Published in final edited form as:

Biomaterials. 2012 April ; 33(12): 3411–3420. doi:10.1016/j.biomaterials.2012.01.011.

Hormone-responsive 3D multicellular culture model of human breast tissue

Xiuli Wang^{1,2} and David L. Kaplan¹

¹Biomedical Engineering Department, Tufts University, 4 Colby street, Medford, USA 02155

²Dalian Institute of Chemical and Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian, China 116023

Abstract

A hormone-responsive 3D human tissue-like culture system was developed in which human primary mammary epithelial cells (MECs) were co-cultured with two types of predominant mammary stromal cells on silk protein scaffolds. Silk porous scaffolds with incorporated extracellular matrix provided a compatible environment for epithelial structure morphogenesis and differentiation. The presence of stromal cells promoted MEC proliferation, induced both alveolar and ductal morphogenesis and enhanced casein expression. In contrast, only alveolar structures were observed in monocultures. The alveolar structures generated from the heterotypic cultures *in vitro* exhibited proper polarity similar to human breast tissue *in vivo*. Consistent with their phenotypic appearance, more functional differentiation of epithelial cells was also observed in the heterotypic cultures, where *casein-α* and *-β* mRNA expression were increased significantly. Additionally, this 3D multicellular culture model displayed an estrogen-responsive physiologically relevant response, evidenced by enhanced cell proliferation, aberrant morphology, changes in gene expression profile and few polarized lumen structures after estrogen treatment. This culture system offers an excellent opportunity to explore the role of cell–cell and cell–substrate interactions during mammary gland development, the consequences of hormone receptor activation on MEC behavior and morphogenesis, as well as their alteration during neoplastic transformation in human breast tissue.

1. Introduction

The mammary gland is a special organ that undergoes natural cycles of proliferation, differentiation and apoptosis, as well as remodeling, throughout life under the cyclical influences of multiple steroid and polypeptide hormones [1]. These developmental events are regulated in response to a precise interplay between epithelial cells and their surrounding microenvironments [1–3]. In addition to the soluble factors recognized for their role in growth control, microenvironments are also comprised of multiple stromal cells as well as insoluble glycoproteins of extracellular matrix (ECM). Growing evidence has indicated an important role played by stromal cells in regulating normal mammary tissue morphogenesis and their aberrant behavior during the progression of breast cancer [1, 4, 5]. However, an

© 2012 Elsevier Ltd. All rights reserved.

Correspondence to: David L. Kaplan.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

explanation for these processes at different levels of cell and tissue complexity remains sparse due to a lack of appropriate model systems for study.

Recently, the advent of three dimensional (3D) culture models has allowed investigators to make significant progress toward characterizing factors involved in the establishment and maintenance of epithelial architecture [6, 7]. In contrast to the limitations inherent to two-dimensional (2D) culture systems, many aspects of organization of mammary epithelial structures were recapitulated *in vitro* when primary mammary epithelial cells or established cell lines were exposed to a 3D physiological exogenous matrix, e.g. collagen, Matrigel™ [7–9]. However, while the use of 3D culture systems has proven to be advantageous in the characterization of the behavior of a single human mammary cell type (especially epithelial cells), these studies have largely ignored the fact that no epithelial cells exist as “isolated islands” in the mammary tissue *in vivo* [10]. It has been demonstrated that mammary stroma, including fibroblasts, adipocytes, endothelial cells and inflammatory cells, comprises over 80% of the cellular population of the mammary gland *in vivo* [10]. Thus, it is critical to develop multicellular culture systems composed of epithelial cells and their stromal counterparts, exploring how paracrine signals or cell-cell interactions affect epithelial behavior during mammary gland development, involution and neoplastic transformation.

Currently, due to improved techniques in cell isolation and culture methodologies, some heterotypic 3D co-cultures comprised of luminal and myoepithelial cells, breast cancer cells and fibroblasts/or endothelial cells/or adipocytes are available [11–14]. Preliminary studies with these models have established the critical role of multiple cell types in mammary epithelial morphogenesis and differentiation. However, it is still challenging to incorporate multiple cell types into one single 3D culture system to mimic the microenvironment found in the native mammary gland tissue more closely *in vitro*. This is important because apparently this type of system would provide an excellent opportunity to allow investigators to explore the molecular mechanisms involved during human mammary gland development or carcinogenesis in a more physiologically relevant manner.

Recently, our laboratory developed a 3D culture system by co-culturing the human mammary epithelial cell line MCF10A with adipocytes and fibroblasts on porous silk protein scaffolds supplemented with a mixed ECM, in which both alveolar and ductal morphogenesis with correct polarity were generated. In addition, these epithelial structures exhibited significantly enhanced functional differentiation in comparison to the monoculture compartment as evidenced by histology and functional analysis [15,16]. In the present study, we describe a more physiologically relevant heterotypic 3D culture system by replacing the immortalized epithelial cell line MCF10A with human primary mammary epithelial cells (HuMECs) isolated from reduction mammoplasty tissue. We hypothesized that primary HuMECs growing in a 3D microenvironment provided by multiple types of stromal cells, ECM molecules as well as silk protein scaffolds, would not only generate mammary tissue-like structures that more closely resemble the *in vivo* mammary morphology, but also contribute to producing a hormone-responsive 3D culture model with an improved differentiated functionality. Towards this goal, the constructed heterotypic 3D culture model was characterized by its growth profile, histology and gene expression. Moreover, its capability of responding to hormone stimulation was also evaluated through estrogen treatment.

2. Materials and Methods

2.1 Cell maintenance culture and differentiation

Primary human mammary epithelial cells (HuMECs, P₂₋₄, Invitrogen, Carlsbad, CA) were initially cultivated in HuMEC serum free Medium (Invitrogen) and fed every other day until

the culture reached approximately 50% confluence. Then the medium was refreshed every day until the cells were ready for subculture (~80–90% confluence). At this timepoint, most of the HuMECs exhibited a “cobblestone”-like morphology. Human mammary fibroblasts (HMFs, P_{3–6}, ScienCell™, Carlsbad, CA) were cultured in low glucose-DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution (all from Invitrogen). Human adipose-derived stem cells (hASCs, P₀) were kindly provided by Prof. Jeffrey Gimble (Pennington Biomedical Research Center, LA), and their maintenance culture and differentiation inducement were conducted as described before [15,16]. For the heterotypic culture experiment, a combined medium composed of an equal volume of HuMEC medium, RMF medium and hASCs differentiation medium was used, which has been previously tested to assure proper growth and behavior of each type of cell in 2D culture system by MTT assay. All cells were cultured at 37°C, 5% CO₂.

