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14. ABSTRACT The mammary gland is comprised of stromal and epithelial cells that communicate with each other through the extracellular matrix (ECM). Disruption of communication between the epithelium and stroma can both induce and promote breast cancer. Crosstalk between the mammary epithelium and stroma is also crucial for the proper patterning and function of the normal mammary gland. It has been proposed that HA may induce malignant transformation in normal cells through interaction with its receptors. We therefore wanted to elucidate its function during normal mammary gland development. The expression of HA in the stroma increased at week 5 and peaked at week 7, the time of puberty coinciding with ductal growth. We observed a decrease with age when the mammary gland achieves mature virginal development (week 9 and 11). The peak of HA expression during the time of puberty led us to hypothesize that HA expression may be estrogen-mediated. Preliminary data suggest a role of estrogen as a mediator for HA expression but the analysis is still ongoing.								
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Introduction

The mammary gland is comprised of stromal and epithelial cells that communicate with each other through the extracellular matrix (ECM). Disruption of communication between the epithelium and stroma can both induce and promote breast cancer (1,2). Crosstalk between the mammary epithelium and stroma is also crucial for the proper patterning and function of the normal mammary gland.

A major component of the ECM is hyaluronan (HA). HA as a negatively charged polymer provides remarkable hydrodynamic characteristics due to its viscosity and its ability to retain water. It therefore plays an important role in tissue homeostasis and biomechanical integrity. HA-induced signaling occurs through receptor interactions, such as CD44 and receptor for HA-mediated motility (RHAMM). These interactions activate numerous downstream signals such as Rac and Ras that mediate the cell's response to numerous processes. As the predominant receptor for HA, the CD44 transmembrane glycoprotein family monitors changes in the extracellular matrix that influence cell proliferation, survival and differentiation (3). The heterogeneity of CD44 protein products is in part generated by post-translational modifications, which differ depending on the cell type and growth conditions. In addition, CD44 transcripts are subject to alternative splicing, which affects predominantly the extracellular stem structure of CD44 proteins (4). Expression of the variant isoforms of CD44 is restricted mainly to a limited selection of epithelia in normal tissue (Figure 1).

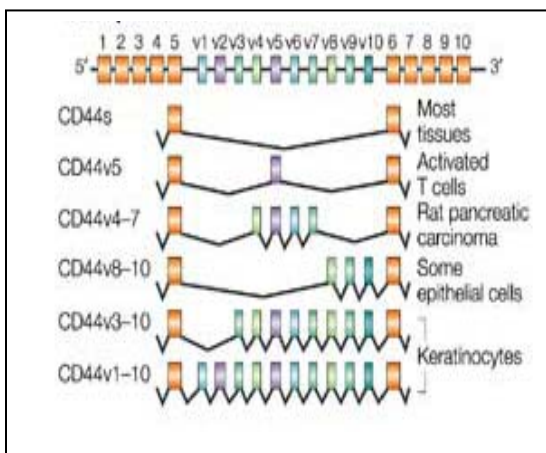


Figure 1: CD44 transcripts

CD44 pre-mRNA is encoded by 20 exons, 10 of which can be regulated by alternative splicing (variant or 'v' exons).

Figure was adapted by Ponta H *et al.*, 2003 (4).

One of the isoforms of CD44, the CD44v6 epitope, has been widely reported to be expressed in human mammary carcinomas (5,6). Hebbard *et al.* examined the expression of CD44v6 epitope in murine mammary glands (7). They showed that CD44c6 expression is first seen during puberty, and that after puberty protein expression followed the estrous cycle.

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

Previously we had tested our hypothesis that HA expression may be estrogen-mediated by comparing HA expression in the postnatal mouse mammary gland of mice that were ovariectomized at 25 days of age (i.e. estrogen-depleted) and implanted with an estrogen pump that released a steady amount of estrogen per day (2.5 $\mu\text{g}/\text{kg}/\text{day}$ E₂) with ovariectomized animals implanted with a vehicle-filled pump. A third group of mice underwent sham operation without any treatments. We had found that there was no HA expression in the periductal stroma of the vehicle-treated ovariectomized animals, while there was a strong HA expression in the sham-operated mice and in the estrogen-treated mice suggesting a role for estrogen as a mediator for HA expression.

