BREAST CANCER METASTASIS TO BONE:
A STUDY OF THE EFFECT OF BONE CELL CONDITIONED MEDIA ON TRIPLE NEGATIVE BREAST CANCER CELLS IN BONE MICROENVIRONMENT

by
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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Mechanical Engineering

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ABSTRACT

Breast cancer is the second leading cause of death in women—behind lung cancer. Statistics have shown that 1 in 36 women in the United States die from the pandemic. Triple negative breast cancer, TNBC, which accounts for 10-20% of all breast cancer subtypes, is classified as aggressive in growth and invasiveness and is associated with poorer short-term prognosis. Due to their independence from hormone receptors, these cancer cells lack effective therapeutics and treatment of patients is limited to chemotherapy. Death occurrence from breast cancer is prevalent in patients with metastatic breast cancer. In the United States, approximately 200,000 cases of metastatic breast cancer and 50,000 cases of non-invasive breast cancer are estimated to be diagnosed annually.

Breast cancer is frequently associated with skeletal metastases. Over 70% of breast cancer metastasis occurs in bone. Bone metastasis causes bone pain, fracture, hypercalcemia, and paralysis. This is because tumor-bone interaction in the bone microenvironment creates a vicious cycle that upsurges bone resorption. While the effect of triple negative breast cancer tumor cells on bone are widely known, the effect of bone cells on metastatic triple negative breast cancer cells is not fully understood. The present study was designed to investigate the effect of bone-conditioned medium
on TNBC using conditioned medium from different lineages of cells within the bone marrow. Conditioned media harvested from new born mice Calvaria, mouse myoblast cell line C2C12, preosteoblast cell line MC3T3, 8-weeks old mice BMSC, 6-months old mice BMSC and osteoclast were all used as treatment to determine the effects on migration of TNBC. Breast cancer cells, MDA-MB-231, extracted from older Caucasian patients, MDA-MB435, extracted from younger Caucasian patients and MDA-MB468, extracted from older African American patients were used as triple negative breast cancer cells. Here, we report that in comparison to the above conditioned medium, myoblast-conditioned medium greatly inhibited these breast cancer cells. However, the degree of inhibition depended on the type of TNBC.

Further investigations were conducted to determine the effect of myoblast-conditioned medium on proliferation and invasion activities of TNBC and it was found that myoblast-conditioned medium greatly inhibited triple negative breast cancer proliferation and invasion of MDA-MB-435. Additionally, results indicated that invasion of MDA-MB-231 and MDA-MB-468 to bone was inhibited under the influence of myoblast-conditioned medium. However, it was observed that minimal myoblast-conditioned medium inhibited triple negative breast cancer proliferation of MDA-MB-231 while it promoted triple negative breast cancer proliferation of MDA-MB-468.
Chapter 1
INTRODUCTION

1.1 Breast Cancer Overview

Breast cancer is the second leading cause of cancer death in women, the first being lung cancer. Statistics show that 1 in 36 women in the United States die from breast cancer [1]. These cancer cells begin in the breast tissue of both men and women; however, it is more prevalent in women than men. The normal female breast is made up mainly of lobules, ducts and stroma. Lobules are milk-producing glands, ducts are tiny tubes that carry the milk from the lobules to the nipple, and stroma are fatty and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels [1] (figure 1.1). The breast, like any other part of the body, consists of billions of microscopic cells that multiply in an orderly manner; cells proliferate to replace the cells that undergo apoptosis [2]. During the proliferation process, a cell makes an extra copy of its entire DNA and then splits into two new cells. A procedure is followed after a cell splits to ensure the DNA has been copied correctly and the new cells can function properly. Two types of genes are important to cell proliferation, oncogene and tumor suppressor genes (Table 1.1). While cells proliferate, error in DNA, also known as mutation, can occur and these mutations are either inherited or spontaneous. Inherited mutations of specific genes, such as the BRCA1 and BRCA2, increase the risk of developing breast cancer. They account for 5-10% of all breast cancer cases in the United States while the spontaneous ones account for as high as 95% [3]. While there are repair mechanisms, a mutation might not cause any
problems, however, failure in repair of error can result in splitting of new cells into more abnormal cells at a fast rate thus forming a tumor [4].

A tumor is a mass of abnormal tissue and there are two types: benign (non-cancerous) and cancerous, which are also known as malignant. Benign tumors are not generally aggressive in growth; however, a continuous growth can cause pressure on organs and surrounding tissue. Malignant tumors, on the other hand, are cancerous and more aggressive tumors that invade and damage surrounding tissue [5].

1.2 Subtypes of Breast Cancer

Similar to normal cells, the growth and spread of cancerous cells is usually fueled by healthy growth factors and hormones in the body. Certain hormones, such as estrogen, progesterone and the human epidermal growth factor receptor 2, HER2, serve an important healthy function but also accelerate the growth of breast cancer tumors. Breast cancer is classified into growth rate depending on their receptors or molecular subtypes. Luminal A, about 40% breast cancer subtype, is the most common of all. Luminal A tumors are estrogen receptor-positive, ER+, and/or progesterone receptor-positive, PR+, but human epidermal growth factor 2 receptor-negative HER2-. This means that they grow in the response to estrogen and/or progesterone but do not overexpress HER2 and are the least aggressive of all subtypes. Luminal B, about 10% to 20% of breast cancers, is similar to luminal A but also overexpress HER2. Luminal B have high proliferation rate. Basal-like is the third subtype and it is also known as triple negative. Constituting about 10% to 20% of all breast cancers subtypes, basal-like lack estrogen and progesterone receptors and do not overexpress HER2 protein. The basal-like tumors are more common in people
with a BRCA1 gene mutation. The tumors are very aggressive, fast growing and are associated with poorer short-term prognosis than the other types. HER2 enriched, about 10% of breast cancers, produce excess HER2 but do not express hormone receptors. Similar to the basal-like tumors, they tend to grow and spread more aggressively than other cancers and are associated with poorer short-term prognosis compared to ER+ breast cancers [6].

1.3 Types of Breast Cancer

When cancerous tumors form, they have a tendency to invade surrounding sites. There are two types of breast cancer: invasive breast cancer and non-invasive breast cancer. The TNM classification of tumors determines how far breast cancer cells have spread within the breast (T), the extent of spread to the nearby lymph nodes (N) and the presence or absence of distant metastases (M). Once the T, N, and M are determined, a stage of 0, I, II, III, or IV is assigned [7]. Cancers that occur in the lining layer or epithelial cells of the breast are called Carcinoma and most breast cancers usually start in the ducts and lobules. The non-invasive breast cancer, carcinoma in situ, is the early stage (stage 0, or I) of cancer that has not grown into deeper tissues in the breast or spread to the other organs in the body though it might develop into an invasive breast cancer if left untreated. Invasive cancer, infiltrating carcinoma, is one that has already gone beyond the originating point through the lymph nodes to other organs. The cancer cells travel through the lymphatic system to other parts of the body either in the mid-stage (stage II or III) or later stage (stage IV) of the disease [1].
Approximately 1 in 8 women in the U.S. will develop breast invasive cancer, *infiltrating carcinoma*, in their lifetime. Presently, over 230,000 new cases of invasive breast cancer have been estimated to be diagnosed for 2013 and only 64,640 new cases of *carcinoma in situ* have been diagnosed. For this year alone, it is predicted that approximately 39,620 women will die from breast cancer [8].

### 1.4 Breast Cancer Migration & Invasion Overview

Breast cancer that remains in the breast rarely results in death, however, when cancerous cells metastasize to vital organs, the life of a patient is threatened. Understanding the mechanism of metastasis is crucial in determining ways of reducing mortality from breast cancer. Metastasis of breast cancer involves two steps; migration and invasion (figure 1.2). Virtually, all cancers, including cancers of the blood and lymphatic system can form metastatic tumors. Breast cancer is a heterogeneous disease, in that, once it is formed within the breast ducts or lobules, it can have multiple pathways of progression to invasive cancer [9]. For instance, ductal breast cancer, which accounts for 80% of all breast cancer diagnoses, progresses from normal pathology through atypical ductal hyperplasia (ADH) to ductal carcinoma *in situ* (DCIS) then invasive ductal carcinoma (IDC) thus leading to metastatic disease [10]. Invasion requires cancer cells to breach the epithelial cell basement member and migrate out of the duct into the surrounding tissue. These cells then invade and move through the walls of nearby lymph vessels or blood vessels, a term known as intravasation. The cells move through the lymphatic system and the blood stream to other parts of the body. These cancer cells then stop moving in small blood vessels called capillaries at a distant location then invade the walls of the capillaries and
migrate into the surrounding tissues, a term known as extravasation. Once in the surrounding tissues, cancer cells multiply to form small tumors known as micrometastases. These micrometastases stimulate the growth of new blood vessels to obtain a blood supply, which is needed to obtain the oxygen and nutrients necessary for continued tumor growth [11].

While these are basic steps of breast cancer cell metastasis to other tissues, it is important to note that there are programs within the tumor cell that determine whether it has the ability to invade [12]. Since metastasis cannot happen without initial migration, a better understanding of the migratory mechanisms used by cells is important for the understanding of some key events influencing mortality in breast cancer [9]. Migration of tumor cells differ from that of normal cells in that while pro- and anti-migratory signals are strictly balanced in normal cells, cancer cells have a preponderance of pro-migratory signals such as overexpression of levels of growth factor receptors. Elaboration of growth factors such as Epithermal Growth Factor, EGF, or cytokines such as Hepatocyte Growth Factor/Scatter Factor, HGF/SF, by breast cancer cells tip the balance in favor of migration [13]. For instance, HGF/SF facilitates migration by activating actin-restructuring enzymes such as Rho kinase and PAK [14] or by enhancing turnover of migratory machinery at spreading edges [15]. Breast cancer migrate through a mechanism called mesenchymal movement, whereby the cancer cells possess an elongated fibroblast-like shape and move rapidly through channels cut into surrounding matrix by matrix-degrading enzymes such as MMPs [16] (figure 1.3)

The secretion of proteases by tumor cells is one of the factors that cause invasiveness while homing receptors, identified on the cell surfaces, are necessary and
sufficient to mediate metastasis formation by tumors. The ability of cancer cells to invade depends on two factors, anchorage-independent survival and homing (figure 1.3). Metastasis genes encode homing receptors, their ligands, associated signaling molecules, and extracellular matrix-degrading proteinases, which jointly cause invasion and anchorage-independent survival [12]. In order words, the extracellular matrix of cancer cells, ECM, plays an important role in cell adhesion, migration, invasion, proliferation and differentiation. Breast cancer cells interact with ECM via the use of cell adhesion molecules, CAMs. The ECM can be divided into two categories: the basement membrane and the interstitial connective tissue [17]. The major components of the basement membranes are type IV and V collagen, laminin, entactin and several glycoproteins, which interact to form a dense network [10]. Breast cancer cells express matrix metalloproteinase, MMPs, a family of highly conserved zinc-dependent endopeptidases, which are capable of degrading the components of the basement membrane and interstitial ECM. This then allows cells to cross ECM barriers and epithelial basement membrane thus allowing intravasation, extravasation and colonization at distant sites [18].

Crossing ECM barriers is one of the steps to invasion. Alterations in cell-matrix and cell-cell adhesion also play an important role in breast cancer cell invasion. Cell adhesions aid in adhesion, organization, communication, and function of epithelial tissues while cell-matrix adhesion proteins enable the anchoring and interaction of cells with the ECM (figure 1.5). Integrins and CD44 are some of the wide range of proteins involved in cell-ECM interaction. However, α6β4 integrin have been said to promote invasion and metastasis by interacting with and regulating the expression of the growth factors ErbB2, EGFR and Ras [19]. In addition, studies
have also shown that β1-integrin has an aberrant expression that can lead to tumor progression and invasion [9]. Overall, while several proteins are necessary for the functionalities of normal cells, these proteins, along with other factors, can enhance the growth, migration and invasion of breast cancer cells. Figures 1.4 and 1.5 depict some factors that influence and facilitate breast cancer invasion. When these cells migrate out of the breast, as outlined above, they invade other tissues in the body. Breast cancer cells are commonly known to metastasize to sites such as the bone, brain, liver and lungs.

