Regulation of Breast Cancer Cells’ Bone-Metastatic Potential by Mechanically Stimulated Osteocytes

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy in Biomedical Engineering
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Abstract

Bone metastasis, the migration of cancers to the bone, occurs in 65-75% of patients with advanced breast cancer and significantly increases patients’ morbidity and mortality. The bone-metastatic cancer cells interact with cells in the bone to disrupt the bone remodeling balance, causing reduced bone quality and other complications while facilitating tumor growth. Bone remodeling cells can be regulated by osteocytes, the major population of cells in the bone that are embedded in the bone matrix, in response to dynamic loading on the bone. Osteocytes also signal to blood vessel-lining endothelial cells that interact closely with cancer cells during early metastasis before a secondary tumor is established in the bone. Therefore, we hypothesized that mechanically stimulated osteocytes may regulate cancer cells directly and via other cells. To investigate, we mimicked what osteocytes experience \textit{in vivo} during bone-loading activities, such as walking, with oscillatory fluid flow. We observed that factors secreted by flow-stimulated osteocytes increase cancer cell migration and survival. Contrasting, signaling from flow-stimulated osteocytes through bone-resorbing osteoclasts or endothelial cells to cancer cells were anti-metastatic. Specifically, it reduced cancer cell migration, survival, and invasion. Factors secreted by flow-stimulated osteocytes also reduced cancer cells’ trans-endothelial migration and endothelial monolayers’ permeability and ability to be adhered by cancer cells. These
demonstrated the capability of mechanically stimulated osteocytes in reducing the bone-
metastatic potential of breast cancer cells by signaling through osteoclasts and endothelial cells. 
Investigating this regulation further can provide novel insights into the potential of bone-loading 
exercise in preventing bone metastasis.
Acknowledgments

The past five years as a graduate student have been a great experience, thanks to everyone who supported me throughout. Without them, I would have never been able to accomplish what I did.

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I also want to thank all my lovely colleagues who made working in the lab exciting. To senior graduate students who were in the lab before me, Chao and Kevin, thank you for creating a friendly lab environment and getting me started in the lab. To the junior graduate students who have assisted me, Christina, Henry, and Madeleine, thank you for all your help and wish you all the best in your own careers! To my undergraduate students, Sheryash, Peter, Jacob, and Candy, thank you for being such wonderful students and friends. This work would not have been possible without you. To my first friend in the lab, Avinash, thank you for making my first months in Toronto filled with adventures and fun. To others in the lab and many of you in third floor Mining Building or fourth floor Rosebrugh Building, thank you for the hallway chats that always cheered me up.

Special thanks to Kevin, who went from being a colleague to a friend, then to my partner for the rest of my life (definitely the most unexpected result from my doctoral study). Thank you for all the supports, laughs, and everything else.

Finally, I would like to thank my parents and sisters for the continuous and unconditional love and support. I know I always have you to count on no matter what happens. Thank you, as well as other family members and friends outside the lab, for balancing my life with funs after experiments. Thank you all!
# Table of Contents

Acknowledgments........................................................................................................ iv

Table of Contents........................................................................................................ v

List of Figures ................................................................................................................. x

Overview of Contributions ........................................................................................... xii

List of Publications .......................................................................................................... xiv

Peer-Reviewed Journal Articles.................................................................................... xiv

Book Chapter .................................................................................................................. xiv

Articles in Review ............................................................................................................. xiv

Chapter 1 .......................................................................................................................... 1

1 Introduction .................................................................................................................. 1

1.1 Motivation ................................................................................................................ 1

1.2 Thesis Overview ..................................................................................................... 2

Chapter 2 .......................................................................................................................... 3

2 Background .................................................................................................................. 3

2.1 Breast Cancer Bone Metastasis ............................................................................. 3

2.2 Metastatic Cascade .................................................................................................. 4

2.2.1 Escape from primary tumor .............................................................................. 4

2.2.2 Arrival at the bone .......................................................................................... 5

2.2.3 Extravasation and interaction with the endothelium ..................................... 8

2.3 Secondary Tumor in the Bone Disrupts Bone Remodeling ................................ 10

2.3.1 Balanced bone remodeling .............................................................................. 10

2.3.2 Vicious cycle of bone metastasis ..................................................................... 16

2.4 Current Treatment .................................................................................................. 18

