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14. ABSTRACT The homeostasis observed in an organ is a consequence of a coordinated multidirectional communication between the epithelium and its microenvironment through physical and biochemical signals. An interruption of this communication can lead to the formation of cancer. One of the major components of mammary gland microenvironment, also called stroma, is hyaluronan (HA). It has been proposed that HA may induce malignant transformation in normal cells through interaction with its receptors. However, HA's function during normal mammary gland development is still unknown and we therefore wanted to elucidate its function during normal development before taking a closer look at its possible functions during breast cancer.					
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Introduction

The homeostasis observed in an organ is a consequence of a coordinated multidirectional communication between the epithelium and its microenvironment through physical and biochemical signals. An interruption of this communication can lead to the formation of cancer. Studies in the mammary gland have shown that normal mammary epithelial cells can be induced by carcinogen-treated stroma to become tumor cells. On the other hand, normal stroma can instruct tumor cells to form a normal mammary gland. These phenomena occur *in vivo* and further support the notion that cancer is a tissue-based disease (1-3).

The mammary microenvironment, also termed stroma, contains many cellular and acellular components. The cellular components include fibroblasts, endothelial cells and cells of the immune system, whereas the acellular stroma consists of extracellular matrix (ECM) components such as type I collagen, fibronectin and hyaluronan (HA) (4, 5). HA plays an important role in tissue homeostasis and biomechanical integrity of tissues due to its viscosity and its ability to retain water (6, 7). HA has also been shown to play an important role for signal transduction in many tissues. HA-induced signaling occurs through receptor interactions, such as CD44 and receptor for HA-mediated motility (RHAMM) (8, 9).

In order to study stromal-epithelial interactions of the mammary gland, either the use of animal models or the use of three-dimensional (3D) culture systems is needed. 3D models for the mammary gland that have been developed so far use one cell type, epithelial cells such as MCF10A cells, and a reconstituted basement membrane, termed Matrigel™, as the acellular stromal component (Figure 1) (10, 11).

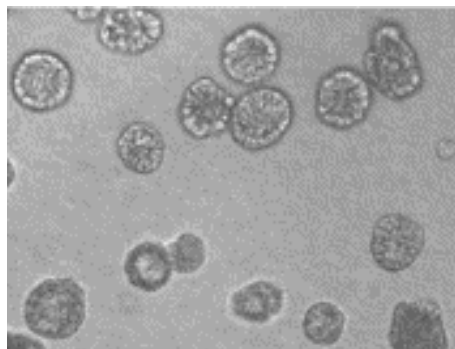


Figure 1: MCF10A grown in Matrigel™.

MCF10A cells grown alone in Matrigel™ exclusively form acinar structures. Figure was adopted from Debnath *et al.*, 2003 (10).

However, these 3D systems exclusively develop acinar structures whereas the majority of breast cancers are classified as of ductal origin. Therefore, we developed a novel 3D culture system that enabled us to obtain alveolar- and ductal-like structures resembling closely the mammary gland morphology *in vivo*.

In addition to our studies using a mouse model, we will now be able to use the 3D *in vitro* model to elucidate HA's role during mammary gland development and breast cancer.

Results

This grant was originally awarded to Alexandra Zoltan-Jones in July 2003 and was transferred to Silva Krause in July 2004. Therefore, this report will initially focus on the data obtained with the original SOW.

Task 1: To examine the effects of perturbing hyaluronan levels on mammary tissue morphology and polarization in a three-dimensional system.

During the short grant period, Ms. Zoltan-Jones was unable to set up a 3D culture system.

Task 2+3: To examine the effects of increased hyaluronan on regulation of cell proliferation via phosphoinositide 3-kinase/PTEN, ILK and β -catenin and on focal adhesion kinase activity.

MCF10A cells and MDCK kidney epithelial cells grown in a standard 2D culture system were used for this study. Using these cells, the effects of up-regulating endogenous HA synthesis on changes in cell characteristics associated with epithelial-mesenchymal transformation (EMT) were examined. It was shown that increased HA production promoted anchorage-independent growth and invasiveness and caused increased phosphoinositide 3-kinase(PI3K)/Akt pathway activity. It was also determined that increased HA expression was sufficient for epithelial-mesenchymal transformation in non-tumorigenic epithelial cells.

Furthermore, it was shown that this transformation by hepatocyte growth factor (HGF) and β -catenin was dependent on HA (12).

Ms. Zoltan-Jones assisted in studies showing that the extracellular matrix metalloproteinase inducer (EMMPRIN) stimulated HA production and promoted anchorage-independent growth and drug resistance in a HA-dependent manner (13, 14). It was shown that increased HA production stimulated focal adhesion kinase activity.

In July 2004, the grant was transferred to Silva Krause and concomitantly a change in the SOW was requested and approved.

Task 1: To examine HA levels in mouse mammary gland tissue during postnatal development.

In order to explore the function of HA during mammary gland development, HA expression was analyzed in the mouse mammary gland at 3, 5, 7, 9, and 11 weeks of age. The expression of HA in the mammary stroma increased at week 5 and peaked at week 7, the time of puberty coinciding with ductal growth (Figure 2). The peak of HA expression during the time of puberty led us to hypothesize that HA expression may be estrogen-mediated.

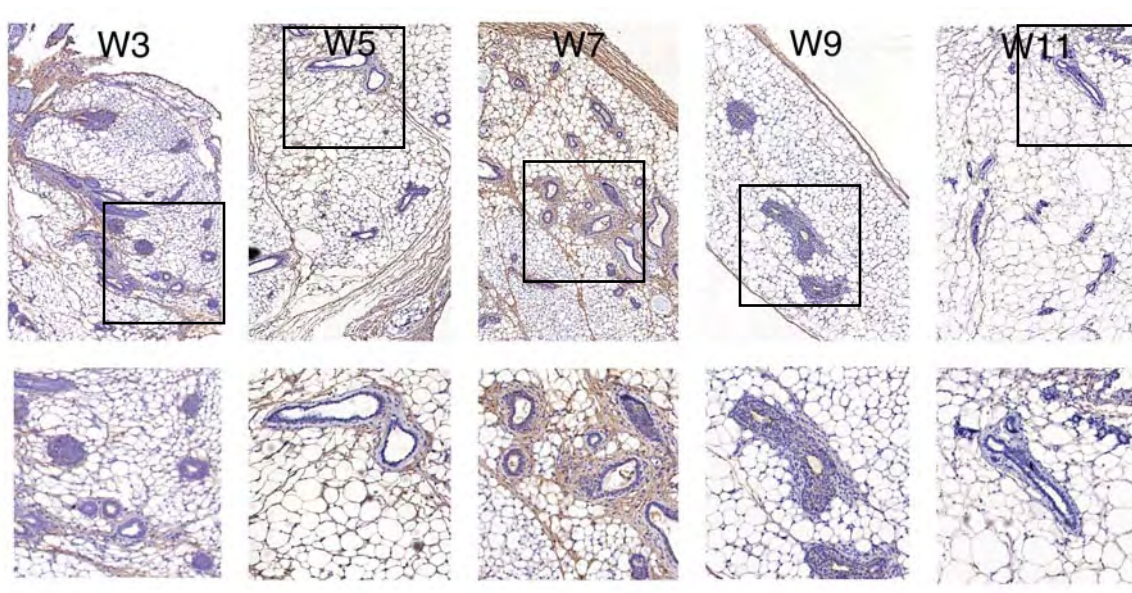


Figure 2: Expression of HA during postnatal mammary development.

HA expression in 3, 5, 7, 9, and 11-week old mice. Panels show histochemistry analysis using biotinylated HA-binding protein (HABP). Bottom panels present magnification of section in top panels as indicated by squares.

