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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

IMPACT OF OSTEOBLAST-DERIVED BONE REMODELING CYTOKINES ON METASTATIC BREAST CANCER CELL DORMANCY

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ABSTRACT

It has been observed that patients in remission from breast cancer often have a reoccurrence of breast cancer at the site of a bone injury. Sometimes this reoccurrence as a metastases occurs many years after a “cure” has been declared. Thus the original cancer likely metastasized but remained dormant. Therefore, it would be beneficial to determine how certain factors in the bone-remodeling environment impact dormancy and metastatic breast cancer cell proliferation and morphology. The aspects of the bone-remodeling environment that make it unique as compared to the homeostatic bone microenvironment include the presence of bone-remodeling cytokines, inflammatory cytokines, and the increased activity for the osteoblasts and osteoclasts.

It is proposed that by observing how certain bone-remodeling cytokines, inflammatory cytokines, and osteoblasts impact breast cancer cell dormancy *in vitro*, a better understanding of how breast cancer reoocurs will be gained. The inflammatory cytokines included IL-6, MCP-1, IL-8, VEG-F and Gro-α [1]. Bone Remodeling Cytokines included TGF-β, IL-6, IL-1β, and PGE2 [2]. Three breast cancer cell lines were used, MDA-MB 231, a highly aggressive line, MDA-MB 231 BRMS11, a metstatic-suppressed variant of the MDA-MB 231 cell line, and MCF7, a non-metastatic line. An MTT assay revealed that the bone remodeling cytokines stimulated more proliferation of breast cancer cells than inflammatory cytokines. In co-culture, MC3T3-E1s, or murine osteoblasts, were cultured for one month to allow them to lay down a matrix. At this point, some cultures were decellularized and breast cancer cells were added. Additionally, some of the cancer cells both with and without osteoblasts received

bone-remodeling cytokines. Proliferation was measured either with a biochemical assay (MTT), or by quantitation of the expression of the green fluorescent protein expressed by the cancer cells. Morphology was examined using confocal microscopy. The bone remodeling cytokines clearly enhanced breast cancer cell proliferation, both on the charged plastic surface of standard tissue culture plates, and on the bone matrix *in vitro*. However, the same effect was not observed when the osteoblasts were present, indicating that these cells may play a key role in the cross-talk between cytokines and breast cancer cells.

TABLE OF CONTENTS

List of Figures……………………………………………………………………………iv

List of Abbreviations..v

Acknowledgements………………………………………………………………………vi

Chapter 1 Introduction………………………………………………………………….1

Chapter 2 Materials and Methods………………………………………………………10

Chapter 3 Results- Proliferation of Breast Cancer Cells In Response to Inflammatory and Bone Remodeling Cytokines…..…..……………………………………………….....14

Chapter 4 Impact of Bone Remodeling Cytokines on MDA-MB 231 BRMS1 Proliferation and Morphology on a Bone Matrix *In vitro*………...……….…………………….…..18

Chapter 5 Impact of Bone Remodeling Cytokines on MCF7 Proliferation and Morphology on a Bone Matrix *In vitro* …………………….……..................................26

Chapter 6 Discussion………………………………………………………...............…32

Appendix A GFP Assay Verification ……………………….……………………...38

Appendix B Morphology of MDA-MB 231……………………………………….....39

BIBLIOGRAPHY……………………………………………………………………….41

LIST OF FIGURES

Figure 1-1: Proliferation of Breast Cancer Cells In Response To Inflammatory and Bone Remodeling Cytokines……………………………………………………………………..17

Figure 2-1: MDA-MB 231 BRMS1 Proliferation on a Bone Matrix *In vitro*…............19

Figure 2-2: Proliferation of MDA-MB-231 BRMS..20

Figure 2-3: Proliferation of MDA-MB 231 BRMS1 in Various *In vitro* Environments..22

Figure 2-4: Morphology of MDA-MB 231 BRMS1 in Response to Cytokines on a Bone Matrix...24

Figure 2-5: DIC Images of Morphology of MDA-MB 231 BRMS1 on a Bone Matrix In Response to Bone Remodeling Cytokines. 25

Figure 3-1: MCF 7 Proliferation on a Bone Matrix *In vitro*...27

Figure 3-2: MCF-7 Proliferation on a Bone Matrix in Response to Bone-Remodeling Cytokines *In vitro*...28

Figure 3-3: Morphology of MCF7 on a bone matrix *in vitro* in response to bone remodeling cytokines...30

Figure 3-4: DIC Images of MCF7 Morphology in response to Bone Remodeling Cytokines on a Bone Matrix with Osteoblasts *in vitro*...31

Figure 4-1: GFP Assay and RFU Reading Verification..38

Figure 5-1: Morphology of MDA-MB 231 Cells in response to Osteoblasts and Bone Remodeling Cytokines...39

List of Abbreviations

BR/ BRC- bone remodeling cytokines
BRMS- MDA-MB 231 BRMS1
CCL2- chemokine ligand 2
FGF- fibroblast growth factor 2
GFP- green fluorescent protein
GROα- growth regulated alpha protein
IL-6- interleukin 6
IL-1β- interleukin 1β
IL-8- interleukin 8
MCP-1- monocyte chemoattractant protein 1
MTT- methyltriazol tetrazolium
OB- osteoblasts
PGE2- prostaglandin E2
RFU- relative fluorescence units
SDF-1- stromal cell-derived factor 1
TNFα- tumor necrosis factor alpha
VEG-F- vascular endothelial growth factor

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Chapter 1

Introduction

In the United States alone, it is estimated that there will be over 232,000 new cases of invasive breast cancer diagnosed this year. Additionally, over 39,000 women are expected to die as a result of breast cancer[1]. Cancer has multiple stages, from stage 1, which is the earliest form, to stage 4, which is the most dangerous and deadly stage. Starting in stage 1, cancer can begin to spread outside of the site of the primary tumor, and by stage four, the cancer has spread to different organs or tissues[1]. Cancer cells break away from the primary tumor, migrate around the body via the blood stream, and eventually break through the endothelium and into the tissue to form a metastasis [3]. Metastases can alter the environment of the organ to severely compromise patient health. For patients with metastatic breast cancer, the most common site of metastasis is the bone. Cancer cells are transported from the breast tissue through the blood stream and integrate into the bone microenvironment, altering it and creating a vicious cycle that results in bone degradation.

The bone microenvironment is influenced largely by two key cell types- osteoblasts and osteoclasts. The osteoblasts lay down bone matrix to make the bone, while the osteoclasts degrade the bone. In a cancer-free environment, both cell types work cooperatively so the overall effect is no net change in the adult bone mass. However, when cancer cells are present, a vicious cycle is initiated in which the osteoclast activity of bone resorption is up-regulated. This resorption of the bone matrix

releases growth factors, which then stimulate tumor cell activity, and the vicious cycle is set in motion. Osteoblasts also play a key role in this bone microenvironment, as there is much remodeling of the matrix occurring[4]. It is thought that their activity may also play a key role in the cross-talk with cancer cells [5], though their activity is not as well known.

Both during and following treatment, a patient may have circulating cancer cells, or some cancer cells which may have settled into a metastatic site but that have entered a state of dormancy. Dormancy is defined as a period in which the cell cycle is arrested, little to no DNA transcription occurs, and only enough mRNA to maintain a vegetative state is translated [6]. In this state, the cancer cells cease dividing, but remain quiescent. During this time, there is no net degradation of the bone due to an increase in osteoclast activity. However, it has been anecdotally reported that following a bone break, or other injury to the bone, the breast cancer cells awaken from dormancy and the metastases become active again. This activity results in a re-initiation of the vicious cycle [3].

Remodeling of the extracellular matrix, increased activity of osteoblasts, and rearrangement of the metastatic niche have been thought to stimulate the awakening from dormancy [5].Due to the observation that bone injuries stimulate cancer cell awakening, it was worthwhile to look at the interaction between the cancer cells, the cytokines involved in bone remodeling after injury and inflammation, and osteoblasts, the main bone cell involved in reforming bone.

In bone-remodeling, four cytokines play a particularly important role in the activity of osteoblasts. IL-6, TNF-α, IL-1β, and PGE2 all work in this environment to stimulate bone remodeling, but they may also work to reactivate cancer cells[2] [5] [3].

The extent to which these cytokines work together and impact the dormancy of metastatic breast cancer cells remains to be explored.

The IL-6 family of cytokines plays a variety of roles. One of the main roles it plays is that of bone formation. In regard to osteoblasts, the IL-6 cytokine family is thought to induce differentiation of osteoblasts [7]. This differentiation includes the up-regulation of certain markers such as alkaline phosphatase and osteocalcin. Upregulation of these factors then enhances bond nodule formation and mineralization of the extracellular matrix it produces. IL-6 is thought to act on osteoblasts mainly through the STAT3 pathway, though some other pathways have been implicated in the interaction between IL-6 and the osteoblasts[7] . Other roles of IL-6 on maturing osteoblasts include reduced proliferation and inhibition of apoptosis. These opposite effects have been noted to occur in the presence of IL-6 when the osteoblasts are less mature. Cancer cells produce certain cytokines when they form metastases. These cytokines include IL-6, along with others, such as parathyroid hormone, that enhance bone resorption by stimulating osteoclastogenesis and diminishing osteoblastogenesis, and the vicious cycle of bone degradation and cancer cell growth commences [8] [9] [10]. Thus, it appears that IL-6 induces a differentiation of osteoblasts depending on the conditions and alters their activity, along with the structure of the bone microenvironment [10].

The role of IL-6 in breast cancer cells is much more complicated and contradictory. For both the MDA-MB 231 cell line and the MCF7 cell line, IL-6 has been shown to inhibit apoptosis and promote proliferation in some cases, while in other cases it promotes apoptosis and inhibits proliferation [11]. IL-6 has been shown to suppress

proliferation of breast cancer cell lines by blocking the G1 phase of the cell cycle. The STAT pathway activation by IL-6 is thought to induce p21, a cell cycle inhibitor. Additionally, it has been noted to have a pro-apoptotic effect by activating various death inducers and p53 [12] .However, IL-6 has also been known to be pro-tumorigenic, and act via the STAT3 pathway. STAT3 is the major effector of IL-6, and when this was blocked, cell growth was noted to have slowed cell growth in culture and in a xenograft model (i.e. human tumor cells transplanted into an immunocompromised mouse) [11] [13]. This model allows for the study of cancer cells in the in-vivo environment. Therefore, the effects of IL-6 have been observed both *in vitro* and in-vivo. Moreover, it has been noted that elevated levels of IL-6 are correlated with a poor prognosis for breast cancer patients [14]. The role of IL-6 remains very unclear. It may act differently depending on concentrations, conditions, and the exact cell lines used.IL-6's dual involvement in the bone remodeling pathway and cancer cell growth make it a good candidate to test in the bone remodeling-metastatic niche.

Another cytokine with dual involvement in bone remodeling and cancer cell growth, is also an interleukin family member. IL-1β is known to effect both breast cancer cells and bone cells. IL-1B mainly serves to inhibit bone formation. It acts on osteoblasts and causes them to produce prostaglandins and certain proteins [15]. The production of these molecules stimulates osteoclast growth and inhibits osteoblast activity.