2.2 Aqueous-derived silk scaffold preparation

Aqueous-derived silk fibroin scaffolds were prepared according to the procedures described in our previous studies [17, 18]. Briefly, a 6.5% (w/v) silk fibroin solution was prepared from *Bombyx mori* silkworm cocoons (supplied by Tajima Shoji Co, Yokohama, Japan) by using Na₂CO₃/LiBr solution. Then, granular NaCl particles were added to the silk fibroin solution, leading to a formation of porous silk scaffolds with a pore size of 500–650 µm. Finally, these scaffolds were cut into small discs (5 mm diameter × 2.5 mm thickness) and autoclaved for cell culture experiments. Before cell seeding, the scaffolds were preconditioned with the culture medium overnight at 37°C, 5% CO₂.

2.3 Three-dimensional culture on silk scaffolds

A mixed Matrigel™-collagen gel was prepared using a 1:1 volume ratio of growth factor reduced (GFR)-Matrigel™ and type I rat tail collagen solution (all from BD Biosciences, San Jose, CA), keeping the final collagen concentration at 1.0 mg/ml. In heterotypic culture, HuMECs, HMFs and pre-differentiated hASCs were mixed with the Matrigel™-collagen solution and seeded on the preconditioned silk scaffolds in a 2:1:1 ratio keeping the number of HuMEC constant (80,000 cells/scaffold). After gelation at 37°C for 2 hours, the cell-loaded scaffolds were transferred into non-cell culture treated 12-well culture plates (BD Biosciences); the combined medium was added gently to avoid disturbing the scaffolds. Monocultures of HuMECs under the same conditions with the same seeding density served as control. All cultures were incubated in a 37°C, 5% CO₂ in a 100% humidified incubator for 2~3 weeks and the medium was changed every other day.

To distinguish the epithelial cells from the stromal cells within the 3D culture system, CellTracker™ DiI and CellTracker™ Green CMFDA (Invitrogen) were applied to label the HuMECs and the stromal cells, respectively, as described before [15]. Morphologic development was photographed by either phase contrast microscopy (Zeiss Axiovert S100, Germany) or confocal laser scanning microscopy (CLSM, Leica SP2, Oberkochen, Germany).

2.4 Cell proliferation and viability on silk scaffolds

Cell proliferation on 3D silk scaffold was determined by DNA content analysis as described in our previous study [10,11]. After harvesting all the samples at indicated time points (store at -80°C), the DNA content was measured by PicoGreen DNA Assay following the protocol provided by the manufacturer (Molecular Probes, Eugene, OR). For some experiments, fluorescence-activated cell sorting (FACS, BD Sciences, Tufts Medical Center, Boston MA) was applied before DNA assay to sort different types of cells in the heterotypic cultures. Samples ($n=3$ per group in the same experiment, three repeats) were measured by a micro-plate fluorometer ($\lambda_{ex}=480$ nm, $\lambda_{em}=530$ nm). Cell viability was assessed by calcein-

AM/EthD-1 staining (Invitrogen) as described previously [19]. Only live cells with intracellular esterase activity digest non-fluorescent calcein-AM into fluorescent calcein, while dead or dying cells containing damaged membranes allow the entrance of EthD-1 to stain the nuclei. Images were captured by CLSM SP2.

2.5 H&E staining

Constructs were harvested and fixed in 4% formaldehyde at indicated time points for histological detection. Paraffin sections (~5 μ m) were prepared by Tufts Medical Center (Boston, MA). Hematoxylin and eosin (H&E, EM sciences, Fort Washington, PA) staining was conducted as before [11]. Images were captured with a Leica DMIRE2 microscope (Germany).

2.6 Immuno-fluorescence and immuno-histochemistry staining

For immuno-fluorescence staining, deparaffinized sections (~5 μ m) were treated with antigen retrieval solution, 0.1% Triton-X-100 solution and 1% blocking serum sequentially (all from Fisher Scientific, Pittsburgh, PA), then they were incubated with the primary antibodies (mouse anti-human) as followings: anti-GM130 (1:80, BD Biosciences), anti-sialomucin (1:20, Abcam, Cambridge, MA), anti-casein (1:20, Abcam), anti-E-cadherin (1:40, Abcam) and anti-collagen IV (1:40, Abcam) overnight at 4°C, followed by incubation with appropriate FITC or TRITC-conjugated goat anti-mouse IgG (1:100, Sigma) as previously described [10,11]. Cell nuclei were counter-stained with propidium iodide (PI, 5 μ g/ml, Invitrogen). Paraffin-embedded human normal breast tissue section (provided by Tufts Medical Center) served as a positive control. Images were captured with CLSM SP2. For immunohistochemistry (IHC) staining of Ki67 (mouse anti-human, 1:80, BD Biosciences), a mouse ABC staining kit (Santa Cruz Biotechnology) was used following the manufacturer's protocol, and the number of positive staining cells in different microscopic fields (at least 8 microscopic fields per slide, 3~5 slides per group) was counted under the microscope Zeiss Axiovert S100.

2.7 Real time quantitative RT-PCR analysis

To prepare samples for RT-PCR analysis, FACS was used to sort different type of cells in the heterotypic cultures for RNA extraction. Total RNA was extracted from the sorted HuMECs and stromal cells at different time points by using an RNAeasy Mini Kit (Qiagen, Valencia, CA) [15,16]. cDNA was synthesized using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA), and real-time PCR was conducted with TaqMan Gene Expression assay kits (Applied Biosystems) to detect the transcript levels of casein- α (Hs_00157136), casein- β (Hs_00914395), ER α (Hs_01046818), and ER β (Hs_01100353). The data were analyzed by ABI Prism 7000 Sequence Detection Systems version 1.0 software [15]. The relative expression level for each target gene was normalized by the Ct value of human GAPDH (HS_99999905) ($2^{-\Delta C_t}$ formula, Perkin Elmer User Bulletin #2). Each sample was analyzed in triplicate.

