THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF BIOCHEMISTRY & MOLECULAR BIOLOGY

MATRIX MANIPULATION AFFECTS ATTACHMENT AND GROWTH OF BREAST CANCER CELLS IN A BONE-LIKE MICROENVIRONMENT *IN VITRO*

SHELBY JEAN FOSTER
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Reviewed and approved* by the following:

Andrea M. Mastro
Professor of Microbiology and Cell Biology
Thesis Supervisor

David Gilmour
Professor of Molecular and Cell Biology
Honors Adviser

Scott B. Selleck
Professor and Department Head of Biochemistry and Molecular Biology

* Signatures are on file in the Schreyer Honors College.

ABSTRACT

Breast cancer is one of the most common and deadly cancers, but treatment of the primary tumor is usually very successful if the condition is realized early. The disease becomes more deadly once metastasis occur sand cancer cells spread from the primary tumor to secondary sites. One of the most common sites of breast cancer metastasis is the skeletal system. Breast cancer metastasis to bone is a painful process for a patient to endure, as breast cancer cells interrupt the healthy balance of buildup and breakdown of the bone. Introduction of breast cancer cells into the bone microenvironment causes an upregulation of bone degradation by increasing the activity of osteoclasts. Osteoblasts, bone forming cells, also are affected. They are unable to make up for the increased degradation, and the breast cancer invasion causes bones to become weak and more susceptible to trauma.

It was hypothesized that specific changes to the bone microenvironment altered the attachment and growth of breast cancer cells within the bone. The aim was to determine the role of the bone extracellular matrix in breast cancer colonization. Experiments were conducted *in vitro* by differentiating mouse osteoblasts for four weeks until they created a bone matrix. In order to distinguish the osteoblasts from their secreted matrix, the cultures were altered through fixation and decellularization. Human metastatic breast cancer cells, MDA-MB-231 and an isologous line that carries a metastasis suppressor gene, MDA-MB-231 BRMS1 were added to the cultures of osteoblasts and attachment and growth of the cancer cells were Monitored. Most notably, breast cancer cells attached and proliferated more on a fixed osteoblast cultures than on decellurized or live osteoblast cultures. It was concluded that the growth was due to a change in

the structure of the matrix after fixation. This result may correlate with a reduction of EPLIN, a protein responsible for fixation of surfaces, in metastatic cancer cells including MDA-MB-231.

A second aim was to test another relevant modification of the bone environment, brought about by estrogen during osteoblast differentiation. Estrogen effects were important to test because breast cancer metastasis to bone occurs most often in women after menopause when less estrogen is produced in the body. Inhibiting estrogen receptors during osteoblast differentiation noticeably changed collagen fiber rearrangement and reduced the total amount of protein produced by the osteoblasts. Estrogen inhibition, however, did not affect breast cancer cell attachment or proliferation on a decellularized matrix, but significant changes in both attachment and proliferation were observed when the live osteoblasts were left intact. ELISA analysis also showed that more of the inflammatory and bone remodeling cytokine IL-6 is produced by MC3T3-E1 cells when they are grown with the estrogen inhibitor and exposed to the breast cancer cells. It was concluded that estrogen inhibition causes an inflammatory environment that is more supportive of breast cancer cells.

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Dr. Mastro, my research adviser, has been a large part of my success at Penn State. I would like to thank her for allowing me to work in her lab for the past three and a half years. Due to my experience in Dr. Mastro's lab, I realized that I wanted to pursue a career in science. Dr. Mastro helped me in many of my accomplishments here, including several research awards and with the opportunity to present my research at a national breast cancer research conference. Within Dr. Mastro's lab, there have been several other influential figures whom I would like to thank. I would not be where I am without the help of Donna Sosnoski – Dr. Mastro's long time lab tech and our "lab mom". Thank you, Donna, for teaching me so many techniques and the ins and outs of a career in science. I would also like to acknowledge several of lab mates, especially Bobby Norgard, Richa Pursnani, Walter Jackson, Jonathan Vincenty, and Adam Mobley for helping with my experiments and being good friends.

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LIST OF ABBREVIATIONS

ECM – Extracellular Matrix

EPLIN – Epithelial Protein Lost in Neoplasm

FBS – Fetal Bovine Serum

- all FBS used in the following experiments was heat inactivated

FGFs – Fibroblast Growth Factors

GFP – Green Fluorescent Protein

IL – interleukin

MCP – Monocyte Chemoattractant Protein

PBS – Phosphate Buffer Saline

PF – Paraformaldehyde

TGFβ – Transforming Growth Factor β

TNFα – Tumor Necrosis Factor α

Chapter 1

Introduction

Breast Cancer

Breast cancer is one of the most well-known cancers because it affects so many people; approximately one in eight women and one in 1,000 men will face breast cancer during their lives. The cancer also has one of the highest death rates among all cancers along with lung and skin cancers. Breast cancer is defined as a malignant tumor made up of a group of invasive cells originating in the breast issue; breast cancer that spreads away from the primary breast site and grows elsewhere in the body is metastatic breast cancer. Primary breast cancer, especially if it is detected early is much easier to treat than metastatic cancer, making routine screening procedures such as mammograms so important (American Cancer Society, 2016). The five year cure rate for localized breast cancer has been reported to be as high as 99%, but this percentage quickly drops as low as 24% with time and greater chance for the cancer to successfully spread to other parts of the body. Many problems arise when breast cancer metastasizes away from the primary breast tumor to secondary sites. The cancer commonly spreads to bone, lung, brain and other sites (DeSantis, 2014).

Metastasis to Bone

The spread of cancer to the skeletal system is one of the most common and devastating side effects of breast cancer. Bone metastases occur in 80% of breast cancer patients with advanced disease. Researchers have yet to fully understand what draws these metastatic cells to the bone environment. Cancer cells must first detach from a primary tumor, activate angiogenesis in order to enter and migrate

through the circulation, and then localize in the bone. Once the cells have successfully established themselves within the bone, a process known as the vicious cycle begins and disrupts normal homeostasis within the bone. The amount and severity of metastases is also used in the staging of breast cancer. Localized breast tumors are associated with higher rates of survival than those with metastases to secondary sites. Of these secondary sites, bone is one of the most common for breast cancer to invade and to colonize (Kozlow, 2005).

Much of the bone is composed of an ECM – nonliving material surrounding the cells within the bone that serves as a scaffold and reservoir for cytokines and other cell secretions. The ECM within the bone is made up of proteins, and a large percentage of this protein is collagen type I (Gentili C, 2009). Collagen is a very large protein with molecular weight ranging from 115 to 130 kilodaltons. Collagenous protein within the bone is very important for tensile strength of the bone due to the unique triple helical structure of the protein. Due to its large size and multiple helices, collagen is one of the few proteins that is assembled outside of the cell (Alberts B, 2002). Other than collagen, the bone matrix also contain non-collagenous glycoproteins such as hyaluronan and proteoglycans. Additionally, the ECM becomes mineralized via calcium deposition from osteoblasts which increases bone hardness. The ECM is a dynamic structure that is constantly receiving different secretions from the surrounding cells and regulating the function of the cells as well (Gentili C, 2009).

Bones are heterogeneous with several different types of cells that play varying roles in bone formation, immune system development, blood clotting, fat storage, and other functions. A healthy bone constantly undergoes remodeling, i.e. break down of old bone and buildup of new bone in order to keep the bones strong and to adapt to the growth of the organism. Cells named “osteoblasts” lay down new bone, while other cells called “osteoclasts” break down old bone. This dynamic environment is thought to be attractive to invasive circulating tumor cells due to the production and release of bone growth and remodeling factors such as transforming growth factor β (TGFβ), tumor necrosis factor α (TNFα), fibroblast growth factors (FGFs), and interleukin 1 (IL-1). IL-6 is also thought to be important to the

differentiation of osteoblasts, leading to eventual matrix maturation. When cancer cells enter the bone environment, they disrupt the normal cycle of bone maintenance beginning the vicious cycle, the initiation and development of cancer metastases in the bone (Canalis, 1988).

When cancer cells begin to proliferate within the bone, beginning the vicious cycle, they produce factors like IL-6, IL-11prostaglandin E2 (PGE2), TNF, MCSF that stimulate the activity of osteoclasts. Cancer cells also induce osteoblast precursor cells to make RANK ligand, further stimulating osteoclast activity (Kozlow, 2005). When osteoclast activity is upregulated, osteoclast resorption of the matrix is constitutively activated along with release of factors like TFGβ, IGFs, FGFs, and BMPs that increase cancer cell growth. By this cycle of crosstalk, introduction of cancer cells into the bone niche creates a cytokine environment that supports bone break down and increased cancer cell proliferation. Additional physical factors within the bone such a generally hypoxic environment, acidic pH, and high extracellular calcium concentration from bone breakdown may attract and promote growth of cancer cells within the bone microenvironment. Hypoxia, for example, has been shown to contribute to resistance to radiation and chemotherapy in patients. This suggests that the factors of the bone environment may actually make the metastatic cancer more difficult to treat (Kingsley, 2007). Continual breakdown of the bone can be an extremely painful process for a patient, leading to weaker bones that are more susceptible to trauma such as bone break (Figure 1) (Guise, 1988).

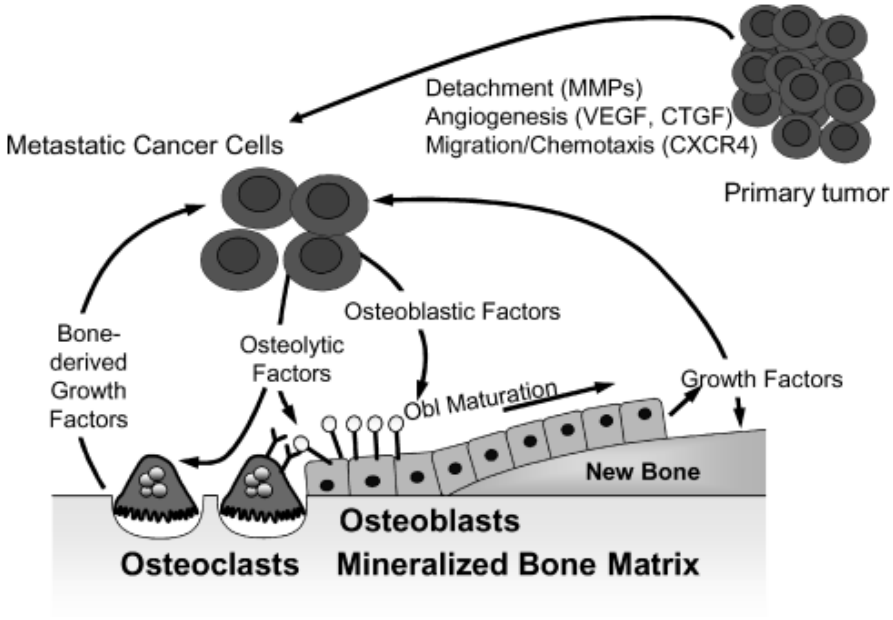


Figure 1. The Vicious Cycle Betwen Breast Cancer Cells and Bone

This figure summarizes the major parts of the process of breast cancer metastasis to the bone. Breast cancer cells detach from a primary tumor and spread to the bone through circulation. Once arriving at the site of the bone, tumor cells produce factors that stimulate osteoclast formation and consequently bone resorption. Taken from Kozlow.

Dormancy

Not all breast cancer cells make their presence known within the bone upon arrival. An additional complicating factor of metastasis to bone is dormancy, a state when cells lie in a quiescent or non-active state. During dormancy, little to no DNA transcription occurs and only enough mRNA to retain this quiescent state is translated. Cell division also occurs to a limited degree, and the cross talk between cancer cells and osteoclasts is downregulated. Osteoclast activity is not increased, and the bone still appears healthy. Possibly, due to the lack of inflammatory signal, dormant breast cancer cells within the bone often go unrecognized for many years (Meltzer, 1990). Cancer therapies do not prove successful against dormant cancer cells, as many patients have experienced recurrence of breast cancer in the bone

years after treatment of the primary tumor has ended. In some instances, breast cancer metastases to bone have been realized more than twenty years after treatment of the primary breast tumor (Omidvari, 2013).

Breast cancer cells can remain in dormancy for years until something “awakens” the cells. Researchers do not fully understand the process of exiting dormancy, but many cases of breast cancer metastasis to bone have been detected after a traumatic event like a bone break. In fact, chronic inflammation diseases such as arthritis have been show to increase instance of metastasis (Das Roy L, 2009). When a bone breaks, inflammatory signals such as IL-6 and MCP-1 are released locally in the bone as well as into the periphery. IL-6 has been shown to inhibit breast cancer cell apoptosis and promote proliferation. Additionally, bone remodeling cytokines such as TNFα and IL-β are upregulated and have been shown to stimulate cancer cell proliferation and awakening from dormancy (Sosnoski, 2015).

Hypotheses and Goals

The overarching goal of this project was to explore why breast cancer spreads to bone and to determine the properties of the bone matrix that make it appealing to metastatic breast cancer cells. More specifically, the project goal was to obtain more information regarding the role of the bone extracellular matrix (ECM) in this process. Based on the observation made by another researcher in the laboratory that breast cancer cells proliferated more readily *in vitro* on a paraformaldehyde fixed, well differentiated culture of osteoblasts than on the same cultures of live cells, it was hypothesized that the osteoblast produced ECM supports the attachment and growth of cancer cells. Additionally, the live osteoblasts may produce something that suppresses cancer cell growth. Because breast cancer and its metastasis is common in women following menopause, it was hypothesized that changes to the bone ECM caused by increasing or decreasing estrogen levels during osteoblast differentiation would affect the growth and attachment of breast cancer cells. Specifically, it was predicted that a lack of estrogen would create a bone

matrix that is more preferable for breast cancer cell colonization. These hypotheses were tested through a series of *in vitro* experiments using a mixed system model – human breast cancer cells and mouse osteoblast cells and their associated matrix. Experiments were conducted using mouse MC3T3-E1 osteoblasts and human MDA-MB-231 breast cancer cells. This model permitted us to distinguish cells, products, and markers produced by the cancer cells and the osteoblasts by use of species-specific antibodies.