In order to test this hypothesis, we compared HA expression in postnatal mouse mammary glands of mice that were ovariectomized at 25 days of age (i.e. estrogen-depleted) and implanted with a vehicle-filled pump with ovariectomized animal implanted with an estrogen pump releasing a steady amount of estrogen ($2.5\mu\text{g/kg/day E}_2$). A third group of mice underwent a sham operation without any treatments. All mice were killed 10 days later and mammary glands were analyzed for HA expression using histochemical analyses. Even though HA expression was not entirely depleted in the mammary glands of ovariectomized mice, there was no expression observed in the periductal stroma (area directly surrounding ductal epithelium) compared to strong expression observed in the other two groups of mice (Figure 3).

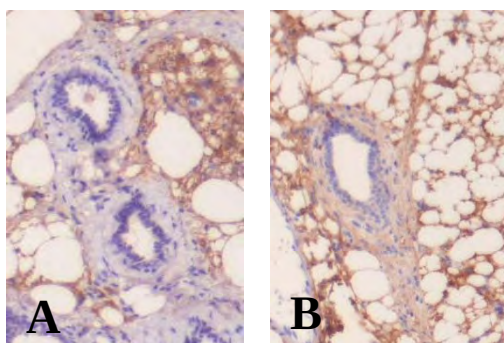


Figure 3: HA expression in periductal stroma.

Panels show histochemical analysis using biotinylated HA-binding protein (HABP) in mammary glands 35 days of age. Panel A shows lack of HA staining in periductal stroma of an ovariectomized mouse compared to a strong HA staining in panel B in the mammary gland of a mouse that had an estrogen pump implanted.

Furthermore, the expression of CD44 and one of its isoforms, CD44v6, were analyzed and shown that the expression differed depending on whether estrogen was depleted or not.

In summary, estrogen had a more subtle effect on HA expression during postnatal mouse mammary gland development than expected. However, the depletion of estrogen in mice was shown to have a profound effect in the expression of HA in the periductal stroma. During postnatal mammary gland development, ductal elongation and branching depend on mesenchymal-induced modifications of the periductal stroma (15). Estrogen may be triggering some of those modifications such as differential expression of HA. In order to further elucidate this phenomenon it could be helpful to examine the branching pattern and measure ductal elongations in the different groups of mice using morphometrical analyses.

Task 2: To examine the effects of perturbing HA levels on mammary tissue morphology.

Initially, due to Ms. Zoltan-Jones' difficulties in establishing a 3D model, we had proposed to switch to an *in vivo* model using fibroblasts that were stably transfected with HA synthase and to place them together with epithelial carcinoma cells under the kidney capsule of SCID mice. We determined that even though the tissue grafts formed ductal-like structures as shown in Figure 4, it took 2 to 3 months to retrieve a recombinant of an appropriate size to evaluate structures.

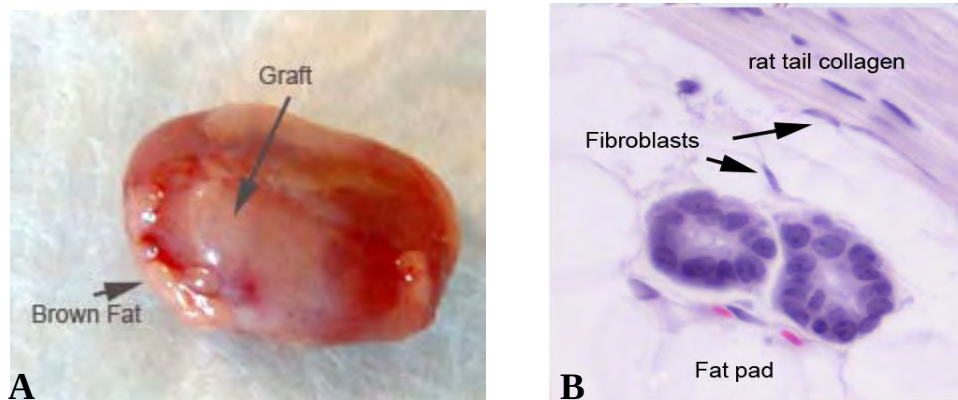


Figure 4: Tissue recombinants.

Panel A shows a kidney with tissue graft consisting of mammary epithelial cells and fibroblasts that was placed under the kidney capsule of rats and formed ductal-like structures as depicted in panel B.

During that time, my mentors Drs. Ana Soto and Carlos Sonnenschein received a grant to design a novel 3D *in vitro* model for the mammary gland. I used this opportunity and pursued the original idea of designing a 3D culture model.

In this model, co-cultures of human fibroblasts obtained from reduction mammoplasty and human mammary epithelial cells, MCF10A cells, were embedded in either a type I collagen gel, the main component of the mammary stroma, or a mixture of type I collagen and Matrigel™ using special 6-well trays (Figure 5). These experiments were carried out for up to 6 weeks.

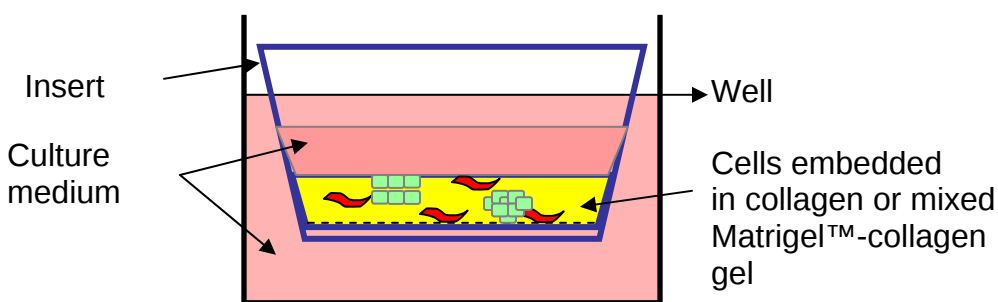


Figure 5: Collagen gel model

For the 3D tissue culture we used special 6-well-trays purchased from Organogenesis, Inc (Canton, MA). Each well contained an insert with a porous membrane on the bottom allowing medium to penetrate. A mixture consisting of cells and either collagen or mixed Matrigel™-collagen was poured into the inserts and was allowed to solidify at 37°C.

Reliable models for both matrices were established within the last year. Histological and ultrastructural analysis confirmed the formation of ductal and alveolar structures. The importance of the stromal cells was apparent in both matrices; in the collagen gels the presence of reduction mammoplasty fibroblasts accelerated the initial formation of epithelial structures and in the mixed Matrigel™-collagen gels the presence of those fibroblasts was necessary for the formation of ductal structures. These models provide an excellent system to study tissue organization, epithelial morphogenesis and breast carcinogenesis. A manuscript summarizing the results was recently submitted to Tissue Engineering. It is currently being revised and will be sent back before April 15. For detailed descriptions of the models, the manuscript was attached to this report under Appendices on page 15.

Furthermore, the 3D model using type I collagen was shown to express HA when fibroblasts were grown alone or in co-culture with MCF10A cells (Figure 6).

