THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

OSTEOBLAST-MEDIATED OSTEOCLASTOGENESIS IN RESPONSE TO BREAST CANCER CELL CONDITION MEDIA

ANDREW E. BARON
Fall 2009

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biochemistry and Molecular Biology
with honors in Biochemistry and Molecular Biology

Reviewed and approved* by the following:

Andrea Mastro
Professor of Microbiology and Cell Biology
Thesis Supervisor

Chen-Pei D. Tu
Professor of Biochemistry and Molecular Biology
Honors Advisor

Scott Selleck, Department Head
Department of Biochemistry and Molecular Biology
Faculty Reader

***Signatures are on file in the Schreyer Honors College**

ABSTRACT

The bone microenvironment is a dynamic system in which bone is constantly being degraded and rebuilt. In addition to being responsible for the building up of bone, osteoblasts also directly influence the bone-degrading cells, osteoclasts. Breast cancer preferentially metastasizes to this bone microenvironment, thus beginning what is referred to as the "vicious cycle." When the cancer cells reach their niche, they secrete parathyroid hormone-related protein (PTHrP), which stimulates osteoblasts to release increased levels of receptor activator to nuclear factor κB ligand (RANKL). This cytokine promotes osteoclast bone resorption activity and differentation. As the osteoclasts continually resorb the bone matrix, they release embedded growth factors, such as transforming growth factor-β (TGF-β), which complete the cycle by promoting tumor cell proliferation.

In addition to causing the vicious cycle, cancer cells also cause osteoblasts to undergo an inflammatory response, which not only inhibits their function, but also causes them to release pro-inflammatory cytokines. These cytokines in turn, promote osteoclastogenesis, or the differentiation and activation of precursor cells in the bone marrow into multinucleated, mature osteoclasts, perpetuating osteolysis.

We hypothesize that the osteoblast inflammatory response occurs after breast cancer cells metastasize to the bone is partially responsible for enhanced levels of osteoclast activity, specifically through increasing the number of differentiated osteoclasts. In this study, the importance of all three cell types in the cycle was emphasized by monitoring osteoclastogenesis in response to various condition media. Pre-osteoclasts were exposed to condition media from osteoblasts, and breast cancer cells as well as combinations of their media for 21 days and then assayed for mature osteoclast markers. The results of these series of experiments demonstrate that medium that includes cytokines of the osteoblastic inflammatory response is capable of increasing osteoclastogenesis compared to baseline values. These data provide important information regarding possible intervention sites for halting the vicious cycle and thereby decreasing the painful osteolysis that is associated with breast cancer bone metastases.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would first like to thank my mother and father for understanding and accepting my commitment to this project and to this lab. You have always shown an interest in my activities and you have been a phenomenal source of inspiration throughout these past 3 years. I know that whenever it seems as if the "research gods" are conspiring against me, I can always pick up the phone and vent my frustration to you two. I cannot begin to tell you how much of an influence you have had on me not only in my collegiate tenure, but over the course of my life. I owe part of everything I accomplish to your faith and trust in me.

I would also like to sincerely thank Yu-Chi Chen and Venkat Krishnan. Since I started working in this lab, you have always jumped at the opportunity to teach me. You are two great people and I am absolutely amazed at the time and effort you put in to your projects. I wish you the best of luck, and I know you will succeed with any future endeavors you undertake, in the lab and in life in general.

Finally, I would like to thank my "lab mom," Donna Sosnoski and Dr. Andrea Mastro, who have both spent countless hours advising me throughout this process. Dr. Mastro, I truly appreciate your patience and optimism as I was beleaguered with failed experiment after failed experiment. You have always encouraged me to continue with my projects even if their initial results seem incomprehensible and your determination is something I envy. Donna, I cannot begin to thank you enough for all the hours of technical advice (and advice on life in general!) you have given me over these 3 years. From the moment I joined this lab, you took me under your wing and helped mold the researcher that I am today. You've always kept my spirits up and have had a profound impact on my experience here.

Thank you all so much!

INTRODUCTION

Breast Cancer

Cancer is the second leading cause of death in the United States, only behind cardiovascular disease, accounting for one in every four deaths annually[1]. In 2008 alone, it was estimated that 1,437,180 new cases of cancer were reported in America[1]. Aside from skin cancer, breast cancer is the most common form of cancer among women. According to the American Cancer Society, one in every eight women will be diagnosed with an invasive form of breast cancer over her lifetime; and astoundingly, one in 35 women will develop terminal breast cancer[2]. In 2009, it is expected that 192,370 cases of breast cancer will be reported and an estimated 40,170 deaths will occur as a result of this illness[3].

Breast tissue is composed mostly of milk-producing lobular cells, ductal cells which act as canals to transport the milk from the lobules to the nipples, and stroma (including fat, muscle, connective tissue as well as blood and lymphatic vessels). Breast cancer usually forms as an adenocarcenoma in the ductal cells (85% of cases), but can occasionally start in the lobules (15%)[4].

If not detected in its early stages, breast cancer often preferentially metastasizes to the bone[5]. If caught early in its development, prior to metastasis, 90% of victims can be cured of breast cancer. However, if the cancer metastasizes to secondary sites, the 5-year survival rate drops to less than 16%[6]. In post-mortem autopsies of breast cancer related deaths, nearly 70% of patients had bone metastases[5]. Men and women living with breast cancer bone metastases usually suffer from hypercalcemia, painful bone lesions, fractures and spinal cord compression[7].

Bone Microenvironment and Metastasis

When a cancer leaves its primary site, it moves to neighboring tissues and enters blood or lymphatic vessels to easily travel to distant organs[8]. Although breast cancer also commonly metastasizes to the liver and lung, it preferentially seeks the skeleton, although the exact reason is not fully known[9].

The human skeleton is composed of two different types of bones: flat bones, like the skull, sternum and scapula, and long bones like the femur, tibia and radius. Long bones have shafts or diaphysis running down the center of the bone and epiphyses at either end. The shafts are filled with bone marrow which contains hematopoietic stem cells, as well as several other cells types. The bone ends are comprised primarily of trabecular or spongy bone[10].

Despite its appearance, the skeleton is a remarkably dynamic organ and is constantly remodeling to cope with environmental stresses. In a healthy individual, there are three cells involved with the maintenance and alteration of bones: osteoclasts, osteoblasts, and osteocytes[11]. Osteoclasts are responsible for resorbing the type 1collagen and hydroxylapatite that compose bone through lysosomal enzymes and hydrogen ions[12]. Osteoclasts are large, multinucleated polar cells. On one side of the polykaryon (a cell that possesses more than one nuclei) is a ring comprised of the protein ostepontin, which secures the osteoclast to the bone matrix prior to bone resorption[13]. Osteopontin also ensures that the degradative enzymes and hydrogen ions are contained within the seal and do not leak into adjacent bone. As the bone matrix is being broken down, it is resorbed into the osteoclast and secreted into the stroma on the opposite side of the osteoclast[13].

In contrast to osteoclasts, osteoblasts are in charge of building new bone. Osteoblasts are derived from mesenchymal stem cells, and when these precursors receive stimulation from

growth factors within the bone microenvironment, they differentiate into pre-osteoblasts and eventually mature osteoblasts[14]. The mature osteoblasts secrete osteoid, an unmineralized collection of proteins that will eventually become bone when mineralized. At this point the osteoblast can either undergo apoptosis or surrounded by the mineralized bone. If the osteoblast remains embedded in the bone matrix, is becomes an osteocyte[15]. The trapped cells reside in their own lacunae, or pits, and although they are incapable of cellular division, continue to secrete bone matrix[15].

Osteoclast Differentiation

Osteoblasts and stromal cells are responsible for regulating the process of osteoclast differentiation into mature, multinucleated, bone resorbing cells. This is referred to as osteoclastogenesis. Both osteoblasts and stromal cells produce the protein receptor activator for nuclear factor κ B ligand (RANKL) and macrophage colony stimulating factor (M-CSF)[16]. Osteoclasts are derived from hematopoietic stem cells along the macrophage lineage, differentiate into pre-osteoclasts, and eventually fuse into multinucleated cells capable of bone resorption[11]. On their surfaces, the pre-osteoclasts express macrophage colony stimulating factor receptor (M-CSFR) and receptor activator for nuclear factor κ B (RANK), the receptor to RANKL.[17]. M-CSF is an important cytokine in the early differentiation from the macrophage/monoctye lineage into the pre-osteoclast cell type, while RANKL is responsible for later differentiation **(Figure 1)**[18]. Once the cells fuse they express a variety of markers, including Tartrate-Resistant Acid Phosphatase (TRAP)[19], which can be detected with commercially available staining kits.

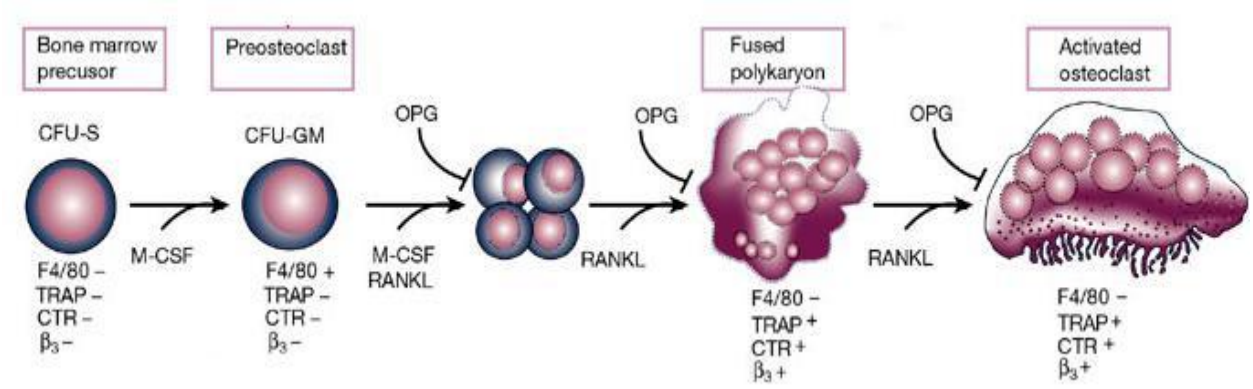


Figure 1: Activated, multinucleated osteoclasts are derived from hematopoietic precursor cells. With the addition of M-CSF to the macrophage lineage cells, pre-osteoclasts are produced, and begin to express different proteins, including RANK. Cells will begin to fuse into polykaryons and begin expressing osteoclast enzymes such as TRAP. Osteoprotegerin (OPG), secreted by osteoblasts, can inhibit differentiation of osteoclasts at any point of the multinucleation process past the pre-osteoclast phase.
Boyle, W.J., et al. "Osteoclast differentiation and activation." *Nature* 432 (2003): 337-342.