1.5 Metastasis to Bone

About two-thirds of patients with cancer will develop bone metastasis [20] and it has been estimated that close to 350,000 people die of bone metastasis each year in the United States. Bones are known to be the most common place for metastasis followed by lungs and liver and bone metastasis is usually more common than primary bone cancers [21]. The incidence of skeletal metastasis from autopsy studies is of 73% in breast cancer, 68% in prostate cancer, 42% in thyroid cancer, 36% in lung cancer, 35% in kidney cancer, 6% in esophageal cancer, 5% in gastrointestinal tract cancers, and 11% in rectal cancer. Patients with advanced breast and prostate cancers almost always develop bone metastases, and the chances are high that, in patients who are originally diagnosed with breast or prostate cancers, the bulk of the tumor burden at the time of death will be in bone [22]. As outlined above, breast cancer is the most common cause of bone metastasis and about 70% of people who die from breast cancer will have radiological evidence of skeletal metastasis before their death and in 40% of cases the bone is the first metastatic site [23].
The process of metastasis formation must entail a strong component of tumor-host interactions [12]. Bone metastasis can occur in any bone in the body but is most commonly found in bones near the center of the body. The most common sites are spine, pelvis, femur, humerus, ribs and skull [24]. As stated previously, breast cancer cells move away from the breast through blood vessels to the bone. They migrate with the bloodstream, while escaping the surveillance of the immune cells, to the bone marrow sinusoids where they reside and multiply [25]. Preferential localization in skeletal segments, which contains red bone, is due to the fact that rich vascularity allows cancer cells to be transported to this site. In addition, reduced blood flow velocity together with the formation of vortices and/or microthrombi, promotes the adhesion and immobilization of the tumor cells on the endothelial cells [26]. Some studies also suggest that bone provides fertile ground for the growth of tumor cells because they are areas of constant cell turnover and growth. Bone cells release substances that may prompt faster cancer growth, and these cancer cells are likely to attach better to bone than other substances in the body [27].

The bone and bone marrow cells contain and express a variety of growth factors, cytokines, enzymes and hormone-like substances, which together with factors produced by cancer cells, make bone microenvironment suitable for cellular implantation and development [28]. Once in the bone marrow, migration within the marrow space, growth, and proliferation of these breast cancer cells depends on number of promoting or inhibiting conditions, primarily on interaction with surrounding bone and bone marrow cells, through the increased expression of adhesion molecules, the availability of space, degree of vascularity, and the type of bone remodeling [24].
1.6 Bone Overview

To fully understand the interaction between breast cancer cells and bone cells in the bone microenvironment, bone properties, modeling and remodeling, and the cells involved must be understood. Bone is a living tissue with blood vessels and is made of living cells that help it grow and repair itself [29]. It also constitutes series of proteins, minerals and vitamins. Together with cartilage, bone makes up the skeletal system of the body that provides structural support for heart, lungs, and marrow and protects the brain, uterus, and other internal organs. It also serves as an attachment site for muscles allowing movements of limbs and is a mineral reservoir for calcium and phosphorous; it stores almost 99% of the body’s calcium and 85% of the phosphorus [30]. 10% of bone mass is made up of organic collagen, about 65% is composed of inorganic hydroxyapatite (calcium and phosphorus) and the remaining 25% comprised of water. Since bone is mostly made of collagen and calcium phosphate, this combination of collagen and calcium makes bone flexible and strong, which in turn helps it withstand stress [31], thus it is known as a mineralized connective tissue that has the same strength as cast iron but is as light as wood.

There are four major types of bone in the body: long bones, which are longer and narrower than flat bones (thinner and curved bones), short bones (roughly cube-shaped) and irregular bones [32] (figure 1.6). Long bones include the femora, tibiae, and fibulae of the legs, the humeri while the flat bones are the cranium (skull), ilium (pelvis), ribs, sternum and scapula. Short bones are triquetral (wrist bones) and anklebones while irregular bones are vertebrae and coxae. These bones have two textures: compact (cortical) bone and cancellous (spongy) bone. The dense outer layer or shell consists of cortical bone that appear smooth and solid while the cancellous
bone is located within the cortical bone and it consists of honeycomb, needle-like, or flat pieces called trabeculae [33] (figure 1.7).

Bone is either formed by intramembranous ossification or endochondral ossification. Intramembranous ossification is the embryonic development of flat bones from an embryonic tissue called mesenchyme that differentiates into osteoblast [33] (figure 1.8) while endochondral ossification, the formation of long bones and most of the other types of bone, is the process where a cartilage model is formed and replaced by bone. Initially, cartilage models are formed through condensation of mesenchymal cells, followed by their differentiation into chondrocytes and secretion of typical cartilage extracellular matrix components [34]. As this hyaline cartilage develops, chondrocytes in the center of the model increase in size, begin to calcify, and differentiate into osteoblasts. Due to this, perichondrium surrounding the cartilage is gradually converted into periosteum or bone collar. Capillaries then invade the center through the nutrient foramen through which osteoblasts, osteoclasts, and nutrients migrate into the spaces. The osteoblasts and osteoclasts work together in that the osteoclast breaks down the remaining cartilage and the osteoblasts secrete osteoid forming trabeculae. As this process continues, the diaphysis begins to elongate and the medullary cavity is formed. Similar to the primary ossification center, vessels bud into the ends of the hyaline cartilage, the epiphysis, forming what is called the secondary ossification center. However, instead of forming the medullary cavity, cancellous bone is formed. The growth plate, containing cartilage, separates the diaphysis and epiphysis. Bone continues to grow through the hypertrophy of chondrocytes within the growth plate. When chondrocytes undergo hypertrophy, they deposit matrix that allows osteoblast to continue to deposit matrix and minerals.
Eventually, in adult bone, the growth plate disappears and the cartilage is fully turned to bone with a little articular cartilage on the ends of the bone [35] (figure 1.9).

1.6.1 Bone Marrow

All types of bone contain hematopoietic tissues, otherwise known as the bone marrow. Bone marrow is the manufacturing site where bone cells are produced and differentiated, and because of the rich nature of this site, as previously stated, breast cancer cells find it attractive. In short bones, bone marrow is located within the trabecular cavities of the spongy bone, and within the medullary cavity in the long bone. It is a soft, sponge-like material that contains stem cells like hematopoietic or blood-forming stem cells (HSC), and marrow stromal cells or mesenchymal stem cells (MSC). HSCs differentiate to form osteoclasts, leukocytes (cells that fight infection), erythrocytes (cells that carry oxygen) and platelets (cells that help blood to clot) [36] while MSCs mostly differentiate into cartilage cells (chondrocytes), bone cells (osteoblasts), muscle cells (myoblast), adipocytes and fibroblasts (figure 1.10).

1.6.2 Bone Remodeling

The ability of bone to sustain the tremendous load placed on it in everyday life depends on constant repair of mechanical microdamage that develops in both cancellous and cortical bone. Approximately 10% of bone is remodeled every year. Bone remodeling is based on the concerted action of resorptive cell (osteoclast) and formative cell (osteoblast) populations in order to replace old bone with new bone. Osteoclasts are gigantic multinucleated cells formed by fusion of mononucleated hematopoietic progenitor cells. The dominating pathway regulating osteoclast differentiation is the RANKL/RANK/OPG pathway. Macrophage colony-stimulating factor, M-CSF, binds to c-fms through the protein, transcription factor PU.1
regulation, to allow monocyte progenitor proliferation and the expression of receptor activator of nuclear factor k B (RANK) [37]. Osteoblasts then promote osteoclast differentiation by expressing RANKL (RANK ligand). This RANKL binds to the membrane receptor RANK, which activates NF-kB signaling leading to the mononuclear osteoclast expression of tartrate-resistant acid phosphatase (TRAP) [38]. While osteoblast can promote osteoclast differentiation, it can also inhibit it by the decoy receptor osteoprotegerin (OPG) [39] (figure 1.11).

During remodeling, osteoclasts release hydrolytic enzymes such as protons and lysosomal hydrolases, which include cathepsin K and metalloproteases that degrade collagen in the matrix. The degraded mineral components are released into the circulation, leaving a space known as resorption pit. After resorption, which usually takes between 2-4 weeks, osteoclasts undergo a programmed death (apoptosis). Osteoblasts come in to lay down new matrix and this usually takes between 4-6 months [40].

Osteoblasts are derived from mesodermal and neural crest progenitor cells. Runt-related transcription factor 2 (Runx2) triggers progenitor cell differentiation into osteoblast lineage [41] by regulating expression of genes encoding osteocalcin, VEGF, RANKL, sclerostin and dentin matrix protein 1 (DMP1) [42]. Osterix, another transcription factor, along with bone morphogenetic proteins (BMPs), growth factors like FDF and IGF, angiogenic factors like endothelin-1, and hormones such as PTH and prostaglandin agonists all modulate osteoblast differentiation [43]. Once formed, osteoblasts express alkaline phosphatase (ALP) and type I collagen, which are both important for synthesis and mineralization of bone matrix [44]. In addition, osteoblasts also produce osteocalcin, osteopontin, and osteonectin, and RANKL; all
required for osteoclast differentiation [45]. Eventually, at the end of their lifespan, osteoblasts undergo terminal differentiation in which they transform into osteocytes and become embedded in the mineralized matrix. These osteocytes then express molecules, such as DMP1, FGF 23 and sclerostin, which are necessary to control bone formation and phosphate metabolism [46] (figure 1.12).

1.7 Metastatic Breast Cancer & Bone Remodeling

Significant deviations from a neutral balance between resorption and formation can result in severe accelerated bone loss or bone gains with possible disastrous consequences in terms of increased fracture risk or compression syndromes [37]. Most cancer metastases are associated with two types of lesion: osteolytic lesions, which are caused by stimulation of osteoclastic activity accompanied by reduced osteoblastic activity [47], and osteoblastic lesions, the expression of increased bone formation around the tumor cells associated with a disequilibrium of the osteolytic activity and altered bone turnover [48]. Most breast cancer metastasis to the bone is associated with osteolytic lesions. When breast cancer cells enter the bone microenvironment, the balanced remodeling process is disrupted (figure 1.13). In bone, breast cancer cells produce a variety of growth factors, of which the most important is the parathyroid hormone-related proteins (PTHrP). Some of the other factors produced by breast cancer cells are IL-1-6-8-11, PGE2, TGFα, TGFβ, EGF, VEGF, TNF, CSF-1, GM-CSF, and M-CSF (table 1.2). These factors directly or indirectly stimulate osteoclastic activity leading to bone resorption [49]. Furthermore, studies have revealed that these factors are more present in metastatic breast cancer than in non-metastatic breast cancer [50]. TGFβ, VEGF, IGFs, BMPs, calcium and
fibroblast-derived factors stimulate the tumor proliferation and increases PTHrP production [51]. When PTHrP is released, it causes upregulation of RANKL and downregulation of OPG [52]. Overall, cancer cells can elicit an increase in osteoblast production of several osteoclastogenic cytokines such as monocyte chemotactic protein-1 (MCP-1) [53].

As outlined above, breast cancer is the second leading cause of death in women and this is due to invasiveness as breast cancer that remains in the breast rarely leads to death. However, of all breast cancer subtypes, triple negative breast cancer cells are associated with poor prognosis, and while all breast tumor types may be treated with chemotherapy, there is a lack of effective therapeutic options for triple negative breast cancer (TNBC). This is because present chemotherapy targets one of the three receptors that are not present in the triple negative cancer cells. Due to their aggressiveness in growth and high invasive nature, TNBC cells, which account for 15% of all breast cancers, must clearly be understood for the development of effective therapies. Previous investigations have shown that repercussion of TNBC metastasis to bone is most often associated with osteolytic lesion, however, little is known about the effect of bone cells on the migration, invasion and proliferation of these cancer cells. Does bone cell-conditioned medium reduce or increase migration, proliferation and invasion of triple negative breast cancer cells within bone? This study examines these questions by hypothesizing that bone cell-conditioned media inhibits triple negative breast cancer cells in bone microenvironment.
Figure 1.1  Simplified version of the anatomy of the breast: Breasts contain about 15-20 glands called lobes, which are connected to the nipple by tubes called ducts. The structure of the lobes and ducts is a bit like the branches of the tree. Breast cancer usually begins in the lobe or duct and is carried through the lymphatic system to other sites.