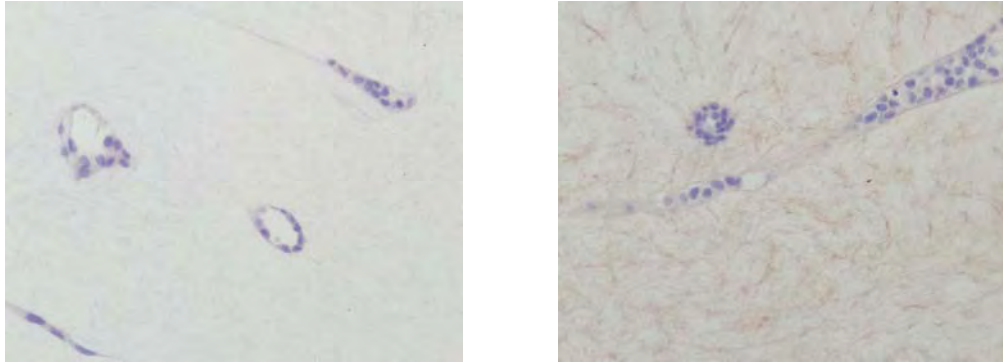


Figure 6: HA expression using histochemical analysis in 3D collagen gels

Histochemical analysis of HA was performed in collagen gels containing human fibroblasts from reduction mammoplasty and human mammary epithelial cells. Panel A shows the negative control with hyaluronidases pretreatment. Panel B shows positive HA staining as depicted in brown.

In summary, we developed two novel 3D *in vitro* models to study stromal-epithelial interactions in the mammary gland. These models provide an excellent system to study tissue organization, epithelial morphogenesis and breast carcinogenesis. As most breast cancers arise in the ducts, we now have the opportunity to study breast cancer *in vitro* because the models allow for the formation of ducts and alveolar structures in an elaborate network closely resembling *in vivo* mammary gland morphology. Furthermore, the models can be used to further elucidate the role of hyaluronan during mammary gland development and carcinogenesis.

Key research accomplishments

- Increased HA was shown to be sufficient for epithelial-mesenchymal transformation (EMT) in non-tumorigenic epithelial cells.
- Induction of EMT by HGF and β -catenin was shown to be dependent on HA.
- Increased HA production causes increased PI3K/Akt pathway activity and stimulated focal adhesion kinase activity.

- EMMPRIN was shown to stimulate HA production and promoted anchorage-independent growth and drug resistance in an HA-dependent manner.
- HA showed a distinct expression pattern during postnatal mouse mammary gland development with a peak in expression at 7 weeks of age.
- Estrogen was identified as a mediator for HA expression in the periductal stroma during mouse mammary gland development.
- One of the HA receptors, CD44 and its isoforms, showed a distinct expression in mammary glands of mice that were ovariectomized when compared to control mice.
- A novel 3D culture model for the mammary gland was successfully established.
- Using this model, ductal and alveolar structures closely resembling those observed in mammary gland morphology *in vivo* were obtained.

Reportable Outcomes

- Publications that have resulted from this research:
 1. **Zoltan-Jones A**, Huamg L, Ghatak S and Toole BP: Elevated hyaluronan production induces mesenchymal and transformed properties in epithelial cells. *J. Biol. Chem.* 278: 45801-45810, 2003.
 2. Marieb E, **Zoltan-Jones A**, Li R, Misra S, Ghatak S, Cao J, Zucker S and Toole BP: Emmprin promotes anchorage-independent growth in human mammary carcinoma cells by stimulating hyaluronan production. *Cancer Res* 64: 1229-1232, 2004.
 3. Misra S, Ghatak S, **Zoltan-Jones A** and Toole BP: Regulation of multi-drug resistance in cancer cells by hyaluronan. *J. Biol. Chem.* 278: 25285-25288, 2003.
 4. **Krause S**, Maffini M, Soto A and Sonnenschein C: A novel 3D in vitro culture model to study stromal-epithelial interactions in the mammary gland. *Tissue Engineering*, *accepted*

- Presentations:

Silva Krause, Rana Zahedi, Carlos Sonnenschein, Mina J. Bissell, Bryan P. Toole, Ana M. Soto (2006). Involvement of Extracellular Matrix (ECM) Components in Mammary Gland Development. DOD Era of Hope meeting, Philadelphia, PA

Silva Krause, Maricel Maffini, Ana Soto and Carlos Sonnenschein (2007), Carcinogenesis in the Third Dimension. Keystone meeting for Tissue Engineering and Development Biology, Snowbird , UT

Silva Krause, Maricel Maffini, Ana Soto and Carlos Sonnenschein (2007), Carcinogenesis in the Third Dimension. Gordon Research Conference on Mammary Gland Biology, Newport , RI

- Degrees obtained:

Alexandra Zoltan-Jones completed her Ph.D. in Cellular, Molecular and Developmental Biology at Tufts University, Sackler School of Graduate Biomedical Sciences

Silva Krause is expected to complete her Ph.D. in Cellular, Molecular and Developmental Biology at Tufts University, Sackler School of Graduate Biomedical Sciences in August 2008

- Personnel receiving pay from research effort:

Alexandra Zoltan-Jones:	July 2003 to June 2004
Silva Krause:	July 2004 to June 2007

Conclusions

It was shown that the increased expression of HA was sufficient for epithelial-mesenchymal transformation in non-tumorigenic epithelial cells in 2D culture systems. Increased levels of HA have been observed in several kinds of tumors and changes as such in the stroma are thought to be sufficient for cancer to arise. Therefore, it is feasible to conclude that those augmented levels can lead to an EMT in the neighboring epithelial cells and allowing them to invade their microenvironment. To confirm this hypothesis, it will be important to repeat those studies in the 3D culture models.

To elucidate HA's roles during postnatal mammary gland development, we have shown that estrogen is a mediator for HA expression, particularly in the periductal stroma. During postnatal mammary gland development, ductal elongation and branching depend on mesenchymal-induced modifications of the periductal stroma (15). Therefore, estrogen-triggered changes in HA expression might be involved in proper branching and ductal elongation of the developing mammary gland.

In addition, we have established a novel 3D culture model for the mammary gland. This model will allow us to study mammary gland development and carcinogenesis *in vitro* while considering the important influence of tissue-level organization.

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Appendices

Unfortunately I cannot attach copies of the publications by Alexandra Zoltan-Jones because I only have pdf versions of the paper. Upon request I am happy to forward the pdfs.

Attached is the version of Silva Krause's manuscript that was originally submitted to Tissue Engineering. The manuscript is currently under revision and is thought to be sent out in it's final version next week (no new experiments will be added to the final version). Upon request we will be happy to send you a reprint.

A novel 3D *in vitro* culture model to study stromal-epithelial interactions in the mammary gland

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Abstract

Stromal-epithelial interactions mediate mammary gland development and the formation and progression of breast cancer. In order to study these interactions *in vitro*, the development of defined 3-dimensional models is essential. In the present study, we have successfully developed a novel 3-dimensional *in vitro* model which allows the formation of mammary gland structures closely resembling those found *in vivo*. Co-cultures of human mammary epithelial cells and human mammary fibroblasts obtained from reduction mammoplasties embedded in either a type I collagen or a mixed MatrigelTM-collagen matrix were grown for up to 6 weeks. Histological and ultrastructural analysis confirmed the formation of ductal and alveolar structures. The importance of the stroma was apparent in both matrices; in the collagen gels the presence of RMF accelerated the initial formation of epithelial structures and in the mixed MatrigelTM-collagen gels the presence of RMF allowed for the formation of ductal structures. This model provides an excellent system to study tissue organization and epithelial morphogenesis. In addition, as the majority of breast cancers are of ductal origin, both models are suitable to study breast carcinogenesis.

Introduction

Mammary gland organogenesis occurs throughout embryonic life, puberty and adulthood by reciprocal interactions between the mesenchyme and epithelium^{1,2}. The mammary gland is a branched tubuloalveolar gland in which an epithelium lines the secretory acini and the ducts. The mesenchyme during fetal life and the stroma during adulthood contain extracellular matrix (ECM) as well as cellular components such as fibroblasts, adipocytes, immune cells and endothelial cells. The main ECM component of the mammary gland is type I collagen but it also contains several glycosaminoglycans and basement membrane components such as type IV collagen and laminins^{3,4}.