IL-1B is also known to be a growth factor and has been shown to clearly promote cancer growth. This cytokine promotes the expression of pro-metastatic genes, such as

genes encoding matrix metalloproteinases, and also induces nearby cells to produce angiogenic proteins and other growth factors, such as IL-6[16].

TNF-α is known to stimulate bone resorption in osteoclasts. It activates the NF-kB pathway, and then promotes osteoclastogenesis. The differentiation of osteoclasts results, and bone resorption is increased in the bone microenvironment [2]. However, it has also been noted that TNF-α promotes differentiation of osteoblasts *in vitro*. This effect was detected via an increased expression of alkaline phosphatase, an osteoblastic differentiation marker, following treatment of the pre-osteoblasts with TNF-α. Additionally, matrix mineralization is also enhanced following treatment with TNF-α. These findings suggest that TNF-alpha plays a key role in bone remodeling [17].

This pro-differentiation factor, TNF-α, is also involved in alteration of breast cancer cell properties that contribute to tumor progression and relapse. It has been seen to cause cancer cells to have more stem-cell like properties. TNF-α increases the expression of transcription factors such as SLUG, which then increases expression of CD44 and Jagged 1. Increased expression of these factors contributes to tumor invasiveness and overall tumor aggression [18].

The fourth molecule addressed is PGE2. This prostaglandin is a small, lipid-like molecule. It is also downstream of IL-1β, and TNF-α, and is thought to have similar effects as these cytokines [19]. Generally, prostaglandins are thought to promote differentiation of osteoblasts and enhance proliferation of breast cancer by contributing to immunosuppression [20].

Inflammatory cytokines may also play a role in the bone microenvironment following bone injury. They are IL-6, GROα, VEG-F, IL-8, and MCP-1 [1]. IL-6 has already been discussed. However, GROα, one of the major chemokines involved in the inflammatory response, is thought to induce proliferation of cancer cells. When GROα receptors are blocked on cancer cells, proliferation is often slowed. However, when elevated levels of this chemokine are present, proliferation and cellular activity is enhanced. Additionally, osteoblasts are known to secrete GROα during the inflammatory response. However, GRO α does not impact the osteoblasts directly [1].

VEG-F also plays a role in the inflammatory response. Many tumor cells produce this cytokine. The production of this cytokine by the tumor cells is thought to lead to resistance to apoptosis, which then leads to increased proliferation and continued growth of the tumor. Additionally, due to the impact of the cytokine on endothelial cells, it is thought that VEGF may play a role in dissemination of tumor cells via circulation. This cytokine has been a therapeutic target, as blocking it has been noted to decrease tumor burden [21].

IL-8 is a fourth cytokine that is involved in the inflammatory response. IL-8 is highly correlated with the presence of human breast cancer tissue. IL-8 is expressed by both tumor cells as well as vascular endothelial cells. When present, IL-8 regulates tumor and vascular endothelial cell activation. This activation then results in control of proliferation, angiogenesis and metastasis. Higher levels of IL-8 and its receptor on breast cancer cells are associated with malignant cancer, indicating this cytokine has a pro-tumorigenic effect [22].

The last cytokine that will be studied in the inflammatory response is MCP-1. MCP-1 is produced by tumor cells, fibroblasts, endothelial cells, and monocytes. This cytokine is known to regulate the migration of macrophages into tumors. Interestingly, transfection of this gene into cancer cells enhances the metastatic potential of the cancer through increased angiogenesis. However, this cytokine also activates monocyte function to combat tumor cells. This cytokine plays a positive role in both the life cycle of the breast cancer cells, as well as the immune system [23].

Another relationship that needs to be considered is that between the breast cancer cells and osteoblasts. As mesenchymal stem cells differentiate into osteoblasts, levels of certain chemokines and cytokines change. The change in expression has an impact on the attraction of breast cancer cells, and also how the breast cancer cells behave. As mesenchymal stem cells differentiate into osteoblasts, the expression of CCL2 increases significantly. CCL2is a chemokine that acts as a chemoattracant to a variety of cell types. Additionally, the level of CCL2 in breast cancer cells is increased when they are co-cultured with the MSCs. The increase in CCL2 results in an increased attraction of the breast cancer cells to bone as the osteoblasts mature [24]. It has also been seen that breast cancer cells may prevent differentiation and alter adhesion molecules on the osteoblasts. There is less matrix mineralization as well, which may alter the ability of the breast cancer cells and the osteoblasts to remain on the matrix [25].

In order to study the impact that bone remodeling cytokines, inflammatory cytokines, osteoblasts, and the bone matrix have on dormant-like breast cancer cells, two main lines of breast cancer cells were analyzed. The human MDA-MB 231 breast cancer

cell line is known to be a very aggressive, and is estrogen receptor positive [26] Due to the aggressive nature, only the morphology of these cells was observed, and not the proliferation. However, a variant of this cell line was used to examine the impact these various groups of cytokines have on proliferation and morphology. The MDA-MB 231 BRMS1 are a metastatically-suppressed variant of the MDA-MB 231 cell line. They are much less aggressive, representing a more dormant-like state. They are also estrogen receptor negative. The second cell line in which both the proliferation and morphology was observed was the MCF7 breast cancer cell line. This cell line is non-metastatic, dormant-like, and unlike the other cell lines, is estrogen receptor positive [27].

One aim of this study was to determine the role of bone remodeling cytokines in breast cancer cell growth on a bone matrix *in vitro*. Also, it was important to determine the relationship the osteoblasts have to the breast cancer cells, and whether they are involved in the signaling between cytokines and breast cancer cells. Due to the fact that bone remodeling cytokines are often responsible for up-regulating the proliferation of cancer cells, and osteoblasts frequently produce these cytokines, it was hypothesized that addition of the bone remodeling cytokines would increase proliferation of the breast cancer cells on the bone matrix. Similarly, many of the inflammatory cytokines are implicated in up-regulation of breast cancer cell proliferation. Therefore, I predicted a similar result of enhance proliferation due to the addition of the inflammatory cytokines. Additionally, I predicted that the osteoblasts would further enhance proliferation of the cancer cells due to the observation that remodeling of the metastatic niche by fibroblasts often awakens cancer cells from dormancy, and the fact that osteoblasts produce

cytokines thought to be responsible for upregulating cell growth.

Chapter 2

Materials And Methods

2.1 Cells and Tissue Culture Conditions

Murine calvaria pre-osteoblasts (MC3T3-E1,) cells were cultured in alpha minimum-essential medium (α-MEM) and 10% fetal bovine serum (FBS), and in an incubator at 37^0C with 5%$CO_{2.}$ These cells were a gift from Dr. Norman Karen of the University of Delaware. Cells were passed every 3-4 days.

MC3T3-E1 cells were plated at 10,000 cells/cm^2 in 6-well plates. Following plating, cells were kept in differentiation media, (α modified eagle's medium (αMEM, Sigma, St. Louis, MI) supplemented with 10% FBS, 50 µg/ml ascorbic acid, 10 mM β-glycerolphosphate) (. Cells were grown for one month.

Three breast cancer cell lines were used in these experiments. MDA-MB 231 breast cancer cells are aggressive, estrogen receptor negative cell line. MDA-MB 231 BRMS1 are a metastatically-suppressed variant of the MDA-MB 231 cell line. MCF7 are non-metastatic, estrogen receptor positive cell line [27]. Each cell line was engineered to express green fluorescent protein. MDA-MB 231cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Cellgro, Herndon, VA), 5% FBS, penicillin (100ug/ml), streptomycin (100 ug/ml solution; Mediatech Inc, Manassas, VA) and Nonessential Amino Acids (NEAA, 100µg/ml, Cellgro, Manassas, VA). MDA-MB 231 BRMS1 were also used and cultured in DMEM/F12, 5% FBS, penicillin (100ug/ml),

streptomycin (100 ug/ml), and Nonessential Amino Acids (NEAA, 100 µg/ml, Cellgro, Manassas, VA). MCF7 cells were cultured in DMEM, 10% FBS, and penicillin (100ug/ml), streptomycin (100 ug/ml),. Each line was kept at 37^{0}C and 5% CO_2.

2.2 Decellularization

After one month, two of the 6-well plates were decellularized.In order to decellularize the matrix laid down by the month-old osteoblasts, wells were initially washed once with 2 mL of PBS. Then, 2 mL of 12mM sodium deoxycholate in 10 mM Tris-HCl at pH8 was added to each well. The plates were incubated at 4^{0}C for 10 minutes. After incubation, the lysing solution was removed, and each well was washed 4 times with PBS.

2.3 Proliferation Assay

To analyze proliferation of the breast cancer cell lines, one of two assays was employed. A Methylthiazol Tetrazolium (MTT) Assay or a quantitation of the GFP expressed by the cancer cells was used.

2.3.1 MTT Proliferation Assay

Breast cancer cells (MDA-MB-231, MDA-MB-231 BRMS, MCF7 cells) were plated in a 24-well plate at a density of 5,000 cells/well in respective media (2.1). Cells were incubated for 24 hours. A baseline level of MTT was carried out 24 hours after plating.

The MTT reagent was purchased from Sigma (St. Louis, Missouri). Culture media were removed. 20µl of 5 mg/ml MTT was added to each well and

incubated for 2 hours at room temperature in culture hood. Media were removed and 200 µl of solubilization solution (10% Triton X-100,0 .1N HCl in anhydrous propanol) was added to each well. Plates were covered with aluminum foil and put on a rotary shaker for 15 minutes. The absorbance was read using the Packard SpectraCount ® plate reader at 550 nm with a reference filter at 620nm.

Immediately following the baseline measurements, both bone remodeling and inflammatory cytokines were added to separate replicate wells. The bone remodeling cytokines were TNF-α (5 ng/mL), IL-1β (10 ng/mL), IL-6 (10 ng/mL), and PGE2 (10 ng/mL) . The inflammatory cytokines were IL-6 (5 ng/mL), MCP-1 (2 ng/mL), IL-8 (0.5ng/mL), VEGF (2 ng/mL), and GRO-α (.25 ng/mL). These concentrations mimic in-vivo bone remodeling and inflammatory conditions respectively[28] [29]. FGF was also added to all wells containing MCF7 cells to keep them dormant [30]. All cytokines were purchased from R&D Systems (Minneapolis, MN)

2.3.2 Quantification of Green Fluorescent Protein (GFP)

Following decellularization, breast cancer cells were added to each well, of both decellularized and non-decellularized plates. Three cell lines were used: MDA-MB-231, MDA-MB-231 BRMS, and MCF 7s. Cells were plated at a density of 4,000 cells/cm^2. After incubating each plate at 37^{0}C for 6 hours, bone remodeling cytokines were added to one decellularized plate, and one non-decellularized plate for each cell line.

24, 48, and 72 hours after addition of the bone remodeling cytokines, wells were washed with PBS. After PBS was removed, 0.5 mL of PBD Buffer

(sterile H_20, Glycerol, N-P40, 5M NaCl, $MgCl_2$, and 1M Tris at pH 7.5) was added to each well. Plates were incubated for 10 minutes at 25 ^{0}C to lyse the cells. Lysate was then transferred to 1.5 mL microcentrifuge tubes and centrifuged for 5 minutes at 9447 x g. 100 µL of supernatant was then transferred to a 96 well plate in triplicate. Absorbance was read using the Infinite M1000 Pro Tecan Plate-Reader at 488-509 nm.

