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The Role of Bone-Derived Osteopontin in the Migration and Stem-Like Behavior of Breast Cancer Cells

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Graduate Program in Anatomy and Cell Biology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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THE ROLE OF BONE-DERIVED OSTEOPONTIN IN THE MIGRATION AND STEM-LIKE BEHAVIOR OF BREAST CANCER CELLS

(Thesis format: Monograph)

by

Graciella Pio

Graduate Program in Anatomy and Cell Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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ABSTRACT

The majority of breast cancer deaths occur due to metastasis, with the most common site of distant breast cancer metastasis being bone. Previous work in our laboratory has established that stem-like breast cancer cells with high aldehyde dehydrogenase (ALDH) activity and expression of a CD44⁺CD24⁻ phenotype have enhanced metastatic capacity. This study tested the hypothesis that bone-derived osteopontin (OPN) promotes the migration and stem-like behavior of breast cancer cells. We demonstrate that bone-derived OPN promotes migration, tumorsphere-forming ability and colony-forming ability of whole population and ALDH⁺CD44⁺CD24⁻ breast cancer cells in bone marrow-conditioned media (an **ex vivo** representation of the bone microenvironment). We observed that cell surface receptors CD44 and RGD-dependent integrins facilitate these behaviors via OPN, potentially through activation of WNK-1, PRAS40 and HSP60 signaling in breast cancer cells. Our analysis of the interactions between bone-derived OPN and breast cancer cells provides valuable insight regarding breast cancer cell metastasis to bone.

**Keywords:** Breast cancer, Metastasis, Bone microenvironment, Osteopontin, Cancer stem cell, CD44, Integrins
CO-AUTHORSHIP STATEMENT

All sphere-limiting dilution assays (SLDA) and colony-forming assays were performed by Matt Piaseczny (MSc Candidate, Allan Lab, Anatomy and Cell Biology, Western University).

Fluorescence-activated cell sorting (FACS) analysis was performed by Kristin Chadwick (London Regional Flow Cytometry Facility, Western University).
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<td>7-Aminoactinomycin</td>
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<td>AF</td>
<td>Alexafluor</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde Dehydrogenase</td>
</tr>
<tr>
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<td>Acute Myeloid Leukemia</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>APC</td>
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<td>Adenosine Triphosphate</td>
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<td>BODIPY™-aminoacetate</td>
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<td>Basic Fibroblast Growth Factor</td>
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<td>Basal Media</td>
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<td>BMCM</td>
<td>Bone Marrow-Conditioned Media</td>
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<td>BMP</td>
<td>Bone-Morphogenetic Protein</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<tr>
<td>CAF</td>
<td>Cancer-Associated Fibroblast</td>
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<tr>
<td>CCAC</td>
<td>Canadian Council of Animal Care</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>Cyclooxygenase-2</td>
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<td>CTGF</td>
<td>Connective Tissue Growth Factor</td>
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<td>DCIS</td>
<td>Ductal Carcinoma <em>In Situ</em></td>
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<td>DEAB</td>
<td>Diethylaminobenzaldehyde</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
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<td>Epidermal Growth Factor</td>
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<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<td>ELISA</td>
<td>Enzyme-linked Immunosorbant Assay</td>
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<td>Epithelial-to-Mesenchymal Transition</td>
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<td>Estrogen Receptor</td>
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<td>Extracellular-Signal Related Kinases</td>
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<td>GTP</td>
<td>Guanosine-5'-Triphosphate</td>
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<tr>
<td>HA</td>
<td>Hyaluronan</td>
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<tr>
<td>HCC</td>
<td>Hepatocellular Carcinoma</td>
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<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid</td>
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<td>Horseradish Peroxidase</td>
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<td>HSP60</td>
<td>Heat Shock Protein 60</td>
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<td>Inhibitor of DNA Binding-1</td>
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<td>Interleukin</td>
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<tr>
<td>kDa</td>
<td>Kilodalton</td>
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<tr>
<td>LCIS</td>
<td>Lobular Carcinoma In Situ</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic Acid</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloprotease</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>NC</td>
<td>Negative Control</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerytherin</td>
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<tr>
<td>Pen/Strep</td>
<td>Pencillin/Streptomycin</td>
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<td>PI3K</td>
<td>Phosphoinositide 3-Kinase</td>
</tr>
<tr>
<td>Ppi</td>
<td>Inorganic Pyrophosphate</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>PRAS40</td>
<td>Proline-Rich Substrate of Akt</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid Hormone-Related Peptide</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor/Activator for NF-κB</td>
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<td>RANKL</td>
<td>Receptor/Activator for NF-κB Ligand</td>
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<td>Stromal Cell-Derived Factor-1</td>
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<td>Side Population</td>
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<td>Skeletal Related Events</td>
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</tr>
<tr>
<td>TAM</td>
<td>Tumor Associated Macrophage</td>
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<td>Transforming Growth Factor β</td>
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<tr>
<td>TNF-α</td>
<td>Tumor-Necrosis Factor-α</td>
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<td>Urokinase-Type Plasminogen Activator</td>
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<td>Vascular Endothelial Growth Factor</td>
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1 INTRODUCTION

Cancer is responsible for 1 in 7 deaths worldwide; it causes more deaths than AIDS, malaria and tuberculosis combined [1]. According to the International Agency for Research on Cancer, there were approximately 14 million new cancer cases in 2012 - by 2030, this number is expected to surge to 21.7 million new cases [1]. This projected increase in cancer prevalence is due to the expanding and aging global population and the adoption of lifestyles known to increase cancer risk, including poor diet, physical inactivity and exposure to carcinogens [1].

Breast cancer is an example of a type of malignancy that is and will remain a major global health challenge [1, 2]. It is currently the most frequently diagnosed cancer amongst women and is the second leading cause of cancer-related deaths in North America. It is estimated that breast cancer cases will also increase in the coming years due to less frequent and delayed pregnancies in women, as well as reduced breast-feeding [1, 2]. Even though we have seen a drastic improvement in the treatment of breast cancer patients over the last 25 years, there remains a deplorable gap in our ability to manage metastatic breast cancer patients. In order to significantly reduce the impact of breast cancer on women worldwide, it is imperative that we focus on researching the biology, prevention and treatment of metastatic disease.

This thesis focuses on investigating the potential mechanisms of breast cancer metastasis to bone, since bone is the most common site of distant metastases in breast cancer patients [3, 4]. Specifically, it aims at elucidating the role of the soluble bone-
derived factor osteopontin (OPN) and its interactions with breast cancer cells that may contribute to the establishment of bone tumor burden in breast cancer patients.
2 LITERATURE REVIEW

2.1 Cancer

Cancer is a group of diseases characterized by dynamic changes in the genome that cause normal cells to evolve progressively to highly malignant derivatives. The multistep nature of tumorigenesis includes the acquisition of sustained proliferative signaling, replicative immortality, the evasion of growth suppressors and cell death, induced angiogenesis and the ability to invade and metastasize to other tissues [5]. These neoplastic characteristics are further amplified by tumor-promoting inflammation and the deregulation of cellular energetics, as well as the tumor cell’s ability to evade the immune response and recruit ostensibly healthy cells to contribute to the progression of the disease [6].

Cancer is currently a major health challenge worldwide and in Canada [7]. Approximately 2 in 5 Canadians are expected to develop cancer in their lifetime and a quarter of Canadians will die from their disease [7]. Cancer is responsible for 30% of all deaths in Canada, making it the leading cause of death and premature death (measured in potential years of life lost) in the country [7]. In addition to the social ramifications this disease entails, cancer has major economic consequences for our country [7]. In 2000, it was the fourth most costly disease in Canada, amounting to $17.4 billion in physician and hospital expenditure and loss of productivity due to premature death [7]. The social and economic toll that cancer has on Canadians is expected to increase as the aging population continues to grow.
2.2 Breast Cancer

Breast cancer is the most frequently diagnosed cancer among Canadian women, accounting for 26.1% of all newly diagnosed cancer cases in 2015 [2]. Discovering and implementing effective treatments for this disease is dependent on elucidating the underlying mechanisms in its formation and progression. Research advances in the past half century have shown that breast cancer is a genetically and clinically heterogeneous disease, arising from the malignant transformation of epithelial cells lining the milk ducts or the milk-producing lobules in the breast [8]. It can be broadly classified into in situ carcinoma and invasive (infiltrating) carcinoma; further sub-classifications are determined based on the cell type of origin, histological representation and molecular phenotypes [9]. Breast cancers classified as in situ (still confined within the duct or lobule of origin) have an excellent prognosis if treated appropriately; when caught at this stage, patients with ductal carcinoma in situ (DCIS) have a 99.2% overall 5-year survival rate [10].

Breast cancer patients diagnosed with invasive carcinoma do not fare as well, since their disease has invaded the surrounding breast tissue and has possibly spread to lymph nodes and other organs. Molecular classification of breast cancers has proven to be particularly useful in stratifying patients based on relative risk of recurrence and progression; these classifications are based on estrogen and progesterone receptor status, human epidermal growth receptor 2 (HER2/neu) status, and proliferative index as measured by Ki67 [9, 11]. One major benefit of these molecular classifications is their use in the determination of which patients are likely to respond to targeted therapies (e.g. tamoxifen or aromatase inhibitors for ER+/PR+ patients and trastuzumab, pertuzumab or
lapatinib for HER2/neu patients) [11]. Despite these recent advances in targeted therapy, breast cancer still ranks second in mortality amongst all other cancers, since patients with metastatic breast cancer have a mere 22% chance of surviving longer than ten years. This is primarily due to the failure of conventional therapy to mitigate and eliminate metastatic disease [2, 7].

2.3 Metastasis

Understanding the underlying molecular mechanisms of the metastatic process is vital to the development of novel therapeutics that can effectively treat metastatic cancer. Past research has helped elucidate the multistep nature of cancer metastasis. Tumor cells first escape the primary tumor and intravasate into the circulatory or lymphatic system. The blood and lymphatic systems are used as transportation routes to the secondary site, where cancer cells can exit the vasculature into the tissue site, at which point they can grow into micrometastases and subsequently to clinically detectable macrometastases [12]. The metastatic process is surprisingly inefficient; Luzzi et al (1998) determined that only 0.02% of a melanoma cell population formed macrometastases in the mouse liver after being injected intraportally. It is the ability to initiate growth after extravasation and to continue to grow into clinically detectable macrometastases that proves most difficult for cancer cells during the metastatic cascade [13].

2.4 Cancer Stem Cells

The cancer cells that do successfully complete the metastatic process are not random. It has been shown that breast cancer tumors exhibit distinct molecular subtypes (luminal A, luminal B, basal-like, HER2-overexpressing, normal breast-like, and claudin-
low) and consist of a hierarchy of phenotypically and functionally different cells [9, 14]. A subpopulation of aggressive cells is believed to exist at the apex of this hierarchy, and these cells have the propensity to initiate the primary tumor and successfully metastasize to adjacent tissues and distant organs [14-17]. These cells have been termed “cancer stem cells” (CSCs) because of their unique ability to self-renew and differentiate into a heterogeneous tumor, contrasting with their non-CSC counterparts that are incapable of tumor propagation [14, 15, 18-20]. Although the cell-of-origin of the CSC has not yet been definitively identified, CSCs are hypothesized to arise via one of three different mechanisms: a normal stem cell may acquire tumorigenic properties through aberrant mutations; a more differentiated progenitor may dedifferentiate into a stem-like state; or mature differentiated cancer cells acquire mutations that result in de-differentiation and co-opting of primitive stem cell signaling pathways, thus forming a CSC. Recent work by multiple groups support the progenitor hypothesis, as it has been shown that subpopulations within breast cancer cells can interconvert between stem-like, basal or luminal phenotypes and CSCs and non-CSCs can be generated after oncogenic reprogramming of differentiated fibroblasts [21, 22].

Stem-like breast cancer cells can be isolated by selecting for specific cell surface markers. Using cells isolated from pleural effusions or primary tumors of breast cancer patients, Al-Hajj et al (2003) identified and isolated tumor-initiating cells by fluorescence-activated cell sorting based on a CD44+CD24−/low phenotype [23]. As few as 100 cells of this phenotype were able to form tumors in immune-compromised mice while tens of thousands of cells with alternate phenotypes failed to form tumors [23]. They also showed that CD44+CD24−/low cells could be serially passaged; these cells where
able to recapitulate the heterogeneous nature of the original tumor, containing CD44⁺CD24⁻/low cells in addition to a myriad of other cellular phenotypes present within the primary tumor [23]. Sheridan et al (2006) later showed that breast cancer cell lines containing >30% of CD44⁺CD24⁻ cells (MDA-MB-231, MDA-MB-436, Hs578T, SUM1315, and HBL-100) express higher levels of pro-invasive genes, including IL-1α, IL-6, IL-8, and urokinase plasminogen activator (UPA) and have highly invasive properties [24].