2.4.1 Breast cancer treatment ............................................................................... 18

2.4.2 Antiresorptive drugs ..................................................................................... 18
2.4.3 Exercise.................................................................................................................. 19

2.5 Mechanical Loading Regulates Bone Remodeling via Osteocytes ......................... 19

2.5.1 Bone mechanical loading.................................................................................... 19

2.5.2 Osteocytes as bone mechanosensors............................................................... 21

2.5.3 Potential role of osteocytes in the mechanical regulation of bone metastasis ..... 22

Chapter 3 ....................................................................................................................... 26

3 Hypothesis and Objectives ....................................................................................... 26

3.1 Rationale and Focus ............................................................................................ 26

3.2 Hypothesis ............................................................................................................ 26

3.3 Objectives ............................................................................................................ 27

Chapter 4 ....................................................................................................................... 28

4 Direct Effect of Flow-Stimulated Osteocytes on Cancer Cells .................................. 28

4.1 Introduction .......................................................................................................... 28

4.2 Methods ................................................................................................................. 29

4.2.1 Cell cultures ...................................................................................................... 29

4.2.2 Oscillatory fluid flow ....................................................................................... 30

4.2.3 Transwell migration ......................................................................................... 32

4.2.4 Apoptosis, proliferation, and viability .............................................................. 32

4.2.5 Adhesion to collagen ....................................................................................... 32

4.2.6 Protein quantification and blocking ................................................................. 33

4.2.7 Statistics ........................................................................................................... 33

4.3 Results .................................................................................................................. 33

4.3.1 Breast cancer cell activities ............................................................................ 33

4.3.2 Osteocyte-secreted VEGF and breast cancer cell migration .......................... 36

4.3.3 Prostate cancer cell activities ......................................................................... 36

4.4 Discussion .............................................................................................................. 38
4.5 Conclusion ................................................................................................................................... 40

Chapter 5 ........................................................................................................................................ 41

5 Osteoclast- and Endothelial-Mediated Effect of Flow-Stimulated Osteocytes on Breast Cancer Cells ................................................................................................................................ 41

5.1 Introduction ................................................................................................................................ 41

5.2 Methods ....................................................................................................................................... 43

5.2.1 Cell cultures ................................................................................................................................ 43

5.2.2 Oscillatory fluid flow on osteocytes ............................................................................................... 43

5.2.3 Osteoclastogenesis ......................................................................................................................... 44

5.2.4 Transwell migration ......................................................................................................................... 44

5.2.5 Trans-endothelial migration .............................................................................................................. 44

5.2.6 Apoptosis ..................................................................................................................................... 45

5.2.7 Statistics ...................................................................................................................................... 45

5.3 Results .......................................................................................................................................... 46

5.3.1 Osteoclast-mediated regulation ...................................................................................................... 46

5.3.2 Endothelial-mediated regulation .................................................................................................... 47

5.4 Discussion .................................................................................................................................... 49

5.5 Conclusion .................................................................................................................................. 53

Chapter 6 ........................................................................................................................................ 54

6 Effect of Flow-Stimulated Osteocytes on Endothelial-Breast Cancer Cell Interaction .............. 54

6.1 Introduction ................................................................................................................................... 54

6.2 Methods ....................................................................................................................................... 56

6.2.1 Cell cultures ................................................................................................................................ 56

6.2.2 Oscillatory fluid flow on osteocytes ............................................................................................... 57

6.2.3 Endothelial permeability ................................................................................................................. 57

6.2.4 Adhesion on endothelial monolayer ............................................................................................... 57
6.2.5 RNA sequencing ................................................................. 58
6.2.6 Invasion assay ................................................................. 59
6.2.7 Statistics ........................................................................ 59
6.3 Results ............................................................................... 60
  6.3.1 Endothelial permeability .................................................. 60
  6.3.2 Cancer cell adhesion on endothelial monolayer ..................... 60
  6.3.3 Gene expression ............................................................... 61
  6.3.4 MMP-9 and FZD4 expression ............................................. 65
  6.3.5 Invasion ......................................................................... 66
6.4 Discussion .......................................................................... 66
6.5 Conclusion .......................................................................... 70