2.8 17 β -estradiol treatment on the constructed 3D cultures

To assess the effect of steroid hormone on the 3D heterotypic culture model constructed above, 10 nM 17 β -estradiol (E2, Sigma) was added to the combined basal medium. This concentration was used according to the previous reports and our cytotoxicity detection before treatment initiation [20]. The medium was changed every second day and fresh E₂ solution was added regularly. After one or two weeks of cultivation with E₂ additions, the 3D cultures from each group were harvested, and their proliferation, histology, phenotype, as well as gene expression, were characterized using the methodologies described above. Untreated 3D culture served as control.

2.9 Statistical Analysis

All reported values were averaged ($n=3$ repeats except for specific experiments where explanations are provided) and expressed as mean $\pm$ standard deviation (SD). Statistical differences were determined by *Student's two-tailed t test* and differences were considered statistically significant at $p<0.05$.

3. Results

3.1 Proliferation of HuMECs and/or stromal cells on silk scaffolds

Similar to our previous study, as a first step in defining the heterotypic culture effect on HuMEC growth, a combined culture medium, in which an equal volume of HuMEC medium, HMF medium and hASCs differentiation medium II was tested through DNA content assay. As shown in Figure.1a, DNA content of the cells (including HuMECs and/or stromal cells) on 3D silk scaffolds increased progressively during two weeks of cultivation. This result indicates the capability of the combined medium in supporting the growth of each type of cell within the 3D constructs. In addition, it seems that the proliferation rate of cells during the second week was much higher than at any other indicated time points. After the cultures were cultivated for up to three weeks *in vitro* (especially by the end of the third week), decreased cellular proliferation was observed, evidenced by the decreased DNA content.

Expression of Ki67, an important marker for cellular proliferation, was also assessed by IHC staining. Compared to the monoculture, a higher percentage of Ki67 positive (Ki67⁺) HuMECs was observed in the heterotypic culture over two weeks of culture. Specifically, after one and two weeks of co-culture with mammary stromal cells, the number of Ki67⁺ HuMECs increased 1.5-fold and 1.8-fold, respectively, in comparison with the monocultures (Fig.1b). This is consistent with the results of DNA content assay above, suggesting a proliferative effect of stromal cells on HuMEC growth. A maximal Ki67 expression of HuMECs occurred during the second week and decreased thereafter through the third week. No significant difference existed between the heterotypic cultures and monocultures by the end of the third week, even though the former exhibited a relatively higher percentage of Ki67⁺ cells.

3.2 Growth profiles of HuMECs on 3D silk scaffolds

Compared to the growth profile exhibited by MCF10A cells under similar culture conditions before [16], the HuMECs on silk scaffolds displayed a delayed onset of organization both in the heterotypic cultures and in the monocultures. At least a five-day period in culture was required to allow the HuMECs to organize into desirable epithelial structures, indicating a more controllable growth pattern of HuMECs in comparison with those immortalized MCF10A cells. By day 7, some acinar and tubule-like structures were generated in the heterotypic culture. These structures could be identified based on their special morphology (Fig. 2a, c). The size of the acinar and duct-like structures in the heterotypic cultures increased over time, especially during the second week. By contrast, only acinar structures were observed in the monoculture of HuMECs (Fig. 2b, d), even though the culture time was prolonged for more than three weeks. This is similar to the monoculture of MCF10A cells (under similar culture condition) that has been reported in our previous studies [15, 16]. With respect to the timeline of acinar structure formation, no obvious difference was observed between the heterotypic culture and monoculture group, suggesting a minor role played by stromal cells in initiating or promoting the morphogenesis of acinar structures in the mammary gland.

The viability of cells on silk scaffolds was also assessed by calcein-AM/EthD-1 staining. After one week of cultivation, the majority of the cells in each group (heterotypic culture and monoculture) exhibited good viability (Fig. 2c, d), suggesting that both the silk scaffolds and the culture matrix provided a suitable microenvironment for the growth of primary HuMECs and stromal cells.

A detailed growth profile of the HuMECs either in the heterotypic culture or in the monoculture was revealed by H&E staining. The HuMECs in heterotypic culture formed both acinar and duct-like structures in the mixed Matrigel™-collagen matrix in contrast to the exclusive formation of acinar structures in the monocultures (Fig. 2e, f). In addition, as early as one week of cultivation, a lumen was observed in the center of some acini and their nuclei were located to the basal side, an indicator of cell polarity (Fig. 3E, F). More importantly, a few terminal duct lobular unit (TDLU)-like structures were found in the heterotypic culture group (Fig. e₁, e₂), while this structure was absent either in the monoculture of HuMECs or in the co-cultures (or tri-cultures) developed by MCF10A cells.

3.3 Morphological characteristics of the epithelial structures formed on 3D silk scaffolds

To further characterize the functional polarity of the epithelial structures, the expression pattern of two important markers, GM130, a Golgi complex marker, and sialomucin (CD164), a glycosylated protein produced by epithelial tissues, were evaluated by immunofluorescence staining. As shown in Figure 3, their expression was mostly focused on the apical side of epithelial cells in the heterotypic culture (Fig. 3b₁–b₃). This type of expression pattern is similar to the pattern exhibited by the human breast tissue *in vivo* (positive control, a₁–a₃), where these two markers are physiologically expressed at the apical side of human mammary acini (Fig. 3a₁–a₃). Interestingly, in contrast to the reverse polarity displayed by the monoculture of MCF10A cells (a phenomenon reported in our previous study) [15], here the acinar structures formed within the monoculture of HMECs exhibited the correct polarity, showing a similar apical distribution pattern of GM130/CD164 although their expression pattern looks less “focused” when compared with that of the heterotypic cultures (Fig. 3c₁–c₃).

