ROLE OF MICROCALCIFICATIONS IN BREAST CANCER BONE MIMICRY

by

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Breast microcalcifications (MCs) are insoluble calcium deposits, that are used as a diagnostic tool to detect breast cancer where up to 93% of cases of ductal carcinoma in situ (DCIS) are determined based on the presence of MCs. Breast MCs are categorized into type I (benign – calcium oxalate (OX)) and type II (malignant – calcium phosphate, hydroxyapatite (HA)) MCs. Breast lesions with the presence of malignant MCs show an increase in the expression of mesenchymal and bone markers, also termed as “bone mimicry”. Breast cancer cells with bone mimicry are known to exhibit propensity to metastasize to bone. Despite correlative evidence linking MCs to bone mimicry and bone mimicry to bone metastasis, the biological role of MCs in promoting invasive phenotype, bone mimicry and subsequent metastasis remains unknown. To address this, our laboratory has developed collagen-inspired extracellular matrices ("ECM-Mimics") recapitulating benign or malignant MC composition observed in MC-positive DCIS patients. We hypothesize that the presence of malignant microcalcifications in the primary breast tumor can promote invasive and bone mimicry phenotypes. We also hypothesize that breast cancer cells pre-conditioned to malignant MCs create a premetastatic niche in the primary breast tumor where breast cancer cells secrete cytokines and chemokines which are important for remodeling the bone microenvironment.

In this MS thesis project, we show that the ECM-mimics are able to deposit and recapitulate the composition of benign (OX) and malignant (HA) microcalcifications found in breast biopsies of cancer patients. When seeded onto the ECM-mimics containing different microcalcifications,
non-metastatic T47D breast cancer cells exhibit higher mRNA expression of mesenchymal and bone markers by qPCR analysis. The conditioned media collected from T47D cells exposed to malignant MCs show higher alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) enzyme activity, which are markers for osteoblast and osteoclast activity, respectively. Furthermore, *ex vivo* human bone explants exposed to conditioned media from the T47D cells seeded on malignant MCs also show significant increase in the bone metabolic activity, ALP enzyme activity, and TRAP enzyme activity of the bones compared to bones exposed to conditioned media from the T47D cells seeded on non-mineralized or benign MC containing ECM-mimics. These results suggest that malignant MCs may play a critical role in metastatic breast cancer progression.
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Preface

Nomenclature

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<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
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<tr>
<td>BC</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>Bi-RADS</td>
<td>Breast Imaging Reporting and Data System</td>
</tr>
<tr>
<td>BMP2</td>
<td>Bone morphogenic protein-2</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone morphogenic protein-4</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSP</td>
<td>Bone sialoprotein</td>
</tr>
<tr>
<td>CCL2</td>
<td>C-C motif chemokine ligand 2</td>
</tr>
<tr>
<td>CCN3</td>
<td>Cellular communication network factor 3</td>
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<td>CITED2</td>
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</tr>
<tr>
<td>CM</td>
<td>Conditioned media</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>C-X3-C motif chemokine receptor 1</td>
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<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor 4</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma <em>in situ</em></td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate-buffered saline</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>Enpp1</td>
<td>Ectonucleotide pyrophosphatase/phosphodiesterase 1</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptors</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
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<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>Hydroxyapatite</td>
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<td>Human epidermal growth factor receptor 2</td>
</tr>
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<td>Intercellular adhesion molecule-1</td>
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<td>IL8</td>
<td>Interleukin-8</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LCIS</td>
<td>Lobular carcinoma <em>in situ</em></td>
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<tr>
<td>MC</td>
<td>Microcalcification</td>
</tr>
<tr>
<td>MEBM</td>
<td>Mammary epithelial cell growth basal medium</td>
</tr>
<tr>
<td>Micro-CT</td>
<td>Micro-computed tomography</td>
</tr>
<tr>
<td>MPC</td>
<td>Malignant pleural effusion cells</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NM</td>
<td>Non-mineralized</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>OX</td>
<td>Calcium oxalate</td>
</tr>
<tr>
<td>p-NP</td>
<td>p-Nitrophenol</td>
</tr>
<tr>
<td>p-NPP</td>
<td>p-Nitrophosphyl phosphate</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptors</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa-B ligand</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple negative breast cancer</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>TWIST1</td>
<td>Twist family bHLH transcription factor 1</td>
</tr>
<tr>
<td>VIM</td>
<td>Vimentin</td>
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</table>
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1.0 INTRODUCTION

1.1 Overview of Breast Cancer

With more than one million cases per year, breast cancer (BC) is the most common type of cancer found in women. It affects 1-in-8 women in the United States\(^1\) leading to required treatments such as surgery, chemotherapy, radiotherapy, and hormone therapy. Within this disease, there are different subtypes based on different biological characteristics such as tumor size, lymph node involvement, expression status of different receptors such as estrogen receptors (ER), progesterone receptors (PR), human epidermal growth factor receptor 2 (HER2), and levels of protein Ki-67 (which contributes to the cancer cell growth rate)\(^2\). These breast cancer subtypes also exhibit different gene expression profiles and different degrees of aggressiveness. A “tumor grade” is usually assigned to the cancer to describe the aggressiveness on a scale of 1 to 3 based on the appearance of cancer cells compared to healthy breast cells and the growth pattern of the cancer cells\(^3\). Both the grade of the tumor and the stage of breast cancer will affect the prognosis of the patient. Grade 1 represents a slight difference in cancer cell appearance compared to normal cells and the growth is slow and well-organized, while grade 2 is moderate, and grade 3 represents significant differences in cancer cells compared to normal cells and the growth is quick and disorganized\(^4\).

The molecular subtypes of breast cancer include luminal A, luminal B, triple negative, and HER2-enriched\(^5\). Both luminal A cancer and luminal B cancer begins in the inner, luminal cells lining the mammary ducts and are most commonly classified as a low to intermediate grade (grade 1 or 2). Luminal B is usually higher in grade when compared to luminal A. Luminal A breast
cancer cells are both ER and PR positive, HER2 negative, and the expression levels of Ki-67 protein are low, which means that the cancer cell growth is slow. Luminal B is ER positive, PR positive, can be HER2 positive or negative, and the levels of protein Ki-67 are high, which means the cancer cell growth is faster compared to luminal A cancer\textsuperscript{6,7}.

Triple negative breast cancer (TNBC) is often classified as a grade 3 cancer and is a more common type of breast cancer found in women that have a BRCA1 gene mutation\textsuperscript{8}. This subtype is both hormone-receptor negative, therefore ER and PR negative, and HER2 negative. HER2-enriched breast cancer is hormone-receptor negative and HER2 positive. This subtype typically has the worst prognosis in comparison to the luminal and triple negative subtypes, but advancements in HER2 targeted therapies have improved the outcome of patients\textsuperscript{9,10}. According to the American Cancer Society, 73\% of breast cancer is luminal A, 11\% of breast cancer is luminal B, 12\% is TNBC and 4\% is HER2-enriched breast cancer\textsuperscript{11}.

1.2 Microcalcifications in Breast Cancer

Mammographic microcalcifications (MCs) are commonly used as a diagnostic tool and are often a common indicator of the early stages of breast cancer\textsuperscript{12}. Breast microcalcifications are small deposits of calcium found within breast tissue\textsuperscript{13}. Statistics show that up to 50\% of all nonpalpable breast cancers are detected exclusively through microcalcifications in mammographic evaluations and up to 93\% of cases of ductal carcinoma \textit{in situ} (DCIS) are determined based on the presence of microcalcifications\textsuperscript{14}.

A Breast Imaging Reporting and Data System (Bi-RADS) is a standardized system that is commonly used to assign a category of malignancy to the breast cancer based on characteristics
such as density, architectural distortions, and microcalcifications\textsuperscript{15}. Microcalcifications in a mammography are examined based on their morphology, size and distribution to classify their risk. The representative images of the different morphologies and distributions of microcalcifications can be seen in Figure 1. “Popcorn-like,” eggshell, or dystrophic morphologies are considered low-risk, and coarse heterogeneous or fine linear morphologies are considered high-risk. When studying the distribution of the microcalcification within the breast tissue, the order of increasing malignancy is as follows: diffuse, regional, clustered, segmental, and linear. A diffuse or regional distribution is considered more random while segmental distribution is microcalcifications found within the breast duct and linear distribution is microcalcifications found along the breast duct. It is necessary to examine all of these characteristics of the microcalcification when evaluating malignancy\textsuperscript{16}.  

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Morphology and Distribution of Microcalcifications found in Breast Tissue\textsuperscript{16}}
\end{figure}

Microcalcifications and their diagnostic markers are important in the detection of breast cancer, but recently the focus has shifted to study microcalcifications association to prognosis, recurrence and HER2 status in breast cancer patients\textsuperscript{16}. A 2-fold increase in risk of a relapse and 2.4-fold increase in mortality is shown in breast cancer patients with microcalcifications compared to those without microcalcifications\textsuperscript{17}. In addition, after analyzing data from 7317 breast cancer patients, it was found that there is a 2.5-fold higher rate of HER2 positivity in patients with microcalcifications\textsuperscript{18}. Many studies consistently highlight the link between microcalcifications and the increased risk of mortality, the increased risk of recurrence, and HER2+ tumors\textsuperscript{16, 17, 18, 19, 20}. 

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1.3 Types of Microcalcifications

There are two types of microcalcifications based on their physical and chemical properties. At present, the difference in types of microcalcifications cannot reliably be seen through mammography, which often leads to the patient requiring a biopsy\textsuperscript{16}. Type I microcalcifications are composed of calcium oxalate (OX), which have a pyramidal structure and appear amber-colored\textsuperscript{21}. This type of microcalcifications is only found in benign tumors, such as non-invasive lobular carcinoma \textit{in situ} (LCIS)\textsuperscript{14}. Type II microcalcifications are a form of calcium phosphate, known as hydroxyapatite (HA), which have irregular faces to form an ovoid or fusiform shape. This type of microcalcifications is associated with both benign or malignant tumors\textsuperscript{14}. Type I microcalcifications are termed “benign microcalcifications” and type II microcalcifications are termed “malignant microcalcifications.”