Chapter 7 ................................................................................. 72

7 Conclusion ............................................................................. 72
  7.1 Summary of Findings ........................................................... 72
  7.2 Limitations and Future Directions ......................................... 75
    7.2.1 Mechanism for the anti-metastatic regulation by flow-stimulated osteocytes..... 75
    7.2.2 Microfluidics device ...................................................... 76
    7.2.3 Animal studies ............................................................. 76
    7.2.4 Potential for vibration therapy ........................................ 77
    7.2.5 Cancer cell on osteocyte mechanosensitivity ..................... 78
  7.3 Conclusion .......................................................................... 78

References ................................................................................ 79

Appendices ............................................................................. 114
  A. Code (R/Bioconductor) ......................................................... 114
    A.1 RNA Sequencing Analysis .................................................. 114
      A.1.1 DESeq2 analysis .......................................................... 114
A.1.2 EdgeR analysis ........................................................................................................115
A.1.3 Gene ontology and KEGG pathway analysis ...........................................................117
A.1.4 Gene expression heat map ......................................................................................121
Copyright Acknowledgements ........................................................................................123
List of Figures

Figure 2-1: Osteocytes in lacuna in trabecular and cortical bones. ........................................ 6

Figure 2-2: The basic multicellular unit during bone remodeling. ........................................... 11

Figure 2-3: Different mechanisms through which cells in the bone may be stimulated when the bone is mechanically loaded. ................................................................. 20

Figure 4-1: Flow chamber (A) schematic, (B) parts, and (C) assembled. .............................. 31

Figure 4-2: Breast cancer cell migration towards osteocyte CM. ......................................... 34

Figure 4-3: Breast cancer cell migration towards osteoblast CM. ....................................... 34

Figure 4-4: Breast cancer cell apoptosis in osteocyte CM. .................................................. 35

Figure 4-5: Breast cancer cell growth in osteocyte CM. ..................................................... 35

Figure 4-6: Breast cancer cell adhesion in osteocyte CM. ................................................... 36

Figure 4-7: VEGF and breast cancer cell migration towards osteocyte CM. ...................... 37

Figure 4-8: Prostate cancer cell migration and apoptosis in osteocyte CM. ...................... 37

Figure 5-1: Osteoclastogenesis in osteocyte CM. .............................................................. 46

Figure 5-2: Breast cancer cell migration towards CM from osteoclasts cultured in osteocyte CM. .................................................................................................................. 47

Figure 5-3: Breast cancer cell apoptosis in osteoclast CM. .................................................. 47

Figure 5-4: Breast cancer cell trans-endothelial migration towards osteocyte CM. .......... 48

Figure 5-5: Breast cancer cell apoptosis in CM from endothelial cells cultured in osteocyte CM. .................................................................................................................. 49

Figure 5-6: Summary of direct and indirect signaling from flow-stimulated osteocytes on breast cancer cell migration and apoptosis. ........................................................................ 50
Figure 6-1: Endothelial permeability in osteocyte CM................................................................. 60

Figure 6-2: Breast cancer cell adhesion to endothelial monolayers conditioned in osteocyte CM. .................................................................................................................................................. 61

Figure 6-3: Breast cancer cell gene expression in static- and flow-osteocyte groups.............. 63

Figure 6-4: KEGG cancer pathway with genes downregulated by breast cancer cells in the flow-osteocyte group. .................................................................................................................................................. 64

Figure 6-5: MMP-9 and FZD4 expression by breast cancer cells in static- and flow-osteocyte groups.................................................................................................................................................. 65

Figure 6-6: Breast cancer cell invasion in static- and flow-osteocyte groups............................ 66

Figure 6-7: Summary of the effects of mechanically stimulated osteocytes on the interaction between endothelial cells and breast cancer cells to reduce the bone-metastatic potential. ....... 67
Overview of Contributions

The work presented here was only possible with guidance from my supervisor, Dr. Lidan You, and numerous help from many of my colleagues. This section describes the contributions each individual made towards the different parts of the projects.