Based on these findings, we continued to explore the role of one of the receptors for HA, CD44 and its isoforms. Western Blot analysis for the most common CD44 isoform, CD44s, which does not include any variant exons, showed no difference in protein expression among the three groups of animals (Figure 2). This was an unexpected result and after studying the literature about the various CD44 isoforms, we learned about a specific isoform, CD44v6, which seemed to be estrogen-mediated as its expression after puberty followed the estrous cycle (7). This was a promising new candidate and Western Blot analysis using an epitope specific to this variant showed us a diminished expression of the CD44v6 protein in our ovariectomized group of mice (Figure 3).

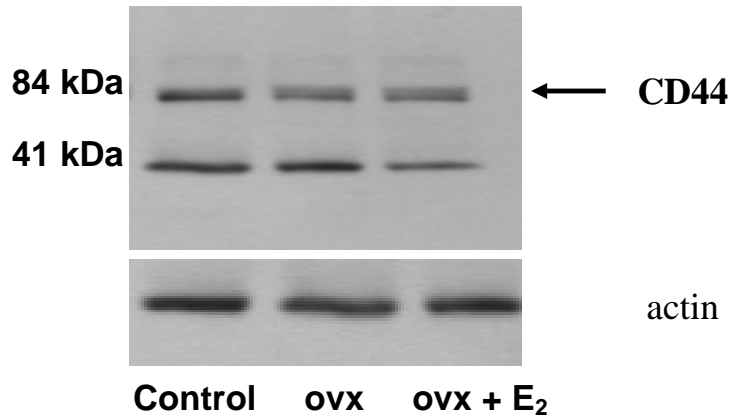


Figure 2: Western Blot analysis of CD44 receptor.

CD44 expression in all three animal groups appears to be similar as depicted in the upper band. The lower band refers the extracellular domain of CD44 only. Actin was used as a loading control.

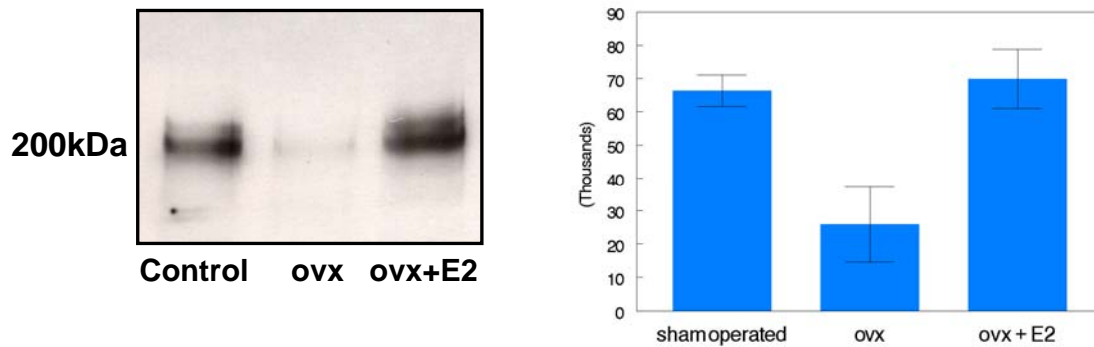


Figure 3: Western Blot analysis of CD44v6 isoform.

CD44v6 protein expression was diminished in the ovariectomized animals compared to the sham-operated mice and the group of mice that was ovariectomized and had the estrogen pump implanted. Quantification on the right panel was prepared using an n of 5.

We then hypothesized that CD44v6 expression might be diminished in myoepithelial cells around the ducts located adjacent to the periductal stroma that showed depleted HA expression in the ovariectomized animals. However, using immunohistochemical analysis for CD44v6, we found no difference in the intensity of staining among the different animal groups (Figure 4).