2.4 Morphology

Breast cancer cells were plated on bone matrix grown from the osteoblasts in the 6-well plates following the methods used for the GFP proliferation assay. Instead of washing the cells with PBS and lysing them with PBD Buffer, cells were imaged under the Olympus Fluoview 300 Confocal Microscope.

2.5 Statistical Analysis

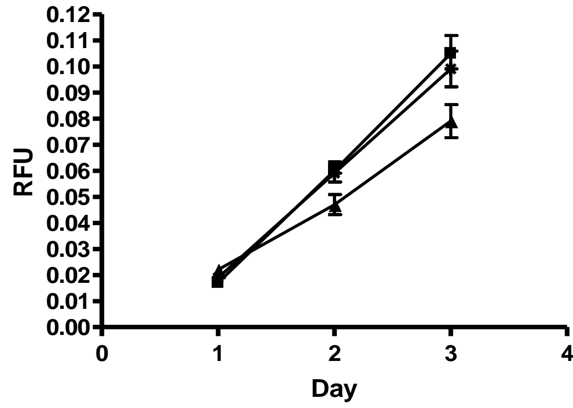
Data for the proliferation assays was analyzed using the GraphPad 4- Iprism. A two way ANOVA was performed in order to determine statistical significance.

Chapter 3

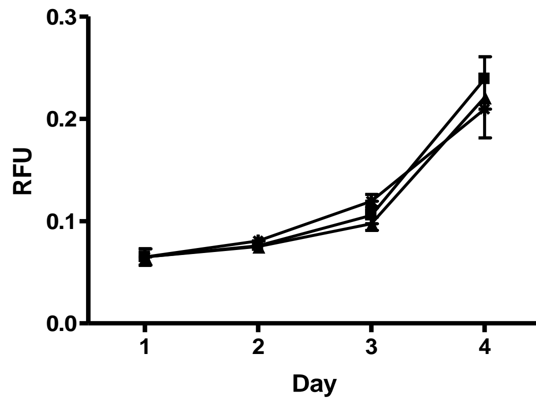
Results- Proliferation of Breast Cancer Cells In Response to Inflammatory and Bone Remodeling Cytokines

Because it was proposed that injury to the bone could have an impact, either directly or indirectly, to the ability of breast cancer cells to proliferate, two groups of cytokines were tested, inflammatory and bone remodeling. To discover if the cytokines had a direct impact on the breast cancer cells, the breast cancer cells were plated in a 96-well plate at a density of 20,000 cells/cm^2. After 24 hours, either the inflammatory cytokines (IL-6, GROα, VEG-F, IL-8, and MCP-1, at the concentrations given in the methods), or the bone remodeling cytokines (TNFα, IL-1β, IL-6, and PGE2 at the concentrations given in the methods) were added to the appropriate wells. Beginning at 24 hours following the initial plating of the breast cancer cells, the proliferation of the three breast cancer cell lines was measured at 24 hour intervals for 72 hours total using the MTT Assay. Proliferation was measured before the cytokines were added, and again 24, 48 and 72 hours following addition of inflammatory and bone remodeling cytokines. As controls, some co-cultures did not have cytokines added to the medium.

a.1



a.2



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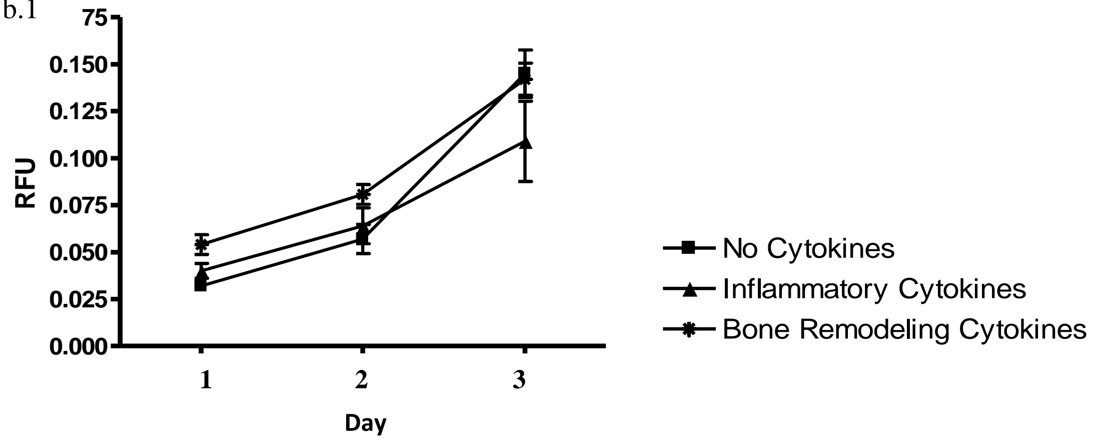

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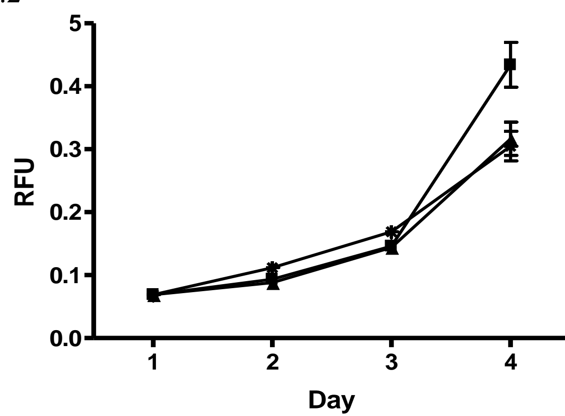

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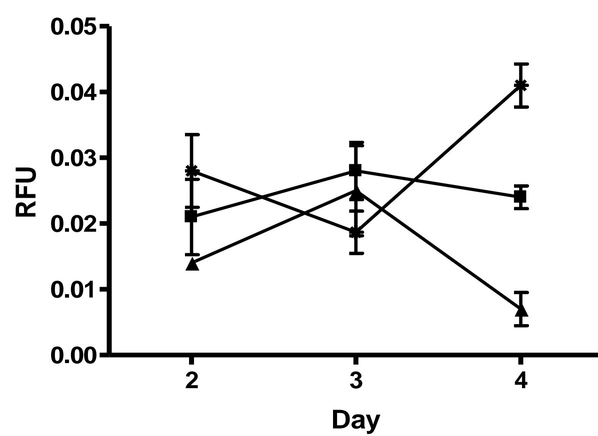

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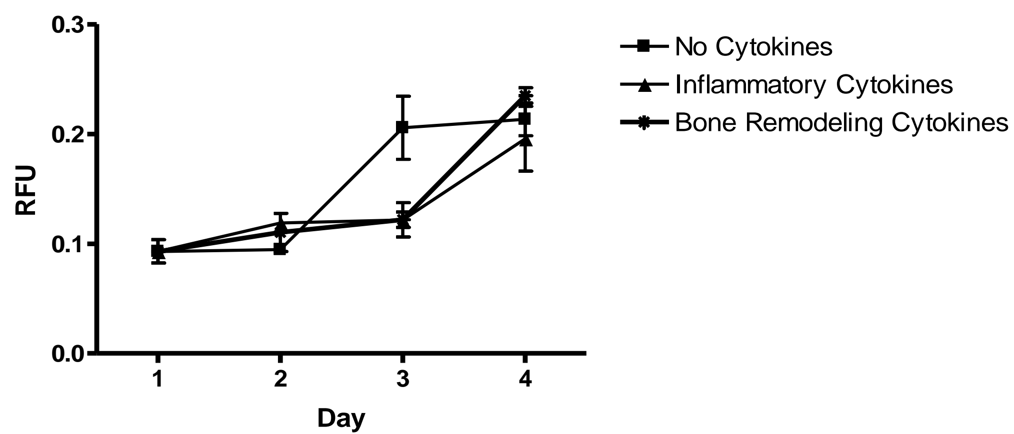

Figure 1-1: Proliferation of Breast Cancer Cells In Response To Inflammatory and Bone Remodeling Cytokines. a.1) MDA-MB 231 proliferation, a.2) MDA-MB 231 proliferation in a repetition of the same experiment b.1) MDA-MB 231 BRMS1 proliferation, b.2) MDA-MB 231 BRMS1 proliferation in a repetition of the same experiment c.1) MCF7 proliferation, and c.2) MCF7 proliferation in a repetition of the same experiment.

Breast cancer cells were plated at a density of 20,000 cells/cm^2 in a 96-well plate and allowed to attach for 24 hours. After this, in some wells, an MTT Assay was done to measure baseline proliferation. The appropriate cytokines were added to replicate wells. An MTT Assay was carried out in separate plates 24, 48, and 72 hours after addition of the cytokines discussed in the above section. Proliferation was measured using an MTT assay. Overall, no significant differences in proliferation were found between the cancer cells treated with bone remodeling cytokines, inflammatory cytokines, and no cytokines.

In the first round of experimentation, Figure 1-1, there were no significant differences found in proliferation in any of the cell lines due to the addition of cytokines, either the inflammatory or the bone remodeling, at the concentrations tested. However, though it was not considered significant, there did seem to be some increase in the MCF7 proliferation on day 4 in the presence of the bone remodeling cytokines.

In order to further verify the results obtained, the experiment was repeated. There were again no significant differences in proliferation of any of the breast cancer cell lines after treatment with the inflammatory or bone remodeling cytokines (Figure 1-1). However, though not significant, it was again seen that proliferation was slightly enhanced on day 4 with the MCF7 cells after treatment with bone remodeling cytokines (Figure 1-1 c). Therefore, these cells, along with the MDA-MB 231 BRMS1 were used in further experiments exploring the impact of bone remodeling cytokines on proliferation. Additionally, though also not considered to be statistically significant, there seems to be some down-regulation of proliferation due to the inflammatory cytokines. However, the lack in significant differences between any of the groups indicates that the cytokines may not act directly on the breast cancer cells. Instead, the bone matrix or osteoblasts may be involved in the communication between the cytokines and the breast cancer cells.

Chapter 4

Impact of Bone Remodeling Cytokines on MDA-MB 231 BRMS1 Proliferation and Morphology on a Bone Matrix *In vitro*

Due to the lack in any significant difference in proliferation between MDA-MB-231BRMS treated with bone remodeling and inflammatory cytokines from the untreated control in the previous experiment, it is possible the bone matrix plays a role in the relationship between the breast cancer cells and the cytokines. Due to the fact that the inflammatory cytokines never appeared to enhance proliferation, it was decided that the bone remodeling cytokines would be the focus of experimentation. In order to investigate the impact of bone remodeling cytokines on MDA-MB 231 BRMS1 proliferation on a bone matrix *in vitro*, osteoblasts were plated in a 6-well plate at a density of 10,000 cells/cm^2. The osteoblasts were cultured in differentiation media for one month. Some of the wells were decellularized, removing the osteoblasts, while some plates were left intact, i.e. osteoblasts remained on the matrix. Breast cancer cells were added at a density of 4,000 cells/cm^2. Bone remodeling cytokines were then added to some of the wells. However, PGE2 was removed from the bone remodeling cytokine group due to the fact that IL-1β and TNFα both already cause production of PGE2 [19]. 24, 48, and 72 hours after plating, proliferation was measured by a GFP assay and morphology was examined by confocal microscopy.

