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**14. ABSTRACT**
Purpose: Characterize the compositional and functional changes in mammary stroma that result from tamoxifen treatment. Approach and Results: 75 mature female Sprague Dawley rats were randomized into three groups of 25 each; Gp1 nulliparous control, and Gp 2 tamoxifen treated (0.5 mg/tamoxifen per kg body weight, s.c. injection for 30 days)and Gp 3 tamoxifen treated (1.0 mg tamoxifen dose). ECM was harvested from the mammary glands of Gps 1 & 3 for biochemical and functional characterizations. The ECM preparations have been subjected to LCMS and MALDI-TOF mass spec. Due to technical difficulties we have also developed two in vitro models to investigate the effects of tamoxifen on mammary stroma. ECM deposited by primary mammary fibroblasts isolated from control and tamoxifen treated rats, or primary control fibroblasts treated with tamoxifen in culture has been utilized for ECM proteomics method development. Optimized conditions demonstrate fibronectin (FN) is downregulated by tamoxifen, in vitro and in vivo; observations consistent with data demonstrating that FN is upregulated during with MEC proliferation and downregulated at times of MEC loss; suggesting that loss of FN may be integral to a tumor suppressive microenvironment. To investigate functional changes in ECM, MDA-MB-231 cells were pre-mixed with control matrix or matrix isolated from tamoxifen treated rats and orthotopically injected into nude mice. Tumor growth was reduced in mice injected with ECM isolated from tamoxifen treated rats, demonstrating that tamoxifen altered ECM in a manner consistent with tumor cell dormancy. This work fulfills the goals of the DOD CDMRP Breast Cancer Research Program by challenging existing paradigms asserting that the protective mechanism of action of tamoxifen is mediated exclusively through action on mammary epithelial or tumor cells. Finally, this work has the potential to identify ECM mediators of tumor cell dormancy and progression that can be targeted for drug development. The development of such drugs is anticipated to synergize with existing drugs that target the tumor cell itself, hopefully providing a significant improvement with respect to disease control.

**15. SUBJECT TERMS**
Tumor microenvironment, epithelia cell –ECM interactions, tumor cell dormancy

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## TABLE OF CONTENTS

**INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Early stage cancers have long been considered to be less aggressive than late stage cancers because they have accumulated fewer mutations required for metastasis. For breast cancer, recent gene-expression profiling data have challenged this paradigm by identifying very early stage cancers with gene expression profiles similar to that detected in fully metastatic cancers. In fact, numerous studies have demonstrated that having genetic mutations required for metastasis is not sufficient to guarantee a successful metastatic event. These studies indicate that the interaction of the tumor cell with its microenvironment is as rate-limiting for tumor cell progression as is the mutational genotype of the tumor cell. Thus, identifying stromal proteins that determine whether a tumor cell will remain dormant or progress is critical for the clinical control of breast cancer. We propose that the functional unit of cancer is the cancer cell plus its microenvironment, and that both compartments comprise the response to interventions that reduce risk for cancer progression. Specifically, we hypothesize that stromal changes actively contribute to the protection afforded by parity and tamoxifen, two strategies proven to reduce breast cancer risk in women. In this study, we will investigate this hypothesis by characterizing the compositional and functional changes in mammary stroma that result from parity and tamoxifen treatment. To this end we proposed to determine whether breast cancer preventive strategies alter the breast environment, resulting in mammary extracellular matrix (ECM) that suppresses metastatic attributes of tumor cells. Parity and tamoxifen induced changes in mammary ECM composition will be determined by proteomics and changes in matrix function will be determined by *in vitro* assays for metastasis. Mammary ECM proteins elevated by tamoxifen and parity will be candidates for mediators of tumor suppression, whereas ECM proteins decreased by tamoxifen and parity will be candidates for mediators of tumor progression. Further, we will extend these studies to determine whether tumor cell-stroma interactions *in vivo* are altered by tamoxifen and pregnancy. Using a xenograft model, tumor growth and ability of tumor cells to induce a host desmoplastic reaction will be evaluated for tumor cells injected into mammary fat pads of nulliparous, parous and tamoxifen treated mice.

**BODY OF REPORT:** This section of the report shall describe the research accomplishments associated with each task outlined in the approved Statement of Work.

## Research Accomplishments Associated with Each Task Outlined in the Approved Statement of Work:

### Task 1-Animal husbandry for female rats with mammary tissue at reduced risk for developing cancer; parous and tamoxifen treated rats

a) Randomize 90 female Sprague Dawley rats, 50 days of age, into three groups; Group 1, nulliparous control, Group 2, tamoxifen treated and Group 3, parous. Throughout the study, animals will be weighed twice weekly to monitor effects of tamoxifen treatment on body weight gain.
b) Breed Group 3 rats. After parturition, normalize pups to 8, wean at 21 days of parturition, and allow mammary glands to regress for one month post weaning.
c) In rats age matched to Groups 1 (nulliparous)& 3 (parous), begin tamoxifen treatment on Group 2. Treat with 0.5 mg tamoxifen/kg body weight, s.c. injection, daily for 4 weeks. Group 1 nulliparous rats will receive daily s.c. injections of vehicle (sesame oil).

d) At end of 4 week treatment (Gp2) and after 1 month post-weaning (Gp3), all groups will be euthanized.
e) Two hours prior to euthanasia, inject rats with 50 mg/BrdU i.p. for incorporation into actively proliferating cells.
f) At time of euthanasia, stage of estrous will be determined by vaginal smear so that inter-animal variation due to estrous can be controlled for across groups. Further, cervical samples will be collected from each rat and used to verify stage of estrous. Based on preliminary studies, it is anticipated that tamoxifen treatment will inhibit cycling.
g) Inguinal mammary glands, with lymph nodes removed will be flash frozen and stored at -80°C for subsequent ECM isolation. Thoracic glands will be harvested for histological analyses.

**Progress Report, Task 1:** Tasks 1 a-g were completed and due to mechanical error, all frozen tissue samples were lost on 7/21/06, before any of the tissue was processed for biochemical characterization. Not only were these DOD samples lost, but every experimental tissue, protein and RNA preparations of the entire laboratory were lost. Independent verification of this freezer crisis can be obtained from Jim Gantner, UCHSC Risk System, at 303-315-2732. As a consequence of this catastrophic loss, we are behind on some Statement of Work timeframes. However, due to a tremendous effort on the part of all members of my laboratory, we have made substantial progress towards the objectives outlined in this grant.