In addition to the expression of GM130/CD164, E-cadherin staining, a marker of cell-cell adhesion [21], was observed in both acinar and duct-like structures (Fig. 4a). More importantly, a basement membrane was also found at the basal side of the acinar structures as evidenced by anti-collagen IV immunostaining, providing further evidence for the tissue-like integrity of the epithelial structures formed in the 3D silk scaffolds (Fig. 4b).

3.4 Functional differentiation of epithelial structures formed on 3D silk scaffolds

To determine whether the presence of stromal cells in the heterotypic culture group could enhance HuMEC functionality as well as morphological differentiation as described above, casein gene expression, a critical indicator for functional differentiation of mammary epithelial cells, was analyzed by real-time RT-PCR. Compared with the monoculture, both α -casein and β -casein mRNA levels were significantly up regulated in the co-cultured HuMECs. In addition, casein expression either in the heterotypic culture or the monoculture increased over time; with nearly a two-fold increased expression observed in the co-cultured HuMECs after two weeks of culture (Fig. 4). Significantly decreased expression of casein genes was found in all the cultures (both heterotypic culture and monoculture) after four weeks cultivation *in vitro* (not shown), which might be correlated with the undesirable viability of the HuMECs during the prolonged culture.

Consistent with the mRNA expression level assayed by real-time RT-PCR, a more intensely stained casein protein was observed in the heterotypic group when compared to the

monoculture (Fig. 5C₁–C₂). This is also consistent with the result obtained from the multicellular culture system of MCF10A cells. The data here provide further evidence that stromal cells present in the heterotypic culture system contribute to the functional differentiation of primary MECs on 3D silk scaffolds.

3.5 E2 effect on the 3D heterotypic culture model

The effect of E2 on epithelial cell proliferation in 3D heterotypic culture and monoculture was evaluated by using both DNA content assay and IHC staining of Ki67. Through both assays, E2 elicited a time-dependent induction effect on HuMEC growth either in the heterotypic culture or in the monoculture. Only DNA content assay is shown (Figure 6a), due to the good agreement between these two assays. Compared to the control group (heterotypic culture without E2 treatment), relatively higher values of DNA content were detected out in the E2-treated heterotypic culture throughout the culture. But the difference between these two groups did not reach statistical significance at the time point of one-week culture ($^{\#}P>0.05$). In contrast, a 1.9-fold increase in growth over the control was observed in the heterotypic culture when treated with 10 nM E2 for up to two weeks ($*P<0.05$). A similar tendency was also observed in the E2-treated monocultures. However, it seemed that the effect of growth enhancement by E2 treatment was more pronounced in the heterotypic culture in comparison with the monoculture, where only a minor increase (around 1.2-fold) was observed by the second week of E2 treatment. This indicates that the effect of E2 probably occurred in part through the stromal-relevant pathway.

Since the concentration of 10 nM E2 conferred a more pronounced growth enhancement according to the cell proliferation assay above, this concentration was then adopted in our following experiments. Histological examination revealed the difference in the appearance of HuMECs in each group. Compared to the control group (E2-untreated), the epithelial cells in the E2-treated heterotypic cultures often appeared hyperplastic, and the epithelial structures, especially acini-like structures, were larger and less compact (Fig. 6b). The number of cell layers in the epithelial structure increased significantly and in some cases, there was a loss of distinction between the lining cells. The distribution of E-cadherin protein between the cells became uneven after the treatment (Fig. 6c). Moreover, few polarized lumen structures were observed in the E2-treated cultures evidenced by the “disorganized” GM130/CD164 expression pattern (Fig. 3d₁–d₃). Thus, all these phenomena suggest the loss of organization of the epithelial structures caused by E2 treatment. A similar morphological alteration also happened in the E2-treated monocultures (data not shown). However, it seemed that the E₂ effect on the monocultures was less significant when compared with the heterotypic culture of HuMECs.

To further assess E2 effect on the 3D heterotypic culture model, a series of gene expressions was used to evaluate functional activity of mammary epithelial cells through real time RT-PCR assay after cell sorting. As shown in Figure 7, a decreased gene expression of *casein α/β* and steroid receptors (*ERα*, *ERβ*) was observed in the E2-treated heterotypic cultures. Specifically, the expression of *casein β* gene after the treatment was down regulated more than the others study groups, reaching only 30% of the control level after two weeks of E2 treatment. No statistical significance exists between the E2-treated and non-treated monocultures, even though a slightly decreased expression of *casein α/β* and steroid receptor gene were detected (data not shown). This supports a suitable responsive capability possessed by the constructed heterotypic culture model.

4. Discussion

In an attempt to more closely simulate the *in vivo* microenvironment in mammary gland tissue, we have previously bioengineered 3D co-culture and tri-culture models *in vitro* by

co-culturing immortalized human mammary epithelial cell line MCF10A and mammary stromal cells on silk scaffolds supplemented with a mixture of collagen/Matrigel™ [15,16]. We have shown that morphogenesis of epithelial structures, such as acinar and duct-like structures could be recapitulated by MCF10A cells within the co-culture or tri-culture compartment. In addition, those epithelial structures generated from the multicellular compartment exhibited more differentiated phenotype and functionality when compared to the monoculture, suggesting a critical role of stromal cells in controlling the behavior of epithelial cells.

However, it should be noted that although MCF10A cells are commonly recognized as a “normal” breast epithelial cell line with a stable karyotype, they are nonetheless cytogenetically abnormal [22]. Specifically, these cells harbor both genetic and epigenetic abnormalities commonly associated with their immortalization and long-term *in vitro* cultivation without the architectural cues from the stromal cells and ECM *in vivo*. For instance, MCF10A cells are negative for estrogen receptor (ER) and express markers commonly related to a basal epithelial phenotype [23]. Thus, interpretation of data from such experimental models still need to be weighed against limitations of the epithelial cells employed.