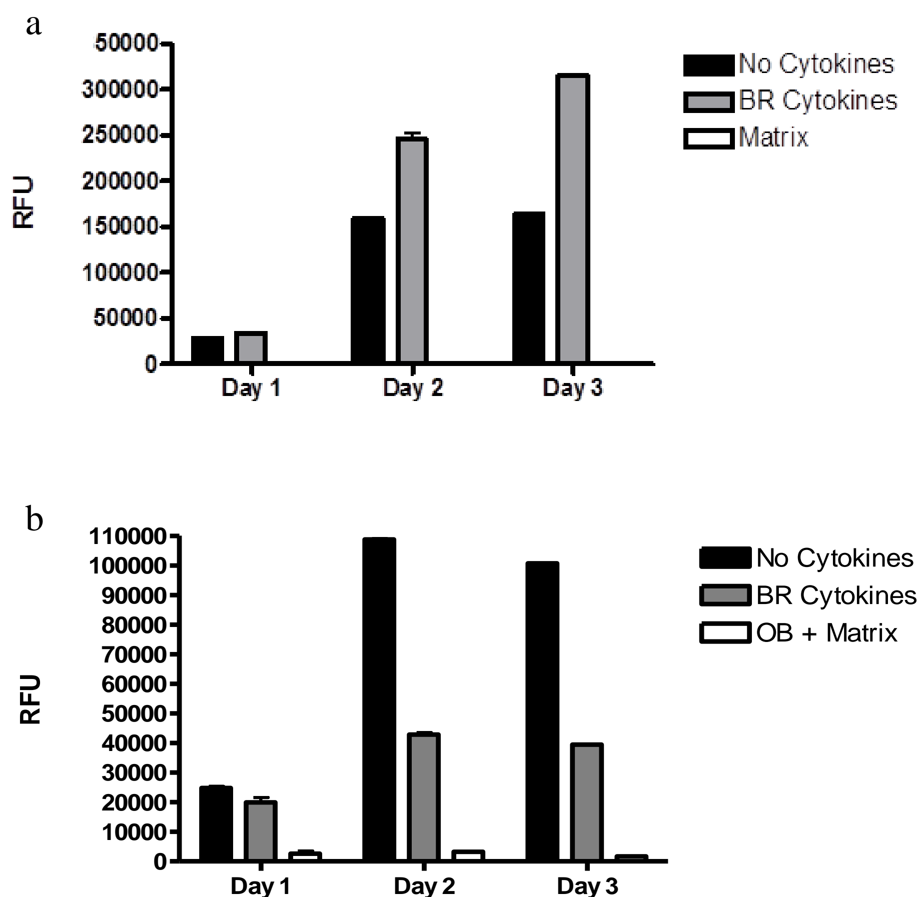

Figure 2-1: MDA-MB 231 BRMS1 Proliferation on a Bone Matrix *In vitro*.
a) MDA-MB 231BRMS proliferation on a decellularized bone matrix b) MDA-MB 231 BRMS1 proliferation on an intact bone matrix.
MDA-MB-231 BRMS were plated on an intact or decellularized matrix, and bone remodeling cytokines were added. A GFP assay was performed in order to examine the proliferation of the cancer cells. The bone remodeling cytokines significantly enhanced proliferation of the MDA-MB-231 BRMS1 cells after 48 and 72 hours when the matrix was decellularized. However, they significantly suppressed proliferation after 48 and 72 hours when the matrix was intact. (N=6, P<.0001)

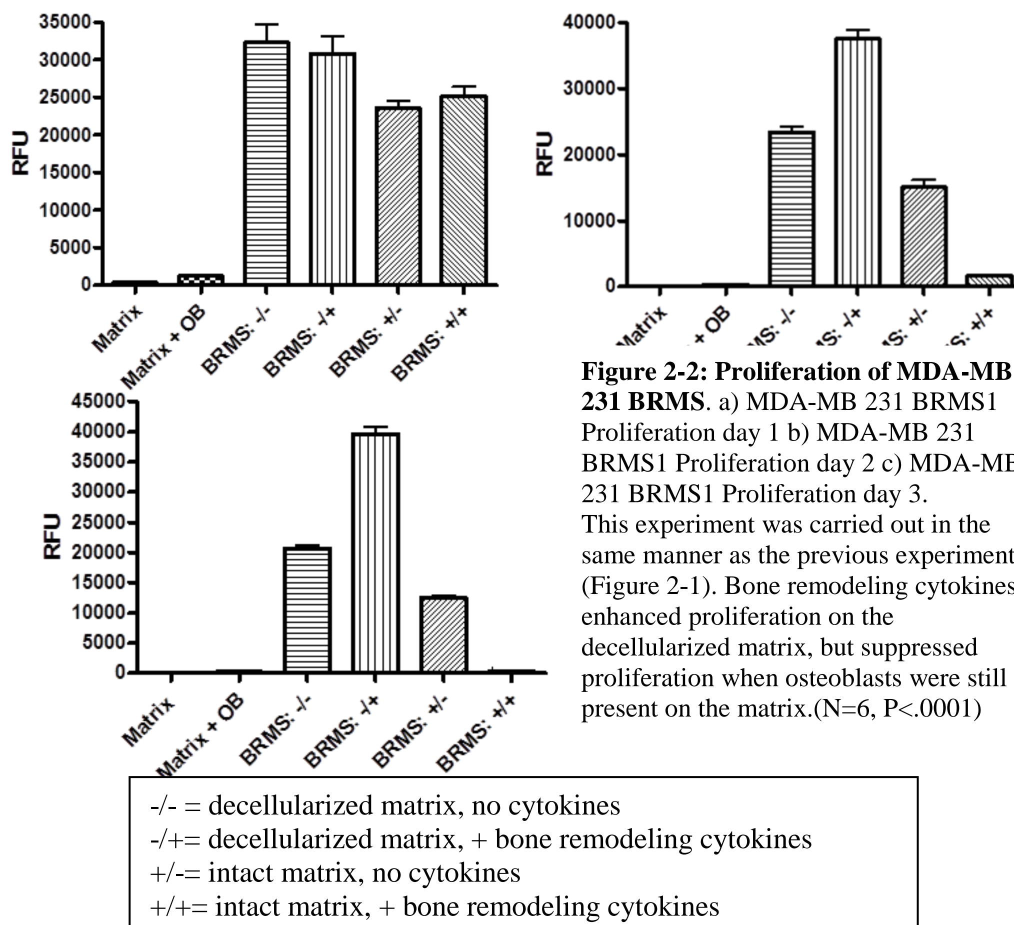

Figure 2-2: Proliferation of MDA-MB-231 BRMS. a) MDA-MB 231 BRMS1 Proliferation day 1 b) MDA-MB 231 BRMS1 Proliferation day 2 c) MDA-MB 231 BRMS1 Proliferation day 3.
This experiment was carried out in the same manner as the previous experiment (Figure 2-1). Bone remodeling cytokines enhanced proliferation on the decellularized matrix, but suppressed proliferation when osteoblasts were still present on the matrix.(N=6, $P<.0001$)

-/- = decellularized matrix, no cytokines
-/+= decellularized matrix, + bone remodeling cytokines
+/-= intact matrix, no cytokines
+/+= intact matrix, + bone remodeling cytokines

From these results, it appears that bone remodeling cytokines enhanced proliferation of the MDA-MB 231 BRMS1 on the decellularized matrix. However, when osteoblasts were present, they suppressed proliferation. Due to this finding, it may be the case that the cytokines influenced the osteoblasts to produce factors that resulted in suppression of proliferation of the breast cancer cells. In order to further verify these findings, another experiment was carried out. The same conditions as were used for Figures 2-1 and 2-2, however, standards were used to calculated the cell count from the relative fluorescent units. This use of a standard curve gave a better idea as to the exact

amount of proliferation, and values could be more easily compared between days. In addition, MDA-MB 231 BRMS1 were also plated on plastic alone in 6-well plates at a density of 4,000 cells/cm^2. The cancer cells were treated with bone remodeling cytokines. Calculation of the exact cell count allowed for a direct comparison between cancer cells on the matrix with osteoblasts, on the matrix alone, and on plastic, providing a better understanding of the role of osteoblasts and the bone matrix. Proliferation of the BRMS was measured using the GFP Assay after 24, 48 and 72 hours of plating.