Beginning 8/16/06, we repeated Tasks 1 a-g, but omitted Group 3, the parous group. Group 3 was omitted because we felt it was important to narrow the scope of the project during this period of lab-reconstruction. Further, we expanded Group 2 to include two different doses of tamoxifen; 1mg/kg and 0.5 mg/kg bodyweight. We were funded to investigate the effects of 0.5 mg/kg body weight only. However, all of our pilot studies were performed with 1.0 mg/kg tamoxifen per kg body weight. Since our pilot study tissue samples were also lost in the freezer-crisis, we felt it important to repeat the 1.0 mg/kg per body weight arm of the study, so that we had a known effective dose to compare the effects of the0.5 mg/kdbw dose. To this end, 54 female SD rats at 70 days of age were randomized into 4 groups of 13 and treated for 30 days; Gp1: control rats treated daily with vehicle based on the volume used for 1.0mg/kg tamoxifen dose (approximately 1 ml solvent/rat/day); Gp2: 1.0 mg/kg tamoxifen daily; Gp3: control rats treated with vehicle based on the volume for 0.5 mg/kg and Gp 4: 0.5 mg/kg tamoxifen daily. Beginning 6/01/08 we began breeding studies to obtain age matched parous and nulliparous rats. These breeding studies have been completed and we now have 10 SD rats per each of these groups.

**Final Report Conclusion for Task 1:** Completed

**Task 2-Histological evaluation of mammary gland morphology and proliferative index of mammary epithelial cells**.

**a)** To minimize differences in gland morphology due to proximity to the nipple, the same area of gland #4 from each animal will be processed for histological analyses. Three classes of gland morphology will be distinguished; primarily ductal, moderately alveolar (less than 10 ascini per lobule), and alveolar (more than 10 ascini per lobule), using previously published criteria.

**b)** Effect of parity and tamoxifen treatment on mammary epithelial cell proliferation will be determined by quantitating BrdU incorporation by immunohistochemical (IHC) methods.

**Progress Report, Task 2**: Task completed. All tissue samples have been processed for histological and IHC evaluation. It is apparent from cervical histology, that the tamoxifen treatment was effective at both doses, as epithelial hyperplasia and hormone stimulation are apparent. Mammary gland histology is also consistent with effective drug treatment. In year 2 we have completed the quantification of histology and IHC for BrdU, and results are described in Figure 1.

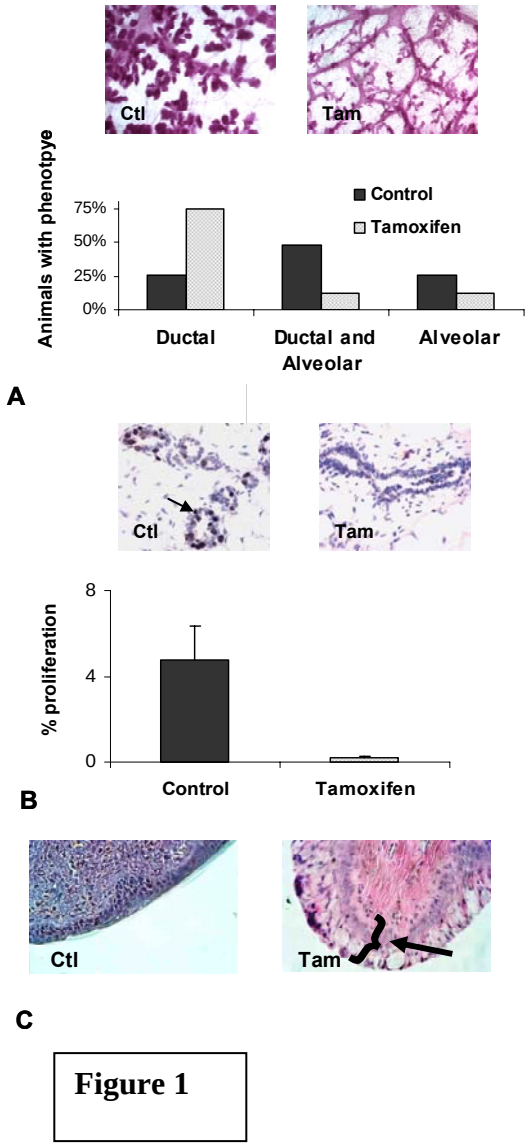



**Figure 1**. Effects of tamoxifen on proliferation of mammary and cervical epithelium. **A, upper panels**) Whole mount mammary gland images from control and tamoxifen treated rats showing loss of alveoli with tamoxifen treatment, 40X. **A, lower panel**) Graph depicting percent of rats in each group with ductal, mixed or alveolar mammary gland morphology. **B, upper panels**) BrdU positive proliferating cells detected by IHC. Arrow depicts brown staining BrdU positive cell, 200X. **B, lower panel**) quantitation of proliferating mammary epithelial cells in control (n=12) and tamoxifen (n=12) treated rats. **C**) Cervical histology of control and tamoxifen treated rats. Arrow shows thick stratum germinativum layer in tamoxifen treated rats consistent with hormone stimulation.

**Figure 1**

**Final Report Conclusion for Task 2:** Completed

**Task 3-Isolation and characterization of endogenous mammary gland ECM (Months 6-24)**

**a)** For quality control purposes, prior to ECM isolation, mammary gland histology will be performed to ascertain overall gland health. If any animal is found to have evidence of infection within the mammary gland, the animal will be eliminated from the study. **Progress Report**: Task completed.

**b)** Endogenous mammary ECM will be isolated from lymph-node free inguinal mammary glands. **Progress Report:** Task completed.

**c)** Perform proteomic analysis of endogenous mammary gland ECM isolated from nulliparous, parous and tamoxifen treated rats using both liquid chromatography mass spectrophotometry (LCMS) and 2-D gel electrophoresis with spot isolation followed by MALTI-TOF-MS.

**Progress Report, Task 3c** In the second year we have focused on both Western blot and proteomic analyses. To test for tamoxifen induced changes in mammary ECM in vivo, several ECM proteins were evaluated by Western blot analysis. Consistent with the in vitro fibroblast data, mammary tissue from tamoxifen treated rats had decreased levels of fibronectin (Fig 2A). In addition, tamoxifen treatment resulted in a modest increase in basement membrane laminin chains of 200 kD and 100 kD detected using a pan-specific antibody for LN 1 and LN 2 (Fig 2A). Western blot analysis also revealed an increase in the interlobular ECM protein collagen 1 (Fig 2B, left panel). Tissue collagen deposition, as measured by Picrosirus red staining, confirmed elevated levels of fibrillar collagen in the mammary stroma of tamoxifen-treated rats (Fig 2B, right panels & Table 1). Further, Western blot analyses suggested a decrease in mammary ECM proteolysis with tamoxifen treatment, as evidenced by an increase in the ratio of high to low molecular weight species of FN, LN1/2, LN5 and collagen 1 (Figs 2A & B). Matrix metalloproteinases-2, an MMP found in high levels in virgin rat mammary glands and implicated in ECM proteolysis, was evaluated in control and tamoxifen treated mammary tissue. Total MMP-2 and active MMP-2 were found to be decreased in mammary tissue of tamoxifen treated rats. Thus, the decrease in ECM proteolysis in tamoxifen treated glands correlated with a concurrent decrease in MMP-2 activity (Fig 2C).

