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Award Number: W81XWH-05-1-0330

TITLE: A Biophysico-computational Perspective of Breast Cancer Pathogenesis and Treatment Response

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REPORT DATE: March 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-03-2006	Annual	01 Mar 05 – 28 Feb 06

4. TITLE AND SUBTITLE
A Biophysico-computational Perspective of Breast Cancer Pathogenesis and Treatment Response

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0330

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pennsylvania
Philadelphia PA 19104-6270

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Apoptosis regulates the pathogenesis and treatment responsiveness of breast tumors yet the molecular mechanisms whereby breast cancer cells resist apoptosis remains unknown. We found that coincident with malignant transformation and in association with an increase in collagen deposition, cross-linking and reorganization, the mammary gland becomes incrementally stiffer and that the elastic modulus of the tissues to which breast tumor cells characteristically metastasize varies widely. We used natural collagen and basement membrane (BM) hydrogels as well as synthetic laminin and BM-cross linked polyacrylamide gels with precisely calibrated compliances, and could show that elevated matrix stiffnesses independently induce mammary cell proliferation, perturb cell-cell integrity, disrupt tissue polarity, and inhibit apoptosis-dependent lumen formation to disrupt mammary tissue morphogenesis. When we varied matrix force within the range we measured for the various metastatic and transformed tissues, we could modify the activity of several key stress response pathways previously linked to apoptosis regulation. Matrix mediated stress pathway regulation profoundly influenced the apoptosis responsiveness of mammary tissue to a diverse array of exogenous death stimuli including chemotherapeutics such as taxol, immune receptor activators including trail and gamma irradiation. Towards delineating a molecular mechanism we were able to demonstrate that matrix stiffness increases the expression and activation of integrins, drives the assembly of mature focal adhesions, and increases Rho GTPase-dependent intracellular contractility. Experiments are now in progress to further explore these findings using novel biomaterials and imaging modalities and organotypic culture manipulations.

15. SUBJECT TERMS
Extracellular matrix, stiffness, mechanical, force, apoptosis, resistance, bioengineering, cross-disciplinary, computational, breast tumor treatment

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	41	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION:

Apoptosis resistance regulates the pathogenesis, and treatment response of breast tumors. Despite concerted effort towards understanding the molecular basis for apoptosis resistance in breast tumors, progress in this area has been frustratingly slow. Lack of advancement may be attributed in part to the current cell autonomous view of breast cancer etiology and treatment responsiveness. What we now know is that the organ microenvironment can and does regulate the therapeutic responsiveness of metastatic tumors (Taylor et al., 2000, Zahir et al., 2004), and that stromal-epithelial interactions influence mammary gland development, tissue homeostasis and breast tumor progression (Unger and Weaver, 2003). Alterations in the mammary gland ECM correlate with changes in mammary differentiation, involution (apoptosis) and tumor progression, and culture experiments clearly show that the stromal ECM can modulate mammary epithelial cell (MEC) growth, differentiation and survival and alter apoptotic responsiveness (Zahir et al., 2004, Truong et al., 2003, Lewis, Truong and Schwartz, 2002). **How the stroma promotes apoptosis-resistant breast tumors remains unclear.**

We have been studying the role of integrin ECM receptors as key regulators of mammary tissue behavior as well as malignant transformation and metastasis. We have been exploring the molecular mechanisms whereby the ECM can regulate mammary tissue homeostasis, invasion and apoptosis responsiveness. We found that integrin expression, organization and activity are consistently altered in breast tumors and that perturbing integrin expression and activity can drive malignant behavior of non-malignant and pre-malignant MECs, and that normalizing integrin activity represses expression of the malignant breast phenotype in culture and in vivo (Unger et al., 2003; White et al., 2004). We also determined that integrins regulate cell survival and modulate the apoptotic responsiveness of mammary tissues to a diverse array of exogenous stimuli including various chemotherapies and immune receptor activators (Weaver et al., 2002, Zahir et al., 2004). We found that integrin-dependent apoptosis resistance and survival are intimately linked to many of the biochemical pathways and mechanisms that regulate tissue organization and specifically tissue polarity. For example, we found that $\alpha 6\beta 4$ integrin directs mammary epithelial cells to assemble polarized mammary tissue structures that display apoptosis resistance to a wide spectrum of apoptotic insults. We are now exploring the underlying mechanisms whereby integrin expression and/or function becomes altered in breast tumors, how integrin modulate the survival of nonmalignant and transformed mammary epithelial cells, what the molecular link could be between integrin-dependent survival and tissue polarity and the clinical relevance of these findings.

We found that prior to malignant transformation the mammary gland exhibits a 'desmoplastic' response that is associated with an incremental and significant increase in global elastic modulus (stiffness) of the gland and elevated/altered expression of integrins and integrin adhesions (Krouskop et al., 1998; Paszek and Weaver 2004; Paszek et al., 2005; unpublished data). Consistent with results from other laboratories we determined that externally-applied mechanical force regulates the behavior and phenotype of multiple cell types including endothelial, fibroblasts, neurons, and MECs (Grinnell, F. 2003, Bershadsky, Balaban and Geiger, 2003, Geiger, B. et al., 2001). Although the mammary gland is not traditionally viewed as a mechanically-regulated tissue, MECs within the ductal tree and alveolus experience passive (isometric) and active mechanical force throughout the lifetime of the mammary gland most notably during development, lactation and involution (Paszek and Weaver, 2004; Samani et al., 2003, Plewes et al., 2000). Similar to other solid tumors, the mammary gland also becomes appreciably stiffer in association with its malignant transformation and mammary epithelial cells within the tumorigenic mammary gland experience an array of additional compression and stress and interstitial associated forces (REF). During the process of metastasis and once at the metastatic site breast tumor cells also encounter an array of external mechanical forces that could conceivably influence their behavior and alter their response to treatment. For example, many of the common metastatic sites for breast cancer differ appreciably with respect to their stiffness and biochemical compositions than a normal mammary gland such as bone (very stiff, high vitronectin), in the vasculature (high pulsatile

pressures, high fibronectin and fibrin), pleural cavity (very compliant with high fibrin composition but also adjacent fibrotic lung could be quite stiff with a high amount of elastin).

Because physical forces so profoundly influence cell proliferation, survival and differentiation of multiple cell types, we maintain that it is critical to understand how mechanical cues could influence mammary tissue behavior and apoptosis responsiveness.

Accordingly, we predict that the physical organization of the ECM (which contributes to its mechanical properties) constitutes an independent regulator of mammary epithelial behavior and apoptosis resistance. Delineating the molecular basis for this phenotype will likely have important consequences for tumor therapy. To rigorously test this idea we are in the process of achieving the following specific aims:

Specific Aim 1. Engineer tractable 3D organo-typic model systems that recapitulate the biophysical properties of primary and metastatic breast tumor tissues, and then use these models to dissect candidate molecular stress-response mechanisms whereby ECM stiffness could regulate apoptosis resistance in culture and in vivo.

Specific Aim 2. Develop xenograft and transgenic mouse models to test whether ECM stiffness regulates apoptotic responsiveness of mammary epithelia in vivo.

Specific Aim 3. Build a computational model that can predict how changes in ECM compliance could influence integrin-dependent apoptosis responsiveness of mammary epithelia and query this model with clinical data.

Specific Aim 4. Develop non-invasive imaging tools that could be used to monitor changes in ECM stiffness or stiffness-induced changes in mammary tissue phenotype.

Summary of Achievements - Proposal Body:

To engineer tractable 3D organo-typic model systems which recapitulate the biophysical properties of primary and metastatic breast tumor tissues.

Measurement of biophysical property of normal and diseased in vivo breast tissue

Our first objective has been to generate versatile culture systems that recreate the biochemical and biophysical microenvironment within which normal and transformed breast cells exist in vivo so that we can test the effect of isometric force on breast cell responsiveness to immune and exogenous therapy. Towards this goal in collaboration with Drs. Susan Margulies and Joyce Wong we have successfully measured the “stiffness” herein defined as the elastic modulus of normal, pre-malignant and transformed breast tissue, the stroma surrounding transgenic breast tumors, and the physical properties of several tissues to which breast tumor cells characteristically metastasize including lung, brain, and liver. The objective of these measurements has been to determine what range of matrix stiffness to which normal and transformed breast epithelial cells interact with in vivo so that we can effectively generate ECMs that recreate the effect of mechanical force on breast tumorigenesis and treatment responsiveness ex vivo for experimental analysis of relevance and molecular pathways.

To achieve this goal we used unconfined compression analysis as well as shear rheology techniques to measure the viscoelastic properties of normal, premalignant, and breast tumor tissue as well as lung,

liver and brain tissue. Results reported for bone, borosilicate glass and tissue culture plastic were obtained from published data bases. Unconfined compression analysis provides a direct measurement of the tissues elastic modulus (see Figure 1) whereas shear rheology yields a shear rheology measurement that can be mathematically transformed to the elastic modulus value. Both sets of tissue measurements yielded comparable stiffness measurements, although we found that our measurements using the Compression Analysis were consistently 20-30% higher than the Shear Rheology measurements. We were able however to measure a consistent and progressive increase in the elastic modulus of breast tissue coincident with malignant transformation using both approaches (see Figure 2). We also determined that lung, brain and liver tissues are significantly stiffer than normal breast tissue. Published values for bone also revealed that this tissue is significantly stiffer than normal breast tissue and indeed than all other tissues within the body.

Towards achieving our goal we worked with Dr. Margulies at the University of Pennsylvania and also with Dr. Wong at Boston University. To facilitate technology transfer and aid in the teaching element of this grant proposal, two of my own bioengineering graduate students from the University of Pennsylvania were sent to MIT and Boston University to be trained by Drs. Cam and Wong on Shear Rheology measurements. While visiting MIT and Boston University, both of my students were also taught the principals of the correct assembly of self assembled peptide gels and introduced to the nuances of collagen gel measurements and PEG/methylcellulose biogel generation.

Once we had successfully been able to make consistent measurements of different normal tissues in vivo and of progressively transformed breast tissues we set about to manipulate natural and synthetic biomaterials to attain stiffness ranges comparable to those we measured in vivo so that we could explore the relevance of our in vivo measurements. Our first goal was to exploit the ready availability of collagen I/recombinant basement membrane gels. By systemically varying the collagen concentration while keeping the laminin or basement membrane concentration constant we were able to vary the matrix stiffness from approx. 100 pascals upwards to 1200 to 1600 pascals. Because the elastic modulus we measured for the transforming breast tissue in vivo varied from 100-140 pascals to 1,200 to 1,500 pascals during the early stages of breast transformation, we initiated studies to examine the effect of modulating collagen/dependent stiffness on mammary epithelial cell phenotype. As can be observed in Figures 3 & 4, increasing matrix stiffness using collagen promoted mammary epithelial cell proliferation and compromised mammary tissue morphogenesis. These results have been reported in a recent publication in Cancer Cell by Paszek et al., 2005 and clearly show for the first time that ONLY physical conditions that are identical in their visco elastic properties or stiffness to that found in the mammary gland in vivo are able to support mammary epithelial morphogenesis. Thus we could show that the mammary gland in a normal mouse (similar to what we have now determined in a human mammary gland) is highly compliant (soft). We also noted that rBM or Matrigel which promotes mammary morphogenesis has a similar soft consistency as do low concentrations of collagen. In marked contrast however, tissue culture plastic substrates such as polystyrene and borosilicate glass are significantly and dramatically stiffer (see Table 1). As can be seen from Figure 3, as the matrix is progressively stiffened tissue morphogenesis becomes substantially compromised. Initial effects include a failure to clear the lumen (a hallmark of DCIS lesions) followed by a destabilization of cell-cell junctions and finally a loss of basal and apical polarity. The consequence of perturbing epithelial morphogenesis is that MECs encountering a highly stiff matrix form continuously growing, abnormal colonies reminiscent of premalignant mammary lesions (Paszek et al., 2005).