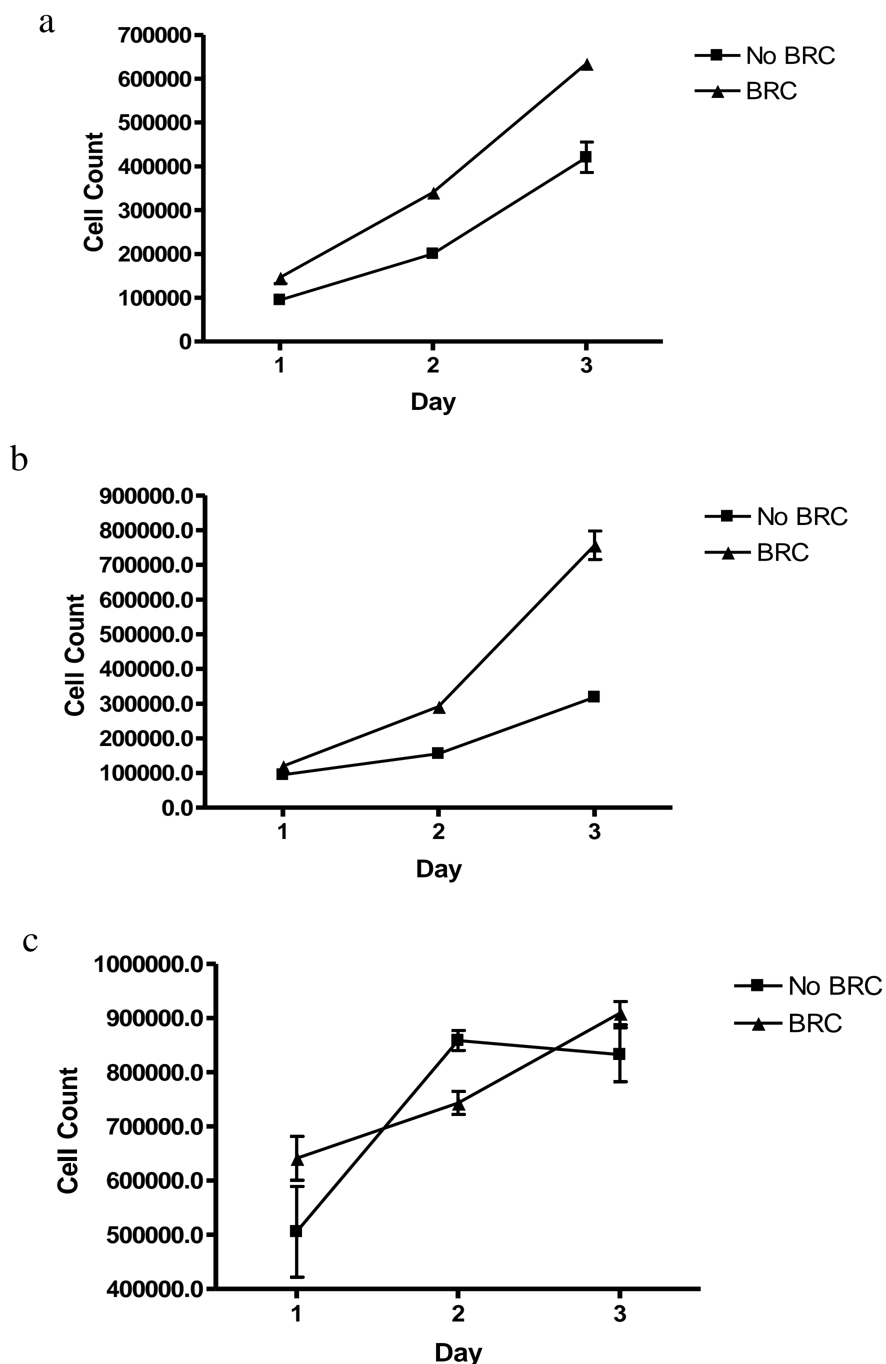

Figure 2-3: Proliferation of MDA-MB 231 BRMS1 in Various *In vitro* Environments
a) Proliferation of MDA-MB 231 BRMS1 on a plastic dish b) Proliferation of MDA-MB 231 BRMS1 on a decellularized bone matrix c) proliferation of MDA-MB 231 BRMS1 on an intact bone matrix. MDA-MB 231 BRMS1 were plated on a plastic dish, decellularized matrix, or an intact matrix. Bone remodeling cytokines were added. A GFP Assay measured proliferation 24, 48, and 72 hours after plating. Bone remodeling cytokines significantly increased proliferation on the plastic dish and the decellularized matrix, but not on the intact matrix. (N=2, P<.0001)

In this experiment, the bone remodeling cytokines significantly enhanced proliferation of the MDA-MB 231 BRMS1 when the cells were in a plastic dish, and when they were on a decellularized matrix. However, there was no change in proliferation with the addition of bone remodeling cytokines when the osteoblasts were also present. This result indicated that the bone remodeling cytokines stimulated proliferation of the breast cancer cells directly. Additionally, the factors in the matrix, such as collagen, and the adherence factors in the cells that respond to collagen and other matrix proteins, may not be altered by the cytokines, as the presence of the bone matrix made no difference in the enhancement of proliferation by the bone remodeling cytokines. However, the osteoblasts must communicate with the breast cancer cells to override the direct impact of the bone remodeling cytokines on the breast cancer cells, as no increase in proliferation was seen when the osteoblasts were present. This observation provides clues as to the relationship between the bone remodeling cytokines, breast cancer cells, and osteoblasts.

In addition to measuring proliferation, morphology of the cell line in the various conditions was observed using confocal microscopy. The same conditions were used when these images were taken as in the previous experiments (Figures 2-1, 2-2, 2-3).

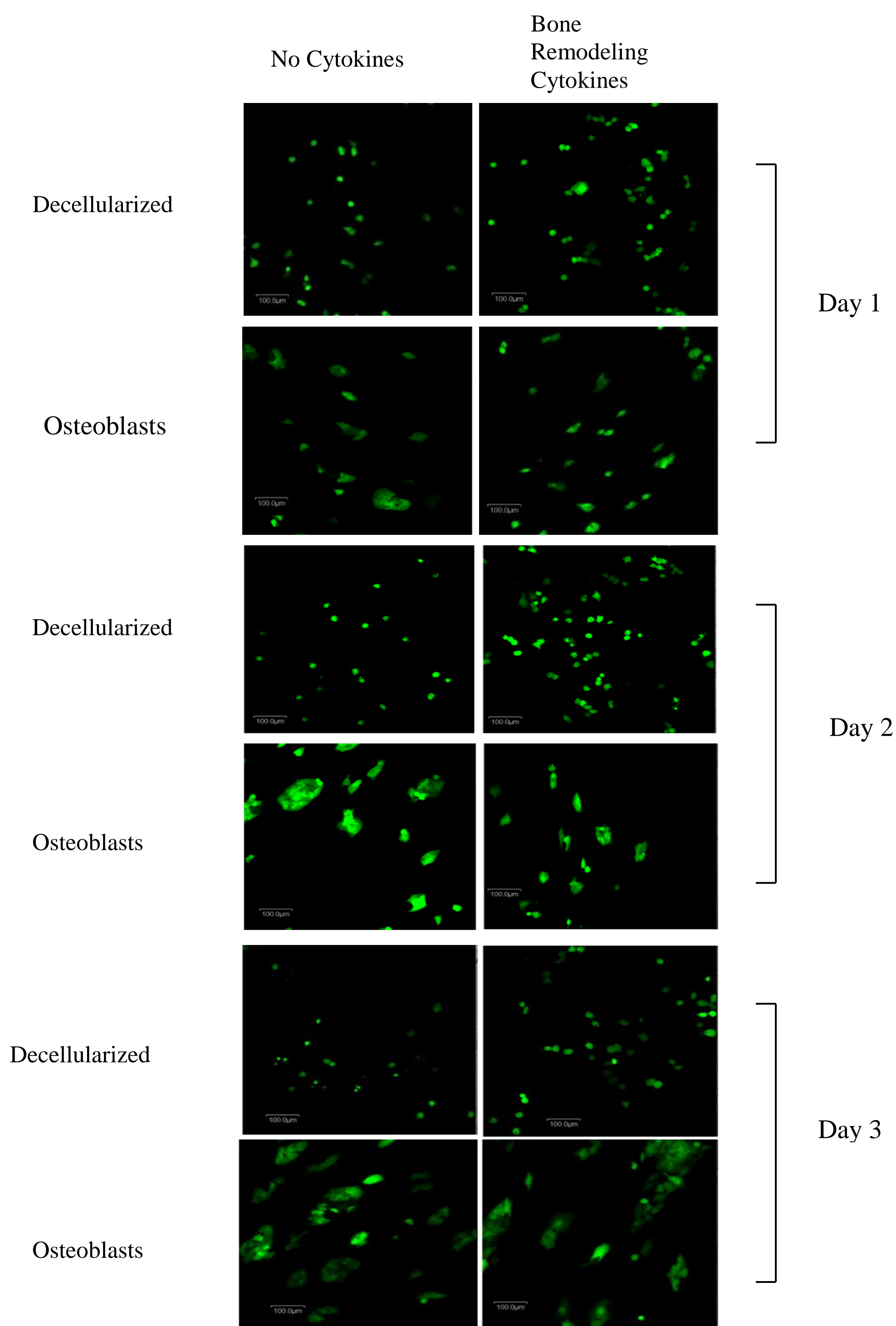

Figure 2-4: Morphology of MDA-MB 231 BRMS1 in Response to Cytokines on a Bone Matrix. MC3T3-E1 osteoblasts were plated at a density of 10,000 cells/cm^2 and grown up for one month. Some 6-well plates were decellularized. Then cancer cells were added at a density of 4,000 cells/cm^2. Bone remodeling cytokines were added. Morphology was observed using confocal microscopy. It appeared that osteoblasts caused cancer cells to spread out, yet the cytokines promote a smaller, more regular spherical shape of colonies.

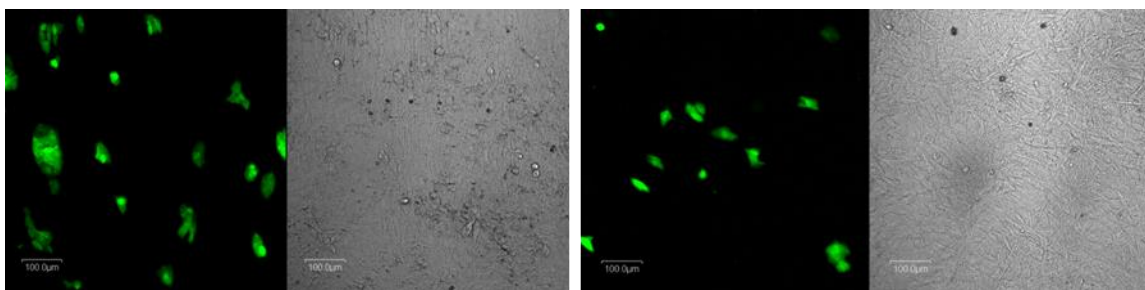

Figure 2-5: DIC Images of Morphology of MDA-MB 231 BRMS1 on a Bone Matrix In Response to Bone Remodeling Cytokines.
The conditions of the experiment were the same as for those in Figure 2-4. Morphology was observed using confocal microscopy. The image on the left displays BRMS, osteoblasts and the matrix without bone remodeling cytokines. The images on the right display BRMS, osteoblasts, and matrix with bone remodeling cytokines present. It appears that the osteoblasts induce the cells to line up, however, the cytokines may prevent this effect.

Both the osteoblasts and the bone remodeling cytokines appeared to influence the morphology of the MDA-MB 231 BRMS1 breast cancer cells. On the decellularized matrix, both with and without cytokines, the cells appear small and spherical. When the osteoblasts were present, the cells were larger, more spread out, and less regular in shape. The cancer cells also aligned with the osteoblast. However, when the bone remodeling cytokines were present with the osteoblasts, the cancer cells appeared somewhat smaller, and there are more cells that retained the small, regular spherical shape (Figure 2-4). Both the osteoblasts and cytokines appeared to influence morphology. DIC, or differential interference contrast, images reveal the structure of the environment. With the DIC images, it appears that the osteoblasts induce the cancer cells to line up. However, there is less lining up when the bone remodeling cytokines were added.

Chapter 5

Impact of Bone Remodeling Cytokines on Proliferation and Morphology of MCF 7 Breast Cancer Cells

In addition to the MDA-MB 231 BRMS1, the MCF7 breast cancer cell line provided a non-metastatic, estrogen-receptor positive, more dormant-like, cell line. Due to the differences between the cell lines, the role of bone-remodeling cytokines on MCF7 proliferation and morphology was also investigated.

Conditions for each experiment with the MCF7 breast cancer cells was identical to those used in the MDA-MB 231 BRMS1 experiment, however, in order to keep the cells in a more dormant-like state, FGF(10ng/mL) was added to all the wells after the cells were plated (methods for reference).

MC3T3-E1 osteoblasts were cultured for one month. The matrix in some wells was then decellularized, and MCF7 breast cancer cells were added at a density of 4,000 cells/cm^2. The bone remodeling cytokines were then added to some of the wells with a decellularized matrix and some of the wells with an intact matrix. Proliferation was measured using the GFP assay 24, 48 and 72 hours after addition of the bone remodeling cytokines.