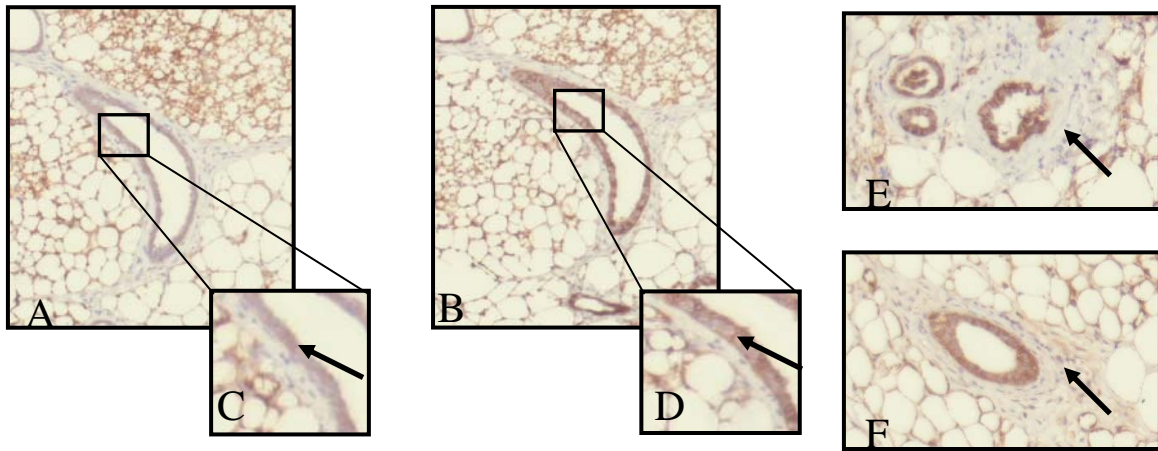


Figure 4: Immunohistochemical analysis of CD44v6 protein expression.

Panel A and B show consecutive sections from control mice that were sham operated. Panel A shows negative control without primary antibody treatment. Despite background staining of adipose tissue, staining of ductal epithelium including luminal epithelial cells and myoepithelial cells seems specific as it is not seen in the absence of the first antibody. Panel C and D display magnifications to highlight staining of ductal epithelium. Panel E shows CD44v6 staining of section from ovariectomized group of mice whereas panel F shows staining of section from the third group of mice that were ovariectomized and had been implanted with an estrogen-containing pump. The CD44v6 protein staining seems similar in all three animal groups.

Estrogen is considered one of the main factors responsible for ductal growth and branching to occur during pubertal mammary gland development. Therefore, depleting estrogen in the developing mouse results in a significantly underdeveloped epithelial tree migrating through the mammary gland fat pad that can be seen in whole mount (Figure 5). It shows a significant difference in the ductal area of vehicle-treated ovariectomized animals compared to the sham-operated mice and the estrogen-treated mice. **Previously, the E₂ dose-response in the mammary gland using several parameters had been measured (8).** The mammary gland showed a non-monotonic dose-response to estradiol for parameters such as number of terminal end buds (TEBs), ductal area, etc. Based on these data we had determined to use 2.5 µg/kg body weight/day 17β estradiol for our experiment as it correlates with the half-maximal response to estradiol (Figure 6). One of the parameters measured, the ductal area in ovariectomized mice that were treated with

estrogen-containing pumps of different estradiol concentrations, was $141.11 \pm 14.82 \text{ mm}^2$ as compared to $58.41 \pm 9.43 \text{ mm}^2$ in vehicle-treated ovariectomized animals (8).