Chapter 2

Materials and Methods

Growth of a "Mature" Bone Matrix Using MC3T3-E1 Osteoblasts

All experiments were conducted using MC3T3-E1 osteoblasts, a murine calvaria pre-osteoblast cell line gifted from Dr. Norman Karin of Pacific Northwest National Laboratories to the Mastro Lab. MC3T3-E1 cells were first isolated by the Kasai Group from the Tohoku Dental University in Japan (Sudo, 1983). The cells were cultured in growth medium (Table 1) to keep them in a pre-osteoblastic state until needed for an experiment where growing a mature osteoblast matrix was required (Wang, 1999). Cells were maintained in growth media, and were passed every three to four days to maintain cell health and prevent contact differentiation. MC3T3-E1 cells were not passed more than 30 passages. After the passage limit was reached, more cells were obtained from a liquid nitrogen stock of the immortal cell line.

Table 1. MC3T3-E1 Growth Medium (100 mL)

Component	Amount
Alpha Minimum-Essential Medium (α-MEM; Mediatech, Inc., Manassas, VA)	89 mL
FBS (Cansera, Rexdale, Ontario)	10 mL
Penicillin-Streptomycin (PenStrep; Mediatech, Inc., Manassas, VA; Penicillin 10,000 µg/mL; Streptomycin 25 µg/mL)100X	1 mL

In order to differentiate the cells into bone forming cells, they were plated in osteoblast differentiation medium (Table 2), which contains β-glycerolphosphate and ascorbic acid in order to facilitate collagen cross linking and matrix mineralization required for bone formation. β-glycerolphosphate induces matrix mineralization by providing necessary phosphate ions to osteoblasts so that the cells can produce hydroxylapatite mineral (Chung CH, 1993). Ascorbic acid acts as a cofactor in order for proline hydroxylation of collagen chains, a necessary step in order to form the helical structure of type I collagen (Shoulders, 2009). It was previously determined that a mature bone matrix was formed after the cells differentiated for approximately four weeks (Mercer, 2004). Osteoblasts were plated at a density of 10,000 cells/cm^2. Medium was changed every three to four days. The cells were kept in a humidified incubator at 37°C and 5% CO2.

Table 2. MC3T3-E1 Differentiation Medium (100 mL)

Component	Amount
Alpha Minimum-Essential Medium (α-MEM)	89 mL
FBS (Cansera, Rexdale, Ontario)	10 mL
Penicillin-Streptomycin (Pen-Strep) 100X	1 mL
Ascorbic Acid	5 mg
β-glycerolphosphate (1 Molar)	1 mL

Fixation

In order to preserve the matrix produced by the differentiated osteoblasts and prevent it from further change, the osteoblast cultures were fixed in some experiments. Fixation is also designed to harden tissues and prevent decomposition (Thavarajah, 2012). Differentiation medium was removed and the cultures were washed with PBS for five minutes. Fixation was carried out with addition of 1% paraformaldehyde diluted in PBS which was allowed to remain on cultures for five minutes at room temperature. After fixation, the cultures were washed three times for five minutes before the addition of any additional cell types for experimental purpose.

Decellularization

In order to remove cells from the matrix but leave the matrix intact, decellularization with deoxycholatic acid (12 mM deoxycholic acid in 10 mM Tris-HCl, pH 8) was performed. Medium was removed from the matrix, and the matrix was washed with PBS for five minutes. Deoxycholate was added to the matrix for ten minutes at 4°C, deoxycholate was removed, and the matrix was washed carefully three times with cold PBS (4°C). Matrices were stored at 4°C in PBS for up to one month for later use..

Alkaline Phosphatase Staining

In order to measure cell differentiation over periods of time with varying cell growth conditions, a stain for the enzyme alkaline phosphatase was used. Alkaline phosphatase is a marker of osteoblast differentiation. Throughout osteoblast differentiation different amounts of the enzyme are present on the cell membrane. The enzyme is responsible for dephosphorylating nucleotides and proteins under basic conditions and provides phosphate for mineralization.

The stain (Table 3) was completed by removing medium, rinsing the cultures with cold PBS, and fixing them with 10% formalin. Formalin was added to the matrices for ten minutes at room temperature. After fixation, the cells were rinsed three times with PBS before addition of the staining reagent. The stain was first filtered with Whatman paper (cellulose, ⌀ 150 mm) and added to cells at room temperature for 30 minutes. After incubation with the stain, samples were rinsed with PBS to remove excess stain and allowed to air dry.

Table 3. Alkaline Phosphatase Stain

Component	Amount
Pre-warmed H_2O	6.25 mL
Napthol	0.0013 g
Tris (0.2 M, pH 8.5)	6.25 mL
Fast Blue RR Salt	0.0075 g

Von Kossa Staining

The von Kossa staining method is used to identify mineralization via deposits of phosphate in the matrix. The stain functions due to a precipitation rection when added silver ions react with phosphate in a sample in the presence of acidic conditions. Mineralization is a measure of matrix maturity and eventual bone hardness and strength (Bonewald, 2003).

To perform the von Kossa stain, medium was removed and cell layers were fixed using paraformaldehyde. After fixation, they were washed with ddH_2O three times before the addition of the von Kossa staining solution. Silver nitrate (5%) was added to the cells, and samples were incubated at room temperature in the dark for 30 minutes. After incubation, samples were rinsed with ddH_2O and incubated under a fluorescent light for one hour.

Estrogen Addition and Depletion

Estrogen was increased in the medium through addition of estradiol. All 10% serum used in media had an initial estrogen concentration of 52 pg/mL. Estradiol was added at a concentration of 200 pg/mL, making the total estrogen concentration 252 pg/mL under these conditions.

In order to prevent osteoblasts from responding to estrogen present in the FBS (52 pg/mL), a receptor antagonist Fulvestrant (ICI-182,780) was used. Fulvestrant was added to the differentiation medium at a concentration of 1 mmol in order to ensure that signaling was inhibited (Long, 1998). The inhibitor was added with each medium change.

Chondrex® Staining for Collagenous and Non-Collagenous Proteins

In order to test the effects of estrogen levels on matrix composition, the Chondrex® stain was performed to measure the amounts of collagenous and non-collagenous protein. The Chondrex® kit contains dyes Sirius Red, which stains for collagenous protein, and Fast Green, which stains for non-collagenous protein. After staining, matrices can be studied via microscopy or dye can be extracted and analyzed via spectrophotometry for protein quantification.

Matrices were decellularized with deoxycholate and fixed with 95% ethanol/5% glacial acetic acid, before dye was added to cover matrices and incubated for 30 minutes at room temperature. Dye was

removed, and matrices were washed with ddH_2O to remove residual unbound dye. At this point, matrices were dried and viewed with a microscope. Alternatively after washing, 1 mL of extraction solution (proprietary) was added and the solution was analyzed via spectrophotometry. Sirius Red absorbance was measured at 540 nm, and Fast Green absorbance was measured at 605 nm (Chondrex, 2013).

Cancer Cells on Osteoblast Bone Matrix

Cancer cell attachment and growth were tested in the experiments as described. Two isologous human breast cancer cell lines were used, MDA-MB-231 and MDA-MB-231 BRMS1. The 231 and the BRMS lines have been engineered to express GFP. The BRMS1 line is a variant of the 231 line engineered to express the BRMS1 (breast cancer metastasis suppressor 1) metastatis suppressor gene. Both cell lines were a gift from Dr. Danny Welsh of the University of Kansas Medical Center, and were maintained in 231 and BRMS1 specific growth media (Tables 4 & 5) in a humidified incubator at 37°C and 5% CO_2. Additionally, booth lines are estrogen receptor negative (Nesaretnam K, 1998). Cells were quantified in experiments below through cell lysis and quantification of GFP through spectrophotometry (see Appendix 1).

MDA-MB-231 GFP

The highly metastatic MDA-MD-231 line originated from a pleural effusion from a 51 year-old white patient (Cailleau, 1978). They were grown in the medium listed in Table 4.

Table 4. MDA-MB-231 Medium (100 mL)

Component	Amount
Dulbecco's Modified Eagle Medium (DMEM; Mediatech, Inc., Manassas, VA)	93 mL
FBS (PAA lab, Etobicoke, Ontario)	5 mL
Non-Essential Amino Acids (NEAA; Mediatech, Inc., Manassas, VA)	1 mL
Penicillin-Streptomycin (Pen-Strep) 100 X	1 mL

MDA-MB-231BRMS1 GFP

The BRMS1 gene, found on human chromosome 11, was identified as being a suppressor of breast metastasis when it was cloned into two highly metastatic breast cancer cell lines, MDA-MB-435 and MDA-MB-231 (Samant, 2014). Addition of the gene to these cell lines significantly reduced metastasis to lung and lymph nodes after injection into the mammary fat pad. These cells are still capable of growing within the bone microenvironment, but their homing ability to secondary sites such as bone is inhibited. The method of metastasis suppression is not yet fully understood (Seraj, 2000). In order to test a model of breast cancer cell dormancy within the bone, BRMS cells were used for the majority of experiments.

Table 5. MDA-MB-231BRMS1 Medium (100 mL)

Component	Amount
Dulbecco's Modified Eagle Medium (DMEM)	46.5 mL
Ham's F12	46.5 mL
FBS (PAA lab, Etobicoke, Ontario)	5 mL
Non-Essential Amino Acids (NEAA)	1 mL
Penicillin-Streptomycin (Pen-Strep) 100 X	1 mL

Isolation of Conditioned Medium

In some experiments, conditioned medium was collected in order to separate the secretions of cells from the matrix and cells. Conditioned medium was collected at the time of medium change; instead of discarding the used medium it was saved. This used medium contained the cytokines and secretions of the cells, which was osteoblasts or osteoblasts and cancer cells depending on the experiments. The used medium was combined in different ratios with fresh medium before it was added back to cells in order to ensure that the cells had sufficient nutrients in addition to the secretions in the old media to be tested.

Statistical Analysis

All statistical analyses were conducted using Prism (GraphPad Software, Inc.). One-way ANOVA tests were used to analyze the number of cancer cells to show significance between numbers of cells on different days or conditions in growth curves. Bonferroni's post test was used to compare conditions, and statistical significance was deemed by a p-value of less than 0.05.

Chapter 3

Investigating the Role of the Bone Matrix in Metastasis

Altering the Matrix

In the spring of 2014, a post-doctoral student in the Mastro Laboratory, Yu-Chi Chen, was studying growth of metastatic cancer cells on cultures of osteoblasts. In order to separate the effects of the osteoblasts from their matrix, Yu-Chi decelluarlarized the cultures (physical removal of the osteoblasts) or fixed (functional removal) them before she added the cancer cells. She compared growth of the cancer cells under these conditions with growth on cultures of live, intact osteoblasts. Yu-Chi observed that the cancer cells grew significantly better on the fixed cultures than on the live or decelluarized ones. This result was surprising; no one expected the removal of osteoblast from the equation to have that much of an effect on the growth of the cancer cells. Thus began the project for this thesis – to determine why the cancer cells grew better on a fixed culture than on a live osteoblast culture.

Physical and Functional Alterations to the Matrix

After growing MC3T3E-1 osteoblasts for four weeks to allow them to make a mature bone matrix, some cultures were left as is with live osteoblasts or fixed with paraformaldehyde before MDA-MB-231 and MDA-MB-231 BRMS1were added at a density of $2x10^3$ cells/cm^2. A three day growth curve was created using the GFP Lysis Protocol (Appendix 1). All experiments were performed in triplicate in order for statistical analysis to be performed.

Both BRMS and 231 cells experienced between three and four times as much growth on fixed cultures at day three than on live cultures (Figure 2, Figure 3). After repeating the experiment several times, it was concluded that fixing the osteoblasts and their matrix allowed the cancer cells to proliferate much faster. This finding raised many questions regarding the structure of the fixed matrix and

competition for nutrients in the medium between cancer cells and osteoblasts. Several hypotheses were developed: 1.) that fixation of the cultures created a matrix structure that was preferred for cancer cell attachment and thus growth, 2.) that cancer cells had more nutrients available in the medium when osteoblasts were dead, so cancer cells grew better, and 3.) osteoblasts secreted a substance that is inhibitory to cancer cells, and fixation of the osteoblasts removes their ability to make the inhibitor.

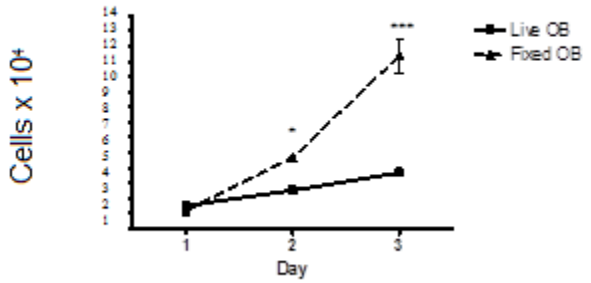


Figure 2. MDA-MB-231 BRMS1 Proliferated More on a Fixed Osteoblast Matrix Than on a Live Osteoblast Culture.