Chapter 4 describes the investigation of direct signalling between flow-stimulated osteocytes and cancer cells. All the flow chamber experiments were performed by me, with initial guidance from Dr. Chao Liu, a PhD candidate in our lab at the time, and occasional assistance from my undergraduate students for summer 2014, Sheryash Dalmia and Peter Gao. Preliminary test on breast cancer cell migration, which initiated this project, was done by Emily Rose, an undergraduate student in our lab at the time, before I joined the lab. I revised the protocol and repeated her observation on cancer cell migration, and then proceeded to investigate cancer cell apoptosis, proliferation, viability, and adhesion. Migration was imaged using a fluorescent microscope, while proliferation and viability were quantified using a flow cytometer, both kindly provided by Professor Jed Davies. Dr. Zhen-Mei Liu from the Davies lab and Dr. Jeffrey Kiernan, a PhD candidate in the Stanford and Davies lab at the time, provided guidance on using the flow cytometer. Sheryash Dalmia and Peter Gao performed some of the migration imaging and quantification and RANKL ELISA experiments; while some of the VEGF ELISA experiments were performed by Dr. Cheryl Druchoc, an exchange student in our lab at the time from McMaster University. Professor Davies also provided the equipment for reading ELISA results and counting cells, and instructions for these were provided by Dr. Zhen-Mei Liu and Dr. Elena Bajenova of the Davies lab. Guidance on statistical analysis was offered by Dr. Kevin Middleton, a PhD candidate in our lab at the time.

Chapter 5 explores the interaction between breast cancer cells and endothelial cells or osteoclasts under signalling from flow-stimulated osteocytes. I performed all the flow chamber and conditioning experiments, with occasional help from Jacob Young, my undergraduate student in summer 2015, and Candy Lam, my undergraduate thesis student from fall 2015 to summer 2016. Initial guidance on osteoclast differentiation and TRAP-staining was provided by Dr. Chao Liu and Dr. Kevin Middleton. Preliminary trials using bone slices for osteoclast-mediated signaling were performed in Dr. Davies’ lab with help from Dr. Zhen-Mei Liu and Birol Ay, although the experiments were incomplete and the results were never presented. I performed all the migration,
apoptosis, and trans-endothelial migration experiments. Confocal images on VE-cadherin staining were obtained by Dr. Kevin Middleton. I wrote the manuscript on selected results from Chapters 4 and 5 and it is published in the *Journal of Cellular Biochemistry* (January 2018).

Chapter 6 further details the results on the effect of flow-stimulated osteocytes on the interaction between endothelial cells and breast cancer cells. I performed all the flow chamber experiments as well as obtained all the results on permeability, adhesion, qPCR, and invasion. Permeability was quantified using a microplate reader provided by Professor Milica Radisic. Adhesion studies using ibidi devices were advised by Liangcheng (Henry) Xu, a PhD candidate in the lab. Protocols on qPCR were provided by Madeleine Barreau, a master student in our lab at the time. Preliminary test on MMP-9 qPCR was done by June Mei, a graduate student in our lab at the time. Additional permeability and invasion studies were attempted using microfluidic devices fabricated by Xueting (Christina) Mei, a master student in our lab, although results from these devices were incomplete and are not presented here. Christina Mei also assisted with the maintenance of endothelial and breast cancer cells. Henry Xu explored the options for analyzing RNA-sequencing data, and I performed the final analysis using R/Bioconductor. Dr. Kevin Middleton provided advice on statistical analysis. I wrote the manuscript on results presented in Chapter 6 and it is published in the *Journal of Cellular Biochemistry* (November 2018).