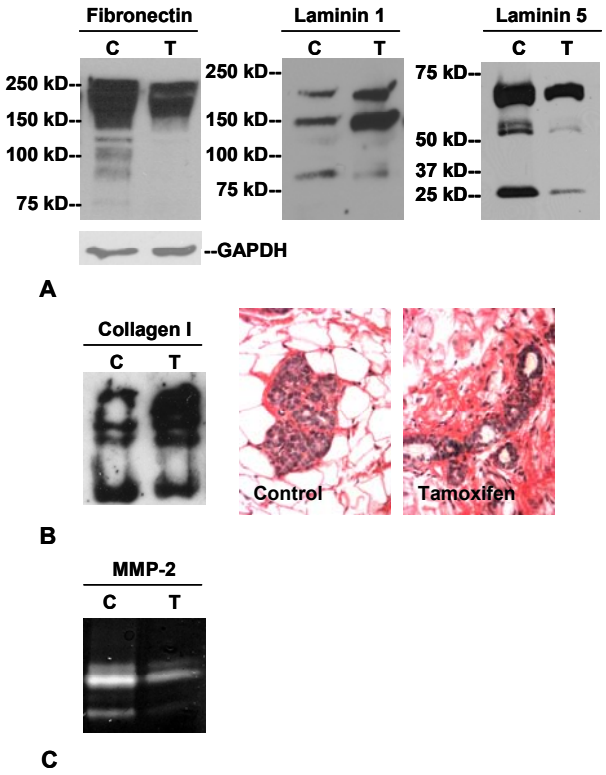



**Figure 2**. ECM proteolysis is reduced with tamoxifen treatment. **A**) Western blot analyses of mammary FN, LN1, and LN5 in control and tamoxifen treated rats. **B**) Fibrillar collagen detected by Western blot (left panel) and in 5µm Picro-sirius red stained mammary gland sections. **C**) MMP-2 activity detected by zymogen assay.

Proteomic analysis of mammary ECM proved to be severely limited by technical limitations. During the course of this funding period, we explored numerous methods to improve ECM proteomic methods. Proteomic analyses of ECM proteins have been hindered by the insoluble and digestion resistant nature of ECM. We explore the utility of combining rapid ultrasonication and surfactant assisted digestion for the detailed proteomic analysis of ECM samples. Our UATD method followed by mass spectrometry analyses increased collagen 1 α1 and α2 chains proteome coverages from less than 5% for both chains to 35% and 39%, respectively (data not shown). When compared to traditional overnight digestion, this optimized method dramatically improved the sequence coverage for collagen I, revealed the presence of hundreds of previously unidentified proteins in Matrigel, and identified a protein profile for ECM isolated from rat mammary glands

that was substantially different to that found in Matrigel. A manuscript describing these results entitle ' **An In-Solution Ultrasonication Assisted Digestion Method for Improved Extracellular Matrix Proteome Coverage**' is in press in the journal of *Molecular and Cellular Proteomics*, and an E-pub print is included in the Appendices.

Using our optimized digestion method, mammary ECM proteins that may contribute to the distinct functional properties of control and tamoxifen ECMs, proteins in the isolated matrices were identified by tandem mass spectrometry. Two hour LC/MS/MS runs on the tryptic digests of these samples where used to identifiy several ECM proteins (**Table 2**). Prominent ECM proteins identified included collagen chains α-1(I), α-2(XIV), and α-2(I), laminins chains α4, β1, β2, and γ1, nidogen 1, and fibrillin 1. In addition, several proteoglycans were identified in the mammary gland, including decorin, perlecan, lumican, biglycan, and mimecan, and periostin. To semi-quantify the ratio of each protein between the two different matrices, the mass spectrometry peak areas were calculated and expressed as a ratio of peak area from tamoxifen ECM compared to control ECM (emPAI ratio). As an additional assessment of protein abundance, the ratio of the number of distinct peptides identified for each protein divided by the total number of peptides identified in the two hour run are displayed as the peptide count ratio. Using this peptide counting label-free quantification approach, surprisingly, suggested that the three fibrillar collagen chains were at lower levels in tamoxifen than control ECM. The laminin subunit β2 was present at higher levels with tamoxifen. The proteoglycans perlecan and mimican were found to be elevated while biglycan, decorin, lumican, and periostin were decreased in tamoxifen ECM.

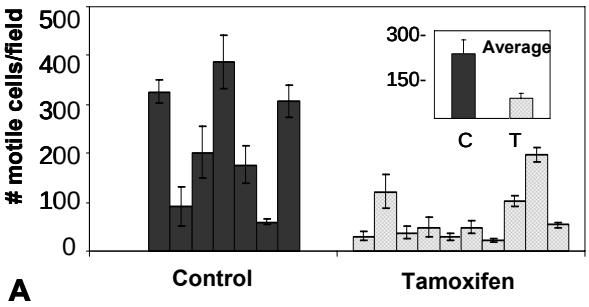
**Table 2** Mammary ECM proteins identified by mass spectrometry-with GAPDH serving as internal control for quantitation