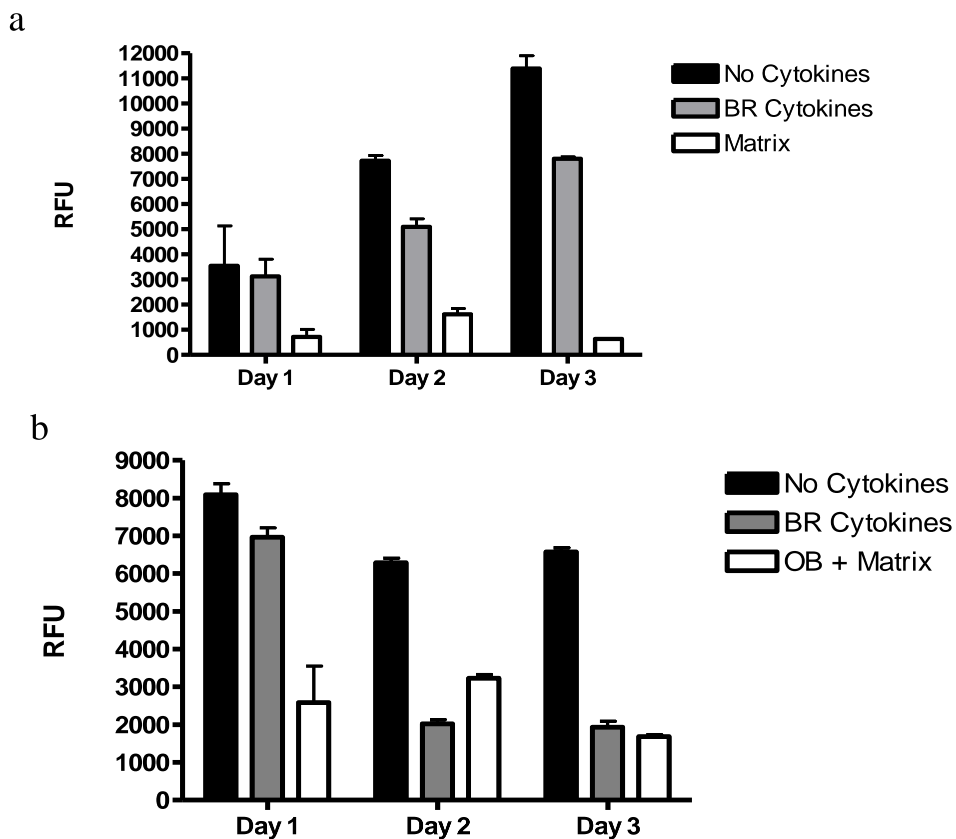

Figure 3-1: MCF 7 Proliferation on a Bone Matrix *In vitro*.
a) proliferation of MCF7 cells on a decellularized bone matrix b) proliferation of MCF7 cells on an intact bone matrix. MCF7 cells were plated on either a decellularized or an intact bone matrix. Bone remodeling cytokines were then added. A GFP Assay was performed in order to examine proliferation of cancer cells on the bone matrix 24, 48 and 72 hours after cytokine addition. Bone remodeling cytokines significantly suppressed proliferation of MFC7s on both the decellulrized and the intact bone matrix on day 2 and day 3. (N=6, P<.0001)

In order to confirm the observed results, a second experiment was done using the same conditions.

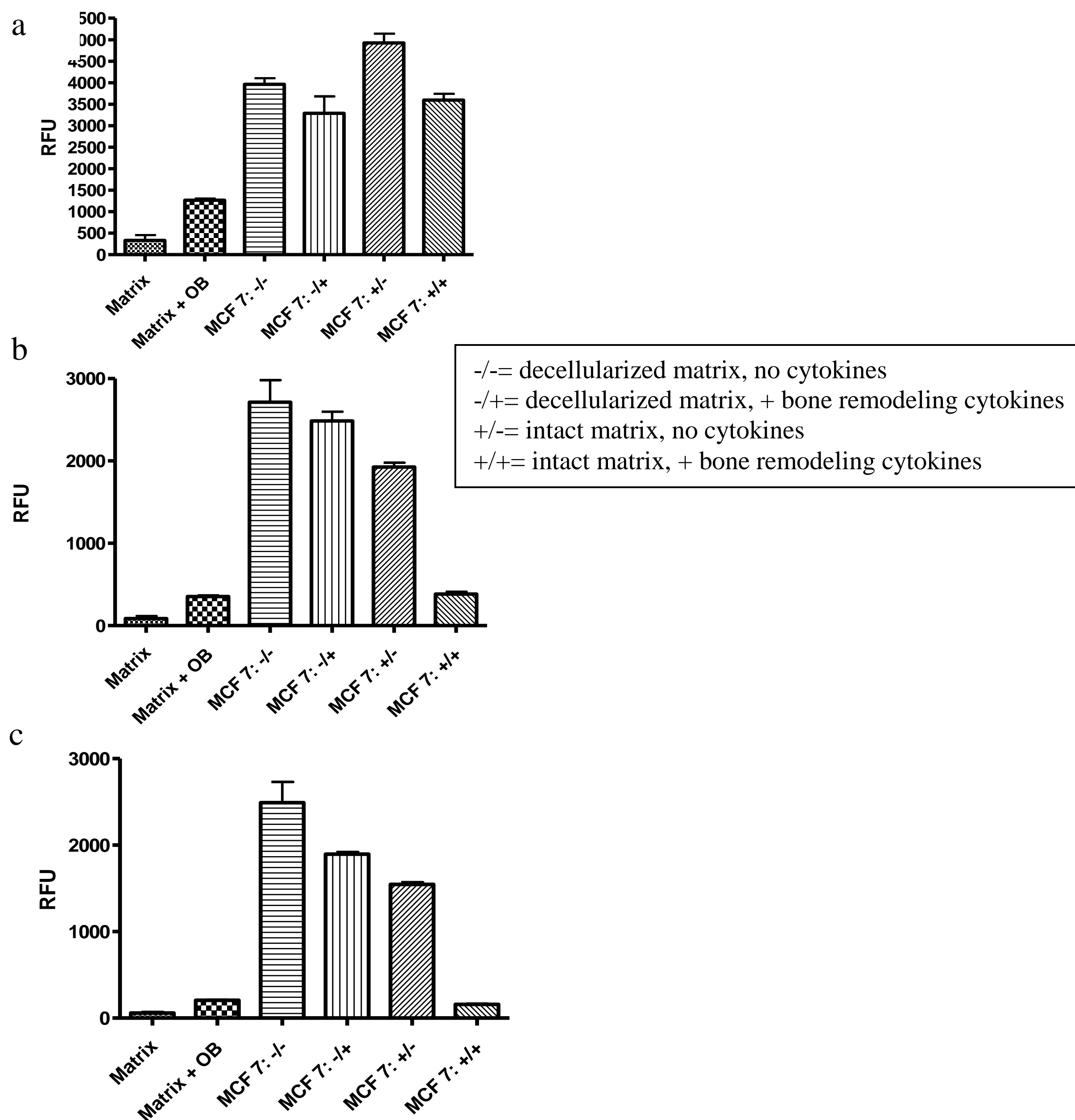

Figure 3-2: MCF-7 Proliferation on a Bone Matrix in Response to Bone-Remodeling Cytokines *In vitro*. a) MCF7 proliferation day 1 b) MCF7 proliferation day 2 c) MCF7 proliferation day 3. MC3T3-E1 cells were cultured in a 6-well plate for one month. Some matrices were decellularized. MCF7 cells were then plated and bone remodeling cytokines were added. Proliferation was measured using a GFP assay 24, 48 and 72 hours after addition of cytokines. Bone remodeling cytokines significantly suppressed MCF7 proliferation on both the decellularized and intact matrix (N=6, $P<.0001$).

The first experiment and the repetition gave similar results, indicating consistency (Figures 3-1, 3-2). On each day, no matter whether the osteoblasts were present on the matrix or not, the bone remodeling cytokines suppressed proliferation of the MCF7 breast cancer cell line. These results do not indicate whether or not there was a cross-talk between the osteoblasts and breast cancer cell lines, or whether the cytokines had a direct impact on the breast cancer cells. Either way, it was clear the cytokines suppressed proliferation of the MCF7 cells.

Morphology of the MCF7 cells was also observed in response to bone-remodeling cytokines in both the presence and absence of osteoblasts on a bone matrix *in vitro*. The conditions for observation of morphology were the same as those used in the previous experiments. Morphology was observed using confocal microscopy 24, 48 and 72 hours after addition of the bone remodeling cytokines to the breast cancer cells.

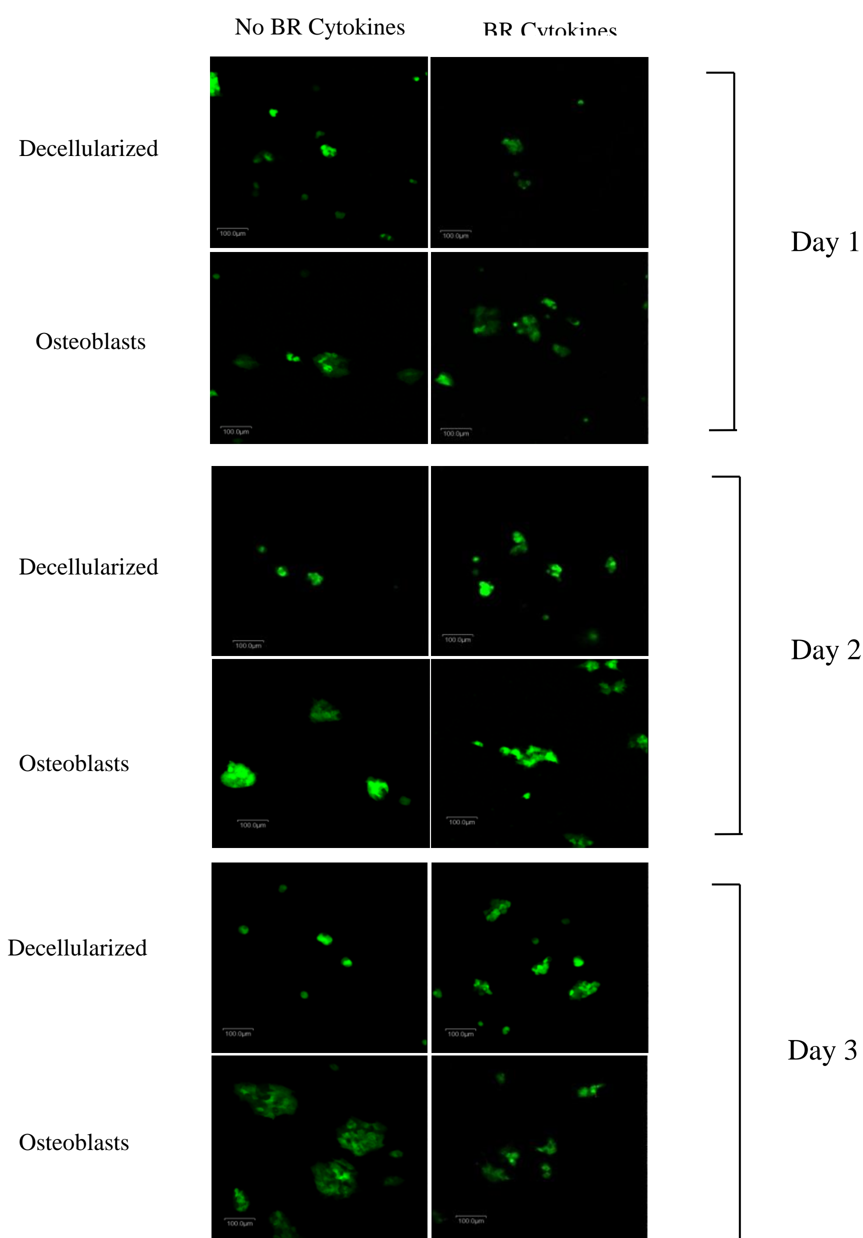

Figure 3-3: Morphology of MCF7 cells on a bone matrix *in vitro* in response to bone remodeling cytokines. MC3T3-E1 cells were plated in 6 well plates at a density of 10,000 cells/cm^2. Cells were cultured in differentiation media, and media was changed every 3-4 days. After one month, some wells were decellularized and breast cancer cells were added. After cells were allowed to attach for six hours, bone remodeling cytokines were added to the appropriate wells. Morphology was observed using a confocal microscope with the GFP-expressing cells 24, 48 and 72 hours after cytokine addition. Size, shape and alignment were influenced.