The value of CD44 as a CSC marker is of little surprise considering that it is capable of promoting multiple tumorigenic properties. CD44 participates in a variety of signaling networks, including the activation of Rho GTPases (integral in cytoskeletal remodeling and invasion) and the PI3K/Akt and MAPK-Ras pathways (promotion of growth, survival and invasion) [25]. CD44 facilitates cell migration by serving as a docking site for matrix metalloproteases (MMPs) and matrix-modifying enzymes that degrade components of the extracellular matrix, such as collagen [25].

Ginestier et al (2007) added an additional component to the breast CSC phenotype by demonstrating that cells with high aldehyde dehydrogenase activity (ALDH⁷hi) isolated from healthy human breast tissue have phenotypic and functional characteristics of mammary stem cells and that ALDH⁷hi human breast carcinoma cells contain a CSC subpopulation [26]. This study also showed that ALDH⁷hi cells are capable of generating tumors when as little as 500 cells are implanted into the mammary fat pad of NOD/SCID mice. Investigation of the relative hierarchy of the ALDH⁷hi and CD44⁺CD24⁻ phenotypes demonstrated that the combined phenotype of ALDH⁷hiCD44⁺CD24⁻ resulted in the greatest tumor-initiating capacity, while cells displaying an ALDH⁷loCD44⁺CD24⁻
phenotype were not tumorigenic even when implanting 50,000 cells/fat pad [26], suggesting that ALDH activity is essential for identifying the tumor-initiating subpopulation in breast cancer cells (Figure 1).

Prior to the elucidation of ALDH’s function as a biomarker for cancer stem cells, ALDH was known to play a self-protective role in normal stem cells. The ALDH enzyme superfamily catalyzes the oxidation of endogenous and exogenous aldehydes into their corresponding carboxylic acids and thus protects organisms from potentially harmful aldehydes [27]. ALDH also offers cellular protection against cytotoxic drugs in both normal mammary stem cells and CSCs; Tanei et al (2009) showed that ALDH$^+$ tumor cells and ALDH expression significantly increased in breast cancer patients following neoadjuvant chemotherapy consisting of paclitaxel and epirubicin and that ALDH$^{high}$ breast tumors are correlated with resistance to neoadjuvant chemotherapy [28]. ALDH also plays a role in the expansion and differentiation of normal and cancer stem cells; when ALDH activity is inhibited, hematopoietic and cancer stem cells shift into a more differentiated state [29-31]. This shift in CSCs increases their sensitivity to chemotherapy and renders them less aggressive [31-33].

The Allan lab has recently pioneered functional characterization of ALDH$^{hi}$CD44$^+$CD24$^-$ breast cancer cells in relation to metastatic behavior. Croker et al (2012) demonstrated for the first time that ALDH$^{hi}$CD44$^+$CD24$^-$ stem-like breast cancer cells have enhanced capacity for cell growth, colony formation, migration, invasion through Matrigel and adhesion to select extracellular matrix components in vitro. Croker et al (2012) also used in vivo studies employing standard experimental metastasis assays,
The primary breast tumor is made up of cancer cells with multiple different cell phenotypes. Stem-like breast cancer cells can be isolated by their high aldehyde dehydrogenase activity and CD44+/CD24− phenotype (ALDH hiCD44+CD24−); these cells have the ability to self-renew and differentiate into a secondary tumor [16, 34].
which involved injection into the tail vein, or spontaneous metastasis assays involving orthotopic injection into the mammary fat pad and showed that the ALDH\textsuperscript{hi}CD44\textsuperscript{+}CD24\textsuperscript{-} phenotype selects for breast cancer cells with enhanced tumorigenic and metastatic capacity relative to non stem-like ALDH\textsuperscript{low}CD44\textsuperscript{-}CD24\textsuperscript{+} breast cancer cells [34].

2.5 Metastatic Organ Tropism

In addition to the findings that only a subset of aggressive cancer cells can successfully metastasize, clinical observations demonstrate that metastatic cells have a specific affinity for certain tissues and organs, a quality referred to as organ tropism [35, 36]. Breast cancer, for example, commonly metastasizes to the lymph nodes, lung, liver, bone and brain [37]. It was first proposed in 1889 by Stephen Paget that certain tissues are predisposed to developing secondary metastases. Paget’s ‘seed and soil’ hypothesis states that metastatic dissemination depends on crosstalk between a subset of cancer cells (the ‘seeds’) and specific organ microenvironments (the ‘soil’) [37]. A cancer cell’s altered genetic or molecular signature and unique cell surface receptors result in a predilection for certain microenvironments, and in turn a favorable niche will provide conditions to promote tumor growth [38].

2.6 Breast Cancer Metastasis to Bone

Of particular interest is breast cancer’s preference for bone, as this is the most common site of metastasis in breast cancer patients. Coleman and Rubens (1987) found that the incidence of bone metastases was significantly higher (69%) than lung (27%) and liver (27%) metastases and that bone was the most common site for first distant relapse [39]. These observations are not unique to breast cancer, as bone is also a common site of
metastasis in myeloma, prostate, thyroid, bladder and lung cancer [40]. Bone metastases pose significant challenges for a patient’s quality of life and treatment of their disease as skeletal metastases are associated with significant morbidity and poor prognosis; breast cancer patients that develop bone metastases survive approximately 22 months post-diagnosis. Breast cancer bone lesions are generally osteolytic in nature; bone homeostasis is disrupted by the upregulation of osteoclast activity, bone resorption dominates and there is little new bone formation [40]. These physiological changes compromise skeletal integrity and result in bone pain, pathological fractures, spinal cord compression and reduced patient mobility [39, 40]. Hypercalcaemia (serum calcium > 3.0 mmol/L) is also a significant metabolic complication in patients and if left untreated, can result in dysfunction of the gastrointestinal tract, renal failure and central nervous system complications such as cardiac arrhythmias [40].

Breast cancer mainly forms bone metastases in the metaphyseal bone of the axial skeleton (ex. vertebrae, ribs, sternum) and the ends of long bones [40, 41]. The metaphysis is largely composed of metabolically active trabecular bone and is surrounded by hematopoietic marrow, fatty marrow and blood vessels [42, 43]. In agreement with Paget’s seed and soil hypothesis, bone metastases of all cancer types occur almost exclusively in active red marrow, an attractive site for metastatic involvement because of its characteristic sinusoidal vascular spaces and relatively easy barrier for tumor cell penetration [3, 40]. Physical properties of the circulation within the bone marrow cavity, such as capillary structure and slow blood flow, likely also contribute to this observed pattern of metastases [40].
In conjunction with the physical properties and blood flow patterns characteristic of metaphyseal bone, bone marrow offers circulating tumor cells an optimal pre-metastatic niche via a wide variety of chemokines, such as stromal cell-derived factor-1 (SDF-1), C-X-C motif chemokine 10 (CXCL10) and other proteins, including osteopontin (OPN) and angiopoietin [44, 45]. Once breast cancer cells arrest in the bone, they influence the activity of osteoclasts and osteoblasts in such a way that results in constitutive osteoclast activation and a down-regulation of osteoblast production of bone matrix, causing sustained bone degradation [3, 46]. In healthy bone, osteoblasts externally express receptor/activator for NF-κB ligand (RANKL), which binds to RANK on the surface of monocytes to promote fusion of several of these cells into a multinucleated osteoclast [42]. Activated osteoclasts adhere to the bone matrix through \( \alpha_v\beta_3, \alpha_v\beta_5 \) and \( \alpha_2\beta_1 \) cell surface integrins and secrete acidic and lysosomal enzymes that cause bone resorption and increased bone turnover [42]. Breast cancer cells have the ability to hijack this mechanism by secreting a variety of cytokines, chemokines and other factors including parathyroid hormone-related peptide (PTHrP), interleukin 1 (IL-1), IL-6, IL-8, IL-11, cyclooxygenase 2 (COX-2) and prostaglandin E2 that have all been shown to stimulate osteoclastogenesis by up-regulating RANKL expression [3, 46]. These secreted factors can influence osteoclast physiology in a RANKL independent manner by binding directly to receptors on the surface of osteoclasts; for example, IL-8 can bind directly to CXCR1 to effect osteoclast differentiation and activity [47].

During breast cancer cell-induced bone resorption, growth factors usually locked in the bone matrix such as transforming growth factor \( \beta \) (TGF-\( \beta \)), insulin-like growth factor (IGF) 1 and 2, fibroblast growth factor (FGF) 1 and 2, platelet-derived growth
factor (PDGF), and bone-morphogenetic proteins (BMPs) are released into the bone microenvironment, creating a niche in which cancer cells can grow [44, 45, 48, 49]. These growth factors also stimulate the secretion of metastasis-promoting factors from breast cancer cells such as connective tissue growth factor (CTGF), IL-11 and PTHrP, thereby creating a vicious cycle of bone resorption and tumor growth [50, 51] (Figure 2).

The bone metastatic niche is also influenced by bone marrow stromal cells and transient cells (erythrocytes, T-cells and platelets) [3, 46]. Mesenchymal stem cells in the bone marrow give rise to stromal cells that can differentiate into osteoblasts, chondrocytes, adipocytes or fibroblasts. These stromal cells are known to support the proliferation, migration, and survival of breast cancer cells, specifically through vascular cell adhesion molecule-1 (VCAM-1). When VCAM-1 is blocked via a neutralizing antibody, bone osteolysis decreases [52]. Additionally, adipocytes are known to secrete IL-6, tumor-necrosis factor-α (TNF-α) and leptin, all responsible for stimulating bone resorption and inhibiting osteoblast proliferation [53, 54], while fibroblasts can secrete inactive MMP-2 that is activated by breast cancer cells to increase invasiveness and migration [55]. Platelets and T-cells may also assist in the establishment of metastases by expressing RANKL secreting factors such as lysophosphatidic-acid (LPA) and tumour necrosis factor α (TNF-α) to further increase osteoclast activity [56, 57]. Interestingly, as bone resorption is up-regulated, TGF-β is released and functions to suppress the activity of T-cells; this suppresses the immune response and may allow breast cancer cells to escape immune surveillance [58]. Notably, the bone marrow represents a rich supportive niche for hematopoietic stem cells [59] and it has been shown that most early-disseminated breast cancer cells in bone have a stem-like phenotype, observations that complement the
Breast cancer cells and the bone microenvironment interact in a vicious cycle.

The bone microenvironment contains multiple cell types, including osteoblasts, osteoclasts and stromal cells that secrete a myriad of growth factors and cytokines to offer a niche conducive to the establishment and progression of breast cancer metastases. Once breast cancer cells arrest in bone, they cause bone degradation via the upregulation of osteoclastic activity. Bone resorption causes more soluble factors to be released into the microenvironment, which further fuel the development of metastases in the bone, creating a “vicious cycle” of bone resorption and skeletal metastases development. Adapted from Sterling et al [3, 45, 46, 60].
CSC hypothesis of cancer metastasis [61]. Specifically, the CD44\(^+\) stem cell-like phenotype has shown increased adherence to human bone marrow endothelial cells in breast cancer [62].

Despite the extensive research conducted in the area of breast cancer metastasis to bone, this condition remains incurable. However, bone-targeted therapies do exist that attempt to reduce and delay skeletal related events (SREs) such as fractures, spinal cord compression and hypercalcaemia. Bisphosphonates were the first agents used clinically to reduce SREs and were introduced over three decades ago [63]. There are multiple different types of bisphosphonates, including clodronate, ibandronate, pamidronate and zoledronic acid; all are structural analogs of inorganic pyrophosphates (PPi) [63, 64]. PPi inhibit calcification and bone resorption by incorporating into sites of active bone remodeling, binding to hydroxyapatite crystals and inhibiting their breakdown [63]. Bisphosphonates also promote osteoclast apoptosis either by becoming incorporated into molecules of newly formed adenosine triphosphate (ATP; this nonhydrolyzable analogue of ATP is cytotoxic to osteoclasts because it inhibits multiple ATP-dependent processes) or by binding to and inhibiting farnesyl pyrophosphate, resulting in the inhibition of the posttranslational modification of multiple different proteins essential for osteoclastic function [63]. Although bisphosphonates substantially relieve skeletal pain and complications in breast cancer patients, absorption in the gastrointestinal tract and subsequent localization is poor and use of the drug is with multiple adverse effects including osteonecrosis of the jaw, atrial fibrillation, hypocalcaemia and acute inflammatory response [63]. More recently, denosomab, a monoclonal antibody that binds to RANKL and inhibits its ability to bind to RANK, has been introduced as a
therapeutic to reduce SREs. Efficacy and side effects of denosumab are similar to those of bisphosphonates and although both drugs are widely used, clinical practice guidelines do not specifically state which therapeutic to use over the other [65]. Unfortunately, neither bisphosphonates nor denosumab have any effect on progression-free or overall survival and are purely palliative in nature [65]. An agent that shows recent promise is everolimus, an inhibitor of mammalian target of rapamycin (mTOR); an exploratory analysis of the effect of everolimus on bone metastases found that patients using everolimus had a significant reduction in bone turnover markers and rates of progressive disease [65]. Even if everolimus proves to be useful in clinical applications, breast cancer treatment is in dire need of therapeutics that prevent and reduce bone metastases in breast cancer patients, rather than just slowing the rate of progression. Elucidation of other players within the bone microenvironment that promote the recruitment of breast cancer cells to the bone and the establishment of bone metastases is therefore essential to the discovery of such therapeutics.