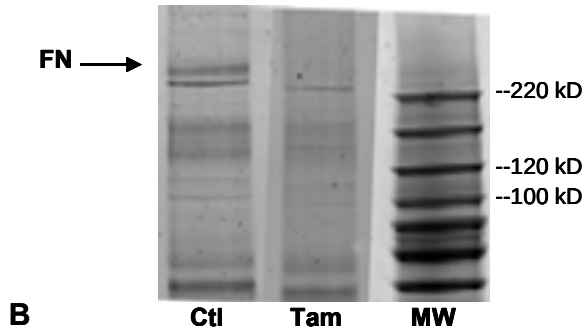
| Accession | Protein | emPAI ratio (T/c) | peptide count ratio (T/c) |
|---|---|---|---|
| Q64467 | Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | 1.00 | 1.10 |
| Q05793 | Perlecan (HSPG) | 1.50 | 1.32 |
| P47853 | Biglycan (Bone/cartilage proteoglycan I) (PG-S1) | 0.52 | 0.83 |
| P11087 | Collagen alpha-1(I) chain | 0.50 | 1.02 |
| Q80X19 | Collagen alpha-1(XIV) chain | 0.80 | 0.95 |
| P02466 | Collagen alpha-2(I) chain | c | c |
| Q01129 | Decorin (Bone proteoglycan II) (PG40) | 0.52 | 0.60 |
| Q9QZZ6 | Dermatopontin (Tyrosine-rich acidic matrix protein) (TRAMP) | 1.00 | 1.65 |
| Q61554 | Fibrillin-1 | 0.83 | 1.04 |
| P97927 | Laminin subunit alpha-4 | T | T |
| P02469 | Laminin subunit beta-1 (Laminin B1 chain) | T | T |
| P15800 | Laminin subunit beta-2 | 3.00 | 0.94 |
| Q61292 | Laminin subunit beta-2 (S-laminin) (S-LAM) | c | c |
| P02468 | Laminin subunit gamma-1 (Laminin B2 chain) | T | T |
| P51886 | Lumican (Keratan sulfate proteoglycan lumican) | 0.76 | 1.06 |
| P09650 | Mast cell protease 1 (CLIP protein) | 1.00 | 1.04 |
| Q62000 | Mimecan (Osteoglycin) | 1.20 | 1.43 |
| O88766 | Neutrophil collagenase (Matrix metalloproteinase-8) (MMP-8) | c | c |
| P10493 | Nidogen-1 (Entactin) | c | c |
| Q62009 | Periostin (Osteoblast-specific factor 2) (OSF-2) | 0.88 | 1.11 |
| P19324 | Serpin H1 (Collagen-binding protein) | 1.00 | 1.10 |

**Final Report Conclusion for Task 3:** Completed

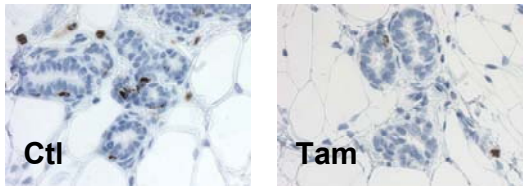
Also not proposed in the original grant, the following experiments, which were a logical extension of our proposed work were performed. To begin to address the question of whether tamoxifen alters mammary stroma, fibroblasts were isolated from mammary glands of control and tamoxifen treated rats. Fibroblast identity was confirmed by positive IHC staining for vimentin and lack of E-cadherin staining (data not shown). In vitro fibroblast motility was assessed as a functional marker of cell activity. While inter-animal variation in mammary fibroblast motility was high, overall, fibroblasts from the tamoxifen treated animals displayed a 3 fold decrease in motility compared to control fibroblasts (**Fig 3A**). This less motile phenotype is consistent with tamoxifen treatment causing fibroblast quiescence.

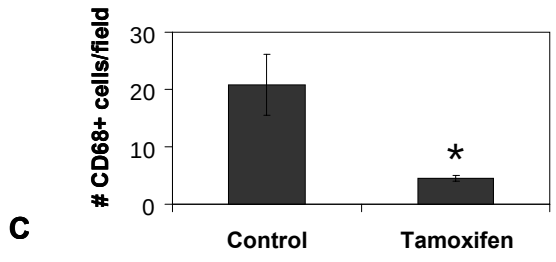
Fibroblasts are the major producers of mammary interlobular and periductal ECM and the motility data suggested that ECM production by fibroblasts might also be altered by tamoxifen treatment. To address this question, primary mammary fibroblasts from rats treated in vivo with vehicle or tamoxifen were cultured on plastic. The ECM secreted basally and incorporated into a three dimensional insoluble matrix was harvested and run on a 1D-gel. A prominent band of ~250kD was differentially expressed (**Fig 3B**). This band was excised, digested and identified as FN by mass spectrometry. Cumulatively, these data show that in vitro, fibroblasts isolated from tamoxifen treated rats are less motile and incorporate significantly less FN into the ECM matrix than fibroblasts isolated from vehicle control rats.




Tissue macrophages are a stromal cell type whose presence has been shown to directly correlate with breast cancer progression in rodent models and in women [38]. To determine whether tamoxifen treatment influenced the macrophage content of the mammary gland, immunohistochemical analysis of the macrophage lysosomal marker CD68 was performed (**Fig 3B, upper panels**). Quantification of CD68 staining demonstrated that CD68 signal was significantly decreased in mammary glands of tamoxifen treated rats (**Fig 3C**).



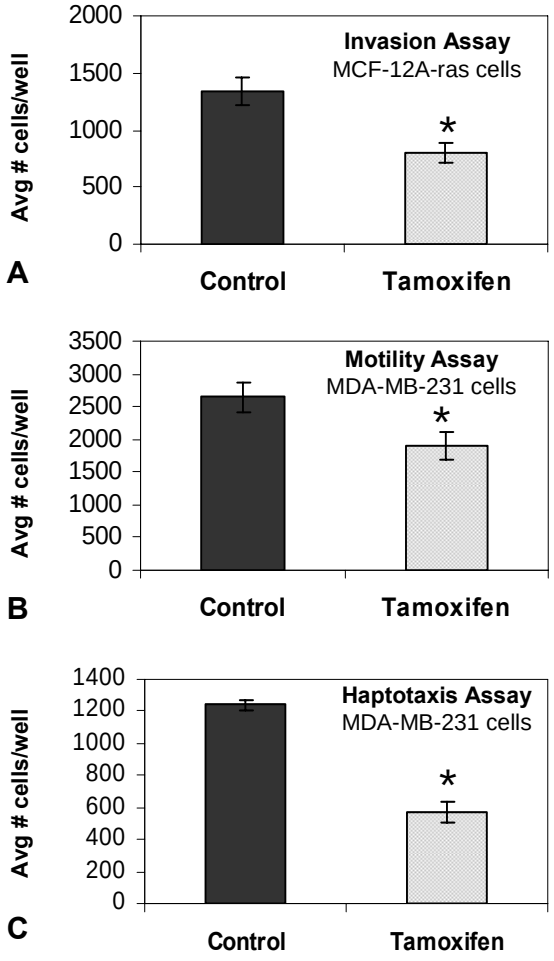




**Task 4-In vitro assays for evaluating ECM-epithelial cell interactions. Cell lines to be**

**evaluated are non-tumorigenic MCF-10A, tumorigenic but non-metastatic, ER positive MCF-7, and metastatic, ER negative MDA-MB-231 cells.**