To inhibit osteoclastogenesis, osteoblasts can secrete the RANK homolog, osteoprotegerin (OPG), which acts as a decoy receptor to RANKL. The OPG binds to RANKL expressed on the surface of osteoblasts and stromal cells, as well as secreted RANKL (sRANKL) to effectively shut down the process of osteoclast differentiation[17]. Furthermore, OPG can inhibit the bone resorbing capabilities of osteoclasts that have already matured and are multinucleated [20].

By using M-CSF, RANKL, and OPG, osteoblasts and stromal cells can effectively control osteoclast differentiation and the resulting bone resorption so that a proper balance can be struck between bone rebuilding and bone degradation.

The Vicious Cycle

When breast cancer metastasizes to the bone, it wreaks havoc on the balance between osteoblast and osteoclast function. In a model proposed by Mundy and Guise, known as the

"vicious cycle," metastatic cancer cells, osteoblasts, and osteoclasts play crucial roles in a self-perpetuating cycle of bone degradation. Breast cancer cells secrete parathyroid hormone-related protein (PTHrP) which stimulates osteoblasts to upregulate their production of RANKL. As the concentration of RANKL rises, more pre-osteoclasts differentiate into activated, multinucleated osteoclasts and resorb the bone matrix. As the matrix is degraded, embedded growth factors such as transforming growth factor-β (TFG- β**)** are released into the extracellular space causing the breast cancer cells to in turn upregulate their production of PTHrP[21]. This continual cycle helps account for the overactive bone resorption that is common in victims of breast cancer bone metastasis **(Figure 2)**.

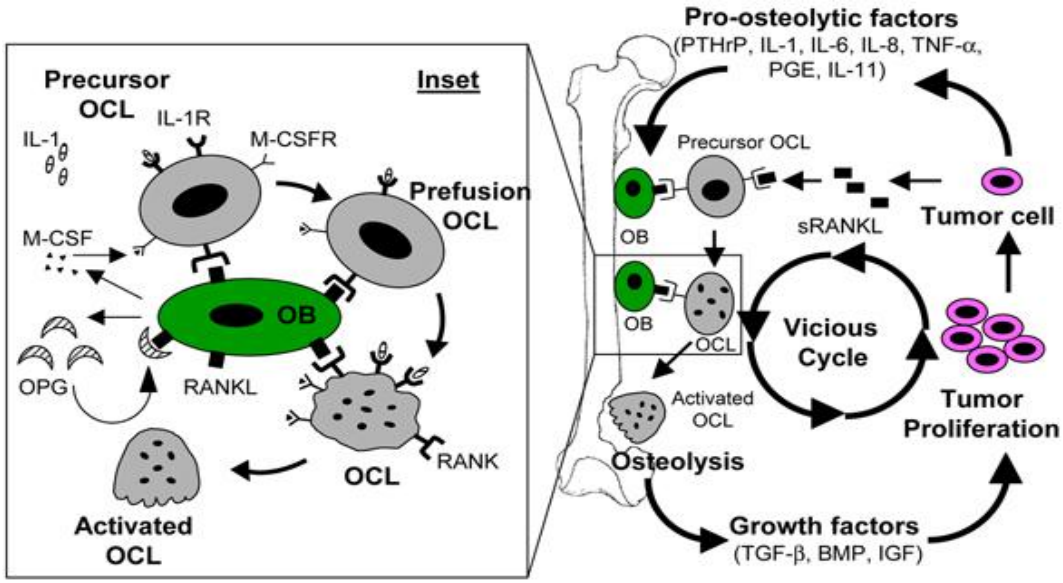


Figure 2: The vicious cycle involves osteoclasts, osteoblasts and cancer cells. When tumor cells invade the bone micro-environment, they secrete a myriad of proteins, including PTHrP, which act on osteoblast. These factors cause an upregulation of osteoblast-produced RANKL, which leads to higher numbers of activated osteoclasts. As the osteoclasts resorb the bone matrix the release growth factors like TGF-β. These growth factors increase tumor proliferation and the cycle can begin anew. In the inset, the critical role osteoblasts have in the life and maturation of osteoclasts is displayed. M-CSF and RANKL secreted by the osteoblast (in green) help move The pre-cursor osteoclast through its differentiation process into an activated, multinucleated cell capable of bone resorption. OPG acts as decoy receptor to RANKL and inhibits osteoclastogenesis. Virk, M.S. and Liberman, J.R. "Tumor metastasis to the bone." *Arthritis Research Therapy*. 9 (5).

The Inflammatory Response

In addition to causing osteoblasts to upregulate their production of RANKL, cancer cells also induce osteoblasts to undergo an inflammatory response[22]. In response to 50% breast cancer condition media, Bussard showed using the Bio-Rad Bio-Plex®Murine Cytokine Assay that murine osteoblasts upregulate their production of several pro-inflammatory cytokines [23]. Specifically, macrophage inflammatory protein-2 (MIP-2), keratinocyte-derived chemokine

(KC), vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), and monocyte chemotactic protein-1 (MCP-1) were all shown to be increased after of osteoblast exposure to breast cancer condition media[23].

A variety of studies have shown that most of these inflammatory cytokines are capable of inducing osteoclastogenesis, or at least play a role in the process. IL-6 has been shown to induce osteoclastogenesis when added with soluble IL-6 receptor[24]. VEGF receptor (VEGFR) is expressed on the surface of pre-osteoclasts; and when VEGF is paired with RANKL *in vitro*, it can induce osteoclastogenesis in peripheral blood monocytes similarly to M-CSF and RANKL. This finding suggests that VEGF might have a role in the early phases of osteoclast differentiation and can even substitute for M-CSF *in vitro*[25]. MCP-1has been shown to not only act as a chemoattractant for bone marrow macrophages[26], but also can stimulate the production of mature osteoclast markers. In RANKL-deficient systems, MCP-1 was found to produce TRAP-positive, calcitonin receptor-positive (another mature osteoclast marker) multinucleated cells that appeared to look like activated osteoclasts. However, these cells were not capable of bone resorption[27].

While the degree to which these cytokines stimulate osteoclastogenesis is still unknown, due to their upregulation during an osteoblastic inflammatory response, their role in the process of osteoclastogenesis cannot be dismissed.

Hypothesis and Statement of Experimental Goals

We hypothesize that as a consequence of bone metastatic breast cancer, osteoblasts will increase osteoclastogenesis due to the release of osteoclastogenic cytokines during the

inflammatory response. This swelling of mature osteoclasts will in turn degrade more bone, and thus perpetuate the vicious cycle. In this study, media containing the inflammatory cytokines that osteoblasts release in response to breast cancer cell invasion was added to murine bone marrow cells. The degree to which this media can induce osteoclastogenesis was quantified and compared to other types of media.

The following experiments were performed to accomplish three specific aims: 1) prove the interconnectedness of all 3 cell types involved in the vicious cycle and emphasize that osteoblasts act as the primary mediators between pre-osteoclasts and activated osteoclasts; 2) observe the effects of inflammatory response media on osteoblast proliferation and determine if any correlation exists between the number of osteoblasts and the resulting number of osteoclasts and; 3) prove that the inflammatory cytokines acts in a synergistic manner in osteoclastogenesis, and not just in an additive fashion.

MATERIALS AND METHODS

Osteoblast and Breast Cancer Cell Culture

MC3T3-E1, an immature murine osteoblast line are capable of differentiation and bone mineralization were a gift from Dr. Norman Karin (university of Delaware)[28]. The immature osteoblasts were grown on T-150 tissue culture flasks in growth media containing α−Minimum Essential Media (α-MEM), 10% fetal bovine serum (FBS) and penicillin 100 U/ml/streptomycin 100 μg/ml (1% Pen-Strep solution) until reaching confluency. At this point they were grown in osteoblast differentiation media (α-MEM, 10% FBS, 1% Pen-Strep Solution, 50 μg/ml ascorbic acid, and 10 mMβ-glycerophosphate) for 10 days, unless specified otherwise. MDA-MB-231are human metastatic breast cancer cells that preferentially metastasize to bone, causing bone lesions[29]. The cancer cells were cultured on aT-150 tissue culture flasks in α-MEM, 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep) solution. Both cell types were incubated at 37°C and 5% CO_2, and received media changes every other day.

Condition Media Preparation

Vehicle and RANKL/M-CSF Medium

1% Pen-Strep solution was mixed with 10% FBS in α-MEM to prepare vehicle medium (VM). The RANKL/M-CSF Medium was prepared in the same fashion, but with the addition of murine recombinant sRANKL (50 or 100 ng/ml, PeproTech Inc.) and M-CSF (50 ng/ml, PeproTech Inc.) to the VM.

Breast Cancer Condition Medium (BCCM)

MDA-MB-231 human breast cancer cells were allowed to grow to confluency on a T-150 tissue culture flask. The medium was decanted and replaced with α-MEM for 24 hours. After this period, the supernatant was collected and centrifuged at 300X g. for 10 minutes. 10% FBS was added and the solution was frozen 0°C until later experimentation.

Osteoblast Condition Medium (OBCM)

Murine MC3T3-E1's were plated on a T-150 tissue culture flask. After ten days of culture in differentiation media, the supernatant was aspirated and 20 ml α-MEM was added. The osteoblasts were incubated for an additional 24 hours at 37°C and 5% CO_2. The supernatant was then decanted, and centrifuged for 10 minutes at 300X g. 10% FBS was added to supplement the medium after the initial incubation.

Osteoblast Breast Cancer Condition Medium (OB-BCCM)

After 10 days of culture in differentiation media, 50% BCCM, 40% α-MEM, and 10% FBS was added to the tissue culture flasks and the cells were incubated for 24 hours. The supernatant was collected, and centrifuged. Due to the prior addition of FBS, no supplementation was required after the medium was collected.

Osteoblast-Osteoblast Condition Medium (OB-OBCM)

After 10 days of growth in differentiation media, 50% OBCM, 40% α-MEM, and 10% FBS were added to the osteoblasts. The flask was incubated at 37°C and 5% CO_2 for 24 after which time,

the supernatant was decanted and centrifuged. Like OB-BCCM, this media did not require any further addition of FBS.

Co-culture Medium

After MC3T3-E1's had differentiated for 10 days, the cells were counted. MDA-MB-231 breast cancer cells, grown as previously described, were then counted as well and the two cell types were co-cultured in a T-150 tissue culture flask at a ratio of 1:10 (MC3T3-E1:MDA-MB-231) in VM. The cells were then incubated for 48 hours. Afterwards, the supernatant was decanted and centrifuged to remove any remaining cells.

OBCM + BCCM

Standard OBCM and BCCM were mixed together without plating the supernatants on MC3T3-E1's, prior to culture with bone marrow cells. This was done to compare OB-BCCM, which was generated by exposing the MC3T3-E1's to BCCM, thereby simulating the supposed synergistic effects of breast cancer bone metastasis on osteoclastogenesis. OBCM + BCCM was generated *de novo*, and therefore was absent of any additional osteoblast inflammatory response cytokines. **Table 1** lists the media used in the following experiments and summarizes their compositions.