Chapter 7 outlines some of the side projects that resulted from this thesis. Candy Lam and Jacob Young did the majority of the experiments mentioned in Section 7.2.5 investigating the effect of cancer-secreted factors on osteocyte mechano-sensitivity. Dr. Kevin Middleton and Christina Mei led the efforts on translating the experiments on the interaction between flow-stimulated osteocytes, endothelial cells, and cancer cells onto microfluidic devices to incorporate real-time signaling (Section 7.2.2). Dr. Kevin Middleton and I wrote a review paper on studying metastasis using microfluidics devices and it is published in the journal *Microsystems & Nanoengineering*. Christina Mei is also working towards investigating the effect of vibration-stimulated osteocytes on breast cancer bone metastasis (Section 7.2.4).

Finally, I would like to acknowledge CIHR (Canadian Institutes of Health Research) Doctoral Research Award (the Frederick Banting and Charles Best Canada Graduate Scholarship), Ontario Graduate Scholarship, and Barbara and Frank Milligan Fellowship for funding my research.
List of Publications

Peer-Reviewed Journal Articles


Book Chapter


Articles in Review


Chapter 1

1 Introduction

1.1 Motivation

Bone metastasis occurs when cancers travel from their primary sites to bone, primarily via blood vessels. It is a severe complication seen in 65-75% of patients with advanced breast cancer (Coleman, 2006). Currently, bone metastasis is incurable and has a median survival of less than 2 years (Yong et al., 2011). Once the cancer cells arrive at the bone microenvironment, they are capable of disrupting the balance between the bone-forming osteoblasts and the bone-resorbing osteoclasts, causing lesions with either excessive bone formation or resorption. This then results in pain, increased fracture risk, and hypercalcemia, which may lead to death in severe cases (Roodman, 2004). Furthermore, the imbalance in bone remodeling also accelerates bone turnover and the release of growth factors stored in the bone matrix, which can in turn facilitate cancer cell growth and survival. Therefore, a vicious cycle of bone metastasis is established where both bone lesions and tumors grow in the bone (Weilbaecher, Guise and McCauley, 2011).

On the other hand, this vicious cycle can potentially be broken by bone-loading exercise, since it is known to regulate bone remodeling (Lanyon and Rubin, 1984). A recent clinical review has shown that exercise is generally associated with improved outcomes in patients with bone metastases (Sheill et al., 2018). In addition, an in vivo study demonstrated a reduction in tumor size when bone-loading was applied to mice with breast cancer cells injected into their bone (Lynch et al., 2013). These studies suggest a promising potential that, in addition to improving patients’ quality of life, exercise may be able to regulate bone metastasis by mechanically stimulating the bone.

Osteocytes are main mechanosensors of the bone. They signal to a variety of cells and orchestrate the bone remodeling balance (Chen et al., 2010). However, very little is known about the role of osteocytes in bone metastasis. Osteocytes are the major cell population that reside in holes in the bone matrix (called the lacuna) and are connected to each other and to blood vessels via canals (called the canaliculi) (Boneswald, 2011). Since this lacunar-canalicular of the bone is filled with interstitial fluid, oscillatory fluid flow occurs when the bone is mechanically loaded during activities such as running (Price et al., 2011). The physiological level of shear stress that
results from this fluid flow stimulates osteocytes to regulate osteoblasts and osteoclasts (Bakker et al., 2001; You et al., 2008; Hoey, Kelly and Jacobs, 2011; Middleton, Al-dujaili, et al., 2017). This regulation by osteocytes may in turn have an impact on the vicious cycle of bone metastasis, in which osteoblasts and osteoclasts are major players. Furthermore, osteocytes have been shown to signal to endothelial cells (Cheung, Simmons and You, 2012; Prasadam et al., 2014), which can potentially affect cancer cell extravasation out of the blood vessel. Therefore, we hypothesize that osteocytes stimulated with oscillatory fluid flow will be able to affect the metastatic potential of breast cancer cells through 1) direct signaling, 2) osteoclasts, and 3) endothelial cells.

In conclusion, this project aims to elucidate the role of osteocytes in the mechanical regulation of breast cancer bone metastasis. We believe that it is a significant part of bone metastasis, but research on it is still limited. We hope that this study will provide insights for potential future studies on the prevention of bone metastasis by bone-loading activities.