2.7 Osteopontin

Osteopontin (OPN) is one of the most abundant non-collagenous extracellular matrix proteins in bone and as such, is a protein of interest when considering the bone as a favorable niche for breast cancer metastases [66]. OPN is a secreted, acidic glycoprophosphoprotein that was discovered independently multiple times. It was first discovered as a 60-kDa malignant transformation-specific phosphoprotein secreted by many different tumorigenic cell types, subsequently rediscovered as bone sialoprotein I, a major non-collagenous component of the bone extracellular matrix, and then identified as a protein associated with T-cell dependent genetic resistance to bacterial infections [67-
The name “osteopontin” was finally introduced to reflect its inherent ability to form bridges between hydroxyapatite (HA) and multiple cell types in bone [70]. OPN is expressed by multiple tissues in the body other than bone including the kidney, brain, gastrointestinal tract, hypertrophic cartilage, dentin and cementum. However the highest levels of the protein in a healthy human body are associated with mineralized tissues and bodily fluids that have high concentrations of calcium, such as breast milk, urine and seminal fluid [71, 72]. In the bone, OPN is produced by osteoblasts, osteoclasts, osteocytes and macrophages [66, 73]. Human OPN can range in size from 41- to 75-kDa due to the myriad of post-translational modifications it undergoes, including phosphorylation, glycosylation, proteolytic cleavage and crosslinking by transglutamination [73]. Its structure consists of various highly conserved regions that are indicative and essential to its various biological functions [72, 73] (Figure 3). It contains a calcium binding motif and many electronegative glutamic and aspartic acid residues that allow it to tightly bind to HA in bone. Binding of OPN to HA renders it a potent inhibitor of mineralization, as it inhibits the growth of HA crystals. It also has RGD (arginine-glycine-aspartate) and SVVYGLR (serine-valine-valine-tyrosine-glycine-leucine-arginine) domains for integrin binding and a heparin binding site that mediates CD44 receptor binding [73, 74]. The ability of OPN to bind to cells that express cell surface receptors such as integrins and CD44 allows it to mediate cell attachment, cell migration, chemotaxis and intracellular signaling in various cell types, including monocytic cells/macrophages, smooth muscle cells, endothelial cells and epithelial cells [75].
The protein OPN contains a number of highly conserved structural features that are indicative of its biological functions. It contains a thrombin cleavage domain, which results in proteolytic cleavage and of OPN into two similarly-sized fragments. The N-terminal fragment contains an aspartate rich domain which allows OPN to bind hydroxyapatite in bone. The RGD and SVVYGLR domains allow for binding to ligands expressing RGD-dependent integrins ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$) as well as SVVYGLR-dependent integrins ($\alpha_4\beta_1$, $\alpha_9\beta_1$). The C-terminal fragment contains two heparin binding domains which assist in the binding of OPN to CD44 and a calcium binding domain. Adapted from Tuck et. al (2003) [76].
OPN expression is observed in many pathophysiological responses and pathological conditions, including cardiovascular disease (e.g. atherosclerosis, and ventricular hypertrophy), kidney diseases (e.g. kidney stone formation), wound healing, fibrosis, autoimmune diseases and multiple different cancers [72, 73, 75]. Its presence in traumatized, diseased and inflamed tissues can be accounted for by heavy secretion from activated macrophages and T-lymphocytes. In these settings, OPN promotes adhesion of T-cells and contributes to type-1 cell mediated immunity by modulating macrophage interleukin expression.

OPN’s role in malignancy is well documented; it is secreted by several types of cancer cells including breast, lung, gastric, ovarian and myeloma cancer cells [77-81]. In these cancers, it has been shown to mediate cell-matrix and cell-cell communication through interactions with a variety of cell surface receptors (i.e. CD44, α9β1, αvβ3 and αvβ5) to activate pathways that contribute to multiple steps in the metastatic cascade [82-87]. Firstly, OPN can protect cells against apoptosis and induce survival and proliferation via in multiple ways, including through NK-κB downstream of the αvβ3 cell surface integrin, the PI3K-Akt axis downstream of CD44 or via epidermal growth factor (EGF)-dependent mechanisms [75]. It has been shown that breast carcinoma cells are protected against apoptosis when they adhere to OPN via αvβ3 [88]. Studies have also shown that OPN can induce angiogenesis by up-regulating vascular endothelial growth factor (VEGF) expression through the activation of PI3K/Akt and ERK-mediated pathways. OPN also has a direct effect on cancer cell migration via CD44 and RGD-dependent cell surface integrins; it has been shown to induce migration via growth factor pathways involving hepatocyte growth factor (HGF), its receptor Met and epidermal growth factor
(EGF), as well as interact with focal adhesion complexes to impact cell motility [83]. OPN signaling via αvβ3 also induces uPA and multiple types of MMP secretion by breast cancer cells that allows them to invade through the basement membrane.

In the establishment of bone metastases specifically, OPN assists in the attachment of breast cancer cells to human bone marrow endothelial cells and thus plays a vital role in the early steps of bone colonization [83]. It also contributes to the up-regulation of bone resorption during breast cancer metastasis to bone as neutralization of OPN can suppress osteoclastogenesis, whereas addition of OPN enhances osteoclastogenesis by up-regulating RANKL and decreasing osteoprotegerin (OPG), an osteoclastogenesis inhibitory factor. OPN’s role as a metastasis-enhancing protein is supported by the observation that OPN-deficient mice have a reduced number of metastases to bone and soft tissue [89]. OPN can also generate a favorable growth environment for breast cancer cells in bone by activating a variety of growth factors, including HGF, TGF-β and bFGF via the upregulation of μPA and IL-11 [83].

OPN has been identified as a clinical prognostic marker; clinical studies have revealed a correlation between elevated plasma OPN levels, increased tumor burden and poor prognosis in cancer patients [83, 84]. The amount of plasma OPN in healthy women ranges from 22 to 122 μg/L, with a median level of 47 μg/L. A higher level of OPN, upwards of 300 μg/L, exists in the plasma of breast cancer patients as well as colon, lung and prostate cancer patients [83, 84, 90]. In breast cancer specifically, high levels of OPN in carcinoma cells are associated with increased metastases to the bone [91]. Although extensive research has demonstrated that tumor-derived OPN can contribute to several
steps of the metastatic cascade, the role of bone-derived OPN in the metastatic progression of breast cancer has yet to be fully investigated.

2.8 Project Rationale

Extensive work has focused on characterizing the role of the bone microenvironment in breast cancer metastasis in hopes that this knowledge would lead to the discovery of clinically applicable agents that could prevent, reduce and slow the growth of bone metastases in breast cancer patients. However, there remain unanswered questions regarding the mechanism in which the bone niche promotes the establishment of breast cancer bone lesions remains unclear. Further work must be done in order to characterize what makes the bone a suitable “soil” for breast cancer cells, and identifying soluble factors in bone marrow that contribute to breast cancer cell recruitment and establishment of metastatic lesions is necessary in this regard. Previous work by Jenny Chu in the Allan lab lead to the establishment of a comprehensive ex vivo model system for investigating the influence of organ-specific soluble factors on the metastatic behavior of breast cancer cells (described in further detail below) [92]. Preliminary work indicated that both whole population and stem-like ALDH^{hi}CD44^{+}CD44^{-} breast cancer cells show enhanced migration towards bone marrow conditioned-media relative to control [92]. The goal of the current project is to investigate the role of bone-derived OPN in the migration and stem-like behavior of whole population and ALDH^{hi}CD44^{+}CD24^{-} breast cancer cells in bone.
3 HYPOTHESIS and OBJECTIVES

3.1 Hypothesis

Bone-derived OPN promotes the migration and stem-like behavior of breast cancer cells.

3.2 Objectives

1. To determine the role of bone-derived OPN in promoting breast cancer migration and stem-like behavior of breast cancer cells.

2. To investigate the receptor-ligand interactions between breast cancer cells and bone-derived OPN and the functional consequences on migration and stem-like behavior.

3. To assess the OPN-associated downstream signaling responses within breast cancer cells in response to bone-conditioned media.
4 MATERIALS and METHODS

4.1 Cell Culture and Reagents

MDA-MB-231 cells [93] were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM/F12+10% fetal bovine serum (FBS). SUM159 cells [94] were obtained from Asterand Inc. (Detroit, MI) and maintained in HAMS:F12 + 5% FBS + 5 μg/mL insulin + 1 μg/mL hydrocortisone + 10mM HEPES. Media/supplements were from Invitrogen (Carlsbad, CA); FBS was from Sigma-Aldrich (St. Louis, MO).

4.2 Bone Marrow-Conditioned Media (BMCM)

Bone marrow-conditioned media (BMCM) was generated as previously described [92]. Briefly, healthy female nude mice (Hsd: Athymic Nude-Foxn1nu; Harlan Sprague-Dawley, Indianapolis, IN) were maintained as per the Canadian Council of Animal Care (CCAC) under a protocol approved by the Western University Animal Use Subcommittee (AUS; #2009-064). Mice (6-12 weeks old) were euthanized and bone marrow was collected by flushing femur cavities as previously described [95]. Aspirates were dissociated by pipetting and cells were washed and plated in DMEM + 10% FBS + pen/strep. Resulting adherent bone marrow stromal cells (BMSCs) were passaged 2-3 times, washed, and exposed to DMEM/F12 + Mito+™ + pen/strep. Conditioned media was collected after 72 hr and stored at -20°C. To account for mouse-to-mouse variability, BMCM from multiple mice was pooled prior to use.
4.3 RayBio® Biotin Label-Based Mouse Antibody Arrays

The Raybio® Biotin label-based mouse antibody array (AAM, BLM-1, RayBiotech Inc, Norcross, GA) were used to assess the expression of 308 murine soluble factors within BMCM. Basal media samples (DMEM/F12 + Mito+™ + pen/strep) or BMCM protein samples (0.12 mg each) were dialyzed and labeled with a biotin-labeling reagent. Free biotin was removed using spin columns, membranes were blocked and samples were added to the membranes and incubated at 4°C overnight. Streptavidin-horseradish peroxidase (HRP) and chemiluminescent detection reagents were used to produce a signal at each capture spot corresponding to the amount of protein bound. Membranes were visualized using ChemiDoc Detection Software (n=3 for each media condition) (BioRad, Hercules, CA). The concentration of resulting proteins of interest present in BMCM was confirmed by Quantikine ELISA (R&D Systems, Minneapolis, MN).

4.4 Immunodepletion of OPN

Osteopontin was immunodepleted from BMCM using a rat anti-mouse osteopontin-specific antibody (R&D Systems). Antibodies were incubated for 20 minutes at RT with Dynabeads Protein G (8 µg OPN-specific antibody per mg of beads; Life Technologies, Burlington, ON). Bead-antibody complexes were then incubated with BMCM for 30 minutes at RT. Resulting bead-antibody-antigen complexes were removed from BMCM using a DynaMag-2 magnet (Life Technologies). The concentration of OPN in depleted BMCM was assessed by Quantikine ELISA kits specific for OPN (R&D Systems). Negative controls included BMCM exposed to beads only (no antibody). GST-tagged human OPN (GST-hOPN), a kind gift from Dr. Ann Chambers, London Regional
Cancer Program, London, Ontario, is a functional representative of native OPN [85]. For rescue experiments, GST-hOPN was added back to BMCM depleted of OPN at the same concentration that was originally depleted from BMCM.