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Because varying collagen concentration also varies ligand presentation and concentration, we developed an alternative and novel approach to vary matrix stiffness of basement membrane biogels for three dimensional organotypic mammary cultures. Briefly, this newly developed method involves seeding mammary cells onto basement membrane laminated/conjugated polyacrylamide gels with precisely

Figure 1. Compression testing device used to measure mammary tissue stiffness

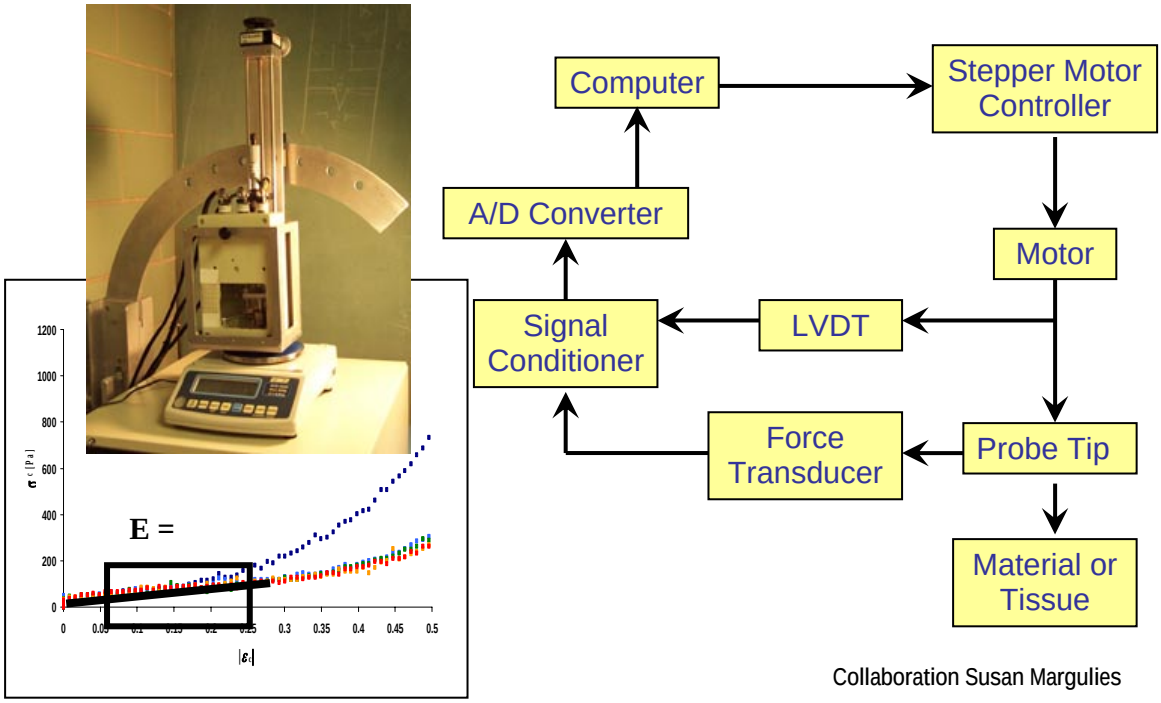
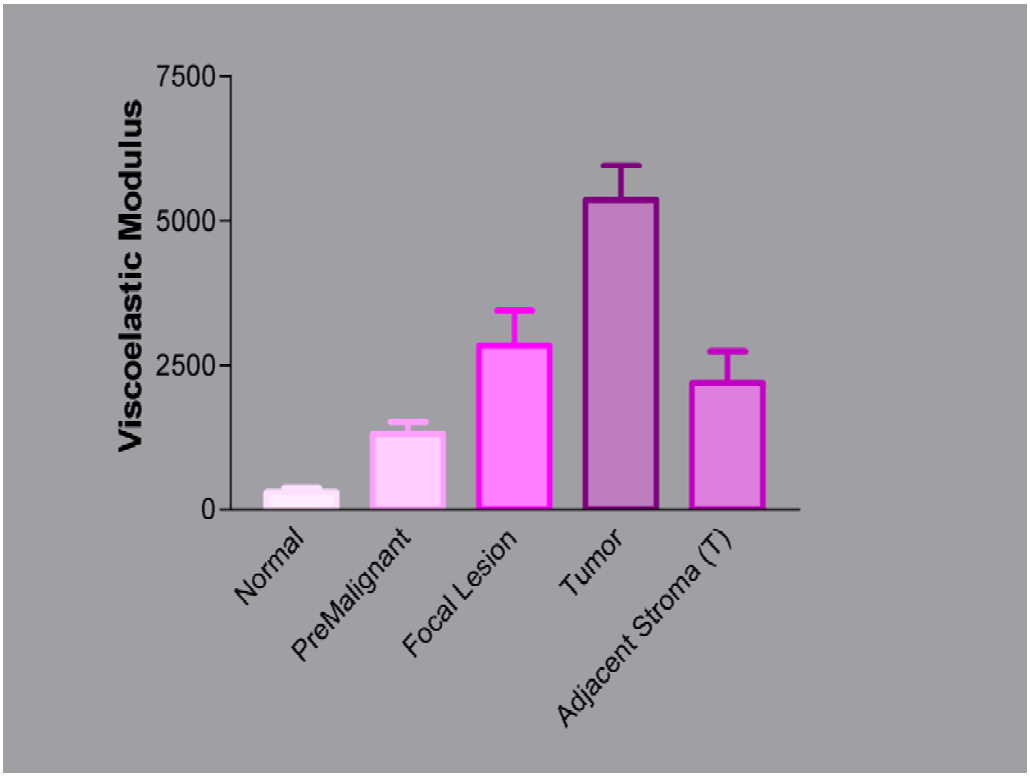


Figure 2. Mammary tissue stiffens in association with malignant transformation



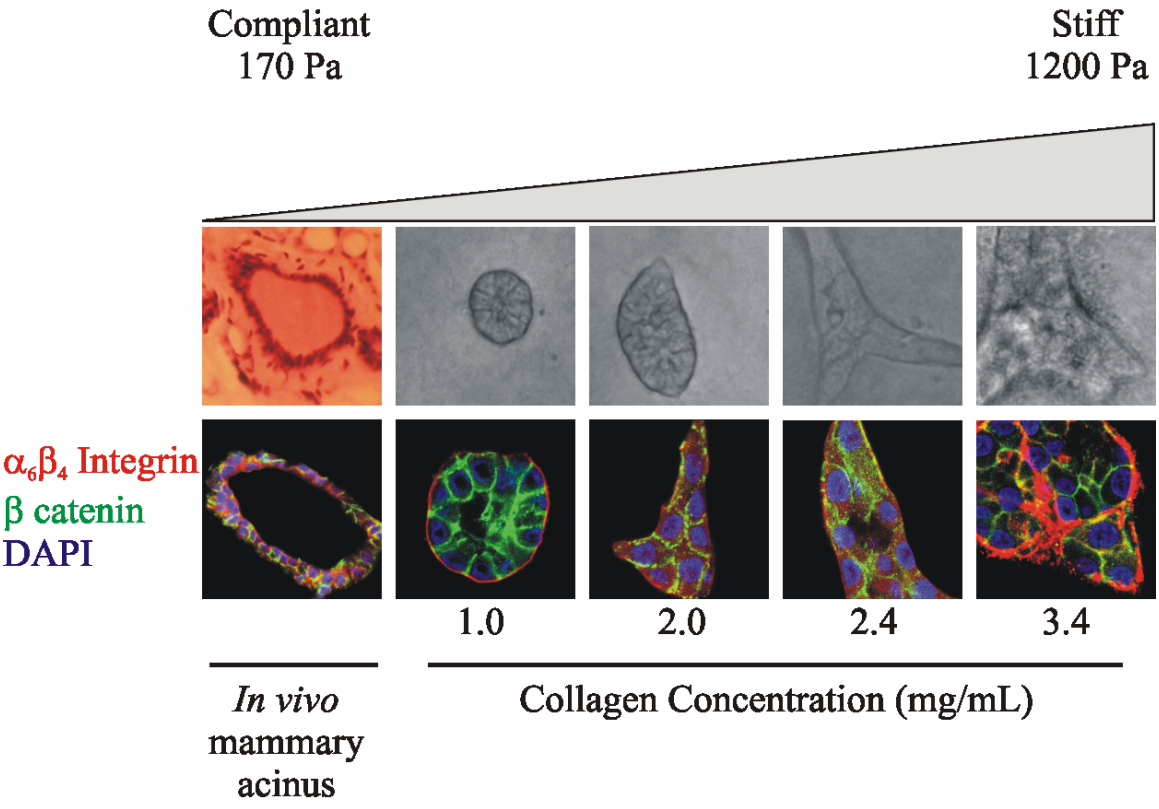


Figure 3. Increasing matrix stiffness by increasing collagen concentration progressively perturbs mammary epithelial tissue morphogenesis

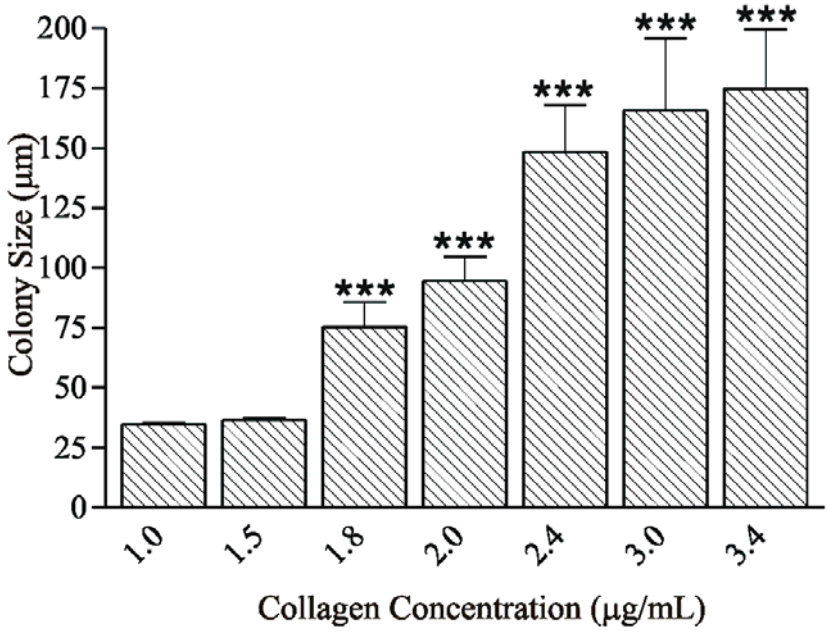
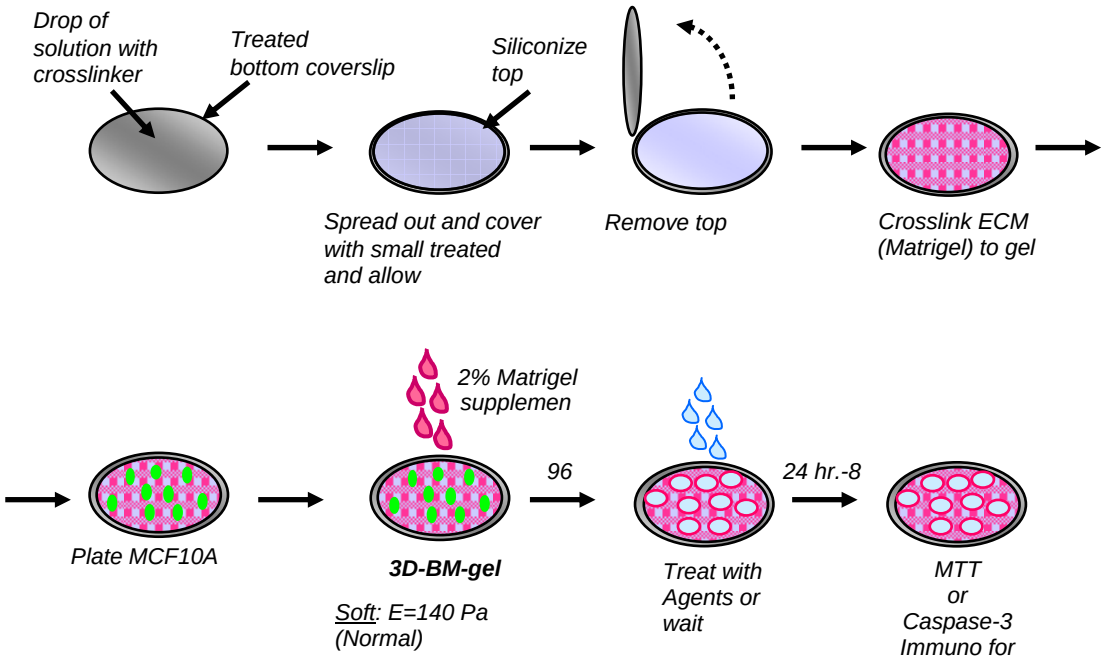


Figure 4. Increasing matrix stiffness by increasing collagen concentration progressively increases tissue size and proliferation.

Table 1. Elastic Modulus of normal breast and tissues to which breast tumors typically metastasize to as compared to properties of typical hydrogel and culture substrata.

Tissue or Material	**Elastic Modulus (Pa)**
Mouse Brain	3520 ± 1897
Mouse Liver	2822 ± 530
Mouse Mammary Gland	166.6 ± 13.6
Matrigel	174.8 ± 37.44
Collagen (2.0 mg/ml)	328 ± 87
Collagen (4.0 mg/ml)	1589 ± 380
Plastic (polystyrene)	$2.28 \times 10^9 - 3.28 \times 10^9$
Glass (soda-lime)	69×10^9

Figure 5. Pseudo- three dimensional basement membrane-conjugated polyacrylamide gel method with constant ligand concentrationto assess effects of matrix stiffness on mammary tissue behavior



calibrated stiffness ranging from 140 pascals upwards to 10,000 to 20,000 pascals and then overlaying the adherent cells with a 3rd dimension of reconstituted basement membrane added to the culture medium, similar to the pseudo 3D method described by Debnath and colleagues (Debnath et al., 2003; see Figure 5). By applying a third dimension of basement membrane in the cell media the cells readily undergo mammary morphogenesis to form acinar structures similar to those assembled by mammary epithelial cells completely embedded within either a complaint recombinant basement membrane or a compliant gels consisting of a combination of collagen I/recombinant basement membrane (see Figure 3). Again an increase in matrix stiffness, even of only a modest range, is sufficient to compromise epithelial morphogenesis and induce mammary epithelial cell growth (see Figure 6). However, because these synthetic conditions only present the matrix stiffening from two dimensions, a large increase in matrix stiffness is required to drive the invasive phenotype. Accordingly, we have been working diligently to develop synthetic, biocompatible materials that can be used to study the effect of precisely varying matrix stiffness in all three dimensions on mammary tissue behavior. Towards this goal we have been working closely with members of Dr. Cam's laboratory at MIT to exploit the availability of self assembled peptide polymers. Although these studies are still in progress we have been able to manipulate these assembled polymers to yield a versatile range of matrix stiffness for experimental manipulations.