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 in 6 well tissue culture plates. They were differentiated for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, some cultures were left unaltered to retain live osteoblasts (live OB) and others were fixed with 1% PF (Fixed OB). Cultures were washed with PBS, and BRMS cells were added at a density of 2,000 cells/cm^2 in osteoblast differentiation medium and allowed to proliferate for 24, 48, and 72 hours. At each point three wells were lysed and assay of GFP was used to quantitate cell number. Fluorescence was converted to cell number by comparison to a standard curve. Shown is the average fluorescence ± the SEM. Values were statistically analyzed via one-way ANOVA and Bonferroni's post test. $P<0.05$

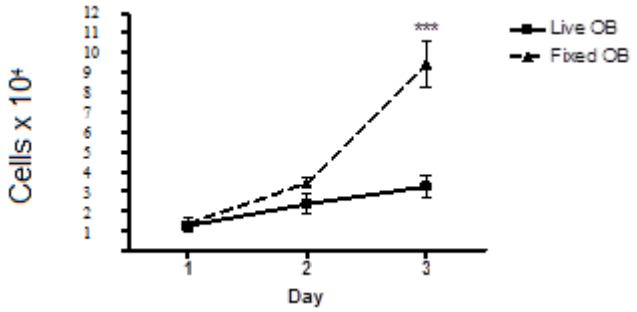


Figure 3. MDA-MB-231 Cells Proliferated More on a Fixed Osteoblast Culture than a Live Osteoblast Culture

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 in 6 well tissue culture platesdifferentiated for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, matrices were unaltered to retain live osteoblasts (live OB) or fixed with 1% PF (Fixed OB). 231 cells were added to the cultures at a cell density of 2,000 cells/cm^2 in osteoblast differentiation medium and allowed to proliferate for 24, 48, and 72 hours. At each time point three wells were analyzed via the GFP lysis assay to quantify cell number. Fluorescence was converted to cell number by comparison to a standard. Cell number was used to create a three day growth. Values were statistically analyzed via one-way ANOVA and Bonferroni's post test. Error bars represent mean ± SEM.

Other growth surfaces for cancer cells were tested to compare to the growth on the fixed and live osteoblast cultures. Osteoblasts were growth for four weeks before the cultures were decellularized, and cancer cells were added to the remaining matrix. For comparison some cancer cells were grown on tissue culture plastic without osteoblasts or matrix. Through the GFP cell lysis assay, three day growth curves were created (Figure 4). BRMS cells proliferated more on decellularized matrix than they did on plastic. To rank the four growth surfaces, BRMS grow fastest on fixed matrix, followed by decellularized matrix, live matrix, and plastic.

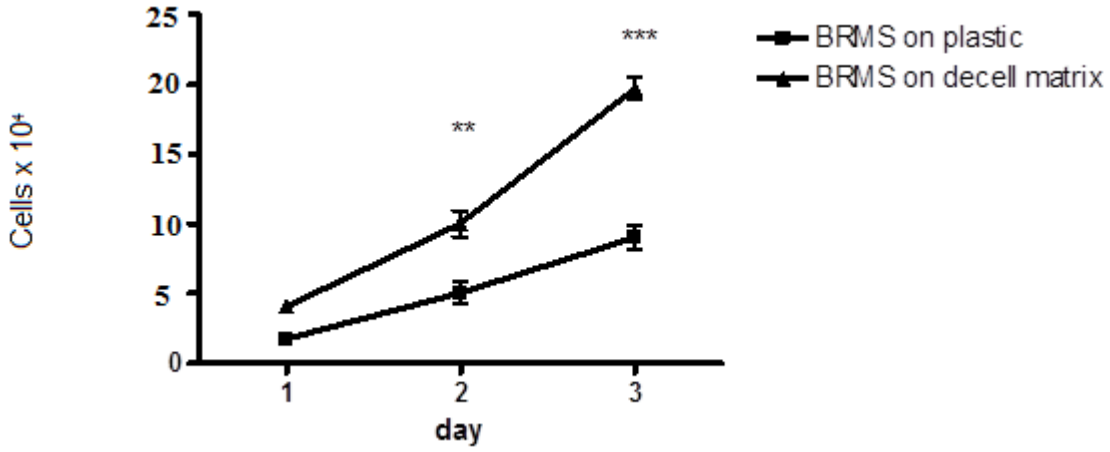


Figure 4. MDA-MB-231 BRMS Proliferated More on Decellularized Matrix than Plastic

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 in 6 well plates and differentiated for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, matrices were decellularized using deoxycholate as described in the methods section. BRMS cells were added to the decellularized matrix (BRMS on decell matrix) or to a standard tissue culture plastic plate (BRMS on plastic) at a cell density of 2,000 cells/cm^2 in osteoblast differentiation medium and allowed to proliferate for 24, 48, and 72 hours. At each time three wells were analyzed via the GFP lysis assay to quantify cell number. Fluorescence was converted to cell number by comparison to a standard.. Values were statistically analyzed via one-way ANOVA and Bonferroni's post test. Error bars represent mean ± SEM.

Osteoblast Production of an Inhibitor of Cancer Cell Growth

In order to test the hypothesis that live osteoblasts suppressed cancer cell growth, an experiment was designed using osteoblast conditioned medium. Medium was collected from a 48 hour old flask of osteoblasts and added to BRMS proliferating on live and fixed osteoblast cultures. This "used medium" contained the secretions of osteoblast cells that could potentially be inhibitory to the growth of cancer cells. The used medium was diluted to 50% with fresh osteoblast differentiation medium before being

added to the proliferating cancer cells to ensure that adequate nutrients would be available. The 50% used medium on lived and fixed matrix conditions was compared to the normal 100% osteoblast differentiation medium on lived and fixed cultures.

The osteoblast conditioned medium still increased proliferation like normal differentiation medium (Figure 5). This finding led to the conclusion that osteoblasts alone did not secrete a factor inhibitory to the growth of cancer cells. However, this experiment did not eliminate the possibility that osteoblasts produced an inhibitory factor in the presence of cancer cells because the used medium was taken from osteoblasts that were not in contact with cancer cells.

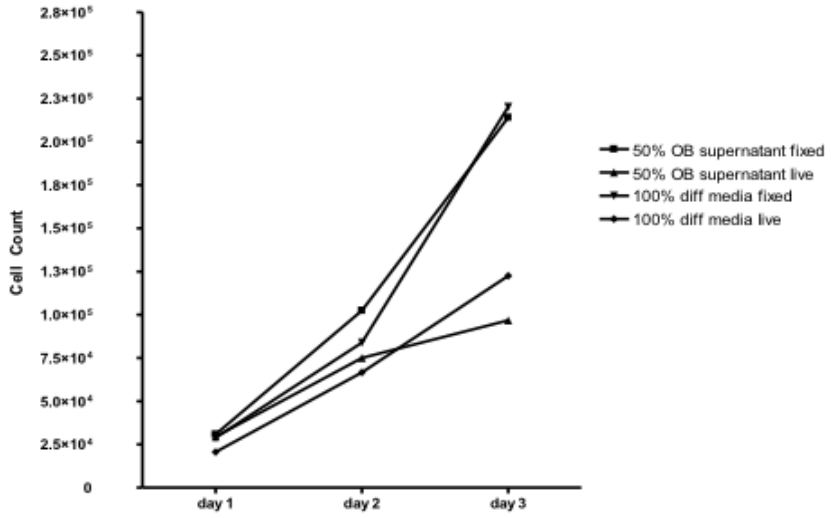


Figure 5. Osteoblasts Alone Did Not Produce An Inhibitor of BRMS Proliferation on Live or Fixed Matrix

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 in 24 well tissue culture plates differentiated for four weeks in order to create a mature matrix. Medium was changed every three days. After four weeks, matrices were unaltered to retain live osteoblasts (live OB) or fixed with 1% PF (Fixed OB). BRMS cells were added to the cultures at a cell density of 2,000 cells/cm^2 in osteoblast differentiation medium (100% diff media live and fixed) or in medium taken from a flask of osteoblasts that had been growing for two days. It was diluted with osteoblast differentiation medium (50% OB supernatant live and fixed). MDA-MB-231 BRMS1 (2x10^3/cm) were added to cultures in diluted medium and allowed to proliferate for 24, 48, and 72 hours. At each time three wells were analyzed via the GFP lysis assay to quantify cell number. Fluorescence was converted to cell number by comparison to a standard. Cell number was used to create a three day growth. Values within the single assay were statistically analyzed via one-way ANOVA and Bonferroni's post test. Error bars are not available because the experiment was not replicated.

In order to test the possibility that osteoblasts when in contact with cancer cells produce inhibitors, culture medium was collected from a three day co-culture of BRMS on four week old osteoblasts. This medium was diluted to 50%, 25%, and 10% with osteoblast differentiation medium.

After allowing the cancer cells to proliferate for three days, the same results as the previous experiment were reported. Supernatant from osteoblasts grown in co-culture with BRMS did not affect the proliferation of BRMS on live or fixed matrix, as no significant changes in growth were recorded with the addition of the supernatant (Figure 6, Figure 7). Taken together the results of these experiments did not support the hypothesis that the osteoblasts produce an inhibitory secretion to BRMS. Another hypothesis needed to be developed in order to understand why cancer cells proliferate more on the fixed osteoblast cultures than the live osteoblast cultures. Matrix composition and structure was studied.

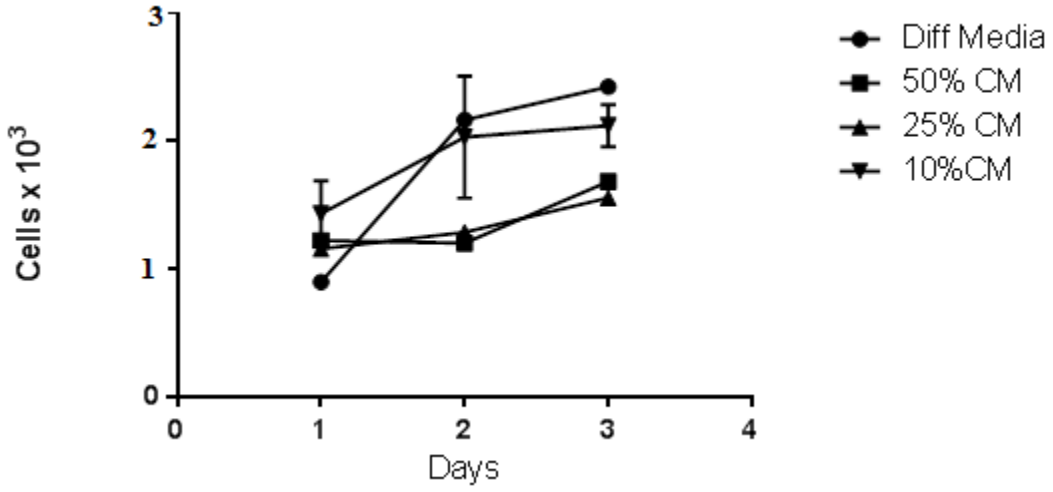


Figure 6. Osteoblasts Grown in Co-culture with BRMS Do Not Produce an Inhibitor of BRMS Proliferation on Live Osteoblast Matrix

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 differentiated for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, matrices were unaltered to retain live osteoblasts. BRMS cells were added to the cultures at a cell density of 2,000 cells/cm^2 in osteoblast differentiation medium (diff media) or in medium taken from a three day co-culture of four week old osteoblasts that was diluted with osteoblast differentiation medium (50% CM, 25% CM, 10% CM) and allowed to proliferate for 24, 48, and 72 hours. At each time point three wells were analyzed via the GFP lysis assay to quantify cell number. Fluorescence was converted to cell number by comparison to a standard. Cell number was used to create a three day growth. Triplicate values from the single experiment were statistically analyzed via one-way ANOVA and Bonferroni's post test.

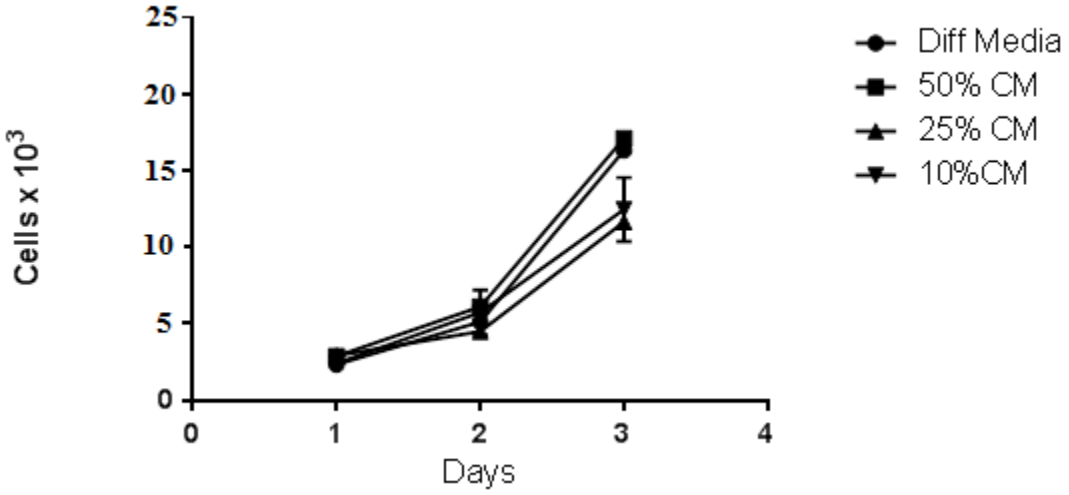


Figure 7. Osteoblasts Grown in Co-culture with BRMS Did Not Produce an Inhibitor of BRMS Proliferation on Fixed Osteoblast Cultures

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 differentiated for four weeks in order to create a mature matrix. Medium was changed every three days. After four weeks, matrices were fixed with 1% PF. BRMS cells were added to the fixed cultures at a cell density of 2,000 cells/cm^2 in osteoblast differentiation medium (diff media) or in medium taken from a three day co-culture of four week old osteoblasts that was diluted with osteoblast differentiation medium (50% CM, 25% CM, 10% CM) and allowed to proliferate for 24, 48, and 72 hours. At each time three wells were analyzed via the GFP lysis assay to quantify cell number. Fluorescence was converted to cell number by comparison to a standard. Cell number was used to create a three day growth. Values were statistically analyzed via one-way ANOVA and Bonferroni's post test.