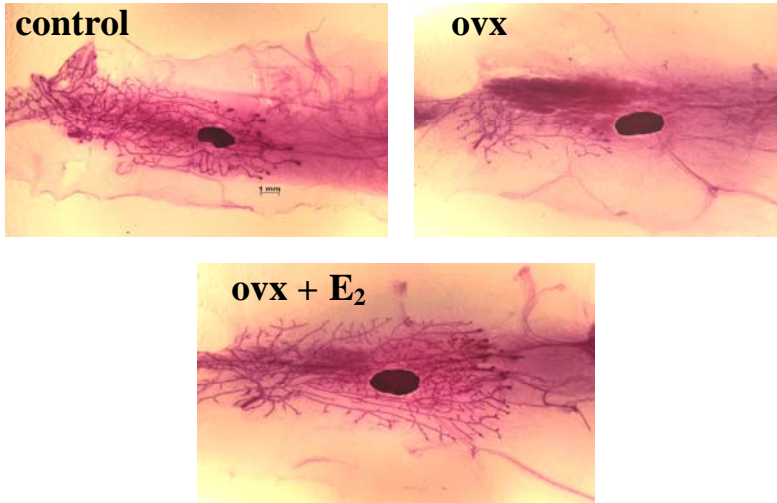


Figure 5: Whole mounts of murine mammary glands of all three experimental animal groups.

The panels highlight the underdeveloped epithelial tree of ovariectomized mice compared to that of control mice and mice that following ovariectomy had an estrogen pump implanted.

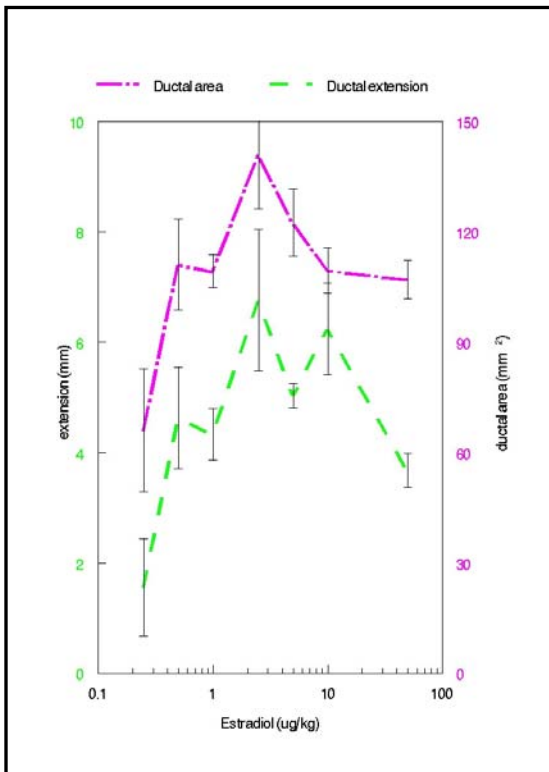


Figure 6: Morphological responses of the mammary gland to different estrogen concentrations.

Mice were ovariectomized at 25 days of age and osmotic pumps were implanted releasing 0, 0.25, 0.5, 1, 5, 10 or 50 $\mu\text{g}/\text{kg}$ body weight/day 17β estradiol. The mice were killed 10 days later and whole mounts using carmine staining were prepared. Images of mammary gland whole mounts were captured and used to measure several parameters including ductal area as measured by the outermost points of the ductal tree extensions (8).

This difference in ductal area demonstrates a 2.4 fold decrease in area in ovariectomized animals compared to estrogen-treated mice. Taking into consideration that the CD44 and CD44v6 proteins are exclusively expressed in the epithelium, we decided to normalize our Western Blot analysis results to the ductal area. For the CD44v6 protein expression, we observed an approximate 2.8 fold decrease in protein expression in ovariectomized animals compared to estrogen-treated mice. Comparing this decrease to the 2.4 fold decrease in ductal area, the decline in CD44v6 protein expression is only due to the decreased amount of epithelium in the ovariectomized mice. However, normalizing the protein expression of CD44 to the ductal area, it results in a 2.4 fold increase of CD44 expression in ovariectomized animals compared to estrogen-treated mice. We are currently optimizing the immunohistochemical analysis for CD44 and hope to confirm this expected increase of CD44 protein expression.