The role of altered matrix force and force sensing in breast tumorigenesis

Although the effect of matrix stiffness on MEC morphogenesis is striking and intriguing – without a clear indication of molecular mechanisms the development of definitive therapeutic targets will not be feasible. Accordingly, we undertook an exhaustive analysis of the effect of matrix stiffness on integrin and growth factor receptor signaling. Much of these results have been published in our Cancer Cell article Paszek et al., 2005 Cancer Cell and have also been discussed in a review article published in 2005 in the Journal of Mammary Gland Biology and Neoplasia Paszek and Weaver – which has a listed date of 2004 but was written and submitted a FULL year later. Here we summarize our major finding which is that matrix force compromises mammary morphogenesis primarily by CHANGING the nature of the integrin adhesion structure formed (see Figures 7 & 8; see also figures in Paszek et al., 2005). Thus matrix force drives the assembly of ^{397}FAK, and vinculin containing focal adhesions that then lead to the recruitment of multiple signaling molecules that enhance signaling through pathways linked to integrins such as ERK kinase. More importantly when we generated a novel beta 1 integrin cluster mutant cells expressing this mutant form of integrin exhibited a phenotypic behavior similar to when they were interacting with a stiffer matrix including loss of proper morphogenesis, enhanced cell spreading and elevated ERK dependent cell proliferation (see Figures 9 & 10).

Encouraged by our striking observation that tissue stiffness was associated with an abnormal tissue behavior in the breast and that tissues stiffen appreciably prior to tumor formation – we also examined the contractility or force-generating behavior of normal versus transformed breast tumor cells. We found that normal cells are finely tuned to the stiffness of their surrounding matrix such that they exert progressively greater and greater cell generated forces in response to increasing matrix stiffness and they apparently do this via activation of the RhoGTPase Rho kinase (see Figure 11) because when we ectopically expressed a constitutively activated RhoGTPase the mammary cells behaved as if they were embedded within a stiffer matrix (Pazek et al., 2005). In contrast, and surprisingly, we also determined that breast tumor cells exert considerably more force towards their matrix than nonmalignant MECs and they do so via elevated activity of Rho and Rock kinases (see Figure 12). More dramatically we could normalize the behavior of these tumors by inhibiting their integrin-dependent, force-linked signaling pathways – including their integrin/growth factor receptor- Rho-ROCK-myosin activity (see Figure 13). These observations may have considerable relevance to understanding and treating breast tumors and portions have already been reported in Paszek et al., Cancer Cell 2005.

Figure 6. Matrix stiffness compromises mammary tissue morphogenesis

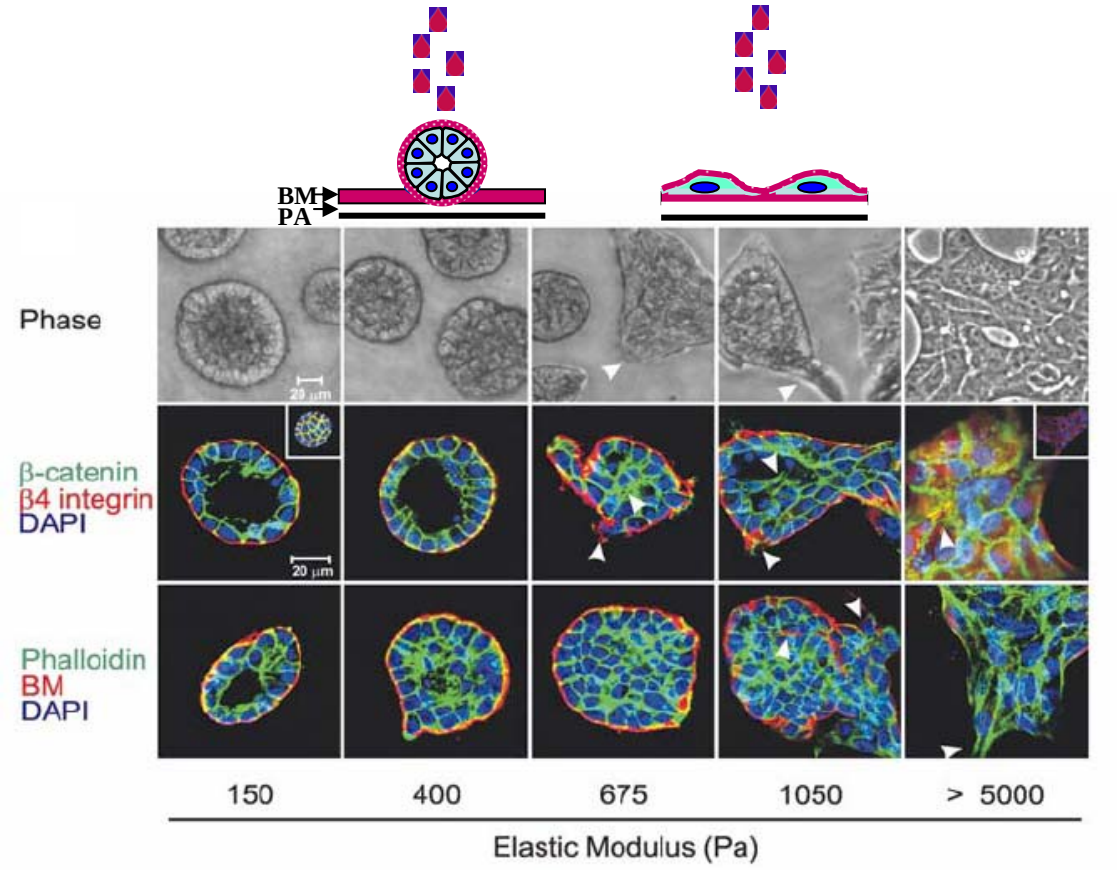


Figure 7. Matrix stiffness in culture regulates the assembly of mature focal adhesions

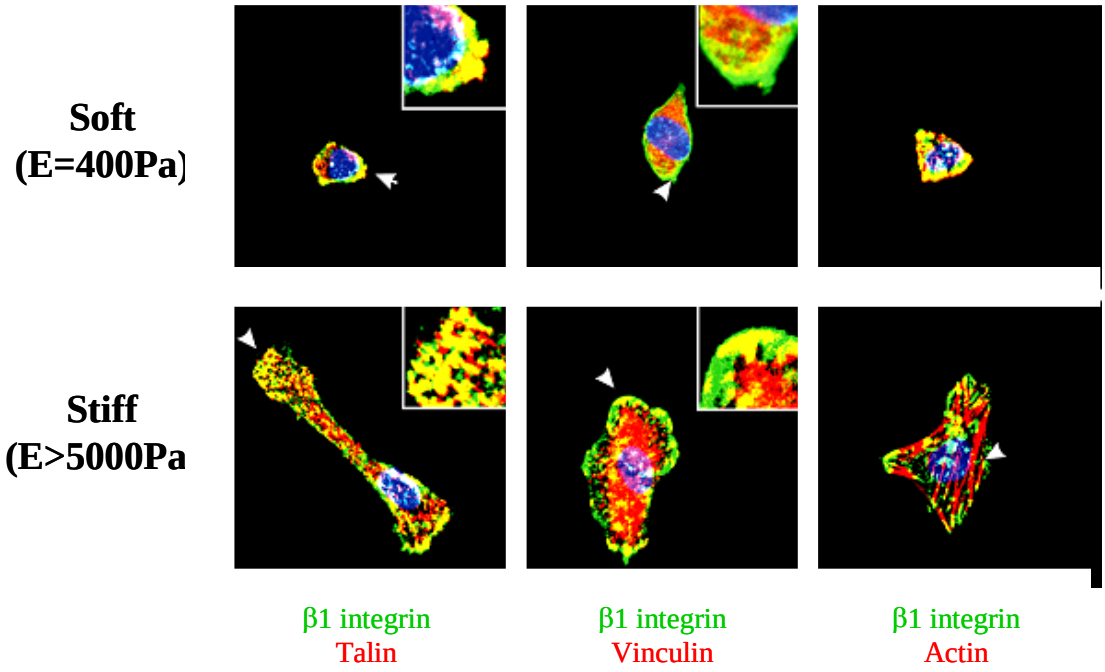


Figure 8. Matrix stiffness in vivo regulates maturation of focal adhesions as indicated by increased 397FAK and vinculin

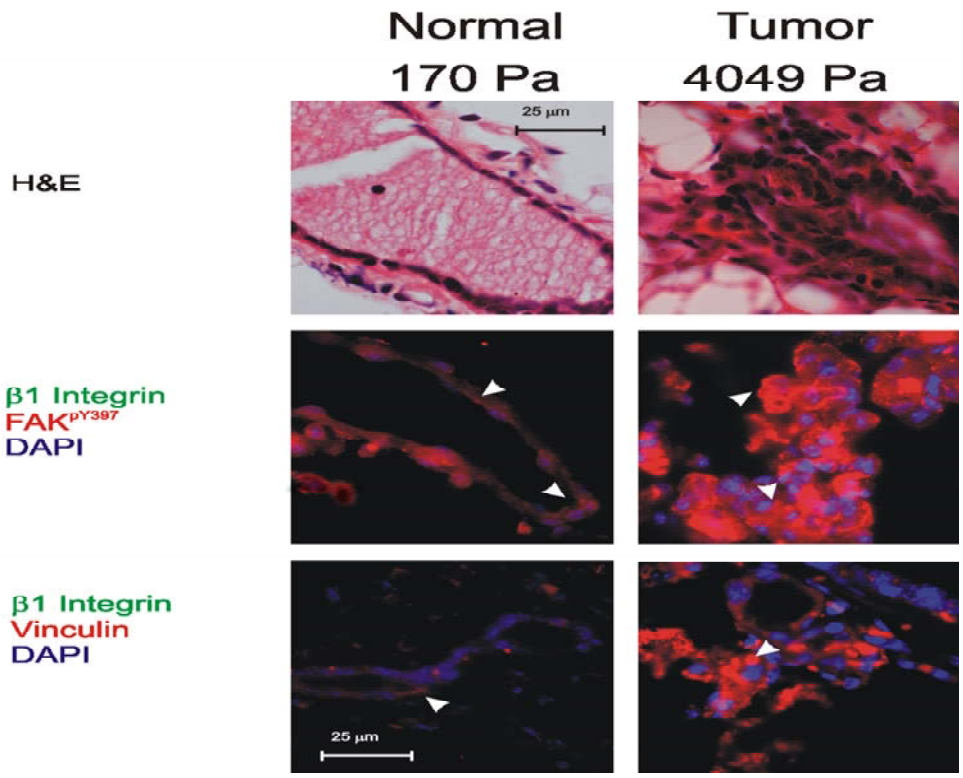
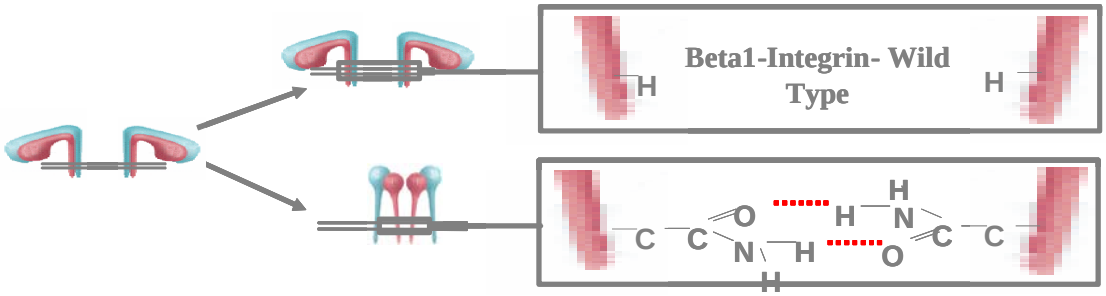


Figure 9. Integrin Clustering Increases maturation of focal adhesions in response to a compliant matrix



A non-polar glycine 744 or hydrophobic valine 737 replaced with a polar asparagine

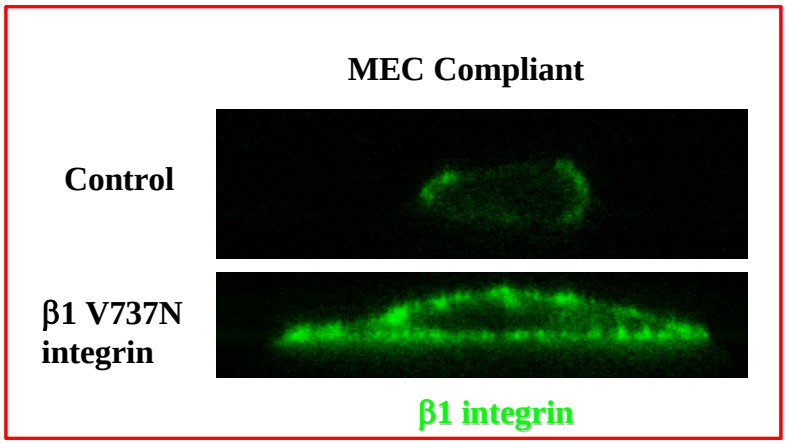


Figure 10. Matrix force modulates mammary epithelial cell fate through functional interactions with RhoGTPase-dependent cell contractility