Structural Changes to the Fixed Cultures Cause the Increase in Cancer Cell Proliferation

The next working hypothesis focused on the possible structural changes to the matrix upon fixation. Fixation was performed with paraformaldehyde, which is known to cross-link proteins (Thavarajah). This structural change could impact the attachment of cancer cells to the matrix and their proliferation. If more cells attach initially, then more would be available to proliferate.

An attachment assay was performed in order to determine if there was any validity to this argument. As described for the growth experiments, cancer cells were added to four week old osteoblast cultures that were either live osteoblasts or fixed osteoblasts. For the attachment assays the cancer cells were added at a higher density than growth assays. 8×10^4 cells/cm^2 were added versus 2×10^3 cells/cm^2. The decellularized matrix and plastic surfaces were also tested. BRMS were added to the surfaces and non-adherent cells were washed off one hour later. The wells were washed three times with PBS at room temperature to remove any cells that did not attach. The cultures were lysed and GFP quantified.

Quantification indicated that cells preferably attached to the fixed cultures, followed by decellularized matrix, live osteoblast cultures, and plastic (Figure 8). About 1.5 times as many BRMS cells attached to the fixed culture than the live culture in one hour, which could potentially lead to more cells with the ability to proliferate at a later time.

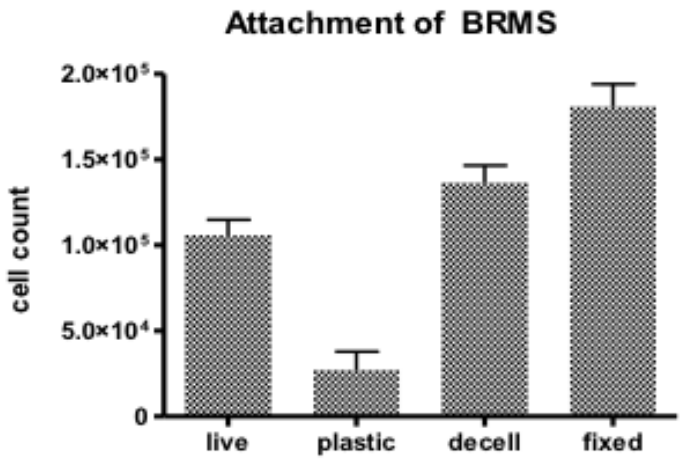


Figure 8. BRMS Attach Most to Fixed Matrix

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 and allowed to differentiate for four weeks. Medium was changed every three days. After four weeks, some cultures were unaltered to retain live osteoblasts, others were fixed with 1% PF, or decellularized with deoxycholate. BRMS cells were added to the cultures or to bare tissue culture plastic at a cell density of 80,000 cells/cm^2 in osteoblast differentiation medium and allowed to attach for one hour before excess cells were washed off with PBS. Three wells per condition were analyzed via the GFP lysis assay to quantify cell number. Fluorescence was converted to cell number by comparison to a standard. Values were statistically analyzed via one-way ANOVA and Bonferroni's post test.

To test the hypothesis that the osteoblasts secreted a stimulatory or an inhibitor factor independent of attachment, an experiment was designed to take cell contact out of the equation. A transwell migration system was used to test soluble factors Osteoblasts were plated in 24 well plates that were compatible with the transwell inserts and allowed to differentiate for four weeks. After four weeks the cultures were either left live, fixed, or decellularized. BRMS cells were added to transwell membrane chambers above the three different types of cultures and plastic. The membranes had 3 um pores which were too small for the cells to pass through but large enough for soluble factors to pass. BRMS were allowed to grow for three days to create a growth curve before the lysis assay was performed in the transwell chambers.

Quantification of the cells within the chambers did not show any significant differences among the four different conditions on the bottom chambers (Figure 9). This finding indicated

that attachment to a physical matrix likely was causing the major difference in proliferation at day three for the live and fixed culture surfaces. However, more cancer cells seemed to have been present on the live matrix than the other surfaces at the day one and two time points than at the day three time point. The cancer cell growth on the fixed osteoblast culture seemed to have caught up and overpassed the live osteoblast culture by day three. This indicates that the cells seem to be proliferating more slowly on the live matrix, possibly due to release of an inhibitory substance from the osteoblasts.

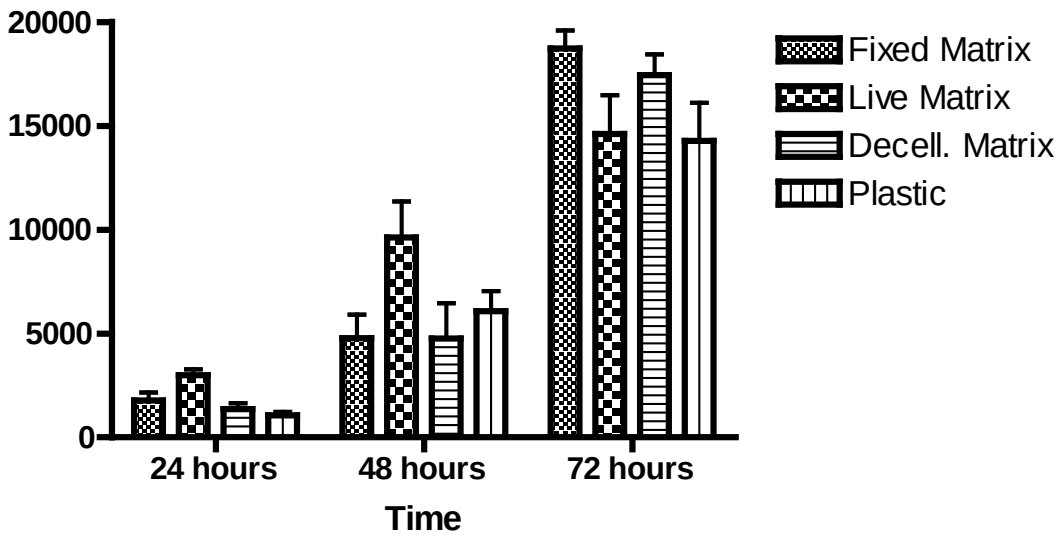


Figure 9. Soluble Factors Alone Did Not Significantly Affect BRMS Proliferation.

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 in the bottom chamber of a transwell system. They were allowed to differentiate for four weeks in order to create a mature matrix. Medium was changed every three days. After four weeks, cultures were left unaltered to retain live osteoblasts, fixed with 1% PF, or decellularized with deoxycholate. BRMS cells were added to the cultures or bare tissue culture plastic at a cell density of 80,000 cells/cm^2 in osteoblast differentiation medium and allowed to attach for one hour before excess cells were washed off with PBS. Three wells per condition were analyzed via the GFP lysis assay to quantify cell number. Fluorescence was converted to cell number by comparison to a standard. Values were statistically analyzed via one-way ANOVA and Bonferroni's post test. Error bars are not available because the experiment was not replicated.

In order to better understand the cancer cell attachment, BRMS were also allowed to attach to the four surface conditions for one hour before fluorescent microscopy was used to photograph the fluorescent cells. Cell body and projections were used as indicators of attachment. Attachment is especially relevant to cancer cells that must escape the initial tumor, travel to the circulation, and then exit the circulation. Once the metastatic cells have reached their secondary site, they must attach quickly or risk being swept away by blood flow or degraded by the immune system (Kozlow). It is important to note that the following images of attachment were analyzed for the cell images; the cells were not quantified. Images were taken using a confocal microscope and FITC filters set for exciation at 476-495 nm and emission at 520-560 nm.

When comparing the attachment to the live (Figure 10) and fixed cultures (Figure 11), BRMS cells had more extracellular projections on the fixed culture. These “feet”, or extensions of the actin cytoskeleton away from the major cell body, showed that the BRMS may have attached better to the fixed osteoblasts than to the live ones. These extracellular projections were not observed on the BRMS attached to the decellularized matrix (Figure 12) or tissue culture plastic (Figure 13).

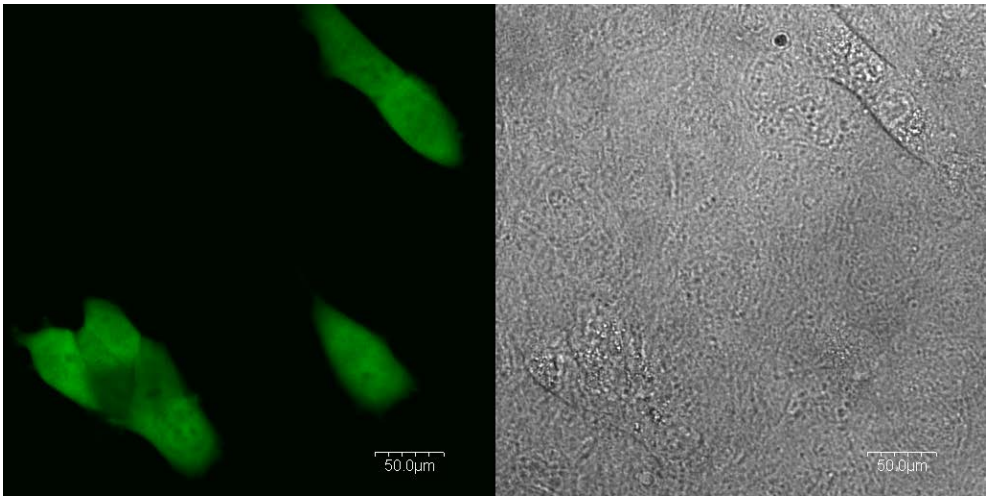


Figure 10. One Hour Attachment of BRMS to a Live Osteoblast Culture

MC3t3-E1 were plated at a cell density of 10,000 cells/cm^2 differentiated for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, matrices were unaltered to retain live osteoblasts. BRMS cells were added to the cultures at a cell density of 80,000 cells/cm^2 in osteoblast differentiation medium and allowed to attach for one hour before images on the left were taken using fluorescent microscopy.

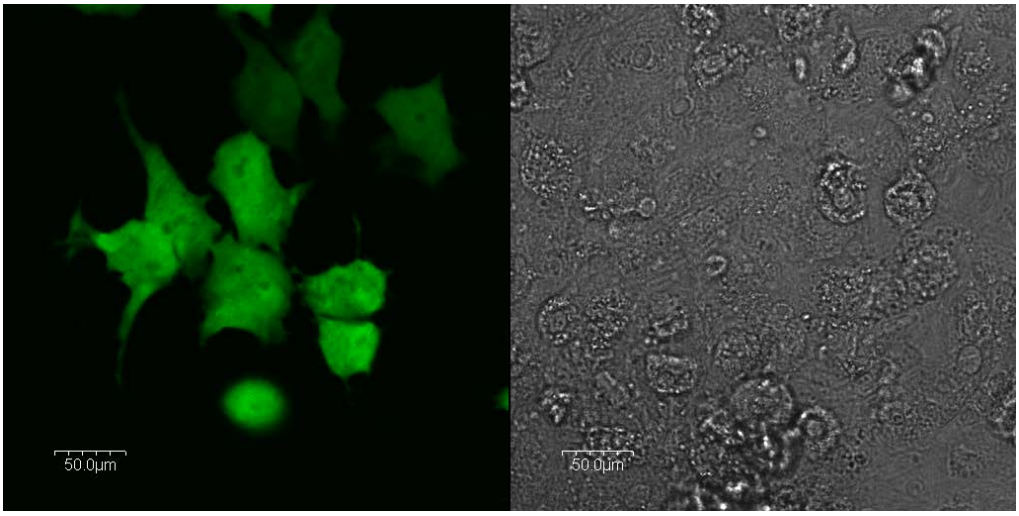


Figure 11. One Hour Attachment of BRMS to a Fixed Osteoblast Matrix

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 differentiated for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, matrices were fixed with 1% PF. BRMS cells were added to the cultures at a cell density of 80,000 cells/cm^2 in osteoblast differentiation medium and allowed to attach for one hour before images were taken using fluorescent microscopy.

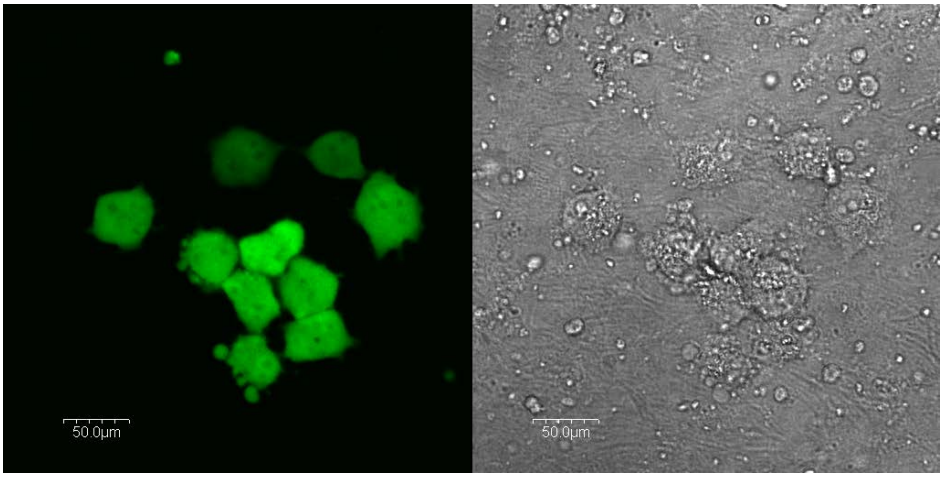


Figure 12. One Hour Attachment of BRMS to a Decellularized Osteoblast Matrix

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 differentiated for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, matrices were decellularized with deoxycholate. BRMS cells were added to the matrices at a cell density of 80,000 cells/cm^2 in osteoblast differentiation medium and allowed to attach for one hour before images were taken using fluorescent microscopy.