4.5 Migration Assays

Transwells® (6.5 mm, 8 µm pore size; Falcon, Corning, NY) were coated with 6 µg of gelatin per well. Osteopontin-depleted or non-depleted BMCM or basal media (DMEM/F12 + Mito+™) were placed in 24-well dishes (n = 3 per condition). MDA-MB-231 or SUM-159 cells (5 x 10^4 cells per well) were plated on top of the gelatin-coated Transwells®, then inserted into 24-well dishes. In experiments involving functional blocking of CD44 or RGD (Arginin-Glycine-Aspartic acid)-sequences, cells were incubated for 30 minutes at RT with a rat anti-human CD44 antibody (10 µg/5 x 10^5 cells, Calbiochem, Mississauga, ON) or an RGD-sequence specific peptide (50 µg/5 x 10^5 cells for MDA-MB-231 breast cancer cells, 100 µg/5 x 10^5 cells for SUM-159 breast cancer cells; Sigma-Aldrich). After 18 hours incubation at 37°C and 5% CO2, Transwells® were removed, fixed and non-migrated cells were removed from the inner surface of the Transwell®. Membranes were then carefully cut from the rest of the Transwell® and migrated cells were stained with DAPI. Five high-powered fields (HPFs) of view were counted for each membrane using ImageJ [National Institutes of Health (NIH), Bethesda, MD] software. Results are expressed as a fold-increase from negative control (n = 3).

4.6 Sphere-Limiting Dilution Assays (SLDA) and Colony-Forming Assays

MDA-MB-231 cells were seeded in 96-well plates for colony-forming assays (Corning, Lowell, Massachusetts) or 96-well ultra-low attachment plates (Corning) for
the sphere-limiting dilution assay (SLDA) in a serial-diluted fashion ranging from 1000 cells/well to 0.001 cells/well. In experiments involving functional blocking, and anti-CD44 antibody (10 µg/5 x 10⁵ cells; Calbiochem) or an RGD-sequence specific peptide (50 µg/5 x 10⁵ cells; Sigma-Aldrich) were used in a similar fashion as in the Transwell™ migration assays. Osteopontin-depleted or non-depleted BMCM or basal media (DMEM/F12 + MITO+™) was added to the wells and cells were incubated with control media or conditioned media for 5 days at 37°C and 5% CO₂. After incubation, each well was scored for the presence or absence of colonies (n = 3) or tumorspheres (n = 3) using L-Calc™ Software (Stem Cell Technologies, Vancouver, British Columbia).

4.7 Flow Cytometry

Cells were grown to 80% confluence in normal growth media, harvested and resuspended at 1 x 10⁶ cells/ml. Cells were then incubated with phycoerytherin (PE)-conjugated CD44 (BD Biosciences, San José, CA), fluorescein isothiocyanate (FITC)-conjugated αvβ3 (R&D Systems), Alexafluor (AF)-488-conjugated αvβ5 (R&D Systems), AF-488-conjugated β1 (R&D Systems) or AF-488-conjugated α9β1 (R&D Systems) antibodies for 1 hour at 4°C. Negative controls included cells only (no antibody) and cells incubated with an isotype-matched IgG-control. Samples were run on a Beckman-Coulter EPICS XL-MCL flow cytometer.

4.8 Fluorescence-Activated Cell Sorting (FACS)

ALDH hiCD44+CD24- and ALDH loCD44-CD24+ cell subpopulations were isolated from the MDA-MB-231 breast cancer cell line as previously described [33, 34]. Briefly, cells were concurrently labeled with 7-amino-actinomycin D (7-AAD), ALDEFLUOR™
assay kit (StemCell Technologies; Vancouver, BC) (Figure 4) and fluorescently-conjugated antibodies including anti-CD44 (clone IM7) conjugated to allophycocyanin (APC) and anti-CD24 (clone ML5) conjugated to phycoerytherin (PE) (BD Biosciences). ALDH activity was used as the primary sort criteria (top ~20%=ALDH\textsuperscript{hi}; bottom ~20%=ALDH\textsuperscript{low}) and the CD44\textsuperscript{+}CD24\textsuperscript{-} phenotype as the secondary sort criteria (top ~10% gated on ALDH\textsuperscript{hi}; bottom ~10% gated on ALDH\textsuperscript{low}) (Figure 5). Cell viability was assessed by 7-AAD staining during cell sorting, and confirmed by trypan blue exclusion post-sorting. FACS-isolated cells were used immediately for \textit{in vitro} assays.

### 4.9 Human Phospho-Kinase Array

MDA-MB-231 cells were incubated in basal media, BMCM and BMCM depleted of OPN and cell lysates were harvested after 2 hr. Protein concentrations were determined with a BioRad DC protein assay (BioRad) and 400 µg of cell lysates were incubated with the Human Phospho-Kinase Array membranes (ARY003B, R&D Systems) overnight at 4°C. According to the manufacturer’s instructions and using kit contents, arrays were washed to remove unbound proteins and a cocktail of biotinylated detection antibodies was applied. Streptavidin-HRP and chemiluminescent detection reagents were used to produce a signal at each capture spot corresponding to the amount of phosphorylated protein bound. Membranes were visualized using ChemiDoc Detection Software (BioRad). Densitometry analysis was performed using the Protein Array Analyzer for ImageJ (n = 3 for each media condition) [96].
Figure 4. Mechanism of the ALDEFLUOR™ assay (StemCell Technologies)

The ALDEFLUOR™ assay is used to identify cells that demonstrate activity of the enzyme aldehyde dehydrogenase. The assay uses a fluorescent, non-toxic reagent, BODIPY™-aminoacetylaldehyde (BAAA) that can diffuse freely into viable cells. BAAA is a substrate for ALDH and is converted into BODIPY™-aminoacetate (BAA⁻), which is retained in the cell due to its negative charge. The active removal of BAA⁻ is inhibited with the use of cold ALDEFLUOR™ assay buffer. The resulting fluorescence of the cell is assessed by flow cytometry. Diethylaminobenzaldehyde (DEAB) is used as a negative control because of its ability to inhibit the activity of ALDH; when DEAB is used, the intrinsically neutral BAAA is free to passively diffuse back out of the cell. The fluorescence of the DEAB-treated cells are used to create the gate for the ALDH<sup>hi</sup> cells. Adapted from Chu and Allan (2011) [18].
Figure 5: Strategy for isolation of MDA-MB-231 ALDH\textsuperscript{hi}CD44\textsuperscript{+}CD24\textsuperscript{−} and ALDH\textsuperscript{lo}CD44\textsuperscript{−}CD24\textsuperscript{+} breast cancer cells.

MDA-MB-231 cells are labeled with 7-AAD, CD44-APC, CD24-PE and the ALDEFLUOR\textsuperscript{TM} assay kit. Cell subsets are isolated using a four-colour protocol on a FACS ARIA III. (A) Cells are first isolated based on expected light scatter and then (B) viability based on 7-AAD exclusion. Cells are then analyzed for (C) ALDH activity; the top 20\% most positive for ALDH activity are deemed ALDH\textsuperscript{hi} and the bottom 20\% for ALDH activity are deemed ALDH\textsuperscript{lo}. Then, (D) ALDH\textsuperscript{hi} cells are selected for the CD44\textsuperscript{+}CD24\textsuperscript{−} phenotype (top 50\%) and (E) ALDH\textsuperscript{lo} cells are selected for the CD44\textsuperscript{−}/lowCD24\textsuperscript{+} phenotype (bottom 50\%).
4.10 Data Analysis

*In vitro* experiments were performed a minimum of three times with three technical replicates within each experiment. Unless otherwise noted, all data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA) using one-way analysis of variance (ANOVA) with Tukey’s or Bonferroni’s post-hoc tests. Values of p<0.05 were classified as being statistically significant.
5 RESULTS

5.1 Bone marrow-conditioned media contains multiple soluble factors that could contribute to breast cancer’s affinity for bone

Assessment of soluble factors present in bone marrow-conditioned media (BMCM) was carried out using the RayBio® Biotin Label-Based Mouse Antibody Array (AAM, BLM-1, RayBiotech Inc), a protein array that tests for the presence of 308 soluble factors of interest. Of the proteins identified in BMCM using the protein array (Figure 6), five were found to be associated with steps of the metastatic cascade (Table 1). Of these, three soluble factors of interest, osteopontin (OPN), matrix metalloproteinase-14 (MMP-14) and insulin-like growth factor-2 (IGF-2) were chosen for further functional validation based on evidence in the literature of their possible involvement in cancer migration and growth.

5.2 Bone-derived OPN promotes human breast cancer cell migration

OPN, MMP-14 and IGF-2 concentrations were analyzed in BMCM samples with Quantikine® ELISA. IGF-2 concentrations were observed to be minimal (below 30 pg/mL) in BMCM samples tested (data not shown). However, OPN and MMP-14 were observed to be present in BMCM samples tested. To assess the effect of bone-derived OPN and MMP-14 on human breast cancer cell migration, OPN was depleted from BMCM using Dynabeads® Protein G (Novex by Life Technologies) and depletion efficiency was confirmed with a Quantikine ELISA (R&D Systems)
Figure 6. Bone marrow-conditioned media contains potential mediators of metastasis

The Raybio® Biotin label-based mouse antibody array (AAM, BLM-1, RayBiotech Inc) was used to identify soluble factors present in BMCM. Membranes were incubated with biotinylated samples of (A) basal media or (B) BMCM at 4°C overnight and visualized using chemiluminescence. Dotted boxes indicate internal positive controls, lined boxes indicate internal negative controls and solid boxes indicate proteins of interest that are potentially involved in the metastatic cascade.
Table 1: Metastasis-associated proteins identified in bone marrow-conditioned media with the RayBio® Biotin label-based mouse antibody array

<table>
<thead>
<tr>
<th>Location on Array (Figure 6)</th>
<th>Protein Name</th>
<th>Function/Involvement in Malignancy</th>
<th>References</th>
</tr>
</thead>
</table>
| 39                          | Basic Fibroblast Growth Factor (bFGF)             | • Overexpressed in breast cancer patients  
• Implicated in osteoblastic bone metastases  
• Regulates neoplastic cell growth          | [90, 97]  |
| 158                         | Intracellular Adhesion Molecule-1 (ICAM-1)        | • Elevated in metastatic breast cancer patients  
• Downregulation leads to a strong suppression of breast cancer cell invasion | [98]       |
| 176                         | Insulin-Like Growth Factor-2 (IGF-2)              | • Up-regulated in triple-negative breast cancer  
• Down-regulation of bone-derived IGF-2 suppresses the growth of bone metastases in prostate cancer | [99, 100] |
| 326                         | Matrix-Metalloproteinase-14 (MMP-14)              | • Modulates migratory ability of breast cancer cells  
• High levels of MMP-14 correlate with bone metastases | [101, 102]|
| 333                         | Osteopontin (OPN)                                 | • Most abundant extracellular matrix protein in the bone  
• Associated with multiple steps of the metastatic cascade, including proliferation, migration, adhesion and invasion | [83, 84, 90]|
(Figure 7A). However, MMP-14 could not be successfully depleted from BMCM using Dynabeads® Protein G and available MMP-14 antibodies (*data not shown*), thus MMP-14 was no longer pursued. The Transwell™ migration assay was then used to assess the migration of MDA-MB-231 and SUM-159 cells to basal media, BMCM and BMCM depleted of OPN. These two breast cancer cell lines were chosen since the Allan lab has previously shown that they both display increased migration toward BMCM [92]. Both cell lines exhibited increased migration toward BMCM compared to basal media (P<0.05, Figure 7B,C) and exhibited significantly decreased migration to BMCM depleted of OPN (P<0.05, Figure 7). In fact, the depletion of OPN from BMCM decreased the migration of both cell lines to a level not significantly different than basal media (P>0.05; Figure 7B,C). To validate that bone-derived OPN was specifically responsible for the observed effects on breast cancer cell migration, recombinant human OPN (GST-hOPN) was added back into BMCM depleted of OPN at the same concentration originally depleted from the BMCM. The addition of GST-hOPN to BMCM rescued the migratory effect of both MDA-MB-231 and SUM-159 cells, causing cells to migrate to similar levels as to non-depleted BMCM (Figure 7B,C). These results demonstrate that bone-derived OPN mediates breast cancer cell migration towards BMCM.