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

In the original proposal by Alexandra Zoltan-Jones it was proposed to use a 3D model to look at changes of HA. However, she could optimize the mode. Therefore, when I became PI for this grant, we proposed to switch to an *in vivo* model stably infecting fibroblasts with HA synthase and placing those together with epithelial carcinoma cells (ECCs) under the kidney capsule of SCID mice. In order to ascertain whether this was a feasible experiment, recombinants between normal epithelium and normal stroma prepared from mammary glands were implanted under the kidney capsule. Preliminary experiments showed that it takes 2 to 3 months get a recombinant of an appropriate size to evaluate structures (Figure 7).

Therefore, concurrent with these experiments, I have also pursued the original goal of developing a 3-dimensional (3D) model for the mammary gland. In this model, both human fibroblasts from reduction mammoplasty as well as human mammary epithelial cells are embedded in a collagen gel consisting of collagen type I, one of the main components of the mammary gland stroma (Figure 8).

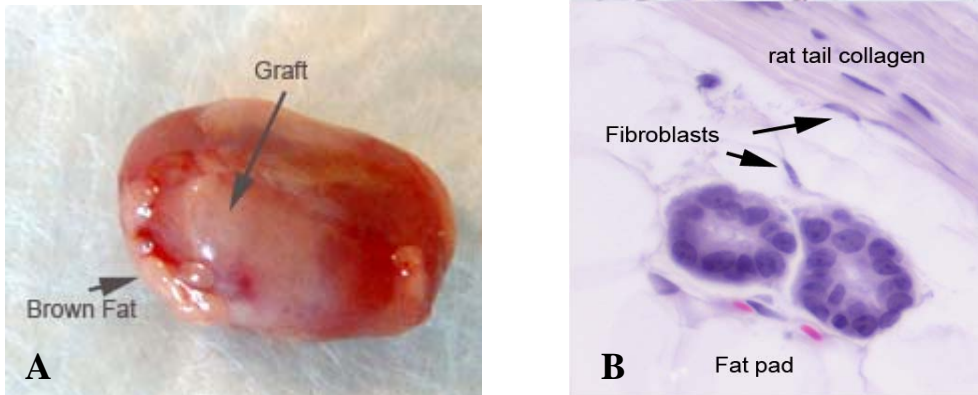


Figure 7: Tissue recombinants

Panel A shows a 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.

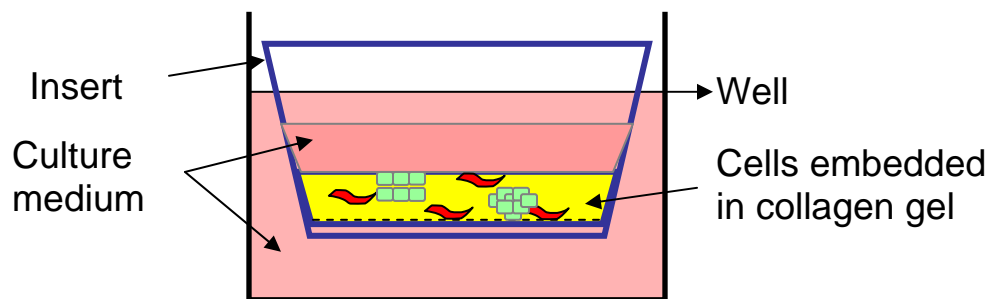


Figure 8: Collagen gel model

For the 3D tissue culture we use special 6-well-trays purchased from Organogenesis, Inc. Each well contains a cup with a porous membrane on the bottom allowing medium to penetrate. Human mammary fibroblasts and epithelial cells are mixed within a collagen type I solution that is poured into the cup and solidifies within half an hour in the incubator at 37°C. After the gels solidified, medium is added onto the top of gel as well as to the bottom of the well. Collagen cultures are usually incubated between one and six weeks.

Using this model we were able to obtain alveolar- and ductal-like structures resembling closely the mammary gland morphology *in vivo* (Figure 8). This model allows us to study mammary gland development and carcinogenesis *in vitro* while considering the important influence of tissue-level organization.