TABLE 1: Summary of Media Preparation

Media Type	Composition
VM	10%FBS, 1% Pen-Strep in α-MEM
RANKl/M-CSF	Prepared identically to VM with the addition of recombinant sRANKL (50 or 100 ng/ml) and M-CSF (50 ng/ml)
BCCM	MDA-MB-231 human cancer cells were grown to confluency. The media was replaced with α-MEM and the cells were incubated for 24
OBCM	MC3T3-E1 murine osteoblasts were grown to confluency and then exposed to osteoblast differentiation media for 10 days. α-MEM then replaced the differentiation media for 24 hours.
OB-BCCM	Differentiated osteoblasts were exposed to 50% BCCM, 50%VM for 24 hours. This medium contains the inflammatory response cytokines osteoblasts undergo in response to breast cancer bone metastasis
OB-OBCM	Differentiated osteoblasts were exposed to 50%OBCM and 50% VM for 24 hours.
Co-Culture	After 10 days of growth in differentiation media, osteoblasts were grown with cancer cells at a 1:10 ratio in VM. After 48 hours, the media was collected.
OBCM + BCCM	OBCM and BCCM supplemented with 10%FBS were added individually without being placed on MC3T3-E1 osteoblasts together.

Bone Marrow and Pre-Osteoclast Isolation

Mice of both genders were collected and euthanized using CO_2. The mice were immediately dipped in Wescodyne to decontaminate the fur. The left and right legs of the mice were removed, and as much fat and muscle was removed with dissection scissors or scapulas. The femurs and tibias were separated from one another and placed in α-MEM until later experimentation. The fibulas were removed entirely and disposed of.

In a sterile hood, the bone ends were removed with dissection scissors and the remaining shaft was placed a 1% Pen-Strep, α-MEM solution. Fresh 1% Pen-Strep, α-MEM solution was aspirated in a sterile 26.5 gauge syringe and then used to flush out the marrow within the shafts into a collecting dish containing 1% Pen-Strep, 10% FBS and α-MEM.

To isolate bone marrow cells, the flushed marrow solution was plated in duplicate on 6-well plates (2 ml per well) at a cell density of 30,000 cells/cm^2 and incubated for 7 days at 37 °C

and 5% CO_2 to allow for cell adherence. The media was carefully changed every other day, so as not to remove too many cells during aspiration.

To isolate non-adherent pre-osteoclasts, the bone marrow was flushed from the shafts and collected as described. The entire solution was cultured on a T-150 tissue culture flask and incubated at 37°C and 5% CO_2 in VM for 24 hours. The supernatant, containing non-adherent cells (bone marrow macrophages/monocytes, or pre-osteoclasts) was then decanted and the cells were centrifuged for 10 minutes at 300X g. The VM was aspirated and the cells were resuspended in 1 ml of 1X red blood cell lysis buffer (eBioscience Cat. No. 00-4333). The solution was allowed to sit on ice for 5 minutes. Five ml of sterile 1X PBS were added to stop the lysis process and the solution was again centrifuged for 12 minutes at 300X g. The supernatant was decanted and the cells were finally resuspended in VM. The cells were then plated in 24-well plates at a density of 45,000 cells/cm^2.

Osteoclastogenesis in Response to Condition Media

After the whole bone marrow isolates had been allowed to adhere for 7 days, 1 ml of VM was removed from each well, and 1ml condition media was added accordingly. This was done so as not to "shock" the cells too much with new condition media. The cells were then cultured for a total of 21 days, with biweekly medium changes.

For pre-osteoclasts, each well (except VM) was supplemented with 100 ng/ml M-CSF and 50 ng/ml sRANKL and incubated for 48 hours to promote cell adhesion. This procedure was done because the usual sources of the osteoclastogenic factors, osteoblasts and fibroblasts, were removed from the cell culture. Therefore, the cytokines had to be added *de novo*. After the 48 hour incubation, half (0.5 ml) of the suspension media was removed and condition media was

added. An additional 100 ng/ml M-CSF and 50 ng/ml sRANKL were added to all media types except VM. The cells were then incubated for 48 hours at 37°C, and 5% CO_2. After the initial 4 days of culture, 100 ng/ml RANKL and 50 ng/ml M-CSF were added to the wells, under the pretext that by this stage, many cells would require less M-CSF in the osteoclast differentiation process.

Media changes and sRANKL and M-CSF supplements were performed biweekly for 21 days. After two weeks of culture, M-CSF was no longer used. We reasoned that after such time, any precursor osteoclasts had already committed to the osteoclast lineage, and would only require RANKL to become multinucleated and mature osteoclasts.

TRAP Staining and Measurements

Medium was aspirated from the cells, and they were fixed in 10% formalin in water. Sigma-Aldrich TRAP stain solution (Kit 387A) was then prepared according to the manufacturer's instructions and added to the wells for 1 hour at 37°C in the dark. TRAP stain was removed and the cells were thoroughly washed with distilled water and allowed to air-dry overnight.

A square of the largest possible area was drawn on the bottom of each circular well to demark the countable area (6.45 cm^2 for 6-well plates and 2.56 cm^2 for 24-well plates) . Using a light microscope at 200X magnification, TRAP-positive, multinucleated (>3 nuclei) were then counted for every well, and the numbers for each condition media type were averaged over duplicate wells.

Alkaline Phosphatase Staining and Measurements

To examine osteoblast numbers, alkaline phosphatase staining was performed (Alk. Phos.). Media was aspirated from the wells and the cells were rinsed with 1X PBS. Cells were then fixed with 10% formalin for 10 minutes at room temperature. The cells were again washed thoroughly with 1X PBS. Alk. Phos. stain (0.0013 g Napthol, 6.25 ml distilled water, 6.25 ml 0.2 M Tris, pH 8.5, 0.0075 g Fast Blue RR salt) was then added to the cells and the plates were incubated at 37℃ for 30 minutes. The stain was removed and the cells were rinsed with distilled water. The wells were allowed to air dry overnight.

After drying, visible Alk. Phos.-positive colonies were counted with the naked eye and marked so as to prevent possible double-counting. The values for each duplicate media type were then averaged.

RESULTS

Experiment 1: Osteoclastogenesis in Response to Conditioned Media

In order to determine the effects of conditioned media on osteoclastogenesis, whole bone marrow isolates from 6-week old C57/BL6 mice were plated and exposed to various supernatants. After 7 days of growth in VM, a variety of supernatant media was added to cells and cultured for 21 days. These supernatants included media collected from MDA-MB-231 breast cancer cells (BCCM), a co-culture of MC3T3-E1 osteoblasts and cancer cells (Co-culture), osteoblasts grown in 50% breast cancer media (OB-BCCM), and osteoblasts grown in 50% osteoblast condition media (OB-OBCM). All supernatant media were diluted in 50% VM. sRANKL (50 ng/ml) and M-CSF (50 ng/ml) were also tested as known inducers of osteoclastogenesis. Because the bone marrow isolates included pre-osteoclasts, stromal cells, and osteoblasts, osteoclastogenesis could potentially occur *in vitro*, without cytokine supplementation. VM was used to simulate this "baseline" value of osteoclast differentiation.

All media types except BCCM notably increased osteoclast differentiation when compared to the VM baseline, with OB-OBCM having the greatest effect. BCCM at 50% appeared to negatively affect osteoclastogenesis **(Table 2, Figure3)**. Co-culture media and OB-BCCM produced a somewhat similar number of multinucleated, TRAP-positive osteoclasts. RANKL/M-CSF also induced a larger amount of osteoclastogenesis as compared to the baseline, which is reasonable given that these two cytokines are normally produced by stromal cells and osteoblasts and are required for osteoclastogensis. OB-OBCM more than doubled the number of mature, TRAP-positive osteoclasts when compared to VM baseline values **(Figure 4)**.

TABLE 2: Osteoclastogenesis in Response to Condition Media

	VM	BCCM	M-CSF/RANKL	OB-BCCM	OB-OBCM	Co-Culture
1st Well	94	12	164	98	249	116
2nd Well	83	20	118	117	198	118
Average	88.5	16	141	107.5	223.5	117

Bone marrow cells from murine tibias and femurs were isolated and plated in 6-well plates. After the cells were plated in VM for one week, they were cultured in various types of condition media for three weeks. Co-culture media, OB-OBCM, OB-BCCM, and BCCM were all diluted to 50% in VM. The cells were then fixed and mature osteoclasts were detected with TRAP stain solution. TRAP-positive, multinucleated (>3 nuclei) were counted over a given area (6.45 cm^2) of each well, and the values were averaged.

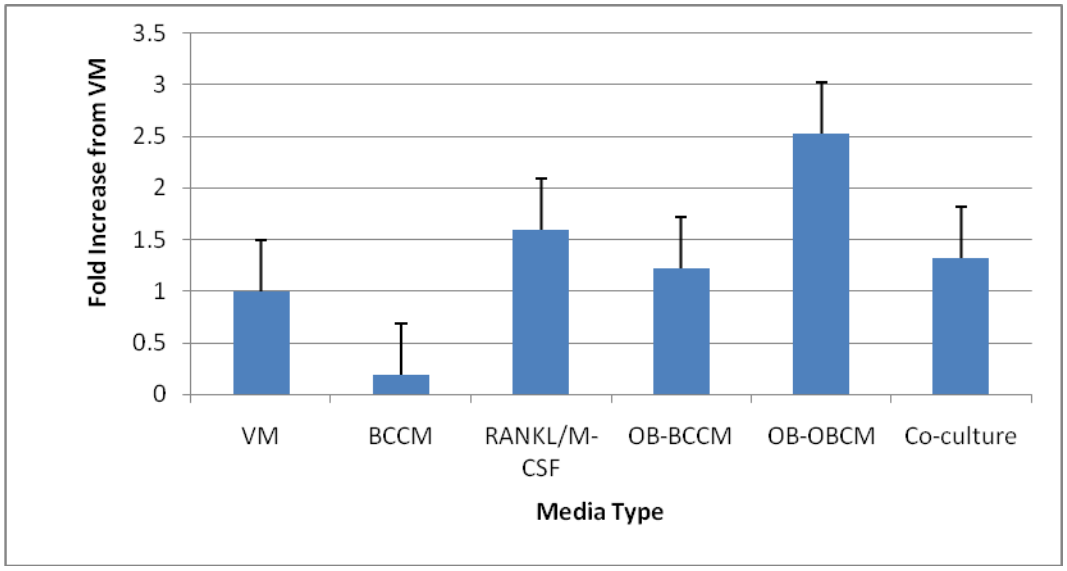
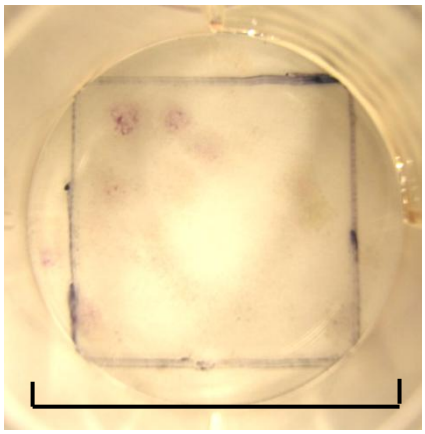
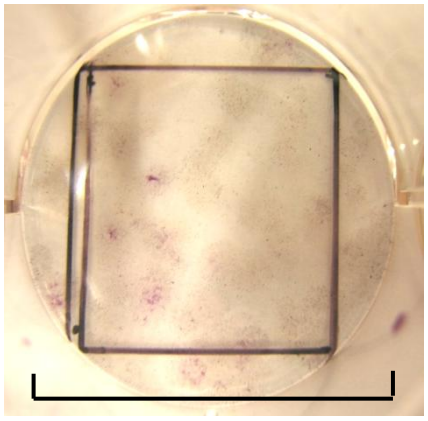


Figure 3: Fold increase of osteoclastogenesis compared to baseline. TRAP-positive, multinucleated cells were counted for each media type and averaged over duplicate plates. Shown are the fold increases of the median values for each conditioned media compared to the average number of osteoclasts induced by the VM baseline. OB-OBCMgave the largest increase of osteoclastogenesis, while BCCM had significantly lower numbers of osteoclasts per well. (n=2, so no meaningful statistical analysis could be performed).