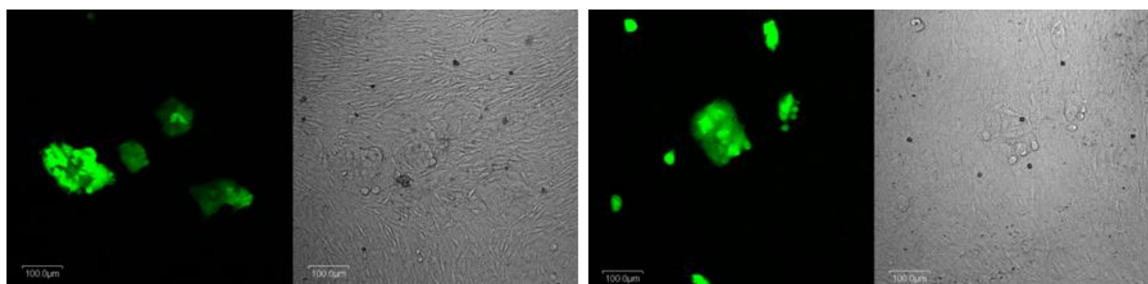

Figure 3-4: DIC Images of MCF7 Cell Morphology in response to Bone Remodeling Cytokines on a Bone Matrix with Osteoblasts *in vitro*. Conditions for this experiment were the same as those used for Figure 3-4. Morphology was observed using confocal microscopy. The image on the left displayed MCF7, osteoblasts and the matrix with bone remodeling cytokines absent. The images on the right were MCF7, osteoblasts, and matrix with bone remodeling cytokines present. Unlike the other cell lines, there appeared to be no lining up with the osteoblasts, whether or not cytokines are present.

It is apparent that the bone-remodeling cytokines, along with the presence of osteoblasts, impacted the morphology of the cells. On the decellularized matrix, with no cytokines added, the cells were small, spherical and unaligned. They appeared to be similar when the cytokines were added on the deceulluarized matrix. However, when osteoblasts were present, but without cytokines, the cells were much larger and more spread out, revealing a less regular shape. With the osteoblasts and bone remodeling cytokines present, the cells again appeared somewhat smaller and more spherical. The cytokines appeared to prevent the cells from spreading out with the osteoblasts as they did when there were no cytokines present (Figure 3-3). When the osteoblasts were present with the matrix, there appeared to be no particular alignment between the breast cancer cells and osteoblasts, indicating possible communication between the osteoblasts and the MCF7 breast cancer cells (Figure 3-4).

Chapter 6

Discussion

This study was designed to answer the question that results from the anecdotal evidence that injury to the bone may reawaken dormant metastatic breast cancer in the bone. In this study, the impact of bone remodeling cytokines on breast cancer cell proliferation and morphology was examined in vitro on a bone matrix. The effect on proliferation and morphology by the bone remodeling cytokines was observed both when the bone matrix was decellularized, and when the osteoblasts remained on the matrix. The cytokines used that are involved in bone remodeling were IL-6, IL-1B, and TNF-α. The two breast cancer cell lines that were the focus of the study were the MDA-MB 231 BRMS1 and MCF7 cells. These cell lines represent less aggressive, more dormant models than the MDA-MB 231 cells [27].

It was hypothesized that the bone-remodeling cytokines would stimulate proliferation of the breast cancer cells, MDA-MB 231 BRMS1 and the MCF7 cells. Additionally, due to the evidence that bone remodeling and fibroblast activity stimulate breast cancer cell proliferation, it was predicted the osteoblasts would also enhance the proliferation of the breast cancer cells [5].However, we found that the bone remodeling cytokines suppressed proliferation of the MCF7 cells, both when osteoblasts were present and absent. This finding suggested that these cytokines were responsible for suppression of cell growth; however, there are a number of factors to be considered. First, all of the cytokines have similar downstream targets, such as PGE2 [19] . Due to the fact that IL-6, IL-1β, and TNFα cytokines were added together, it is possible that the downstream

transcription factors were made in a higher concentration than usual, and the higher concentration resulted in suppression of proliferation. Additionally, it could be the case that these cytokines resulted in the transcription of other cytokines. Under in-vivo conditions, these cytokines would not be built-up, but would instead diffuse away from the cells and enter into the blood stream. Instead, these factors produced by the cells could not diffuse away, and likely built up in concentrations higher than would normally be observed in-vivo. This scenario may be the reason for the suppression of proliferation under *in vitro* conditions.

Morphology was also observed in the experiment aimed at determining the impact of bone remodeling cytokines on MCF7 breast cancer cell proliferation on an *in vitro* bone matrix. On the decellularized matrix, both with and without the bone remodeling cytokines, the MCF7 cells took on a more regular, small, spherical shape. However on the bone matrix with osteoblasts, the MCF7 cells appeared to spread out more. The cancer cells appear larger, and they also appear to cluster together more than when they osteoblasts are absent from the environment. The presence of osteoblasts suggested that there was communication between the breast cancer cells and the osteoblasts to induce this type of morphology. This communication between the cell types could alter adhesion factors and result in the difference in morphology. These results are consistent with the literature, as osteoblasts have been found to enhance prostate cancer, a cancer commonly compared to breast cancer, cell adhesion to bone via the SDF-1/CXCR4 pathway [31]. This finding could also hold true for the breast cancer cell lines observed in this experiment. Furthermore, when the bone remodeling cytokines were added with the osteoblasts present, the breast cancer cells did not appear quite as

large or grouped together, and instead, they took on an appearance more similar to those on the decellularized matrix. This observation is consistent with what has been reported in the literature. IL-6 has been found to downregulate E-cadherin, which is found on MCF7 cells [32]. The more spread out nature of the cells in the absence of cytokines on the matrix with osteoblasts could indicate that the cancer cells attached better to the matrix when osteoblasts were present. However, addition of the cytokines may have decreased this attachment, resulting in a more spherical conformation. This finding may indicate that the cytokines interfered with communication between the osteoblasts and breast cancer cells, or they had an impact of their own on cell adhesion factors. The result of cytokines on adherence factors may play a role in why breast cancer cells seem to awaken from dormancy following injury to the bone, and warrants further investigation.

The results of the MDA-MB 231 BRMS1 cell line proved to be more complicated than the results of the MCF7. When the osteoblasts were absent, on the decellularized matrix, the bone remodeling cytokines clearly stimulated proliferation of the MDA-MB 231 BRMS1 breast cancer cells. However, when the osteoblasts were present, along with the cytokines, the cytokines suppressed proliferation. This result suggests that the osteoblasts and breast cancer cells communicated, and this communication was impacted by the cytokines. It could be the case that without interference of the osteoblasts, the bone remodeling cytokines stimulated proliferation of the MDA-MB 231 BRMS1. However, when the osteoblasts were present, the cytokines induced the osteoblasts to produce factors that overrode the effect of the cytokines on the breast cancer cells. These factors have suppressed proliferation or deactivated the signaling of the bone remodeling cytokines on the breast cancer cells.

The role of the bone matrix was further explored in this experiment by analyzing what role the bone matrix played in the communication with the breast cancer cells and the bone remodeling cytokines. In the experiment done with the MDA-MB 231 BRMS1 plated on plastic, the bone remodeling cytokines appeared to stimulate proliferation just as much as when the breast cancer cells were plated on the decellularized matrix. This finding suggested that the bone remodeling cytokines acted directly on the cancer cells, without the aid of matrix factors, to stimulate proliferation. Interestingly, this significant difference was not seen in the MTT assay done previously. This contradiction could be due to the difference in sensitivity of the two assays. In the GFP assay, cancer cells were plated at a density of 4,000 cells/cm^2, while in the MTT proliferation assay, cancer cells were plated at a density of 15,625 cells/cm^2. Another difference was that the MDA-MB 231 BRMS1 medium was used for the MTT proliferation assay, while the MC3T3-E1 differentiation medium was used in the GFP proliferation assay. Further exploration of why this difference occurred could provide clues as to the impact of cell to cell communication, density, or the importance of the serum concentration.

Changes in the morphology of the MDA-MB 231 BRMS1 cell line was also observed. A similar phenomenon occurred with the MDA-MB 231 BRMS1 as with the MCF7 cells. The cells appeared as small, regular spheres on the decellularized matrix, both in the presence and absence of bone remodeling cytokines. However, they spread out and grouped together much more when the osteoblasts were present. On day 1 and day 2, when the cytokines were added in the presence of osteoblasts, the cells spread out less than when there were no cytokines added and the osteoblasts were present. This effect of the cytokines with the intact matrix was diminished on day 3, which could be

due to the short half-life of the bone remodeling cytokines. However, in the literature, it is also cited that IL-6 is responsible for down regulating E-cadherin. E-cadherin is not expressed by the MDA-MB 231 BRMS1 line, so the difference in effect of the cytokines when osteoblasts are present between the MDA-MB 231 BRMS1 and the MCF7 cells was unsurprising [32]. Additionally, when osteoblasts were present, the breast cancer cells lined up with the osteoblasts. This change in shape and alignment could be due to the fact that there were gaps in the matrix, and the BRMS settled into these holes more easily than they attach to the collagen. In order to confirm this, imaging of the matrix and osteoblasts would need to be done to see exactly what the cells are attached to. However, the alignment could also mean that there was communication between the breast cancer cells and the osteoblasts. There could also be adhesion factors or chemokines, such as SDF-1, that are impacted by the presence of osteoblasts that results in alignment of the cancer cells with the osteoblasts, and change in morphology of the MDA-MB 231 BRMS1 cell line [31].

Another interesting is the difference between the two cell lines. The growth of the MCF7 cells when treated with bone remodeling cytokines on the decellularized matrix was suppressed, while the growth of the MDA-MB 231 BRMS1 when treated with bone remodeling cytokines on the decellularized matrix was enhanced as compared to either breast cancer cell line on a decellularized matrix without addition of bone remodeling cytokines. This difference could signify that the two lines have differing pathways in response to the bone remodeling cytokines.