Different analysis techniques such as Raman spectroscopy, mid-Fourier transform infrared (FTIR) spectroscopy and energy dispersive X-ray microanalysis have been used in recent years to further distinguish the chemical differences between benign and malignant microcalcifications\textsuperscript{16}. A study conducted by Baker \textit{et al.}\textsuperscript{22} reported a significant negative correlation between carbonate content in microcalcifications and the tumor grade. This was determined from 236 breast microcalcifications in 110 patients through FTIR spectroscopy analysis\textsuperscript{22}. Another study conducted by Scimeca \textit{et al.}\textsuperscript{21} showed that a high content of magnesium in the crystal lattice of microcalcifications correlated to increased malignancy using energy dispersive X-ray microanalysis\textsuperscript{21}.
1.4 Microcalcifications and Expression of Mesenchymal Markers in Breast Cancer Cells in Primary Tumor

Epithelial-mesenchymal transition (EMT) is a key promoter of cancer metastasis\textsuperscript{23}. Cancer cells that undergo EMT show enhanced mobility, invasion and resistance to apoptotic stimuli\textsuperscript{24}. EMT is a process where epithelial cells develop mesenchymal properties, which could include changes in characteristic gene expression, fibroblastic morphology and increased motility, which enable cells to break through the basal membrane and travel to other places in the body\textsuperscript{25}. Through \textit{in vitro} and \textit{in vivo} studies, it has been demonstrated that breast tumors go through the EMT process, where they phenotypically lose intercellular adhesion molecules (E-cadherin) and epithelial biomarkers (cytokeratins) and they gain mesenchymal markers including vimentin (VIM), TWIST, and nuclear $\beta$-catenin\textsuperscript{23}. E-cadherin is a tumor suppressor gene that plays a role in cell adhesion as a component of the adherens junctions\textsuperscript{26}. Cytokeratins are intermediate filament proteins that provide mechanical integrity enabling a cell to withstand stress\textsuperscript{27}. Vimentin is classified as a type III intermediate filament that plays a role in cell motility and migration\textsuperscript{28}. TWIST is a transcription factor involved in embryonic development, tumorigenesis, and tumor metastasis\textsuperscript{29}. $\beta$-catenin acts as a transcription co-factor when it translocates from the cytoplasm to the nucleus to stimulate the expression of pro-growth genes and tumor formation\textsuperscript{30}.

To assess mesenchymal characteristics of breast cancer cells surrounding microcalcifications, a study conducted by Scimeca \textit{et al.} compared 4 different groups: benign lesions with microcalcifications, \textit{in situ} carcinomas with microcalcifications, infiltrating ductal carcinomas with microcalcifications and infiltrating ductal carcinomas without microcalcifications. Immunohistochemical analysis of patient samples revealed that the rate of VIM-positive cells in malignant breast lesions with microcalcifications was significantly higher
compared to benign lesions with microcalcifications. Also, when making a direct comparison between infiltrating ductal carcinomas with and without microcalcifications, the rate of VIM-positive cells is significantly higher when microcalcifications are present. The results in this study also showed translocation of β-catenin from the membrane to the cytoplasm and to the nucleus increased significantly in malignant lesions with microcalcifications. This shows that breast cancers cells surrounded by malignant microcalcifications express mesenchymal markers, suggesting these cells undergo EMT\textsuperscript{21}.

1.5 Microcalcifications and Expression of Bone Markers in Breast Cancer Cells in Primary Tumors

The mechanisms underlying the formation of microcalcifications in breast cancer still remain to be elucidated\textsuperscript{31}. There have been numerous mechanisms postulated,\textsuperscript{13} one of the hypotheses is that pathological mineralization such as those found in soft tissues like breast result from mimicking physiological mineralization mechanisms\textsuperscript{32}. Physiological mineralization is a regulated process where calcium salts are deposited during the development of hard tissues such as bone and teeth\textsuperscript{33}. The hardness and strength of bones is dependent on a tightly regulated mineralization process\textsuperscript{34}. In the process to maintain the structural matrix and strength of bones, bones are constantly being remodeled where osteoclasts are absorbing mineralized bones while osteoblasts are forming bone matrices that will become mineralized in a balanced micro-remodeling pit\textsuperscript{35}. Mineralization can become pathological if the process becomes insufficient or excessive or when minerals are deposited in soft tissue\textsuperscript{32}.  

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Some clinical studies have tested this mineralization hypothesis by investigating the expression of bone markers in the breast cancer patient biopsies in the presence and absence of microcalcification\textsuperscript{21, 36}. The bone markers studied include bone morphogenic protein-2 (BMP2), bone morphogenic protein-4 (BMP4), Runt-related transcription factor 2 (RUNX2), receptor activator of nuclear factor kappa-B ligand (RANKL), and osteopontin (OPN)\textsuperscript{21, 36}. BMP-2 and BMP-4 are known osteogenic factors that promote the differentiation of mesenchymal stem cells into osteoblasts\textsuperscript{35}. RUNX2 is a key transcription factor for osteoblast differentiation and an essential component in the formation of bone matrices\textsuperscript{37}. RANKL is a cytokine that binds to the RANK receptor to regulate osteoclast activation, differentiation and survival\textsuperscript{38}, and OPN is an extracellular matrix protein that participates in the formation of hydroxyapatite during osteogenesis\textsuperscript{39}.

Bonfiglio \textit{et. al.} showed an increase in the expression of bone markers (BMP2, BMP4, RUNX2, RANKL, and OPN) in breast lesions with microcalcifications compared to those without microcalcifications\textsuperscript{36}. The study compared 4 different groups: benign breast lesions with microcalcifications, benign lesions without microcalcifications, malignant breast lesions with microcalcifications, and malignant lesions without microcalcifications. It was shown that expression of BMP2, BMP4, and OPN increased in breast lesions with microcalcifications compared to breast lesions without microcalcifications. It was also shown that the expression of RUNX2 in malignant lesions with microcalcifications is elevated compared to the other three groups, while the expression of RANKL in malignant lesions with microcalcifications was shown to be significantly higher compared to benign lesions with and without microcalcifications\textsuperscript{36}. This study strengthens the claim that these breast cells begin to display an osteoblast-like phenotype.
and provides correlative evidence that links microcalcifications to bone mimicry. Cancer cells that display bone mimicry have a higher chance of metastasizing to bone\textsuperscript{21, 36, 40}.

1.6 Breast Cancer Metastasis to Bone

The leading cause of breast cancer-associated death is distant metastasis. Of the half million patients that die from breast cancer each year, over 90% die from metastasis\textsuperscript{41}. Metastasis occurs when cancer cells move away from the primary tumor, spread systematically, and settle in secondary sites\textsuperscript{41}. Bone, liver, lung, and brain metastases are common sites in breast cancer. Figure 2 shows the most common locations of distant metastasis in breast cancer based on the molecular subtype: luminal, HER2, and TNBC. Bone is the most common site of distant metastasis in luminal breast cancer at 49\%\textsuperscript{42}.

![Figure 2. Locations of Breast Cancer Metastasis Based on Molecular Subtypes\textsuperscript{42}](image)

Breast cancer cells metastasizing to bone show enhanced expression of bone markers. It has been shown that the expression of 15 proteins in breast cancer cells promotes metastasis to bone including: IL-11, TWIST1, OPN, CXCR4, ICAM-1, cadherin-11, osteoactivin, bone sialoprotein (BSP), CCN3, CCL2, CITED2, CTGF, CX3CR1, adrenomedullin, and Enpp1\textsuperscript{40}. These proteins are normally expressed in the bone microenvironment and play a role in one or more of
the following functions: cell adhesion, cell proliferation and differentiation, chemokine signaling, and/or bone mineralization and remodeling\textsuperscript{40}. Table 1 summarizes the functional role of each of the 15 factors listed and their possible involvement in bone metastasis\textsuperscript{40}.

<table>
<thead>
<tr>
<th>Functional Role</th>
<th>Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Adhesion</td>
<td>Cadherin 11, ICAM-1, CX\textsubscript{3}CR1, Osteopontin, Osteoactivin, CCN3</td>
</tr>
<tr>
<td>Cell Proliferation &amp; Differentiation</td>
<td>IL-11, CTGF, CITED2, TWIST1, Adrenomedullin, CCN3, Osteoactivin, Enpp1</td>
</tr>
<tr>
<td>Chemokine Signaling</td>
<td>CCL-2, CXCR4, CX\textsubscript{3}CR1, Osteopontin</td>
</tr>
<tr>
<td>Bone Mineralization &amp; Remodeling</td>
<td>BSP, Enpp1</td>
</tr>
</tbody>
</table>

The expression of these osteogenic genes allows breast cancer cells to mimic osteogenic cells, termed as “bone mimicry.” This bone mimicry can lead to the promotion of the homing and survival of breast cancer cells in the bone microenvironment. These cells have a preference to metastasize to bone facilitating osteotropic metastasis. This provides correlative evidence that links bone mimicry to bone metastasis\textsuperscript{40}.

