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

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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 the stromal cells in regulating normal mammary tissue morphogenesis and their aberrant behavior during the progression of breast cancer [1,4,5]. However, an 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 architectures [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^{Tm} [7–9]. However, while the use of 3D culture systems has proven to be advantageous in the characterization of 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 the 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].

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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 the investigators to explore the molecular-based 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 was 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 described 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. Toward 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, P_{2-4}, 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 time point, 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, P_0) 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% CO_2.

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 Na_2CO_3/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% CO_2.

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 h, 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% CO_2 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, BostonMA) 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 μm) 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 microspope (Germany).

2.6. Immuno-fluorescence and immunohistochemistry staining

For immuno-fluorescence staining, deparaffinized sections (~5 μm) 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 counterstained with propidium iodide (PI, 5 μg/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 exaction. 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) ($2^{\Delta Ct}$ formula, Perkin Elmer User Bulletin #2). Each sample was analyzed in triplicate.

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

To assess the effect of steroid hormone on the 3D heterotypic culture model constructed above, 10 nM 17β-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