L (Biotin)

Anti-MUC 1 (rabbit monoclonal) (Abcam)	IgG	1.53µg/mL	0.1% Triton-100, 20 min	horse	Biotinylated anti-rabbit IgG
Isotypic control (goat anti-mouse) (Abcam)	IgG2a	1.1 µg/mL	0.1% Triton-100, 20 min	horse	Biotinylated anti-rabbit IgG
Isotypic control (goat anti-mouse) (Abcam)	IgG	1.53 µg/mL	0.1% Triton-100, 20 min	horse	Biotinylated anti-rabbit IgG
Anti-cytokeratin 5 [K5] (rabbit monoclonal) (Abcam)	IgG	1 µg/mL	Citrated buffer x1, heated for 20min	horse	Biotinylated anti-rabbit IgG
Anti-cytokeratin 19 [CK19] (mouse monoclonal) (Abcam)	IgG2a	1 µg/mL	Citrated buffer x1, heated for 20min	horse	Biotinylated anti-mouse IgG

Staining of the 3-D *In Vitro* Models by Confocal Microscopy Analyses

Paraffin-embedded 3-D *in vitro* models were cut in 6µm sections and prepared as for standard IHC as above. Sections were incubated with primary antibody MUC 1 (Abcam, UK) overnight at 4ºC. On the second day sections were further incubated with secondary antibody - biotinylated anti-rabbit IgG in PBS for 30 minutes at room temperature. Then samples were rinsed in PBS twice for 5 minutes and incubated with avidin biotin complex solution (ABC), (Vectastain, Vector Laboratories, UK) for 20 minutes at room temperature. Samples were further developed with TSA/Fluorescein (PerkinElmer, USA) for 5 minutes and washed in PBS twice for 5 minutes. In the following step sections were washed in PBS and blocked again with goat serum (Sigma, UK) (for α actin) for 10 minutes. Sections were then incubated with primary antibody α actin (Abcam, UK) for 1 hour at room temperature and following PBS washes, sections were further incubated with secondary antibody - Goat anti-mouse IgG H&L (Biotin), for 30 minutes at room temperature. Samples were rinsed in PBS twice for 5 minutes and incubated with avidin biotin complex (ABC) solution for 20 minutes at room temperature. Then samples were developed with TSA/Cy5 (PerkinElmer Life and Analytical Sciences, Inc. Boston, USA) for 5 minutes and washed in PBS twice for 5 minutes. Finally, sections were mounted with DAPI - 4', 6-diamidyno-2-fenyloindol (Sigma, UK). Images of double fluorescent staining were captured using a confocal fluorescent microscope (Leica Microsystems, Wetzlar, Germany), where α actin was stained red and Muc 1 was stained green. Nuclei were counterstained by 6-diamidino-2-phenylindole (blue nuclei).

Results

Multiple structures that resemble duct and lobules of human breast *in vivo* were observed in the established 3-D *in vitro* models by the use of H&E and immunohistochemistry staining. Structures were further stained with different keratins in order to reveal and compare the different patterns of keratin staining in normal and cancerous breast tissue. [8-10]. In our models, K5 was detected in both luminal and myoepithelial cells of normal 3-D *in vitro* model of the breast (Figure 1),

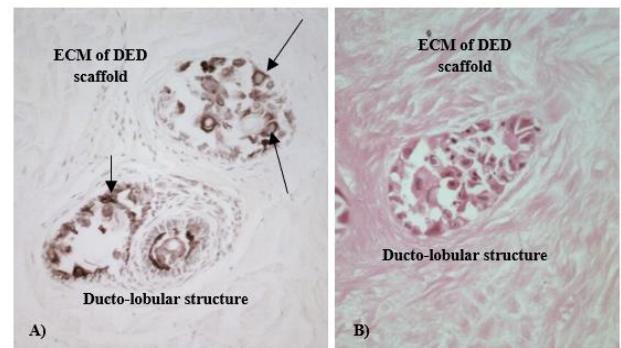whereas K19 was present in 3-D *in vitro* model of cancerous breast (Figure 2).