1.7 Summary

Overall, breast microcalcifications are commonly used as a diagnostic tool where they are examined based on their size, morphology, and distribution. Breast MCs are categorized into two categories based on their chemical composition: type I (benign – calcium oxalate) and type II (malignant – hydroxyapatite). When breast cancer cells are surrounded by these malignant microcalcifications, they begin to gain mesenchymal markers (VIM, TWIST, N-cadherin) and bone markers (RUNX2, RANKL, BMPs, OPN) that are normally expressed in the bone
microenvironment. Breast cancer cells expressing bone markers is termed “bone mimicry” and the breast cancer cells will exhibit a propensity to bone. As shown in Figure 2, bone remains a major site of distant metastasis across all molecular subtypes of breast cancer. BC cells have been shown to express 15 proteins that are normally expressed in the bone microenvironment which promotes metastasis to bone. The link between microcalcifications and bone mimicry and the link between bone mimicry and bone metastasis is known, but what remains unknown is the biological role that microcalcifications may play in metastatic breast cancer progression to promote an invasive phenotype, bone mimicry, and subsequent metastasis to bone.
2.0 OBJECTIVE

Currently, there is evidence that links the presence of microcalcifications to bone mimicry and bone mimicry to bone metastasis; however, the biological role of microcalcifications in promoting an invasive phenotype and subsequent metastasis remains unknown. With this gap, we look to examine whether microcalcifications play a direct role in breast cancer progression and pre-metastatic niche promoting bone metastasis. With this in mind, we hypothesize that in the primary tumor, the presence of malignant microcalcifications creates a microenvironment where the breast cancer cells acquire a bone-like phenotype. We also hypothesize that breast cancer cells pre-conditioned to malignant MCs create a premetastatic niche in the primary breast tumor where breast cancer cells secrete factors which are important for remodeling the bone microenvironment.

To test this hypothesis, three research questions were posed as follows:

1. Can we engineer extracellular matrices that deposit microcalcifications mimicking the composition of benign (OX) and malignant (HA) microcalcifications as seen in breast cancer patient biopsies?

2. Can the exposure of breast cancer cells to malignant (HA) microcalcifications induce mesenchymal and bone mimicry phenotypes?

3. Can the factors secreted by the breast cancer cells preconditioned to malignant (HA) microcalcifications affect the bone microenvironment?

Experiments were designed to test the three major research questions posed. A schematic diagram capturing the experimental design to study the role of microcalcifications in breast cancer bone mimicry is shown in Figure 3.
Figure 3. Schematic diagram of the overall experimental design to study the role of microcalcifications in breast cancer bone mimicry.
3.0 MATERIALS AND METHODS

3.1 Materials

Table 2. List of Materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Company, Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3.2 Fabrication of ECM-mimics</strong></td>
<td></td>
</tr>
<tr>
<td>Chitosan</td>
<td>Sigma C3646</td>
</tr>
<tr>
<td>Gelzan CM (Gellan Gum)</td>
<td>Sigma G1910</td>
</tr>
<tr>
<td><strong>3.3 Preparation of Buffers</strong></td>
<td></td>
</tr>
<tr>
<td>Tris(hydroxymethyl) aminomethane</td>
<td>Sigma 252859</td>
</tr>
<tr>
<td>Maleic Acid</td>
<td>Acros Organics 125230010</td>
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<td>Sodium Hydroxide (NaOH)</td>
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<td>Calcium Chloride</td>
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<td>Sodium Oxalate</td>
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<td>Sigma S9888</td>
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<tr>
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<td>Sigma S8875</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>Sigma P5405</td>
</tr>
<tr>
<td>Di-potassium Hydrogen Phosphate Trihydrate</td>
<td>Sigma P5504</td>
</tr>
<tr>
<td>Magnesium Chloride</td>
<td>Fisher Chem 428/M-13448</td>
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<tr>
<td>1.0M Hydrochloric Acid</td>
<td>Sigma 258148</td>
</tr>
<tr>
<td>Sodium Sulfate</td>
<td>Sigma 238597</td>
</tr>
<tr>
<td><strong>3.6 Cell Lines and Culture Conditions</strong></td>
<td></td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle Medium</td>
<td>Mediatech, Manassas, VA, USA – 10-013-CV</td>
</tr>
<tr>
<td>Penicillin Streptomycin Solution</td>
<td>Mediatech, Manassas, VA, USA – 30-002-CI</td>
</tr>
<tr>
<td>Fetal Bovine Serum</td>
<td>Gibco, Mexico – 10437-028</td>
</tr>
<tr>
<td>TrypLE Express</td>
<td>Gibco, Mexico – 12604-021</td>
</tr>
<tr>
<td>Mammary Epithelial Cell Growth Basal Medium (MEBM)</td>
<td>Lonza, Walkersville, MD, USA – CC-3151</td>
</tr>
<tr>
<td>MEGM Mammary Epithelial Cell Growth Medium SingleQuots Kit (0.4% BPE, 0.1% hEGF, 0.1% Insulin, 0.1% Hydrocortisone, and 0.1% GA-1000)</td>
<td>Lonza, Walkersville, MD, USA – CC-4136</td>
</tr>
<tr>
<td>Trypsin EDTA</td>
<td>Mediatech, Manassas, VA, USA – 25-053-CI</td>
</tr>
<tr>
<td><strong>3.7 Human Bone Explant Culture and Maintenance</strong></td>
<td></td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA) Fraction V</td>
<td>Fisher Scientific, NJ, USA – BP1600</td>
</tr>
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</table>
### 3.10 RNA Extraction & Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

<table>
<thead>
<tr>
<th>GeneJET RNA Purification Kit</th>
<th>Thermo Fisher Scientific, Vilnius, Lithuania – K0731</th>
</tr>
</thead>
<tbody>
<tr>
<td>iTaq Universal SYBR Green One-Step Kit</td>
<td>Bio-Rad, CA, USA - 1725151</td>
</tr>
<tr>
<td>Primers</td>
<td>Integrated DNA Technologies, OR, USA</td>
</tr>
</tbody>
</table>

#### 3.11 Resazurin Reduction Assay

Resazurin, sodium, salt | Acros Organics 418900010 |

#### 3.12 Human Bone Explant Assay

Paraformaldehyde 32% solution | EMS 15714-S |

#### 3.13 Alkaline Phosphatase (ALP) Enzyme Activity Assay

1-step PNPP Substrate System | Sigma 37621 |

#### 3.14 Tartrate-Resistant Acid Phosphatase (TRAP) Enzyme Activity Assay

Acid Phosphatase Leukocyte Kit | Sigma 387A-1KT |

### 3.2 Fabrication of ECM-mimics

Extracellular matrix (ECM)-mimics were fabricated as described previously\(^43\). The solutions used to fabricate the ECM-mimics include 1% (w/v) chitosan (a cationic polymer) in 1% (w/v) acetic acid and 1% (w/v) gellan gum (an anionic polysaccharide). The solutions were introduced in 10 mL syringes positioned perpendicular to each other and fed through a 3-way stopcock with a needle connected to the end (as seen in Figure 4a). The syringes were wrapped in heating jackets set to 70°C and secured into syringe pumps set to a flow rate of 50 mL/h. As the solutions are fed through the stopcock, the chitosan and gellan gum react to form polyelectrolytic complex fibers. Fibers flowed from the needle, as seen in Figure 4b, and were collected vertically onto a plastic coverslip from left to right until 10 mL of each solution was used. The syringes were refilled with 10 mL of each solution, the slide was then rotated 180 degrees, and the process was repeated to create an ECM-mimic hydrogel (Figure 4d). The ECM-mimics were air-dried over 2 days and stored at ambient temperature until further use (Figure 4e).
3.3 Preparation of Buffers

To deposit benign, calcium oxalate microcalcifications into ECM-mimics, an oxalate buffer was prepared. A Tris-maleate buffer solution was prepared first from a 0.2M solution of Tris acid maleate (24.2g of tris(hydroxymethyl) aminomethane + 23.2g of maleic acid in 1L) and a 0.2M solution of sodium hydroxide (NaOH). Fifty mL of the 0.2M solution of Tris acid maleate and 54mL of 0.2M NaOH were combined and diluted to a total of 200mL to create the Tris-maleate buffer solution with a neutral (7.4) pH. This solution was then used to prepare the 2mM calcium chloride and 2mM sodium oxalate buffers. The oxalate buffer was prepared by mixing equal amounts of the 2mM calcium chloride and 2mM sodium oxalate buffers.

To deposit malignant, hydroxyapatite microcalcifications, 1X simulated body fluid (SBF) was prepared\textsuperscript{44}. In a plastic bottle, 350 mL of ion-exchanged, distilled water was heated to 36.5°C
(± 1.5°C). Once heated to the appropriate temperature, the reagents in Table 3 were added one by one in sequential order, making sure each reagent was completely dissolved before adding the next reagent.

### Table 3. List of Reagents for SBF Buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride</td>
<td>4.0175 g</td>
</tr>
<tr>
<td>Sodium Hydrogen Carbonate</td>
<td>0.1775 g</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>0.1125 g</td>
</tr>
<tr>
<td>Di-potassium Hydrogen Phosphate Trihydrate</td>
<td>0.1155 g</td>
</tr>
<tr>
<td>Magnesium Chloride</td>
<td>0.1555 g</td>
</tr>
<tr>
<td>1.0M Hydrochloric Acid</td>
<td>19.5 mL</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>0.146 g</td>
</tr>
<tr>
<td>Sodium Sulfate</td>
<td>0.036 g</td>
</tr>
</tbody>
</table>

Once the sodium sulfate was completely dissolved, the electrode of a pH meter was inserted into the solution. With the solution of the temperature maintaining between 35°C and 38°C (preferably 36.5°C ± 0.5°C), Tris-reagent (Tris-hydroxymethyl aminomethane) was added until the pH became 7.30 ± 0.05. Making sure the temperature was maintained at 36.5°C ± 0.5°C, Tris-reagent was added until the pH reached 7.45. Once the pH had risen to 7.45 ± 0.01, 1M hydrochloric acid (HCl) was added dropwise to the solution until the pH decreased to 7.42 ± 0.01. Alternating the addition of Tris-reagent and 1M HCl, Tris-reagent was dissolved in the 7.42-7.45 pH range until a total of 3.059g of Tris-reagent had been added to the solution. Once all the Tris-reagent was dissolved, the pH of the solution was dropped to 7.40 using 1M HCl and the temperature was adjusted to exactly 36.5°C on the condition that the rate of the solution temperature did not increase or decrease by 0.1°C/min. Ion-exchanged, distilled water was added to make up the volume to 500mL. The SBF was checked for any precipitate and placed in 4°C until further use.
3.4 Depositing Microcalcifications on ECM-mimics

Each dried ECM-mimic (0.75cm x 0.5cm) was placed in a 24-well plate where it was sterilized in 70% ethanol under UV for 1 h, washed with Dulbecco’s phosphate-buffered saline (DPBS) three times and then hydrated in DPBS at 37°C for 3 h. Subsequently, the ECM-mimics were placed in 50 mL tubes with a corresponding oxalate or SBF buffer over 10 days under shaking conditions at 37°C. The preparation of the buffers is described in Section 3.3. To deposit malignant, hydroxyapatite microcalcifications into the ECM-mimics, the ECM-mimics were placed in 30 mL of SBF. To deposit benign, calcium oxalate microcalcifications into the ECM-mimics, the ECM-mimics were placed in 30 mL of the oxalate buffer. Figure 5 below provides a schematic diagram of the process to deposit microcalcifications into the ECM-mimics.