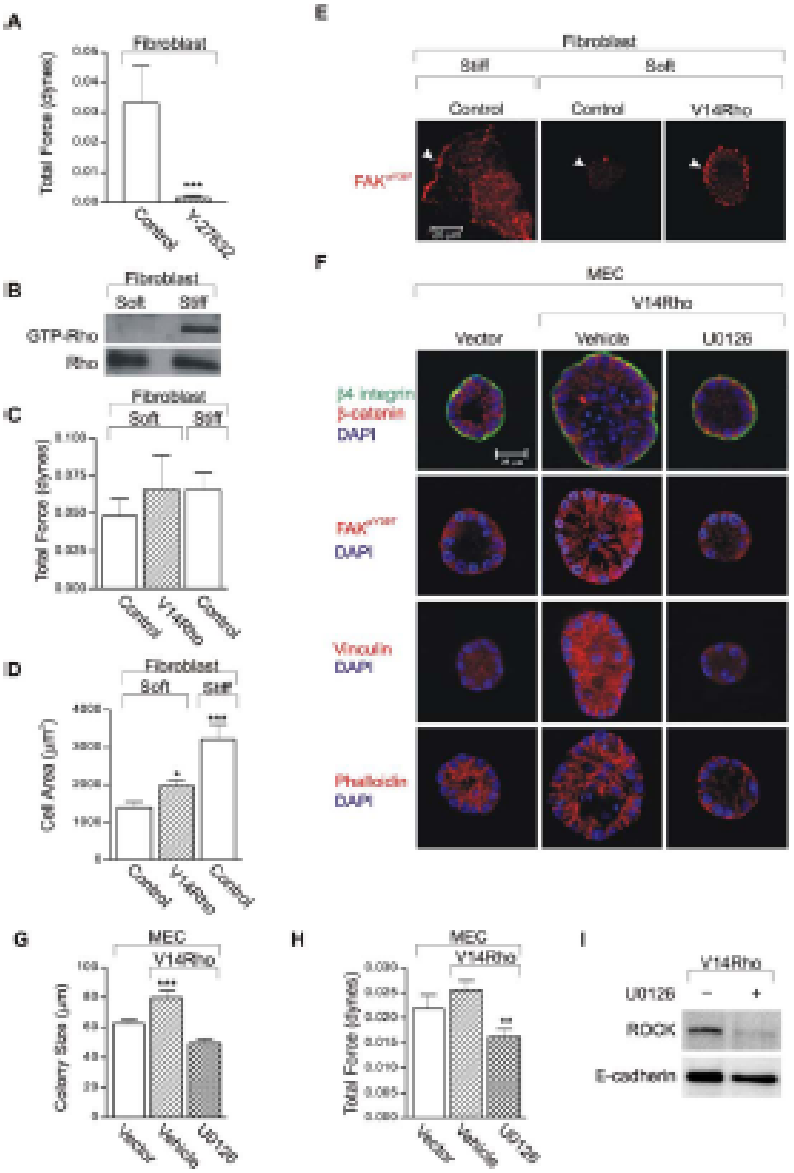


Figure 4. Matrix stiffness induces integrin clustering to enhance GF-dependent ERK activation and Rho-generated tension, increase growth and perturb tissue morphology

A: Cell force quantified by TFM in fibroblasts on a FN gel, treated with vehicle or the ROCK inhibitor Y-27632.

B: Representative immunoblot and quantification of immunoprecipitated Rhotekin-associated Rho (GTP-Rho) and total Rho (Rho), in fibroblasts on soft and stiff FN gels.

C: Cell force using TFM of vector control (Control) and V14Rho-expressing fibroblasts on soft (450 Pa) and stiff (5600 Pa) FN gels.

D: Cell area of vector control (Control) and V14Rho-expressing fibroblasts on soft and stiff FN gels.

E Confocal IF images of FAK^{pY397} (red) in control or V14Rho-expressing fibroblasts.

F: Confocal IF images of β4 integrin (green), co-stained with β-catenin (red), FAK^{pY397}, vinculin (red), actin (red), and nuclei (blue), in MEC colonies in a compliant (175 Pa) BM for 16 days, expressing either a vector (Vector) or active Rho (V14Rho) treated with vehicle or the MEK-1 inhibitor U0126.

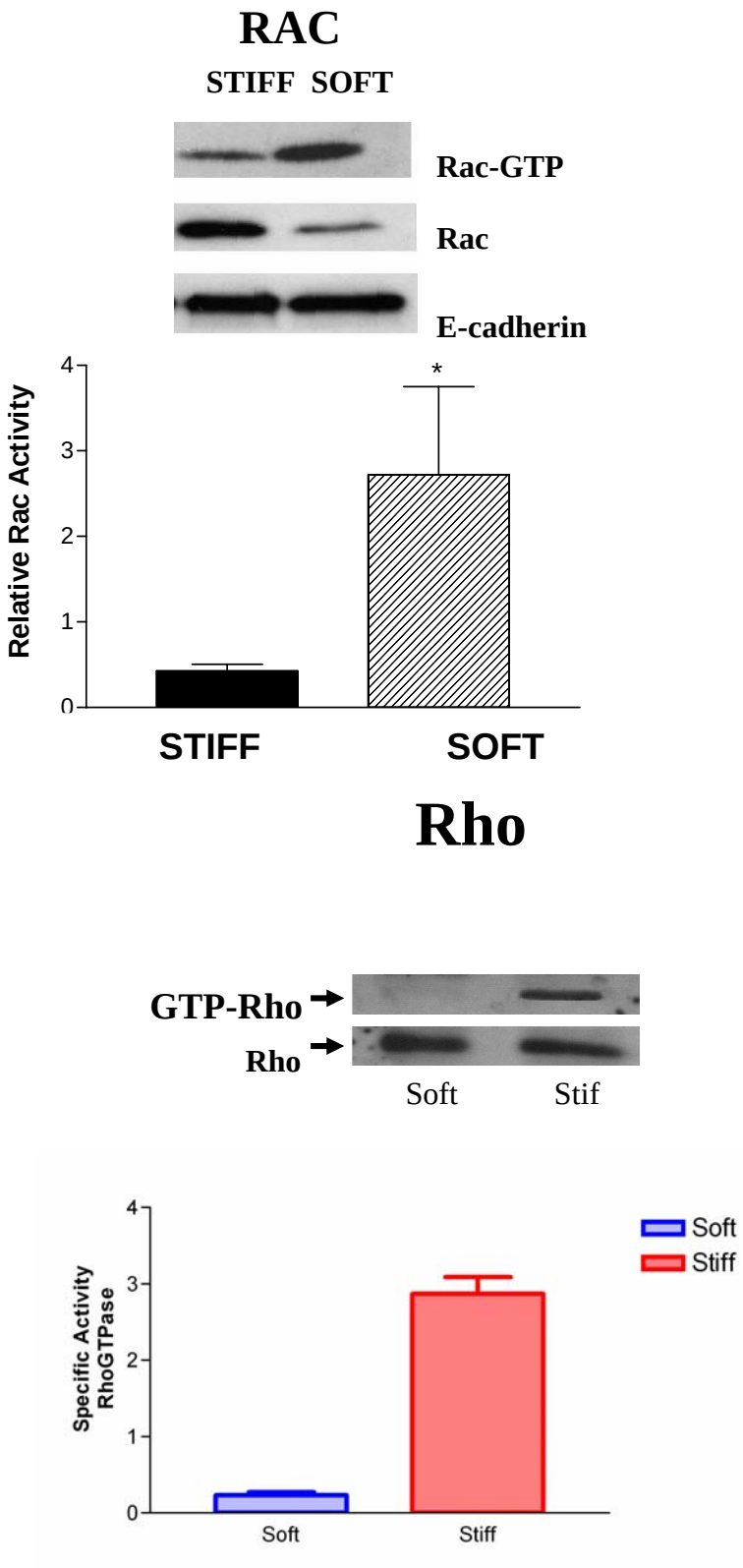
G: Colony size of MECs expressing vector (Vector) or active Rho (V14Rho) treated with vehicle or U0126, in BM for 16 days.

H: Total force (TFM) exerted by V14Rho MECs on soft gels treated with vehicle or U0126.

I: Representative immunoblot of ROCK expressed in V14Rho MECs treated with vehicle or U0126.

Results in G are mean ± SEM of three experiments; A, C, and D represent ≈50 measurements. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

Figure 11. Matrix Stiffness Modulates the activity of Rac and Rho GTPases



Friedland et al., Unpub & Paszek et al Cancer Cell 2005

Figure 12. Functional association between malignant transformation and RhoGTPase-dependent force

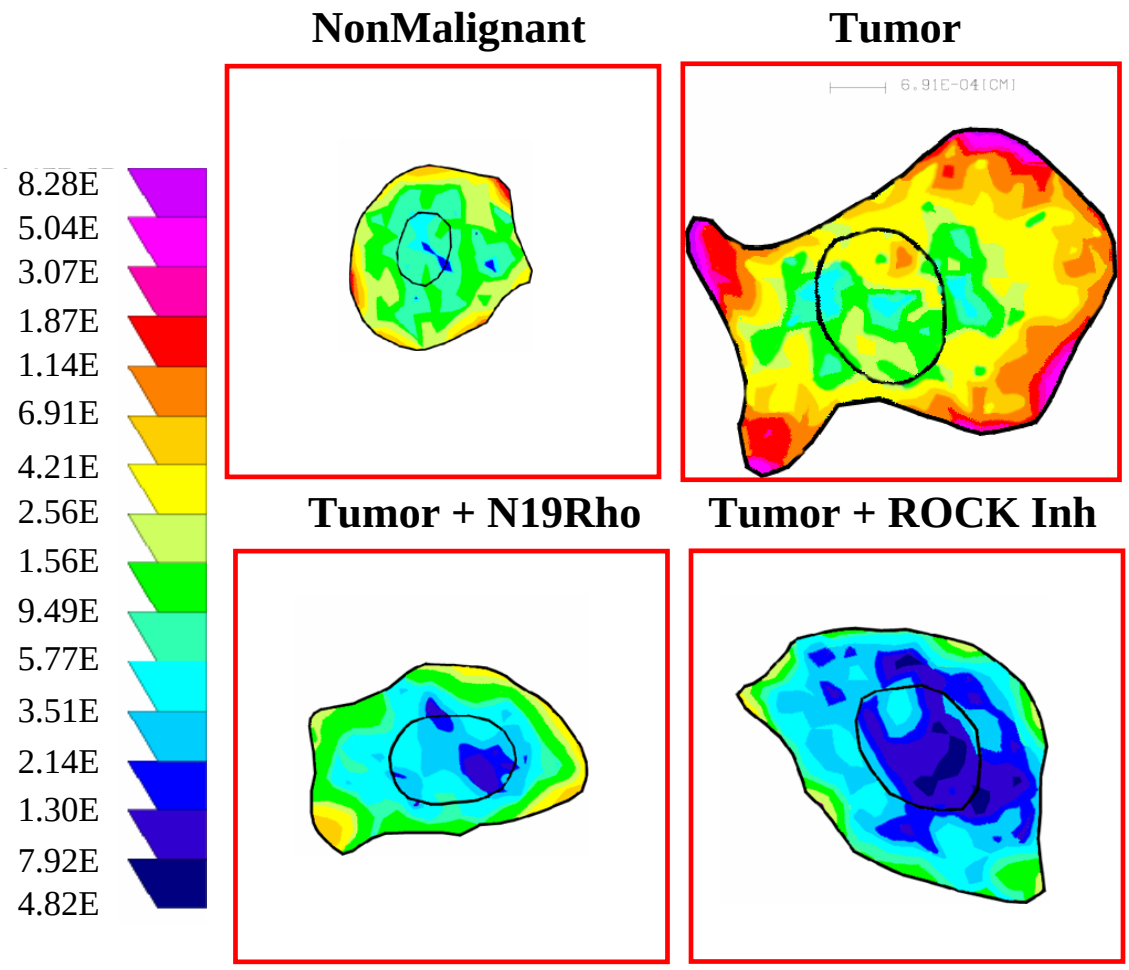
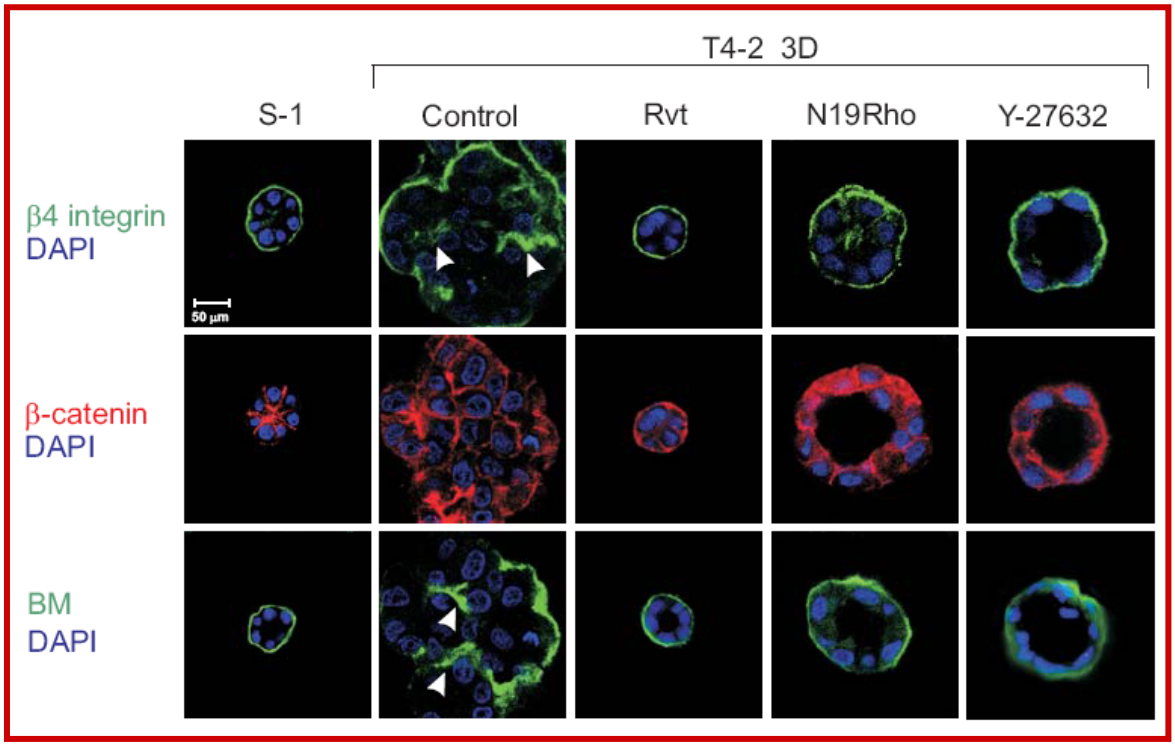