Figure 1: K5 staining in 3-D *in vitro* model of normal human breast; **A)** K5 was positively stained (arrows) for both luminal and myoepithelial cells; **B)** H&E of the same area, mag. x 400.
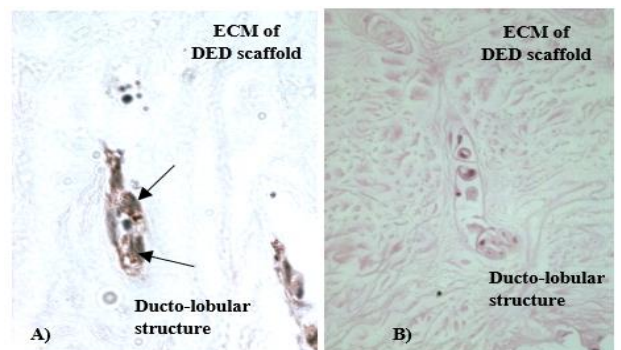

Figure 2: K19 staining in 3-D *in vitro* model of human breast cancer; **A)** K19 positive C2321 cells are indicated by arrows; **B)** H&E of the same section, mag. x 200.

To verify the reliability of 3-D *in vitro* model of the breast α actin and MUC 1 biomarkers were used in both immunohistochemical and confocal fluorescent microscopy analyses. Biomarker MUC 1 that is normally expressed by luminal cells, was detected inside ducto-lobular structures of 3-D *in vitro* models and the staining was most intense in the centre of cellular formations (Figure 3). The positive staining of α actin,

a biomarker that is normally expressed by myoepithelial cells of the breast, was detected outside ducto-lobular structures formed in 3-D *in vitro* models (Figure 4). Spatial organization of myoepithelial cells in relation to luminal cells was observed, particularly in 3-D *in vitro* model of normal breast.
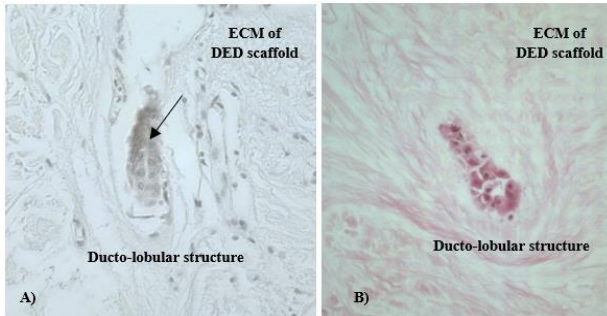

Figure 3: NTERT cells grown in 3-D in vitro model stained with anti MUC1 antibody. **A)** Cells formed ducto-lobular structures inside the DED scaffold (arrow); **B)** H&E staining of the same section, mag. x200.
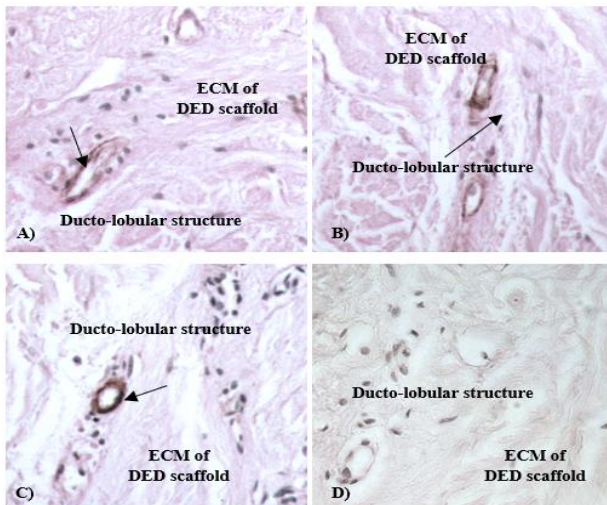