Figure 5. Process to Deposit Micocalcification into the ECM-mimics

3.5 Characterization of benign (OX) and malignant (HA) MCs by RAMAN Spectroscopy

Three samples of each ECM-mimic: malignant (HA), benign (OX), and non-mineralized were used to examine the chemical composition of the microcalcifications. The samples were flash-frozen in liquid nitrogen, lyophilized, and stored at ambient temperature until further use. A
XploRA PLUS confocal Raman microscope (HORIBA Scientific, NJ, USA) and LabSpec6 software (Spectroscopy Suite Software, Version 6, HORIBA Scientific) was used to perform Raman spectroscopy on the ECM-mimics. A 638 nm laser, with a grating of 1800 gr/mm and a 10% filter, was used to set the spectral range between 100 cm\(^{-1}\) and 3800 cm\(^{-1}\). Setting the objective to 100x, the point laser was used to determine an area of the ECM-mimic where there was a high density of microcalcification. Silicone was used as a standard with a peak of 520.7 cm\(^{-1}\) for calibration, and the Raman shifts were corrected accordingly. A spectrum from three separate samples were taken from each experimental group to display reproducibility within the group. The Raman spectrum of the non-mineralized ECM-mimics was used as the baseline correction curve to correct for any shifts that may be seen due to the chemical makeup of the ECM-mimics rather than the microcalcifications.

### 3.6 Cell Lines and Culture Conditions

T47D cells are a human, non-metastatic ER+ (luminal) breast cancer cell line. T47D cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) containing 1% Penicillin Streptomycin solution (PenStrep) and 10% Fetal Bovine Serum in 5% CO\(_2\) at 37°C. Cells were passaged at 70% confluency using TrypLE Express.

Patient-derived malignant pleural effusion cells (MPCs) were provided by Dr. Donnenberg (University of Pittsburgh, School of Medicine) and maintained in Mammary Epithelial Cell Growth Basal Medium (MEBM) containing 0.4% BPE, 0.1% hEGF, 0.1% Insulin, 0.1% Hydrocortisone, and 0.1% GA-1000 in 5% CO\(_2\) at 37°C. Cells were passaged at 70% confluency using 0.25% Trypsin EDTA.
3.7 Human Bone Explant Culture and Maintenance

Human bone explants were obtained from Dr. Vera Donnenberg (University of Pittsburgh, School of Medicine). The bone pieces were healthy human bones from the lumbar vertebrae. The bones were cleaned (Figure 6) and aliquoted into 50 mL tubes where they remained completely submerged in DMEM containing 5 mg/mL of Bovine Serum Albumin (BSA) Fraction V and 1% Penicillin Streptomycin Solution (PenStrep). The bones were stored at 4°C until being used for an assay. Media changes occurred every 2-3 days. To cut the bones into smaller pieces, a small amount of bone media was used to cover the bottom of a tissue culture plate and forceful, downward cuts were made on the bone using a scalpel avoiding unwanted trauma to the bones. A resazurin reduction assay was conducted once every 2 weeks to test the viability of the bones (Section 3.11).

Figure 6. Cleaned Human Bone Explants
3.8 T47D Cell Seeding on ECM-mimics

On the tenth day of depositing microcalcification, the ECM-mimics were removed from their respective buffers and placed into a non-adhesive, sterile 24-well cell culture plate for seeding. Four experimental groups were defined to study the role of microcalcification including malignant (HA) ECM-mimics, benign (OX) ECM-mimics, control (NM) ECM-mimics, and 2D monolayer control where T47D were cultured in 24-well plates.

To prepare the ECM-mimics, non-mineralized ECM-mimics were sterilized for 1 h and hydrated for 3 h prior to the seeding. The mineralized ECM-mimics were removed from the 50mL tubes and placed in wells. Excess moisture or droplets was carefully removed from the ECM-mimics using a pipette.

To prepare the cell suspension for seeding, T47D cells were trypsinized, counted and suspended on T47D cell culture media as a concentration of 200,000 cells per 20 µL. The corresponding amount of master mix was aliquoted and centrifuged for 3 min at 1200 rpm. The media was removed and the cells were resuspended in the required volume of media.

Ten µL of the cell suspension was seeded onto each ECM-mimic and incubated at 37°C for 15 min repeating cell seeding cycle one more time with another 10 µL of the cell suspension followed by incubation at 37°C for 5 min. The plate was removed from the incubator for a second time, and 20 µL of media was pipetted onto the corner of the ECM-mimic. The plate was then incubated for 1 h, followed by addition of 500 µL of media to each well.
The cells were cultured on 2D or different ECM-mimics (n=9/experimental group) over 6 days. Media changes occurred once every 2 days. The conditioned media (CM) was collected and used for assays or flash frozen immediately at -80°C for future use. Fresh media (500 μL) was carefully added to each well. On day 6, the ECM-mimics were either fixed for immunofluorescent staining or processed for RNA extraction (Figure 7).

3.9 Processing ECM-mimics for RNA Extraction

On day 6 of the culture, the ECM-mimics were processed for RNA extraction. To start, the media was collected from each well, and the cell-seeded ECM-mimics were washed with DPBS. The ECM-mimics were then transferred to a new 24-well plate where they were trypsinized with TrypLE. The trypsin solution was swished rapidly to detach the cells from the ECM-mimics and subsequently neutralized with media. Three ECM-mimics within an experimental group were pulled together to obtain enough RNA for one sample; therefore, the cell suspension of three separate wells was placed in a microcentrifuge tube and centrifuged down to a pellet. Without
disrupting the pellet, the solution was removed and discarded. Lysis buffer (obtained from the GeneJET RNA Purification Kit) was added to the tubes and the pellet was resuspended. The samples were now prepared for RNA extraction or stored in -80°C for later use.

3.10 RNA Extraction and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

The ECM-mimics were examined under a Zeiss PrimoVert brightfield microscope (Carl Zeiss Microscopy LLC, NY, USA) qualitatively and three cell-seeded ECM-mimics were pulled together to obtain a similar number of cells for each replicate of extracted RNA (Section 3.9). Total RNA was extracted from each of the pulled samples using the GeneJET RNA Purification Kit according to the manufacturer’s protocol provided. Quantitative PCR was performed using a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, CA, USA) and the iTaq Universal SYBR Green One-Step Kit (BioRad 1725151). Three samples (n=3) were used for each of the following experimental groups: malignant (HA) ECM-mimics, benign (OX) ECM-mimics, control (NM) ECM-mimics, and 2D control of breast cancer cells. GAPDH was used as the internal control. The primer sequences (Integrated DNA Technologies, OR, USA) used for the mesenchymal markers (VIM and TWIST), bone markers (RUNX2 and BMP2), and cytokine markers (IL6, IL8, and RANKL) along with GAPDH are seen in Table 4 below. The delta-delta Ct method ($2^{-\Delta\Delta Ct}$) was used to calculate the relative fold changes of the gene expressions for the samples. Duplicates of the samples were run on the plate for technical replicates. The Ct values of the duplicate samples was averaged. The $\Delta$Ct of the sample is the difference between the average Ct values of the sample (n=2) and the average Ct values of the GAPDH within the 3 samples of
the experimental group (n=6). The ΔΔCt is the difference between the ΔCt of the sample and the average ΔCt of the control (NM) ECM-mimic samples. Two independent experiments were conducted to perform qRT-PCR and both independent results showed reproducibility.

3.11 Resazurin Reduction Assay for Measuring Metabolic Activity of Human Bone Explants

In this assay, living cells reduce resazurin, a nonfluorescent dye, to resorufin, a strongly fluorescent dye, through mitochondrial reductase. The metabolic activity of the human bone explants was measured using a spectrophotometer where the fluorescence intensity is directly proportional to the number of viable cells. To prepare for the assay, human bone explants were cleaned, cut (as described in Section 3.7) and weighed. Bones of similar weight were placed in 1.5
mL Eppendorf tubes. A 500 μL resazurin solution stock (10X) (0.1 mg/mL of resazurin (salt) in DPBS) diluted in bone media (DMEM + 5 mg/mL BSA Fraction V + 1% PenStrep) (10% v/v) was added to each tube containing a bone explant. The tubes were attached to a rotator to ensure homogeneity and placed in an incubator (in 37°C at 5% CO₂) for 4 h. After 4 h, 100 μL of the resazurin solution was taken from each tube and was placed into a 96-well plate in duplicates. The fluorescence (Excitation: 530 nm, Emission: 590 nm) was measured on a SpectraMax M5 spectrophotometer (Molecular Devices LLC, CA, USA). The fluorescent readings of the experimental groups were compared against the blank solution (10% resazurin solution with no cells).