A



B



C

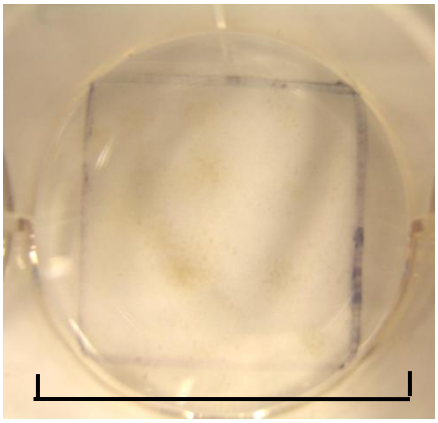


Figure 4: A visualization of TRAP positive cells with various condition media. Clusters of TRAP + cells visible from the naked eye appear purple/fuschia**.** A) Vehicle Media (89% α-MEM, 1% Pen-Strep, 10% FBS) acted as an baseline control. B) OB-OBCM induced noticeably higher levels (2.5X) of osteoclastogenesis compared to VM. C) However, 50% BCCM and 50% VM actually decreased the number of osteoclasts per well. Shown are 6.45cm^2 areas in 6-well plates where scale bars represent 3.38 cm.

Experiment 2: Effects of Condition Media on Osteoblasts and Osteoclasts

Whole bone marrow isolates from green fluorescent protein (GFP) Balb/Cmice of an estimated age of 6 months were cultured in 6-well plates and allowed to grow for 7 days in VM. Cells were then exposed to OBCM, OB-OBCM, or OB-BCCM diluted with 50% VM for 3 weeks. The cells were then fixed and stained separately for osteoblast and osteoclast markers.

All three supernatants taken from MC3T3-E1 osteoblasts increased the number of Alk. Phos.-positive colonies compared to the VM baseline **(Table 3).** OB-OBCM was the most effective inducer of osteoblast colony formation, more than doubling the amount of countable colonies Interestingly, OB-BCCM was found to be more effective than OBCM alone in osteoblast proliferation **(Figures 5 and 6)**.

On separate plates, TRAP staining was performed on bone marrow isolates exposed to the same condition media. Again, all three supernatants proved to be more successful in osteoclast differentiation than VM alone **(Table 4).** Contrary to expected results, OBCM generated the largest number of TRAP-positive, multinucleated osteoclasts, nearly tripling the baseline values. OB-BCCM was the least effective supernatant in osteoclastogenesis induction, although still produced more than twice the number of mature osteoclasts as VM (**Figure 7).** In experiment 2, even under identical growth conditions, OB-BCCM and OB-OBCM produced much fewer TRAP-positive osteoclasts than in Experiment 1. This may likely be attributed to the age of the mice used in the experiment. The mice in Experiment 2 most likely had less osteoblasts and pre-osteoclasts in the marrow than in younger growing mice.

TABLE 3: Osteoblast Proliferation in Response to Condition Media

	VM	OBCM	OB-BCCM	OB-OBCM
1st Well	27	39	44	63
2nd Well	33	38	47	58
Average	30	38.5	45.5	60.5

Bone marrow cells were extracted from Balb/C mice of approximately 6-month old mice. After cells were allowed to adhere to the well bottoms for 7 days, condition media was added a and changed biweekly for an additional 2 weeks. All three supernatants were diluted to 50% with VM. Alk. Phos. staining was then performed and the number of Alk. Phos.-postive colonies were counted over an area of 6.45 cm^2 with the naked eye.

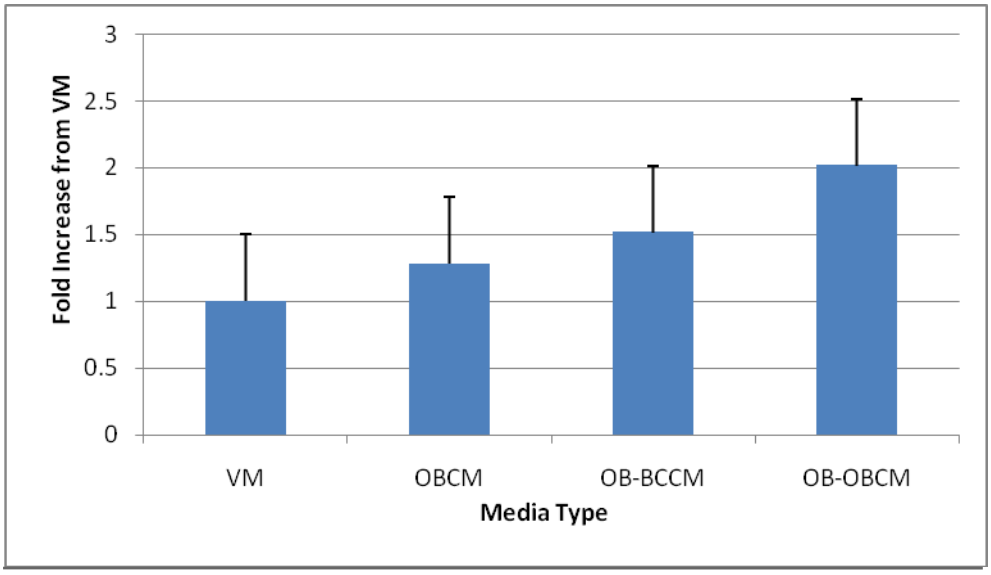


Figure 5: Condition Media increased the number of Alk. Phos. colonies compared to VM alone. Alk. Phos.-positive colonies were counted and averaged over duplicate plates. The median values were then compared to the VM baseline to assess the fold increase of Alk. Phos.-positive colonies generated by each conditioned media. OB-OBCM had the greatest effect on the number of osteoblasts, doubling the value of the VM baseline value. OB-BCCM produced 1.5X's the number of Alk. Phos.-positive colonies as compared to VM and more than OBCM alone. (n=2, so no meaningful statistical analysis could be performed).

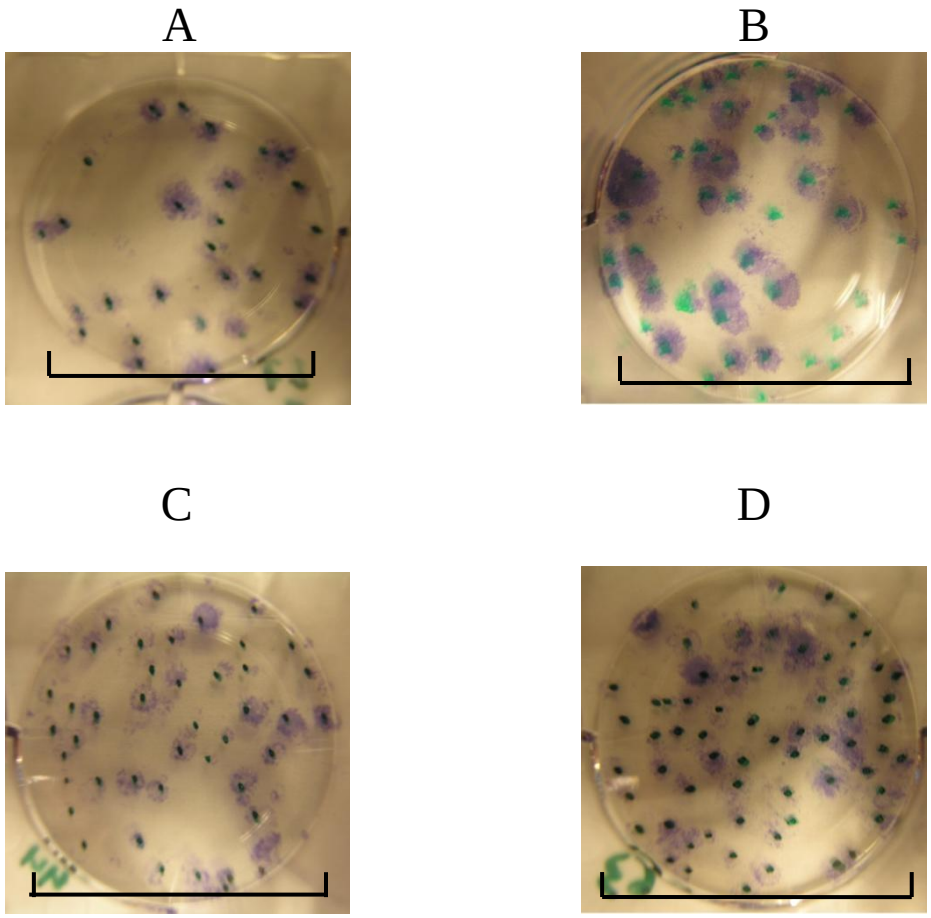


Figure 6: A visual representation of increased osteoblast proliferation when exposed to condition media. Colonies that tested positive for Alk. Phos. (purple) were counted with the naked eye and marked with a green marker to prevent double counting. A) VM produced the lowest number of osteoblast colonies per well (average of 30). B) OBCM increased the number of colonies by 1.25X's, while C) OB-BCCM induced Alk. Phos.-positive osteoblast colony formation by 1.5X. D) OB-OBCM had the greatest effect. Scale bars represent 33.8 mm. See **Table 3** for data values of each conditioned medium

TABLE 4: The Effect of Conditioned Media's Effects on Osteoclastogenesis

	VM	OBCM	OB-BCCM	OB-OBCM
1st Well	30	77	64	84
2nd Well	34	93	70	79
Average	32	85	67	81.5

Bone marrow cells were extracted from Balb/C mice of an unknown age and cultured in 6-well plates. Cells were allowed to settle for 7 days, after which condition media was added accordingly. As was done for the osteoblast portion of this experiment, OBCM, OB-BCCM and OB-OBCM were diluted with 50% VM. After 3 weeks, the cells were fixed and stained for TRAP activity. Multinucleated TRAP-positive cells were counted and averaged over duplicate plates.