Surrogate animal models have contributed extensively to our understanding of how breast cancer arises and progresses. However, detailed interactions between cells and tissues are difficult to study in the animal. To reduce this complexity, epithelial cells in monolayer cultures have been commonly used to study breast cancer. However, these 2-dimensional (2D) cultures neither resemble the structure nor the function of the mammary epithelium *in vivo*^{5,6,7} and are inadequate for assessing the role of the stroma in this tissue. The importance of the stroma as a target for mammary carcinogenesis was recently highlighted by using tissue recombination techniques^{8,9}. Namely, normal mammary epithelial cells can be “induced” to become tumor cells by exclusive treatment of the stroma with a carcinogen^{8,9}. In a complementary approach, it was also shown that normal stroma was able to induce epithelial tumor cells to form normal mammary ducts¹⁰. These phenomena further emphasized the need to include stromal components in *in vitro* studies of the mammary gland. Thus, it is important to use an *in vitro* surrogate model that would mimic as closely as possible the structure and function of the human breast to study the initial steps of normal development and the carcinogenic process in this organ.

A 3-dimensional (3D) model of the human breast would provide the opportunity to combine several cell types in the context of a microenvironment that resembles *in vivo* conditions. Organ explants, a 3D culture developed in the 1960s and 70s, uncovered the direct effects of specific factors such as lactogenic hormones on mammary cells while allowing for the study

of tissue-level interactions ¹¹. However, the usefulness of these cultures is limited by the short life of the explants.

Based on previous studies showing rat liver differentiation on floating collagen gels ¹², Emerman *et al.* pioneered a culture system of primary mouse mammary epithelial cells placed on floating collagen membranes in which these cells maintained morphological features such as secretory activity under the influence of lactogenic hormones throughout 4 weeks of culture ¹³. Later, Li and colleagues introduced the use of reconstituted basement membrane (rBM), a solubilized basement membrane extracted from a mouse chondrosarcoma, as a matrix used to culture breast epithelial cells in 3D ¹⁴. This represented a significant improvement over 2D models to study the interactions among epithelial cells and between epithelial cells and basement membrane components ^{15,16}. However, mammary epithelial cells embedded in rBM develop only spherical structures resembling breast acini while the mammary gland is mainly comprised of ductal structures. Because more than 90% of human mammary carcinomas arise from ductal epithelium ¹⁷, it is paramount to design a 3D model in which ductal as well as alveolar structures are formed.

The aim of the current study was to develop a 3D heterotypic human breast model containing the two most prominent mammary cell types, epithelial cells and fibroblasts, within a defined matrix that would resemble that of the normal breast *in vivo*. Using ultrastructural and histological analyses, we observed ductal and alveolar structures closely resembling the *in vivo* mammary morphology.

Material and methods

Chemicals and cell culture reagents

Hydrocortisone, cholera toxin, insulin, methyl salicylate and carmine were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), DMEM/F12 and penicillin-streptomycin solution were obtained from Gibco/Invitrogen (Carlsbad, CA). Equine serum and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT). Bovine type I collagen was purchased from Organogenesis (Canton, MA). Epidermal growth factor (EGF), Matrigel™ and rat tail type I collagen were purchased from BD Biosciences (San Jose, CA). Formalin was obtained from Fisher Scientific (Atlanta, GA).

Cell maintenance

All cells used in these experiments were maintained and expanded in cell culture plastic flasks (Corning, Corning, NY). Non-tumorigenic human mammary epithelial MCF10A cells were grown in DMEM/F12 containing 5% equine serum, 20ng/ml EGF, 0.5µg/ml hydrocortisone, 0.1µg/ml cholera toxin, 10µg/ml insulin and 1% penicillin/streptomycin solution. Human mammary fibroblasts obtained from reduction mammaplasties (RMF) were grown in DMEM containing 10% FBS. All cells were incubated at 37°C and 6% CO₂. For co-culture experiments, a combined medium (1 part of MCF10A medium and 1 part of RMF medium) was used. The combined medium was previously tested in tissue culture flasks containing each cell type alone to assure proper growth and behavior of cells.

3D cell culture

Type I collagen was used at a concentration of 1mg/ml according to Paszek *et al.*¹⁸. The mixed Matrigel™-collagen gel was prepared using a 1:1 ratio of Matrigel™ and type I collagen keeping the final collagen concentration at 1mg/ml. In co-cultures, MCF10A and RMF were seeded in a 3:1 ratio using 300,000 MCF10A cells and 100,000 RMF. This ratio mimics the *in vivo* numbers of epithelial cells to fibroblasts¹⁹. The same cell number was seeded for each cell type cultured independently. Cells were suspended in collagen or Matrigel™-collagen mixture and seeded into 35mm well inserts of a six-well plate (Organogenesis, Canton, MA) as depicted in Figure 1. Cultures were maintained for one to six weeks and the medium was changed every two to three days.

Gel processing

On the day of harvest, the gels were cut into pieces. One piece was fixed overnight in 10% phosphate-buffered formalin, paraffin-embedded and used for histological analysis. Another piece was whole-mounted onto a slide and fixed overnight in 10% phosphate-buffered formalin for morphometric analysis. The last piece was fixed overnight in 2.5% glutaraldehyde, pH 7.3, buffered using 0.1M sodium cacodylate and used for electron microscopy.

Whole mount staining

The gels that were fixed to a glass slide overnight were changed to 70% ethanol and stained with Carmine Alum overnight following a protocol described by Maffini *et al.* with modifications⁹. After staining, the whole mounts were dehydrated in 70%, 95% and 100% ethanol, cleared in xylene and mounted with Permount™ (Fisher Scientific, Atlanta, GA).

Confocal microscopy

Whole-mounted gels were analyzed using a Zeiss LSM 510 system. The HeNe 633nm/5mW laser was used for data acquisition due to the autofluorescence of carmine dye at this

wavelength. Alveolar and ductal structures in collagen and mixed Matrigel™-collagen gels were scanned with a 20x objective lens and 8 bit depth images with a resolution of up to 1044x1044 pixels were taken. The data were three-dimensionally reconstructed using Zeiss software.

Immunohistochemical analysis

All primary antibody concentrations and supplier information are listed in Table 1. An antigen-retrieval method using microwave pretreatment and 0.01M sodium citrate buffer (pH 6) was used²⁰. The antigen-antibody reaction was visualized using the streptavidin-peroxidase complex, with diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO) as the chromogen. Counterstaining was performed with Harris' hematoxylin. Images were captured using a Zeiss Axioscope 2 plus microscope (Carl Zeiss MicroImaging Inc, Thornwood, NY).

Transmission electron microscopy (TEM)

Fixed samples were washed with 0.1M sodium cacodylate buffer and post fixed in 1% osmium tetroxide buffered by 0.1M sodium cacodylate for an hour on ice. Gels were dehydrated in graded ethanol, cleared with propylene oxide and infiltrated with a 1:1 mixture of propylene oxide and epon followed by another infiltration with a 1:2 mixture of propylene oxide and epon. Finally, the gels were infiltrated with 100% epon before embedding them in fresh epon and curing them at 60°C for 24 to 48h. Following polymerization of the resin, thick sections were produced using a Reichert-Jung Ultracut E microtome and stained with toluidine blue to determine orientation. The blocks were then thin sectioned at 70 to 90nm and mounted onto copper grids. Grids were stained with 3% uranyl acetate and were examined at various magnifications using a Philips CM-10 electron microscope at 80 KV.