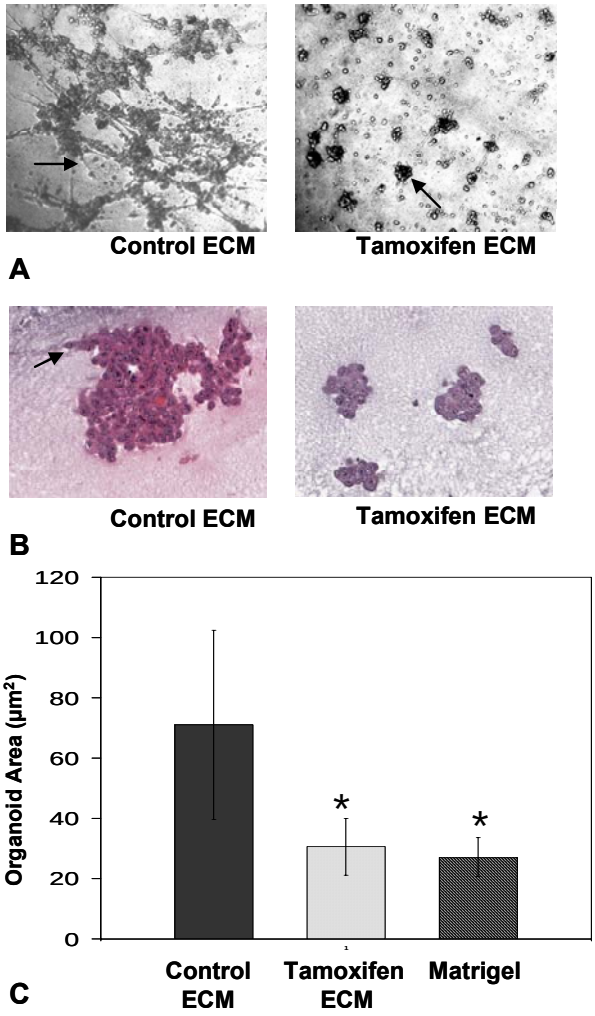
**Year 2, Months 12-24**

- **a.** Prepare mammary ECM, using 6 rats per group per matrix preparation. **Progress Report:** Matrices have been made in triplicate.
- **b.** In 2D culture, treat cells with endogenous mammary gland matrices at 0, 10, 20 and 40 µg/ml to determine effects of matrices on epithelial cell proliferation and apoptotic indices at 24 and 48 hours post plating, using $^{3}$H-thymidine incorporation assays and apoptosis IHC assays. Experimental conditions to be plated in quadruplicate. **Progress Report**: proliferation was evaluated using Brdu incorporation and differences in proliferation were not observed in MDA-MB-231 cells treated with the respective matrices (data not shown).
- **c.** Quantify effects of endogenous mammary on ability of cells to fill in 'wound' in standard scrape assay. Matrices will be tested at 0, 10, 20 and 40 µg/ml. **Progress Report**: In progress.
- **d.** Quantify effects of endogenous mammary matrices on motility and invasion, using ECM preparations as substratum for transwell filter motility and invasion assays. Assays performed in quadruplicate. For motility assays, filters will be coated with 10µg/ml of the respective matrices. For invasion assays, filters will be coated with 200 µg/ml of the respective matrices. **Progress Report:** In a transwell filter invasion assay, MCF-12A-Ras cells had a three fold reduction in invasiveness on tamoxifen ECM compared to control ECM (**Fig 4A**), corroborating the 3D organoid results suggesting that the tamoxifen ECM fails to support tumor cell invasion. With MCF-12A-Ras cells, differences in motility were not observed (data not shown). These functional analyses were extended to another breast cancer cell line, the highly metastatic human breast cancer MDA-MB-231 cells. Even these very aggressive cells had a subtly altered 3D phenotype; on the tamoxifen ECM; MDA-MB-231 cells were more clustered and cuboidal in appearance than when plated on the control ECM, indicating induction of an epithelial-like morphology (data not shown). Consistent with these data, MDA-MB-231 cells also had reduced motility on the tamoxifen ECM (**Fig 4B**), but no change in invasion (data not shown), suggesting a partial suppression of the metastatic phenotype. Further, we evaluated the chemoattractant properties of the isolated mammary ECM by



replacing the serum in the lower chamber with mammary ECM as the chemoattractant. Using MDA-MB-231 cells, the tamoxifen ECM displayed a three fold reduction in haptotactic properties (**Fig 4C**).

**e.** Use endogenous mammary gland matrix as substratum for epithelial cell organization in 3-D organoid (mammosphere) assays. Each conditions to be tested in quadruplicate. Organoids will be harvested at two time points; 5 and 10 days post plating. Twenty-four hrs prior to harvesting, organoids will be treated with 10µm BrdU. Organoids will be fixed in 4% paraformaldehyde, embedded, and sectioned into 5 µm sections. Immunohistochemical analyses for markers of cell proliferation, apoptosis, cadherin localization, and apical/basal epithelial cell polarity will be performed. **Progress Report:** to date, organoid size and proliferation have been characterized. In order to quantitate organoid size, we worked with the Prostate Histology lab to develop an automated computer assisted program to objectively determine organoid size on hundreds of organoids at one time. We have found that proliferation of cells in 3D culture appears unaffected by source of ECM but size of organoids was significantly decreased when cells were plated onto tamoxifen ECM (**Fig 5**).




**Task 4 Future Directions:** 3D organoids will be characterized for polarity markers by IHC.
**Final Report Conclusion for Task 4:** All Completed with the exception of ICH evaluation of organoids for polarity markers, which is ongoing.

**Task 5- Test whether parity and tamoxifen treatment produce a microenvironment suppressive of tumor development.**
**Year 3, Months 24-36**

- **a.** Thirty six Nu/Nu athymic female mice will be randomized into three groups of 12 mice each; Gp1 Nulliparous, Gp2, tamoxifen treated and Gp3, parous, and treated as described in Aim 1, tasks a, b, and c.
- **b.** At one month post weaning (Gp3) and after 4 weeks tamoxifen treatment (Gp 2), mice in all groups will be injected into the left and right mammary fat pads of inguinal gland #4 with 2 x $10^6$ MDA-MB-231 cells in 20 µl of media.