Image adapted from Breakthrough Breast Cancer
Figure 1.2  Breast cancer migration and invasion: Breast cancer cells migrate to surrounding stroma through a mechanism called EMT, epithelial-mesenchymal transition. The cancer cells then invade blood vessel and are transported with the bloodstream to targeted sites. At targeted site, they take on their original shape through a mechanism called MET, mesenchymal-epithelial transition. These cancer cells multiply to form small tumors called micrometastases. Micrometastases stimulate blood vessels to obtain blood supply. Published in [85]
Figure 1.3  Ductal breast cancer migration progressions: 1. Normal ductal tissue is defined by a thin layer of epithelial cells. Mutation leads to excessive cell proliferation that can cause cancerous cell growth. While cancerous cells fill up space, a reduction in myoepithelial cells occurs and cancerous cells begin to breach the epithelial cell basement membrane and invade surrounding stroma. 2. Epithelial cells lose adhesion 3. and there is an epithelial-mesenchymal transition 4. The surrounding extracellular matrix is remodeled by growth factors in breast cancer cells 5. and along with influence from the breast cancer cells microenvironment, 6. culminate breast cancer migration 6. Published in [9]
Figure 1.4 Factors influencing tumor cell invasiveness. The scheme depicts the mechanisms of anchorage-independence and homing induced by osteopontin. Anchorage-independence is supported by osteopontin-c, which induces oxidoreductases that generates ATP (left). Osteopontin also induces homing, which entails chemotaxis, haptotaxis, spreading, and invasion. Chemotaxis is induced when the C-terminal domain binds to CD44 variant. Haptotaxis is mediated by the N-terminal through integrin αvβ3. The ligand spread by cancer cells alters the intracellular signal transduction initiates the secretion of proteases, which allows invasion into targeted site. Published in [12]
Figure 1.5  The legend above illustrates the major growth factors, proteins and molecules involved in normal cell-cell and cell-matrix adhesion. However, alterations in this cell-matrix and cell-cell adhesion also play an important role in breast cancer cell invasion. CD44, α6β4 integrin, and β1-integrin have been known to promote invasion and metastasis by interacting with and regulating the expression of some of the growth factor in breast cancer cells. Published in [9]
Figure 1.6  Types of Bone: There are four major types of bones: A. Long bones, such as femora, tibia, fibulae, humeri B. Short bones, such as wrist bones and anklebones. C. Flat bones are the skull, pelvis, ribs, scapula, and sternum. D., Irregular bones like the vertebrae and coxae. These bones develop either by intramembranous ossification or endochondral ossification.

Image adapted from Bones and Skeletal Tissues

[http://classes.midlandstech.edu/carterp/Courses/bio210/chap06/lecture1.html]
Figure 1.7 Right: Cortical bone, 80% of the weight of human skeleton, is the osseous tissue that supports the body. It consists of Haversian carnal, which entails concentric tubes of bone matrix (the lamellae) surrounding a central Haversian canal that serves as a passageway for blood vessels and nerves. Above: Cancellous bone is another osseous tissue that houses metabolic activities. It is softer, weaker, more flexible and highly vascular in nature compared to the cortical bone.

Image adapted from Bones and Skeletal Tissues [http://classes.midlandstech.edu/carterp/Course/s/bio210/chap06/lecture1.html]
Figure 1.8  Intramembranous ossifications: The formation of flat bone. Bone is formed from mesenchymal cells. Small groups of adjacent mesenchymal cells cluster together to form nodules and differentiate into osteoblasts. Osteoblasts then lay down and mineralizes bone matrix, osteoid. Vascularization occurs which leads to the formation of cortical bone, periosteum and cancellous that containing the red bone marrow.

Image adapted from Bones and Skeletal Tissues

[http://classes.midlandstech.edu/carterp/Courses/bio210/chap06/lecture1.html]
Endochondral ossifications: The formation of long and other types of bones. Bone is formed from hyaline cartilage model. Periosteum replaces perichondrium in the primary ossification center after chondrocytes increase in size, calcify, and differentiate into osteoblasts. The diaphysis begins to elongate and the medullary cavity is formed. Similar to the primary ossification center, vessels bud into the ends of the hyaline cartilage, the epiphysis, forming what is called the secondary ossification center, where cancellous bone is formed. The growth plate, containing cartilage, separates the diaphysis and epiphysis. Right: Growth Plate; further conversion of cartilage to bone.

Image adapted from Bones and Skeletal Tissues [http://classes.midlandstech.edu/carterp/Courses/bio210/chap06/lecture1.html]
Figure 1.10  Bone marrow stem cells: hematopoietic stem cells and multipotent mesenchymal stem cells. Hematopoietic stem cells give rise to red blood cells, platelets, and white blood cells. Osteoclasts are also developed from hematopoietic cells. Mesenchymal stem cells give rise to osteoblasts, chondrocytes, myoblasts, marrow stroma, fibroblasts and adipocytes.

Image adapted from Abcam [www.Abcam.com]
Figure 1.11  Simplified version of Osteoblast and Osteoclast differentiation. Wnt signaling allows MSCs to differentiate into osteoblasts. DKK-1 binding to Wnt receptor gives rise to osteoblast proliferation and differentiation. On the other hand, RANK ligand, RANKL, expressed by mature osteoblasts bind to RANK, which diverts the hematopoietic stem cells down the pathway of osteoclast differentiation. OPG secreted by osteoblasts inhibits osteoclast activity.

Image adapted from Nature Medicine

[http://www.nature.com/nm/journal/v13/n2/fig_tab/nm0207-133_F1.html]
Figure 1.12  Bone Remodeling. Bone is remodeled by the activation of osteoclasts that resorb bones. Osteoclasts release protons and lysosomal hydrolases that breakdown collagen and proteins. The resorption process takes about 2-4 weeks. Once breakdown is complete, osteoblasts come in, in reversal, to lay down new osteoid. The new osteoid is then mineralized and this process takes up to 6 months. Osteoblasts either remain on top of the bone (lining cells) or are embedded in the matrix (osteocytes).

Image adapted from American Journal of Neuroradiology

[http://www.ajnr.org/content/29/10/1816/F1.expansion.html]
Figure 1.13  The bone microenvironment A. under normal bone remodeling; B. in the presence of osteolytic bone metastasis.  

A. Osteoblasts secrete factors to activate osteoclasts that resorb bone and these osteoblasts form produce new osteoid.  

B. Breast cancer in the bone secrete PTHrP, cytokines, and growth factor that increase RANKL and pro-osteoclastogenic cytokines and decrease OPG, which results in increased osteoclast formation, bone degradation and suppressed osteoblast differentiation. Published in [52]
Table 1.1. Two types of genes important to cell growth, how they normally work and how they work when mutated. Susan G. Komen for the Cure
[http://ww5.komen.org/uploadedfiles/Content_Binaries/806-368a.pdf]

<table>
<thead>
<tr>
<th>Type of gene</th>
<th>How it should work</th>
<th>How it works when mutated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oncogene</td>
<td>It “turns on,” or starts normal cell division and growth.</td>
<td>The gene does not stop cell growth when it should and the cell grows out of control.</td>
</tr>
<tr>
<td>Tumor suppressor gene</td>
<td>It “turns off,” or stops normal cell division and growth.</td>
<td>The gene does not work and cell growth continues out of control.</td>
</tr>
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Table 1.2. Factors in the metastatic bone microenvironment that effect osteolysis.  

<table>
<thead>
<tr>
<th>Factor</th>
<th>Source</th>
<th>Target</th>
<th>Effect on Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH</td>
<td>Serum</td>
<td>Osteoblast</td>
<td>Increase in RANKL</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Cancer cell</td>
<td>Osteoblast</td>
<td>Increase in RANKL</td>
</tr>
<tr>
<td>COX-s/PGE2</td>
<td>Osteoblast &amp; cancer cell</td>
<td>Osteoblast &amp; Cancer Cell</td>
<td>Increase in RANKL; Increase in MMPs in Cancer Cells</td>
</tr>
<tr>
<td>IL-1</td>
<td>Macrophages, monocytes &amp; cancer cell</td>
<td>Osteoblast</td>
<td>Increase in RANKL</td>
</tr>
<tr>
<td>IL-11</td>
<td>Osteoblast</td>
<td>Osteoblast</td>
<td>Increase in RANKL</td>
</tr>
<tr>
<td>TNFα</td>
<td>Macrophages &amp; endothelial cell</td>
<td>Osteoblast</td>
<td>Increase in RANKL</td>
</tr>
<tr>
<td>IGF</td>
<td>Serum</td>
<td>Osteoblast</td>
<td>Increase in RANKL</td>
</tr>
<tr>
<td>FGF</td>
<td>Stromal cells</td>
<td>Osteoblast</td>
<td>Increase in RANKL</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Osteoblast, Cancer Cells and Matrix Release</td>
<td>Osteoblast</td>
<td>In Osteoblast, increase in COX-2, cytokines; in cancer cell, increase in PTHrP</td>
</tr>
<tr>
<td>PDGF</td>
<td>Osteoclast, cancer cell, platelets, megakaryocytes</td>
<td>Osteoblast</td>
<td>Increase in osteoblast proliferation, decrease in osteoblast differentiation, and decrease in osteoblast adhesion</td>
</tr>
<tr>
<td>Vitamin D/Calcium</td>
<td>Serum</td>
<td>Osteoblast</td>
<td>Increase in RANKL if deficient</td>
</tr>
<tr>
<td>Estrogen</td>
<td>Serum</td>
<td>Osteoblast</td>
<td>In osteoblast, increase OPG production, increase in collagen synthesis, decrease in cytokines, decrease in apoptosis; in osteoclast, increase in apoptosis</td>
</tr>
<tr>
<td>RANKL</td>
<td>Osteoblast</td>
<td>Osteoclast</td>
<td>Increase in osteoclastogenesis</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoblast</td>
<td>Osteoclast</td>
<td>Decrease in osteoclastogenesis</td>
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<td>Osteoblasts and cancer cells</td>
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<td>IL-8</td>
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<td>Osteoblast, cancer cells</td>
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<td>Increase in osteoclastogenesis</td>
</tr>
<tr>
<td>MCP-1</td>
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<td>Osteoclast</td>
<td>Increase in osteoclastogenesis</td>
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<td>VEGF</td>
<td>Osteoblast, cancer cells and endothelial cells</td>
<td>Osteoclast</td>
<td>Increase in osteoclast formation</td>
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<tr>
<td>MMPs</td>
<td>Osteoblast, cancer cells and endothelial cells</td>
<td>Matrix</td>
<td>Matrix degradation</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>Osteoclast</td>
<td>Matrix</td>
<td>Matrix degradation</td>
</tr>
</tbody>
</table>
Chapter 2

MATERIALS AND METHODS

2.1 Experimental Design & Overview

The effect of bone-conditioned media on breast cancer in bone microenvironment was observed by conducting a migration, proliferation and invasion test. Migration, proliferation and invasion activities of triple negative breast cancer cells were observed under 100%, 50%, and 25% concentrations of different bone-conditioned media. A scratch assay, discussed in section 2.4, was used to monitor the movement of cells while a standardized MTT assay, discussed in section 2.5, was used to monitor cell growth. BD BioCoat Matrigel invasion chamber was used to determine invasion ability of TNBC. The BD Matrigel matrix serves as a reconstituted basement membrane in vitro, blocking non-invasive cells from migrating through the membrane.