To further improve the 3D epithelial culture models constructed in our previous study, a more physiologically relevant, heterotypic 3D culture model was developed by incorporating primary human MECs rather than MCF10A cells into the co-cultures of fibroblasts and adipocytes. This culture model is an extension of our previously study, and represents several significant advances over the previously described models due to the special way of reconstituting the 3D microenvironment of the normal mammary gland *in vitro*. For example, the use of normal primary MECs rather than immortalized cell lines provides reasonable assurance of a more intact genetic profile and signaling pathway of epithelial cells [6, 24]. In addition, it allows us to evaluate the effects of steroid hormones on MECs *in vitro* with a higher fidelity. This is important because a number of studies have demonstrated that steroid hormone-receptor systems participate in mammary gland morphogenesis, functional differentiation, apoptosis as well as neoplastic transformation [25, 26].

To characterize the heterotypic 3D model *in vitro*, similar to our previous study, we first evaluated the influence of mammary stromal cells on the growth of HuMECs. A proliferative effect on HuMECs was observed in current culture compartments as determined by DNA content and anti-Ki67 staining. This result somewhat contradicts our previous study in which an inhibitory effect of stromal cells on the proliferation of MCF10A cells was observed [15]. This difference can be explained by the different properties between these two types of MECs employed in our studies. It has been shown that the effect produced by stromal cells on MECs was highly dependent on the type of epithelial cells, the characteristics of the stromal cells as well as the culture methodology utilized [12, 27]. Unfortunately, a detailed mechanism accounting for these effects still remains unknown. After three weeks of cultivation, decreased cell proliferation of epithelial cells was observed in each group. This might be due to the growth arrest of HuMECs once they formed differentiated tissue-like structures where decreased oxygen delivery becomes an issue within the scaffold during the prolonged culture under the static conditions.

Both acinar and duct-like structures were generated by the HuMECs in the heterotypic culture in comparison to the exclusively formed acinar structures within the monocultures. This morphological profile was similar to our previous reported co-culture or tri-cultured model where similar acinar and duct-like structures were observed with the presence of one or two types of stromal cells, and further confirmed the robust potential of the stromal cells

in reconstituting the microenvironment of the mammary gland *in vivo*. However, it's worth pointing out that terminal duct lobular unit (TDLU)-like structure, an important structural and functional unit of human mammary gland tissue *in vivo*, was exclusively generated in the current heterotypic culture compartment. TDLU is a terminal portion of the lactiferous duct and its lobular branches are embedded in specialized, hormonally responsive intralobular stroma *in vivo*. It's has been suggested that more than 90% of abnormal proliferations of the breast tissue arise at this locus, including ductal carcinoma *in situ* [28–30]. Thus, the formation of TDLU-like structures not only reveals distinct characteristics possessed by primary HuMECs in comparison with the immortalized epithelial cell line, but also predicts greater implications for this 3D multicellular culture model in exploring breast morphogenesis and carcinogenesis.

Cell polarization is a critical feature of mammary glandular epithelium *in vivo*, and thus it has become an important parameter to evaluate the tissue architecture achieved in 3D culture model [31]. Here, acinar structures with correct polarity were observed in both the heterotypic culture and monocultures, accompanied with cell connections at the lateral side and basement membrane formation at the basal side. In contrast to a reversed polarity exhibited by the 3D monoculture of MCF10A cells described before [15, 16], the observation here further supports that the response of epithelial cells on 3D culture matrix is not only related to the culture microenvironment but also highly dependent on the cell type. In comparison with the MCF10A cell line, HuMECs are more differentiated, which could explain their distinct polarity even though they were cultured under similar culture conditions.

Consistent with the polarity differentiation exhibited by HuMECs in the multicellular culture, significantly improved expression of casein gene (α , β) was also detected when compared with the monocultures. This further highlights the central role of stromal cells in inducing MEC functional differentiation. It should be noted that the expression profile of casein genes, especially casein- β in the monoculture of HuMECs, was distinct from the monoculture of MCF10A cells where no casein- β gene transcript was detected in our previous study [15]. This reveals a distinct potentiality between these two types of MECs with respect to their functional differentiation. Accordingly, the time window of increased casein expression in the current culture compartment lasted for up to three weeks. This was much longer than the culture models with MCF10A cells, further supporting the more differentiated functionality possessed by the HuMECs.

It has been well known that human breast tissue *in vivo* is able to respond to circulating ovarian hormones, such as estrogen and progesterone in an accurate manner and undergoes phases of proliferation, differentiation, as well as regression, during aging [20, 32]. Ovarian hormones also likely play a key role in the etiology and biology of breast cancer [33, 34]. Since our results above support a well-differentiated morphology and functionality of the constructed 3D multicellular cultures, in this study we further explored whether the current model responded to stimulation by estrogen. Estrogen (E2) is a potent growth factor for normal breast epithelium [35, 36]. In agreement with this, our data showed that average proliferation was significantly higher in the E2 treated heterotypic culture of HuMECs. Moreover, a disturbed polarity in the acini-like structures, one of symptoms of epithelium de-differentiation, was also observed after E2 treatment, which might be correlated to the highly proliferative activity of the treated HuMECs.

Mechanisms of estrogen-induced mitogenic effects are currently unclear. However, since an extensive body of work has shown that the effect of E2 on epithelial cells is mediated through steroid receptors [29, 33, 35], the expression of two forms of estrogen receptor (ER) genes, namely *ER α* and *ER β* , was analyzed. We found that the expression of *ER α* , *ER β*

significantly decreased in the heterotypic culture after two weeks of treatment with E2. This result is coincident with a report by Eigélie et al, in which freshly isolated human breast tissue was employed and enhanced mitogenic responsiveness and down-regulated steroid receptors observed after the treatment of estrogen [37]. These results indicate that the 3D heterotypic culture model constructed in the present study mimics breast tissue *in vivo*, exhibiting its potential to respond to ovarian hormones in a more physiologically relevant manner.