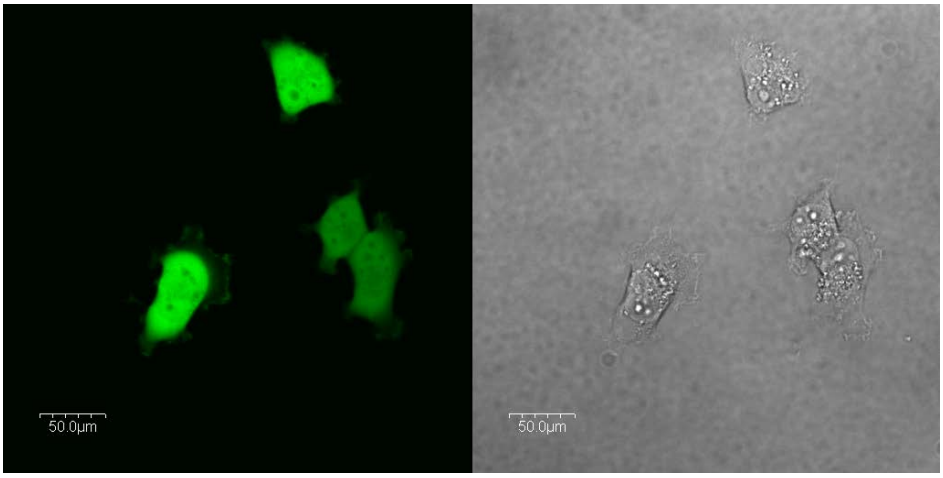


Figure 13. One Hour Attachment of BRMS to Tissue Culture Plastic

BRMS cells were added to bare tissue culture plastic at a cell density of 80,000 cells/cm^2 in osteoblast differentiation medium and allowed to attach for one hour before images were taken using fluorescent microscopy.

Chapter 4

The Role of Estrogen

Breast cancer and thus its metastasis to bone occurs most often in post-menopausal women. One of the most significant aspects of menopause is a decrease in the amount of estrogen that the body produces from the ovaries. The decrease in estrogen is directly to the development of osteoporosis because after menopause bone breakdown is more prevalent than bone formation. Even before menopause, low hormone levels can result in a decrease in bone mass (Zeleniuch-Jacquotte A, 2004). Estrogen has become an increasingly important area of focus within breast cancer research, especially since incident rates of estrogen-receptor positive breast cancers have increased historically over the past two decades (DeSantis, 2014).

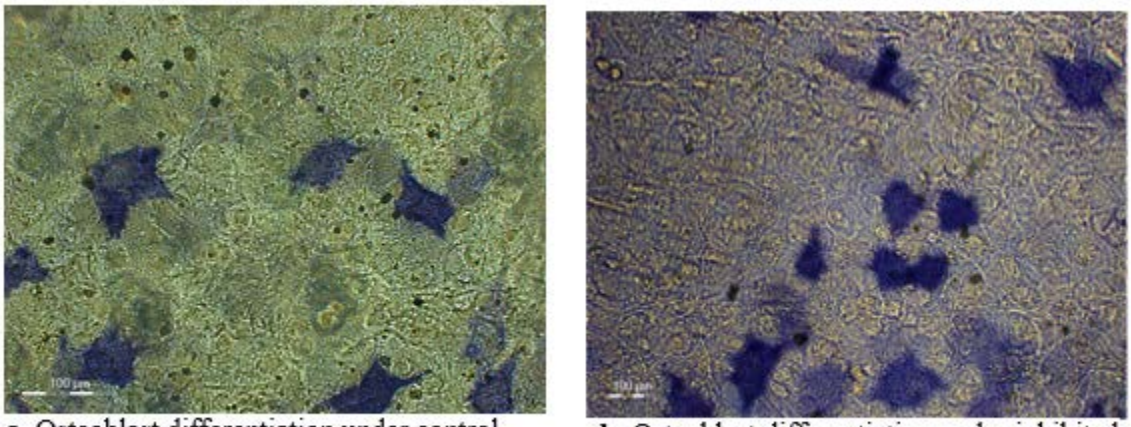
After observing that bone matrix modification via fixation affects cancer cell attachment and proliferation, a more natural modification to the osteoblasts was investigated in relation to bone metastases. The following experiments sought to learn how estrogen inhibition or supplementation affected the formation of osteoblast bone matrices and how this affected breast cancer cell attachment and proliferation.

Effects of Estrogen on Bone

Alkaline Phosphatase (Differentiation)

Because osteoblast differentiation is critical to proper matrix formation, it was measured in relation to estrogen. Alkaline phosphatase, an enzyme important to osteoblast differentiation, was measured by staining of the osteoblast matrix.

Osteoblasts were differentiated for four weeks with normal differentiation medium (Figure 14 a.) or with the addition of the ICI estrogen aromatase inhibitor (Figure 14 b.). Staining for alkaline phosphatase indicated that estrogen inhibition did not seem to affect the differentiation of the osteoblasts.



a. Osteoblast differentiation under control estrogen concentration

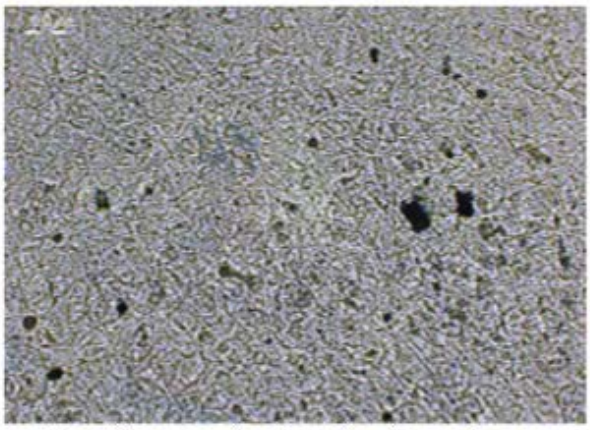
b. Osteoblast differentiation under inhibited estrogen concentration

Figure 14. Estrogen Inhibition Does Not Affect Alkaline Phosphatase Production.

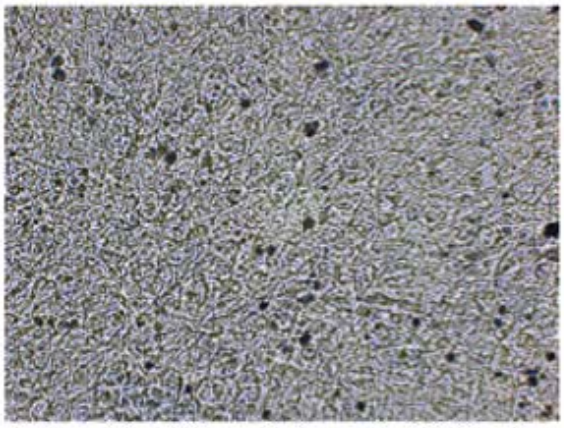
MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 in 24 well plates and differentiated under control estrogen concentration (52 pg/mL) or with the estrogen inhibitor (ICI)for four weeks in order to create a mature matrix. Medium was changed every three days. After four weeks, cells were stained with the alkaline phosphatase staining protocol to measure cell differentiation. Images were taken by light microscopy. The difference in color between the two images was due to different amounts of light exposure and is not significant.

Mineralization (Von Kossa)

In order to test matrix maturation, matrices were stained for phosphate deposition via the von Kossa stain. Phosphate deposits, or mineralization, are an important measure of matrix hardness. Mineralization typically does not appear until osteoblasts have differentiated for at least 20 days (Bonewald). Mineralization was tested under normal osteoblast differentiation conditions or with the addition of the estrogen inhibitor. No changes in mineralization were observed (Figure 15).



a. Osteoblast matrix mineralization under control estrogen concentration



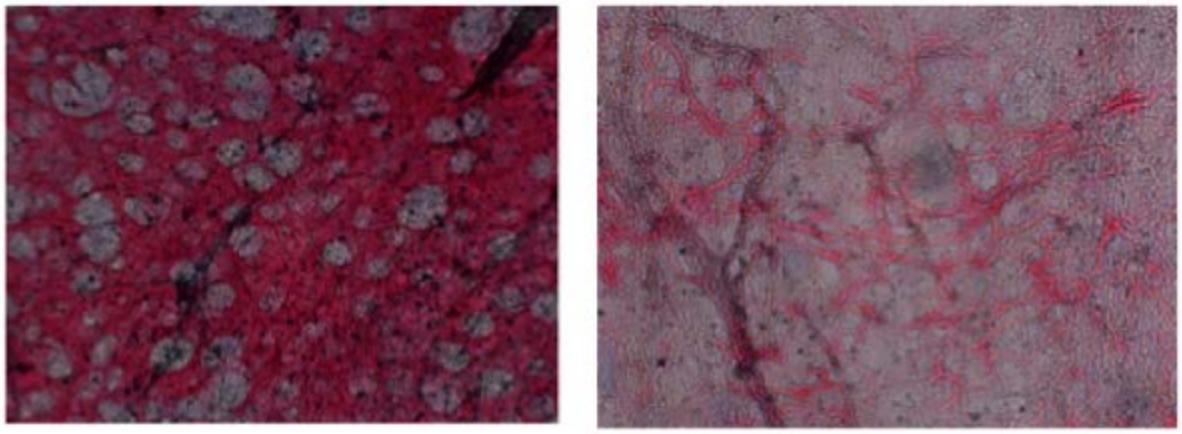
b. Osteoblast differentiation under inhibited estrogen concentration

Figure 15. Estrogen Does Not Affect Osteoblast Matrix Mineralization.

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 in 24 well plates and differentiated under control estrogen concentration (52 pg/mL) or with the estrogen inhibitor (ICI) for four weeks. Medium was changed every three days. After four weeks, cells were stained with the von Kossa staining protocol to measure matrix mineralization. Images were taken by light microscopy.

Protein Modification (Chondrex & CNA35)

To learn more about the structure of the matrix and how it is affected by estrogen, two different methods of looking at proteins within the matrix were used. The Chondrex® staining protocol was used to measure collagenous and non-collagenous proteins. A bacterial tag (CNA35) for collagen that was fluorescently labeled with an Alexa Fluor (488 nm) was also used to visualize the matrix; this way of looking at the matrix became especially useful when observing cancer cells on the matrix as the green cancer cells stood out well against the red labeled matrix. Both stains were conducted on matrices with varying estrogen levels.



a. Chondrex® stain under control estrogen concentration

b. Chondrex® stain under inhibited estrogen concentration

Figure 16. Chondrex Stained Cultures of Osteoblasts Grown Under Different Estrogen Conditions.

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 in 24 well plates and differentiated under control estrogen concentration (52 pg/mL) or with the estrogen inhibitor (ICI) for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, matrices were decellularized and stained via the Chondrex® protocol. Images were taking using light microscopy.

The Chondrex® stain showed less red color on the matrix grown under estrogen inhibition, indicating that less collagen was created when osteoblast were grown without estrogen than when they were grown with the base amount of in the differentiation medium control (Figure 16).

The Chondrex stain was also quantified®, and estrogen levels within a matrix were directly related to the amount of collagen and total protein within the matrix (Figure 17). When osteoblasts were grown with the estrogen inhibited, they produced less total protein than the control. Increasing the estrogen with estradiol increased the total protein production. The same trend was seen for both collagenous and non-collagenous protein as well.

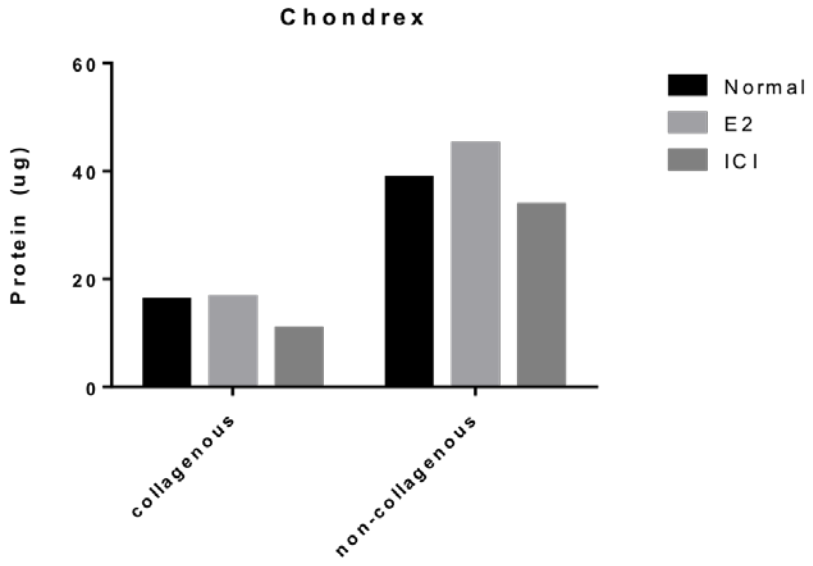


Figure 17. Protein Production Directly Relates to Estrogen Concentration

Osteoblasts were plated at a cell density of 10,000 cells/cm^2 in 24 well plates and differentiated under control estrogen concentration (52 pg/ml), with the estrogen inhibitor (ICI), or estrogen supplementation (252 pg/mL) for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, matrices were decellularized and stained via the Chondrex® protocol. Images were taking using light microscopy. Experiment was performed three times in triplicate; graph made from average values.

Additionally, matrices were stained using CNA35 probe for collagen on control, estrogen inhibited, and estrogen supplemented cultures (Figure 18). This stain showed that collagen fibers were rearranged based on estrogen levels. CNA35 stain was performed on decelluarlized matrix, and placement of cells based on collagen free “holes” (see arrow in 18 b.) which indicated cells in the matrix seemed most irregular on the estrogen inhibited matrix. When osteoblasts were grown under estrogen inhibition, arrangement of fibers appeared much more irregular than the control and estrogen supplemented matrices.