Figure 4: α actin positive staining for NTERT cells grown in 3-D *in vitro* model. **A)**, **B)**, **C)** staining was detected outside ducto-lobular structures formed in 3-D *in vitro* models (arrows), mag x 200; **D)** H&E of the same section, mag x400.
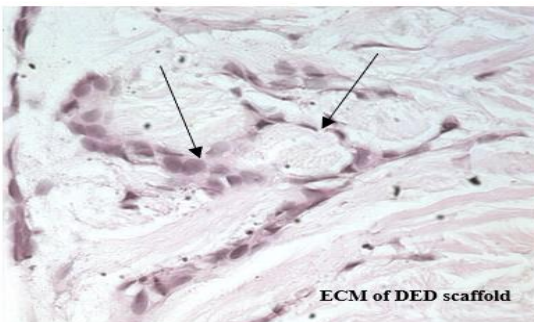

Figure 5: H&E staining for 3D in vitro cell model established using C2335 cells. Cells were formed colonies similar to the ducto-lobular structures observed in normal 3-D model of the breast, but cancer cells were more loosely connected, irregular and largely disorganized. Cells with different sizes and shapes were visible with large, variably shaped nuclei (arrows); mag. x 400.

Some breast cancer colonies seen in the cancerous 3-D model were similar to the ducto-lobular structures observed in normal 3-D model of the breast, but the former cells were more loosely connected, irregular and largely disorganized (Figure 5).

IHC staining with anti-α-actin and anti-MUC 1 confirmed the presence of ducto-lobular structures in 3-D *in vitro* model of normal breast. The enhancement of detection was achieved with the use of a double immunofluorescent staining method that was employed for further 3-D model characterization and validation. The ducto-lobular structures found in 3-D *in vitro* models of breast, were double-stained with anti-MUC 1 antibody (green) and anti–α-actin (red), whereas nuclei of the cells were counter stained using DAPI. Expression of α-actin and MUC 1 in 3-D *in vitro* model of normal breast (Figure 6) correlated with the staining of control breast tissue section presented in (Figure 7). In breast cancer models established using C2335 cells, cells were also reorganized into structures which are similar to ducto-lobular arrangement observed in 3-D model of normal breast (Figure 8), however, the boundary between the inner and outer layer, was not that clear as seen in NTERT cells.
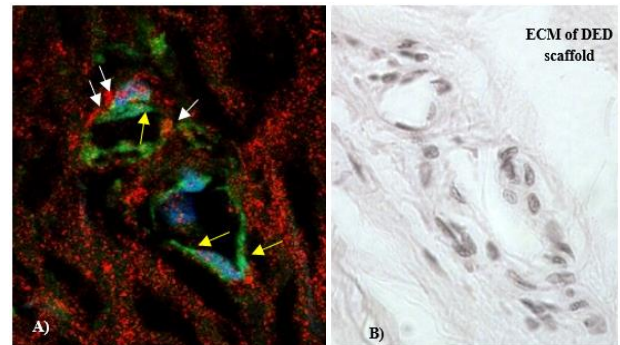

Figure 6: A) Confocal microscopy analyses of NTERT cells grown in 3-D *in vitro* model, stained with α-actin (red) and MUC 1 (green); white arrows represent α actin, whereas yellow arrows represent MUC 1; mag. x 400; **B)** H&E staining of the same area.
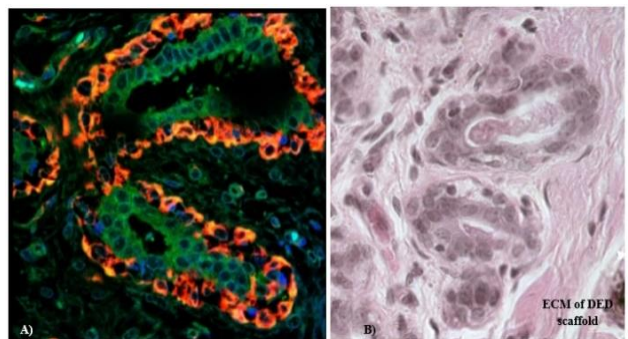