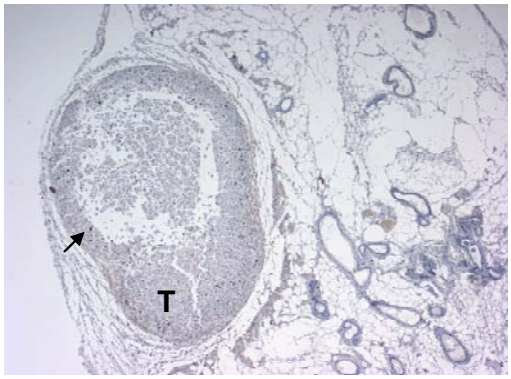
**c.** Tumor growth will be measured twice weekly using calipers and tumor volume calculated as $4/3\ \pi R^2 h$. At six weeks post injections, tumors will be excised with a small border of mammary tissue attached, weighed and final volume calculated. Half of the primary tumors will be flash frozen in liquid nitrogen for ECM isolation and the other half fixed and processed for IHC analyses.

**d.** The question of whether MDA-MB-231 tumor cells induced a desmoplastic reaction in their surrounding stroma will be evaluated by IHC of tumor/mammary gland border sections. IHC for smooth muscle actin, tenascin, collagen I, endothelial specific marker CD31, and for neutrophile granulocyte infiltration will be evaluated.

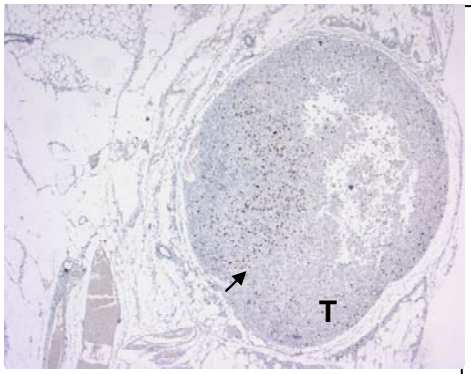
**e.** To determine whether the microenvironment influences deposition of ECM in the proliferating tumor, ECM will be extracted from the resulting tumors for proteomic analyses.

**Progress Report, Task 5: Animal model studies to demonstrate that tamoxifen treatment and parity alter the mammary tumor environment to one less-supportive of tumorigenesis.**

**1.** Using mammary ECM isolated from V and T treated rats for Task 3c, we performed an unscheduled but highly related study. Mammary ECM was mixed with human mammary tumor MDA-MB-231 cells and injected into the 4$^{th}$ mammary gland of immunocompromised mice. The objective was to determine whether tamoxifen altered the ability of mammary ECM to support tumor growth and metastasis in a xenograft model. Our rationale was based on prior success using this model system to investigate the role of mammary ECM on tumor cell fate (McDaniel 2006). Tumors were harvested at two time points post injection; at 5 days (n=5 mice per group) and 6 weeks (n=15 mice per group). For the 5 day time point, our objective was to determine if tumor cell proliferation was altered by the source of ECM that the tumor cells were mixed with prior to mammary fat pad injection (i.e. V vs T ECM). To this end, 5 mice per group were injected with BrdU 2hrs prior to tumor harvest. Examples of two of these small tumors with brown BrdU positive proliferating cells are shown in **Fig 6**.




Tumor cells injected with control matrix




Tumor cells injected with tamoxifen matrix

**Fig 6.** Images of xenograft tumors that developed from MDA-MB-231 cells injected with control ECM (left panel) and tamoxifen ECM (right panel). Arrows depict BrdU positive cells, 40X.

Computer assisted quantization of BrdU incorporation demonstrated no significant differences between groups in proliferation at this time point.

Results from the 6 week tumor study were reported in year 1 and can be seen in Fig 7. These data show that tumor cell pre-mixed with mammary ECM isolated from tamoxifen treated rats tamoxifen matrix develop smaller tumors in an orthotopic model fro breast cancer than when pre-mixed with mammary ECM isolated from nulliparous mammary glands.

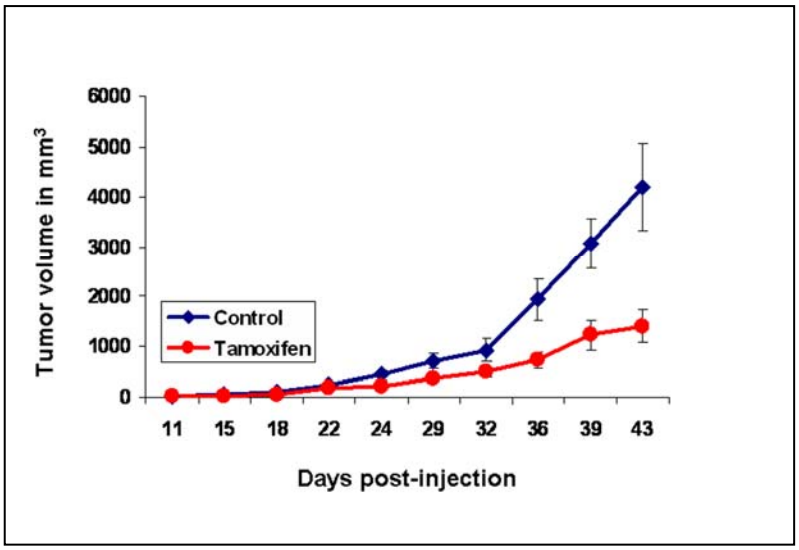



**Fig 7. MDA-MB-231 cells were mixed with control mammary ECM or mammary ECM from tamoxifen treated rats, injected into mammary fat pad #4 and tumor growth monitored twice-weekly.**

To determine whether the elevated levels of fibronectin in control mammary ECM influence tumor size in this xenograft model, as suggested by the 3D organoid data, MDA-MB-231 cells were treated with 20 µg/ml fibronectin prior to mammary fat pad injection (Fig 8). Tumor size was increased by fibronectin across all time points, however, because of large variation in tumor size within a group, these data did not reach statistical significance.

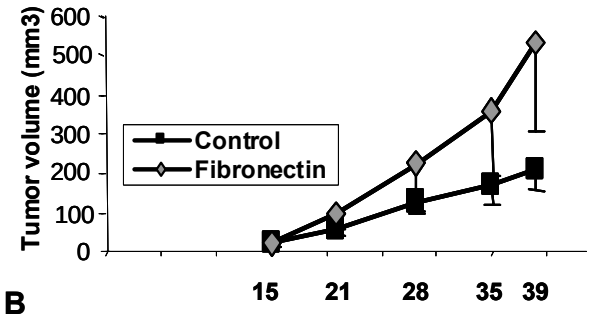



**Fig 8. MDA-MB-231 tumor cells mixed with 20ug/ml fibronectin had a trend towards larger tumor size, consistent with fibronectin contributing to larger tumor seize observed with control ECM, N= 10 per group.**

Compared to nulliparous rats, parity confers a significant 'protective effect' against chemical carcinogen-induced rat mammary tumorigenesis [26, 27], modeling the protective effect of parity observed in women [28, 29]. To test the role of parous mammary microenvironment in this 'protective effect', primary human ductal carcinoma *in situ* (DCIS), MCFDCIS cells were injected into nulliparous or parous mammary fat pad of age-matched mice. Our data show considerable decrease in tumor growth in the parous mice compared to nulliparous mice (Fig.9A), indicative that the parous mammary environment is 'tumor suppressive'. In addition, nulliparous mammary ECM supported the ability of normal mammary epithelial cells to form complex 3D organoid structures compared to parous mammary ECM, which suppressed 3D organoid development (Fig.9B), suggesting that ECM is responsible in part for the 'protective effect' of parity [19].