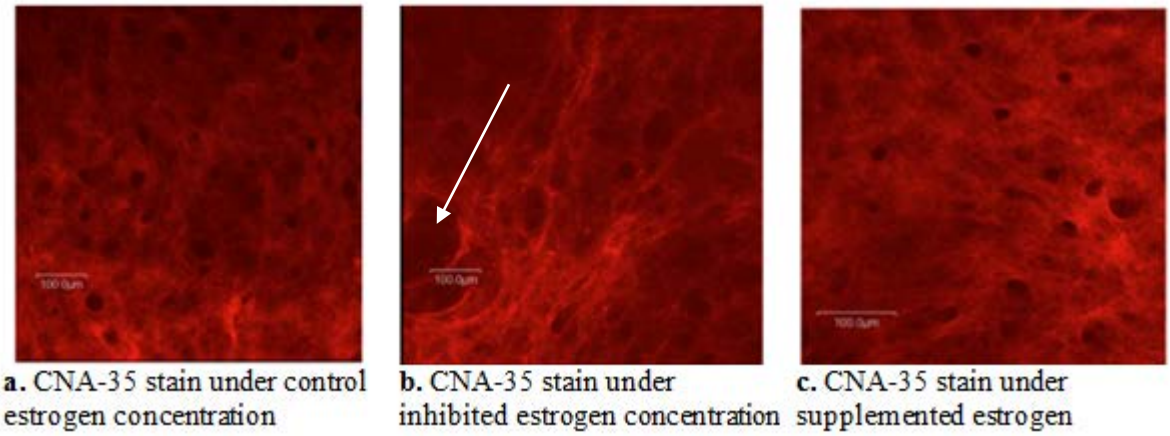


Figure 18. Estrogen Inhibition During Osteoblast Differentiation Alters Collagen Fiber Structure

Osteoblasts were plated at a cell density of 10,000 cells/cm^2 in 24 well plates and differentiated under control estrogen concentration (52 pg/mL) with the estrogen inhibitor (ICI), or estrogen supplementation (252 pg/mL) for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, matrices were decellularized and stained with the CNA35 collagen probe. Images were taking using fluorescent microscopy.

Attachment and Growth of Cancer Cells to Estrogen Modified Matrices

After observing that estrogen affected the structure of the osteoblast derived matrix, attachment and growth of metastatic breast cancer cells on the cultures from different estrogen conditions were tested.

This pilot experiment was designed to compare the growth of MDA-MB-231 BRMS1 on decellularized osteoblast matrices that were grown under estrogen inhibition or supplementation (Figure 19). BRMS cells proliferated on estrogen supplemented or inhibited osteoblast matrices that had been decellularized for three days. Quantification based on analysis of percent area fraction of GFP in fluorescent microscopy images showed that the cancer cells proliferated at similar rates on the two different surfaces; estrogen did not affect growth on the decellualized matrices.

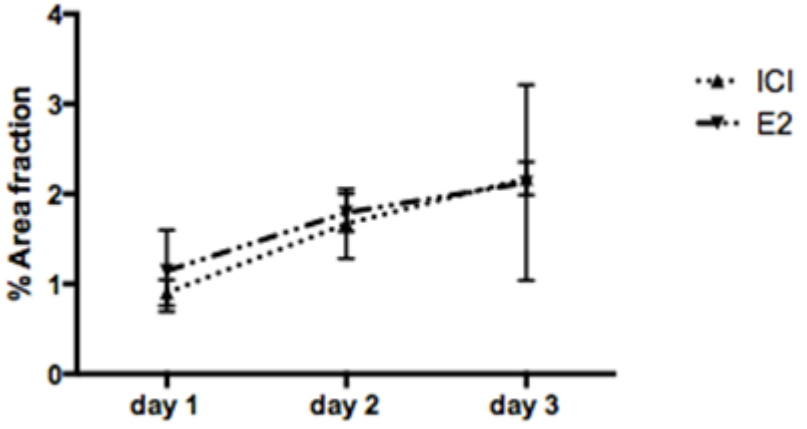


Figure 19. Estrogen Inhibition or Supplementation Does Not Affect Growth of MDA-MB-231 BRMS1 on a Decellularized Matrix

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 in 8 well chamber slides and differentiated with the estrogen inhibitor (ICI) or estrogen supplementation for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, matrices were decellularized using deoxycholate. MDA-MB-231 BRMS1 GFP were added at a density of 2x10^3 cells/cm^2 and allowed to proliferate for 24, 48, and 72 hours. Three fields per time point and condition were analyzed via ImageJ for percent area fraction. Graphed values are averages analyzed via one way ANOVA and Bonferroni post test.

The previous experiment was repeated to confirm validity using the cell growth quantification procedure used in earlier experiments – cell lysis and GFP quantification. MC3T3-E1 cells were differentiated in the control estrogen level, with the estrogen inhibitor, or estradiol supplementation. The cell layers were decelluarlized in order to test cancer cell growth on the matrix alone (Figure 20). The results were the same as the pilot experiment; BRMS proliferated at the same rate regardless of what estrogen levels were present during osteoblast matrix production.

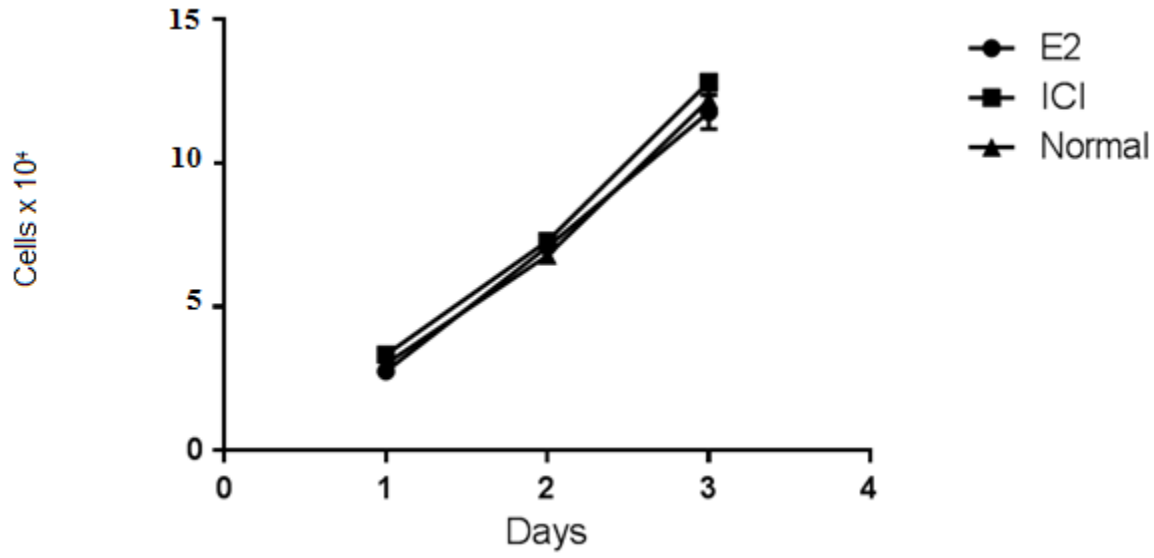


Figure 200. Estrogen Inhibition or Supplementation Did Not Affect MDA-MB-231 BRMS1 Proliferation on Decellularized Matrix

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 in 24 well tissue culture plates and differentiated under control estrogen concentration (52 pg/mL), with the estrogen inhibitor (ICI), or estrogen supplementation (252 pg/mL) for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, matrices were decellularized using deoxycholate. MDA-MB-231 BRMS1 GFP were added at a density of 2x10^3 cells/cm^2 and allowed to proliferate for 24, 48, and 72 hours At each time point three wells were analyzed via the GFP lysis assay to quantify cell number. Fluorescence was converted to cell number by comparison to a standard. Cell number was used to create a three day growth. Values were statistically analyzed via one-way ANOVA and Bonferroni's post test.

After no differences in proliferation on the varying estrogen decellularized osteoblast matrices were observed, experiments wereperformed to observe whether a difference in attachment to the different surfaces existed.

A six hour attachment assay, similar to the experiment referred to above in Figure 8 was performed. MC3T3-E1 cells were allowed to differentiate for four weeks in control estrogen (52 pg/mL), with the estrogen inhibitor (ICI, 1 mmol) or with estrogen supplementation via estradiol (252 pg/mL).

After four weeks the cell cultures were decellularized, and BRMS cells were added and allowed to attach for six hours before the GFP lysis assay was performed (Figure 22).

Throughout the time course assay, there was not significantly greater attachment to one surface compared to either of the other two. After repetition of this experiment, it was concluded that structural changes to the matrix caused by different estrogen osteoblast differentiation conditions did not affect the attachment of MDA-MD-231 BRMS1 cells.

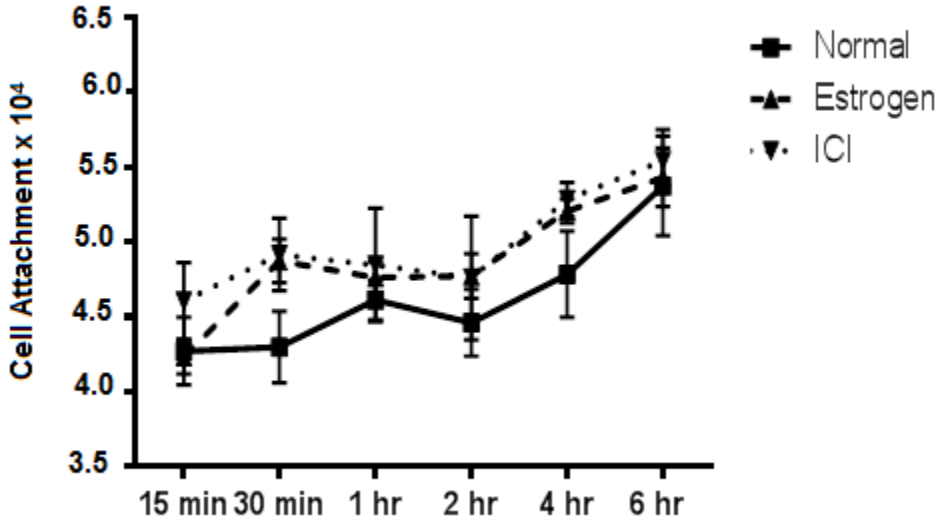


Figure 21. MDA-MB-231 BRMS1 Do Not Attach Differently to Osteoblast Matrices Based on Estrogen Concentration

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 in 24 well tissue culture plates and differentiated under control estrogen concentration (52 pg/mL), with the estrogen inhibitor (ICI), or estrogen supplementation (252 pg/mL) for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, matrices were decellularized using deoxycholate. MDA-MB-231 BRMS1 GFP were added at a density of 8x10^4 cells/cm^2 and allowed to for the allotted times. At each point three wells were washed once with PBS and then analyzed via the GFP lysis assay to quantify cell number. Fluorescence was converted to cell number by comparison to a standard. Cell number was used to create a three day growth. Values were statistically analyzed via one-way ANOVA and Bonferroni's post test.

To confirm that attachment was the same to the three different estrogen osteoblast cultures, cancer cells were allowed to attach to the surfaces for six hours and then stained for actin. A fluorescent tag red Alexa fluor 488 for actin was used to stain for attachment of the cells to the extracellular matrix via actin. The stain was performed on MDA-MD-231 and MDA-MB-231 BRMS1 cells, which both attached similarly to the three different matrix conditions. Little to no difference in actin structure was observed between the three different cultures (Figure 22).

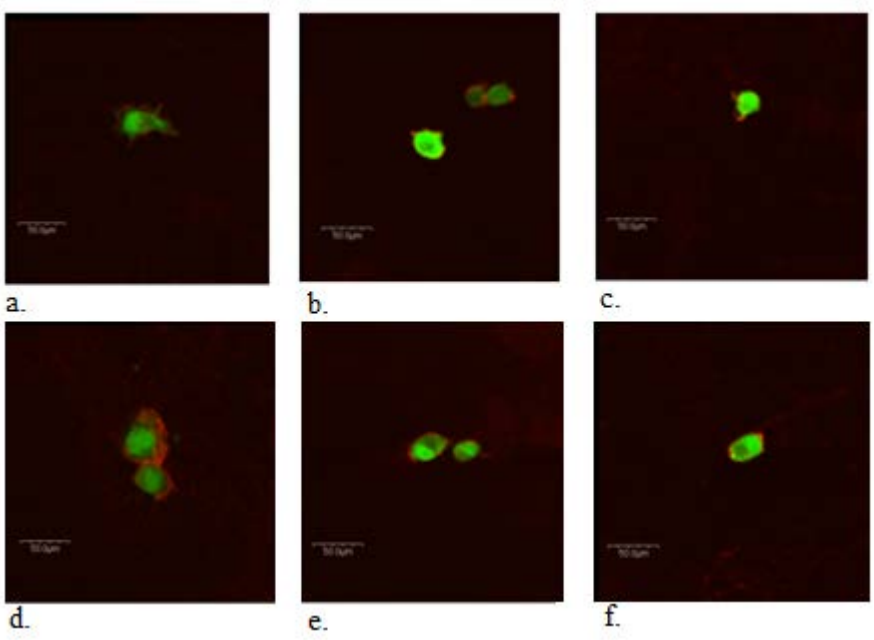


Figure 22. Estrogen Alteration of Osteoblast Matrices Does Not Affect Actin Filament Structure for MDA-MB-231 and MDA-MB-231 BRMS Attachment

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 in 8 well chamber slides and differentiated under control estrogen concentration, 52 pg/mL (a., d.), with the estrogen inhibitor, ICI at 1mmol (b., e.), or estrogen supplementation, 252 pg/mL, (c., f.) for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, matrices were decellularized using deoxycholate. MDA-MB-231 (a. ,b. ,c.) MDA-MB-231 BRMS1 (d., e., f.,)were added at a density of 2x10^3 cells/cm^2 and allowed to attach for 6 hours. After 6 hours, cancer cells on matrices were fixed with 1% PF and stained with phallodin for actin.