To explain the effect of estrogen on mammary epithelial cells, several investigators hypothesized that in normal human breast tissue, steroidal receptor (+) cells and proliferative cells are separate cell populations. Basically, only differentiated cells are ER or progesterone receptor (PR)(+), but they are non-proliferative. The majority of proliferating cells (undifferentiated cells) are adjacent to the ER/PR (+) cells. Thus, the ER/PR(+) cells potentially control the proliferation activity of epithelial cells via juxtacrine and/or paracrine signals [37, 38]. This could be used to explain the effect of E2 on the constructed 3D culture model observed in the present study. It is worth pointing out that the role of stromal cells should not be ignored during estrogen treatment. Reports concerning expression of steroidal receptors in normal mammary stromal cells have been conflicting [39, 40]. Here, our preliminary study showed that most of fibroblasts presented in the 3D heterotypic culture were ER(+). Additionally, they secreted higher hepatocyte growth factor (HGF), a potent mitogenic factor [41, 42], in response to the stimulation of estrogen (data not provided). It means they might be able to respond to the stimulation by estrogen, and produced HGF-mediated mitogenic effects on HuMECs. Accordingly, a relatively lower proliferative activity was observed in the monocultures when compared with the multicellular cultures, presenting experimental evidence to support that stromal effects might also contribute to the mitogenic effect produced by hormone treatment. This result is somewhat consistent with one report by Zhang et al. (43), in which estrogen treatment of breast fibroblasts enhanced proliferation and induced tubulogenesis in MECs when cocultured in collagen gels, and these effects were demonstrated to be highly dependent on stromal production of HGF [9,43].

In addition to the enhanced cellular proliferation and down-regulated expression of steroid receptors exhibited by the heterotypically cultured HuMECs after estrogen treatment, significant morphological and functional alterations were also observed, such as hyperplasia of HuMECs, a loss of lumen structure and disorganized cellular polarity, as well as decreased expression of casein gene transcripts. All of these data support the de-differentiation of epithelial cells after estrogen treatment. This result is somewhat consistent with some previous studies, in which a promoting role of hormone treatment in mammary carcinogenesis was observed based on up-regulating Bax and Bcl-2 expression [44]. In addition, significant hyperplasia of mammary ducts and alveoli was observed when estrogen was applied alone or combined with androgen in rat mammary gland tissue [45]. Therefore, our results suggest a suitably reflective hormone responsive capability of the current heterotypic culture model, but also identify some potentially negative effects of ovarian hormone treatment.

5. Conclusions

A hormone-responsive 3D human tissue-like culture system was developed in which human primary MECs were cultivated with two types of predominant mammary stromal cells (human fibroblasts and adipocytes) on silk scaffolds. Silk porous scaffolds with extracellular matrix (collagen-Matrigel™) incorporation provided a compatible environment for epithelial structure formation. These epithelial structures included alveolar and ductal structures and exhibited a higher degree of differentiation evidenced by their improved morphological

phenotype and functional activity. More importantly, this 3D multicellular culture model displayed an estrogen-responsive capability in a physiologically relevant manner, which has not been reported in our previous studies. Thus, this culture system offers an excellent opportunity to explore the role of cell–cell and cell–substrate interactions during mammary gland development, the consequences of hormone receptor activation on MEC behavior and morphogenesis, as well as their alteration in neoplastic transformation of breast tissue.

Acknowledgments

We appreciate the significant technical contribution by Tufts Medical Center and in particular Prof. Carlos Sonnenschein, Ana Soto and Maricel V. Maffini. This work was supported by Philip Morris International (CS), the NIH P41 (EB002520) Tissue Engineering Resource Center (TERC) and the NFSC (31100689). We thank Prof. Jeff Gimble, Pennington Research Center, for the contribution of hASCs.

References

1. Howlett AR, Bissell MJ. The influence of tissue microenvironment (stroma and extracellular matrix) on the development and function of mammary epithelium. *Epithelial Cell Biol.* 1993; 2:79–89. [PubMed: 8353596]
2. Roskelley CD, Bissell MJ. The dominance of the microenvironment in breast and ovarian cancer. *Semin Cancer Biol.* 2002; 12:97–104. [PubMed: 12027581]
3. Haslam SZ, Woodward TL. Host microenvironment in breast cancer development: epithelial-cell-stromal-cell interactions and steroid hormone action in normal and cancerous mammary gland. *Breast Cancer Res.* 2003; 5:208–215. [PubMed: 12817994]
4. Schor SL, Schor AM. Phenotypic and genetic alterations in mammary stroma: implications for tumour progression. *Breast Cancer Res.* 2001; 3:373–379. [PubMed: 11737888]
5. Maffini MV, Soto AM, Calabro JM, Ucci AA, Sonnenschein C. The stroma as a crucial target in rat mammary gland carcinogenesis. *J Cell Sci.* 2004; 117:1495–1502. [PubMed: 14996910]
6. Ip MM, Darcy KM. Three-dimensional mammary primary culture model systems. *J Mammary Gland Biol Neoplasia.* 1996; 1:91–110. [PubMed: 10887483]
7. Krause S, Maffini MV, Soto AM, Sonnenschein C. A novel 3D in vitro culture model to study stromal-epithelial interactions in the mammary gland. *Tissue Eng Part C Methods.* 2008; 14:261–271. [PubMed: 18694322]
8. Pickl M, Ries CH. Comparison of 3D and 2D tumor models reveals enhanced HER2 activation in 3D associated with an increased response to trastuzumab. *Oncogene.* 2009; 28:461–468. [PubMed: 18978815]
9. Shaw KR, Wrobel CN, Brugge JS. Use of three-dimensional basement membrane culture to model oncogene-induced changes in mammary epithelial morphogenesis. *J Mammary Gland Biol Neoplasia.* 2004; 9:297–310. [PubMed: 15838601]
10. Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods.* 2003; 30:256–268. [PubMed: 12798140]
11. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-associated fibroblasts direct tumour progression of initiated human prostatic epithelium. *Cancer Res.* 1999; 59:5002–5011. [PubMed: 10519415]
12. Darcy KM, Zangani D, Shea-Eaton W, Shoemaker SF, Lee PP, Mead LH, et al. Mammary fibroblasts stimulate growth, alveolar morphogenesis, and differentiation of normal rat mammary epithelial cells. *In Vitro Cell Dev Biol Anim.* 2000; 36:578–592. [PubMed: 11212143]
13. Shekhar MP, Werdell J, Tait L. Interaction with endothelial cells is prerequisite for branching ductal-alveolar morphogenesis and hyperplasia of preneoplastic human breast epithelial cells: regulation by estrogen. *Cancer Res.* 2000; 60:439–449. [PubMed: 10667599]
14. Proia DA, Kuperwasser C. Reconstruction of human mammary tissue in a mouse model. *Nat Protoc.* 2006; 1:206–214. [PubMed: 17406234]