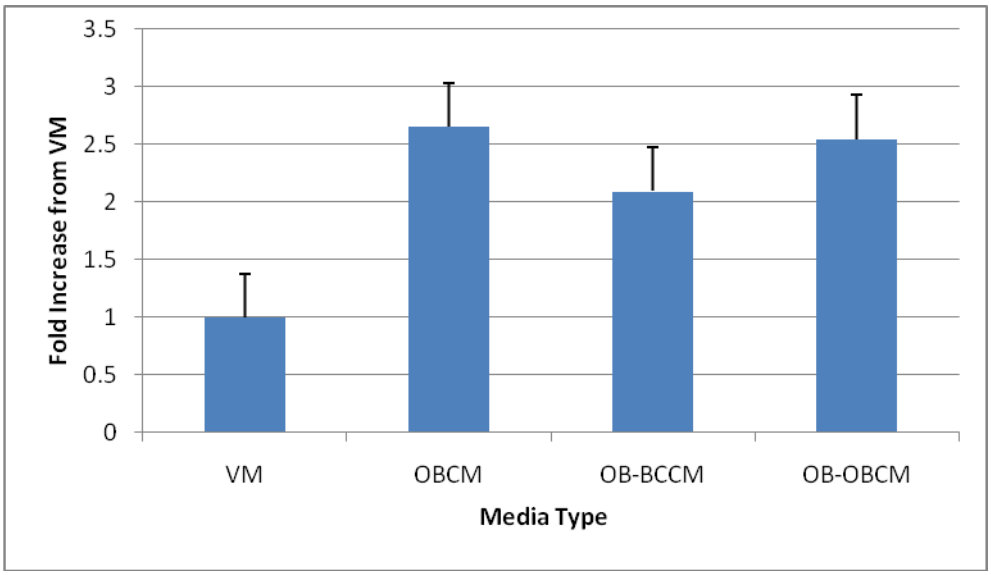


Figure 7: Condition media increased osetoclastogenesis compared The baseline VM. After 3 weeks of culture, TRAP staining was performed and multinucelated osteoclasts expressing TRAP were counted. All three condition media types induced osteoclastogenesis at greater levels than VM alone. (n=2, so no meaningful statistical analysis could be performed).

Experiment 3: Pre-osteoclast Differentiation

To determine the effects of condition media on osteoclast formation without the presence of osteoblasts and other cell types that reside in the bone marrow, only non-adherent pre-osteoclasts, isolated from Tg(CAG-EGFP)B5 Nagy/J GFP mice were cultured and exposed to condition media. Because the cells normally responsible for producing RANKL and M-CSF were absent in this experiment, 100 ng/ml M-CSF and 50 ng/ml RANKL were added for the first 4 days of culture to all media types except VM. After this period, 100 ng/ml RANKL and 50 ng/ml M-CSF were added until the cells had been growing for a total of 2 weeks, at which point only 100 ng/ml RANKL was added at each media change. OBCM, OB-OBCM and OB-BCCM were added at 100% in this experiment, and not diluted with VM as in Experiments 1 and 2. A titration of BCCM was also performed to observe the level of osteoclastogensesis at varying concentrations of the media. Finally, an OBCM + BCCM mixture was given to pre-osteoclasts to determine if the synergestic effects OB-BCCM were caused by the inflammatory response generated when cancer cell media was cultured with the MC3T3-E1's or if the two media types were simply inducing osteoclast formation in an additive manner. Cells were incubated at 37°C, and 5% CO_2 for 3 weeks and were then fixed in 10% formalin and stained for TRAP activity. The confluency at the time of staining was also noted and averaged over triplicate plates. **Table 5** displays the composition of each media type tested in this experiment.

Interestingly, although VM did not receive any supplements of sRANKL or M-CSF, it was capable of inducing osteoclastogenesis in non-adherent cells **(Table 6)**. This was possibly due to osteoclastogenic factors in the serum used throughout the experiment, or possibly remaining osteoclastogenesis-inducing cells (stromal cells or osteoblasts) that were present with the non-adherent cells at the time of plating. RANKL and M-CSF media produced very few

TRAP-positive osteoclastogenesis, indicating possible experimental error, since the combination of cytokines has been proven to induce osteoclastogenesis in murine pre-osteoclast cells[30]. Each supernatant generated more osteoclasts per well than VM alone, with OBCM being the most successful, although the consistency of the data over the triplicate wells was poor. At 12.5%, BCCM was the least effective inducer of osteoclastogenesis. However, when the concentration was doubled it produced the second largest number of mature osteoclasts. OBCM 87.5% + BCCM 12.5% generated fewer osteoclasts than VM or RANKL and M-CSF supplementation, and was nearly half as successful as the OB-BCCM supernatant, intended to represent the inflammatory response osteoblasts undergo when breast cancer cells metastasize to the bone **(Table 6)**.

Conlfuency was most decreased in the 25% BCCM wells, while not affected at all at 12.5% BCCM. A 10-15% reduction in confluency was also noted for the OBCM 87.5% + BCCM 12.5% when compared to OB-BCCM, OB-OBCM, and OBCM **(Table 6)**.

A phenomenon of "giant" osteoclasts was also observed in some condition media types. These enormous multinucleated osteoclasts were composed of 20 or more nuclei and usually took up almost the entire field of vision at 200X magnification. OBCM, and 25% BCCM, were the only media types that generated the "giant" osteoclasts **(Figure 8)**. It was noted that these polykaryons tended to grow near one another in clusters, and were never discovered alone (that is, they were always found in regions of greater cell density).

TABLE 5: Composition of Media Added to Pre-osteoclasts

Media Type	VM	OBCM	BCCM
VM	100%	-	-
RANKL/M-CSF Media	100%	-	-
OB-BCCM	50% (on OB's)	-	50%
OB-OBCM	50% (on OB's)	50%	-
OBCM	-	100%	-
OBCM + 12.5% BCCM	-	87.5%	12.5%
25% BCCM	75%	25%	-
12.5% BCCM	87.5%	12.5%	-

VM (89% α-MEM, 10% FBS, 1% Pen-Strep) was initially created as a negative control, but due to stray RANKL/M-CSF producing cells present in the isolated non-adherent cells or possibly due to osteoclastogenic factors present in the serum, pre-osteoclasts were able to differentiate into osteoclasts even without supplemental RANKL and M-CSF. OB-OBCM was prepared by adding 50% OBCM and 50% VM to confluent and mature MC3T3-E1 murine osteoblasts for 24 hours. OB-BCCM was prepared in the same fashion , but 50% BCCM was used in place of OBCM. OBCM + 12.5% BCCM was made by mixing OBCM and BCCM *de novo* without exposing the media to the murine osteoblasts. 12.5% and 25% were diluted and VM. All media types except VM were supplemented with RANKL (50 ng/ml for the first 4 days of culture and then 100 ng/ml throughout the rest of the experiment) and M-CSF (100 ng/ml for the first 4 days, 50 ng/ml for the following 10 days, and then 0 ng/ml until the cells were fixed and stained).

TABLE 6: Effects of Condition Media on Pre-osteoclast Cells

	VM	RANKL/ M-CSF Media	12.5% BCCM	25% BCCM	100% OBCM	100% OB-OBCM	100% OB-BCCM	OBCM + 12.5% BCCM
1st Well	43	72	17	92	134	75	63	26
2nd Well	73	12	37	111	201	66	82	44
3rd Well	34	12	26	106	44	63	74	53
Avg. TRAP+ Cells	50.0 ± 16.7	32.0 ± 26.7	26.7 ± 6.90	103 ± 7.33	125 ± 55.3	68.0 ± 4.67	73.3 ± 6.57	40.3 ± 10.3
Avg. Confluency	70%	100%	100%	60%	80%	85%	85%	70%

As in Experiments 1 and 2, TRAP-positive, multinucleated were counted over a marked off area for each media type and averaged over triplicate wells. Contrary to prior assumptions, cells cultured in VM were able to undergo osteoclasto-genesis. RANKL/M-CSF media was not able to generate osteoclasts as successfully as VM, suggesting the possibility of experimental error. At a concentration of 12.5%, BCCM was even less successful than RANKL/M-CSF media. When this was doubled however, twice as many osteoclasts were produced compared to VM. 100% OB-BCCM induced a greater degree of osteoclastogenesis compared to OBCM + 12.5% BCCM, highlighting the importance of the inflammatory response in the vicious cycle. On average,100% OBCM generated the largest number of osteoclasts, but the data over three wells was sporadic and not closely grouped. (n=3. The data were analyzed by one-way ANOVA. The only treatment shown to be statistically significant ($p < 0.05$) was 12.5% BCCM compared with 100% OBCM).