Figure 7: A) Confocal fluorescent microscopy analyses of the control breast tissue section stained with α-actin (red) and MUC 1 (green); mag. x 400; **B)** corresponding H&E staining of the same breast tissue sections.
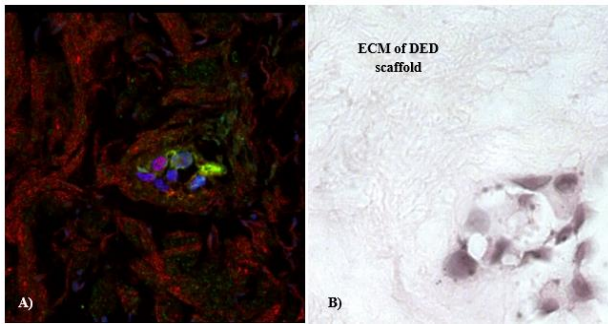
Figure 8: Confocal fluorescent microscopy analyses of α-actin (red) and MUC 1 (green) from 3D *in vitro* breast cancer model. **A)** 3-D *in vitro* model of C2335 breast cancer cells, stained with α-actin (red) and MUC 1 (green); cells were organized in arrangements similar to ducto-lobular structures observed in normal 3-D *in vitro* model, but the inner and outer layer was not clearly separated; mag. x 400; **B)** corresponding H&E staining of the same area.

Discussion

New physiologically relevant and cost effective *in vitro* cellular models are needed to gain a better understanding of the mechanism of human tissue pathology, and to estimate dosage of new therapies, to assess the efficacy, or toxicity of drugs, as well to evaluate drug penetration into tissue. Current progress in tissue engineering have improved the diversity and quality of 3-D *in vitro* models which take them one step closer to the *in vivo* situation. Each of them has some advantages and limitations:

Spontaneous cell aggregation is a technique where the 3-D spheroids can be formed by culturing cells on artificial substrates that stimulate cellular differentiation and maintain cellular function or by spontaneous cell aggregation where spherical cellular conglomerates are generated. Multicellular tumour spheroids are very small structures that are composed with tumour and extensive ECM cells structured in a 3-D arrangement. A number of breast cancer cells and breast cell lines have the ability to spontaneously aggregate during cell culture, to form tissue-like spheroids, such as: human breast carcinoma cell MDA-MB-435, breast carcinoma cell line DU4475 [11, 12]. The multicellular aggregate formation correlates with increased cancer survival and metastasis *in vitro* and is an important feature of metastatic cancer cells. In the spheroid model proliferating cells are usually present near the surface or on the surface and this is caused by mass transport limitations at greater depths, lack of nutrient penetration and toxic metabolic waste build up [13, 14]. For that reason, this model effectively resembles the situation observed in micrometastases prior to vascularization and intervascular microregions seen in large tumours [5]. The advantage of the spontaneous cell aggregation model is that they are cheap and require little work for preparation. The disadvantage is that spontaneous cell aggregation process can only occur in a small number of cell types, also some cells forms clusters instead of spheroids.

Another technique that enables growing cells as 3-D aggregates or spheroids is a liquid overlay culture. Special conditions need to be created, where adhesive forces between the cells are greater than between the cell and a substrate it is plated on. A spinner flask and gyrators are other methods that prevent cell adherence by inhibiting meaningful contact with the culture vessel wall. Liquid overlay techniques that prevents matrix deposition is the simplest way to achieve such conditions. The advantage of this technique is that many cell lines will undergo spontaneous aggregation. Moreover, even in tumour cell lines that do not aggregate or are difficult to aggregate, spheroid formation can be induced. In this process cells are grown in media over an agar base. Initially cells that are cultured on the agar or reconstituted basement membrane (Matrigel) migrate towards each other and form spheroids. In the following step, cells are growing on the substratum but do not adhere to it and spheroid increases in size [15]. Liquid overlay cultures produce spheroids that maintain the cellular composition and differentiation as seen in tissue *in vivo*. They can form lobulo-alveolar structures and exhibit differentiated functions such as the capacity to secrete milk.