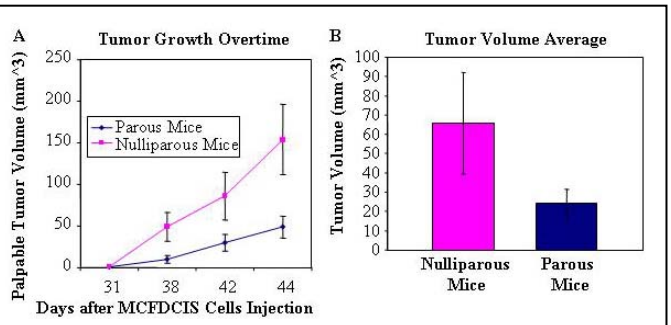



**Figure 9A**. A) Time course of MCFDCIS tumor cell growth in SCID mouse orthotopic model. B) Quantitation of tumors from histological sections at study end.

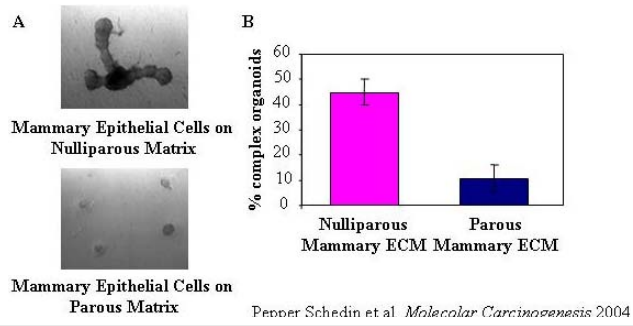



**Figure 9B**. A) Organoid complexity of non-transformed murine mammary epithelial FSK-3 cells plated on mammary ECM isolated from nulliparous and parous rats, 40X.

**Final Report Conclusion for Task 5:** All work completed with the exceptions of Task 5d and 5e, which are partially completed and work is ongoing.

**KEY RESEARCH ACCOMPLISHMENTS**: Bulleted list of key research accomplishments emanating from this research.

1. Extensive method development for ECM proteomics.
2. Identification of numerous, previously uncharacterized ECM proteins present in normal and tamoxifen treated mammary glands.
3. Methods development for quantitation of 3D organoid size.
4. Xenograft models for the study of tumor cell–ECM interactions.
5. Identification of pleiotrophic effects of tamoxifen on mammary stoma including; inhibition of fibroblast motility and FN production, a decrease in macrophage number, a reduction in MMP-2 activity, markedly less proteolysis of ECM proteins, and reduced levels of FN.
6. Identification that reduced fibronectin levels and reduced extracellular matrix turnover appear to be hallmarks of the quiescent mammary microenvironment. These data may provide insight into attributes of a mammary microenvironment that facilitate tumor dormancy.
7. Evidence that the parous mammary microenvironment does not support tumorigenesis in an orthotopic xenograft model of breast cancer and that mammary ECM isolated from mammary glands of parous rats is less supportive of 3D organoid development that mammary matrix isolated from glands of nulliparous rats. These observations suggest that changes in the mammary microenvironment may contribute to the protective effect of parity on breast cancer risk.

**REPORTABLE OUTCOMES**: Provide a list of reportable outcomes that have resulted from this research to include:

1. **Meeting Abstracts:**
   A. TAMOXIFEN TREATMENT FUNCTIONALLY ALTERS THE RAT MAMMARY STROMA, INDICATING A ROLE FOR THE

MICROENVIRONMENT IN TUMOR SUPPRESSION. R. Hattar, S. McDaniel, P. Schedin, The 25th Congress of the International Association for Breast Cancer Research, Montreal, Canada, Sept 15-18, 2006.

b. TAMOXIFEN TREATMENT FUNCTIONALLY ALTERS THE RAT MAMMARY STROMA SUGGESTING A ROLE FOR THE ECM IN TUMOR SUPPRESSION. Pepper Schedin, Rhonda Hattar, Shauntae McDaniel, Sandra Biroc, Kirk Hansen and Anthony Elias. Era of Hope Meeting, Baltimore Maryland, June 25-28, 2008.

c. A METHOD FOR IMPROVED EXTRACELLULAR MATRIX PROTEOME COVERAGE. Kirk C. Hansen, Lauren Kiemele, Ori Maller, Jenean O'Brien, Aarthi Shankar, Jaime Fornetti, and Pepper Schedin. Keystone Symposia Extrinsic control of Tumor Genesis and Progression, Vancouver, British Columbia, March 2009.

d. TAMOXIFEN INDUCES PLEIOTROPHIC CHANGES IN MAMMARY STROMA RESUTING IN SUPRRESION OF TUMORIGENIC PHENOTYPES. \ Ori Maller,Rhonda Hattar, Kirk C. Hansen, Karla J. Hedman, Scott Lucia, Storey Wilson, and Pepper Schedin, AACR 100th Annual Meeting –Tumor Biology Session, Denver, CO, April 2009.