Morphometric analysis

Four to five experiments for each condition were analyzed and for each experiment, three arbitrarily chosen fields at 10x magnification per section were examined. Images were captured using a Zeiss Axioscope 2 plus at 10x and 3900 dpi and analyzed with the Zeiss Axiovision version 4.4 software. The following parameters were measured per section: total number of ductal and alveolar structures, total number of cells per structure and number of Ki67 positive cells within those structures. Proliferating epithelial cells were expressed as %Ki67 positive cells per total epithelial cell number. Morphometric analysis was carried out in collagen and mixed Matrigel™-collagen gels.

Statistics

SPSS software package 15.0 (SPSS Inc., Chicago, Il) was used for all statistical analyses. Independent samples t-tests were used to compare morphological parameters and proliferation index in both type of matrices and in the different culture conditions. For all statistical tests, results were considered significant at $p < 0.05$. All results are presented as mean $\pm$ SEM.

Results

Breast morphogenesis in collagen gels

Co-cultures of MCF10A cells and RMF

After 24h, MCF10A cells and RMF were evenly distributed throughout the gels mostly as single cells or small two to three cell aggregates (Figure 2A). Epithelial structures began to organize after three days in culture and by the fifth day, an incipient ductal network was observed (Figure 2A).

After one week in culture, MCF10A cells formed numerous alveolar and ductal structures in the presence of RMF (Figure 2B, C) and after two and three weeks, the ductal structures formed an elaborate network with ducts increasing in length and complexity (Figure 2C) due to an increase in cell number mainly during the first week. At the same time, cells became morphologically polarized in both ducts and alveoli, i.e. their nuclei adopted a basal location (Figure 3A), and E-cadherin was expressed in the epithelial cell-to-cell contacts (Figure 3A). By the fourth week, some MCF10A cells and RMF were still proliferating and the ductal networks became more complex (Figure 2B, C). After six weeks, the epithelial structures occupied the majority of the gel area, although most of the epithelial ductal cells no longer proliferated. In addition, the epithelial structures no longer resembled ductal structures and polarity was lost (Figure 2B, C).

Using confocal microscopy, the presence of lumina in ductal and alveolar structures was observed as early as one week in culture. Between two (Figure 4) and four weeks in culture, lumina were present in a larger number of structures.

MCF10A cells grown in collagen gels

MCF10A cells cultured alone showed delayed onset of organization when compared to the co-cultures with RMF (Figure 2A). MCF10A cells alone required at least five days in culture

to become arranged into epithelial structures (Figure 2A) indicating that fibroblasts promote the initial cell-cell communication and organization.

After one week in culture, MCF10A cells formed alveolar structures and a ductal network (Figure 2B, C). After two weeks, these cells formed organized alveolar and ductal structures similar to those observed in co-culture with RMF. Cells in most epithelial structures appeared polarized and lumen formation was frequently observed in alveoli and some ducts. After three and four weeks, the ductal network became more distinct, the ducts elongated further and the gels contained a complex ductal network with few alveolar structures (Figure 2B, C). Similar to what was observed in co-culture with RMF, after six weeks of incubation, abundant epithelial structures were observed. The ductal structures seemed less organized, their outlines no longer defined and it appeared as if they had “collapsed” (Figure 2B, C).

RMF grown in collagen gels

After one week of incubation, RMF proliferated sparsely in collagen gels (Figure 2B, C). However, after two weeks in culture, the cell number increased considerably and the cells were homogeneously distributed throughout the gel. After three and four weeks in culture, RMF proliferated further. After six weeks, RMF maintained their homogeneous distribution throughout the gel (Figure 2B, C).

Morphometric analysis

With regard to ductal structures there was no significant difference whether MCF10A were grown alone or in co-culture with RMF (Figure 5A). However, there was an increase in the number of alveolar structures in collagen gels when MCF10A cells were grown in co-culture with RMF compared to when MCF10A cells were grown alone. This increase was significant after one week ($p < 0.05$) with a similar pattern persisting after two weeks in culture (Figure 5B).

Characterization of alveolar and ductal structures

As mentioned above, two, three and four week time points consistently resulted in ducts and alveoli resembling a normal mammary gland. To further characterize these epithelial structures, we chose the two week time point.

Pan-keratin staining revealed that alveolar and ductal structures consisted exclusively of epithelial cells (Figure 3A). Furthermore, E-cadherin staining, a marker of cell-cell adhesion, was observed in both alveolar and ductal structures (Figure 3A). Type IV collagen staining, a marker for basement membrane, was not observed in alveolar or ductal structures regardless of whether or not RMF were present in the culture (Figure 3A). In the co-cultures of MCF10A cells and RMF, the number of proliferating epithelial cells, as revealed by Ki67 staining, was almost twice as high at one week of incubation compared to two weeks of incubation ($p < 0.05$) (Figure 3B). When MCF10A cells were grown alone, a similar trend was observed ($p = 0.09$). This observation is consistent with previous studies using Matrigel™, whereby once cellular polarity was established, mammary epithelial cell proliferation decreased²¹. Few MCF10A cells and RMF continued to proliferate throughout week six (data not shown). Thus, we observed a similar morphological and immunohistochemical staining pattern in cultures of MCF10A cells alone and when co-cultured with RMF.

Ultrastructural analysis using TEM revealed the existence of several junctional complexes such as tight junctions and desmosomes in polarized epithelial cells (Figure 6A). However, a basement membrane surrounding the alveolar and ductal structures was not observed, which is consistent with the lack of type IV collagen staining shown in Figure 3A.

Breast morphogenesis in mixed Matrigel™-collagen gels

Co-cultures of MCF10A cells and RMF

As Matrigel™ is extensively utilized in 3D cultures of epithelial cells, a matrix consisting of a mixture of Matrigel™ and type I collagen in a 1:1 ratio keeping the final collagen

concentration at 1mg/ml was used. These cultures were incubated for one, two and three weeks. After one week in culture, most of the structures were alveolar and few ductal structures were observed in the mixed gels (Figure 7A, B). However, the number of ducts increased with time and ductal networks started to develop after three weeks in culture (Figure 7B). Furthermore, polarized ductal and alveolar structures could be observed after two and three weeks in culture (Figure 7A).

MCF10A cells grown in mixed Matrigel™-collagen gels

MCF10A cells grown alone exclusively formed alveolar structures in the mixed Matrigel™-collagen gels in contrast to the morphology observed in collagen gels (Figure 7A, B). No ductal structures were observed at any time point analyzed. After two and three weeks in culture, the number of alveolar structures increased but the size of each structure remained the same. In addition, as early as one week in culture, the alveolar structures were polarized, i.e. their nuclei were located basally, and maintained their polarity throughout the duration of the experiment (Figure 7A).

RMF grown in mixed Matrigel™-collagen gels

In contrast to the homogeneous distribution of RMF observed in collagen gels, these cells formed clusters in mixed Matrigel™-collagen gels that were often arranged into star-like shapes (Figure 7A, B). After two and three weeks in culture, the clusters of RMF increased in size as the RMF continued to proliferate.

Morphometric analysis

When MCF10A cells were grown in co-culture with RMF a significant decrease of ductal structures in the mixed Matrigel™-collagen gels by about ten-fold after one week ($p < 0.05$) and almost seven-fold after two weeks ($p < 0.05$) compared to collagen gels was observed (Figure 5A). Interestingly, there was a significant increase in the number of alveolar structures by more than two-fold after one week ($p < 0.05$) with a similar trend after two

weeks ($p=0.053$) in co-culture compared to the number of alveolar structures present in collagen gels (Figure 5B).