However, the disadvantage of this model is that adhesion behaviour can be altered as cells have contact with the substratum. The type of media and ECM used are important factors for promoting and supporting cell growth and differentiation. It has been shown that, thick matrices promote differentiation, whereas thin layers of 3-D ECM tumour cells fail to show differentiated phenotypes and form disorganized and apolar cellular masses [16]. It is also important that other media components do not obstruct cell differentiation process. A further limitation is that this method produces only a small number of spheroids [5].

Spinner flask is a common method used for growing cells as a suspension culture in liquid media. This method enables to cultivate greater numbers of spheroids in dynamic suspension when the static environment of liquid overlay cultures is not vital. Spinner flasks are stirred tank bioreactors where mixing maintains the cells in suspension. In this system fluid movement facilitates mass transport of nutrients to, and also wastes from, the spheroids. The disadvantage of this model is that due to high shear forces spheroids architecture is not as well preserved. Also, fragile cells can be damaged by higher fluid turbulence, which affects membrane integrity and cell metabolism. Spinner flask culture developed in the 1970s was the most commonly used method when culturing larger numbers of multicellular spheroids [17]. Roller tubes and gyratory shakers are other methods that are employed for culturing cells in 3-D models.

The gyratory rotation technique is another method that implicates placing cell suspension with media in an Erlenmeyer flask. Erlenmeyer flask is rotated in a gyratory rotation incubator till the time where spheroids of required size are created. In this technique cells have no contact with any substrates [5].

Another technique is a scaffold-based culture system where 3-D *in vitro* models of the breast can be produced by growing cells on prefabricated scaffolds. In this model cells attach to and migrate along fibres of the scaffold. When cells grow and divide, they manage to fill the scaffold interstices and create 3-D cultures [18]. The properties of obtained cultures can be influenced by the constituents of the scaffold. Collagen and other ECM proteins as well as growth factors are the most frequently used scaffolds. Also, different types of cells such as: established cell lines, primary cells and pieces of tissue can be used in this system [19].

The limitation of this model is its high costs and difficulty when seeding and culturing sensitive cell lines.

In recent times, prefabricated 3-D biodegradable engineered scaffolds represent one of the most promising experimental method for mimicking the structure and properties of tissue *in vivo*. These scaffolds are composed of synthetic polymers that form 3-D physical support and natural molecules. Models can be used for both: cell culture (*in vitro)* and tissue regeneration (*in vivo)*. They provide physical and structural support bringing great potential in reproducing not only the structural but also natural physical environment of a living tissue [20]. The biggest limitation of this model is the expense.

Finally, the Rotary Cell Culture System, developed by National Aeronautics and Space Administration (NASA), demonstrates a revolutionary model that simulates microgravity for cells cultured in media, in a dynamic fluid suspension that is mixed by minimal hydrodynamic forces. In this system, the culture flask rotates whole on its horizontal axis and provides end over end mixing of the cells. Shear forces and fluid turbulences are minimized as the flask (vessel) is entirely filled with media. Aeration is facilitated through a semi permeable membrane that eliminates bubbles, which are normally responsible for hydrodynamic forces production. Reduced turbulence affected the physical properties of a culture, like production of spheroids larger in size, considerably more differentiated morphologically and phenotypically than in the ones cultured in spinner flasks. In this model multiple cell types can be co-cultured. The biggest limitation of this model is its expense [5].

Each of the represented models has its advantages and restrictions. The 3-D *in vitro* model proposed in this study is an inexpensive alternative to commercially available systems, where the 3-D architecture of tissue was recreated and maintained successfully. In our model cells are surrounded by a natural environment of human dermis, where nutrients are naturally transported from media. Therefore, there is no mass transport limitations at greater depths, or problem with lack of nutrient penetration and toxic metabolic waste build up that are often seen in other commercially available 3-D *in vitro* models. DED scaffold used in our model provides natural physical and structural support creating *in vivo* like environment of a living tissue. This promising technique has offered a low-cost solution to culture a variety of cell types in a well-defined environment under 3-D culture conditions.