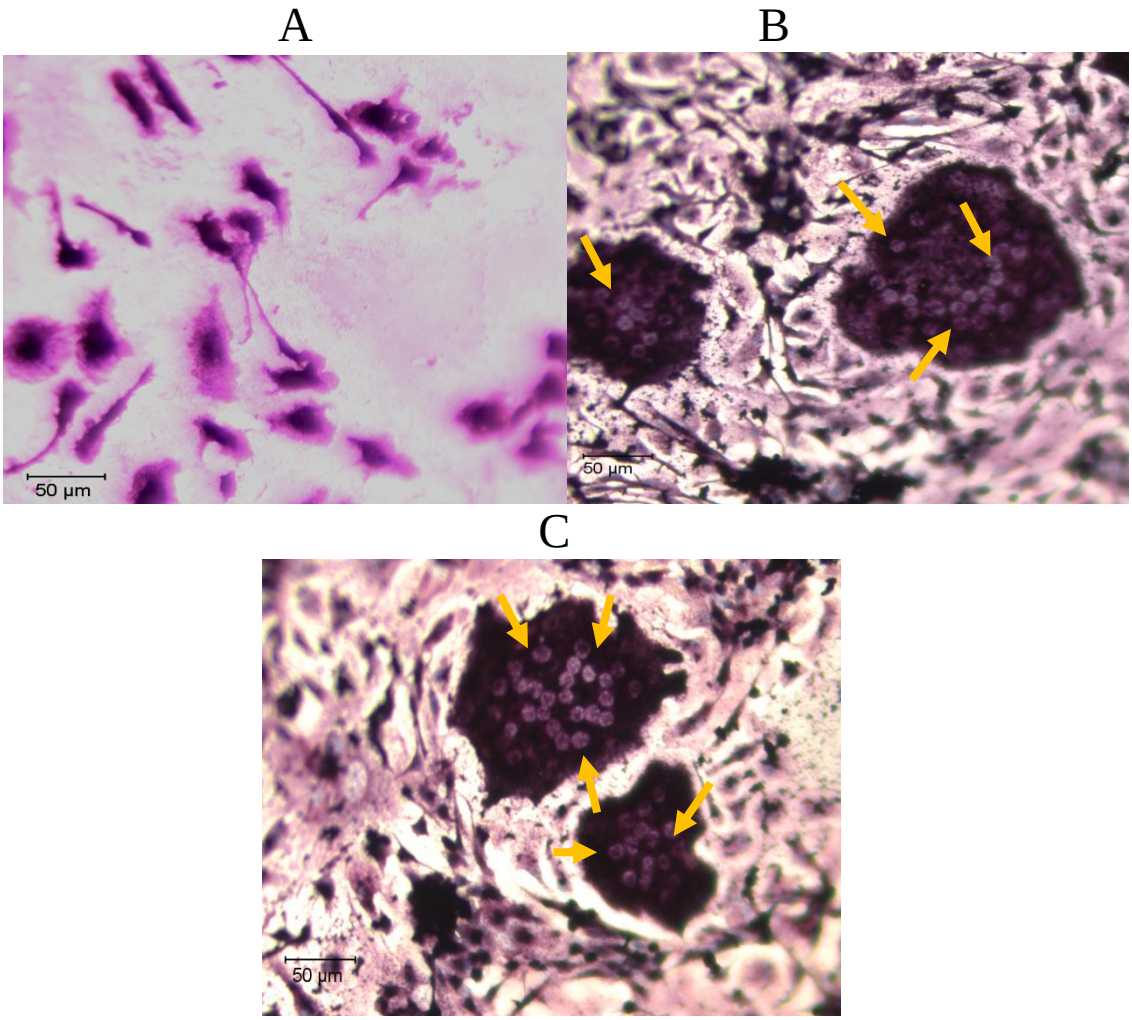


Figure 8: Giant osteoclasts generated by 25% BCCM and OBCM. A) Image of pre-osteoclast cells cultured in VM for 3 weeks. The cells are TRAP-positive, but only contain a single nucleus. B) OBCM was capable of yielding giant, TRAP+, multinucleated osteoclasts. Orange arrows show the several examples of nuclei with the polykaryons. C) 25% BCCM was also capable of producing the giant osteoclasts. Orange arrows again point out nuclei within the cell. Cells of this size often grew closely to one another and were always found in densely populated areas of the 24-well plate.

Experiment 4: OBCM and BCCM Titration

The difficulty in interpreting the results of the pervious data was that the percentages of the various media were not always the same. For a more straightforward comparison and to better assess the most effective concentration of OBCM and BCCM capable of inducing osteoclastogenesis, a medium titration was performed. Non-adherent pre-osteoclasts were isolated from 6-week old Tg(CAG-EGFP)B5 Nagy/J GFP mice. After a red blood cell lysis was performed, cells were plated at 45,000 cells/cm^2 in 24-well plates using VM, 100 ng/ml M-CSF, and 50 ng/ml RANKL. The cells were incubated for 48 hours at 37°C and 5% CO_2, after which, half the media was replaced with OBCM and BCCM at varying concentrations (diluted with VM) and 100 ng/ml M-CSF and 50 ng/ml RANKL were added to the wells (except for VM alone). After an additional 48-hour incubation, the media was entirely aspirated and replaced with the respective condition media. From this time point, condition media was changed every other day and 50 ng/ml M-CSF and 100 ng/ml RANKL were added to each well. After 14 days of culture, only 100 ng/ml of RANKL was added during media changes. A RANKL (50 ng/ml for first 4 days, and 100ng/ml thereafter) and M-CSF (100 ng/ml for the first 4 days and 50ng/ml thereafter until 2 weeks) media was also tested. After 21 days of culture, the cells were fixed and stained for TRAP activity.

In contrast to Experiment 3, VM was not capable of inducing osteoclastogenesis when exposed to pre-osteoclasts **(Table 7, Figure 9)**. The RANKL/M-CSF media produced very few osteoclasts per well, although they were still present. An interesting trend was noticed when comparing the levels of OBCM and BCCM. As the concentration of OBCM increased, the average number of TRAP-positive, multinucleated cells decreased. For BCCM, as the concentration increased, the effect was just the opposite **(Table 7, Figure 9)**. However, due to the small numbers of TRAP-positive cells per well, further experimentation must be performed.

Surprisingly, although the number of multinucleated TRAP-positive cells per well was rather low, high levels of TRAP-positive single-nucleus cells were noticed. After this observation, the wells were recounted for the number of TRAP-positive single-nucleus cells per field of vision. 5 randomly chosen fields were viewed at 200X magnification the number of single-nucleus TRAP-positive cells was averaged over the 5 fields. This number was then averaged over the triplicate plates for each condition media. Interestingly, the titrational effects of OBCM and BCCM on the number of TRAP-positive cells remained partially true. OBCM was the most effective at inducing osteoclastogenesis at 50%. BCCM however was more effective at producing TRAP-positive single-nucleus cells at 50% and 25% than at 12.5% **(Table 8, Figure 10)**. However, BCCM still produced less TRAP-positive, single-nucleus cells per field at all concentrations than OBCM did. In this experiment, VM alone was able to generate some TRAP-positive, single-nucleus cells, unlike TRAP-positive, multinucleated cells.

TABLE 7: Effects of Condition Media Titration on Osteoclastogenesis

	VM	RANKL/M-CSF Media	OBCM			BCCM		
			12.5%	25%	50%	12%	25%	50%
1st Well	0	1	1	0	8	4	0	0
2nd Well	0	0	0	9	6	2	1	2
3rd Well	0	4	0	1	16	0	6	3
Avg.	0	2.5	0.33	3.33	10	4.33	2.33	1.67

Isolated murine pre-osteoclasts plated at 45,000 cells/cm^2 and incubated for 21 days in varying amounts of OBCM, and BCCM. All media, except for VM, received RANKL and M-CSF supplementation. After three weeks, the cells were fixed and stained for TRAP activity. The TRAP-positive, multinucleated osteoclasts were counted and averaged over three wells. It should be noted that the number of TRAP-positive osteoclasts per well was much lower than in Experiment 3.

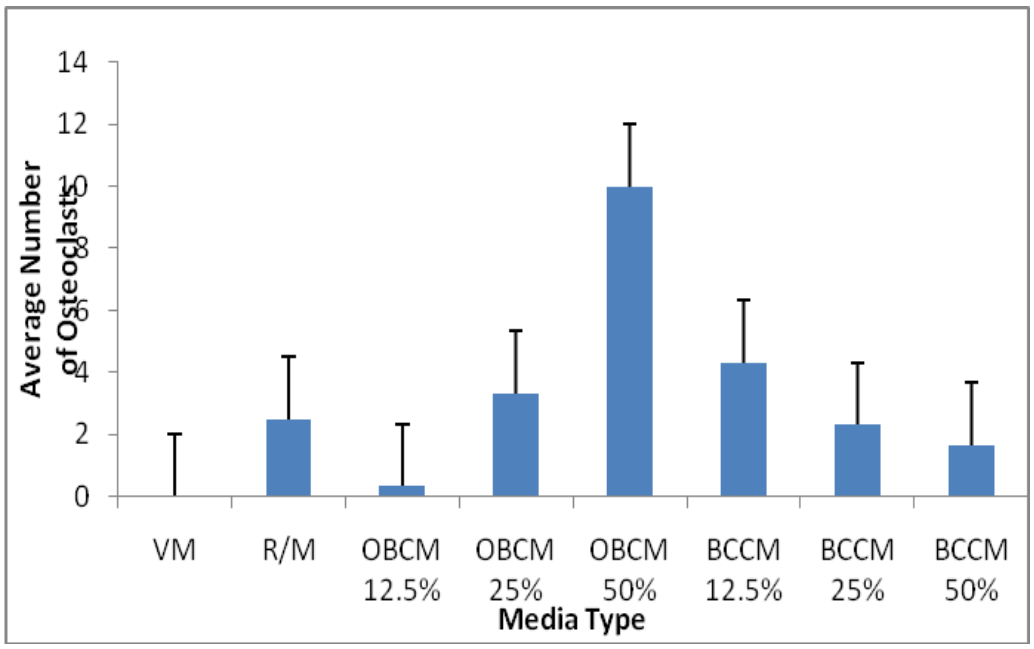


Figure 9: Condition media was titrated and osteoclastogenesis was then measured. Pre-osteoclasts were cultured in 24-well plates and after the cells were allowed to settle, condition media and RANKL/ M-CSF supplements were added. OBCM had a dose-dependent effect on osteoclastogenesis, while BCCM produced a negative correlation as the supernatant concentration was increased. R/M denotes RANKL/ M-CSF medium. (n=3. Due to the overall low number of cells per well, statistics were not performed).

TABLE 8: Effects of Condition Media Titration on Single-nucleus TRAP+ Cell Formation

	VM	RANKL/M-CSF Media	OBCM			BCCM		
			12.5%	25%	50%	12%	25%	50%
1st Well	0.8	0.6	0.4	0.6	1.8	0	0	0.4
2nd Well	2.7	2.2	4.4	3.0	1.4	1.2	3.8	3.4
3rd Well	0.6	1.8	0.4	4.0	9.4	2.2	2.0	2.2
Avg.	1.4	1.1	1.7	2.5	4.2	1.1	1.9	2.0

Pre-osteoclasts were exposed to varying concentrations of OBCM and BCCM and stained for TRAP activity at the end of 21 days of incubation. Each value represents the average of the 5 randomly chosen fields of vision for each well. These numbers were then averaged over triplicate plates.

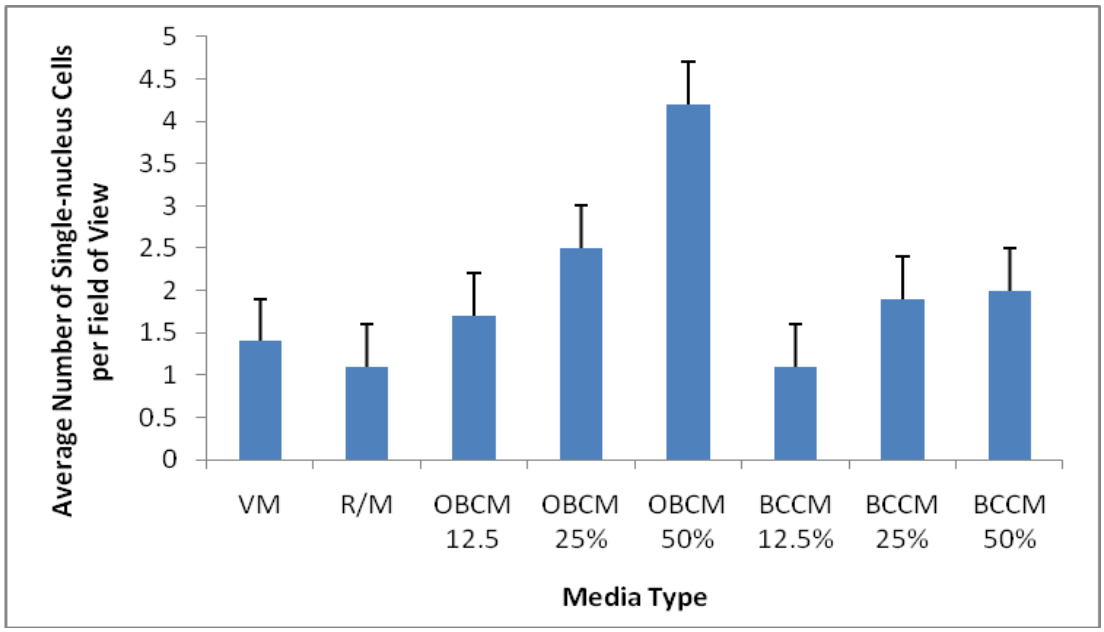


Figure 10: The number of single-nucleus TRAP-positive cells increases as the concentration of BCCM is increased. Single-nucleus TRAP-positive cells were counted in 5 randomly chosen fields of vision at 200X magnification. The 5 values were averaged for each well and then averaged again over triplicate plates. Like for TRAP-positive, multi-nucleated cells, OBCM generated higher levels of single-nucleus TRAP-positive cells as the concentration was increased. BCCM did not follow the same pattern as for the TRAP+ multinucleated portion of Experiment 4, and was actually most effective at producing TRAP-positive cells at 50%. R/M denotes RANKL/M-CSF medium. (n=3. Due to the overall low number of cells per well, statistics were not performed).