2.2 Materials

The triple negative breast cancer cells, MDA-MB-231, MDA-MB-435, and MDA-MB-468, were purchased from America Type Culture Collection located in Manassas, VA, USA. The preosteoblast cell line, MC3T3, and myoblast cell line, C2C12, were also purchased from America Type Culture Collection. The MTT solution, MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MW = 414, Component A), was purchased from Invitrogen (Camarillo, CA).
DMSO, Dimethyl Sulfoxide, was purchased from Fisher Scientific (Fair Lawn, NJ), sodium bicarbonate (NaHCO₃), disodium hydrogen phosphate (Na₂HPO₄), potassium chloride (KCl), sodium chloride (NaCl) and potassium phosphate monobasic, (KH₂PO₄) were all purchased from Fisher Scientific (Fair Lawn, NJ). Dulbecco’s Modified Eagle’s Medium (DMEM) and fetal bovine serum (FBS) were purchased from Corning Cellgro (Manassas, VA). L-Glutamine and penicillin/streptomycin were both purchased from Corning Cellgro (Manassas, VA). The cycloheximide used as a negative treatment for the proliferation assay was purchased from Sigma-Aldrich. Trypsin was also purchased from Corning Cellgro (Manassas, VA). Finally, hydrochloric acid, (HCl), used to adjust the pH level of prepared medium was also purchased from Fisher Scientific (Fair Lawn, NJ). The female 8-weeks and 6-months old mice (C57BL/6J) from which bone marrow stem cells and osteoclast were extracted from were purchased from the Jackson lab (Maine, USA).

Other tangible materials such as the flasks in which cells were cultured, the 6-well plates used for the scratch assay were all purchased from Celltreat (Shirley, MA). The 96-well plates used for the MTT assay was purchased from Nalge Nunc International (Rochester, VA), and the BD BioCoat Matrigel Invasion Chambers 24-well plates were purchased from the BD Biosciences lab (Bedford, VA).

2.3 Cell Culture

2.3.1 Growth Medium Preparation

To make the medium used in cell culturing, Dulbecco’s Modified Eagle’s Medium (DMEM) power and sodium bicarbonate were mixed in sterilized water. In a
sterilized environment, 10% fetal bovine serum, 0.5% L-Glutamine and 0.5% penicillin/streptomycin were added with the solution and filtered using a 500ml 0.45µm bottle top filter purchased from Celltreat (Shirley, MA). When needed, pH of solution was adjusted to 2.4 by adding a few drops of HCl.

2.3.2 Trypsin Solution Preparation

Trypsin was prepared by combining EDTA, pure trypsin and phosphate buffered saline (PBS). PBS was prepared by mixing Na₂HPO₄, KCl, NaCl and KH₂PO₄ in water. The solution was then sterilized. About 250ml of Trypsin was made by combining 1.6g of EDTA buffer power, purchased from Sigma-Aldrich, with 25ml of trypsin in 225mls of PBS. The solution was filtered using a 250ml 0.45µm filter purchased from Celltreat (Shirley, MA).

2.3.3 Cell Counting

A hemacytometer was used to count the number of cells that were seeded. Cells were mixed well in medium using a serological pipette and 1ml was extracted from the sample. 100µl was further extracted from the new sample and mixed in an Eppendorf tube containing 100µl trypan blue. A sample was drawn and filled in the hemacytometer. Using a 10X objective of the microscope and a hand tally counter, unstained cells were counted within 4 sets of squares with a square equivalent to number of cells x 10⁴ / ml. The number of cells per ml was calculated by averaging the numbers obtained from each square and then multiply the average by 2 to adjust for the trypan blue dilution.

2.3.4 Cell Seeding & Culture

In a cell culture hood, MC3T3 cells (preosteoblast) and C2C12 cells (myoblast) were seeded at a density of 1.8 x10⁶ in T-75 flasks containing 15 mls growth medium.
The cells were then incubated for two days, allowing the cells to grow in the medium containing necessary nutrients, growth factors, and hormones. After two days, cells reach confluency at a density of $8 \times 10^6$. Cells are usually split 1:2 ratio with 3 mls of trypsin solution. The triple negative breast cancer cells: MDA-MB-231, obtained from a 51-year old Caucasian, MDA-MB-435, obtained from a 31-year old Caucasian and MDA-MB-468, obtained from a 51-year old African America, were seeded at a density of $4.0 \times 10^6$ in T-75 flasks containing 15 mls growth medium. In two days, cells reach confluency at a density of approximately $2 \times 10^7$, and plated 1:2 ratio. All cells were incubated at 39.5 °C 5% CO$_2$, and 95% humidified air.

2.4 Primary Cell Extraction from Mice

2.4.1 Calvaria Osteoblast Extraction

Calvaria were dissected and removed from newborn mice and were subjected to four sequential 15 minute digestions in digestions in an enzyme mixture containing 0.05% trypsin (Gibco BRL) and 1.5U/ml collagenase P (Boehringer Mannheim, Germany) at 37°C on a rocking platform. Cells were then extracted by adding DMEM containing 10% fetal calf serum (FCS), 100U/ml penicillin and 100µg/ml streptomycin. Cells were then resuspended and centrifuged in DMEM, filtered through a 70-mm cell strainer and diluted 1:1 with 0.04% trypan blue in PBS. Viable cells were counted using hemacytometer and plated at a density of $1.5 \times 10^4$ cells/cm$^2$ in 35-mm culture plates in DMEM. Medium was changed regularly. The medium used contained modified minimum essential medium containing 10% FCS (Gibco BRL), 25mg/ml ascorbic acid (Sigma-Aldrich) dissolved in water, and 4mM β-glycerol
phosphate (Sigma-Aldrich) dissolved in PBS. At a week of culture, medium was obtained for experiment.

For bone marrow and osteoclast primary cell extraction, dead 6-months and 8-weeks old mice were subjected to a Wescodyne bath and a 70% EtOH bath. The hind limbs were dissected and with the muscles and metaphyseal ends removed, the marrows were flushed. Cells were gently dissociated from marrow by passing an 18-gauge needle through the marrow and these cells were syringe filtered through a 70µM filter.

2.4.2 Osteoclast Extraction

Cells were counted and spun at 1000rpm in 4°C for 5 minutes and resuspended in osteoclast media. Osteoclast media made from the combination of 50ml plating media, 10µl Rank-ligand and 6µl m-CSF. Plate media was made with 90ml α-MEM, 10 ml FBS, and 1ml Penn-Strep. Rank-ligand consisted of a mixture of 40µl sterile ddH₂O, and 10µl Rank-ligand (Peprotech) and the m-CSF was made by mixing 40µl sterile H₂O and 10µm-CSF (Peprotech). After 5 minutes, cells were resuspended in new osteoclast media and plated at a density of 1x10⁶/500µl in 48 well dishes. Media was changed occasionally and cells were monitored for osteoclast development. After approximately 10 days, cells were stained for TRAP.

2.4.3 Bone Marrow Stromal Cell Extraction

Leftover cells from the marrow were plated with plating media in 24 well plates at a density of 5x10⁶ per ml. The plate media was changed on day 3 and a differentiation media was added on the 7th day of seeding. Mixing and sterilizing 90ml of α-MEM, 10 ml FBS, and 1ml Penn-Strep, 800µl β-glycerol phosphate and 200µl ascorbic acid prepared differentiation media. The ascorbic acid used to media
consisted of 50mg ascorbic acid in 2ml of sterilized ddH$_2$O while the β-glycerol phosphate solution was prepared by dissolving 2.16g β-glycerol phosphate in 10ml PBS and sterilizing the solution.

2.5 Scratch Assay Methodology

Breast cancer cell line MDA-MB-231 was grown to confluence in a T-75 flask. The cells were then split and seeded with 2mls growth medium at a density of $2 \times 10^6$ in 6-well plates (surface area of 962mm$^2$). The cells were left for a day to reach 90% confluency and growth medium was replaced with different concentrations of conditioned media harvested from bone cultured cells and primary cells. 100%, 50%, 25% and 0% of total volume of growth medium (2mls) was replaced with conditioned media and there were 3 samples per concentration ($n=3$). 10% of FBS was then added to total volume of conditioned media such that in a 100% concentration sample (2mls of complete conditioned media), 200µl of FBS was added (figure 2.1.1). A 200µl pipette tip was then used to create a vertical scratch line between cells in each well. Cell images were taken to measure cell migration (closing of the scratch line) immediately after the scratch was created (0 hours) and every 12-hour interval for two days. This method was also used for other breast cancer cells: MDA-MB-435 and MDA-MB-468. Measurements were taken using Photoshop, an adobe software.

2.6 MTT Assay Methodology

MTT assay was used to measure breast cancer cell proliferation from day 0 of preparation to day 2. MDA-MB-231 cells were seeded at a density of 5000 in 96 well plates in 250µl of media. Cells were grown in 0%, 50%, 25% and 100% conditioned
media of harvested from C2C12 cells. Similar to the scratch assay, 10% of FBS was then added to total volume of conditioned media such that in a 50% concentration sample (1ml of complete conditioned media and 1ml of fresh growth media), 100µl of FBS was added. Additional cells with pure growth media were subjected to two concentrations of cycloheximide (0.5µl and 2.5µl). There were a total of 9 samples (n=9) per concentration and treatment (table 2.2). Cells were placed in the incubator to adhere to plate for 3 hours and 150µl medium was extracted from each well, leaving 100µl media. 12mM MTT stock solution was prepared by adding 5mg of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MW = 414, Component A) to 1mL of sterile PBS. 10µl of 12mM MTT stock solution was added to each well and allowed to incubate for 4 hours. After 4 hours, 85µl of medium (now purple) was removed, leaving 25µl of medium in each well. 50µl was then added to each well and mixed thoroughly with pipette, and incubated for 10 minutes. Each sample was mixed well after incubation and proliferated cells were counted using a microplate reader with absorbance at 540nm. This method was also used for MDA-MB-435 and MDA-MB-468 breast cancer cell lines.

2.7 Invasion Assay Methodology

24-well BD BioCoat Matrigel Invasion Chamber plates containing 12 inserts was used to study the invasive property of MDA-MB-231, MDA-MB-435 and MDA-MB-425 in the absence and presence of different concentrations: 100%, 50%, 25%, of C2C12 (myoblast) media. Three different media were prepared prior to experiment, rehydrating medium, serum-free medium and the normal growth medium. Rehydrating or plain medium was prepared by mixing DMEM power and NaHCO₃ in
sterilized water. Serum-free medium had a mixture of DMEM power, \( \text{NaHCO}_3 \), L-Glutamine, and penicillin/streptomycin while in addition to the serum-free medium, the complete growth medium had FBS. The 24-well chamber plate was removed from the -20°C storage and allowed to come to room temperature 37°C. 0.5ml plain medium was added to the interior of the inserts and bottom wells and allowed to rehydrate for 2 hours in incubator. After rehydration, 0.750ml of complete growth medium was added to well with inserts. 25,000 serum-free breast cancer cells were suspended in each insert and different concentrations (0%, 50%, 25% and 100%) conditioned media harvested from C2C12 cells were added to the cells (table 2.3). The plate was incubated for 22 hours and all medium was gently discarded. Non-invading cells (cells on the upper surface of the insert) were removed by gently scrubbing with a cotton tipped swab twice. A stain solution was prepared by adding methanol (20%) to crystal violet (methanol fixes cells, crystal violet stains them). 0.5ml of crystal violet solution was added to well and 150µl to insert and allowed to stain cells for 45 minutes. Crystal violet solution was slowly removed with pipette and inserts were washed three times and allowed to dry for 24 hours. Images of stained invaded cells were taken and counted using imageJ.