Figure 13. Inhibiting cell-generated tension normalizes tumor phenotype

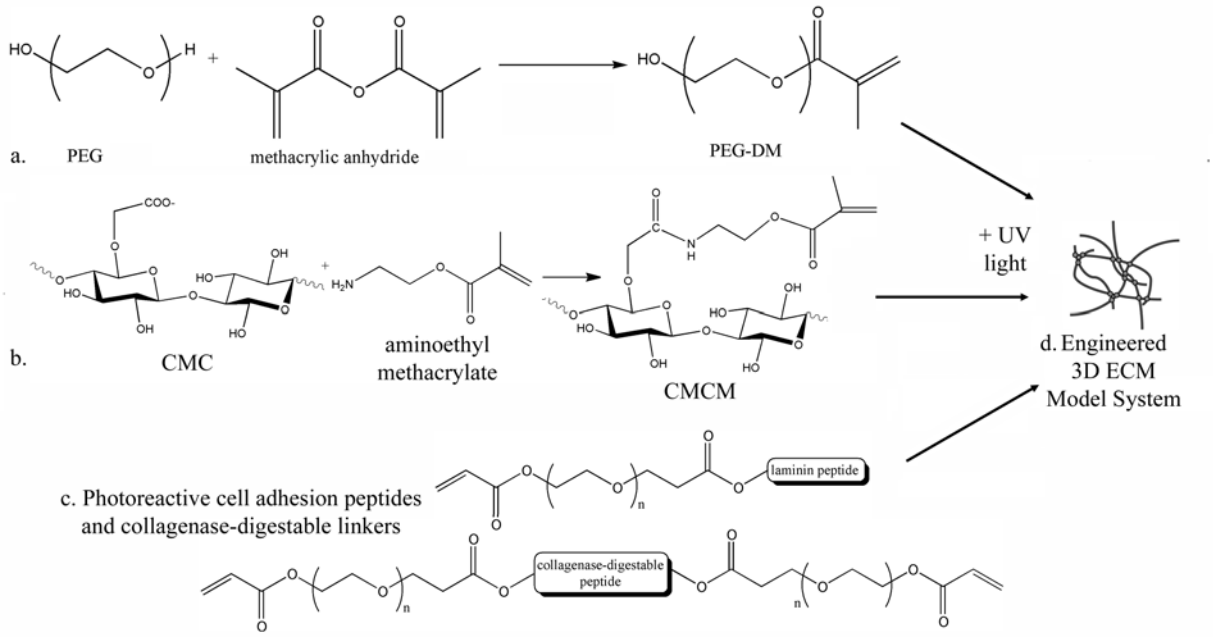


Development of alternate synthetic 3D model systems that recapitulate the biophysical and biochemical properties of primary and metastatic breast tumor tissues. Conducted in collaboration with Drs. Wong and Leach at Boston University and University of Maryland.

Our research effort in this past year have been geared towards successfully exploiting currently available natural collagen type I and collagen/reconstituted basement membrane hydrogels with calibrated stiffness. As mentioned above, to alter the matrix compliance we were compelled to vary the collagen concentration of these hydrogels. Unfortunately, varying collagen concentration complicates experimental interpretation because it not only alters matrix stiffness but also changes the concentration of the presenting ligand and its orientation to the mammary cell. Furthermore, natural biomaterials such as collagen I can be dramatically modified by the embedded mammary epithelial cells, which also can contribute significantly to experimental variability and difficulty in interpreting results. Indeed, natural biomaterials such as collagen and fibrin are notoriously heterogenous to begin with and this alone can and will dramatically complicate experimental interpretations. To address these issues, as described earlier, we developed a novel pseudo-three dimensional culture model to study the effect of matrix stiffness on mammary tissue morphogenesis and apoptotic behavior that applied protein laminated polyacrylamide (PA) gels in which the bis acrylamide cross linker concentration was varied to derive a dynamic and large range of matrix stiffness. Although this synthetic system proved quite useful and permitted us to draw experimental inferences with regards to the effect of matrix force on mammary cell and tissue behavior (see results discussed above) these synthetic gels also have limited experimental applicability. For example, these laminin-ligated PA gels do not represent a true three dimensional matrix system because we cannot fully embed the cells within the PA gels due to its toxicity. Also because of the toxicity effect the PA gels cannot be used to study the effect of matrix stiffness on mammary tissue responsiveness to therapy in vivo. Finally, PA gels cannot be readily remodeled and reoriented by mammary cells and the organization of the gel material is significantly different from that of the natural matrix surrounding a mammary tissue in the mammary gland. Accordingly, over the past year we have focused our efforts on developing novel engineered three-dimensional (3D) extracellular matrix (ECM) model systems with improved versatility for studying the role of matrix force on mammary tissue behavior in both two and three dimensions as well as in culture and in vivo. Ultimately, our goal is to develop a tractable model system in which biochemical, spatial and mechanical variables can be precisely and independently manipulated, that can be tested in vivo in animal models. Accordingly, we have been working with members of Dr. Cam's laboratory at MIT to examine the usefulness of self assembled peptide gels. Thus far we have been able to generate YIGSR and RGD-conjugated self assembled peptide gels and work out conditions that progressively increase the stiffness of these gels. We have been able to demonstrate that mammary epithelial cells remain viable within these gels and can be growth to form colonies. However, preliminary data are not encouraging. At present it appears that even with the addition of exogenous laminins or reconstituted basement membrane matrix mammary organoids do not acquire tissue polarity in these peptide gels. Ongoing studies that are currently being prepared for publication suggest that these peptide gels do not support the necessary matrix remodeling required by developing mammary tissues for their proper tissue morphologies and behavior. Indeed, one of our collaborators has been using similar peptide gels to study effects on angiogenesis and determined that these gels do not support multicellular morphogenesis if conditions require gel remodeling. Such a scenario is not unfamiliar in the world of synthetic biomaterials which do not easily become remodeled by cells. To avoid such difficulties researchers have incorporated peptides and proteins with proteolytic residues. Unfortunately, such strategies mean that these synthetic gels are not easily adapted to biochemical studies due to their expense.

To remedy this situation we have begun to explore alternative biomaterials development. In this regard we have joined forces with Drs. Joyce Wong at Boston University of Jennifer Leach (who just

Figure 14. Generation of biochemically-defined 3D PEG gels for organotypic mammary tissue assays and testing for the effect of matrix force on tissue behavior in vivo



recently moved to take up a faculty position at the University of Maryland) and together we are predicting that laminin peptide conjugated synthetic hydrogels with collagenase-digestible peptide crosslinkers or optimally recombinant simple proteins will comprise a more optimal model ECM system for studying the role of matrix force on mammary tissue behavior. In the past year we have made considerable progress towards developing the chemistry and mechanical properties of these newly engineered ECM model systems and we are now studying the biocompatibility of these synthetic matrices and completing a comprehensive analysis of the effect of matrix stiffness on normal and malignant MEC behavior in 2D versus 3D.

Based on published methods (Leach et al; West et al), we have synthesized a novel engineering ECM model system from photocrosslinkable synthetic polymers. Our work has focused on two polymers: polyethylene glycol (PEG) and carboxymethylcellulose methacrylate (CMC) (see Figure 14a). PEG is highly hydrophilic, flexible, readily modified, non-degradable and biocompatible; as such, PEG derivatives are commonly used in tissue engineering scaffolds and drug delivery devices. CMC, a cellulose derivative, has similar properties as those listed for PEG, but has added chemical versatility due to a greater number of readily modified groups along its backbone. To crosslink these polymers into hydrogel scaffolds, we have chosen a versatile and biocompatible photocrosslinking procedure (see Figure 14a). This procedure has two steps: the first step modifies the polymer with photoreactive methacrylate groups to yield PEG dimethacrylate (PEGDM; see Figure 14) and CMC methacrylate (CMCM; see Figure 14). The second step crosslinks the polymer under physiological conditions and exposure to low intensity UV light (see Figure 14d). To render the hydrogels adhesive and degradable to cells, we incorporate photoreactive laminin-derived and collagenase-digestable peptides (see Figure 14c). Reaction variables, such as relative PEGDM and CMCM concentration, are being explored as methods to modulate hydrogel mechanical properties.

The physicochemical properties of enzymatic degradation rate, swelling and gel porosity are critically linked to the gel composition and degree of crosslinking. The characterization of these hydrogel properties is vital, these parameters will dictate the hydrogel mechanical properties as well as the cells' ability remain viable within and remodel their matrix microenvironment. Specifically, the degradation rate of the hydrogel must occur on a physiologically relevant time and length scale such that the bulk of the hydrogel remains intact while cells are able to digest areas of hydrogel local to their surroundings. Therefore, studies are underway to measure the enzymatic degradation rate of the hydrogels of varying composition in an effort to correlate synthesis variables with degradation. Studies to date have focused on measuring the baseline degradation of CMC-based hydrogels in cellulase and collagenase solutions. Degradation in cellulase (a non-mammalian enzyme) provides insight into the hydrogel structure-physicochemical properties, while collagenase (our targeted enzymatic degradation mechanism) will serve as a predictor for subsequent MEC remodeling studies. We have also characterized the hydrogel swelling ratio (an indirect measure of crosslink density) and mechanical properties; studies of gel porosity (diffusion of solutes of various molecular weight) and MEC viability are in progress.

To study the effect of ECM stiffness on apoptosis responsiveness of a mammary epithelium in culture, and in vivo. These studies were conducted in collaboration with Dr. Eric Bernhard from the Radiation Biology Department at the University of Pennsylvania. .

In the latter part of this first year of grant funding we exploited the newly developed basement membrane-conjugated PA gels with precisely controlled matrix stiffness developed earlier in the year for our studies aimed at exploring putative functional links between the physical properties of the extracellular matrix microenvironment and the cells apoptotic regulatory mechanisms. To achieve this goal we conducted studies of mammary epithelial cell apoptotic responsiveness on gels of high

compliance, representing the normal mammary gland OR alternately regions of primary mammary tissue dramatically remodeled by MMPs or within the core of a necrotic breast tumor, and compared this behavior to the death responsiveness of mammary epithelial cells interacting with a matrix of considerable stiffness, analogous to what we measured in primary tumors, particularly at the leading edge of a tumor where the breast cells interact with the surrounding desmoplastic, collagen rich matrix, or with the matrix found in liver or in parts of the brain or bone. From these studies we deduced that matrix stiffness strongly modifies the ability of mammary epithelial cells to respond to exogenous apoptotic insults including chemotherapies such as taxol, immune receptor activators such as trail and stress insults such as gamma irradiation. Mammary tissue structures interacting in three dimensions with a highly compliant laminin rich matrix exhibit apoptosis resistance to all three types of apoptotic stimuli whereas those interacting with a stiffer matrix die much more readily (see Figures 15, 16, 17 & 18) . It should be noted however, that the enhanced death responsiveness of mammary tissues interacting with a stiffer matrix in three dimensions is still greatly lower than that exhibited by cells grown on traditional tissue culture plastic, consistent with the differential responsiveness of cells lines to apoptotic insults in xenograft studies in vivo. Not only could we show a differential apoptotic responsiveness of mammary tissues in response to death cues but subsequent studies revealed that mammary epithelial cells acquire the greatest enhanced to apoptotic death insults after 4 days of culturing on a compliant matrix, coincident with the acquisition of tissue polarity (see Figure 20) Furthermore, our data suggest that differential apoptotic sensitivities are NOT merely due to altered proliferation regulation or differential cell cycle dynamics (see Figures 19-21. Instead, we found that cells interacting with a stiffer matrix showed enhanced activation of JNK kinases in response to exogenous death stimuli (see Figure 23) and that inhibiting JNK activity could temper this heightened sensitivity. Studies are now underway to further explore this potential link between JNK signaling, apoptosis regulation of matrix compliance.

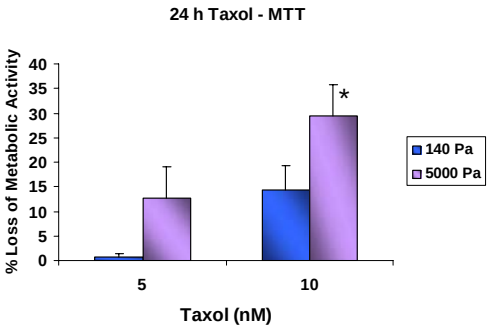
Transgenic animal studies designed to test whether ECM stiffness could influence apoptosis regulation in vivo. These studies have been conducted in collaboration with Dr.s Gasser and Kissil.