### 3.12 Human Bone Explant Assay

Once the viability of human bone explants was confirmed with the resazurin reduction assay, human bone explants were randomized into groups of 4 based on similar weight and baseline metabolic activity and placed in a 96-well plate. The experimental groups were as follows: conditioned media (CM) collected from T47D cells seeded on malignant (HA) ECM-mimics, benign (OX) ECM-mimics, control (NM) ECM-mimics, 2D monolayers, and control bones in bone media without cancer cell conditioned media (n=4 for each group). A 50/50 dilution (100 μL) of cell conditioned media (collected from the cancer cell seeding on different ECM-mimics) and bone media were placed in each well with a bone. The plate was placed in an incubator (in 37°C at 5% CO₂) under shaking conditions for 6-days to expose the human bone explants to cancer cell conditioned media (100μL) from different ECM-mimics or 2D cultures. Media changes occurred once every 2 days. The conditioned media exposed to bones was again collected, labeled
as “Bone CM”, and used for assays or flash frozen immediately at -80°C for future use. Fresh media (100μL) was replaced in each well. On day 6, the resazurin reduction assay was repeated (Section 3.11). The human bone explants were then washed with DPBS and fixed in 4% paraformaldehyde for 24 h. The bone samples were stored in 70% ethanol for future analysis (Figure 8). This protocol was developed in our lab using previous ex-vivo model systems of cancer-bone cell interactions for reference46.

![Experimental Groups:]
- Malignant (HA) Conditioned Media
- Benign (OX) Conditioned Media
- Control (NM) Conditioned Media
- 2D BC Cell Conditioned Media
- Control Bone without Conditioned Media

Figure 8. Exposing human bone explants to breast cancer cell conditioned media

### 3.13 Alkaline Phosphatase (ALP) Enzyme Activity Assay

Alkaline Phosphatase (ALP) assay was performed to measure activity of ALP enzyme, which is a marker for osteoblast activity and bone formation46. The assay was conducted using the conditioned media collected from breast cancer cells (T47D as well as patient-derived MPCs) exposed to the ECM-mimics (BC CM) and the conditioned media collected after culturing the human bone explants in 50% cancer cell conditioned media (Bone CM). An ALP enzyme substrate solution (1-step PNPP (p-nitrophenyl phosphate) Substrate System), pH ~8, and conditioned media from different groups were combined in a 2:1 ratio in a 96-well plate. The plate was placed in an
incubator (in 37°C at 5% CO₂) for 30 minutes where the ALP enzyme hydrolyzed p-nitrophenyl phosphate (p-NPP) to p-nitrophenol (p-NP)⁴⁷. The absorbance was measured at 405 nm wavelength using a SpectraMax M5 spectrophotometer (Molecular Devices LLC, CA, USA). The absorbance measurements were analyzed by subtracting the average background absorbance of the ALP enzyme substrate alone and subsequently dividing the absorbance by the extinction coefficient of p-NP (ε₄₀⁵ = 12,500 mol⁻¹ L⁻¹ cm⁻¹) to determine the concentration of p-NP hydrolysis product (mol/L). The rate of substrate hydrolysis (mol/L*min), or the ALP enzyme activity, was then calculated using the incubation time of 30 min⁴⁶.

\[
\text{ALP enzyme activity} \left( \frac{\mu\text{mol}}{L \times \text{min}} \right) = \frac{\text{absorbance} \times 10^6 \times 1 \text{ cm}}{\text{extinction coefficient} (\varepsilon_{405})(M^{-1} \text{ cm}^{-1}) \times \text{time} (\text{min})}
\]

### 3.14 Tartrate-Resistant Acid Phosphatase (TRAP) Enzyme Activity Assay

Tartrate-Resistant Acid Phosphatase (TRAP) assay was performed to measure the activity of the TRAP enzyme, which is a marker for osteoclast activity and bone resorption⁴⁶. The assay was conducted using the conditioned media from breast cancer cells exposed to different ECM-mimics (BC CM) and the conditioned media collected from human bone explant assay (Bone CM). A TRAP enzyme substrate solution (1-step PNPP Substrate System and Acid Phosphatase Leukocyte Kit), pH 5.5, and conditioned media were combined in a 2:1 ratio in a 96-well plate. The plate was placed in an incubator (in 37°C at 5% CO₂) for 60 minutes. Sodium tartrate in the TRAP solution converted p-NPP to p-nitrophenol. The reaction was stopped using 1 M NaOH where p-nitrophenol was converted to p-nitrophenolate to shift the pH to alkaline pH and the absorbance was measured at 405nm wavelength using a SpectraMax M5 spectrophotometer.
(Molecular Devices LLC, CA, USA). The absorbance measurements were analyzed by subtracting the average background absorbance of the TRAP enzyme substrate alone and subsequently dividing the absorbance by the extinction coefficient of p-NP ($\varepsilon_{405} = 12,500 \text{ mol}^{-1} \text{ L}^{-1} \text{ cm}^{-1}$) to determine the concentration of p-NP hydrolysis product (mol/L). The rate of substrate hydrolysis (mol/L*min), or the TRAP enzyme activity, was then calculated using the incubation time of 60 min.\(^{46}\)

$$TRAP \text{ enzyme activity} \left( \frac{\mu \text{mol}}{L \times \text{min}} \right) = \frac{\text{absorbance} \times 10^6 \times 1 \text{ cm}}{\text{extinction coefficient} (\varepsilon_{405})(M^{-1} \text{cm}^{-1}) \times \text{time (min)}}$$

**3.15 Micro-computed tomography (micro-CT) Analysis**

To evaluate the bone volume and architecture of the fixed bone samples after exposure of human bone explants to BC CM from different ECM-mimics (Section 3.12), micro-CT analysis was conducted by Dr. Hongshuai Li (University of Iowa, Department of Orthopedics and Rehabilitation). The micro-CT scans were performed using a Skyscan1272 micro-CT (Micro Photonics Inc, PA, USA) with the following settings: energy 70KV, intensity 142μA, integration time 200ms, and isotropic voxel size of 10μm. During image acquisition, a 0.5mm filter was applied. Raw data was reconstructed to 2D images by CT reconstruction software, NRecon (Nrecon Software, Bruker Corporation). Regions of interest including the trabecular bones within the bone samples were defined for analysis in CT-Analyser (Bruker Micro-CT Software, Blue Scientific). Multiple bone microarchitectural parameters were determined for analysis including bone fraction (bone volume/tissue volume (BV/TV)). 3D representative images were rendered using CTVol (3D.SUITE Software, Bruker Corporation) to display reconstructed results.
3.16 Statistical Analysis

GraphPad Prism 9.1 (GraphPad Software, Inc.) was used to conduct statistical analysis on all data. The data was represented as mean ± standard error of mean (SEM). A one-way analysis of variance (ANOVA) and Tukey’s multiple comparison test were used to determine any statistical significance between the means of the independent experimental groups. The statistical differences were indicated as follows: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
4.0 RESULTS AND DISCUSSION

4.1 ECM-mimics recapitulate chemical composition of benign (OX) and malignant (HA) microcalcifications found in breast cancer patients.

As described in Section 3.2, chitosan and gellan gum interacted to form fibrous strands and were collected onto a plastic coverslip. This creates a highly aligned, fibrous, three-dimensional hydrogel that mimics a collagen-like hierarchical structure\(^{43}\). The hydrogels are able to mimic native collagen displaying a dark and light banding pattern at the nanoscale and fibrils at the microscale\(^{43}\). Collagen is a major extracellular matrix component that acts as a template for inorganic mineral deposition in hard tissues such as bone\(^{49}\) and is one of the major ECMs deposited in breast cancer patients\(^{50}\). These fibrous hydrogels also recapitulate soft tissue microenvironment present in breast cancer and are hereafter termed as “ECM-mimics.”

Raman spectroscopy was conducted on malignant (HA) ECM-mimics, benign (OX) ECM-mimics and non-mineralized (NM) ECM-mimics. As seen from Figures 9a-9c, the malignant (HA) ECM-mimics, benign (OX) ECM-mimics, and non-mineralized ECM-mimics (n=3 for each group) showed very similar Raman shifts within their respective groups, confirming reproducibility of deposition of different microcalcifications into the ECM-mimics. Figures 10a and 10c displays the Raman shifts of each experimental group, which is representative of their chemical structure. The malignant (HA) ECM-mimics showed a Raman shift at 963 cm\(^{-1}\) and a broad band around 1000 cm\(^{-1}\) with distinguishable peaks at 1039 cm\(^{-1}\) and 1075 cm\(^{-1}\) (Figure 10a). The benign (OX) ECM-mimics exhibited shifts at 914 cm\(^{-1}\), 1490 cm\(^{-1}\), and 1652 cm\(^{-1}\) (Figure 10c).
Figure 9. Reproducibility of ECM-mimics (a) Malignant (HA) ECM-mimic reproducibility (b) Benign (OX) ECM-mimic reproducibility (c) Control (NM) ECM-mimic reproducibility