2.8 Data Analysis

Photoshop was used to measure cell migration, a plate reader was used to measure cell proliferation and imageJ was used to count invasive cells. Experiments were repeated three times and all data collected from tests were analyzed using a single factor Anova embedded in Microsoft excel followed by Tukey’s Honestly Significant
Difference test. $P < 0.05$, denoted *, and $P < 0.01$, denoted **, were accepted as significant.
Table 2.1: Scratch Assay Methodology. Breast cancer cells subjected to different concentrations of conditioned media as depicted in table below.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conditioned Media Quantity</th>
<th>Growth Media Quantity</th>
<th>FBS Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0 ml</td>
<td>2ml</td>
<td>0ml</td>
</tr>
<tr>
<td>25%</td>
<td>0.5ml</td>
<td>1.5ml</td>
<td>0.05ml</td>
</tr>
<tr>
<td>50%</td>
<td>1ml</td>
<td>1ml</td>
<td>0.1ml</td>
</tr>
<tr>
<td>100%</td>
<td>2ml</td>
<td>0ml</td>
<td>0.2ml</td>
</tr>
</tbody>
</table>
Table 2.2: MTT Assay Methodology. Breast cancer cells subjected to treatments and concentrations of conditioned media as depicted in table below.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conditioned Media Quantity</th>
<th>Growth Media Quantity</th>
<th>FBS Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 ml</td>
<td>2ml</td>
<td>0ml</td>
</tr>
<tr>
<td>0.5µl Cycloheximide</td>
<td>0 ml</td>
<td>2ml</td>
<td>0ml</td>
</tr>
<tr>
<td>2.5µl Cycloheximide</td>
<td>0 ml</td>
<td>2ml</td>
<td>0ml</td>
</tr>
<tr>
<td>25% Conditioned Media</td>
<td>0.5ml</td>
<td>1.5ml</td>
<td>0.05ml</td>
</tr>
<tr>
<td>50% Conditioned Media</td>
<td>1ml</td>
<td>1ml</td>
<td>0.1ml</td>
</tr>
<tr>
<td>100% Conditioned Media</td>
<td>2ml</td>
<td>0ml</td>
<td>0.2ml</td>
</tr>
</tbody>
</table>
Table 2.3: Invasion Assay Methodology. Breast cancer cells subjected to treatments and concentrations of conditioned media as depicted in table below.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conditioned Media Quantity in Insert</th>
<th>Serum-Free Media Quantity in Insert</th>
<th>Growth Media Quantity in Well</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0 ml</td>
<td>0.50ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td>25%</td>
<td>0.125ml</td>
<td>0.375ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td>50%</td>
<td>0.25ml</td>
<td>0.25ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td>100%</td>
<td>0.50ml</td>
<td>0ml</td>
<td>0.5ml</td>
</tr>
</tbody>
</table>
Chapter 3

EFFECT OF BONE CELL-CONDITIONED MEDIA ON TRIPLE NEGATIVE BREAST CANCER MIGRATION WITHIN BONE

3.1 Introduction & Rationale

As discussed in chapter 1, the skeleton is the first and most common distant metastatic site for breast cancer and bone metastases cause significant morbidity [54]. Current statistics show that 70% of all breast cancer that spread away from the primary sites goes to the bone and the mechanism of breast cancer-induced osteolysis most often consists of a stimulation of osteoclast-mediated bone lysis [55]. Of all breast cancer subtypes, the basal-like breast cancer, also known as triple negative, accounts for approximately 10-20% of all breast cancer cases [56] and patients exhibit a more aggressive clinical course, have a higher rate of distant recurrence and a poorer prognosis than women with other breast cancer subtypes [57]. While more investigations are being conducted to examine the reason why breast cancer cells metastasize to the bone, as highlighted in section 1.3, the bone marrow seems to be the residential area for these cells. This is because the bone marrow is highly vascular in nature and there is a constant cell turnover and growth. In addition, bone cells are known to release substances that may prompt faster cancer growth, and allow these cancer cells to attach better to bone than other sites in the body.

While metastasis is prevalent in people with breast cancer, much effort has been dedicated to understanding the effects of these cells on bone cells. These
investigations have resulted in findings indicating that breast cancer cells inhibit the growth of osteoblast-like cell (which originates from the differentiation of bone marrow stem cells). In addition, current studies show TGFβ promotes the ability of breast cancer cells to increase osteoblast sensitivity to various osteolytic agents [58]. However, little attention has been given to the reverse effect of bone cells on breast cancer within the bone microenvironment. Since the bone marrow contains bone precursor cells, this current study seeks to understand the effect of these cells on breast cancer in bone. This study is conducted by examining the effects of bone cell-conditioned media on breast cancer migration, proliferation and invasion. It is postulated that triple negative breast cancer cell migration is inhibited by the presence of conditioned media of bone cells within the microenvironment. Understanding the effect of these cells on triple negative breast cancer migration within bone can lead to the development of effective therapeutic measures that reduce bone disease that occur from breast cancer metastasis.

3.2 Methods & Results

To determine the effect of bone-cell conditioned media on breast cancer migration, scratch assay methodology (explained in chapter 2) was used. Triple negative breast cancer cells were exposed to 100%, 50%, 25% concentrations of bone cell conditioned media. A line was created to divide TNBC cells and migration activity was monitored for 48 hours.

3.2.1 Myoblast-Conditioned Medium Inhibited MDA-MB-231 Migration

MDA-MB-231, which was extracted from a 51-year old Caucasian patient was used as TNBC cells. Conditioned media of C2C12, a mouse myoblast cell line, was
used as treatment because of its behavior similarities to mesenchymal stem cells. MDA-MB-231 cells were exposed to 100%, 50% and 25% concentrations of myoblast-conditioned media and migration activity was analyzed for 48 hours. It was found that migratory activity of MDA-MB-231, under the influence of different myoblast conditioned media concentrations reduced in comparison to migratory activity in the absence of treatment (figure 3.1). Results also indicated that the migration activity of the breast cancer cells was immediately impacted in presence of the conditioned medium; that is the lag in migration began immediately. Lastly, results demonstrated that the higher the concentration of the conditioned media, the slower the migration.

3.2.2 Preosteoblast-Conditioned Medium Inhibited MDA-MB-231 Migration

Since initial results from current studies demonstrated that myoblast inhibit triple negative breast cancer migration, the same experiment was conducted using conditioned media from other bone cells. This was done to determine if the effect on TNBC cell migration was dependent on the type of bone cell-conditioned media used. MC3T3, osteoblast precursor cells, or preosteoblast, were used because they arise from mesenchymal stem cells. Results indicate that just like the C2C12, preosteoblast cells significantly inhibit breast cancer cell migration within the bone (figure 3.2). Again, result indicated that different concentrations of preosteoblast conditioned media inhibited in the migratory activities of MDA-MB-231 such that the higher the concentration of MC3T3, the slower the migratory activity. However, though both bone cell lines negatively affect breast cancer migration, it was observed that myoblast has a greater impact on cancer cell migration than the preosteoblast cell line.
3.2.3 BMSC-Conditioned Medium Inhibited MDA-MB-231 Migration

So far, individual single cell lines derived from mesenchymal stem cells have been used to determine the effect of bone cell conditioned medium on breast cancer migration. However, other cells such as adipocytes, chondrocytes can be derived from the bone marrow mesenchymal stem cells (figure 1.9). Therefore, a test to determine the overall effect of bone marrow stromal cells on triple negative breast cancer migration must be conducted. Conditioned media from primary bone marrow stromal cells were extracted from 8-weeks and 6-months old mice for the experiment. Age was used as a factor to determine the effect of BMSC-conditioned medium on breast cancer cell migration. This is because at a young age, the bone undergoes a modeling process and the amount of bone that is gained during adolescence is the main contributor to peak bone mass [59]. 8-weeks mice are known to be adolescent mice with higher bone density, in contrast to the older 6-months mice. As one develops in age, the bone density begins to drop. Several studies have shown that human bones reach peak density at age 30 and start to lose mass thereafter [60]. Not only do breast cancer cells metastasize to the bone obtain nutrients, they also find it attractive because of space [24]. However, lower bone density is associated with increased space in the bone marrow. In accordance with the hypothesis that breast cancer migration was influenced by the presence of BMSC conditioned media. Results revealed that conditioned media from both BMSCs inhibited MDA-MB-231 cell migration (figure 3.3). However, an immediate and greater impact on TNBC migration was observed in the presence of conditioned media from 6-months old mice than that of 8-weeks old mice despite the lower bone density (figure 3.4).
3.2.4 Calvaria-Conditioned Medium Inhibited MDA-MB-231 Migration

While this current study shows that BMSC conditioned media inhibit breast cancer migration in both the young and adults, it was further hypothesized that osteoblast conditioned medium inhibits breast cancer migration within in the bone. Previous studies have been dedicated to understanding that effect of breast cancer on osteoblast and these studies have shown that metastasized breast cancer cells secret PTHrP that allows for an increase in RANKL expression by osteoblasts (table 1.1.2). However, little effort has been dedicated to the study of the reverse effect of osteoblast on breast cancer cells. Since cells in the Calvaria, also known as skull bone, are 98% osteoblast, conditioned medium from this site was used to measure the effect of bone cells on breast cancer migration. Results demonstrated that indeed the migration activity of breast cancer MDA-MB-231 is significantly reduced under the influence of Calvaria conditioned medium (figure 3.5).

3.2.5 Medium-High Concentrations of Osteoclast-Conditioned Medium Promoted MDA-MB-231 Migration

As mentioned previously in the first chapter, embedded in the bone marrow are hematopoietic cells that differentiate into osteoclast with the secretion of RANKL by osteoblast. When osteoclast is activated, it resorbs bone while the osteoblast lay down new osteoid in the resorption pit as part of a balanced cycle. However, when breast cancer metastasizes to the bone, it disrupts the bone remodeling cycle. Metastatic breast cancer is often associated with osteolytic lesion in which osteoclast differentiation is increased through an increased level of RANKL secreted by the osteoblast. While the effect of breast cancer on osteoclast is widely known, the effect of osteoclast on breast cancer is currently unknown. This current study has
demonstrated that conditioned media from bone forming cells inhibit breast cancer migration within bone. Thus, the effect of conditioned media from bone resorbing cells on TNBC migration was determined. Primary osteoclast conditioned media was used to test the hypothesis that osteoclast-conditioned media inhibit TNBC migration within bone. Result showed that contrary to the hypothesis, osteoclast actually promoted breast cancer migration but this is dependent on the concentration of osteoclast-conditioned media (figure 3.6). MDA-MB-231 with a pure osteoclast conditioned medium (100% concentration) and mixed medium (50% concentration) promoted breast cancer migration in comparison to the control after 24 hours. However, no difference occurred in MDA-MB-231 sample with 25% concentration in comparison to the control.

3.2.6 Effect of Osteoclast-Conditioned Medium & Myoblast-Conditioned Medium on MDA-MB-231 Migration

As illustrated above, results showed that osteoclast-conditioned medium promoted breast cancer while osteoblast-conditioned media inhibited breast cancer cells. A two-part test was conducted to determine the effect of a mixture of osteoclast-conditioned medium and myoblast conditioned medium on breast cancer migration. C2C12 was chosen because of its similarities to mesenchymal stem cells and its ability to further differentiate into osteoblast. The first part of the test was done by equally dividing the total concentration of conditioned media into two such that osteoclast conditioned medium was half and myoblast was the other half (50% osteoclast CM and 50% myoblast CM in pure 100% concentration). The mixture was combined to test the hypothesis that the combination of these two factors would inhibit breast cancer migration. In accordance with the hypothesis, results indicated that migration activity
of MDA-MB-231 was significantly reduced as the concentration of conditioned medium was increased. However, results also indicated that this effect occurred after 12 hours (figure 3.7).

A second step test was conducted by replacing normal growth medium with osteoclast-conditioned that were exposed to myoblast cells medium during cell culture. Normal growth medium in seeded myoblast cells was replaced with osteoclast-conditioned medium and after two days of culture, conditioned medium was collected and used for migration experiment. Results showed that there was no significant difference in the migration activity of MDA-MB-231 under the influence of this conditioned media (figure 3.8).