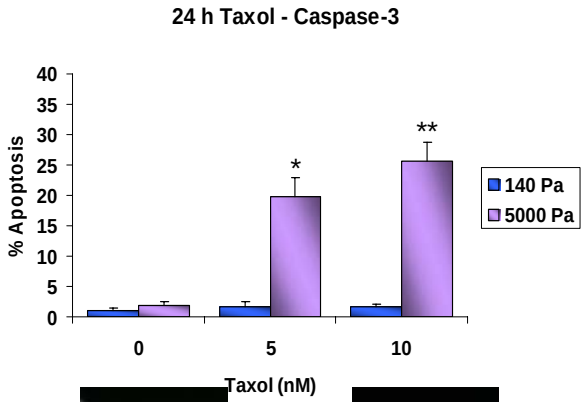
Organotypic culture studies indicated that matrix stiffness altered mammary cell and tissue behavior by altering integrin clustering to drive maturation of focal adhesions. The relevance of this phenotype has yet to be tested in vivo. Accordingly, we outlined our plan to develop animal models to test the effect of matrix stiffness on mammary tissue behavior in vivo. Towards this goal we have designed and thereafter developed a CRE-LOX transgenic mouse expression vector for our V737N beta 1 spontaneous clustering integrin. Studies are now in progress to establish stable mouse ES lines so that we can begin our mouse colony derivation. In addition, we have secured and begun testing the effect of lysyl oxidase activity on collagen mediated cross linking and matrix stiffening. Towards this goal we have obtained a lysyl oxidase antibody as well as an expression construct to a constitutively active lysyl oxidase and BAPN compound which is a specific lysyl oxidase inhibitor. In the next year we hope to begin experimentation with these various lysyl oxidase reagents as a means to determine the effect of manipulating matrix stiffness in vivo on mammary tumorigenesis and treatment responsivness.

To assemble and generate cell biology and published data required for basic computational model. These studies have been conducted in collaboration with Dr. Hammer from the Department of Bioengineering at the University of Pennsylvania and Dr. Tobias at the University of Pennsylvania Bioinformatics Center.

In the past year we have established versatile methods to isolate high quality RNA from our protein-laminiated-conjugated basement membrane PA gels so that we can begin to prepare and analysis gene expression patterns induced in mammary epithelial cells by changes in matrix stiffness. We have also been working diligently with Dr. Hammer and two co supervised graduate student Matt Paszek and

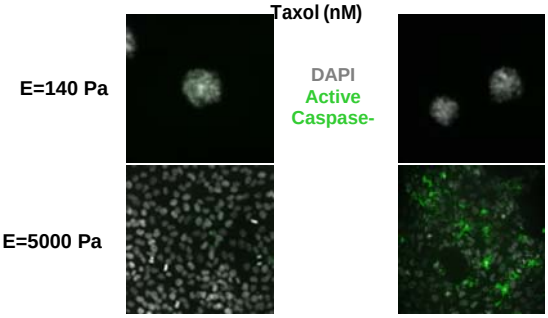
Figure 15. Matrix stiffness enhances apoptosis induction in response to chemotherapeutics such as Taxol





n=3; *p<0.05

MECs were grown for 4 days on PA gels and treated with apoptotic agents for 24 hours before performing an MTT assay or staining for activated caspase-3.



n=4;

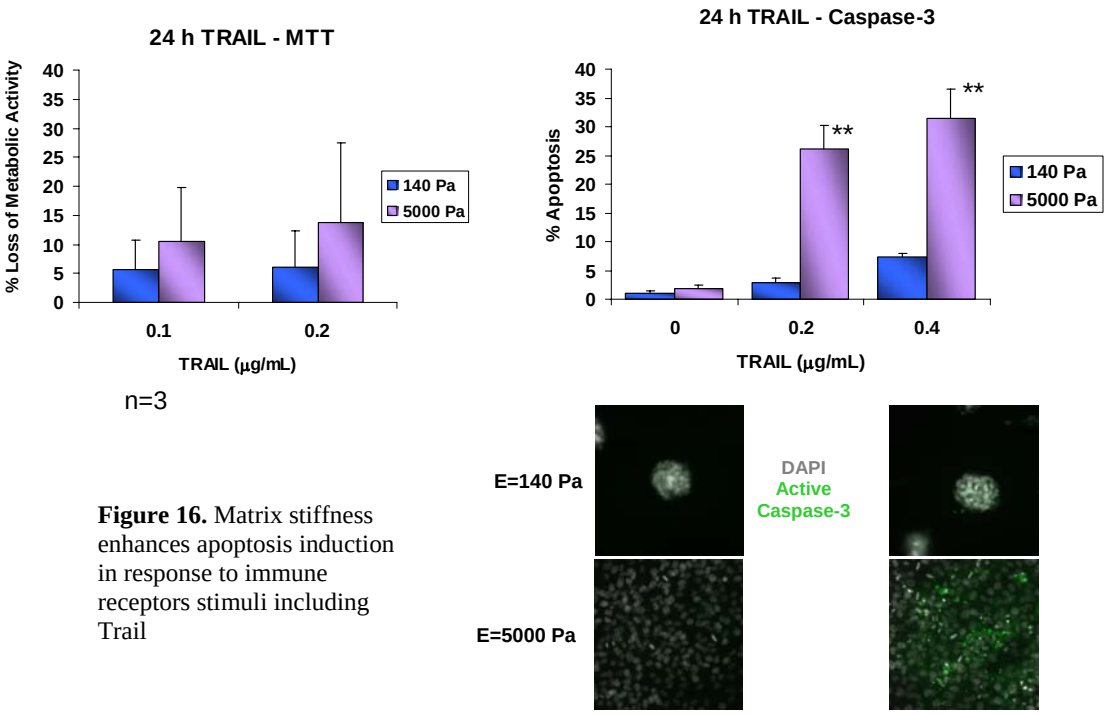


Figure 16. Matrix stiffness enhances apoptosis induction in response to immune receptors stimuli including Trail

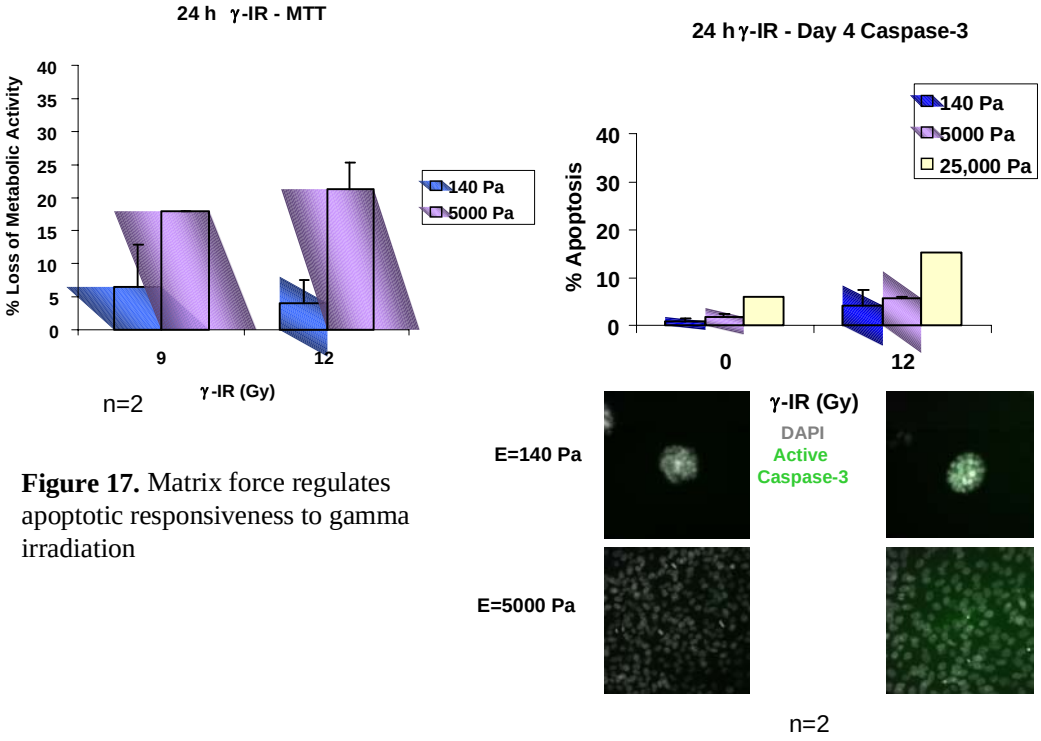
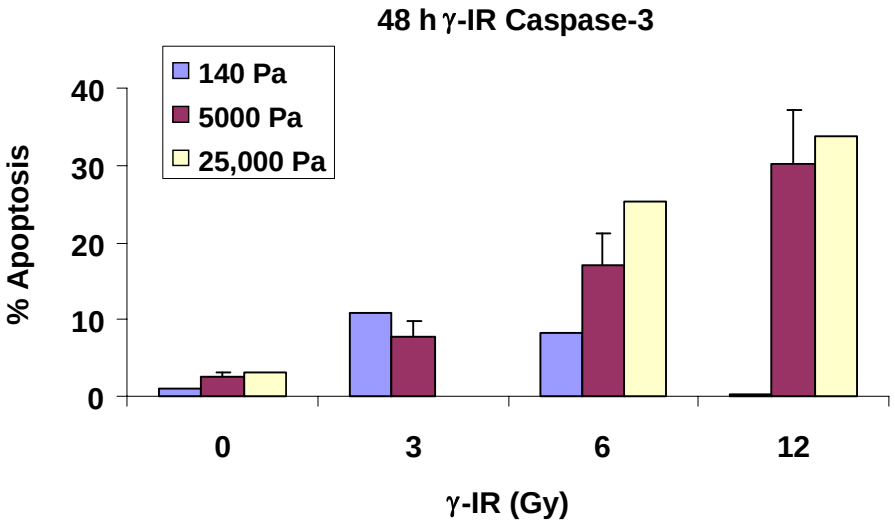


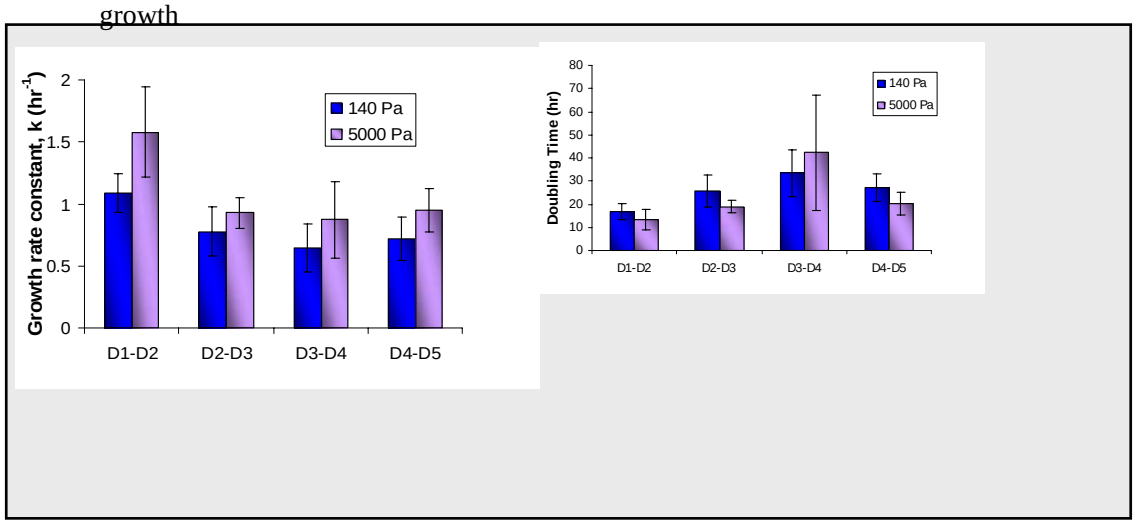
Figure 17. Matrix force regulates apoptotic responsiveness to gamma irradiation

Figure 18. Matrix force regulates apoptotic responsiveness to gamma irradiation



140+5000 Pa, n=3; 25,000 Pa, n=1

Figure 19. Matrix force does NOT regulate the rate of mammary epithelial cell growth



n=4; p=0.40 n=4; p=0.40

The growth rate constant, k, was derived using the equation: $N/No = e^{kt}$, where No is the initial cell number, and N is the cell number at time t. The doubling time, t_d, was calculated using the derived k and setting N/No = 2 in the following equation: $t_d = (\ln 2)/k$.