5.3 Bone-derived OPN promotes stem-like behavior of breast cancer cells

We next wanted to investigate if bone-derived OPN mediates the stem-like behavior of breast cancer cells, since the stem-like subpopulation of breast cancer cells has been shown to play a role in the metastatic process. We used a sphere limiting dilution assay (SLDA) to assess the effect of bone-derived OPN on the tumorsphere-
forming capacity of breast cancer cells. The SLDA tests the ability of cells to form tumorspheres in vitro, a marker for cell “stemness”. Whole population MDA-MB-231 cells show increased tumorsphere-forming capacity in BMCM compared to basal media (P<0.05, Figure 8A). This tumorsphere-

![Figure 7](image_url)

**Figure 7. Bone-derived OPN promotes the migration of MDA-MB-231 and SUM-159 human breast cancer cells**

(A) Goat anti-mouse OPN primary antibody was incubated for 20 min at RT with DynaBeads® Protein G (EMD Millipore) prior to addition and incubation with BMCM for 30 min at RT. The beads-antibody-antigen complex was removed with a DynaMag™-2 magnet. Concentration of OPN in BMCM, BMCM with Dynabeads® only (no antibody) and BMCM depleted of OPN (ΔOPN) was tested with Quantikine® ELISA (R&D Systems). Significance determined by one-way ANOVA and Tukey’s post-hoc test. (B) MDA-MB-231 and (C) SUM-159 human breast cancer cells were plated on gelatin
coated Transwells™ (5 x 10⁴ cells/well; 8µm pore size) before placement into either basal media (DMEM/F12 + Mito⁺), BMCM, BMCM ΔOPN or BMCM ΔOPN with GST-hOPN added. Plates were incubated at 37°C at 5% CO₂ for 18 hr. Transwells™ were then fixed with 1% gluteraldehyde and stained with DAPI. Five high-powered fields of view (HPFs) were captured/Transwell™ and migrated cells were enumerated through the use of ImageJ software (NIH). Data are presented as mean ± SEM (N = 3; fold change from negative control of basal media). Significance determined with one-way ANOVA and Tukey’s post-hoc test; * = significantly different from basal media; ϕ = significantly different from BMCM; δ = significantly different from BMCM ΔOPN; P< 0.05.
forming capacity decreases when BMCM is depleted of OPN (P<0.05, *Figure 8A*), suggesting that bone-derived OPN mediates this function in MDA-MB-231 cells. We also investigated the role of bone-derived OPN in the limiting dilution colony-forming ability of MDA-MB-231 cells. Whole population MDA-MB-231 cells show increased colony-forming abilities in BMCM compared to basal media (P<0.05, *Figure 8B*). When cells are exposed to BMCM depleted of OPN, their colony-forming capacity decreases significantly (P≤0.05, *Figure 8B*), suggesting that bone-derived OPN also plays a role in the colony-forming ability of breast cancer cells in a limiting dilution fashion.

As previously discussed, stem-like ALDH<sup>hi</sup>CD44<sup>+</sup>CD24<sup>−</sup> breast cancer cells are important mediators of metastasis to multiple different organs, including bone [17, 34]. We therefore also investigated if bone-derived OPN mediates the stem-like phenotype of these breast cancer cells. We have previously shown that this subpopulation of MDA-MB-231 cells is responsible for the increased migration to BMCM, compared to their non-stem like ALDH<sup>lo</sup>CD44<sup>−</sup>CD24<sup>+</sup> cells [92]. Thus, we first investigated if bone-derived OPN influenced the migration of this stem-like population. We observed that FACS-isolated ALDH<sup>hi</sup>CD44<sup>+</sup>CD24<sup>−</sup> cells showed significantly increased migration toward BMCM compared to basal media (P<0.05, *Figure 8C*). When OPN is depleted from BMCM, the ALDH<sup>hi</sup>CD44<sup>+</sup>CD24<sup>−</sup> subpopulation shows decreased migration, suggesting that bone-derived OPN influences the migration of these stem-like cells. The non stem-like ALDH<sup>lo</sup>CD44<sup>−</sup>CD24<sup>+</sup> cells did not display significantly increased migration to BMCM or BMCM depleted of OPN compared to basal media (P>0.05, *Figure 8C*), as expected from our previous work [34, 92]. We also observed that the tumorsphere-forming capacity of ALDH<sup>hi</sup>CD44<sup>+</sup>CD24<sup>−</sup> breast cancer cells decreases in BMCM
Figure 8. Bone-derived OPN promotes the stem-like behavior of MDA-MB-231 breast cancer cells

Whole population MDA-MB-231 cells were plated in a limiting dilution fashion for 7 days in basal media (DMEM/F12 + Mito\textsuperscript{+}), BMCM or BMCM ΔOPN on (A) ultra-low adhesion 96-well plates for sphere-limiting dilution assays or (B) on a normal 96-well plates for limiting dilution colony-forming assays. (C) FACS-isolated ALDH\textsuperscript{hi}CD44\textsuperscript{+}CD24\textsuperscript{−} or ALDH\textsuperscript{lo}CD44\textsuperscript{+}CD24\textsuperscript{+} cells from the MDA-MB-231 cell line were plated on gelatin-coated Transwells\textsuperscript{®} (5 x 10\textsuperscript{4} cells/well; 8µm pore size) before placement into either basal media (DMEM/F12 + Mito\textsuperscript{+}), BMCM or BMCM ΔOPN. Plates were incubated at 37\textdegree C at 5% CO\textsubscript{2} for 18 hr. Transwells\textsuperscript{TM} were then fixed with 1% gluteraldehyde and stained with DAPI. Five high-powered fields of view (HPFs) were captured/Transwell\textsuperscript{®} and migrated cells were enumerated with ImageJ software (NIH) (D) FACS-isolated ALDH\textsuperscript{hi}CD44\textsuperscript{+}CD24\textsuperscript{−} and ALDH\textsuperscript{lo}CD44\textsuperscript{+}CD24\textsuperscript{+} cells were also used in the SLDA as in (A). Data are presented as mean ± SEM (N = 3; fold change from negative control of basal media). Significance (P < 0.05) was determined with one-way ANOVA and a Bonferroni’s post-hoc test (SLDA and colony formation) or a Tukey’s post-hoc test (migration); * = significantly different from basal media; ϕ = significantly different from BMCM; δ = significantly different from BMCM ΔOPN.
depleted of OPN compared to BMCM (P<0.05, *Figure 8D*), suggesting that these stem-like cells are responsible for the tumorsphere-forming capacity of MDA-MB-231 cells in BMCM and that bone-derived OPN may mediate this.

### 5.4 Breast cancer cell migration promoted by bone-derived OPN is mediated by CD44 and RGD-dependent cell surface integrins

In order to investigate the mechanism by which bone-derived OPN promotes the migration of breast cancer cells in BMCM, we chose to explore the functional role that select cell surface receptors play in this interaction. OPN is known to influence multiple steps in the metastatic cascade through the cell surface receptor CD44, as well as multiple different cell surface integrins, including αvβ1, α9β1 αvβ3 and αvβ5 [83]. Thus, we first evaluated the expression of CD44, β1, α9β1 αvβ3 and αvβ5 on the MDA-MB-231 and SUM-159 cell lines. Flow cytometry indicated that both cell lines are positive for the expression of CD44 and these four cell surface integrins (*Figures 9 and 10)*.

Next, we investigated the role of these cell surface receptors on the migration of breast cancer cells using an anti-CD44 functional blocking antibody (Calbiochem) or the Arg-Gly-Asp (RGD) peptide (Sigma-Aldrich). OPN contains an RGD recognition sequence that is recognized by a number of cell surface integrins, including the ones examined; therefore incubating the cells with the RGD-sequence specific peptide prior to use in functional assays should block the recognition of OPN via the RGD sequence [85, 103]. We observed that blocking CD44 on the MDA-MB-231 and SUM-159 cells significantly decreases migration to BMCM compared to migration of untreated cells (P<0.05, *Figure 11A, 11C*). The migration of CD44-blocked MDA-MB-231 to BMCM was reduced to a level similar of untreated cells to BMCM depleted of OPN, suggesting that
Figure 9. MDA-MB-231 cells express OPN receptors including CD44 and multiple different cell surface integrins.

Representative histograms are shown from flow cytometry characterization of MDA-MB-231 cells incubated with (A) phycoerytherin-conjugated anti-CD44, (B) AlexaFluor-488-conjugated anti-integrin β1, (C) fluorescein isothiocyanate-conjugated anti-integrin αvβ3, (D) AlexaFluor-488 anti-integrin αvβ5 or (E) AlexaFluor-488 conjugated anti-integrin α9β1 antibodies (black) for 1hr at 4°C compared to cells incubated with an isotype-matched IgG-control (white). Samples were analyzed on a Beckman-Coulter EPICS XL-MCL flow cytometer.
Figure 10. SUM-159 cells express OPN receptors including CD44 and multiple different cell surface integrins.

Representative histograms are shown from flow cytometry characterization of SUM-159 cells cells incubated with (A) phycoerytherin-conjugated anti-CD44, (B) AlexaFluor-488-conjugated anti-integrin β1, (C) fluorescein isothiocyanate-conjugated anti-integrin αvβ3, (D) AlexaFluor-488 anit-integrin αvβ5 or (E) AlexaFluor-488 conjugated anti-integrin α9β1 antibodies (black) for 1hr at 4°C compared to cells incubated with an isotype-matched IgG-control (white). Samples were analyzed on a Beckman-Coulter EPICS XL-MCL flow cytometer.
Figure 11. Promotion of breast cancer cell migration by bone-derived OPN is mediated by the cell surface receptor CD44 and various RGD-dependent cell surface integrins

(A) MDA-MB-231 and (B) SUM-159 cells were blocked with an anti-CD44 antibody for 30 min and plated on gelatin coated Transwells™ (5 x 10⁴ cells/well; 8µm pore size) before placement into basal media (DMEM/F12 + Mito⁺), BMCM or BMCM ΔOPN. Plates were incubated at 37°C at 5% CO2 for 18 hr. Transwells™ were then fixed with 1% gluteraldehyde and stained with DAPI. Five high-powered fields of view (HPFs) were captured/Transwell™ and the migrated cells were enumerated through the use of ImageJ software (NIH). (C) MDA-MB-231 and (D) SUM-159 cells were blocked with an RGD sequence-specific peptide for 30 min and used in the Transwell™ migration assay as described above. Data are presented as mean ± SEM (N = 3; fold change from negative control of basal media). Significance was determined with one-way ANOVA and Tukey’s post-hoc test: * = significantly different from basal media; φ = significantly different from BMCM; δ = significantly different from BMCM ΔOPN; P< 0.05.
CD44 is responsible for the effect of bone-derived OPN of breast cancer cell migration (P>0.05, Figure 11A). In contrast, CD44-blocked SUM-159 cell migration to BMCM was significantly lower than untreated SUM-159 cell migration to BMCM depleted of OPN (P< 0.05, Figure 11C). This suggests that CD44 may mediate the interaction of SUM-159 with bone-derived OPN as well as other, as yet uninvestigated soluble CD44-ligands within the BMCM.

Our results also indicate that blocking the RGD-recognition sequence on MDA-MB-231 and SUM-159 cells significantly reduces the migration to BMCM compared to migration of untreated cells (P<0.05, Figure 11B, 11D). RGD-blocked MDA-MB-231 cells showed significantly decreased migration to BMCM compared to untreated cells to BMCM depleted of OPN (P<0.05, Figure 11B). These results suggest that RGD-dependent integrins may mediate the interaction of MDA-MB-231 with bone-derived OPN and other soluble factors that interact with integrins within the BMCM. In contrast, RGD-blocked SUM-159 cells showed similar migratory levels to BMCM as untreated cells to BMCM depleted of OPN, suggesting that RGD-dependent cell surface integrins mediate the interaction of SUM-159 cells with bone-derived OPN (P>0.05, Figure 8D).

5.5 Promotion of breast cancer stem-like behavior by bone-derived OPN is mediated through CD44 and RGD-dependent cell surface integrins

Given that CD44 and RGD-dependent integrins mediate the interaction between breast cancer cells and bone-derived OPN to influence migration to BMCM, we wanted to explore if these cell surface receptors also influence the stem-like behaviour of breast cancer cells in response to bone-derived OPN. An anti-CD44 blocking antibody (CaliBiochem) and RGD-sequence specific peptide (Sigma Aldrich) were used to block
MDA-MB-231 in the SLDA. Both CD44-blocked and RGD-blocked MDA-MB-231 cells showed decreased tumorsphere-forming capacity when exposed to BMCM and BMCM depleted of OPN compared to untreated cells exposed to BMCM depleted of OPN (P<0.05, Figure 12A, 12B), suggesting that both CD44 and RGD-dependent integrins can mediate the tumorsphere-forming capacity of breast cancer cells.

5.6 Bone-derived OPN causes phosphorylation changes in migratory and stem-like related pathways in breast cancer cells

We have shown that bone-derived OPN influences the migratory ability and stem-like behavior of breast cancer cells via CD44 and RGD-dependent cell surface integrins. Considering this, we wanted to begin to investigate the effect of bone-derived OPN on downstream pathways within breast cancer cells. For this, we used the Human Phospho-Kinase Array (R&D Systems), which simultaneously detects the relative site-specific phosphorylation of 43 kinases and upregulation of 2 related total proteins. We exposed MDA-MB-231 cells to basal media ((DMEM/F12 + MITO+), BMCM and BMCM depleted of OPN for 2 hr prior to harvesting cell lysates for use with the array. Figure 13 shows 15 different kinases and 1 protein that were shown to be significantly different between treatments. Results show that BMCM causes the up-regulation of WNK-1, PRAS40 and HSP60, compared to basal media (P≤0.05; Figure 13) and BMCM depleted of OPN (P≤0.05; Figure 13). Table 2 includes further details about these proteins of interest. We are currently in the process of validating these findings by immunoblotting.
Figure 12: Promotion of breast cancer cell tumorsphere-forming capacity by bone-derived OPN is mediated by the cell surface receptor CD44 and various RGD-dependent cell surface cell surface integrins.