**3.2.7 Degraded Myoblast-Conditioned Medium had no Effect on Breast Cancer Migration**

While cells obtain nutrients from growth medium, precautions were taken to determine if results were unique or if TNBC’s reduced activity was as a result of inadequate nutrients from conditioned media. A test to measure the originality of the results was conducted by boiling up the myoblast-conditioned medium for two hours at 70°C. This degraded the proteins secreted by the cells to the medium. After two hours, test was conducted by placing the boiled medium on MDA-MB-231 to measure its effect on migration. Results indicate that there is no difference in migration activity (figure 3.9), which validates all our results. Therefore, migration activities of breast cancer cells are impacted by growth factors secreted by different bone cells used as treatments.
3.2.8 Myoblast-Conditioned Medium Inhibited MDA-MB-435 & MDA-MB-468 Migration

Tests were also conducted on other triple negative breast cancer cells: MDA-MB-435 and MDA-MB-481 to validate results (figure 3.10 & figure 3.11). Myoblast-conditioned medium was used because of similarity to mesenchymal stem cells and its significant impact on MDA-MB-231 migration. MDA-MB-231 and MDA-MB-435 share basal-like properties and are classified as post-epithelial-mesenchymal transition (post-EMT) cells due to vimentin positivity [65] while MDA-MB-468, also a basal-like cell, expresses cytokeratin [66]. Results indicated that myoblast-conditioned medium inhibited both MDA-MB-435 and MDA-MB-468 migratory activities.

3.3 Discussion

A balanced cycle in bone remodeling is essential to ensure a healthy bone. However, the presence of external factors such as metastasized tumor cells can hinder a balanced cycle resulting in a vicious cycle [61]. In most cases, metastatic breast cancer cause osteolytic lesion, an increased osteoclast activation that leads to an increase in bone resorption bone. Severe bone pain, fracture, hypercalcemia, and paralysis arise from lack of balance between osteoclast and osteoblast activities [62]. The progression of breast cancer metastasis is broken down into three categories: migration, proliferation and invasion. Once tumor cells have broken into the blood vessel and are carried to the bone, they migrate and proliferate within the bone. Migration abilities are not only dependent on internal factors secreted by the tumor cells but also conditions within the extracellular space. While the cause of breast cancer metastasis to the bone marrow is still undergoing investigations, it is widely
accepted that breast cancer cells metastasize to the bone marrow because of its high vascularity feature. In addition, tumor cell migration within the bone microenvironment remains elusive. The mechanism of lytic defects from metastatic foci is suggested to be caused by pressure upon bone from expanding tumor mass, as well as influence from substances elaborated by tumor cells [63]. Consequently, with higher migration activities of breast cancer cells within the bone, higher pressure is exerted on bone by these tumor cells. Therefore, the current study presents a novel investigation of the migratory abilities of MDA-MB-231 within the bone microenvironment under the influence of different concentrations of bone cell-conditioned media. Our hypothesis that the migratory abilities of triple negative breast cancer within bone microenvironment would be inhibited by conditioned medium of bone cells had mixed results.

Results of the tested hypothesis reveal both a positive and negative impact of conditioned media on breast cancer migratory behavior. An expected result was found in breast cancer cells with myoblast and preosteoblast conditioned media. As previously explained, conditioned medium from these cells was observed to inhibit the migratory activities of breast cancer cells within the bone. However, the impact on migratory activities of these cancer cells was greater under the influence of myoblast conditioned media than the conditioned media from preosteoblast. Previous studies have shown age-related changes in mesenchymal stem cells, located in the bone marrow, include loss of differentiation potential, loss of proliferation potential, increases in senescent cell numbers and loss of in vivo bone formation [64]. Therefore, since age is negatively correlated with bone density, the effect of conditioned media of bone marrow stromal cells from younger and older generations
on migratory activities of breast cancer was observed. Contrary to expectations, an increased inhibition of migratory activity of TNBC cells exposed to BMSC conditioned media from 6-months mice was observed. However, further analysis revealed that osteoblast populated Calvaria conditioned media extracted from newborn mice greatly inhibited the migratory activity of MDA-MB-231 within the bone. These results suggest that that reduced inhibition of migratory activity of breast cancer cells in younger generations are not caused by growth factors secreted by osteogenic cells but by growth factors secreted by other cells within the bone marrow.

Since most studies have shown that breast cancer cells allow for increased osteoclast, a test was conducted to determine the effect of osteoclast conditioned medium on breast cancer migration. Contrary to what was expected, osteoclast conditioned medium promoted breast cancer migration. This suggests that osteoclast and breast cancer cells work together. However, the effect on migratory activity of breast cancer cells depends on the combination process of myoblast-conditioned media and osteoclast-conditioned media. In the situation where breast cancer cells were exposed to conditioned media of myoblast and osteoclasts, there was a lag in migration (figure 3.7). There was no difference in migratory activity of breast cancer cells under the influence of osteoclast-conditioned media that have previously been exposed to myoblast cells.

While migration was inhibited in the presence of myoblast-conditioned medium, unlike the results from MDA-MB-231, no significant difference was observed in migratory activities of MDA-MB-468 and MDA-MB-435 with 25% concentration of myoblast conditioned media at 48 hours. Results indicated an unexpected decrease in migratory activity of MDA-MB-468 with 25% concentration of
myoblast-conditioned media at 36 hours. Results also demonstrated that the impact of myoblast-conditioned media on migratory activity were less in MDA-MB-435 cells than MDA-MB-231 and MDA-MB-468. Analysis of these results suggests that the degree of impact of bone-conditioned media on TNBC migration may depend on the type of TNBC.

Lastly, there was no effect of degraded myoblast-conditioned medium on migratory activity of MDA-MB-231. This suggests that bone cells, as a result of secreted proteins and growth factors, created the effects observed on TNBC migration.

Currently, literature shows that cytokines in osteoblast-conditioned medium help promote migration of breast cancer cells within bone. However, these studies are limited to MCF-7 [67]. In contrast to triple negative cancer cells, MCF-7, derived from a pleural effusion of malignant breast cancer [68]. They are characterized as hormone-dependent human breast cancer that contain functional estrogen receptors and respond to estrogen, which induces them to form, migrate and proliferate [69]. Overall, our novel study has revealed the migratory behavior of triple negative (basal-like) breast cancer under the influence of different concentrations of growth factors secreted by various cells within the bone marrow. This study can be a potential step in resolving bone metastasis.
Figure 3.1  Myoblast-conditioned medium inhibited MDA-MB-231 migration. Conditioned Media (CM) from C2C12 were collected and at different concentrations, replaced with MDA-MB-231’s normal growth medium. Normal growth medium was used as control. 3 independent experiments were performed (n=3) and averages were plotted with standard deviation as error bars. All concentrations were significantly different relative to control with ** denoting p<0.01.
Preosteoblast conditioned mediums inhibited MDA-MB-231 migration. Conditioned Media (CM) from C2C12 were collected and at different concentrations, replaced with MDA-MB-231’s normal growth medium. Normal growth medium was used as control. 3 independent experiments were performed (n=3) and averages were plotted with standard deviation as error bars. All samples except sample with 25% CM at 48 hours were significantly different from the control with ** denoting \( p<0.01 \).
Figure 3.3  Conditioned medium of bone marrow stromal cell harvested from 8-weeks old female mice inhibited MDA-MB-231 migration. Conditioned Media (CM) from BMSC were collected and at different concentrations, replaced with MDA-MB-231’s normal growth medium. Normal growth medium was used as control. 3 independent experiments were performed (n=3) and averages were plotted with standard deviation as error bars. At all concentrations of CM from 24-48 hours, migration activity of MDA-MB-231 was significantly reduced relative to control, * denotes significantly different (p<0.05), ** denotes significantly different (p<0.01).
Figure 3.4 Conditioned medium of bone marrow stromal cell harvested from 6-months old female mice inhibited MDA-MB-231 migration. Conditioned Media (CM) from BMSC were collected and at different concentrations, replaced with MDA-MB-231’s normal growth medium. Normal growth medium was used as control. A. Shows a graphical representation of results. 3 independent experiments were performed (n=3) and averages were plotted with standard deviation as error bars. At all concentrations of CM, migration activity of MDA-MB-231 was immediately slowed down relative to control, ** denotes significantly different (p<0.01).
Figure 3.5  Conditioned medium of Calvaria harvested from newborn mice inhibited MDA-MB-231 migration. Conditioned Media (CM) from Calvaria were collected and at different concentrations, replaced with MDA-MB-231’s normal growth medium. Normal growth medium was used as control. 3 independent experiments were performed (n=3) and averages were plotted with standard deviation as error bars. All concentrations of CM, immediately affected the migration activity of MDA-MB-231 relative to control, ** denotes significantly different (p<0.01).
Figure 3.6  Medium-High concentrations of Conditioned medium of Osteoclast harvested from mice promoted MDA-MB-231 migration. Conditioned Media (CM) from osteoclast culture were collected and at different concentrations, replaced with MDA-MB-231’s normal growth medium. Normal growth medium was used as control. 3 independent experiments were performed (n=3) and averages were plotted with standard deviation as error bars. Only 100% and 50% concentrations of osteoclast CM promoted the migration of MDA-MB-231 after 24 hours relative to control, * denotes significantly different (p<0.05), ** denotes significantly different (p<0.01).
Figure 3.7  Medium-High concentrations of mixture of Osteoclast conditioned medium and myoblast conditioned medium inhibited MDA-MB-231 migration. Normal growth medium on seeded myoblast cells was replaced with osteoclast-conditioned medium and after two days of culture, conditioned medium was collected and at different concentrations, replaced with MDA-MB-231’s normal growth medium. Normal growth medium was used as control. 3 independent experiments were performed (n=3) and averages were plotted with standard deviation as error bars. Only 100% and 50% concentrations of conditioned medium inhibited MDA-MB-231 migration after 12 hours relative to control, ** denotes significantly different (p<0.01).
Figure 3.8  Myoblast exposed osteoclast-conditioned medium had no effect on MDA-MB-231 migration. Normal growth medium in seeded myoblast cells was replaced with osteoclast-conditioned medium and after two days of culture, conditioned medium was collected and at different concentrations, replaced with MDA-MB-231’s normal growth medium. Normal growth medium was used as control. 3 independent experiments were performed (n=3) and averages were plotted with standard deviation as error bars. At all concentrations there was no effect of conditioned media on migration of MDA-MB-231.
Figure 3.9  Boiled Myoblast-conditioned medium had no effect on MDA-MB-231 migration. Conditioned Medium (CM) from C2C12 was boiled at 70°C for two hours to degrade proteins secreted by cell in medium. Boiled medium was collected and at different concentrations, replaced with MDA-MB-231’s normal growth medium. Normal growth medium was used as control. 3 independent experiments were performed (n=3) and averages were plotted with standard deviation as error bars. There was no significant effect on cancer migration.
Figure 3.10 Myoblast-conditioned medium inhibited MDA-MB-435 migration. Conditioned Media (CM) from C2C12 were collected and at different concentrations, replaced with MDA-MB-231’s normal growth medium. Normal growth medium was used as control. 3 independent experiments were performed (n=3) and averages were plotted with standard deviation as error bars. Myoblast-conditioned medium inhibited MDA-MB-435 immediately after 0 hours. All concentrations except for minimal myoblast-conditioned medium (25%) were significantly different relative to control with ** denoting (p<0.01).
Figure 3.11  Myoblast-conditioned medium inhibited MDA-MB-468 migration. Conditioned Media (CM) from C2C12 were collected and at different concentrations, replaced with MDA-MB-231’s normal growth medium. Normal growth medium was used as control. 3 independent experiments were performed (n=3) and averages were plotted with standard deviation as error bars. All concentrations were significantly inhibited the migration of MDA-MB-468 after 12 hours, except for minimal concentration of treatment (25%) at 24 and 48 hours. Relative to control, significantly different ** denotes (p<0.01).
Chapter 4

EFFECT OF MYOBLAST-CONDITIONED MEDIUM ON TRIPLE NEGATIVE BREAST CANCER PROLIFERATION WITHIN BONE

4.1 Introduction & Rationale

Triple negative breast cancer cells are breast cancer subtypes that are known to be aggressive in proliferation and invasion. TNBC patients have a higher rate of relapse with distant metastases and a poorer prognosis than women with other breast cancer subtypes [70]. Due to the aggressiveness in proliferation of these cancer cells, less than 30% of women with metastatic TNBC survive 5 years, and almost all die of their disease despite adjuvant chemotherapy, which is the mainstay of treatment [71]. Proliferation occurs from the primary site- the breast and an increase in proliferation leads to invasion of the blood vessel. Once at the targeted site, breast cancer cells continue to aggressively proliferate to migrate to fill up space and this leads to the reduction of normal bone cells. Previous studies have shown that TGFβ, VEGF, IGFs, BMPs, calcium and fibroblast-derived factors stimulate the tumor proliferation. However, proliferation of these breast cancer cells also depends on number of promoting or inhibiting conditions, primarily on interaction with surrounding bone. Although anti-HER2 antibodies, Herceptin, estrogen receptor antagonists and aromatase inhibitors have improved treatment of many breast cancer subtypes, the treatment of TNBC remains a great challenge [72] thus, in identifying potential therapeutic targets for triple negative breast cancer, it is important to understand the
interaction of the breast cancer with bone marrow stromal cells. It is hypothesized that myoblast-conditioned medium inhibits triple negative breast cancer proliferation within bone.