When MCF10A cells were grown alone, the number of ductal structures comparing both gel types was significantly different after both one week ($p<0.01$) and two weeks ($p<0.01$) (Figure 5A). Furthermore, the number of alveolar structures increased significantly by more than ten-fold after one week ($p<0.001$) and two weeks ($p<0.001$) in culture compared to collagen gels.

Characterization of alveolar and ductal structures

The epithelial structures in the mixed MatrigelTM-collagen gels were characterized using the same markers as in the collagen gels. Pan-keratin staining revealed that alveolar and ductal structures consisted exclusively of epithelial cells (Figure 3A). Positive E-cadherin staining provided further evidence that epithelial cells formed cell-cell junctions. Alveolar and ductal structures were able to form a basement membrane as evidenced by positive type IV collagen staining. TEM analyses further confirmed the existence of a basement membrane (Figure 6B). This is in contrast to the results obtained with collagen gels. However, both histological and ultrastructural analysis revealed that the basement membrane was discontinuous (Figure 6B).

When MCF10A cells were grown in co-culture with RMF, the number of proliferating cells decreased significantly between one and two weeks in culture ($p<0.05$) (Figure 3B). A comparable observation was made when MCF10A cells were grown alone ($p<0.05$) (Figure 3B). After two weeks of incubation, the number of proliferating cells was similar to those found in collagen gels (Figure 3B).

Cell-matrix interactions in 3D cultures

Collagen gels had an approximate thickness of 5mm at the beginning of the experiments and contracted to about 1mm within 7-10 days when MCF10A cells and RMF were seeded in co-culture. Gels containing either MCF10A cells or RMF alone contracted less rapidly but also

considerably over time and reached a thickness similar to those observed in co-cultures but several days later. In contrast, mixed Matrigel™-collagen gels contracted only very little if at all. Remarkably, collagen gels contracted non-homogenously forming a thicker inner center surrounded by a thinner outer ring (Figure 8). Thus, cells formed different epithelial structures depending on their location in the gel. The outside ring contained more alveolar structures with long cords of epithelial cells on the extreme periphery, whereas ductal structures developed mostly in the center of the gel. Preliminary data suggest that matrix elasticity varies between the center and the periphery of the gel. The presence of diverse epithelial structures further emphasizes the importance of physical properties in tissue morphologies^{22,23}. The data presented herein were obtained by analyzing structures present in the center of the gels. They represented morphologies similar to those observed *in vivo* and were consistently reproduced.

Discussion

Here we report the characterization of a novel human breast surrogate model for the study of normal and diseased breast development *in vitro* while preserving the 3D architecture characteristic of the organ *in vivo*. This system consists of a 3D co-culture of human breast epithelial cells and human breast fibroblasts embedded within i) a defined matrix of type I collagen, i.e. the major ECM component of the mammary gland, and ii) a matrix of type I collagen and Matrigel™. In the former, mostly branched ducts developed, while in the latter alveoli and ducts were formed, highlighting the importance of matrix composition for the understanding of tissue organization. Furthermore, this human *in vitro* 3D model proved to be reliable and stable for at least four weeks in culture.

Epithelial structures obtained in collagen gels did not form a basal lamina in contrast to those observed in mixed Matrigel™-collagen gels. Emerman *et al.* reported that cells grown on floating collagen gels formed continuous basal lamina¹³. However, these authors observed occasional myoepithelial cells in their cultures which might have been unintentionally included during the initial cell collection¹³. Myoepithelial cells are known to secrete basal lamina components²⁴. The presence of a basement membrane surrounding alveolar structures

in mixed Matrigel™-collagen gels confirms previous observations found when mammary epithelial cells were grown in Matrigel™^{15,16}. Our results extend this finding by showing

that the ductal structures obtained in the co-cultures were also able to form a basement membrane.

We consistently observed a reduced amount of fibroblasts in the MCF10A cells and RMF co-cultures in both collagen gels and mixed Matrigel™-collagen gels when compared to RMF grown alone. Even though 100,000 RMF were seeded in all of these gels, few of these fibroblasts were observed after one and two weeks of co-culture with MCF10A cells. However, in a similar experiment when skin keratinocytes were seeded together with RMF, numerous fibroblasts were observed throughout the gel even after one week (manuscript in preparation), indicating that MCF10A cells may selectively inhibit RMF from rapidly proliferating during the first weeks in culture. More extensive studies are needed to characterize this phenomenon.

It has been reported that mammary epithelial cells grown in reconstituted basement membrane only form acini^{15,16}. The fact that in our model MCF10A cells grown alone in collagen gels were able to form ductal structures indicates that Matrigel™ may have inhibited MCF10A cells from elongating and forming ducts. This inhibitory effect observed in the mixed Matrigel™-collagen gels was significantly diminished by the presence of RMF. This might be due to the presence of the discontinuous basement membrane observed in the epithelial structures formed in the presence of RMF, indicating that RMF play a role in inducing tubule formation by locally degrading basement membrane. This inference is supported by the fact that during early normal development of the lung, salivary gland and kidney, the continuous basal lamina becomes discontinuous during periods of extensive elongation and branching^{25,26,27}. Alternatively, the absence of ductal formation when epithelial cells were grown alone in the mixed Matrigel™-collagen gels could be due to the different elastic properties of this matrix compared to collagen gels. As mentioned above, both matrices have different contraction properties and physical forces play an important role in morphogenesis and cell fate^{22,28}.

MCF10A cells always formed ducts in the presence of RMF in both collagen and the mixed Matrigel™-collagen gels. Contrary to the results reported by Tsai *et al.*²⁹, the ductal structures that developed in our model were part of a complex network and not just isolated ducts.

In the long-term task of characterizing a relevant and reliable 3D surrogate model for breast development and carcinogenesis, researchers are just now at the initial data-gathering stage of developing co-cultures of two or more cell types in a mostly defined microenvironment. The two 3D cultures containing stromal and epithelial cells described herein are relevant surrogate models to study breast biology. Using these models we were able to observe ducts, which are the main structure of the virgin mammary gland and the structure where the acini and the majority of breast cancer arises in humans. As a result, this model will allow us to perturb single components of the stroma (its cellular and ECM components) and the epithelium to assess their individual contributions to normal tissue organization, tumor formation and its reversal.

Acknowledgments

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Figure legends

- Figure 1:** Schematic representation of the 3D *in vitro* model. Each well of the 6-well-plate has an insert containing the cells embedded either within the collagen or mixed MatrigelTM-collagen gel.
- Figure 2:** Ductal and alveolar structures obtained in collagen gels. A) Using H&E and whole mount analyses, a five day time course reveals a delayed onset of the formation of ductal and alveolar structures when MCF10A cells are grown alone compared to the co-culture with RMF. The ductal network is clearly seen in the whole mount preparations. H&Es (B) and whole mounts (C) of collagen gels with MCF10A cells and RMF in co-culture as well as each cell type alone cultured for one to six weeks. Note the lumen formation (B, arrows) in epithelial structures after two and three weeks and the complex ductal network (C) that begins to form as early as one week of incubation. Scale bars: H&Es = 50µm, whole mounts = 100µm.
- Figure 3:** Characterization of epithelial structures. A) Ductal and alveolar structures obtained in both gel types were characterized using immunohistochemical analysis for epithelial origin (pankeratin staining), adherens junctions (E-cadherin staining), cell proliferation (Ki67 staining), and basement membrane (type IV collagen staining). Note that only in the mixed MatrigelTM-collagen gels, a basement membrane could be observed surrounding epithelial structures (arrows). arrowhead indicates polarized structure, scale bar = 50µm. B) Quantitative analysis of percentage of Ki67 positive epithelial cells in both gel types after one and two weeks of incubation. a indicates $p = 0.09$, * indicates statistical significance, $p < 0.05$.
- Figure 4:** Lumen formation in epithelial structures. Representative image taken from a whole mount of MCF10A cells and RMF in collagen gels after two weeks in culture. Consecutive pictures within a z stack arrangement acquired by standard confocal microscopy analysis revealed the presence of a lumen. Scale bar = 50µm.
- Figure 5:** Morphometric analysis of ductal and alveolar structures in both collagen and mixed MatrigelTM-collagen gels. A) The number of ductal structures was significantly different between the two gel types after one and two weeks in co-cultures and when MCF10A cells were grown alone. Note the total absence of ductal structures when MCF10A cells were grown in mixed gels B) In co-cultures of MCF10A cells and RMF, a statistically significant increase was observed after one week with a similar trend after two weeks of incubation (a: $p = 0.053$). The number of alveolar structures in mixed MatrigelTM-collagen gels shows a significant increase compared to those in collagen gels after one and two weeks of incubation when MCF10A were grown alone. Note that there was no statistically significant difference between week one and two in the number of ductal (A) and alveolar (B) structures for each gel type whether