MDA-MB-231 human breast cancer cells were blocked with (A) anti-CD44 antibodies or (B) an RGD sequence-specific peptide for 30 minutes prior to plating in a limiting dilution fashion on ultra-low adhesion 96-well plates for 7 days in basal media (DMEM/F12 + Mito⁺), BMCM or BMCM Δ OPN in the sphere-limiting dilution assay. Significance determined with a two-way ANOVA and a Bonferroni’s post-hoc test; * = significantly different from basal media; ϕ = significantly different from BMCM; δ = significantly different from BMCM ΔOPN; P<0.05.
Figure 13. Bone-derived OPN causes phosphorylation changes in migration- and stem cell-related signaling pathways in breast cancer cells

MDA-MB-231 human breast cancer cells were incubated in basal media, BMCM or BMCM ΔOPN and cell lysates were harvested after 2 hr. Cell lysates were incubated with the Human Phospho-Kinase Array membranes (ARY003B, R&D Systems) overnight at 4°C. Biotinylated detection antibodies were applied and membranes were visualized using chemiluminescence. Densitometry analysis was performed using the Protein Array Analyzer for ImageJ and significance (P<0.05) was determined with a Tukey’s test; * = significantly different from basal media; φ = significantly different from BMCM; δ = significantly different from BMCM ΔOPN. Only kinases and proteins with significantly different phosphorylation or expression between at least two treatments are shown.
<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Phosphorylation Site</th>
<th>Function/Involvement in Malignancy</th>
<th>References</th>
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</table>
| WNK-1        | T60                  | • Serine/threonine protein kinase  
• Participates in cell proliferation and migration  
• Involved in differentiation of neural progenitor cells | [104]      |
| PRAS40       | T246                 | • Acts at the intersection of the Akt and mammalian-target of rapamycin (mTOR)-mediated signaling pathways  
• Acts downstream of OPN to influence cell migration | [83, 105]  |
| HSP60        | -                    | • Increased levels of HSP60 observed in multiple different cancers, plays an essential role in malignant cell survival  
• Interaction between HSP60 and β-catenin promotes metastasis | [106]      |
6 DISCUSSION

One in nine Canadian females are expected to develop breast cancer in their lifetime and one in 32 of all Canadian females are expected to die of breast cancer [7]. About 85% of all breast cancer patients that die from their disease are expected to harbor bone metastases [107]. Bone metastases pose an extreme burden for breast cancer patients, resulting in pain, fractures and hypercalcaemia. The current clinical course of management lacks treatments that cure, reduce or prevent malignant skeletal lesions [107].

Elucidation of the steps involved in the metastatic cascade is vital for the development of therapeutics that can increase progression-free and overall survival of breast cancer patients. So far, scientific research efforts have shown that the process of metastasis is highly inefficient and that subsets of malignant cells with a stem-like phenotype are most likely to form metastases in a secondary site [13, 23, 34, 39]. Moreover, breast cancer repeatedly metastasizes to specific organs in the human body. This observed organ tropism implies a role for the microenvironment in the establishment and progression of metastases in distant organs [12, 37].

Bone is the most common site of secondary growth in metastatic breast cancer patients, suggesting an environment particularly conducive to the development of metastases [60]. It is now clear that both soluble and insoluble factors within the bone microenvironment contribute to the progression of malignant disease. This thesis focuses on the soluble factors within the bone microenvironment that promote the formation of breast cancer lesions in the bone. We used an ex vivo representation of the bone microenvironment to study soluble factors in bone and hypothesized that one specific
soluble factor, OPN, promotes the migration and stem-like behaviour of breast cancer cells in BMCM and that the interaction of bone-derived OPN with breast cancer cells activates downstream intracellular signaling responses.

6.1 Summary of Experimental Findings

1. Bone marrow-conditioned media (BMCM) contains multiple soluble factors that could assist in the establishment of breast cancer metastases in bone, such as osteopontin (OPN), basic fibroblast growth factor (bFGF), intracellular adhesion molecule-1 (ICAM-1), insulin-like growth factor-2 (IGF-2) and matrix-metalloproteinase-14 (MMP-14).

2. Bone-derived OPN promotes the tumorsphere and colony-forming abilities in MDA-MB-231 breast cancer cells.

3. Bone-derived OPN promotes the migration and tumorsphere-forming abilities of stem-like ALDH<sup>hi</sup>CD44<sup>+</sup>CD24<sup>−</sup> MDA-MB-231 breast cancer cells to BMCM.

4. Promotion of MDA-MB-231 and SUM-159 breast cancer cell migration and MDA-MB-231 breast cancer cell tumorsphere-forming capacity by bone-derived OPN is mediated by the cell surface receptor CD44 and various RGD-dependent cell surface cell surface integrins.

5. Bone-derived OPN causes alterations in migration- and stem cell-related signaling pathways in MDA-MB-231 breast cancer cells, including activation of HSP60 and phosphorylation of PRAS40 and WNK-1.

6.2 Implications of Experimental Findings
This study supports the hypothesis that bone-derived OPN promotes the migration and stem-like behaviour of breast cancer cells via CD44 and RGD-dependent cell surface integrins and affects the phosphorylation and activation of downstream signaling pathways in breast cancer cells (Figure 14). There exists in the literature a wide breadth of knowledge on the effect of tumor-derived OPN in multiple steps of the metastatic cascade, however much less research has been conducted on the effect of host-derived OPN in the tumor microenvironment.

It is thought that host-derived OPN differs from tumor-derived OPN both structurally and functionally. Current literature suggests that tumor-derived OPN is less phosphorylated than host-derived OPN [83]. One study showed that transformation of normal rat kidney cells changes OPN from highly phosphorylated to less phosphorylated and OPN produced by Ras-transformed fibroblasts is less phosphorylated than OPN produced by osteoblasts [108]. Kazanecki et al (2007) proposed that the differing phosphorylation profiles of host-derived and tumor-derived OPN affects its function and that the less phosphorylated, tumor-derived OPN is more effective at promoting cancer progression by protecting cells from the immune response, inhibiting apoptosis and increasing anchorage independence and metastasis while host-derived OPN serves as a chemoattractant for multiple cells of the immune system, such as macrophages, that inhibit tumor growth when recruited to the lesion site [109, 110]. It is also suggested that tumor-derived OPN better supports invasiveness while host-derived OPN promotes adhesion to components of the extracellular matrix such as fibronectin and vitronectin.
Bone-derived osteopontin promotes the migration of breast cancer cells toward bone as well as the stem-like behavior of breast cancer cells in bone by interacting with cells via the CD44 cell surface receptor and the RGD-dependent cell surface integrins.
Our work helps elucidate the contribution of host-derived OPN – specifically bone-derived OPN – to the metastatic progression of breast cancer within the bone.

6.2.1 Bone-Derived OPN Promotes the Migration of Breast Cancer Cells

Firstly, we showed that host-derived OPN, specifically soluble bone-derived OPN, can in fact contribute to the metastatic potential of breast cancer cells by increasing their migration towards bone-conditioned media. This finding is supported by the work of others; Nemoto et al (2001) found that OPN-deficient mice displayed less colonization and growth of injected melanoma cells to bone than wild-type mice [89]. Chakraborty et al (2008) showed similar results, demonstrating that OPN-null mice have a slower growth rate of tumors compared to wild type mice [89, 111]. These studies, taken together with our results, suggest that OPN increases breast cancer cell migration to and colonization in bone, thus promoting breast cancer bone metastases.

Both the MDA-MB-231 and SUM-159 breast cancer cells displayed reduced migration towards BMCM depleted of OPN compared to non-depleted BMCM. However, levels of migration toward BMCM depleted of OPN were still significantly higher than migration toward basal media, suggesting that other factors within the BMCM may be contributing to the migration of breast cancer cells. The RayBio® Biotin label-based mouse antibody array identified multiple soluble factors that could be contributing to this observed migratory effect. One such protein is matrix metalloproteinase-14 (MMP-14; MTI-MMP). MMP-14 can be membrane bound or found in the soluble form; the type 1 transmembrane MMP is expressed by both stromal cells and breast cancer cells and undergoes shedding to produce multiple soluble active and inactive fragments [112]. Multiple different types of MMPs are known to contribute to
carcinogenesis through extracellular matrix remodeling and cancer cell migration. Elevated levels of soluble MMP-14 specifically have been detected in the plasma of breast cancer patients and it is known to be a critical protein in cancer progression as it is responsible for invasion through collagen networks and collagenolysis [113]. Tobar et al (2014) showed that MMP-14 produced by bone marrow stromal cells sheds epithelial endoglin, a membrane bound glycoprotein that has tumor suppressor effects by attenuating the expression of TGF-β1. By silencing endoglin, MMP-14 enhances the migratory properties of human breast cancer cells [101]. Zarrabi et al (2011) have shown that MMP-14 can crosstalk with CD44, activating major players in migratory signaling pathways downstream of CD44 such as MAPK and PI3K [114].

Intercellular adhesion molecule (ICAM)-1 is also of interest when considering breast cancer migration toward BMCM. ICAM-1 is expressed on the surface of endothelial cells, leukocytes and cancer cells and can also be shed into the circulation in soluble form. It is an immunoglobulin involved in facilitating adhesion of immune cells to endothelia in the healthy human body [115]. However, extensive research shows that ICAM-1 also plays a role in malignancy. Elevated levels of soluble ICAM-1 are present in breast cancer patients and recently, Schröder et al (2011) showed that ICAM-1 expression is significantly associated with poor prognosis in breast cancer patients, specifically a poorly differentiated phenotype, negative ER status and positive lymph node involvement [116]. Binding of soluble ICAM-1 to target cells has been shown to modulate migration and angiogenesis via the activation of signaling cascades involving MAPK and Src, thus it is possible that ICAM-1 plays a role in the migration of breast cancer cells to BMCM [117].
6.2.2 Bone-Derived OPN Promotes the Stem-Like Phenotype of Breast Cancer Cells in Bone

In this study, we also assessed the ability of bone-derived OPN to influence the stem-like behaviours of limiting dilution tumorsphere-forming ability and colony-forming ability of breast cancer cells in BMCM. To assess these properties, we used the sphere-limiting dilution assay (SLDA) and the colony-forming assay. The SLDA uses a limiting-dilution analysis to quantify the number of tumorsphere initiating cells present in populations grown in nonadherent conditions [118] and the colony-forming assay measures the ability of cells to differentiate and proliferate into colonies at very low numbers. To our knowledge, our study is the first to show that bone-derived OPN promotes the tumorsphere-forming capacity and colony-forming ability of breast cancer cells.

In normal tissues, stem cells reside in a specialized niche that influences their stem-like properties and differentiation fates via cell-to-cell interactions, various secreted soluble factors and extracellular matrix components [59, 119]. This niche helps maintain homeostasis, influencing a stem cell’s ability remain quiescent, self-renew, or differentiate; thus carefully regulating the size and activity of the stem cell pool [59, 119]. The bone marrow is known to provide an optimal niche for the maintenance of hematopoietic stem cells (HSCs). HSCs migrate toward and reside within the endosteal region of bone that lies between the bone and bone marrow interface [59]. It has been shown that contact between osteoblasts and HSCs at the endosteal surface and within trabecular bone is particularly important in modulating HSC proliferation and hematopoiesis [59, 120]. It has recently become clear that OPN is integral in mediating the effects of osteoblasts on HSCs within healthy bone. Nilsson et al (2005) have shown
that osteoblast-secreted OPN promotes migration and lodging of HSCs within the endosteal region; when transplanting HSCs into a normal hematopoietic environment, they settle almost exclusively into the endosteal region of the bone, however when transplanting HSCs into an OPN$^{-/-}$ environment, there is a random distribution of the cells throughout the bone marrow [121]. Studies by this group also suggest that OPN maintains hematopoietic progenitor cell (HPCs) quiescence within the bone marrow [121]. HPCs were isolated from wild type and OPN$^{-/-}$ mice fed bromodeoxyuridine (BrdU) continuously for four weeks; HPCs harvested from wild type animals did not show any incorporation of BrdU, suggesting that HPCs were maintained in the quiescent state, whereas almost all HPCs isolated from OPN$^{-/-}$ mice displayed incorporation of BrdU [121].

Similar to healthy stem cells within the normal bone marrow niche, stem-like cancer cells are also influenced and maintained by the microenvironment in which they reside. Stromal cells play a major role in maintaining a microenvironment suitable for cancer stem cells; these cell types include osteoblasts, osteoclasts, cancer-associated fibroblasts (CAFs), infiltrating leukocytes, mesenchymal stem cells (MSCs), and tumor associated macrophages (TAMs) which interact with CSCs via direct contact or by secreting various growth factors and cytokines [122].