15. Wang X, Zhang X, Sun L, Subramanian B, Maffini MV, Soto A, et al. Preadipocytes stimulate ductal morphogenesis and functional differentiation of human mammary epithelial cells on 3D silk scaffolds. *Tissue Eng Part A*. 2009; 15:3087–3098. [PubMed: 19338449]
16. Wang X, Sun L, Maffini MV, Soto A, Sonnenschein C, Kaplan DL. A complex 3D human tissue culture system based on mammary stromal cells and silk scaffolds for modeling breast morphogenesis and function. *Biomaterials*. 2010; 31:3920–3929. [PubMed: 20185172]
17. Hofmann S, Knecht S, Langer R, Kaplan DL, Vanjak-Novakovic G, Merkle HP, et al. Cartilage-like tissue engineering using silk scaffolds and mesenchymal stem cells. *Tissue Eng*. 2006; 12:2729–2738. [PubMed: 17518642]
18. Mauney JR, Nguyen T, Gillen K, Kirker-Head C, Gimble JM, Kaplan DL. Engineering adipose-like tissue in vitro and in vivo utilizing human bone marrow and adipose-derived mesenchymal stem cells with silk fibroin 3D scaffolds. *Biomaterials*. 2007; 28:5280–5290. [PubMed: 17765303]
19. Wang X, Wang W, Ma J, Guo X, Ma X. Proliferation and differentiation of mouse embryonic stem cells in APA microcapsule: A model for studying the interaction between stem cells and their niche. *Biotechnol Prog*. 2006; 22:791–800. [PubMed: 16739963]
20. Zhuang YH, Saaristo R, Ylikomi T. An in vitro long-term culture model for normal human mammary gland: expression and regulation of steroid receptors. *Cell Tissue Res*. 2003; 311:217–226. [PubMed: 12596041]
21. Gumbiner BM. Regulation of cadherin adhesive activity. *J Cell Biol*. 2000; 148:399–404. [PubMed: 10662767]
22. Soule HD, Maloney TM, Wolman SR, Peterson WD, Brenz R, McGrath CM, et al. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res*. 1990; 50:6075–6086. [PubMed: 1975513]
23. DiRenzo J, Signoretti S, Nakamura N, Rivera-Gonzalez R, Sellers W, Loda M, et al. Growth factor requirements and basal phenotype of an immortalized mammary epithelial cell line. *Cancer Res*. 2002; 62:89–98. [PubMed: 11782364]
24. Ip MM, Masso-Weleh RA, Shoemaker SE, Shea-Eaton WK, Ip C. Conjugated linoleic acid inhibits proliferation and induces apoptosis of normal rat mammary epithelial cells in primary culture. *Exp. Cell Res*. 1999; 250:22–34. [PubMed: 10388518]
25. Stingl J. Estrogen and progesterone in normal mammary gland development and in cancer. *Horm Cancer*. 2011; 2:85–90. [PubMed: 21761331]
26. Asselin-Labat ML, Vaillant F, Sheridan JM, Pal B, Wu D, Simpson ER, et al. Control of mammary stem cell function by steroid hormone signalling. *Nature*. 2010; 465:798–802. [PubMed: 20383121]
27. Sadlonova A, Novak Z, Johnson MR, Bowe DB, Gault SR, Page GP, et al. Breast fibroblasts modulate epithelial cell proliferation in three-dimensional in vitro co-culture. *Breast Cancer Res*. 2005; 7:R46. [PubMed: 15642169]
28. Urbanski S, Jensen HM, Cooke G, Mcfarlane D, Shannon P, Kruikov V, et al. The association of histological and radiological indicators of breast cancer risk. *Br J Cancer*. 1988; 58:474–479. [PubMed: 2849977]
29. Lee S, Mohsin SK, Mao S, Hilsenbeck SG, Medina D, Allred DC. Hormones, receptors, and growth in hyperplastic enlarged lobular units: early potential precursors of breast cancer. *Breast Cancer Res*. 2006; 8:R6. [PubMed: 16417654]
30. Petersen OW, Gudjonsson T, Villadsen R, Bissell MJ, Rønnov-Jessen L. Epithelial progenitor cell lines as models of normal breast morphogenesis and neoplasia. *Cell Prolif*. 2003; 36 Suppl 1:33–44. [PubMed: 14521514]
31. Paszek MJ, Weaver VM. The tension mounts: Mechanics meets morphogenesis and malignancy. *J Mammary Gland Biol Neoplasia*. 2004; 9:325–342. [PubMed: 15838603]
32. Joshi PA, Jackson HW, Beristain AG, Di Grappa MA, Mote PA, Clarke CL, et al. Progesterone induces adult mammary stem cell expansion. *Nature*. 2010; 465:803–807. [PubMed: 20445538]
33. Frech MS, Halama ED, Tilli MT, Singh B, Gunther EJ, Chodosh LA, et al. Deregulated estrogen receptor alpha expression in mammary epithelial cells of transgenic mice results in the development of ductal carcinoma in situ. *Cancer Res*. 2005; 65:681–685. [PubMed: 15705859]