Proliferation of cancer cells on estrogen modified matrix was studied in addition to cell attachment. The following experiments were conducted similar the experiments described in legend to Figure 2. Osteoblasts were culturedfor four weeks under conditions of normal estrogen (52 pg/mL), inhibited estrogen (ICI,1 mmol), or supplemented estrogen (252 pg/mL). After four weeks, the cultures were decellularized using deoxycholate. MDA-MD-231 BRMS were then added to the and allowed to proliferate for three days (Figure 23). No significant difference in BRMS proliferation was observed between the different estrogen cultures.

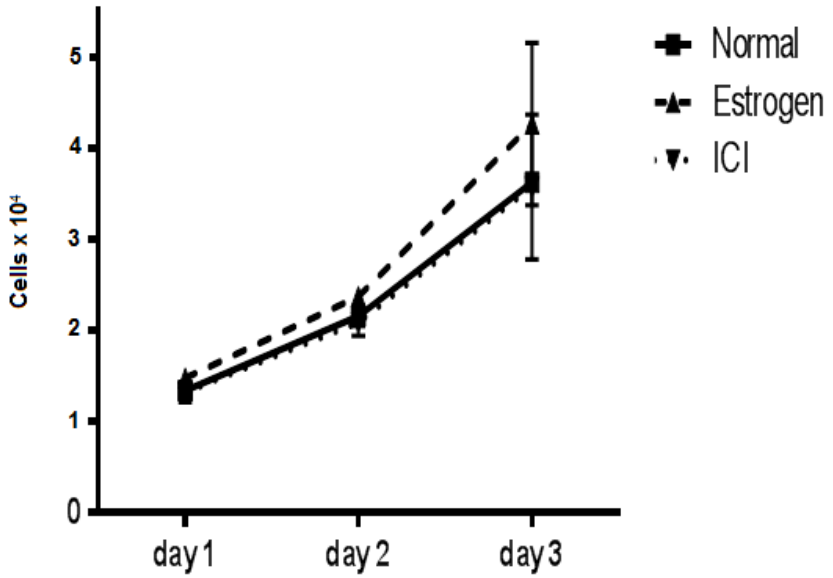


Figure 23. Estrogen Inhibition or Supplementation Does Not Affect MDA-MB-231 BRMS1 Proliferation on a Decellularized Matrix.

Osteoblasts were plated at a cell density of 10,000 cells/cm^2 in 24 well plates slides and differentiated under control estrogen concentration, with the estrogen inhibitor, or estrogen supplementation for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, matrices were decellularized using deoxycholate. MDA-MB-231 BRMS1 GFP were added at a density of 2x10^3 cells/cm^2 and allowed to proliferate for 24, 48, and 72 hours. At each time point three wells were analyzed via the GFP lysis assay to quantify cell number. Fluorescence was converted to cell number by comparison to a standard. Cell number was used to create a three day growth. Values were statistically analyzed via one-way ANOVA and Bonferroni's post test

It was hypothesized that decellularization could might removeosteoblast factors created in response to the various estrogen conditions, and the lack of these factors could potentially affect cancer cell growth. Thus, additional experiments were conducted on live osteoblast cultures, identical to the previous experiment except for the lack of decellularization. Live osteoblasts remained intact when breast cancer cells were added to cultures to test for the possibility that an inhibitor of cancer cell growth was produced by the live osteoblasts dependent on the estrogen condition that was available during osteoblast differentiation (Figure 24). Interestingly, a significant difference in BRMS proliferation was detected between the estrogen deprived and estrogen supplemented cultures. It appeared that osteoblasts grown with higher levels of estrogen were able to suppress cancer cell proliferation, compared to cultures grown under reduced estrogen concentrations.

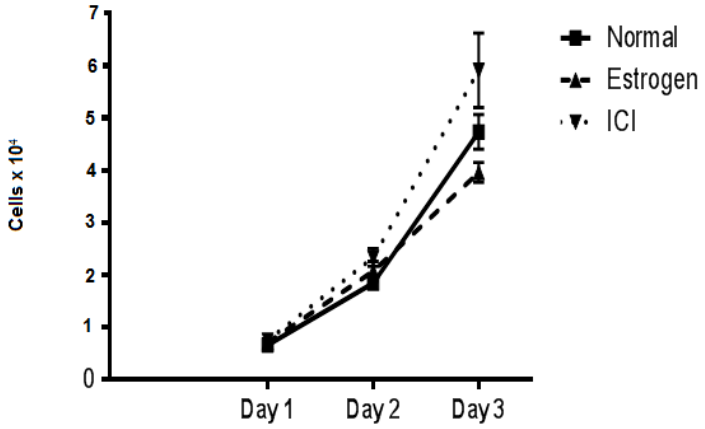


Figure 24. Estrogen Inhibition During Osteoblast Differentiation Inhibited MDA-MDA-231 BRMS1 Proliferation on a Live Osteoblast Culture

MC3T3-E1 were plated at a cell density of 10,000 cells/cm^2 in 24 well plates slides and differentiated under control estrogen concentration, with the estrogen inhibitor, or estrogen supplementation for four weeks in order to create a mature bone matrix. Medium was changed every three days. After four weeks, matrices were unaltered to retain live osteoblasts. MDA-MB-231 BRMS1 GFP were added at a density of 2x10^3 cells/cm^2 and allowed to proliferate for 24, 48, and 72 hours. At each time point three wells were analyzed via the GFP lysis assay to quantify cell number. Fluorescence was converted to cell number by comparison to a standard. Cell number was used to create a three day growth. Values were statistically analyzed via one-way ANOVA and Bonferroni's post test.

Chapter 5

Effects on Inflammatory Cytokine Production

In order to test the hypothesis that the live osteoblasts within the bone matrix create an inhibitor of cancer cell growth, ELISA was used to analyze bone remodeling cytokines. MCP-1 and IL-6 were chosen due to their production by osteoblasts during the bone buildup. These bone remodeling cytokines are also known to be attractive to cancer cells, stimulating them to grow within the bone microenvironment (Sosnoski, 2015).

MC3T3-E1 cells were differentiated for four weeks in order to create a mature matrix under normal, inhibited, and supplemented estrogen conditions. After four weeks, osteoblasts were co-cultured with MDA-MB-231BRMS. BRMS cells were added in osteoblast differentiation medium with addition of estrogen supplement or inhibitor in the respective conditions. Supernatants were collected after 24, 48, and 72 hours and used for ELISA analysis.No difference in MCP-1 secretion was detected for the three estrogen conditions, however, IL-6 secretion increased with estrogen receptor inhibition (Figure 25 & 26)

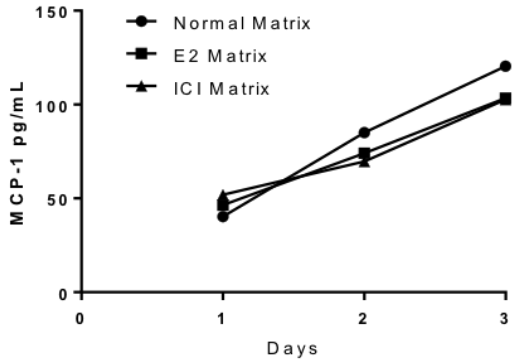


Figure 25. Estrogen Inhibition or Supplementation Did Not Affect MCP-1 Secreion During Co-Culture of MC3T3-E1 with MDA-MB-231 BRMS1

MC3T3-E1 cells were differentiated for four weeks in order to create a mature matrix under normal, inhibited, and supplemented estrogen conditions. After four weeks, osteoblasts were co-cultured with MDA-MB-231BRMS. BRMS cells were added in osteoblast differentiation medium with addition of estrogen supplement or inhibitor in the respective conditions. Supernatants were collected after 24, 48, and 72 hours and used for ELISA analysis. Experiments were conducted once, so no statistics are available. Given values are averages.

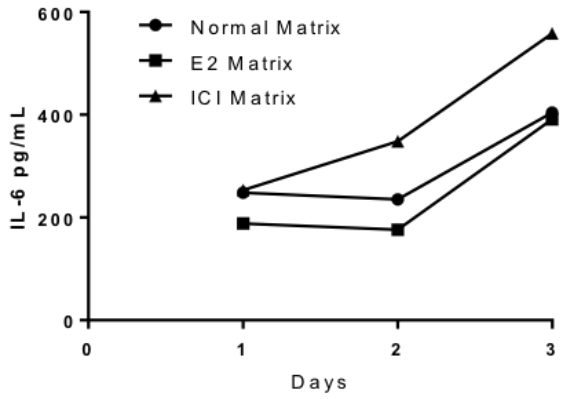


Figure 26. Estrogen Inhibition Increased IL-6 Secretion During Co-Culture Of MC3T3-E1 with MDA-MB-231 BRMS1

MC3T3-E1 cells were differentiated for four weeks in order to create a mature matrix under normal, inhibited, and supplemented estrogen conditions. After four weeks, osteoblasts were co-cultured with MDA-MB-231BRMS. BRMS cells were added in osteoblast differentiation medium with addition of estrogen supplement or inhibitor in the respective conditions. Supernatants were collected after 24, 48, and 72 hours and used for ELISA analysis. Experiments were conducted once, so no statistics are available. Given values are averages.

Chapter 6

Discussion and Future Directions

The extracellular bone matrix plays an important role in the process of breast cancer metastasis to bone. By studying the interaction between metastatic breast cancer cells and the ECM, possible triggers of metastasis to bone and factors that both promote and inhibit tumor cell proliferation can be discovered. The experiments described previously may have several implications for the future of breast cancer metastasis to bone research and potentially treatment as well.

Experiments performed to determine the growth and attachment of BRMS cells on fixed versus live osteoblast cell cultures showed that the cancer cells prefered the fixed cells and matrices. The results

of the experiments did not provide an explanation of why the cells preferred a fixed surface, but it may have to do with the process of epithelial mesenchymal transition (EMT) that occurs when cancer cells break away from the primary tumor and travel to a secondary site (Burridge, 1992). A cytoskeletal protein called EPLIN, epithelial protein lost in neoplasm, crosslinks actin, stabilizes cytoskeletal filaments and inhibits cell motility. This inability to move could potentially aid cancer cells in firmly attaching to and thus proliferating on a fixed matrix (Takeichi, 2008). Interestingly, EPLIN is downregulated in several types of cancer cells including breast cancer cells. EPLIN forced expression in MDA-MB-231 cells reduced migration of invasion, thus potentially improving outcome for patients (Wen G Jiang, 2008). These data have interesting implications for the interpretation of the results obtained from the experiments described in this thesis. Fixation of the matrix could potentially aid metastatic breast cancer cells in the EMT. The BRMS cells should also express low levels of EPLIN, as they are an isologous line to the MDA-MB-231 cell line. Fixation of the matrix may help the cancer cells successfully attach and grow even without high levels of EPLIN, as the fixed matrix should already be more suitable for their attachment and growth.

Fixation experiments could also potentially have different outcomes based on different fixation methods. Most fixation in the experiments described inthis thesis occurred via paraformaldehyde exposure. Formaldehyde fixation works by reacting with primary amines to form Schiff bases with amides, thus forming hydroxymethyl compounds. These hydroxymethyl groups condense and consequently crosslinking within the protein. However, certain proteins have a greater affinity for formaldehyde than others. For example, formaldehyde binds well to tyrosine rings, but can also effectively bind to phenylalanine or tryptophan in the absence of tyrosine. Proteins that are not rich in these amino acids may not fix as well as others (Thavarajah, 2012). Osteoblast bone matrix may not be the suitable tissue sample for fixation by paraformaldehyde because it is so rich in collagen. Collagen is mostly made up of glycine, proline, and hydroxyproline; it does contain an abundance of tyrosine or the

other previously mentioned amino acids (Shoulders, 2009). Fixation could have also been performed using aldehydes, oxidizing agents, or metallic group fixatives.

Early experiments exploring cancer cell growth on fixed and live osteoblast matrices led to the hypothesis that breast cancer cell proliferation was dependent on the structure of the surface that they grew on. The Chondrex stain and fluorescent collagen labeling showed that protein levels and visible collagen arrangement, respectively, were altered when osteoblasts were differentiated without access to estrogen. Because the cancer cells proliferated more on a matrix altered by paraformaldehyde fixation, it was proposed that proliferation would also be affected by a structure altered by changing the osteoblast matrix via varying estrogen concentration. Proliferation and attachment assays on decellularized matrix, however, did not appear to be affected by the estrogen levels during osteoblast differentiation. In order to stimulate a more realistic and more similar to an *in vivo* type of bone metastasis environment and account for more variables with estrogen experiments, the osteoblasts were left intact and experiments were repeated. Differences in BRMS attachment and proliferation were observed on live osteoblast cultures grown with various estrogen conditions; cells attached more and grew more on the estrogen inhibited osteoblast culture. Thus, it was hypothesized that osteoblasts respond differently to cancer cells depending on estrogen availability during differentiation. Due to the increase in BRMS proliferation on osteoblast layers grown with the estrogen inhibitor compared to the cultures grown with higher amounts of estrogen, it was thought that the estrogen-altered environment stimulated osteoblasts to produce bone remodeling cytokines that stimulate BRMS growth. Additionally, it is possible that estrogen inhibition could cause osteoblasts to produce inflammatory cytokines that may also stimulate the growth of BRMS.