Figure 20. Matrix force does not alter cell cycle regulation

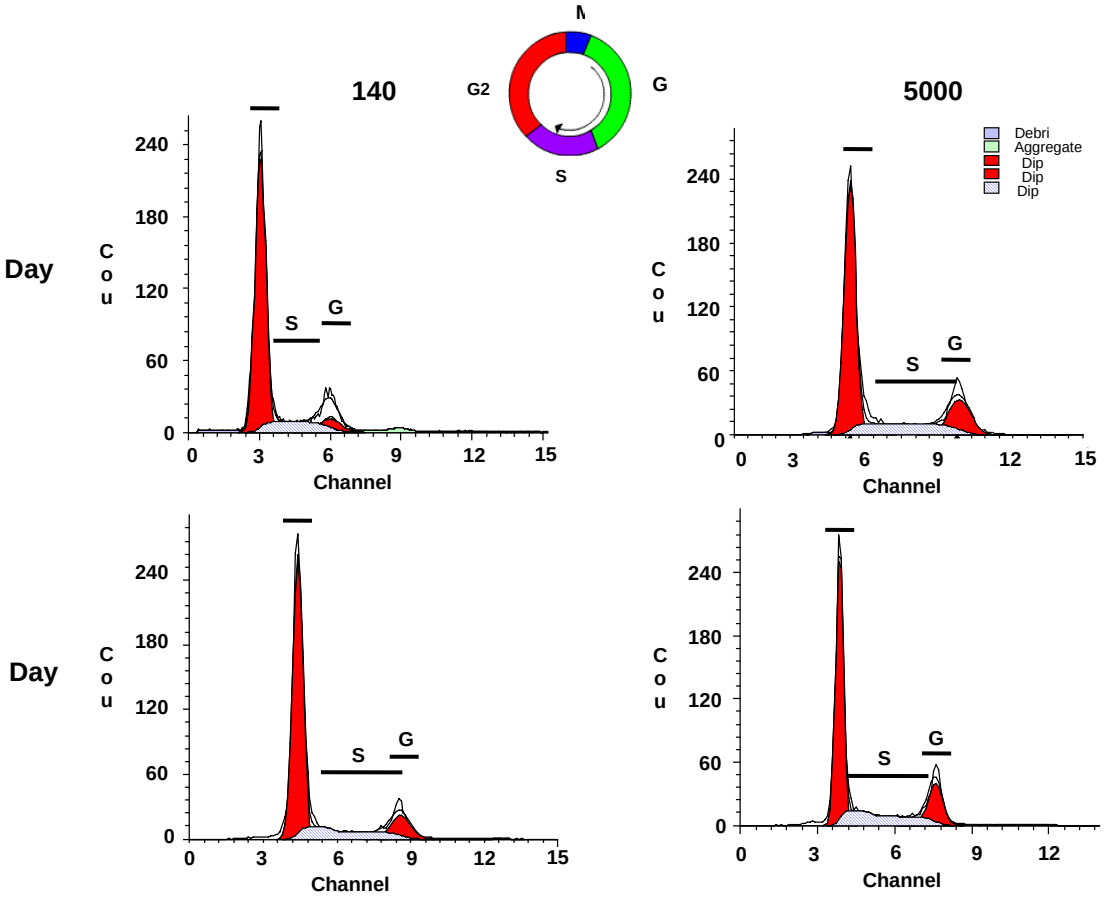
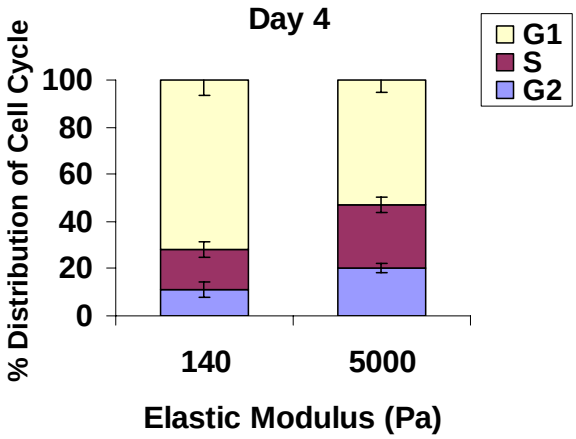


Figure 21. Matrix force does not alter cell cycle regulation

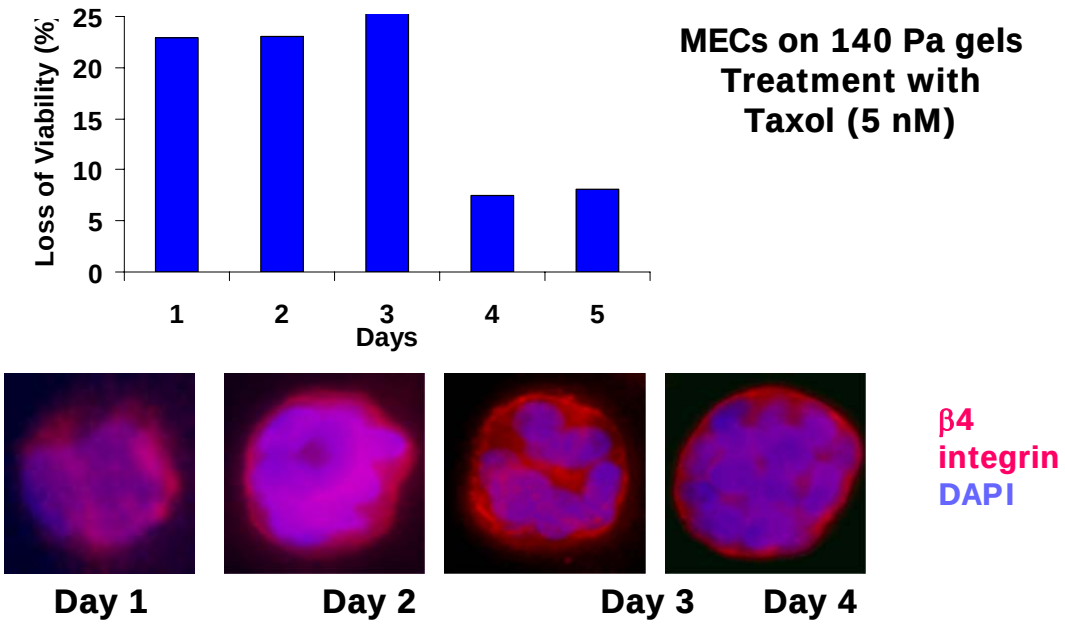


G2+S (%):	27.9 ± 6.3	46.8 ± 5.1

n=3; p=0.07

MECs were grown for 4 days on PA gels, fixed with 96% ethanol, stained with propidium iodide and analyzed for DNA content using the FACS scan.

Figure 22. Matrix compliance promotes apoptosis resistance by four days of 3D basement membrane culture, coincident with acquisition of tissue polarity illustrated by basal relocalization of



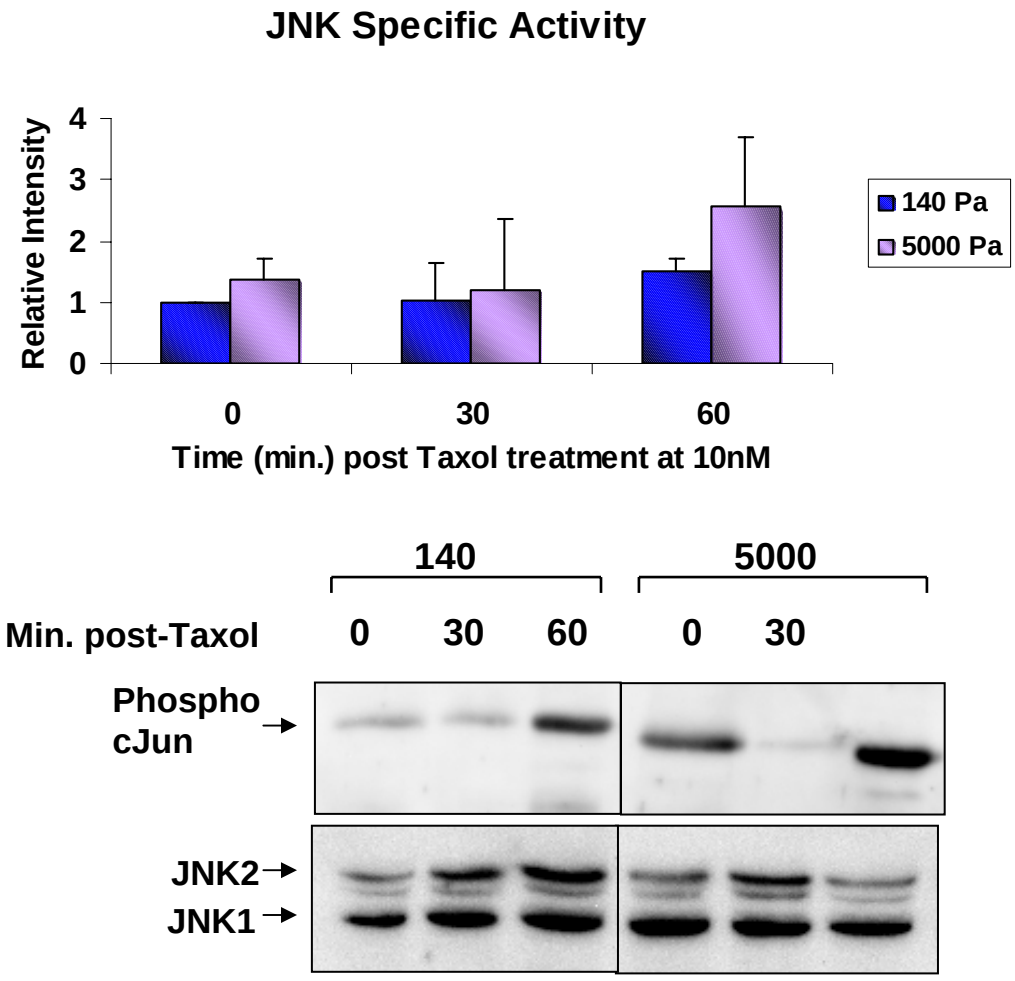


Figure 23. Matrix force regulates stress-dependent JNK activation and increases the specific activity of JNK

KellyYee to derive deterministic models of matrix and force regulated integrin adhesion dynamics and ERK-dependent growth and contractility. We anticipate that within the next year or so we will have working models that can be tested experimentally.

KEY RESEARCH ACCOMPLISHMENTS:

Task 1: *Engineer tractable 3D organo-typic models that recapitulate the biophysical properties of primary and metastatic breast tumor tissues and use these models to dissect candidate molecular mechanisms whereby ECM stiffness could regulate apoptosis resistance in culture and in vivo*

PART A. Development of natural 3D ECM models that recapitulate the biophysical properties of primary normal and malignant metastatic breast tissues.

- **Completed** experimental measurements using compression analysis of normal and malignant mouse breast tissue and that of metastatic sites of breast cancers including the brain, lung and liver.
- **Completed** experimental measurements using shear (indentor) analysis of normal and malignant mouse breast tissue.
- **Completed** initial orientation and analysis measurements of AFM analysis of normal mouse breast tissue.
- **Completed** experiments to manipulate nature ECM gel stiffness to recapitulate the range of stiffness/forces normal and malignant MECs would experience in vivo in both primary normal and malignant breast and in known metastatic tissues by keeping BM concentration constant and increasing collagen concentration.
- **Completed** experimental measurements using compression/indentor analysis of BM/collagen gel preparations for a broad range of matrix stiffness as above.
- **Completed** experimental measurements using shear modulus analysis of BM/collagen gel preparations for a broad range of matrix stiffness as above.
- **Completed** manipulation of natural ECM gel stiffness – keeping BM and collagen concentration constant through ECM cross linking induced through addition of metabolizable ribose.
- **Completed** rheological analysis of ribose cross linked BM/collagen gels using shear modulus measurements.
- **Completed** the generation of constitutively activated lysyl oxidase retroviral and lentiviral expression constructs
- **Completed** successful establishment of nontransformed fibroblast lines expressing a recombinant constitutively active lysyl oxidase
- **Completed** the analysis of the effect of changing ECM stiffness on the morphogenesis behavior of normal MECs in BM/collagen gels.
- **Initial studies completed** to assess the effect of increasing ECM stiffness on the morphology of normal MECs in BM/collagen gels that have been progressively cross-linked through chronic additional of ribose.
- **Completed** the analysis of the effect of increasing ECM stiffness on the growth behavior of normal MECs in BM/collagen gels.
- **Preliminary studies completed** to assess the effect of increasing ECM stiffness on the apoptosis sensitivity of normal MECs to three common classes of agents including chemotherapies (taxol, doxorubicin), immune receptor stimuli (trail, TNFalpha) and gamma irradiation.

PART B. Development of synthetic 3D model systems that recapitulate the biophysical and biochemical properties of primary and metastatic breast tumor tissues. Studies have been conducted in collaboration with Drs. Wong at Boston University and Cam at MIT.

- **Wong Laboratory successfully completed** pilot studies to test the feasibility of generating photo-activatable PVC gels for easy manipulation of matrix stiffness in 3D.
- **Two Weaver graduate students** Jennifer Leight and Kandice Johnson visited MIT and Boston University in the early winter of 2005 and as a results have been **successfully trained** to work with self-assembling peptide gels.
- **Exhaustive preliminary studies have been completed** by the Weaver laboratory to assess the biocompatibility of self-assembling peptides for conducting an assessment of the effect of matrix stiffness on MEC behavior in 3D.
- **Successful transfer of technology and methods** from the Wong and Cam laboratories to the Weaver laboratory students and technical staff to conduct compliance measurements on self-assembling peptide gels.
- **Successful establishment** of 3D methods to modulate the compliance of self-assembling peptide gels in the Weaver laboratory.
- **Successfully methods establishment** to make reproducible and valid measurement of the biophysical properties of self-assembling peptide gels in the Weaver laboratory.
- **Successfully incorporation** of YIGSR and RGD peptides via stable cross-linking and transfer of self-assembling peptides from the CAM laboratory to the Weaver laboratory to test efficacy for induction of mammary morphogenesis.
- **Completion of methodology** to establish PVD synthetic gels cross-linked to RGD and YIGSR peptides in Wong laboratory.
- **Successful demonstration** of biocompatibility of PVD synthetic gels to fibroblasts and also to smooth muscle cells
- **Successful shipments** of unconjugated and RGD and YIGSR-conjugated PVD and PEG gels from the Wong laboratory to the Weaver laboratory and preliminary studies completed to assess the biocompatibility of the synthetic matrices to human MECs.