DISCUSSION

Increased bone resorption as an adverse effect of breast cancer bone metastasis can be explained by the vicious cycle originally proposed by Guise[21]. As metastatic breast cancer cells invade the bone microenvironment they shift the normal equilibrium between bone resorption and bone accretion when osteoblast function is inhibited, continual osteoclast activity continues to not only resorb bone, but release TGF-β, upregulating PTHrP production in cancer cells[31]. These findings demonstrate the role of all three cell types (osteoblasts, osteoclasts and metastatic cancer cells) involved directly in the vicious cycle and reinforce the idea of the inflammatory response.

In the first experiment, whole bone marrow isolates were cultured in the presence of various condition media. OB-BCCM and co-culture media induced a similar number osteoclasts to one another, signifying that the inflammatory response that the osteoblasts undergo may be triggered by condition media and direct cell-to-cell interaction. Co-culture media was slightly more effective at stimulating osteoclastogenesis, but the two media cannot be directly compared because the supernatants were not assayed for their contents, and the concentration of pro-inflammatory cytokines must have been unequal. However, the increase in osteoclastogenesis can likely be attributed to cytokines resulting from the inflammatory response.

OB-OBCM, the most prolific inducer of osteoclast differentiation, was able to increase the number of mature osteoclasts to more than 2.5 fold above the baseline with VM. A possible explanation for this effect could be the method by which the condition medium was created. When the medium was added to the MC3T3-E1's, it contained 50% OBCM and 50% VM. The OBCM was created by adding α-MEM to murine osteoblasts for 24 hours. It is possible that the serum-free conditions could have induced a form of an inflammatory response in the MC3T3-

E1's. Furthermore, while MC3T3-E1's do not secrete RANKL to any extent, the serum-free conditions could have caused an upregulation of the secreted protein. Finally, MC3T3-E1's do secrete M-CSF, an important factor in the initial stages of osteoclast differentiation[32]. The OB-OBCM media essentially contained a "double-dose" of osteoblast-derived osteoclastogenic cytokines, making it so effective at inducing osteoclastogenesis.

In experiment 1, it was seen that BCCM at 50% was inhibitory in regards to osteoclastogenesis with whole bone marrow isolates. Because osteoblasts were present in the bone marrow isolates, the condition medium could have hypothetically caused the osteoblasts to secrete pro-inflammatory cytokines. However, at 50%, the concentration of BCCM may have inhibited certain important steps in the osteoclast maturation process or may have even suppressed the release of osteoclastogenic cytokines.

In another experiment, bone marrow-derived osteoblast differentiation was studied to further determine the role of osteoblasts. All condition media was capable of increasing osteoblast colonies compared to VM. These data prove interesting because in work done by Mercer, et al., it was found that when MC3T3-E1 murine osteoblasts were cultured in breast cancer condition media alone, osteoblast differentiation markers were down-regulated[33]. However, in this experiment when the MC3T3-E1's were cultured with BCCM for 24 hours and the supernatant was then added to bone marrow isolates, a 1.5 fold *increase* of Alk. Phos. colonies was observed compared to the average baseline value for VM.

Initially these results supported the hypothesis that the synergistic effects of OB-BCCM would increase osteoblast differentiation, thereby leading to more cells secreting pro-inflammatory cytokines. However, when TRAP-positive, multinucleated osteoclasts generated from bone marrow cells were exposed to the same condition media were counted, the correlation

between the number of osteoblast colonies and the number of polykaryons was not as concrete. For example, while OBCM produced the fewest number of Alk. Phos.-positive colonies of the three condition media, it was most effective at inducing osteoclastogenesis. Nonetheless, although the relationship between osteoblast colonies and TRAP-positive osteoclasts was not directly parallel, an overall trend of increased Alk. Phos.-positive colonies and TRAP-positive osteoclasts was observed and sheds light onto the importance of osteoblasts in the vicious cycle.

Because the baseline secretions of RANKL, M-CSF and other osteoclastogenic factors produced by cells isolated from the bone marrow, it was decided to remove them as much as possible. In the next experiment, RANKL and M-CSF were added.

In order to verify the synergistic effects of OB-BCCM on osteoclastogenesis, it was necessary to prove that the condition medium was more capable of promoting osteoclast differentiation than the sum of its parts added together. When OBCM and BCCM were added at 87.5% and 12.5% respectively, they did not induce the same level of osteoclastogenesis as 100% OB-BCCM (essentially 50% OBCM and 50% BCCM). While the concentrations of these condition media were not identical, these initial results signify the importance that the inflammatory response (OB-BCCM) has on osteoclastogenesis. Added *de novo*, without exposure to MC3T3-E1's, OBCM + 12.5% BCCM did not possess the same levels of pro-inflammatory cytokines present in OB-BCCM, and was therefore not as effective at the induction of osteoclastogenesis. At the time of staining, the average confluency of the OB-BCCM wells was 80%. The average confluency of the OBCM + BCCM wells was 15% less than the OB-BCCM average, signifying the possible permissive effects the inflammatory response may have on cell growth. The fewer number of cells in a well would directly decrease the possible number of mature osteoclasts.

Perplexingly, the medium that stimulated the greatest amount of osteoclast differentiation in this experiment was 25% BCCM. At 50% BCCM had an adverse effect on osteoclastogenesis in whole bone marrow isolates. In addition, at a concentration 12.5%, BCCM was found to be the least effective inducer of osteoclastogenesis when exposed to non-adherent pre-osteoclasts. However, Guo et al. demonstrated that MDA-MB-231 condition medium had a bell curve effect on the degree to which RAW 264.7 murine pre-osteoclasts differentiated into mature, multinucleated osteoclasts **(Figure 11)**. Guo, et al. showed that alone, MDA-MB-231 condition media was not capable of inducing osteoclastogenesis, but if the RAW264.7 cells were primed with RANKL before they were exposed to the condition media, supplemental osteoclastogenic cytokines were not required to induce osteoclast differentiation[27]. Guo, et al found that a concentration of roughly 10% BCCM increased the number of osteoclasts to the greatest number. Raising the concentration of BCCM past this point only decreased osteoclastogenesis. It is possible that there is an optimal concentration of BCCM for primary pre-osteoclasts and the RANKL and M-CSF supplementation undertaken throughout this experiment acted in a manner similar to Guo, et al.'s pre-conditioning treatment. Unfortunately, because only two concentrations of BCCM were studied, such a curve cannot yet be established.

Given the possible inhibitory effects of BCCM at 50%, as seen in experiment 1, the final experiment was carried out in an attempt to establish a titration curve of the medium. Although the overall numbers of TRAP-positive, multinucleated cells per well was extremely low, a correlation between the concentration of OBCM and the number of multinucleated osteoclasts was seen, potentially signifying the crucial role that osteoblasts, and not cancer cells (as increasing the concentration of BCCM was actually deleterious) play in the direct stimulation of osteoclastogenesis. A titration of conditioned media is important to better compare the

osteoclastogenic effects of each supernatant as directly as possible. In the final experiment, the concentrations of OBCM and BCCM were not equal in the two media that were compared. OB-BCCM essentially contained 50% OBCM and 50% BCCM while the other medium was composed of 87.5% OBCM and 12.5% BCCM.

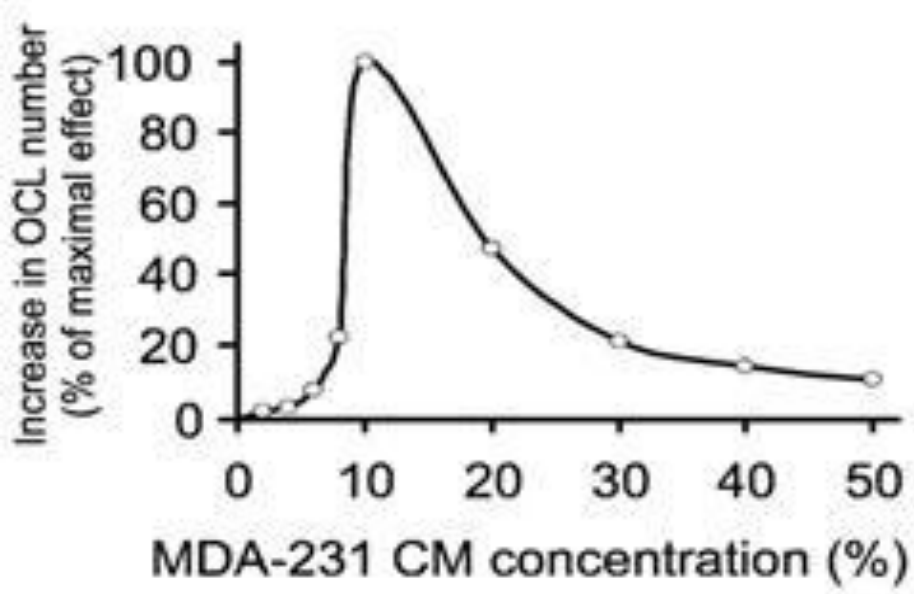


Figure 11: Guo, et al. determined that 10% BCCM stimulated the highest number of osteoclasts to differentiate and stain positively for TRAP. RAW 264.7 murine pre-osteoclasts were treated with RANKL (50 ng/ml) for 3 days and then exposed to varying concentrations of BCCM. After an additional 2 days of culture with the condition media, the RAW cells were fixed and stained for TRAP. A possible titration effect may be possible with the primary pre-osteoclasts isolated in this experiment, but not enough data points exist.
Guo, et al. "Osteoclast precursors acquire sensitivity to breast cancer derived factors early in differentiation." *Bone.* 43(2008): 386-393.