It is known that decreased estrogen during menopause affects bone structure. Osteoblasts and osteoclasts both have estrogen receptors and are affected by the amount of estrogen present. Bone resorption increases, causing a general loss of bone mass and a greater tendency for bone fracture. The bones become more fragile, and when bone trauma occurs inflammatory cytokines are produced. Prior to menopause when estrogen levels in the body are higher, estrogen regulates the expression of IL-6. Lower

IL-6 expression creates an environment less likely to attract metastatic breast cancer cells. It is unclear, however, if estrogen affects bone resorption or bone formation more (H. Kalervo Väänänen, 1996). Another study carried out with mice which had osteoclasts with the estrogen receptor knocked out showed that estrogen plays an important role in osteoclast differentiation. Estrogen is vital for initial formation of osteoclasts, but it is less important for mature osteoclasts. Researchers concluded that estrogen inhibits the vitality of osteoclasts and in turn decreases bone resorption activity (Imai Y, 2009). All of this information makes it more understandable why no changes were seen in breast cancer cell growth or attachment on the decellularized matrix. The bone cells have an important role in creating cytokines that can be recognized by cancer cells. It seems that the change in bone structure alone is not enough to significantly affect the cancer cells. Although literature supports the idea that bone structure changes significantly when estrogen levels are decreased, in terms of breast cancer metastasis to bone, the effect of estrogen on the bone cells may be more important. Data from experiments discussed prior indicate that estrogen plays a role on the osteoblasts, as breast cancer cells attached and proliferated more when osteoblasts were differentiated with the estrogen inhibitor. Estrogen inhibition also caused an increase in production of the inflammatory cytokine IL-6. Future experiments should be carried out to account for bone forming and bone resorbing cells in order to get a more complete picture of which cell type could potentially affect breast cancer cell attachment to bone matrix and proliferation on a bone matrix.

This project may have benefitted from more overall experiments with live osteoblasts cultures instead of decellularized matrix. Although it is apparent that estrogen inhibition changes the collagen structure of the bone and decreases the amount of total protein created by osteoblasts, it seems that these structural changes alone did not significantly affect the breast cancer cells. In an attempt to separate the growth surface, the bone matrix, from the cells and their sections, experiments may have become too focused on an in *in vitro* model. Results from a decellularized matrix may not be very translatable to an *in vivo* system because cells will always be present within the bone matrix in a living model. It may be

possible that a combination of the cell growth surface and the cell secretions change the attachment or proliferation of cancer cells, but separating them entirely is difficult and may not be useful. Additionally, carrying out experiments longer than four weeks may be valuable. Much information is already known about how estrogen affects bone formation, but there is still much to be learned about how these differences in bone density and structure affect the metastasis of breast cancer cells to the bone environment. It would be interesting to learn more about how breast cancer cells move through the bone; perhaps changes to collagen fiber arrangement may change how the cancer cells navigate the bone environment before they settle and grow. Implications of structure change via estrogen and how they affect dormancy should also be studied. Perhaps an estrogen deprived bone environment is less conducive to dormant cells, while an estrogen rich environment is more likely to lead to cells becoming dormant.

Currently, several therapies for breast cancer focus on altering the amount of estrogen present in the body. An important part of diagnosis of breast cancer is classifying the type of breast cancer cells and whether that type of breast cancer cell has estrogen receptors. In order to treat breast cancers that are estrogen receptor positive, procedures such as oophorectomy or treatment with aromatase inhibitors are prescribed in order to reduce the estrogen within the body. While these treatments may be successful in treating a primary breast tumor, results from experiments described above may bring other side effects to light. Inhibiting estrogen receptors on osteoblasts may create a bone miroenvironment that is conducive to metastasis. A loss in bone density, leading to possible osteoporosis or bone fracture, is listed as a side effect of taking aromatase inhibitors to treat breast cancer. This side effect, however, does not currently account for possible induced bone metastases (Zeleniuch-Jacquotte A, 2004).A decrease in bone density will create an inflammatory environment within the bone as the bone tries to remodel itself. While the primary tumor is treated with the estrogen inhibitor, cells may travel to the bone and lie dormant. Once the primary tumor has successfully been treated, the patient would typically stop taking the estrogen inhibitor and their bone density would increase. Throughout this process, the breast cancer cells may remain quiescent within the bone. An eventual bone break or other traumatic event may eventually

reawaken these metastasized cells, leading to a recurrence of the initial disease within the bone. By treating breast cancer patients with estrogen inhibitors, doctors may be setting up patients for eventual relapse by recurrence of the cancer within the bones.

Lastly, all experiments regarding estrogen were conducted using breast cancer cells that were estrogen receptor negative. It would be interesting to see if estrogen receptor positive cells, such as the human cell line MCF-7, would respond differently than the 231 cells on osteoblast matrices differentiated in varying estrogen. Future experiments should also aim to produce a human-human model instead of the mouse-human model used in these experiments. By using human osteoblasts and human cancer cells, a more realistic model of what happens in breast cancer metastasis in humans could be achieved. Experiments eventually could be repeated in the bioreactor, a 3-D growth chamber that simulates a more *in vivo* type environment.

Appendix A

GFP Cell Quantification Assay

1. Rinse cells with PBS.
2. Add 0.5 mL PBD Buffer (Table 6).
3. Scrape cells into 2 mL Eppendorf tubes with cell scraper.
4. Incubate on ice for 10 minutes.
5. Spin at 17,000 g for 5 minutes.
6. Aspirate supernatant and put into a clean tube. Freeze at -20°C if storing.
7. Load 100 uL/well into 96 well transparent flat bottom plate.
8. Load GFP cell standard.
9. Run Samples on Tecan Infinite M1000 Pro Plate Reader.
    a. Turn on machine, monitor, and computer.
    b. Start Icontrol software and click on instrument name. Initialization process will happen automatically.
    c. Drag fluorescent intensity scan from side bar into center screen.
        i. Set GFP reading: excitation (488 nm) and emission (509 nm).
        ii. Set band at 5 nm.
        iii. Set top and gain from the well with the first point of the standard curve.
    d. Place plate in Tecan tray without cover.
    e. Start measurement. Excel will open automatically and transport data.

Table 6. PBD Buffer (100 mL)

Component	Amount
Tris (1 M, pH 7.5)	5 mL – 12.114 g
$MgCl_2$ (100 mM)	2 mL – 0.01904 g
$NaCl_2$ (5 M)	2 mL – 0.5844 g
NP40	1 mL
Glycerol	10 mL
Sterile ddH_2O	80 mL

Appendix B

ELISA Assay for Cytokine Quantification

****Note: When working with antibodies or proteins, be sure to keep them out at room temperature as little as necessary and keep them on ice.****

Day 1:

1) Create capture antibody and dilute to necessary concentration with Ngai's buffer (15 mM Na_2HCO_3, pH 9.6). *Be sure to avoid repeated freeze/thaw cycles of "stock" antibody/protein. Always dilute the antibody/protein to a "working stock" concentration and use this "working stock" for subsequent dilutions.*
2) Add 50 µl working solution antibody to each well (is easiest to use the multi-channel pipettor).
3) Seal plate using plate lid and parafilm and incubate overnight at 4ºC in a closed humidified container (in a Tupperware box, place a ddH_2O moistened paper towel on the bottom and cover the container tightly).

Day 2:

4) Blocking:
    a) Wash ELISA plate 4x @ 1 min. each with PBS/Tween, pH 7.0 (washing step, PBS/Tween is made using 500 ml 1x PBS to 500 µl Tween 20).
    b) Shake plate out into the sink between each wash and tap plate on paper towels after final wash to remove excess solution.
    c) Add 200 µl of 1% BSA dissolved in PBS (blocking step. 1% BSA is made at 1g BSA to 100 ml PBS).

d) Seal plate using plate lid and parafilm, put in a closed humidified container, and incubate for at least 2 hours at room temperature.

5) Apply standards and samples to plate:

a) Wash ELISA plate 3x @ 1 min. each with PBS/Tween, pH 7.0.

b) Shake plate out into the sink between each wash and tap plate on paper towels after final wash to remove excess solution.

c) Add 100 µl standard or sample to respective wells (standards should be done in duplicate and samples should be done in triplicate, if possible.). **Note: When performing serial dilutions to create standards, be certain not to cross-contaminate less concentrated dilutions by using a new pipette tip for each dilution. i.e. The same pipette tip can be used when going "up" in dilution (e.g. from 4 mg/ml to 8 mg/ml), however a new pipette tip must be used when going "down" in dilution (e.g. from 8 mg/ml to 4 mg/ml). Also, samples should be "cold" thawed in a refrigerator and be sure to use the "working stock" dilutions of a protein to avoid repeated freeze/thaw cycles of the "stock" protein. Proteins should only be kept out as long as necessary and should be kept on ice.*

d) Seal plate using plate lid and parafilm, put in a closed humidified container, and incubate overnight at 4ºC.

Day 3:

6) Wash ELISA plate 4x @ 1 min. each with PBS/Tween, pH 7.0.

7) Shake plate out into the sink between each wash and tap plate on paper towels after final wash to remove excess solution.

8) Create a working detection antibody solution in 1% BSA dissolved in PBS. *Be sure*

to avoid repeated freeze/thaw cycles of "stock"antibody/protein. Always dilute the antibody/protein to a "working stock" concentration and use this "working stock" for subsequent dilutions.

9) Add 100 µl detection antibody to each well.
10) Seal plate using plate lid and parafilm, put in a closed humidified container and incubate for 2 hours at room temperature.
11) After 2 hours, wash the plate 6x @ 1min. each with PBS/Tween, pH 7.0.
12) Shake plate out into sink between each wash and tap plate on paper towels after final wash to remove excess solution.
13) Dilute NeutrAvidin Horseradish Conjugate 1:1000. Working stock is 1 µl in 1 ml PBS.
14) Add 100 µl NeutrAvidin HRP to each well.
15) Seal plate using plate lid and parafilm, put in a closed humidified container and incubate for 30 min. at room temperature.
16) Wash plate 8x @ 1 min. each with PBS/Tween, pH 7.0.
17) Shake plate out into sink between each wash and tap late on paper towels after final wash to remove excess solution.
18) Prepare ABTS substrate by adding 100 µl hydrogen peroxide (3%) with 10 ml ABTS. *Be sure to "cold" thaw ABTS.*
19) Add 100 µl ABTS substrate to each well.
20) Incubate for 60-90 min. at room temperature in the dark unsealed. Aluminum foil is loosely placed around the plate to keep the plate in the dark.
21) Read the plate at 405 nm using an ELISA plate reader.

Be sure to use good pipetting and laboratory techniques with this procedure for consistency.

Suggested ELISA Antibody / Protein Stock and Aliquoted Concentrations

Table 7. Capture Antibodies

Cytokine	Diluent	Stock Concentration	Working (aliquoted) Concentration	ELISA Concentration
IL-6	Sterile PBS	500 ug/mL	50 ug/mL	2 ug/mL in 50 uL
MCP-1	Sterile PBS	500 ug/mL	100 ug/mL	0.4 ug/mL in 100 uL

Table 8. Protein Standards

Cytokine	Diluent	Stock Concentration	Working (aliquoted) Concentration
IL-6	1% BSA in PBS	20 ug/mL	50 ug/mL in 100 uL
MCP-1	1% BSA in PBS	10 ug/mL	5 ug/mL in 100 uL

Table 9. Detection Antibodies

Cytokine	Diluent	Stock Concentration	Working (aliquoted) Concentration	ELISA Concentration
IL-6	TBS pH 7.3 (20 mM Trizma base, 150 mM NaCl containing 0.1% BSA)	50 ug/mL	50 ug/mL	100 ng/mL in 100 uL
MCP-1	TBS pH 7.3 (20 mM Trizma base, 150 mM NaCl containing 0.1% BSA)	50 ug/mL	50 ug/mL	100 ng/mL in 100 uL

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ACADEMIC VITA

Academic Vita of Shelby Foster
Sjf5224@psu.edu

EDUCATION

The Pennsylvania State University - Schreyer Honors College *August 2012 – May 2016*
State College, Pennsylvania
Bachelor of Science, Biochemistry & Molecular Biology; Minor, Technical Writing

- Relevant coursework including lab courses requiring proficiency in PCR, gel electrophoresis, western blotting, microscopy, bacterial culture and plasmid mapping, DNA and protein purification, etc.

WORK EXPERIENCE

Research Assistant *May 2015 – August 2015*
Pfizer Oncology Research Unit, Pearl River, NY
Dr. Ken Geles Lab

- Investigated acinar development of primary lung cancer cells in a 3-D growth environment through culturing cells in Matrigel, western blotting, immunofluorescence, and qRT-PCR
- Worked independently on an individual research project and presented data at a group meeting

Undergraduate Researcher *January 2013 – Present*
The Pennsylvania State University, State College, PA
Dr. Andrea Mastro Lab

- Studied the interaction between metastatic breast cancer cells, osteoblasts, and the bone matrix
- Trained in sterile technique, tissue culture methods, small scale bioreactors, and mouse handling
- Co-authored article published in *Clinical and Experimental Metastasis*
    - Sosnoski, D., Norgard, R., Grove, C., Foster, S., & Mastro, A. (2015). Dormancy and growth of metastatic breast cancer cells in a bone-like microenvironment. *Clinical & Experimental Metastasis,32*(4), 335-344.
- Poster Presentation at American Association for Cancer Research Annual Meeting, April 2015
    - "Matrix Manipulation affects attachment and growth of breast cancer cells in a bone-like microenvironment *in vitro*." Abstract No. 4017

Peer Tutor in Writing *August 2013 – Present*

Penn State Learning, The Pennsylvania State University, State College, PA

Resident Assistant *August 2014 – Present*

The Pennsylvania State University, State College, PA

LEADERSHIP & AWARDS

Science LionPride President, Service Director *April 2013 – Present* Student ambassador group to the Eberly College of Science involved in community outreach, recruiting potential Penn State Science Students, interaction with Eberly College of Science Alumni

Sigma Xi Undergraduate Research Award *January 2016*

Gail Folena-Wasserman Undergraduate Research Scholarship Recipient *April 2014*

Student Leadership Scholarship Recipient *December 2013*