Task 2: *Develop xenograft and transgenic mouse models to test whether ECM stiffness regulates apoptotic responsiveness of mammary epithelia in vivo*

PART A. Xenograft studies to test whether ECM stiffness could regulate apoptosis responsiveness of a mammary epithelium in vivo. These studies were conducted in collaboration with Dr. Eric Bernhard from the Radiation Biology Department at the University of Pennsylvania.

- **In collaboration with the Bernhard laboratory the Weaver group has successfully completed** an assessment of the gamma radiation sensitivity of single nonmalignant and tumorigenic MECs embedded within compliant rBM gels using short term viability as an end point
- **Successful completion** of assessment of the gamma radiation sensitivity of nonmalignant and tumorigenic colonies of nonmalignant and tumorigenic MECs embedded within compliant rBM gels using short term viability as an end point.
- **Successful completion** of assessment of the gamma radiation sensitivity of single nonmalignant and tumorigenic MECs embedded within compliant rBM gels ex vivo using long-term re-growth assays as the end point.

- **Successful completion** of the assessment of the gamma radiation sensitivity of colonies of nonmalignant and tumorigenic MECs embedded within compliant rBM gels ex vivo using long-term viability assessment AND clonogenic survival assessment as the end point.

PART B. Transgenic animal studies designed to test whether ECM stiffness could influence apoptosis regulation in vivo. These studies have been conducted in collaboration with Dr.s Gasser and Kissil.

- **Successful design** of CRE-LOX conditional expression vector for b1 integrin cluster mutant
- **Successful generation** of CRE-LOX b1 integrin cluster mutant expression construct for generation of transgenic mouse model.

Task 3: *Build a computational model that can predict how changes in ECM compliance could influence integrin-dependent apoptosis responsiveness of mammary epithelia and query this model with clinical data*.

PART A. To assemble and generate cell biology and published data required for basic computational model. These studies have been conducted in collaboration with Dr. Hammer from the Department of Bioengineering at the University of Pennsylvania and Dr. Tobias at the University of Pennsylvania Bioinformatics Center.

- **Successful completion** of studies to assess the state and type of integrin behavior and adhesions formed in natural and synthetic matrices of varying compliance
- **Successful completion** of analysis of effect of matrix compliance/stiffness on ERK signaling in natural and synthetic matrices of varying compliance
- **Completion of preliminary studies** to assess the effect of varying matrix compliance/stiffness on JNK signaling
- **Completion of preliminary studies** to assess the effect of varying matrix compliance/stiffness on PI3 kinase signaling.

PART B. Generate a simple cell adhesion computational model based upon published values from the literature and data generated using culture models. These studies are now in progress and will be reported under reportable outcomes for the 2007 Annual Report for this grant.

PART C. Incorporate mechanical force values and assumptions into the basic adhesion model. These are future studies that have now been initiated and will be reported in future Annual Reports as reportable outcomes.

PART D. Initiate modeling studies using micro array data sets from the cell culture models developed by my group. These studies are being conducted by the Weaver laboratory in collaboration with Dr. Dan Hammer of the Bioengineering Department at the University of Pennsylvania and Dr. John Tobias in the University of Pennsylvania Bioinformatics Center.

- **Successfully developed methodology** to extract high grade RNA from 2D and 3D matrices of varying matrix compliances
- **Successfully purified and prepared** samples for micro array analysis from 3D compliant matrices

PART E. Initiate pilot studies to analyze micro array data sets and clinical samples from neoadjuvant breast cancer clinical trial data using a simple model generated using gene data from the culture system. These are studies that will not be initiated for a few years after the earlier studies have been completed.

Task 4: *Develop non-invasive imaging tools that could be used to monitor changes in ECM stiffness or stiffness-induced changes in mammary tissue phenotype.*

PART A. Proof of principal studies for imaging sensitivity using 3D culture models. These are studies that will not be initiated for a few years after the earlier studies have been completed.

PART B. Set up and initiate screening trials with peptide libraries for identification of novel stiffness markers in the ECM. These are studies that will not be initiated for a few years after the earlier studies have been completed.

Reportable Outcomes:

A. Manuscripts

1. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D, Hammer DA, **Weaver VM.** Tensional homeostasis and the malignant phenotype. Cancer Cell 2005; 8(3): 241-254.

2. Paszek, M. and **Weaver V.M.** The tension mounts: mechanics meets morphogenesis and malignancy. J Mammary Gland Biol Neoplasia. 9:325-42, 2004. **NOTE:** This manuscript was written and submitted AFTER funding was received by the DOD BCRP Scholar Award and therefore this grant has been credited towards the support of this article – however the publication schedule of this journal is almost ONE full year behind schedule.

3. Johnson KR, Leight JL, **Weaver VM.** "Demystifying three-dimensional force and tissue morphogenesis." Methods in Cell Biology: Cell Mechanics. Academic Press, San Diego, Submitted.

B. Abstracts

1. Zahir, N., Paszek, M., Johnson, K.R., Lakins, J.N., Lynch, L., Rozenberg, G.I., Dembo, M., Hammer, D.A., Margulies, S.S., and Weaver, V.M. Tensional homeostasis and malignant transformation of a mammary epithelium. Keystone Meeting, 2005, Banff, Canada.

2. Johnson KR, Lakins JN, Friedland JC, Weaver, VM. Dynamic and reciprocal link between forces and PTPs in normal and malignant mammary behavior. Poster Presentation. BMES Annual Fall Meeting, Baltimore, MD, 2005.

3. Leight, J., Chatterjee, C., Zahir, N., Nuth, M., Lakins, J., Bernhard, E., and Weaver, V.M. Force-dependent apoptosis resistance and breast tumorigenesis. BMES Baltimore, MA, 2005.

4. N. Zahir, J. Leight, B. Alston-Mills, and V.M. Weaver. Matrix stiffness differentially regulates Jun Kinase to modulate apoptosis responsiveness of a mammary epithelium. Abstract submitted to the American Society for Cell Biology, 45th Annual Meeting, San Francisco, 2005.

5. Leach, J.B., Leight, J.L., Johnson, K.R., Zahir, N., Paszek, M.J., Sieminski, A., Spirio, L., Wong, J.Y., and Weaver, V.M. Engineered 3D models to study force-dependent mammary morphogenesis and malignancy. ASCB 45th annual meeting, San Francisco, CA, 2005.

6. Paszek, M.J., Zahir, N., Johnson, K.R., Dembo, M., Hammer, D.A., and Weaver, V.M. Mechanical crosstalk and feedback between integrins and ERK. ASCB 45th annual meeting, San Francisco, CA, 2005.

C. Oral Meetings Presentations:

1. **Weaver, V.M.** Tensional force, cell signaling and epithelial differentiation and survival. Gordon Conference on Radiation Oncology, Ventura, CA, February 2, 2005.

2. **Weaver, V.M.** Tissue polarity and the mechanism of epithelial resistance to chemotherapy, Keystone Meeting on Microenvironment in Tumor Induction, Banff, Alberta, Canada, February 7, 2005.

3. **Weaver, V.M.** Tensional regulation of tissue morphogenesis and malignant transformation, Lorne Cancer Conference, Melbourne, Australia, February 12, 2005.

4. **Weaver, V.M.** Tumor microenvironment in the 3rd Dimension, AACR Major Symposium on Stromal-epithelial interactions, Anaheim, CA, April 18, 2005.

5. **Weaver, V.M.**Microenvironmental forces driving malignant transformation and metastasis, Joint IRCC and EMBO Alpine Spring Conference on 'The invasive growth program: Concepts and technologies, Torino, Italy, May 27, 2005.

6. **Weaver, V.M.** Spatial-mechanical regulation of life and death of a mammary epithelium, 38th Annual Meeting of the Japanese Society of Developmental Biology, Sendai, Japan, June 3, 2005.

7. **Weaver, V.M.**Tissue architecture and Pathology, Era of Hope DOD Meeting, Philadelphia, PA, June 11, 2005.

8. **Weaver, V.M.** Force-dependent spatial regulation of signaling and morphogenesis, Gordon Conference on Mechanisms of Cell Signaling, Hong Kong University of Science and Technology, Hong Kong, China, June 14, 2005.

9. **Weaver, V.M.** Implications of matrix remodeling on tumor progression, NCI special meeting on Mouse Models of Human Cancers Consortium, New Brunswick, New Jersey, June 29, 2005.

10. **Weaver, V.M.** Spatial-Mechanical regulation of mammary morphogenesis and malignancy, ASCB-ECI Conference on Engineering Cell Biology, Washington, Seattle, July 16, 2005.

11. **Weaver, V.M.** A biophysical perspective of epithelial morphogenesis, malignancy and treatment responsiveness AACR Special Conference on Cancer, Proteases, and the Microenvironment, Bonita Springs, FL, December 2, 2005.

D. Invited Institutional Presentations:

1. **Weaver, V.M.** The tension mounts: mechanisms meets mammary morphogenesis and malignancy, Department of Cell Biology, UMass Medical School, Worcester, MA, March 23, 2005.

2. **Weaver, V.M.**Tension, Tumors and Trauma, NIH NIDCR, Bethesda, MA, April 7, 2005.

3. **Weaver, V.M.**Form, fate and flexibility, Vontz Center, University of Cincinnati College of Medicine, Cincinnati, OH, May 12, 2005.

4. **Weaver, V.M.** Tensional-homeostasis and morphogenesis, Center for Developmental Biology, Kobe, Japan, June 7, 2005.

5. **Weaver, V.M.** Spatial-mechanical signaling and tumorigenesis, Science Lecture series, Jefferson Institute of Molecular Medicine, Philadelphia, PA, October 3, 2005.

6. **Weaver, V.M.** A Spatial-Mechanical Perspective of Mammary Morphogenesis, Malignancy and Treatment, Dept Surgery, USCF, San Francisco, CA, October 25, 2005.

7. **Weaver, V.M.** The tension mounts: mechanics meets morphogenesis and malignancy, MD Anderson, Houston, TX, November 2, 2005.

8. **Weaver, V.M.** A Biophysical Perspective of Mammary Gland Development and Tumorigenesis, The Cancer Institute of New Jersey, New Brunswick, NJ, February 22, 2006

Progress Summary and Conclusions

Over the past year we have made considerable progress towards achieving our stated goals. Using several complimentary approaches we have systemically measured the changes in matrix stiffness associated with malignant transformation of an experimental mammary tumor model in vivo (transgenic MMTV Her2/neu as well as the elastic modulus of common metastatic breast cancer tissues. To determine the relevance of these stiffness changes to mammary tissue phenotype and behavior we have spent considerable time and effort towards developing both natural and synthetic biomaterials for organotypic culture studies. Thus, we have used an array of culture models including collagen gels, collagen/basement membrane or laminin hydrogels, 2D acrylamide gels, as well as self assembling peptide gels and newly developed PEG/methylcellulose gels with defined ECM compositions and a range of calibrated stiffness with a similar magnitude and range to what we and others have detected in the gland during transformation. Using these natural and synthetic materials we have made considerable progress towards exploring the role of ECM stiffness on mammary tissue phenotype and apoptosis responsiveness. We have thus far been able to demonstrate that matrix stiffness profoundly influences the behavior of normal and transformed mammary epithelial cells. We have specifically demonstrated that matrix stiffness alters mammary cell proliferation by enhancing the magnitude and duration of ERK activation in response to growth factors such as epidermal growth factor, and modulates the apoptotic responsiveness of mammary epithelial cells to chemotherapeutics, immune stimuli and gamma irradiation possibly by modulating stress signaling pathways. Towards delineating a molecular mechanism we thus far have found that ECM stiffness independently induces expression and activation of integrins and alters Rho GTPases. By independently increasing collagen stiffness in 3D tissues and the stiffness of basement membrane-laminated PA gels we could show that ECM stiffness perturbs cell-cell integrity, disrupts tissue polarity, inhibits apoptosis-dependent lumen formation and promotes an invasive phenotype. We have also made good progress towards the establishment of animal models to study the effect of matrix force on mammary tumor behavior in vivo including the establishment of

transgenic expression vectors which mimic the changes induced by force in vivo on integrin clustering. Towards establishing a computational model we have been working diligently with Dr. Hammer and two graduate students Matt Paszek and Kelly Yee to create a deterministic model of mechanically-regulated adhesion-dependent mammary epithelial cell growth regulation and integrin-dependent signaling. Finally, we have been working with colleagues to develop appropriate technology for imaging of live tissues – initially for the assessment of matrix stiffness changes in animal models and thereafter for assessment of clinical breast tumor patient samples. For these imaging experiments we are applying imaging elastography and sonoelastography methodologies that exploit MRI and ultra sound technology respectively. Although, our initial statement of work stated that we would use and/or develop quantum dot technology – we were rightfully critiqued for being too ambitious and feel that the ready availability of MRA and ultrasound imaging approaches constitutes a more tractable approach to developing imaging modalities to assess changes in matrix stiffness surrounding developing human breast lesions. Below we summarize and highlight our results for assessment of our current progress. The results of this past years efforts have been presented at national and international conferences (see list of abstracts) and have also been published in peer reviewed journals and as review articles. Additional observations are currently being completed and prepared for subsequent submission for peer reviewed publication and will be reported in next years progress report summary.

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1. Taylor, S.T., J.A. Hickman, and C. Dive, *Epigenetic determinants of resistance to etoposide regulation of Bcl-X(L) and Bax by tumor microenvironmental factors.* J Natl Cancer Inst, 2000. **92**(1): p. 18-23.
2. Zahir, N. and V.M. Weaver, *Death in the third dimension: apoptosis regulation and tissue architecture.* Curr Opin Genet Dev, 2004. **14**(1): p. 71-80.
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