The present study has emphasized the significance of cytokines of the inflammatory response in the role of osteoclastogenesis. In whole bone marrow isolates, OB-BCCM significantly increases the number of mature osteoclasts, as well as osteoblast colonies. This information reveals the complex nature of the vicious cycle and the interconnections between

breast cancer cells, osteoblasts and osteoclasts. It is important that all aspects of the bone microenvironment receive attention in regards to breast cancer bone metastases.

Future experimentation of this topic should focus on several issues. The first and perhaps most important problem facing the experiment was the lack of a defined control and very few standardization steps. Condition media should be titrated with isolated pre-osteoclasts and normalized curves should be generated to better understand the most appropriate concentrations of OBCM, OB-BCCM and OB-OBCM to be added. Experiment 4 addressed this partially, but due to experimental variation, and possible staining problems, cannot be trusted as completely accurate data. Additionally, a cytokine array should be performed on the aforementioned supernatants to determine the levels of osteoclastogenic cytokines. While each batch of condition media would undoubtedly contain varying levels of the cytokines, due to differences in initial osteoblast population, or the exact amount of media added to the cells, a range could be established to better comprehend exactly what is being added to the whole bone marrow isolates or non-adherent precursor cells.

The link between Alk. Phos.-positive colonies and TRAP-positive osteoclasts should also be investigated to a further extent. Additional media types should be tested on both the osteoclasts and osteoblasts to better get an idea of all the changes the condition media types stir up when exposed to the bone microenvironment *in vivo*.

Also, although preliminary results were intriguing, OB-BCCM and OBCM+BCCM should be tested at equal concentrations of media types. Currently, while the levels of media are similar, they are not exact and can therefore not be directly compared. The concentrations to add the two media types could be determine by a standard curve, as previously mentioned.

Finally, a comparison of the pro-inflammatory cytokines induced by the inflammatory response should be undertaken to better understand the role that each one has in the process of osteoclastogenesis. Although each cytokine has been proven to elicit TRAP-positive multinucleated cells *in vitro* the concentrations of the cytokines, osteoclast cell line and overall experimental procedure have all been different. A more specific study in which all parameters would be set equal would allow us to better comprehend the role of these inflammatory cytokines in osteoclastogenesis. Such an experiment was tested, adding equal concentrations of pro-inflammatory cytokines to pre-osteoclasts, with and without RANKL and M-CSF. However, due to the result of poor staining, the TRAP-positive cells could not be counted. Unfortunately, time constraints prohibited any further investigation into this area of research.

In summary, the results of the experimentation described in this thesis, although not definitive, highlight the importance of osteoblasts and the inflammatory response they undergo. This response increases the concentration of osteoclastogenic cytokines and perhaps even the number of osteoblasts present in the bone microenvironment. Finally, an elevated number of osteoclasts causes increased bone resorption, releasing embedded growth factors, thus completing the cycle. The results of this experiment emphasize the role of all three types of cell involved in the vicious cycle and highlight their interconnectedness.

REFERENCES

[1] American Cancer Society. Breast Cancer Facts and Figure 2008-2008. Atlanta: American Cancer Society 2008.

[2] American Cancer Society. Overview: Breast Cancer. Atlanta: American Cancer Society, 2009.

[3] American Cancer Society. Breast Cancer Facts and Figure 2009-2010. Atlanta: American Cancer Society 2009.

[4] Dickson, RB, RG Pestell, ME Lippman. Cancer of the breast. In: Devita, VT, S Hellman, SA, Rosenberg, eds. Cancer – principles and practice of oncology. 7th edition. Philadelphia: Lippincott Williams and Wilkins; 2005.

[5] Rubens, RD, GR, Mundy. *Cancer and the Skeleton*. London: Martin Dunitz: 2000.

[6] Welch, D.R., P.S. Steeg and C.W. Rinkler-Schaeffer. 2000. Molecular biology of breast cancer metastasis: Genetic regulation of human breast carcinoma metastasis. *Breast Cancer Res.* 2(6) 408-416.

[7] Osteen, RT. Breast Cancer, 251-68 *The American Cancer Society's Clinical Oncology,* ed. R.E. Lenhard, R.T. Osteen, T Gansler,. American Cancer Society, Atlanta, 2004.

[8] Price, J.E. *The Breast Comprehensive Management of Bengign and Malignant Disorders*. K.I. Bland, ed., St. Louis: Saunders, Vol. 1. 2004.

[9] BreastCancer.org. Where Breast Cancer Might Come Back and How to Detect It. Ardmore, PA: BreastCancer.org 2008.

[10] Favus, M.J. (ed.), Christakos, S. (ed.), Goldring, S.R. (ed.), Holick, M.F. (ed.), et al. *Primer on Metabolic Bone Diseases and Disorders of Mineral Metabolism: An Official Publication of the American Society for Bone and Mineral Research* 4th Edition. Lippincot, Williams and Wilkins. 1999.

[11] Bilezikian, J.P.(ed.), Raisz, L.G. (ed.), G.A. Rodan (ed.). *Principles of Bone Biology.* San Diego: Academic Press; 2002.

[12] Väänänen H, Zhao H, Mulari M, Halleen J. "The cell biology of osteoclast function". *J Cell Sci* 113(3): 377–81.

[13] Takahash, N., Ejiri, S., Yanagisawa, S., Ozawa, H. "Regulation of osteoclast polarity." *Odontology* 95(1): 1-9.

[14] Minguell, J.J., Erices, A., P. Conget. "Mesenchymal stem cells." *Exp Biol Med.*226 (2001): 507-520.

[15] "osteocyte." *Encyclopædia Britannica.* 2009. Encyclopædia Britannica Online. 10 Nov. 2009 <http://www.britannica.com/EBchecked/topic/434280/osteocyte>.

[16] Bai,Y., et al. "Inhibition of RANK/RANKL signal transduction pathway: A promising approach for osteoporosis treatment." *Medical Hypotheses* 71(2): 256-258.

[17] Suda T., Takahashi N., Udagawa N., Jimi E., Gillespie M.T., Martin T.J. "Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families". *Endocr. Rev.* 20 (3): 345–57.

[18] Boyle, W.J., Simonet, W.S., Lacey, D.L. "Osteoclast differentiation and activation." *Nature* 432 (2003): 337-342.

[19] van de Wijngaert, F.P., Burger, E.H. "Demonstration of tartrate-resistant acid phosphatase in un-decalcified, glycolmethacrylate-embedded mouse bone: a possible marker for (pre)osteoclast identification." *Journal of Histochemistry and Cytochemistry* 34 (10): 1317-1323.

[20] Atkins, G.J., et al. "Osteoprotegerin inhibits osteoclast formation and bone resorbing activity in giant cell tumors of bone." *Bone* 28 (4): 370-377.

[21] Guise, T.A.. "Molecular Mechanisms of Osteolytic Bone Metastasis. 2000. Skeletal Complications of Malignancy: Supplement to *Cancer*. 88 (12): 2892-2898.

[22] Kinder, M. 2004. The Cytokine Connection Between Metastatic Breast Cancer and Osteoblasts.*The Pennsylvania State University: Schreyer Honors College Thesis.*

[23] Bussard, K.M. 2008. The Role of Osteoblast-Derived Inflammatory Cytokines in Bone Metastatic Breast Cancer. *The Pennsylvania State University: Ph.D. Thesis.*

[24] Udagawa, N., Takahashi, N., Katagiri, T., Tamura, T., Wada, S., Findlay, D.M., Martin, T.J., Hirota, H., Taga, T., Kishimoto, T. "Interleukin (IL)-6 induction of osteoclast differentiation depends on IL-6 receptors expressed on osteoblastic cells but not on osteoclast progenitors." *Journal of Experimental Medicine.* 182 (1995): 1461-1468.

[25] Aldridge, S.E., Lennard, T.W.J., Williams, J.R., Birch, M.A. "Vascular endothelial growth factor acts as an osteolytic factor in breast cancer metastases to bone." *British Journal of Cancer.* 92 (8): 1531-1537.

[26] Ito, A., et al. "Role of CC chemokine receptor 2 in bone marrow cells in the recruitment of macrophages into obese adipose tissue." *The Journal of Biol Chem.* 283 (2008): 35715-35723.

[27] Kim, M.S., Day, C.J., Selinger, C.I., Magno, C.L., Stephens, S.R.J., Morrison, N.A. "MCP-1-induced human osteoclast-like cells are tartrate-resistant acid phosphatase, NFATc1, and calcitonin receptor-positive but require receptor activator of NFκB ligand for bone resorption." *The Journal of Biol Chem.* 281 (2006): 1274-1285.

[28] Quarles, L.D., Yohay, D.A., Lever, L.W., Caton, R., Wenstrup, R.J. "Distinct proliferative and differentiated stages of murine MC3T3-E1cells in culture: An *in vtiro* model of osteoblast development." *Journal of Bone and Mineral Resarch.* 7 (6): 638-692.

[29] Yoneda, T., Sasaki, A., Mundy, G.R. "Osteolytic bone metastasis in breast cancer." *Breast Cancer Research and Treatment.* 32 (1): 73-84.

[30] Guo, Y., Tidemann, K., Khalil, J., Russo, C., Siegel, P., Komarova, S. "Osteoclast precursors acquire sensitivity to breast cancer derived factors early in differentiation." *Bone*. 4 (2008): 386-393.

[31] Mundy, G.R. "Metastasis to bone: causes, consequences, and therapeutic opportunities." *Endocrine Rel Cancer.* 7 (2000): 271-284.

[32] Elford, P.R.. Felix, R., Cecchini, M., Trechsel, U., Fleisch, H. "Murine osteoblastlike cells and the osteogenic cell MC3T3-E1 release a macrophage colony-stimulating activity in culture." *Calcified Tissue International*. 41 (1987): 151-156.

[33] Mercer, R.R., Miyasaka, C., Mastro, A.M. "Metastatic breast cancer cells suppress osteoblast adhesion and differentiation." *Clinical and Experimental Metastasis.* 21 (5): 427-435.

ACADEMIC VITA

Drew Baron
126 North Barnard Street (rear)
State College, PA 16801
Aeb240@psu.edu

Education
Bachelor of Science Degree in Biochemistry and Molecular Biology, the Pennsylvania State University, Fall 2009
Minor in Spanish
Honors in Biochemistry and Molecular Biology
Thesis Title: Osteoblast-mediated Osteoclastogenesis in Response to Breast Cancer Cell Condition Media
Thesis Supervisor: Dr. Andrea Mastro

Related Experience:
Hershey Medical Center Summer Preceptorship
Summer 2008

Awards:
Dean's List (Fall 2005, Fall 2007, Spring 2008, Fall 2008, Spring 2009, Fall 2009)

Presentations/Activities:
Technology Director for American Society for Microbiology
Morale Committee Member for IFC/Panhellenic Penn State Dance Marathon 2007, 2008
Entertainment Captain for IFC/Panhellenic Penn State Dance Marathon 2009
Co-founder of Independent Fundraising Group, A-7, for Penn State Dance Marathon
Team Leader for Relay for Life 2007, 2008