4.2 Methods & Results

As a standard assay, MTT cell proliferation assay was used to determine the proliferation or growth rate of triple negative breast cancer cells that are exposed to myoblast-conditioned medium (figure 2.2). The proliferation activities of triple negative breast cancer (MDA-MB-231, MDA-MB-435 and MDA-MB-468) were observed. Six groups of nine samples of 5000 MDA-MB-231 cells were placed in 3 96-wells labeled days 0-3. Two groups were treated with two concentrations of cycloheximide, a negative control, three groups were treated with three different concentrations of myoblast-conditioned medium that was extracted from 2-day cultured C2C12 cell line and the last group was left as the control. All cells were exposed to Tetrazolium dye, MTT (3-(4,5-Dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromides, which reduced to insoluble purple formazan in the mitochondria of living cells. DMSO used as a solubilizing agent to dissolve the formazan, and the concentration is determined by optical density at 540 nm.

4.2.1 Minimal Concentration of Myoblast-Conditioned Medium inhibits MDA-MB-231 Proliferation

The purpose of this experiment was to determine whether the triple negative MDA-MB-231 increase or decrease in growth under the influence of myoblast-conditioned medium. Data generated from experiment by microplate reader demonstrated an expected growth in MDA-MB-231 in the absence of treatment.
However, contrary to prospects, among the group exposed to myoblast-conditioned medium, analysis suggested a significant proliferation inhibition in MDA-MB-231 exposed to the lowest concentration of treatment. A similar result was also observed in MDA-MB-231 exposed to higher concentration of negative control (figure 4.1).

4.2.2 Minimal Concentration of Myoblast-Conditioned Medium Promotes MDA-MB-468 Proliferation

A test was conducted on MDA-MB-468 to determine if the effect is the same across all triple negative breast cancers or specific to MDA-MB-231 cells. In accordance to the experimental setup explained above, measurements were taken at an absorbance level of 540nm. Data collected demonstrated a significant increase in proliferation of MDA-MB-486 cells with the lowest concentration of myoblast-conditioned medium. There was also a significant decrease in proliferation of MDA-MB-486 cells that were exposed to higher negative control concentration in day 2 (figure 4.2).

4.2.3 Myoblast-Conditioned Medium Inhibits MDA-MB-435 Proliferation

A final test to measure proliferation of TNBC was done by examining the effect of myoblast-conditioned medium on the proliferation activity of MDA-MB-435 within bone. In accordance to postulation, analysis of data collected by the microplate reader indicated that myoblast-conditioned medium inhibited the proliferation of MDA-MB-435. This was independent of the concentration of myoblast-conditioned media. In addition, as expected, cycloheximide, the negative control, inhibits the growth of these TNBC cells. However, the effect was not immediate but was observed after day 0.
4.3 Discussion

The necessity to develop new therapeutics to resolve proliferation of triple negative cancer cells within the bone starts with an understanding of their interaction with bone cells in the bone microenvironment. Numerous studies have examined different factors contributing to the proliferation of cancer cells. While Wnt signaling regulates cell proliferation, survival, and differentiation, studies have shown that it also plays key roles in embryonic development and tumorigenesis [73]. Sex-determining region Y-box 4, SOX4, is known to play a role in the Wnt signaling pathway in cancers [74]. However, it is important to note that Wnt signaling as depicted in figure 2.0 is needed for the formation of osteoblast and highly active in the bone marrow. This has led to a number of studies that examine the effect of osteoblast-conditioned medium on the proliferation of breast cancer cells. Numerous studies have revealed that conditioned medium collected from primary human osteoblast cultures has a pronounced stimulatory capacity on some human mammary tumor cells [75]. Literature shows that osteoblast-conditioned medium promotes the proliferation of MDA-MB-231. According to this study, osteoblasts secrete several cytokines and growth factors, some of which are responsible for the stimulation of triple negative breast cancer cells growth. Factors that are currently identified include IL-1, IL-6, VEGF, TGF- β and PDGFs [76]. Furthermore, a study examined whether osteoblast-conditioned medium sensitizes breast cancer cells to the effect of anti-proliferative effects of imatinib, a cancer medication. Data from this study shows that while osteoblast-conditioned medium promotes proliferation of cancer cells, it also influences the response of both triple negative and hormone-dependent breast cancer cells to imatinib; a mechanism mainly caused by expression of PDGF [77].
While these studies on the proliferation of triple negative cancer cells exist, the majority has specifically focused on the effect of factors expressed by osteoblast and Wnt signaling. However, our results from previous experiments conducted within the scope of this project have suggested that myoblast-conditioned medium inhibited triple-negative breast cancer cell migration (figure 3.1). Further experiments were done to examine the effect of this medium on triple negative breast cancer proliferation within bone. Our data suggest that among the three groups of TNBC: MDA-MB-231, MDA-MB-468, MDA-MB-435, myoblast-conditioned medium significantly inhibited MDA-MB-435 proliferation. An inhibition effect was observed in MDA-MB-231 but only by a minimum concentration of myoblast-conditioned. Conversely, a minimum concentration of myoblast-conditioned medium aided the proliferation of MDA-MB-468 cells. The effect of myoblast-conditioned medium on both MDA-MB-468 and MDA-MB-231 proliferation was observed only on day 2. Overall, this study showed that the effect of myoblast-conditioned medium depends on the type of TNBC.
Figure 4.1  Minimal myoblast-conditioned medium inhibited MDA-MB-231 proliferation. Conditioned Media (CM) from C2C12 were collected and at different concentrations, replaced with MDA-MB-231’s normal growth medium. Normal growth medium was used as control and cycloheximide as negative control. 3 independent experiments were performed (n=9) and averages were plotted with SEM as error bars. High concentration of cycloheximide inhibited proliferation on all days and low concentration of myoblast-conditioned medium inhibit proliferation on day 2 relative to control with ** denoting p<0.01.
Figure 4.2  Minimal myoblast-conditioned medium promoted MDA-MB-468 proliferation. Conditioned Media (CM) from C2C12 were collected and at different concentrations, replaced with MDA-MB-468’s normal growth medium. Normal growth medium was used as control and cycloheximide as negative control. 3 independent experiments were performed (n=9) and averages were plotted with SEM as error bars. High concentration of cycloheximide inhibited proliferation on day 2 and low concentration of myoblast-conditioned medium promoted proliferation on day 2 relative to control with ** denoting significant difference  p<0.01.
Figure 4.3  Myoblast-conditioned medium inhibited MDA-MB-435 proliferation. Conditioned Media (CM) from C2C12 were collected and at different concentrations, replaced with MDA-MB-435’s normal growth medium. Normal growth medium was used as control and cycloheximide as negative control. 3 independent experiments were performed (n=9) and averages were plotted with SEM as error bars. Cycloheximide and myoblast-conditioned inhibited proliferation of MDA-MB-435 after day 0 except for high level concentration of myoblast-conditioned medium, which inhibited proliferation only on day 2. Relative to control, * denoted significant difference (p<0.05) and ** denotes significant difference  p<0.01.
Chapter 5

EFFECT OF BONE CELL CONDITIONED MEDIA ON INVASIVE POTENTIAL OF TRIPLE NEGATIVE BREAST CANCER TO BONE MIRCOENVIRONMENT

5.1 Introduction & Rationale

Worldwide, over 1.3 million cases of invasive breast cancer are diagnosed, and more than 450,000 women die from breast cancer annually [78]. In the United State, approximately 200,000 cases of invasive breast cancer and 50,000 cases of in situ breast cancer are diagnosed annually. Over 40,000 women die from breast cancer each year [79]. Despite this high number, there has been a decrease in mortality from the disease mainly because of improved adjuvant therapy. However, while these therapeutic interventions have been prescribed for a number of breast cancer cases, it is not the case for TNBC. TNBC accounts for 10-15% of all breast cancers. As stated in previous chapters, patients with TNBC have a poor outcome compared to the other subtypes of breast cancer. Incidence of TNBC in African American women is two to three times higher than other ethnic groups, although the reason for this has not been elucidated [80]. TNBC is associated with aggressiveness and high invasiveness, thus, there is a clear need for a greater understanding of the subtype of cancer at all levels and for the development of better therapeutics. While in our current study, myoblast-
conditioned medium has proven to inhibit TNBC proliferation and migration, it is postulated that the conditioned medium also inhibits the invasive potential of TNBC to bone.

5.2 Methods & Result

A 24-well Matrigel plate was used to assess the behavior of breast cancer invasion to bone under the influence of myoblast-conditioned media. The BD BioCoat Matrigel Invasion Chamber is widely used to study cell invasion of malignant cells. The invasion chambers consist of cell-cultured 8-micron pore inserts that are coated with BD Matrigel™ matrix. The matrix provides cells with the conditions that allow assessment of their invasive capacity in vitro. It serves as a reconstituted basement membrane in vitro, occluding the pores of the membrane and blocking non-invasive cells from migrating through the membrane. 25,000 TNBC cells were placed in the inserts above the chambers that had the myoblast-conditioned medium. After a day of incubation, images were taken to determine how many TNBC cells penetrated through basement membrane to the chamber containing myoblast-conditioned media.