2. **Other:** Research highlighted in The Breast Cancer Research Program 2007 Report.
3. **Presentations:**
    a. P. Schedin, Invited Speaker, Gordon Research Conference on Basement Membrane, *'Plasticity of the mammary gland extracellular matrix and breast cancer progression'*, Barga, Italy, June 18, 2006.
    b. P. Schedin, Visiting Professor, The Cellular and Molecular Basis of Disease, Interdepartmental seminar series, University of New Mexico, '*Plasticity of the mammary gland extracellular matrix and breast cancer progression'*. Albuquerque, New Mexico, Sept 22, 2006.
    c. *Identification of the Extracellular Matrix Proteomes Responsible for Breast Cancer Metastasis and Tumor Cell Dormancy*, Butcher Symposium on Genomics and Biotechnology, The Future of Biomedicine in Colorado, A Workshop of the Possible, Friday, November 16, 2007 Westin Hotel, Westminster, CO.
    *d.* *The Inflammatory Milieu Permits Metastasis in Pregnancy-Associated Breast Cancer,* Laboratory of Cancer Biology and Genetics Seminar Series, NIH, Bethesda, MD, December 13, 2007.
    e. *The pro-inflammatory milieu of breast involution as a cancer prevention target,* Hematology–Oncology Grand Rounds, Mount Sinai School of Medicine, January 9, 2009
    f. *Physiologic Mammary Gland Involution Promotes Breast Cancer,* Department of Genetics, Cell Biology and Anatomy Grand Rounds, University of Nebraska Medical School, January 14, 2009
    *g.* *Information Conveyed by ECM Proteins in the Mammary Gland,* Keystone Symposia on Extrinsic Control of Tumor Genesis and Progression, Vancouver, British Columbia, Mar 15, 2009.
    h. *Tamoxifen induces pleiotrophic changes in mammary stroma resulting in suppression of tumorigenic phenotypes.* Short Talk, AACR 100th Annual Meeting –Tumor Biology Session, Denver, CO, April 2009.

i. HHS/NIH AYAO Biology Working Group, Bethesda, MD, June 9-10, 2009

4. **Research Opportunities Applied for:**
    a. Rhonda Hattar, Predoctoral Training Grant, DOD, 5/2006, entitled 'Using SERMS and Parity as Tools to Investigate the Tumor-Suppressive Microenvironment'. Not funded.
    b. Ori Maller, DOD Breast Cancer Predoctoral Traineeship Award titled "Identifying 'tumor suppressive' extracellular matrix mediators and determining their ability to induce tumor cell dormancy'. Submitted March 2009.
    c. Schedin, Pepper, PI, DOD Idea Award entitled 'Defining the dormant tumor microenvironment for breast cancer prevention and treatment'. Submitted April 2009.
5. **Training:**
    a. Rhonda Hattar, Masters Graduate training in Cell, Stem Cell and Developmental Biology Program, 2005-2008.
    b. Or Maller, PhD Graduate training in Cancer Biology, 2006-present
    c. Preceptor, Thomas Sweed, 2nd Year UCHSC Medical Student, Louise Coulter Foundation Scholarship Recipient, Summer, 2007.
    d. Preceptor, Reema Mallick, 1st Year Medical Student, Cancer Center Student Research Fellow, Summer, 2007.
    e. Preceptor, Neena Gupta, 2nd year medical student at Virginia Commonwealth, Cancer Center Student Research Fellow, Summer Semester, 2008.
    f. Preceptor, Aarthi Shankar, Cancer Center Student Research Fellow, Summer Semester, 2008.
    g. Preceptor, Nicole Snelgrove, Cancer Center Student Research Fellow, Summer Semester, 2009.
6. **Manuscripts:**
    a. Rhonda Hattar, Ori Maller, Shauntae McDaniel, Kirk C Hansen, Karla J Hedman, Traci Lyons, Scott Lucia, R Storey Wilson Jr, and **Pepper Schedin**. Tamoxifen induces pleiotrophic changes in mammary stroma resulting in extracellular matrix that suppresses transformed phenotypes. *Breast Cancer Res.* Jan 27: 11(1)R5 [Epub ahead of print], 2009 [Highly Accessed Article]
    b. Kirk C. Hansen, Lauren Kiemele, Ori Maller, Jenean O'Brien, Aarthi Shankar, Jaime Fornetti, and **Pepper Schedin**. An Ultrasonic Assisted Digestion Method for Improved Extracellular Matrix Proteome Coverage. Mol Cell Proteomics. 2009 Apr 7. [Epub ahead of print] PMID: 19351662
    c. Lauren Kiemele, Ori Maller, Jenean O'Brien, Aarthi Shankar, **Pepper Schedin**. Kirk C. Hansen. Quantitative analysis of mammary ECM proteins differentially regulated by tamoxifen and parity. In preparation.

**CONCLUSION:** In this grant, we hypothesized that it would be possible to gain insight into the role of the microenvironment in tumor cell dormancy by utilizing treatments (or conditions) that target the mammary epithelial cells directly. Our rationale was that because the functional unit of the mammary gland is the epithelial cell plus it's ECM, any condition that causes a change in epithelial cell function (such as tamoxifen treatment) would cause a compensatory and reciprocal change in the surrounding stroma. Our results are consistent with this hypothesis and are already providing insight into the 'tumor suppressive microenvironment'. Specifically, reduced fibronectin

levels and reduced extracellular matrix turnover appear to be hallmarks of the quiescent mammary microenvironment. Our continued work in the area of ECM proteomic development is another independent accomplishment that is likely to greatly benefit the tumor microenvironment field.

**REFERENCES:** List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine*, etc.).

Bissell, M. J. and M. A. Labarge (2005). "Context, tissue plasticity, and cancer: are tumor stem cells also regulated by the microenvironment?" Cancer Cell **7**(1): 17-23.

McDaniel, S. M., Rumer, K.K., Biroc, S.L., Metz R.P., Singh, M., Porter W., and Schedin, P. (2006). "Remodeling of the Mammary Microenvironment after Lactation Promotes Breast Tumor Cell Metastasis." Am J Pathol **168**(2).

O'Farrell, P. H. (1975). "High resolution two-dimensional electrophoresis of proteins." J Biol Chem **250**(10): 4007-21.

Ross, P. L., Y. N. Huang, et al. (2004). "Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents." Mol Cell Proteomics **3**(12): 1154-69.

Schedin, P., T. Mitrenga, et al. (2004). "Mammary ECM composition and function are altered by reproductive state." Mol Carcinog **41**(4): 207-20.

**APPENDICES**:

Rhonda Hattar, Ori Maller, Shauntae McDaniel, Kirk C Hansen, Karla J Hedman, Traci Lyons, Scott Lucia, R Storey Wilson Jr, and **Pepper Schedin**. Tamoxifen induces pleiotrophic changes in mammary stroma resulting in extracellular matrix that suppresses transformed phenotypes. *Breast Cancer Res.* Jan 27: 11(1)R5 [Epub ahead of print], 2009 [Highly Accessed Article]

Kirk C. Hansen, Lauren Kiemele, Ori Maller, Jenean O'Brien, Aarthi Shankar, Jaime Fornetti, and **Pepper Schedin**. An Ultrasonic Assisted Digestion Method for Improved Extracellular Matrix Proteome Coverage. Mol Cell Proteomics. 2009 Apr 7. [Epub ahead of print] PMID: 19351662

**SUPPORTING DATA**: none provided