Recent work has implicated OPN in the maintenance of “stemness” in different types of cancers. Pietras et al (2014) showed that OPN was able to promote stemness in proneural glioblastoma [123]. This study used two functional assays to measure stemness; (1) the drug efflux-based side population (SP) assay, which was previously described to enrich for stem-like tumor initiating cells in human and murine glioma by
their ability to exclude Hoechst dye via ABC transporter activity [124] and (2) the colony-forming assay following irradiation, as well as the expression of a panel of stem-cell markers, including Nanog, Sox2, Oct4 and Id1. This study found that OPN had the ability to increase the amount of cells displaying the SP phenotype within glioma primary cultures, suggesting that OPN was necessary to induce the stem-like phenotype. They also found that glioma primary cultures treated with OPN displayed up-regulated Nanog, Sox2, Oct4 and Id1 stem cell markers. Finally, glioma primary cultures treated with OPN formed more colonies in a colony-forming assay following irradiation relative to control cells.

Cao et al (2015) showed that OPN promotes a cancer stem cell-like phenotype in hepatocellular carcinoma cells. They found that OPN expression is correlated with hepatocellular carcinoma (HCC) SP fractions that display high self-renewal and tumorigenic properties and was highly expressed in dormant cells, spheroids and chemoresistant cancer cells [125]. They also found that HCC cells exhibited a dramatically decreased ability to form colonies and spheroids in colony-forming assays and tumorsphere-forming assays when OPN is knocked down with an shRNA construct in HCC cells [125]. These cells also displayed a reduction in stemness-related genes, including HIF-1α, OCT4, Nanog, CK19, BMI-1 and Notch1 and decreased tumorgenicity in immunodeficient mice [125].

The aforementioned studies by Pietras et al (2014) and Cao et al (2015) focused on OPN derived from carcinoma cells however, given the role of the microenvironment in maintaining CSC phenotype and activity and our data showing that bone-derived OPN promotes the tumorsphere and colony-forming capabilities of breast cancer cells in vitro,
it is possible that OPN within the bone microenvironment also plays a crucial role in metastatic tumor progression by maintaining the stemness of breast cancer cells that have metastasized to bone. Our work proposes a novel function for bone-derived OPN in the maintenance of breast cancer cell "stemness" and thus suggests a role for bone-derived OPN in the establishment and maintenance of heterogeneous breast cancer tumors in bone.

6.2.3 The Role of CD44 and RGD-Dependent Cell Surface Integrins in the Bone-Derived OPN Mediated Migration and Stem-Like Behavior of Breast Cancer Cells

Given the observed functional role of bone-derived OPN in promoting the migration and stem-like behaviour of breast cancer cells, we next explored the mechanism by which bone-derived OPN influences these functions of breast cancer cells. Our results demonstrate that bone-derived OPN influences the migration and tumorsphere-forming ability of breast cancer cells via the cell surface receptor CD44 and RGD-dependent cell surface integrins.

CD44 is encoded by 20 exons, 7 of which make the extracellular domain of the standard form of CD44 (CD44s). Variants of CD44 arise through the alternative splicing of at least 12 of the 20 exons [126]. Cell surface expression of CD44 on macrophages and osteoclasts has been shown to effect normal cell motility through interaction with OPN [127, 128]. Receptor-ligand interactions between CD44 and OPN have also been implicated in malignancy, specifically related to migration as well as other steps in the metastatic cascade. Weber et al first showed in 1996 that OPN interacts with CD44 and then went on to show that the interaction between CD44 and cancer cell-secreted OPN promotes the migration of tumor cells to specific sites of metastasis formation [129, 130].
Katagiri et al (1999) later showed that OPN is able to bind to several CD44 variants in an RGD-dependent and independent manner on both the N-terminal and C-terminal fragments of OPN to promote cell spreading, motility and chemotactic behavior [131]. Khan et al (2005) then showed that incubating T1NT tumorigenic human breast cancer cells with monoclonal antibodies recognizing all isoforms of CD44 or the specific variants of CD44 v6 or v9 resulted in a significant decrease in OPN-mediated cell migration and that OPN enhances CD44 expression at both the mRNA and protein level and increases the localization of CD44 to the cell membrane surface [132]. Our lab also recently showed that breast cancer cells preferentially migrate toward lung-derived OPN in a CD44-dependent manner [92].

The CD44-OPN interaction is also thought to facilitate OPN’s effect on cancer cell stemness. The ability of OPN to maintain glioma stemness phenotypes in the perivascular niche is shown to occur in a CD44-dependent manner; mouse models of glioma that were CD44/- or CD44+/- showed better survival compared to controls, an effect shown to be mediated through downstream enhancement of HIF-2α activity [123]. The current study adds to this breadth of knowledge, showing that bone-derived OPN influences breast cancer cell migration and the tumorsphere-forming ability of breast cancer cells via CD44.

The RGD-dependent cell surface integrins include α5β1, α8β1 and all αv-containing integrins. These integrins recognize ligands that contain the RGD motif (Arg-Gly-Asp), mainly found in components of the extracellular matrix such as OPN, fibronectin, vitronectin and nephronectin [133]. The interaction between OPN and these cell surface integrins is used in multiple different settings in the healthy body, including
facilitating the migration and adhesion of cell types such as osteoclasts, multiple different types of epithelial cells and smooth muscle cells via clustering and functional activation of the focal adhesion kinase complex [134]. Cell surface integrins also prove particularly important in malignancy, as increased expression of these integrins on tumor cells enhances their tumorigenic properties, specifically migration and adhesion. OPN-mediated migration in breast cancer has been shown to involve many of these integrins, including αvβ1, αvβ5 [135, 136] and α8β1 [137]. The cell surface integrin that seems to be most commonly associated with OPN’s effect in malignancy is the αvβ3 heterodimer. Increased expression of αvβ3 during breast cancer tumorigenesis and progression makes tumor cells more responsive to OPN and thus enhances tumorigenic properties such as migration and adhesion [86]. Interestingly, it has been shown that breast cancer cell lines can express variable amounts of different cell surface integrins and thus depend on certain integrins over others in their migratory and adhesive functions. Wong et al (1998) showed that the highly metastatic MDA-MB-435 breast cancer cell line expresses high levels of αvβ3 while the non-metastatic MCF-7 breast cancer cell line expressed significantly lower levels of αvβ3 [138]. Similarly, van der Pluijm et al (1997) found higher levels of expression of α2β1, α3β1, α5β1 and αvβ3 in the highly metastatic MDA-MB-231 breast cancer cell line compared to the less malignant MCF-7, T47D and ZR75-1 breast cancer cell lines [139] and found that use of the GRGDS peptide blocked the adhesion of MDA-MB-231 to bone matrix molecules. Tuck et al (2000) then showed that the highly metastatic MDA-MB-435 breast cancer cell line depends more on the αvβ3 heterodimer for OPN-mediated migration than αvβ5 or β1 integrins, whereas the non-
metastatic breast cancer cell lines, 21PT and 21NT, migrated in an αvβ5 or β1-dependent and an αvβ3-independent manner [140].

In keeping with these studies, our results show cell line-specific migratory responses when the OPN-CD44 or the OPN-RGD-dependent cell surface integrin interactions are abrogated. The MDA-MB-231 cells display significantly decreased migration to BMCM depleted and non-depleted of OPN when the RGD motif is blocked compared to when the CD44 cell surface receptor is blocked. In contrast, the SUM-159 cells display the opposite effect; they show significantly reduced migration when the CD44 cell surface receptor is blocked compared to when the RGD motif is blocked. Flow cytometry analysis revealed that both cell lines express significant amounts of CD44 and similarly moderate amounts of RGD-dependent β1, αvβ3 and αvβ5 and RGD-independent α9β1, suggesting that the differences in migratory function observed could be dependent on the expression of other cell surface integrins on the MDA-MB-231 such as α5β1 and α8β1.

We also showed that blocking RGD-dependent cell surface integrins on MDA-MB-231 cells attenuates their tumorsphere-forming capacity. These results are supported by those of Cao et al (2015), who showed that OPN promotes the stem cell-like phenotype of HCC cells via the αvβ3 cell surface integrin, which in turn activates the downstream transcription factor NF-κB and hypoxia-related protein HIF-1α [125].

6.2.4 Bone-Derived OPN Causes Phosphorylation and Expression Changes in Migratory and Stem Cell-Related Pathways in Breast Cancer Cells

OPN influences a number of signaling pathways via communication with CD44 and RGD-dependent cell surface integrins to mediate steps of metastatic cascade,
including migration, cell proliferation and survival, adhesion and angiogenesis. Soluble OPN can promote the migration, proliferation and survival of tumor cells via \( \alpha v\beta 1, \alpha v\beta 3, \alpha v\beta 5 \) and CD44 while adhesion and angiogenesis are promoted via \( \alpha v\beta 3 \) [83]. The interaction of OPN with \( \alpha v\beta 1 \) and \( \alpha v\beta 5 \) typically result in the upregulation of paxillin, Crk and Ras while signaling via \( \alpha v\beta 3 \) activates Src and the focal adhesion kinase complexes and their downstream effectors such as Erk1/2, uPA, NF- \( \kappa B \), OPG and the FAK and PI3K/Akt axis [83]. Interaction of OPN with CD44 activates the CD44-ERM complex, as well as promoting the phosphorylation of Akt and the activation of MAPK [83]. The migratory response of cells to OPN also includes the upregulation of epidermal growth factor (EGF) and hepatocyte growth factor (HGF) and induces EGF receptor (EGFR) receptor expression and tyrosine kinase activity as well as an increase in the kinase activity of the HGF receptor Met [140, 141].

Given the effect of OPN on multiple downstream signaling pathways within tumor cells, we wanted to explore the effect that bone-derived OPN has on the phosphorylation of common kinases and the activation of 2 related proteins in MDA-MB-231 human breast cancer cells using the Human Phospho-Kinase Array (R&D Systems). This array contained proteins that are commonly activated and phosphorylated in response to soluble OPN, including Akt, EGFR, Erk1/2 and FAK. With use of this array, we identified three novel factors that appear to be influenced by bone-derived OPN. Our results indicate that the presence of bone-derived OPN causes significant increases in the phosphorylation of PRAS40 and Wnk-1 and an upregulation in the expression of HSP60. We have yet to validate the results of the array with immunoblotting, however these three
proteins are interesting candidates for the downstream signaling that may be activated in breast cancer cells in response to bone-derived OPN.

### 6.2.4.1 PRAS40

PRAS40 is a proline-rich substrate of Akt and mTORC1. It acts at the intersection of the Akt and mammalian target of rapamycin (mTOR) signaling pathways and has been used in studies as a marker for PI3K pathway activation. The PI3K-Akt signaling pathway regulates many normal cellular processes including cell motility, proliferation, cell growth and cell survival. It has been extensively studied in relation to carcinogenesis and the aberrant expression of many components within this pathway has been implicated in numerous human cancers [142]. Andersen et al (2010) found that expression of phospho-PRAS40 was up-regulated in 40% of primary breast cancer samples and correlates with PI3K-Akt signaling and thus predicts Akt inhibitor sensitivity in triple-negative breast cancer tissues [143]. As previously mentioned, OPN activates the PI3K/Akt/mTOR pathway via interaction with αVβ3 to promote migration, invasion and cell survival and proliferation thus it is likely that bone-derived OPN may also indirectly cause increased phosphorylation of PRAS40 [144].

### 6.2.4.2 WNK1

WNK1 is a seronine-threonine protein kinase that is widely expressed in many human tissues. Most research on the WNK protein kinase family has focused on their role in the regulation of ion transporters in the kidney and extrarenal tissues, however growing evidence suggests that they are involved in various signaling cascades related to human cancer. WNK1 is involved in the MAPK cascade and has been shown to be involved in EGF-dependent stimulation of ERK1/2 and ERK5 in neural progenitor cells and HeLa
cells [104, 145]. Knockdown of WNK1 in these cell types lead to the suppression of ERK1/2 by EGF and resulted in greatly reduced cell growth and migration [104]. WNK1 is also known to phosphorylate Smad2 and negatively affects Smad-mediated gene expression, which results in suppression of TGF-β signaling. TGF-β is integral in inducing the epithelial-to-mesenchymal transition (EMT) thus loss or inactivation of WNK1 activity could promote EMT of epithelial tumor cells [146]. WNK1 is also linked to Rho GTPases, which control the dynamics of the cytoskeleton and are integral in cell migration and invasiveness [147, 148]. Notably, upregulation of WNK1 in neural tumor cells has been correlated with increased invasiveness. WNK1 is also a substrate of Akt, suggesting that it may play a role in the PI3K/Akt pathway [145]. Its role in this pathway could cause WNK1 to be indirectly phosphorylated by the interaction of bone-derived OPN with αvβ3 on breast cancer cells, and thus could contribute to breast cancer migration, invasiveness and cell growth downstream of OPN.