5.2.1 Myoblast-Conditioned Medium Inhibit Breast Cancer Invasion to Bone

Invasive cells secrete proteases that enzymatically degrade the BD Matrigel matrix and enable invasion through the membrane pores. Using ImageJ to count the number of cells that had invaded, analysis showed that myoblast-conditioned media significantly inhibited all three TNBC cells: MDA-MB-231 (figure 5.1), MDA-MB-468 (figure 5.2), and MDA-MB-435 (figure 5.3).
5.3 Discussion

Studies have shown that breast cancer invasion of distant sites is not usually a random process. However, factors responsible for the invasion of breast cancer cells to the bone are still being determined. As a target, bone is unique in that it is continuously being remodeled under the influence of local and systemic growth factors that are mostly embedded in the bone matrix. Several studies have demonstrated that conditioned medium harvested from resorbing rat Calvaria contain growth-stimulating activity for tumor cells. A specific study was recently conducted to demonstrate whether osteoblastic cells produce agents able to recruit MDA-MB-231. In this chemoinvasion assay experiment conducted, results reveal a fluctuating invasion activity of these breast cancer tumors in response to osteoblast-conditioned medium; with an initial decrease then an increase in activity [81]. While available data points to chemokines and cytokines produced by breast cancer cells as key players in invasive abilities [82], cytokines also produced by osteoblasts have been suggested to aid the invasion of breast cancer to the bone microenvironment [83]. To validate this hypothesis, a study was conducted to determine some of the molecules involved in the cross-talk of osteoblasts and cancer cells in the tumor microenvironment. It was found that breast tumor-associated osteoblasts express large amounts of CXCL5 proteins that aid the migration, invasion and epithelial–mesenchymal transition of MDA-MB-231. However, these CXCL5 proteins had no influence on the TNBC cancer cell proliferation [84]. While numerous data exists for the effect of osteoblast cells on the invasive behavior of TNBC cells to bone, little effort has been dedicated to
understanding the effect of myoblast on breast cancer invasion of bone. Results from this current study suggest that myoblast-conditioned medium significantly inhibited TNBC cells invasion of bone. While this effect was observed across the three TNBC tested, results demonstrated that myoblast-conditioned medium had a greater effect on the invasion of MDA-MB-435 than it did on the other two TNBC (MDA-MB-231 and MDA-MB-468). The least reduction in invasiveness under the influence of myoblast-conditioned medium was observed in MDA-MB-231.
Myoblast-conditioned medium inhibited MDA-MB-231 invasion. Conditioned Media (CM) from C2C12 were collected and at different concentrations, replaced with MDA-MB-231’s normal growth medium. Normal growth medium was used as control. A. Graphical representation of results. 3 independent experiments were performed (n=3) and averages were plotted with SEM as error bars. B. Number of invaded MDA-MB-231 cells. Myoblast-conditioned medium inhibited MDA-MB-231 invasion of bone. All concentrations were significantly different relative to control with ** denoting p<0.01.
Figure 5.2  Myoblast-conditioned medium inhibited MDA-MB-468 invasion. Conditioned Media (CM) from C2C12 were collected and at different concentrations, replaced with MDA-MB-468’s normal growth medium. Normal growth medium was used as control. A. Graphical representation of results. 3 independent experiments were performed (n=3) and averages were plotted with SEM as error bars. B. Number of invaded MDA-MB-468 cells. Myoblast-conditioned medium inhibited MDA-MB-468 invasion of bone. All concentrations were significantly different relative to control with ** denoting \( p<0.01 \).
Figure 5.3  Myoblast-conditioned medium inhibited MDA-MB-435 invasion. Conditioned Media (CM) from C2C12 were collected and at different concentrations, replaced with MDA-MB-435’s normal growth medium. Normal growth medium was used as control. A. Graphical representation of results. 3 independent experiments were performed (n=3) and averages were plotted with SEM as error bars. B. Number of invaded MDA-MB-435 cells. Myoblast-conditioned medium inhibited MDA-MB-435 invasion of bone. All concentrations were significantly different relative to control with ** denoting p<0.01.
Chapter 6

SUMMARY AND PROSPECTUS

Breast cancer is the second leading cause of death among women, following lung cancer. Statistics have shown that 1 in 36 women in the United States die from the pandemic [1]. Though this type of cancer can occur in women and men, it is more prevalent in women. Breast cancer incidence among males account for only 1% but as high as 99% in females. An increasing effort in the study of breast cancer is gradually unveiling the cause of the illness. Errors or mutations associated with oncogene and tumor suppressor genes have been traced to the development of abnormal cell growth that can eventually lead to breast cancer. Invested efforts in determining effective therapeutics have suppressed death occurrence in breast cancer patients. However despite the amount of dedicated efforts, therapeutic interventions for triple negative breast cancer subtype is lacking. This is because most therapeutic interventions target hormones that are not present in this subtype of breast cancer.

There are two types of breast cancer: invasive and non-invasive. In the United State, approximately 200,000 cases of invasive breast cancer and 50,000 cases of in situ breast cancer are diagnosed annually. Over 40,000 women die from breast cancer each year [79]. Death of breast cancer patients occurs as a result of breast cancer that has migrated away from primary site, the breast, and invaded other sites. Breast cancer
cells migrate through a mechanism called mesenchymal movement. A process in which the cancer cells possess an elongated fibroblast-like shape and move rapidly through channels cut into surrounding matrix by matrix-degrading enzymes such as MMPs [16]. Once these cancer cells breach the epithelial cell basement membrane, they invade the blood vessel and move along the blood steam. They then slowly reduce movement in capillaries at a distant location, invade the walls of the capillaries and migrate into targeted sites. At targeted sites, breast cancer cells assume their original shape by undergoing a mesenchymal-epithelial transition.

Due to its degree of vascularity and variety of growth factors, bone is the most targeted site of breast cancer cells that migrate away from the breast. The most common locations within the bone are the spine, pelvis, femur, humerus, ribs and skull. About 70% of people who die from breast cancer will have radiological evidence of skeletal metastasis before their death and in 40% of cases, the bone is the first metastatic site [23]. Most death of breast cancer patients with metastatic tumor cells occurs because of the disruption of the balance of osteoclast and osteoblast activities during bone remodeling. Most tumor cells have been identified to work with osteoblasts to increase the production of osteoclasts. With an increase in osteoclast activity at a normal osteoblast activity level, bone is resorbed more than new osteoid is laid down [49]. While the consequence of breast cancer in bone is widely studied, minimum efforts have been devoted to understanding the reverse effect of bone cells on triple negative tumor cells. Understanding the interaction of triple negative breast cancer with bone cells within the bone microenvironment can result in the
development of effective therapeutics to reduce metastasis and death occurrence in breast cancer patients. The effect of bone cells on breast cancer migration, proliferation and invasion is not fully known, but it is hypothesized that bone cell-conditioned media will inhibit triple negative breast cancer migration within the bone.

Current study revealed that triple negative breast cancer cell inhibition and promotion depends on the type of bone cell-conditioned medium exposed to the tumor cells. Conditioned medium from C2C12 myoblasts (a cell line that behave like mesenchymal stem cells) was found to inhibit MDA-MB-231, MDA-MB-468 and MDA-MB-435. However, a greater inhibition effect occurred in MDA-MB-231, triple negative breast cancer cells that were extracted from older Caucasian patients. To validate this result, more bone-conditioned media was tested on TNBC cells. Conditioned medium from MC3T3 (preosteoblast cells that arise from mesenchymal stem cells) also inhibited MDA-MB-231. However, conditioned medium from osteoblasts (cells that are differentiated from preosteoblast) showed a greater degree of inhibition of TNBC migration. Literature suggests that osteoblast-conditioned medium promote breast cancer cell migration. However, these studies are limited to the use of MCF-7. MCF-7 cells differ from TNBC in that they are breast cancer cells derived from pleural effusion of malignant breast cancer [68]. They are characterized as hormone-dependent human breast cancer that contain functional estrogen receptors and respond to estrogen, which induces them to form, migrate and proliferate [69].

While metastatic breast cancer cells are primary found in the bone marrow, which contains several stems cells, understanding the interaction between all bone
marrow stromal cells and breast cancer cells is important. This current study revealed that BMSC-conditioned medium inhibited MDA-MB-231 migration. Additionally, age is a factor in the degree to which BMSC-conditioned medium inhibits the tumor cells. Contrary to expectations, BMSC-conditioned medium extracted from older mice was observed to have inhibited MDA-MB-231 migration more than BMSC-conditioned medium extracted from younger mice (figure 3.3 &3.4). BMSC were then differentiated into osteoclast to determine the effect of osteoclast on MDA-MB-231 migration. Results revealed that only medium-high concentrations of osteoclast-conditioned medium slightly promoted breast cancer cells after 24 hours (figure 3.6). When MDA-MB-231 cells were treated with a mixture of myoblast and osteoclast conditioned media, migration was inhibited after 12 hours. However, when osteoclast-conditioned medium was exposed to myoblast cells prior to being used as a treatment for MDA-MB-231 cells, there was no effect on migration. This result suggests that the osteoclasts secreted proteins that suppressed the tumor-migration inhibitory proteins secreted by myoblasts. Overall, results gathered from analysis suggest that TNBC cells most likely migrate within the bone during the first phase of bone remodeling when osteoclasts are formed and active. Results also suggest that these tumor cells reduce migration during the second phase of bone remodeling when osteoblasts are active.

Due to nutrient deprivation of high-populated breast cancer cells within a space, breast cancer cells tend to migrate to look for space and nutrients. Triple negative breast cancer cells are known to be aggressive in proliferation and invasion.
Observations within this study have revealed that myoblast-conditioned medium have a high impact on triple negative breast cancer migration within bone. Thus, in understanding the effect of this conditioned medium on TNBC proliferation and invasion, it postulated that myoblast-conditioned medium inhibits the TNBC proliferation and invasion activities.

Results indicated that the effect of myoblast-conditioned medium depends on the type of TNBC cell under observation. Myoblast-conditioned medium greatly inhibited triple negative breast cancer proliferation and invasion of MDA-MB-435 TNBC cells. Results also suggested that invasion of triple negative breast cancer cells to bone was inhibited under the influence of myoblast-conditioned medium in MDA-MB-231 and MDA-MB-468. It was further noticed that minimal myoblast-conditioned medium inhibited MDA-MB-231 proliferation while it promoted MDA-MB-468 proliferation.

In summary, C2C12 myoblasts, found in the bone marrow, behave like mesenchymal stem cells in that they have the ability to differentiate into bone forming cells (figure 1.9). These cells are widely used across bone studies. This current study has demonstrated preliminary knowledge of the effect of conditioned-medium from this myogenic cell on the migration, proliferation, and invasive activities of triple negative breast cancer cells. Further experiments to determine the proteins secreted/expressed by myoblasts that are responsible for these effects must be conducted. This will expand the knowledge of the effect of bone-conditioned media on triple negative breast cancer cells, which can eventually lead to the discovery of
effective therapies. Physical activity and exercise are among the key preventative strategies to prevent bone loss and also reduce the risk of fractures in population [86]. Different training trails have been carried out among breast cancer patients and some positive effects have been observed in areal bone mineral density [87]. According to the research conducted by Cornell biomedical researchers, weight-bearing exercise might have anti-cancer effects. In their study, it was found that mechanical loading inhibits expression of Runx2, a transcription factor that stimulates osteolysis and breast cancer growth [88]. However, further experimentation must be conducted to confirm this finding. A future direction of our current study is to determine whether exercise affects the effect of bone-cell conditioned media on breast cancer migration, proliferation and invasion. These findings can lead not only to effective therapeutic measures but also exercise routine treatments for breast cancer patients.
REFERENCES


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### APPENDIX

A. EFFECT OF MYOBLAST-CONDITIONED MEDIUM ON MIGRATORY ACTIVITY OF MDA-MB-231

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B. EFFECT OF PREOSTEOBLAST-CONDITIONED MEDIUM ON MIGRATORY ACTIVITY OF MDA-MB-231

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C. EFFECT OF BMSC-CONDITIONED MEDIUM HARVESTED FROM 8-WEEK OLD MICE ON MIGRATORY ACTIVITY OF MDA-MB-231

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D. EFFECT OF BMSC-CONDITIONED MEDIUM HARVESTED FROM 6-MONTH OLD MICE ON MIGRATORY ACTIVITY OF MDA-MB-231

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### E. EFFECT OF CALVARIA-CONDITIONED MEDIUM HARVESTED FROM NEWBORN MICE ON MIGRATORY ACTIVITY OF MDA-MB-231

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F. EFFECT OF OSTEOCLAST-CONDITIONED MEDIUM HARVESTED FROM MICE ON MIGRATORY ACTIVITY OF MDA-MB-231

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# G. EFFECT OF OSTEOCLAST & MYOBLAST CONDITIONED MEDIUM ON MIGRATORY ACTIVITY OF MDA-MB-231

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<td><img src="image5.png" alt="Image" /></td>
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<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
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H. EFFECT OF MYOBLAST EXPOSED OSTEOCLAST-CONDITIONED MEDIUM ON MIGRATORY ACTIVITY OF MDA-MB-231
## I. EFFECT OF PROTEIN FREE MYOBLAST-CONDITIONED MEDIUM ON MIGRATORY ACTIVITY OF MDA-MB-231

<table>
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<td><img src="image17" alt="Image" /></td>
<td><img src="image18" alt="Image" /></td>
<td><img src="image19" alt="Image" /></td>
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J. EFFECT OF MYOBLAST-CONDITIONED MEDIUM ON MIGRATORY ACTIVITY OF MDA-MB-468
K. EFFECT OF MYOBLAST-CONDITIONED MEDIUM ON MIGRATORY ACTIVITY OF MDA-MB-435

<table>
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