The biology of normal luminal and myoepithelial cells is the key to understand breast cancers. Terminal ducto-lobular unit in the breast is the structure from which breast cancers predominantly arise. This structure is made up of two types of epithelial cells; the inner layer of luminal cells that could be milk secreting cells and an outer basal layer composed of contractile myoepithelial cells. The two layers of cells are separated from the interstitial stroma by an intact basement membrane [21, 22]. The glandular luminal epithelial cells are polarized with specific basolateral and apical membrane. Luminal cells that line the apical surface of the normal breast duct have secretory properties. The myoepithelial cells that have both contractile muscle and epithelial properties play a part in the formation of basement membrane. For the reason that carcinoma of the breast arises mainly in the luminal epithelial compartment, not much attention was paid in the past to the role of the surrounding cells in mammary gland [22-24]. The fact that intact myoepithelial cells are significant factors of normal breast development and differentiation is well known now and myoepithelial cells are gaining more attention in cancer research [22-24].

Several myoepithelial proteins demonstrated the ability to inhibit epithelial tumour formation. Examples of proteins that have tumour suppressive activities are: α-actin, K5, α 6 integrin, connexin 43, myoepithelium-derived serine proteinase inhibitor (MEPI), relaxin, maspin, and activin [22, 25-31]. Paracrine interactions between luminal and myoepithelial cells are known to be important for establishing epithelial cell polarity, regulation of cell cycle progression, and inhibition of cell migration and invasion. Better understanding of myoepithelial cell function and their role in tumour progression may lead to their exploitation for cancer therapy and prevention [32].

3-D *in vitro* systems have been proven to be important experimental tools for cancer research. Serving as low-cost screening platforms they are important models to study the mechanisms of tumour growth, its progression, tumour cell loss of differentiation and the response to therapeutics. When cells are cultured in 2-D environment they lose important signals from ECM, which plays fundamental function in normal homeostasis [33]. Growing cells in 3-D conditions enable restoring some of the important signals. In this study culturing normal and cancer cells in 3-D model of the breast allowed phenotypic discrimination between nonmalignant NTERT cells that formed polarized ducto-lobular structures and malignant proliferating mammary cells that were mainly disorganized and nonpolar. Some breast cancer colonies seen in the cancerous 3-D model were similar to the ducto-lobular structures observed in normal 3-D model of the breast, but the former cell were more loosely connected, irregular and largely disorganized. All cell phenotypes could be distinguished in the 3-D *in vitro* model of the breast cancer, such as non-proliferating, proliferating and necrotic cells, similar to the situation observed in tumours *in vivo*. Interactions between DED and epithelial cells enabled development of both normal and neoplastic breast 3-D *in vitro* models.

The 3-D *in vitro* systems proposed in this study can serve as reliable *in vitro* models of normal breast and breast cancer. Standard IHC staining and confocal microscopy analyses confirmed the presence of ducto-lobular structures in the model of normal breast. In this study we compared the expression of keratins, the major structural proteins of epithelial cells, in both normal and cancerous 3-D *in vitro* models. Keratins are divided into two groups of acidic type I (K9-K20) and basic type II (K1-K8) keratins and its main function is to maintain the epithelial cell integrity of cytoskeleton [34-36]. They also play important role in cell signalling, stress responses and apoptosis [35, 37]. Keratins have a defined distribution pattern in epithelial tissues [38, 39]. Cells lining the lumen of normal breast ducts express keratins: 8, 18, 19 and 7 whereas the basal cell layer of the mammary duct express keratins: 5/14, 5/6 or 17. Myoepithelial cells will express: K5, K14, K17, smooth muscle actin, calponin, p63 [40] (Table 2).