MCF10A cells were grown alone or in co-culture with RMF. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

- Figure 6:** Transmission electron microscopy of epithelial structures. Cell-cell junctions such as tight junctions (white arrows) and desmosomes (arrowheads) are present in epithelial structures in both A) collagen gels and B) mixed MatrigelTM-collagen gels. A basement membrane (black arrows) was observed only in mixed MatrigelTM-collagen gels (B). N = nucleus, n = nucleolus, v = vacuole; scale bars = 1 μm .
- Figure 7:** Ductal and alveolar structures obtained in mixed MatrigelTM-collagen gels. H&Es (A) and whole mounts (B) of gels with MCF10A cells and RMF in co-culture and cultured independently for one to three weeks. Ductal structures formed exclusively in the presence of RMF. Scale bars: H&Es = 50 μm , whole mounts = 100 μm .
- Figure 8:** Schematic representation of the time-dependent contraction of collagen gels. A newly cast gel shows a homogeneous thickness throughout the gel whereas the contracted gel shows a thicker inner center surrounded by a thinner outside ring after several days.

Table 1: List of primary antibodies used in immunohistochemical analyses.

Primary Antibody	Source	Dilution
Mouse anti pankeratin	Sigma-Aldrich (St. Louis, MO)	1:1000
Rabbit anti Ki67	Vector (Burlingame, CA)	1:3000
Mouse anti E-cadherin	Novocastra (Newcastle, UK)	1:150
Mouse anti collagen type IV	DakoCytomatin (Glostrup, DM)	1:50