Future Studies

Several follow up experiments could provide more answers to the question of how these bone remodeling cytokines, bone matrix, and osteoblasts impact breast cancer cells. In order to determine whether the bone remodeling cytokines are stimulating the osteoblasts to produce factors that then suppress the proliferation of the MDA-MB 231 BRMS1, a conditioned media experiment could be done. Osteoblasts could be treated with the bone remodeling cytokines, then the cytokines could be neutralized with antibody, and the collected media could be used to make conditioned medias. Treating the cancer cells with this media on a decellularized matrix could provide clues as to whether or not the osteoblasts secreted factors to suppress proliferation. Additionally, a cytokine array could be done to investigate what additional factors were being produced by both the osteoblasts and the breast cancer cells in response to bone remodeling cytokines.

Further experimentation with attachment and morphology could also provide good clues as to the relationship between the cell types and the cytokines. Imaging the matrix could allow one to see how exactly the breast cancer cells were conforming to the matrix, and how the osteoblasts impacted this. Visualizing the attachment factors, such as FAK and the cadherins, could also reveal more about how bone remodeling cytokines and osteoblasts impact breast cancer cell attachment to bone. Overall, it is evident the bone remodeling cytokines impact both the morphology and proliferation of breast cancer, and looking further into this relationship could provide answers as to why proliferation is upregulated and cancer cells awaken from dormancy.

Appendix A

GFP Assay and RFU Reading Verification

In order to verify the GFP assay was successful, and the plate reader was getting an accurate reading of cell number, a standard was used. The standards were made so the first data point had a value of 5 x 10^5 cells, the second value had half the number of cells, etc, all the way down to a value of 488 cells. The cells were lysed in the PBD buffer and the RFU was analyzed. As the cell count was halved, so was the RFU value, validating the GFP assay. The PBD buffer successfully lysed the cells, and the RFU was proportionate to the cell count.

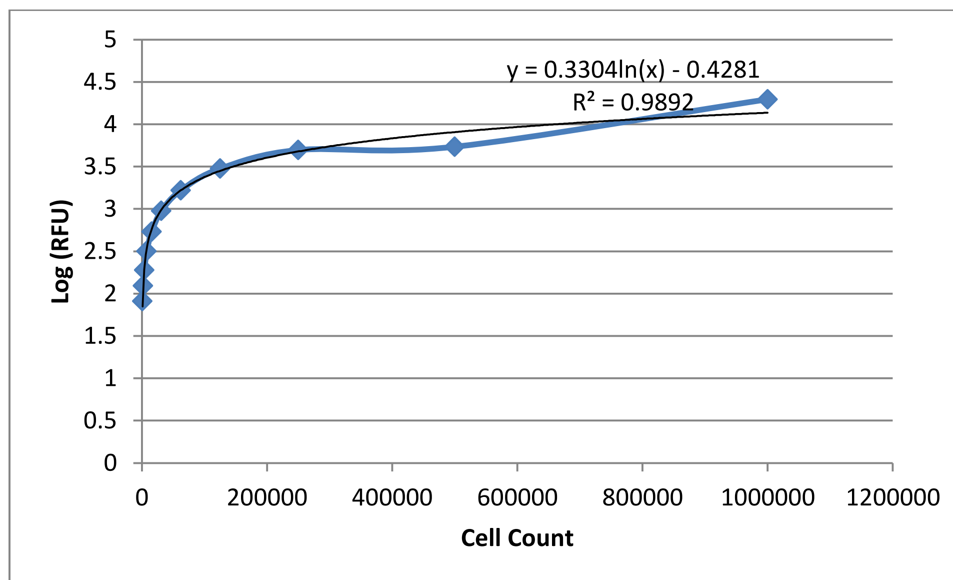

Figure 4-1: GFP Assay and RFU Reading Verification. MDA-MB 231 BRMS1 were serially diluted, starting with 5 x 10^5 cells. They were lysed with PBD Buffer and the lysate was read using the Infinite M1000 Pro Tecan Plate-Reader at 488-509 nm. As the cell count was halved, so was the RFU value, indicating a successful GFP assay.

Appendix B

Results of MDA-MB 231 Cell

Impact of Bone Remodeling Cytokines on MDA-MB 231 Morphology on a Bone Matrix *In vitro*

Although the MDA-MB 231 breast cancer cell line was not the focus of the study, the morphology of the cells in response to osteoblasts and bone remodeling cytokines was observed. This cell line is known to be very aggressive, and due to the focus on a more dormant model, this cell line was not explored as in-depth. The morphology could provide clues as to the relationship between the cytokines, the osteoblasts and the cancer cells. In this experiment, MC3T3-E1, murine osteoblast, were plated at a density of 10,000 cells / cm^2 and grown up for one month. Following this period, some wells were decellularized, and the MDA-MB-231 breast cancer cells were plated at a density of 4,000 cells/cm^2. After adhering to the matrix for several hours, they were treated with bone remodeling cytokines. This experiment was done to observe how the bone remodeling cytokines impacted the morphology of the MDA-MB 231 cells when they were on a bone matrix, and revealed whether or not the osteoblasts also played a role in this relationship. The following images represent the breast cancer cells in the various experimental conditions.

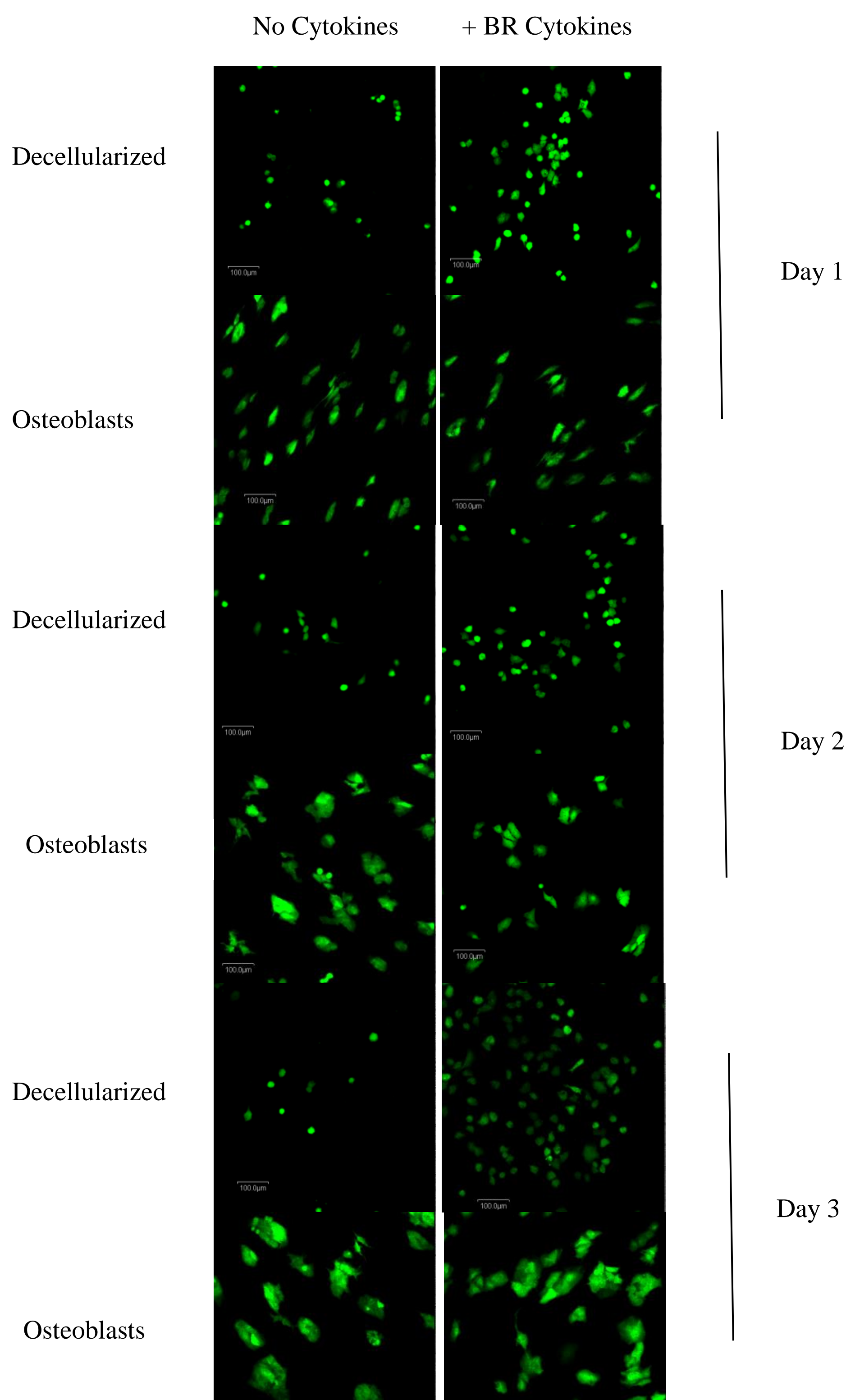

Figure 5-1: Morphology of MDA-MB 231 Cells in response to Osteoblasts and Bone Remodeling Cytokines. The conditions for this experiment were the same as those described for the morphology examination of the other two cell lines (Figures 2-4, 3-3). It is apparent that the osteoblasts induce the cells to spread out, but when the cytokines are also present, they reverse this effect. Also, the breast cancer cells appear to be lining up with the osteoblasts on the bone matrix.

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Academic Vita

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Education

The Pennsylvania State University — Expected Graduation May 2014
Schreyer Honors College
Bachelor of Science in Biochemistry & Molecular Biology
Minor in Economics

Relevant Coursework:

General Chemistry & Labs	Genetic Analysis	Biostatistics
Organic Chemistry & Lab	General Biochemistry	Genetics
Microbiology & Lab	Immunology	Medical Microbiology

Thesis: The Effects of Bone-Remodeling Cytokines on the Interaction Between Breast Cancer Cells and Osteoblasts on a Bone Matrix *In vitro*

- The laboratory of Dr. Andrea M. Mastro

Experience

University of Massachusetts Medical School, Department of Biochemistry — Worcester, MA
Research Assistant, The Carruthers Lab — Summers 2011, 2012

- Created glucose transport protein mutants, measured protein expression, and ran transport assays

Beth Israel Deaconess Medical Center, Department of Anesthesiology — Boston, MA
Observer, Dr. John Pawlowski — December 2012

- Shadowed in thoracic, orthopedic, plastic, and OBGYN operating rooms

Marlboro Hospital, Department of Cardiology — Marlboro, MA
Observer, Dr. Daniel Carlucci — June 2011

- Attended clinical appointments, visits to the ICU, and minor surgeries

The Pennsylvania State University, Department of Economics — University Park, PA
Undergraduate Teaching Assistant — 2012- Present

Activities

Penn State Club Gymnastics Team

- Treasurer — 2012-2013
- Penn State Dance Marathon (THON) Organization Chair — 2011-2012
- Penn State Dance Marathon (THON) Organization Dancer — 2013
- Competitor on balance beam and floor exercise

Global Medical Brigades — 2011- Present

- Spring Break Trips to Panama and Nicaragua
  - Set up medical clinics and treated patients in rural villages

Special Olympics Volunteer — 2008- Present

Honors

Phi Beta Kappa — 2013
Dean's List — 2010- Present
Stiles Scholarship — 2012
Shigley Award — 2011