Similarly, breast-associated adenocarcinomas express the same markers as the normal mammary gland. One of the main features of transition from normal to cancerous state is the alteration in the cytoskeletal structure [41]. Breast cancers express either luminal or basal keratins.

Breast carcinoma originates from luminally differentiated epithelial cells where 85% carcinomas are ductal (luminal type and basal type) and 15% lobular. Both lobular and ductal carcinomas arise in the terminal ducto-lobular unit [40]. Dissimilarity in keratins expression are often used to distinguish lobular from ductal carcinomas. K5 and 10 are commonly expressed by lobular carcinomas. In contrast, they are absent or expressed at low levels in most ductal carcinomas in situ (DCIS) [40]. In this study the expression of K5 and 19 was studied. Table 2 represents different pattern of keratin expression in non-malignant and cancerous breast tissue.

Table 2: Keratins expression in non-cancerous breast tissue, where "-" represents negative staining, "+" represents weak positive staining, "++" represents positive staining and "+++" represents strongly positive staining.

		Normal tissue		Malignant tissue	
Neutral-basic or acidic		Acidic	Neutral-Basic	Acidic	Neutral-Basic
Type of keratin		19	5	19	5
Breast Complex epithelia	Basal and myoepithelial cells	++	+++	++	-
	Luminal cells	+++	+	++	-

Multiple structures that resemble duct and lobules of human breast *in vivo* were observed in the established 3-D *in vitro* models. Structures were further stained with different keratins in order to reveal and compare the different patterns of keratin staining in normal and cancerous breast tissue. Normal mammary epithelial cells express mainly K5 whereas breast cancer cells produce K19 [8-10]. In our models K5 was detected in both luminal and myoepithelial cells of normal 3-D *in vitro* model of the breast (Figure 1), whereas K19 was present in 3-D *in vitro* model of cancerous breast tissue (Figure 2). One of the features of cancer cell is that it losses the specific differentiated appearance of normal cell over the time. The nuclei of malignant cells become large, which indicates the increased synthesis of DNA [33]. Malignant cells are also less rigorously regulated by cell-cell and cell-matrix interactions in comparison to normal cells. They are less adhesive than normal cells due to reduced expression of cell surface adhesion molecules. Cancer cells are less firmly attached to each other or to normal cells, they also poorly adhere to the ECM. They are rounder than normal cells and can easily metastasize and invade surrounding tissue [5, 42]. Morphological changes between normal and cancer cells cultured in 3-D *in vitro* models include alteration in the nuclear envelope. Breast cancer cells have enlarged nuclei (Figure 3) in comparison to the normal cells (Figure 1). Increase in nuclear volume is usually from the increased chromosome content and increased DNA synthesis [33].

The ducto-lobular structures formed in the normal breast tissue model were composed of two types of epithelial cells, the inner cell layer that show luminal cells characteristic (positively stained with MUC 1 biomarker), and an outer basal layer that exhibit myoepithelial cells characteristics (positively stained with α actin biomarker). Similar structures were observed in breast cancer model but staining with α actin and MUC 1 show only uncompleted differentiation. These disturbed cells organization are commonly seen in breast cancers, where cancerous mammary cells have an impaired ability to differentiate to myoepithelial

and alveolar cells [43]. The phenotype of breast cancer cells, their reaction to proliferation and differentiation stimuli, as well as mammary cells polarity is also altered [44-46]. Moreover, many breast cancers lack myoepithelial cells and even if the myoepithelial cells are detected they are often loosely connected and irregular, whereas luminal cells are largely disorganized [47-51]. The cell-cell interactions and complex 3-D network of cell–matrix successfully formed in 3-D *in vitro* models presented in this study is of a great value. 3-D models successfully recapitulate both normal and pathological tissue architecture and offer physiologically relevant systems to study the disease in breast tissue.

Conflicts of Interest

None.

Abbreviations

α-actin: alpha smooth muscle actin
DED: de-epidermalised dermis
ECM: extracellular matrix
K: keratin

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