Figure 1

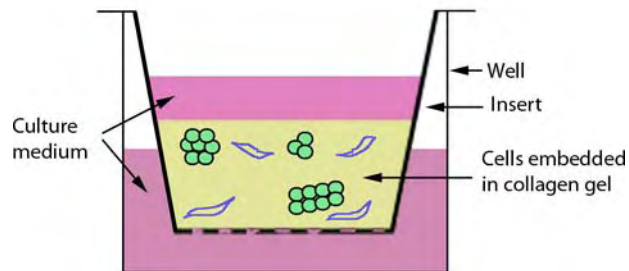


Figure 2

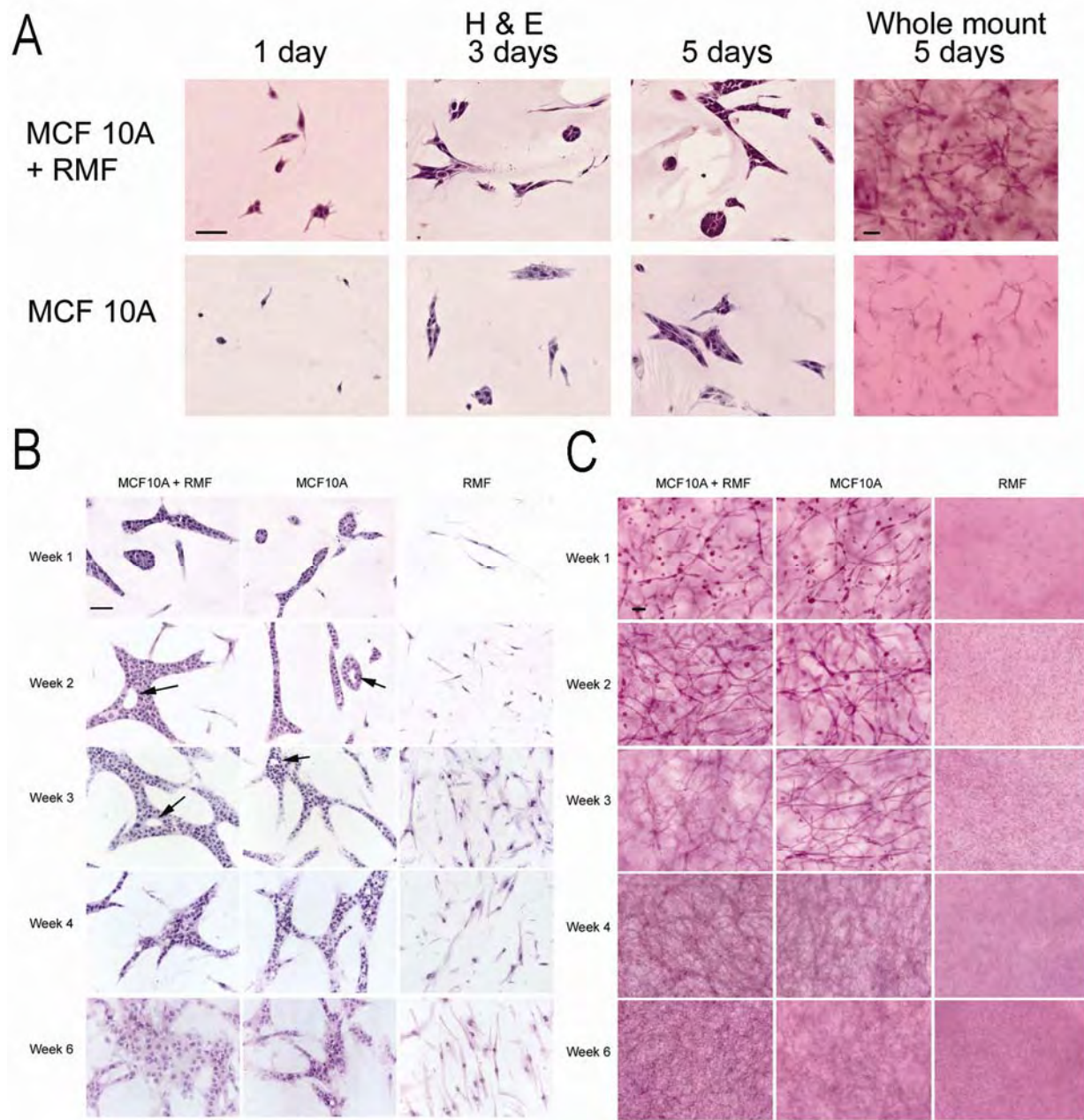


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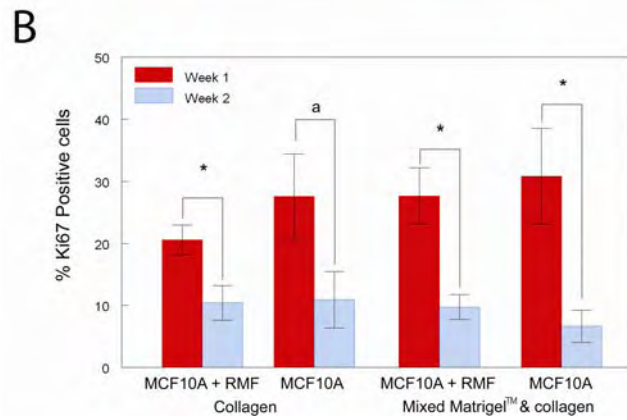
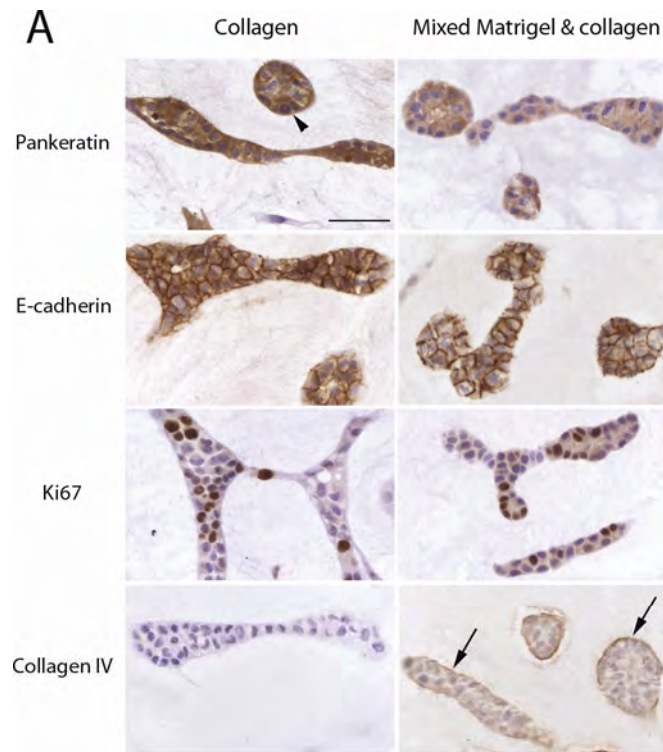


Figure 4

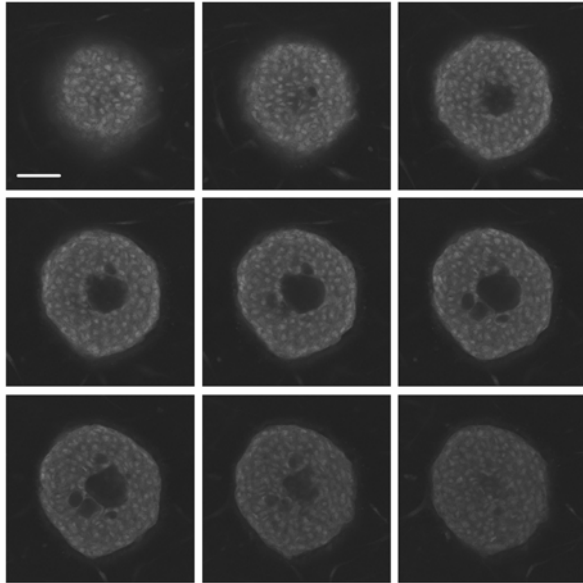


Figure 5

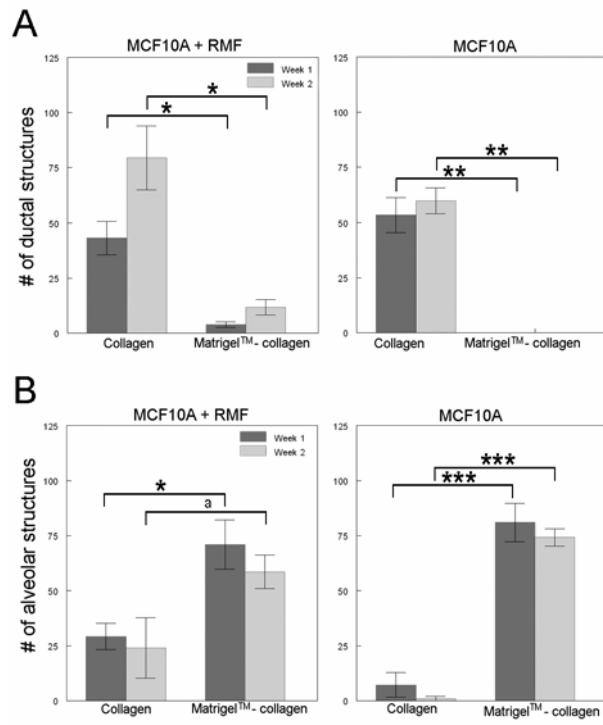


Figure 6

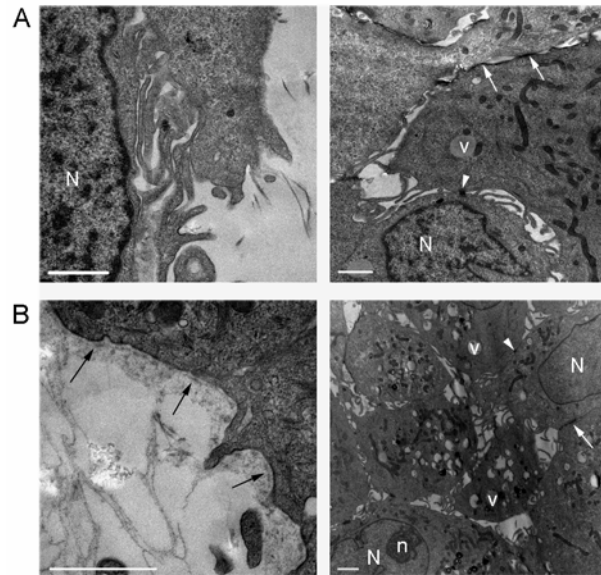


Figure 7

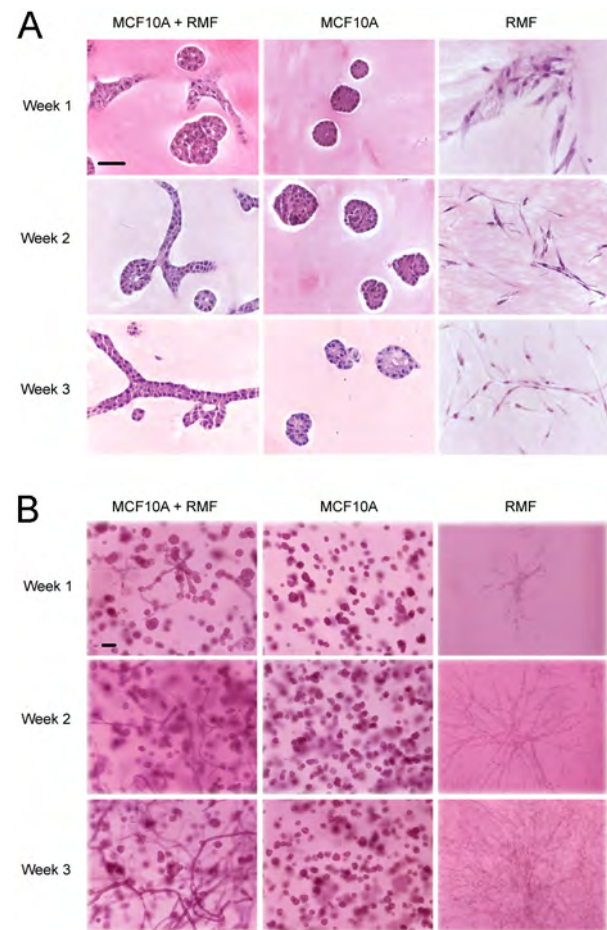


Figure 8