Malignant microcalcifications found in breast cancer patient biopsies (inset in Figure 10b shows hematoxylin and eosin staining) show a shift at 960 cm\(^{-1}\) along with an overlapping band seen at 1028 cm\(^{-1}\), 1061 cm\(^{-1}\) and 1075 cm\(^{-1}\) (Figure 10b)\(^{51}\). This corresponds closely to the chemical composition of the microcalcifications in the malignant (HA) ECM-mimics (963 cm\(^{-1}\) and a broad band around 1000 cm\(^{-1}\) with distinguishable peaks at 1039 cm\(^{-1}\) and 1075 cm\(^{-1}\) (Figure 10a)). The shift observed at 960 cm\(^{-1}\) represents the \(v_1\) phosphate-oxygen stretching vibration and the 1028 cm\(^{-1}\), 1061 cm\(^{-1}\), and 1075 cm\(^{-1}\) shifts represent the six \(v_3\) phosphate-oxygen stretching vibrations (Table 5)\(^{52}\). Benign (OX) microcalcifications in patient biopsies show vibrational features characteristic of calcium oxalate dihydrate where Raman shifts appear at 912 cm\(^{-1}\), 1477 cm\(^{-1}\), and 1632 cm\(^{-1}\) (Figure 10d)\(^{51}\). These results are similar to the Raman shifts of the calcium oxalate microcalcification deposited on the benign (OX) ECM-mimics (914 cm\(^{-1}\), 1490 cm\(^{-1}\), and 1652 cm\(^{-1}\)) (Figure 10c). The Raman shifts in benign microcalcifications represent the carbon-carbon stretching (912 cm\(^{-1}\)) and the carbon-oxygen symmetric and asymmetric stretching within the chemical structure of the calcium oxalate dihydrate (Table 5)\(^{52}\).
Figure 10. Raman spectra comparison of (a) malignant (HA) and (c) benign (OX) MCs in ECM-mimics vs. (b) malignant (HA) and (d) benign (OX) MCs in breast biopsies

Table 5. Comparison of Raman shifts between microcalcifications deposited in ECM-mimics and microcalcifications observed on breast biopsies

<table>
<thead>
<tr>
<th>Malignant (HA) MCs</th>
<th>Benign (OX) MCs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental Raman Shift (cm⁻¹)</strong></td>
<td><strong>Literature Raman Shift (cm⁻¹)</strong></td>
</tr>
<tr>
<td>962.8</td>
<td>960</td>
</tr>
<tr>
<td>Broad band ~1000 w/ peaks at 1039.2 and 1074.5</td>
<td>Overlapping peaks at 1028, 1061, and 1075</td>
</tr>
<tr>
<td><strong>Benign (OX) MCs</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Experimental Raman Shift (cm⁻¹)</strong></td>
<td><strong>Literature Raman Shift (cm⁻¹)</strong></td>
</tr>
<tr>
<td>914.4</td>
<td>912</td>
</tr>
<tr>
<td>1489.8 and 1652.2</td>
<td>1477 and 1632</td>
</tr>
</tbody>
</table>
The minor differences between the vibrational features found in the literature and our experimental results could be due to the interaction occurring between the microcalcification and the ECM-mimics, slightly shifting the peaks up or down. Overall, Raman spectroscopy results confirm that the microcalcification deposited into the ECM-mimics displayed a physiologically similar chemical composition to microcalcifications found in biopsies of breast cancer patients.

4.2 Breast cancer cells exposed to malignant (HA) microcalcifications exhibit migration.

Throughout the 6-day seeding, the T47D cells seeded onto the ECM-mimics began to exhibit characteristic behaviors when exposed to their respective groups. The control (NM) ECM-mimics formed a monolayer or sheath on the ECM-mimic with no noticeable movement seen in the cells throughout the 6 days. The cells exposed to benign (OX) ECM-mimics and malignant (HA) ECM-mimics formed small clusters or colonies. There was no noticeable movement seen in the cells exposed to the benign (OX) microcalcifications while the cells exposed to the malignant (HA) microcalcifications appeared to migrate towards the edges of the ECM-mimics and migrate off the ECM-mimics during the end of 6-day cultures. Brightfield microscopy images, taken at 4X, (Figure 11) display these characteristic behaviors of the cells exposed to different conditions.
Figure 11. T47D cell characteristics throughout 6-day seeding of (a) control (NM) ECM-mimics (b) benign (OX) ECM-mimics and (c) malignant (HA) ECM-mimics. Bright field microscopy images taken at 4X.

It has been reported that tumor cell clusters produce metastases more efficiently than single cells and tumor cell clusters can rapidly traverse and travel as a unit to distant sites from the primary tumor\textsuperscript{53}. Specifically analyzing the molecular properties of breast cancer cells, collective cell migration has been seen in carcinomas where they maintain their epithelial cell-cell adhesion when invading adjacent tissue\textsuperscript{53}. Similar properties are seen in the ER+ T47D breast cancer cells exposed to microcalcifications where throughout the 6-day culture, they start to form clusters on the ECM-mimics (Figure 11b and 11c). Evidence from in vivo studies has shown that there is
varying epithelial mesenchymal transition (EMT) hybrid states from epithelial to completely mesenchymal status explaining why tumors may maintain their cell-cell adhesion while displaying invasiveness and metastatic potential\textsuperscript{54}. It was observed during the seedings that the breast cancer cells exposed to the malignant microcalcifications tended to migrate off the ECM-mimics or seed along the border of the ECM-mimics suggesting the cells may be undergoing a degree of EMT (Figure 11c). Further analysis of the EMT markers was required to study this association.

4.3 Malignant (HA) microcalcifications induce mesenchymal phenotype and bone mimicry in breast cancer cells.

After exposure to the ECM-mimic for 6 days (Figure 7), RNA was extracted from the breast cancer cells to evaluate expression of EMT markers (TWIST, VIM) and bone markers (RUNX2, BMP2) using quantitative RT-PCR. TWIST expression, an EMT marker, increased significantly in the breast cancer cells exposed to malignant (HA) ECM-mimics compared to the cells exposed to benign (OX) ECM-mimics and control (NM) ECM-mimics. This increase in TWIST expression was seen in both the T47D cells exposed to the malignant (HA) ECM-mimics (Figure 12a) and the patient-derived MPCs exposed to the malignant (HA) ECM-mimics (Figure 12c). The mRNA expression of vimentin was also significantly higher in the patient-derived MPCs from the malignant (HA) ECM-mimics compared to the cells exposed to the benign (OX) and control (NM) ECM-mimics (Figure 12c). Similar trends were observed for expression of the bone markers, RUNX2 and BMP2. In both the T47D cells and patient MPCs exposed to malignant (HA) ECM-mimics, there is a significant increase in the expression of RUNX2 and BMP2 compared to the benign (OX) and control (NM) groups (Figure 12b and 12d).
Figure 12. Increased (a) EMT markers and (b) bone markers from mRNA of ER+ T47D cells exposed to malignant (HA) ECM-mimics. Increased (c) EMT markers and (d) bone markers from mRNA of ER+ Patient MPCs exposed to malignant (HA) ECM-mimics (Mean ± SEM, n=3, mean of each column is being compared to control (NM) ECM-mimics using ANOVA and Tukey’s multiple comparison test, *p<0.05, **p<0.01)

These results provide evidence that the exposure of breast cancer cells to malignant microcalcifications can induce mesenchymal and bone mimicry phenotypes. TWIST is one of the key transcriptional factors needed for EMT activation to induce invasion and metastasis. It has been shown that the overexpression of TWIST in luminal breast cancer cells transitioned the morphology of the cells to a basal-like phenotype and increased both the migration activity (3-fold) and invasiveness (14-fold) of the cells observed in an *in vitro* wound healing assay and Matrigel-coated Boyden chambers, respectively. It has also been shown that breast cancer cells
in the lesions with the presence of microcalcifications exhibit mesenchymal characteristics (i.e. vimentin and nuclear β-catenin)\textsuperscript{21}. Our data provide evidence that the exposure of ER+ luminal cells to malignant microcalcifications upregulates the TWIST and VIM mesenchymal markers (Figure 12a and 12c).

It has also been shown that breast lesions with the presence of microcalcifications overexpress bone markers\textsuperscript{36}. BMPs and RUNX2 are important molecular players in the bone microenvironment since they induce mesenchymal stem cell differentiation and regulate bone remodeling, respectively\textsuperscript{56, 57}. In breast cancer, it has been shown that BMP2 induces EMT and promotes tumor angiogenesis while the upregulation of RUNX2 highly correlates to bone metastatic potential\textsuperscript{56, 57}. The bone mimicry that is induced by BMP2 and RUNX2 creates a ‘seed’ that is favorable for a specific soil (the bone microenvironment)\textsuperscript{56}. The data presented in Figures 12b and 12d shows an increase in RUNX2 and BMP2 expressions when exposed to malignant microcalcifications suggesting that malignant microcalcifications can induce bone mimicry in breast cancer cells. Many studies in breast cancer to bone metastasis suggest that breast cancer cells displaying bone mimicry have a strong propensity to metastasize to bone\textsuperscript{56}. Thus, we speculate that the presence of malignant microcalcification may enhance propensity of breast cancer cells to bone metastasis by acquisition of bone mimicry and thus, enhanced homing and survival in the bone microenvironment.
4.4 Breast cancer cells exposed to malignant (HA) microcalcifications secrete factors implicated in bone metastasis.