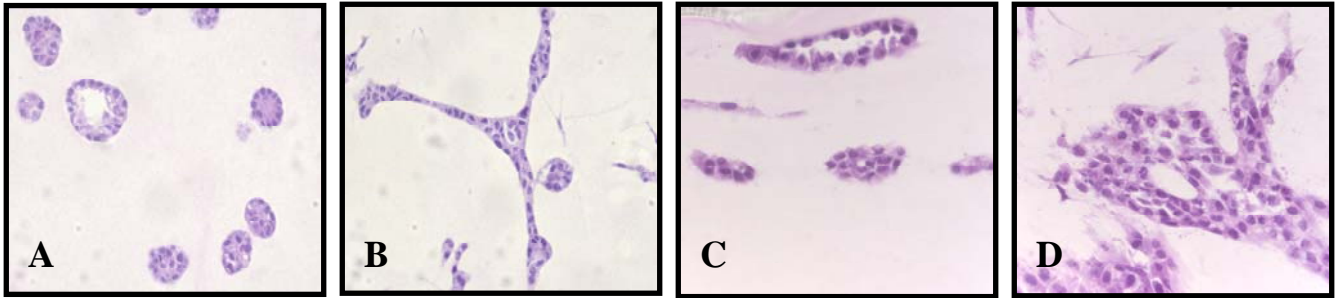


Figure 9: 3D tissue culture model to study mammary gland development and carcinogenesis

Using the 3D model as described in figure 7, alveolar-like structures (panel A) and ductal-like structures could be obtained (panel B-D) using human fibroblasts from reduction mammoplasty and human mammary epithelial cells.

Preliminary data showed that HA is expressed in our collagen gels containing only fibroblasts or fibroblasts together with epithelial cells (Figure 9). Therefore we will be able to perturb HA levels in this 3D model by either knocking it down using siRNA constructs or to overexpress it by transfecting fibroblasts with HA synthase.

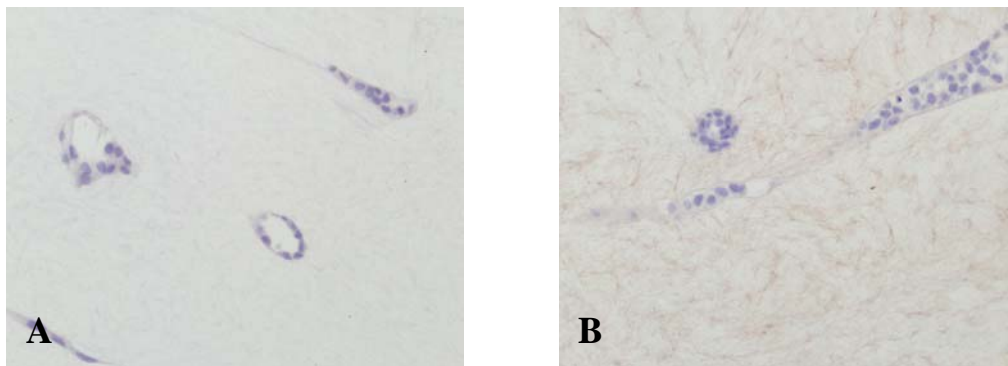


Figure 10: HA expression using histochemistry in 3D collagen gels

Histochemical analysis of HA 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 in collagen gels.

Key research accomplishments

- Estrogen was identified as a mediator for HA expression in the periductal stroma during mammary gland development.
- One of the HA receptors, CD44 and its isoforms, shows a distinct expression in mammary glands of mice that were ovariectomized when compared to sham-operated and E₂-treated mice.
- The tissue recombinant model works but takes too long to produce results. Therefore, we are establishing a 3D *in vitro* model.
- Using the 3D model, we obtained structures that closely resemble the mammary gland morphology *in vivo*.
- HA is expressed in the 3D model. This lends further support to use this *in vitro* model for determining HA's role in mammary gland development and/or carcinogenesis.

Conclusions

We have shown that estrogen is a mediator for HA expression during postnatal mammary gland development. Further elucidating HA's role during normal mammary gland development will give important insights into possible functions during mammary gland carcinogenesis where HA has been shown to be overexpressed.

Furthermore, we have established a 3D 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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