34. Daniel AR, Hagan CR, Lange CA. Progesterone receptor action: defining a role in breast cancer. *Expert Rev Endocrinol Metab.* 2011; 6:359–369. [PubMed: 21857868]
35. Clarke RB. Human breast cell proliferation and its relationship to steroid receptor expression. *Climacteric.* 2004; 7:129–137. [PubMed: 15497901]
36. Arendt LM, Evans LC, Rugowski DE, Garcia-Barchino MJ, Rui H, Schuler LA. Ovarian hormones are not required for PRL-induced mammary tumorigenesis, but estrogen enhances neoplastic processes. *J Endocrinol.* 2009; 203:99–110. [PubMed: 19635758]
37. Eigélie N, Harkonen P, Erkkola R. Effect of estradiol and medroxyprogesterone acetate on morphology, proliferation and apoptosis of human breast tissue in organ cultures. *BMC Cancer.* 2006; 6:246. [PubMed: 17044944]
38. Anderson E. The role of oestrogen and progesterone receptors in human mammary development and tumorigenesis. *Breast Cancer Res.* 2002; 4:197–201. [PubMed: 12223124]
39. Parmar H, Young P, Emerman JT, Neve RM, Dairkee S, Cunha GR. A novel method for growing human breast epithelium in vivo using mouse and human mammary fibroblasts. *Endocrinology.* 2002; 143:4886–4896. [PubMed: 12446616]
40. Palmieri C, Saji S, Sakaguchi H, Cheng G, Sunters A, O'Hare MJ, et al. The expression of oestrogen receptor (ER)-beta and its variants, but not ERalpha, in adult human mammary fibroblasts. *J Mol Endocrinol.* 2004; 33:35–50. [PubMed: 15291741]
41. Tahara Y, Ido A, Yamamoto S, Miyata Y, Uto H, Hori T, et al. Hepatocyte growth factor facilitates colonic mucosal repair in experimental ulcerative colitis in rats. *J Pharmacol Exp Ther.* 2003; 307:146–151. [PubMed: 12954797]
42. Nicoleau C, Benzakour O, Agasse F, Thiriet N, Petit J, Prestoz L, et al. Endogenous hepatocyte growth factor is a niche signal for subventricular zone neural stem cell amplification and self-renewal. *Stem Cells.* 2009; 27:408–419. [PubMed: 18988709]
43. Zhang HZ, Bennett JM, Smith KT, Sunil N, Haslam SZ. Estrogen mediates mammary epithelial cell proliferation in serum-free culture indirectly via mammary stroma-derived hepatocyte growth factor. *Endocrinology.* 2002; 143:3427–3434. [PubMed: 12193555]
44. Xie B, Tsao SW, Wong YC. Sex hormone-induced mammary carcinogenesis in the female Noble rats: expression of bcl-2 and bax in hormonal mammary carcinogenesis. *Breast Cancer Res Treat.* 2000; 61:45–57. [PubMed: 10930089]
45. Zhang J, Sun Y, Liu Y, Sun Y, Liao DJ. Synergistic effects of androgen and estrogen on the mouse uterus and mammary gland. *Oncol Rep.* 2004; 12:709–716. [PubMed: 15375489]

Fig.1.

(a) DNA quantification analysis by PicoGreen™ DNA assay showed cells proliferating over time in different experimental groups. (b) Quantitative analysis of Ki67⁺ staining HuMECs counted in different microscopic fields. A promotional effect of stromal cells on HuMEC proliferation was observed in the heterotypic cultures. A significant difference between the heterotypic culture and monoculture was observed after one and two weeks culture *in vitro* (* $p < 0.05$), while no difference exists between them by the third week (# $p > 0.05$)

Fig.2. Growth profile and viability of epithelial structures developed by the HuMECs on silk scaffold (day 8–10). CLSM images showed both alveolar and duct-like structures formed in the heterotypic cultures (a), while only alveolar structures were observed in the monocultures (b). Viability of HuMECs in each group detected by viability staining (c,d). H&E staining revealed morphological characteristics of the epithelial structures formed by HuMECs in different groups (e,f). TDLU-like structures were also generated in the heterotypic culture (e₁,e₂). (Arrow notes duct-like structure, asterisk notes alveolar structure, arrowhead note TDLU-like structure).

Fig.3.

Immunostaining with CD164 (red) and GM130 (green) showed a correct polarity of the alveolar structures formed by the heterotypic culture on silk scaffold (b₁-b₃), which is similar to the positive control of human breast tissue (a₁-a₃). The alveolar structure generated by the monoculture exhibited a similar apical distribution pattern of GM130/CD164, but their expression pattern looks less “focused” when compared with that of the heterotypic culture (c₁-c₃). After two weeks culture with 10 nM E2 addition, the epithelial structures in the heterotypic cultures lost their organized polarity significantly (d₁-d₃).

Fig.4. Morphological characteristics of the epithelial structures formed by HuMECs on silk scaffold: positive E-cadherin (a) and collagen IV (b) staining indicated the formation of tight junctions and basement membrane (arrow noted), suggesting the integrity of the epithelial structures formed by the 3D culture models.

Fig.5.

Transcript expression levels of α -casein (a) and β -casein (b) by real-time RT-PCR at the indicated time points. A significant increase in the expression of both α and β -casein was detected in the heterotypic cultures ($n=3$, $*p<0.05$). Consistent with the result of RT-PCR, a more intensely stained casein protein was observed in the heterotypic cultures (c₁) in comparison with the monoculture group. The image of c₃ was the negative control of immunostaining (anti-casein antibody was replaced by PBS). The cellular nuclei were counterstained with PI (red).

Fig.6.

Effect of E2 on the cellular proliferation of HuMECs in the heterotypic culture assayed by PicoGreen DNA assay. A significantly enhanced cell proliferation was observed after two weeks cultivation with E2 ($*p < 0.05$). DNA quantification analysis by PicoGreen™ DNA assay showed cells proliferating over time in different experimental groups.

Fig.7.

Effect of E2 on the transcript expression of *casein α/β* and *ERα/β* genes in 3D cultured-HuMECs was assayed by real time RT-PCR after FACS. Untreated cultures served as control. A decreased gene expression of *casein α/β* and *ERα/β* was observed in the E2-treated heterotypic cultures ($p < 0.05$). Specifically, the expression of *caseinβ* gene after the treatment was down regulated more evident than the others detected. No statistical significance exists between the E2-treated and non-treated monocultures ($p > 0.05$).