To further support malignant microcalcification-induced bone mimicry phenotype, cytokine expressions were evaluated through quantitative RT-PCR using the RNA obtained from the breast cancer cells seeded on NM, OX and HA-ECM-mimics (Figure 7). RANKL expression was significantly higher in breast cancer cells exposed to malignant (HA) microcalcifications in comparison to the control (NM) and benign (OX) groups. This increase was seen in both the ER+ T47D cells (Figure 13a) and ER+ patient MPCs (Figure 13b) exposed to malignant (HA) ECM-mimics. There was also a significant increase of Interleukin-6 (IL6) and Interleukin-8 (IL8) mRNA expression in the ER+ T47D cells and the ER+ patient MPCs exposed to malignant microcalcifications (Figure 13a and 13b) in comparison to the benign (OX) and control (NM) groups.
Figure 13. Increased (a) cytokine mRNA expression of ER+ T47D cells exposed to malignant (HA) ECM-mimics. Increased (b) cytokine mRNA expression of ER+ patient MPCs exposed to malignant (HA) ECM-mimics (Mean ± SEM, n=3, mean of each column is being compared to control (NM) ECM-mimics using ANOVA and Tukey’s multiple comparison test, *p<0.05, **p<0.01)

The results show that malignant microcalcifications enhance cytokine expressions in breast cancer cells. IL6 plays an important role in tumor development where it promotes EMT by enhancing the recruitment of mesenchymal stem cells to human breast cancer cells and upregulates CXCR4 to promote metastasis to bone. Case studies have shown that CXCR4 expression plays a pivotal role in predicting bone metastasis in primary breast cancer. IL8 also plays a role as a pro-inflammatory cytokine, that promotes tumor cell invasion and metastasis. In addition,
RANKL is a secreted protein produced by bone marrow stromal cells, osteoblasts, and osteocytes. It has been shown that RANKL can induce the migration of normal mammary epithelial cells and in vivo studies have shown that the inhibition of RANKL/RANK signaling has reduced metastasis to bone suggesting that RANKL/RANK-regulated migration plays a role in bone metastasis. It has also been shown that malignant breast lesions exposed to microcalcifications overexpress RANKL in comparison to benign lesions with and without microcalcifications. Thus, our results showing an increase of IL6, IL8, and RANKL mRNA expression in T47D cells and patient-derived primary MPCs exposed to malignant microcalcifications (Figure 13) suggest that malignant microcalcifications induce exposed breast cancer cells to secrete cytokines implicated in bone metastasis.

To confirm these mRNA expression results, the conditioned media collected from breast cancer cells exposed to different ECM-mimics was used to further examine the ALP and TRAP enzyme activity (Figure 7). The conditioned media from the ER+ T47D cells exposed to malignant (HA) microcalcifications showed a significantly higher ALP enzyme activity and TRAP enzyme activity. The malignant (HA) conditioned media had a mean ALP enzyme activity of 2.78 µmol/L*min (Figure 14a) and a mean TRAP enzyme activity of 0.73 µmol/L*min (Figure 14b) while the enzyme activity of all other groups was below 0.6 µmol/L*min and 0.1 µmol/L*min, respectively. Similar trends were exhibited in the patient MPCs where the malignant conditioned media had significantly higher ALP and TRAP enzyme activities of 1.86 µmol/L*min and 0.68 µmol/L*min (Figure 14c and 14d) respectively.
Figure 14. Increased (a) ALP enzyme activity and (b) TRAP enzyme activity of conditioned media collected from ER+ T47D exposed to malignant (HA) microcalcification. Increased (c) ALP enzyme activity and (d) TRAP enzyme activity of conditioned media collected from patient-derived MPCs exposed to malignant (HA) microcalcification (Mean± SEM, n=4, mean of each column is being compared to control (NM) CM using ANOVA and Tukey’s multiple comparison test, **p<0.01, ****p<0.0001)

The ALP enzyme plays an important role in the formation of hard tissue and aids in the synthesis of hydroxyapatite by providing inorganic phosphate. Based on the biological activity described, an ALP enzyme activity assay is often used as a marker for osteoblast activity or bone formation. Furthermore, ALP levels have been studied as a diagnostic marker in breast cancer patients to predict bone metastasis, where elevated ALP activity could be an indication of
metastasis. A study analyzing the serum ALP activity of 388 breast cancer patients showed that patients with advanced stages of the disease displayed higher ALP levels. In addition, multiple studies have displayed a strong association between increased ALP activity and bone metastasis presentation.

TRAP enzyme activity is a marker for osteoclast activity or bone resorption. Up to 94% of breast cancer patients with bone metastasis show increased TRAP activity, suggesting TRAP levels can also be a useful diagnostic marker for breast cancer metastasis to bone. Our data shows that the conditioned media from breast cancer cells exposed to malignant microcalcifications display increased ALP and TRAP enzyme activity (Figure 14) compared to those exposed to benign or no microcalcifications. These results further suggest that malignant microcalcifications can create the pre-metastatic niche preparing breast cancer cells (the seed) to improve their interaction within the bone microenvironment (the soil).

4.5 Factors secreted by the breast cancer cells preconditioned to malignant (HA) microcalcifications affect the bone microenvironment.

To further test if conditioned media (CM) collected from breast cancer cells exposed to malignant microcalcifications affect the bone microenvironment, we used human bone explants collected from healthy individuals (Dr. Donnenberg, University of Pittsburgh, School of Medicine). Once received, the bones were cleaned, cut and weighed. As shown in Figure 15, an initial resazurin assay was performed at Week 0 when the bones were received. Subsequently, the bones with similar weights and metabolic activity were submerged in DMEM containing 5 mg/mL of BSA Fraction V and 1% Penicillin Streptomycin (PenStrep) Solution. No other factors, such as
ascorbic acid, were added to ensure the bones remained relatively dormant and bone remodeling was not activated. The human bone explants were stored at 4°C until further use. Every 2 weeks, a resazurin assay was conducted to monitor the metabolic activity of the bones. Figure 15 displays a significant decrease in the bone metabolic activity after transferring the human bone explants to DMEM with BSA Fraction V and PenStrep at Week 0. Figure 15 also shows that the human bone explants maintained a relatively constant baseline metabolic activity over the span of a month. The goal of the bone maintenance conditions was to prevent bone turnover while keeping the bones relatively viable, which was achieved. These quality controls were set to minimize the effect of confounding variables when examining the bone microenvironment. The objective of the human bone explant assay was to examine the changes in the bone microenvironment when exposed to T47D conditioned media, specifically the effect on the bone metabolic activity, ALP and TRAP enzyme activity, and bone structure when cancer cells were cultured in the absence or presence of benign or malignant microcalcifications.

Figure 15. Human bone explants remain viable over extended period of time. (Mean ± SEM, n=20, ANOVA and Tukey’s multiple comparison test, ***p<0.001, ****p<0.0001)
We compared the metabolic activity of bone explants before and after exposure to conditioned media of T47D cells cultured on 2D monolayers or on different ECM-mimics. A resazurin reduction assay was conducted on the human bone explants before and after culturing them in conditioned media for 6 days with change of media every 2 days (Figure 8). As seen in Figure 16a, all the human bone explants showed a similar average metabolic activity before their exposure to conditioned media. After exposure to the respective conditioned media for 6 days, there were no significant changes in the metabolic activity of bone explants in the control bone group without conditioned media, T47D 2D CM group, the control (NM) CM group, and the benign (OX) CM group; however, the bones cultured in the malignant (HA) CM had a significant increase in their metabolic activity. Figure 16b displays the fold change in the bone metabolic activity of bone explants in each group before and after 6-day culture in the respective conditioned media (calculated as the ratio of the final fluorescence intensity reading from the resazurin reduction assay to the initial fluorescence intensity reading). It can again be seen that the bones exposed to the malignant (HA) CM had a significantly higher (>3-fold) difference while the bones from other groups showed similar metabolic activity before and after culture in the respective conditioned media.
Figure 16. Bone metabolic activity (a) increased significantly when cultured in the malignant (HA) CM. Empty bars represent baseline bone metabolic activity before culture in the respective CM while solid bar represent bone metabolic activity after 6-day culture in the respective conditioned media; control bones without CM were cultured in DMEM containing 5mg/mL BSA and 1% Penicillin/streptomycin. (b) Bone explants exposed to malignant (HA) CM showed greater than 3 fold increased metabolic activity (Mean ± SEM, n=4, mean of each column is being compared to control (NM) CM using ANOVA and Tukey’s multiple comparison test, ***p<0.001)

In preclinical and clinical research, the metabolic activity of bones can be measured through bone turnover markers. The bone turnover rate increases during bone metastasis when cancer cells invade the bone marrow and interfere with normal bone cells to locally release cytokines and growth factors. The overall rate of bone turnover is a coupled balance between bone formation and resorption; therefore, markers of bone turnover are often separated into bone formation markers and bone resorption markers. During bone metastasis, this balance between the bone forming osteoblasts and the bone resorbing osteoclasts is disrupted causing highly elevated bone turnover activity. In this study, the viable human bone explants exposed to malignant (HA) CM showed increased metabolic activity suggesting the factors secreted by the breast cancer cells preconditioned to malignant microcalcification elevated the bone turnover
activity of the bone microenvironment. To analyze this further, osteoblastic (ALP) and osteoclastic (TRAP) enzyme activity was investigated using the conditioned media collected from the human bone explant metabolic activity assay (Figure 8).

Compared to the control bone explants cultured in DMEM containing only BSA without CM and those cultured in the control (NM) CM and the benign (OX) CM, the malignant (HA) CM showed a significantly higher ALP enzyme activity (107-fold) and TRAP enzyme activity (100-fold) than the bone explants cultured in the control (NM) CM. The average ALP enzyme activity and TRAP enzyme activity of the malignant (HA) CM group was 9.1±0.076 µmol/L*min and 1.5±0.012 µmol/L*min while the average of all the other groups was below 0.1 µmol/L*min and 0.3 µmol/L*min, respectively (Figure 17b and 17d). The average TRAP enzyme activity of the benign (OX) CM group is slightly elevated at 0.3 µmol/L*min in comparison to the control bone without CM and the control (NM) CM group, which are both less than 0.05 µmol/L*min (Figure 17d).
Figure 17. (a) Increased ALP enzyme activity (19-fold) of malignant (HA) T47D cell CM from Figure 14. (b) ALP enzyme activity increased significantly (107 fold) when human bone explants were cultured in the 50% diluted malignant (HA) CM. (c) Increased TRAP enzyme activity (12.5-fold) of malignant(HA) T47D cell CM from Figure 14. (d) TRAP enzyme activity increased significantly (100 fold) when human bone explants were cultured in the 50% dilute malignant (HA) CM (Mean ± SEM, n=4, mean of each column is being compared to control (NM) CM using ANOVA and Tukey’s multiple comparison test, ****p<0.0001)
It is often difficult to use bone scans and conventional radiography to evaluate bone metastasis due to their low specificity and low sensitivity, respectively\(^7^1\). As an alternative, bone markers, including ALP and TRAP enzyme activity, have recently been investigated since they can reveal the interaction of cancer cells with osteoblasts and osteoclasts\(^7^1\). When comparing breast cancer patients with and without bone metastases, it has been shown that patients with bone metastases have a median bone-specific ALP concentration of 154 U/L while patients without bone metastases have a median concentration of 22.8 U/L\(^7^2\). TRAP has also been used as a marker for metastatic bone disease where patients with bone metastasis display an increased TRAP activity over patients without bone metastasis\(^7^3\). It was also seen that the increased TRAP activity in the patients with bone metastasis is directly correlated to the number of bone metastases\(^7^3\). Both ALP and TRAP enzyme activities have significant prognostic value as biomarkers in breast cancer patients with bone metastasis\(^7^4, 7^5\).