6.2.4.3 HSP60

Finally, heat shock protein 60 (HSP60) is a chaperone protein that is essential in assisting many different newly synthesized proteins fold into their native forms. Research focused on HSP60’s role in disease has mostly shown its role in innate immunity and cardiac ischemia, however increased HSP60 expression is also observed in a wide variety of cancers, including prostate, ovarian, pancreatic and large bowel carcinoma [149]. Cellular distribution of HSP60 changes during carcinogenesis; while it is normally found in the mitochondria, instead it accumulates in extramitochondrial sites, such as the cytosol, plasma membrane, and secretory vesicles [150, 151]. HSP60 has also been shown to accumulate in lymph node-invading tumor cells, possibly suggested a role for
HSP60 in metastasis, and to accumulate in tumor cells as a result of treatment with the systemic agent 5-azacytidine, conferring tumor cell resistance [149]. Tsai et al (2009) recently demonstrated that overexpression of HSP60 induces metastasis in head and neck cancer cell lines by interacting with β-catenin and activating its downstream targets, such as MMP-14 [106]. Our results from the Human Proteome Profiler array suggest bone-derived OPN may also have an effect on the expression of HSP60 in breast cancer cells.

6.3 Limitations to the Study

This study’s methods rely heavily on the use of an ex vivo model system as a surrogate for the bone microenvironment. While this system provided an excellent platform in which to study the effect of bone-derived OPN on the migration and stem-like behaviour of breast cancer cells, this approach is not without limitations.

Firstly, this model only allows the examination of soluble factors within the bone marrow microenvironment and excludes insoluble components such as the extracellular matrix and the various cell types that are present in bone, including osteoblasts, osteoclasts, osteocytes, hematopoietic and mesenchymal stem cells, T cells, erythrocytes and platelets. These insoluble factors are known to influence the metastasis of breast cancer cells within bone marrow and would be valuable factors to include when examining the effect of bone-derived OPN on breast cancer metastasis to bone [3].

Secondly, the BMCM is harvested from nude mice and thus consists of murine soluble factors. When using this model, it is assumed that these murine soluble factors can interact with human breast cancer cells. The mouse genome and human genome are highly homologous; of the 4,000 genes that have been studied, less than ten exist in one species but not in the other [152]. Many murine factors are capable of interacting with
and stimulating human cells however, it is known that some murine factors are not capable of being recognized by human cells and are not compatible with human signaling pathways [153]. Thus, this model may not be ideal for studying the effect of all soluble factors within the BMCM on breast cancer cell malignant cell behavior. Additionally, the use of nude mice excludes a significant proportion of the immune system contribution to breast cancer cell metastasis in the bone. The immune system is particularly important when studying the effects of OPN on breast cancer cells, as OPN is known to recruit macrophages that can inhibit tumor growth at the site of metastasis [83]. However, the use of the nude murine platform is beneficial in that the results obtained can be closely related to previous and future in vivo studies in the same mouse strain.

Finally, the results of this study rely on the use of immortalized breast cancer cell lines as opposed to primary human breast cancer cells. Although our cells are routinely authenticated, cell lines have the ability to mutate in vitro as a result of being maintained outside of their native environment (tissue plastic vs. the extracellular matrix) and due to their inherent lack of fully functioning DNA repair mechanisms. Therefore although immortalized cell lines are well-characterized, commercially available, and easy to grow in culture, they still may not accurately represent the true behavior that might be seen if primary patient-derived breast cancer cells were used.

6.4 Future Directions

While this study contributes to the breadth of knowledge implicating OPN in the metastasis of breast cancer to bone, some unanswered questions remain that should be addressed to help form a more complete picture of breast tumorigenesis in the bone microenvironment.
Firstly, rescue experiments should be performed with respect to OPN’s effect on the tumorsphere-forming and colony-forming capacity of breast cancer cells in BMCM. OPN should be added to BMCM that has been depleted of OPN and used in the SLDA and colony-forming assay to show that OPN is solely responsible for the reduction in the tumorsphere- and colony-forming capabilities of breast cancer cells in BMCM.

Secondly, it would be beneficial to show the localization of OPN in the bone and its cell surface receptors and integrins on in vivo breast cancer bone metastases to support the conclusion that bone-derived OPN is interacting with CD44 and RGD-dependent cell surface integrins to facilitate the metastatic process.

Additionally, in order to appropriately conclude that bone-derived OPN results in the phosphorylation of PRAS40 and WNK1 and the upregulation of HSP60, immunoblotting needs be performed with cell lysates exposed to basal media, BMCM and BMCM depleted of OPN. Immunoblotting would support the findings of the Human Proteome Profiler array, as the array’s results alone are not robust enough to draw definite conclusions. It would also be interesting to repeat the array with other breast cancer cell lines (i.e. SUM-159) to identify any differences in cellular signaling in response to bone-derived OPN. As shown in the results of this study, MDA-MB-231 and SUM-159 cells both respond to the presence of bone-derived OPN but their response seems to depend more heavily on one cell surface receptor/integrin over another in a cell line-specific manner. It is possible that the different cell lines have differing cell signaling responses to OPN as well.

It would also be beneficial to our conclusions to show the effect of bone-derived OPN on breast cancer metastases in vivo. Some studies have used OPN knockout mice to
explore the metastatic behavior of breast cancer in relation to OPN, however these models have all excluded the effect on the immune system as they have used nude mice. Repeating these studies in an OPN knockout mice strain using a syngeneic injection model would be interesting, as it has been hypothesized that the recruitment of macrophages to the tumor site by OPN can slow tumor growth and thus may also play a role in initially inhibiting the development of bone metastases [66, 154].

Finally, it is clear that other soluble factors within the BMCM can influence breast cancer cell migration to bone; although migration was significantly reduced in response to BMCM depleted of OPN compared to non-depleted BMCM, migratory levels were still higher than migration toward basal media. Other factors to explore include MMP-14 and ICAM-1.
7 CONCLUSIONS

This thesis investigated the role of bone-derived OPN in the metastatic behavior of breast cancer cells and their penchant for bone as a secondary growth site. Our work has shown that bone-derived OPN is integral to the metastatic cascade culminating in the establishment of skeletal breast cancer metastases. Bone-derived OPN promotes the migration of breast cancer cells to bone marrow and thus, assists in attracting breast cancer cells to bone. It also contributes to maintaining the stem-like behavior of breast cancer cells in bone marrow and thus, may promote the establishment of metastases in bone. This thesis also suggests that bone-derived OPN activates a downstream signaling response in breast cancer cells involving the phosphorylation of PRAS40 and WNK-1, as well as the activation of HSP60.

Broadly, this thesis focused on the contribution of the bone microenvironment to metastatic breast cancer. While the cancer research community has known about specific metastatic patterns and organ preferences that breast cancers exhibit for over a century, only recently have efforts focused on the role of the secondary site or “soil” in promoting and supporting metastatic growth at these sites. It is becoming increasingly clear that the microenvironment, specifically the bone, offers an optimal niche for the establishment and progression of metastases; our study shows that bone-derived soluble factors – specifically OPN – contribute to this process, possibly by interacting with stem-like breast cancer cells. This knowledge is valuable to our efforts in attempting to treat metastatic disease in breast cancer patients, as targeting the OPN-breast cancer stem cell interaction in addition to using chemotherapies and radiotherapies could help mitigate breast cancer bone metastases.
REFERENCES


52. Michigami, T., N. Shimizu, P.J. Williams, M. Niewolna, S.L. Dallas, G.R. Mundy, and T. Yoneda, *Cell-cell contact between marrow stromal cells and*


Appendix A: Animal Protocol Approval

Western

AUP Number: 2009-064
AUP Title: Role of ALDH+/CD44+ stem-like cells in breast cancer progression and treatment
Yearly Renewal Date: 10/01/2014

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2009-064 has been approved, and will be approved for one year following the above review date.
This AUP number must be indicated when ordering animals for this project.
Animals for other projects may not be ordered under this AUP number.
Purchases of animals other than through this system must be cleared through the ACVS office.
Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals.
Please consult directly with your institutional safety officers.
Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

The University of Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, London, Ontario, CANADA – N6A 5C1
PH: 519-661-2111 ext. 86768 • FL 519-661-2028
Email: auspc@uwo.ca • http://www.uwo.ca/animal
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- **B**: Biotin Label
- **N**: Nega
- **E**: Eucine

**Target:**

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**Note:**

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Appendix C: R&D Human Phospho-Kinase Array Target List

- Akt (S473)
- Akt (T308)
- AMPK alpha1 (T174)
- AMPK alpha2 (T172)
- beta-Catenin
- Chk-2 (T68)
- c-Jun (S63)
- CREB (S133)
- EGF R (Y1086)
- eNOS (S1177)
- ERK1/2 (T202/Y204, T185/Y187)
- FAK (Y397)
- Fgr (Y412)
- Fyn (Y420)
- GSK-3 alpha/beta (S21/S9)
- Hck (Y411)
- HSP27 (S78/S82)
- HSP60
- JNK pan (T183/Y185, T221/Y223)
- Lck (Y394)
- Lyn (Y397)
- MSK1/2 (S376/S360)
- p27 (T198)
- p38 alpha (T180/Y182)
- p53 (S15)
- p53 (S392)
- p53 (S46)
- p70 S6 Kinase (T421/S424)
- PDGF R beta (Y751)
- PLC gamma-1 (Y783)
- PRAS40 (T246)
- Pyk2 (Y402)
- RSK1/2/3 (S380/S386/S377)
- Src (Y419)
- STAT2 (Y689)
- STAT3 (S727)
- STAT3 (Y705)
- STAT5a (Y694)
- STAT5a/b (Y694/Y699)
- STAT5b (Y699)
• STAT6 (Y641)
• TOR (S2448)
• WNK-1 (T60)
• Yes (Y426)
Curriculum Vitae - Graciella Pio

EDUCATION

Honors BSc                   London, ON
Western University            September 2008-June 2013
• Achieved an Honors Specialization in Biology with a Major in Medical Cell Biology

RESEARCH EXPERIENCE

Honors Thesis Project, Allan Lab,
Department of Anatomy and Cell
Biology, Western University   London, ON
September 2012-April 2013
• Research area: cellular and molecular biology of metastasis
• Identified two soluble factors that attract whole population breast cancer cells to bone marrow

Obstetrics and Gynecology Clinic,
St. Michael’s Hospital        Toronto, ON
May 2011-August 2011
• Research area: reproductive and infectious diseases, maternal fetal medicine
• Research roles: managed various ongoing clinical studies, organized information sessions for potential study participants, recruited clinic patients as potential study participants, organized and analyzed data

Sinclair Lab, Department of Biology,
Western University            London, ON
September 2010-April 2011
• Research area: animal physiology, biology of arthropods in cold environments
• Responsible for general laboratory and fly maintenance

SCHOLARSHIPS and AWARDS

Scholarships
• Ontario Graduate Scholarship, (15,000/year) 2014-2015 (accepted)
• CIHR Strategic Training Program in Cancer Research and Technology Transfer Studentship ($9,050/year), 2013-2015 (accepted)
• Translational Breast Cancer Unit Studentship from the London Regional Cancer Program ($9,050/year), 2014-2015 (accepted)
• Western Graduate Scholarship ($4500/year), 2013-2015 (accepted)

Awards
• CIHR Institute of Cancer Research Travel Award for the Canadian Cancer
Research Conference ($1,000) (accepted)

- Dean’s Honor List (UWO), 2011-2013

PUBLICATIONS

Pio GM, Piaseczny M, Xia Y, Goodale D and Allan AL. Bone-derived osteopontin mediates the migration and stem-like properties of breast cancer cells. [In preparation for International Journal of Cancer]

ORAL AND POSTER PRESENTATIONS

External Presentations


Internal Presentations

- **Pio GM**, Chu JE, Xia Y and Allan AL. Identification and functional implications...

PROFESSIONAL EXPERIENCE

Teaching Assistant, Oral Histology, Western University
London, ON
September 2014-April 2015

- Course topic: histology of the oral mucosa and tissue

Teaching Assistant, Medical Sciences 4900 F/G, Western University
London, ON
September 2013-December 2014

- Course topic: laboratory techniques with focus on animal models of human disease, real time PCR, biochemical assays, histology and medical imaging

Teaching Assistant, Translational Models of Cancer 4461b, Western University
London, ON
January 2014-April 2014

- Course topic: histology, prognosis, biomarkers, imaging and sites of metastasis of main cancer types including historical perspectives of how findings in the basic sciences have led to better treatments for cancer