Comparing the fold changes of the T47D CM (1X) and the bone CM (0.5X) for the ALP and TRAP enzyme activity, there was a significant increase seen in the fold change of the malignant (HA) CM group compared to the control (NM) CM group. For the ALP enzyme activity, the fold change increased from 19-fold in the malignant (HA) T47D CM (1X) to 107-fold in the malignant (HA) bone CM (0.5X) (Figure 17a and 17b). For the TRAP enzyme activity, the fold change increased from 12.5-fold in the malignant (HA) T47D CM (1X) to 100-fold in the malignant (HA) bone CM (0.5X) (Figure 17c and 17d). The fold change of the malignant (HA) bone CM is much higher showing that osteoblast and osteoclast cell populations in the bone explants are being stimulated. It is speculated that either pre-existing osteoclasts and osteoblasts are being activated more or late-stage osteoblasts and osteoclasts are being pushed to finish differentiation; however, histology staining needs to be conducted to examine relative population
of osteoblasts and osteoclasts after exposure to different CM. Taken together, our results showing increased TRAP and ALP enzyme activity of CM collected from human bone explants exposed to malignant (HA) CM correlate well with preclinical and clinical studies in patients with and without bone metastasis, thereby suggesting the plausible role of malignant microcalcifications in risk of bone metastasis.

Since we observed enhanced activity of both ALP (marker of osteoblastic/bone forming activity) and TRAP (marker of osteoclastic/bone resorption activity), we further conducted micro-CT analysis on the fixed bone samples to evaluate the structural changes in the bone explants exposed to conditioned media of T47D cells cultured on 2D monolayers or on different ECM-mimics (Dr. Hongshuai Li, University of Iowa, Department of Orthopedics and Rehabilitation). The bone explants cultured in DMEM containing BSA without any conditioned media were used as controls. Figure 18 shows that all the human bone explants exposed to breast cancer cell conditioned media had a decrease in bone fraction (BV/TV) in comparison to the control bone group without conditioned media exposure. Figures 19a – 19e are representative 3D images displaying the structure of the human bone explants from each of the experimental groups after exposure to conditioned media from T47D cancer cells exposed to different ECM-mimics or 2D monolayers.
Figure 18. Decreased bone fraction in bone samples exposed to conditioned media of T47D cells exposed to different ECM-mimics or 2D culture (Mean ± SEM, n=4, mean of each column is being compared to control bone w/o CM using ANOVA and Tukey’s multiple comparison test, **p<0.01, ***p<0.001)

Figure 19. Representative 3D images of micro-CT of the bone structure from each experimental group

The decrease in the bone fraction (Figure 18) of the bones exposed to conditioned media indicates a loss of bone volume, or bone resorption compared to the control bones that were not exposed to any conditioned media suggesting that the factors secreted by cancer cells have the ability to remodel the bone microenvironment, specifically enhancing bone resorption. Tumor cell factors can act on the bone to cause dysregulation and subsequent destruction and formation76. Although bone metastasis can be osteoblastic or mixed, osteolytic is the most common form of bone metastasis in breast cancer76. In the case of osteolytic metastasis, breast cancer cells
collaborate with the osteoclasts to cause active resorption of the bone matrix\textsuperscript{77}. It was interesting and somewhat unexpected that we did not observe any significant changes between the different groups exposed to conditioned media even though our earlier results showed significantly higher metabolic activity for the bones exposed to malignant (HA) breast cancer conditioned media (Figure 16) in comparison to the other groups. We speculate that 6 days of exposure to the bone explants could be too long and we may have missed the peak resorption window for HA-conditioned media while other experimental groups slowly caught up to the bone resorption levels of HA conditioned media. A potential suggestion would be to study the structural changes in the bones exposed to different conditioned media at earlier time points.

Another possible explanation to the volume loss across all the bones exposed to conditioned media is to consider if all of the conditioned media has an acidic pH. In an acidic environment caused by the conditioned media, osteocytic osteolysis could be triggered, which does not require osteoclasts. During osteocytic osteolysis, osteocytes resorb bone minerals through acidification surrounding osteocytic lacunae. This could lead to bone loss in the absence of osteoclast activity\textsuperscript{78, 79, 80}. Overall, the malignant microcalcifications precondition breast cancer cells to secrete factors with the ability to remodel the bone microenvironment.
Breast cancer is the most common cancer found in women, affecting 1 in 8 women in the United States. A common diagnostic tool used to detect breast cancer is mammographic microcalcifications. Statistics show that up to 93% of cases of ductal carcinoma in situ (DCIS) are determined based on the presence of microcalcifications. There are two types of microcalcifications based on their chemical composition. Type I composed of calcium oxalate is considered a “benign microcalcification”, and type II composed of hydroxyapatite (calcium phosphate) is considered a “malignant microcalcification.”

Breast lesions that are surrounded by malignant microcalcifications gain mesenchymal markers such as vimentin and nuclear β-catenin, suggesting breast cancer cells are acquiring an invasive phenotype by undergoing EMT. Breast lesions surrounded by malignant microcalcifications also begin to display bone mimicry, where the breast cancer cells express bone markers such as RUNX2 and BMPs that are usually found in the bone microenvironment. Studies unrelated to microcalcifications suggest that the breast cancer cells with bone mimicry show greater propensity to bone metastasis. Specifically, 15 proteins, normally found in the bone microenvironment, have been identified in breast cancer cells as a signature that promotes metastasis to bone. Indeed, bone is a major site of metastasis across all molecular subtypes of breast cancer. In the luminal subtype of breast cancer, 49% of advanced breast cancers metastasize to bone.

Currently, clinical studies in breast cancer patient biopsies suggest a link between microcalcifications and bone mimicry while preclinical and clinical studies suggest a link between bone mimicry and bone metastasis. However, the biological role of malignant microcalcifications
in creating a pre-metastatic niche to promote bone metastasis remains to be elucidated. Do microcalcifications play a biological role in promoting an invasive phenotype and subsequent metastasis? We hypothesized that in the primary tumor, the presence of malignant microcalcifications creates a microenvironment where the breast cancer cells acquire bone mimicry. We also hypothesized that breast cancer cells pre-conditioned to malignant microcalcifications create a premetastatic niche in the primary breast tumor where breast cancer cells secrete factors important for remodeling the bone microenvironment.

To create a 3D model that simulates the soft tissue microenvironment in breast along with the presence of benign and malignant microcalcifications, we fabricated ECM-mimic hydrogels recapitulating the nano- to micro-scale structure of native collagen. Raman spectroscopy confirmed that the microcalcifications deposited in the ECM-mimics have similar chemical composition to those found in breast cancer patient biopsies.

The T47D cells as well as patient-derived primary MPCs exposed to the malignant (HA) microcalcifications showed an increase in the mRNA expression of the mesenchymal markers (TWIST and VIM) and bone markers (RUNX2 and BMP2). This shows that malignant (HA) microcalcifications can induce bone mimicry in breast cancer cells. Additionally, the cytokine (RANKL, IL6 and IL8) expressions increased significantly in the breast cancer cells exposed to malignant (HA) microcalcifications in comparison to the benign (OX) and control (NM) groups. The conditioned media from the malignant (HA) ECM-mimics group also showed significantly higher ALP and TRAP enzyme activity. Taken together, the results show that malignant microcalcifications can induce breast cancer cells to secrete bone metastatic factors.

Human bone explant assays revealed 3-fold higher metabolic activity of the bones in the malignant (HA) CM group as well as significantly higher ALP and TRAP enzyme activity in the
malignant (HA) bone conditioned media compared to conditioned media from benign and non-mineralized ECM-mimics. Furthermore, micro-CT analysis showed that the bone structure of the bones exposed T47D conditioned media experienced a decrease in bone fraction indicating bone resorption although we did not see significant differences in bone volumes for different conditioned media.

In the future, we would like to analyze protein expression of the mesenchymal markers and bone markers of the breast cancer cells since all of our data is only at the mRNA level currently. We would also like to evaluate the mRNA and protein expression of osteoprotegerin (OPG) and RANKL in the breast cancer cells exposed to different microcalcifications. To prevent excessive resorption, OPG binds to RANKL inhibiting the binding of the RANK receptor. Thus, the ratio of OPG/RANKL would give a better understanding of the balancing act between osteoblasts and osteoclasts to determine which one might be more dominant. In addition, kinetic studies on the ALP and TRAP enzyme activity using conditioned media collected on days 2, 4, and 6 may provide some useful information on the timeline of breast cancer cells acquiring bone mimicry phenotypes as well as secretion of cytokines capable of remodeling the bone microenvironment. This may provide some explanation to our observation of similar bone resorption with no significant differences across bones exposed to different conditioned media. Nonetheless, the results presented in this dissertation using novel ECM-mimics as a three-dimensional model suggest that malignant microcalcifications may play a role in metastatic breast cancer progression and warrants further investigation into underlying biological mechanisms.