1.2 Thesis Overview

This thesis is divided into seven chapters. Chapter 2 provides the background of this research, including the bone-metastatic cascade and the mechanical regulation of bone remodeling by osteocytes. Chapter 3 outlines the focus, hypothesis, and objectives of this study. Chapters 4-6 are research chapters. Chapter 4 presents results of the direct impact of flow-stimulated osteocytes on breast cancer cell migration, apoptosis, proliferation, viability, and adhesion to collagen. Chapter 5 discusses the anti-metastatic potential of osteoclast- and endothelial-mediated signaling from flow-stimulated osteocytes. Chapters 4 and 5 are based on my published journal article “Mechanical regulation of breast cancer migration and apoptosis via direct and indirect osteocyte signaling” (Ma, Lam, et al., 2018). Since endothelial cells interact closely with cancer cells in early metastasis before a secondary tumor is established in the bone, Chapter 6 builds on Chapter 5 and further investigates the effect of flow-stimulated osteocytes on the interaction between endothelial cells and breast cancer cells. Contents in Chapter 6 are published in my journal article “Mechanically stimulated osteocytes reduce the bone-metastatic potential of breast cancer cells in vitro by signalling through endothelial cells” (Ma, Xu, et al., 2018). Finally, Chapter 7 concludes the findings of this research while discussing limitations and future directions.
Chapter 2

2 Background

2.1 Breast Cancer Bone Metastasis

Breast cancer is the most common malignant disease affecting women worldwide, with approximately 570,000 women dying from breast cancer in the year 2015 (World Health Organization, 2018). With recent advancements in cancer treatment, the 5-year survival rate for primary breast cancer is now more than 90% (Steeg, 2016). Metastasis, the migration of cancer to distant sites, is responsible for the majority of mortality associated with breast cancer. Breast cancer cells predominantly metastasize to the lungs, liver, bone, and brain. In particular, bone metastasis is incurable and occurs in 65-75% of patients with advanced breast cancer (Suva et al., 2011). The 5-year survival rate after the diagnosis of bone metastasis is less than 15% (Svensson et al., 2017) and has not improved in the past 10 years (Steeg, 2016).

The most common sites of breast cancer bone metastasis is the spine, followed by ribs, sternum, pelvis, femur, and others (Kakhki et al., 2013). Once a secondary tumor is established in the bone, it leads to severe skeletal-related events such as pathological bone fractures, bone marrow aplasia, pain, spinal cord compression, and hypercalcemia (Roodman, 2004). Breast cancer cells that have metastasized to the bone are capable of interacting with bone remodeling cells to disrupt the balance between bone resorption and bone formation. This causes lesions that are 1) osteolytic, with excess bone resorption, 2) osteosclerotic, with excess bone formation, or 3) a mixture of both. Lesions from bone metastases are typically mixed, with approximately 85% of breast cancer bone metastasis lesions being osteolytic (Kozlow and Guise, 2005). In either type of lesions, the bone is significantly weakened and becomes more prone to fracture. Bone metastasis is also the most common cause of pain from cancer (Mercadante, 1997). The origin of this pain may be inflammation, when tumor cells secrete cytokines that stimulate intraosseous nerves, or mechanical, when tumor growth in the bone exerts pressure. Furthermore, spinal cord compression occurs in 10-20% of patients, when the metastatic site happens to be the spine, and can permanently paralyze patients. Finally, severe hypercalcemia (14 mg/dL), caused by the accelerated release of calcium from the bone, may lead to gastro-intestinal, renal, and central nervous system dysfunctions. This, in turn, causes altered mental state, coma, cardiac arrythmias,
and renal failure. Most importantly, although there are bone-targeting drugs that assist in managing pain and skeletal-related events, these drugs do not cure bone metastasis or prolong the survival of patients (Fontanella et al., 2015).

2.2 Metastatic Cascade

2.2.1 Escape from primary tumor

Although bone metastasis is common in certain types of cancers, it is in fact a highly inefficient multi-step cascade that requires the metastasizing cancer cells to undergo a series of evolution and selection. Therefore, the more metastatic cells in a tumor often have higher rates of genetic mutations or epigenetic modifications (Fidler, 2003). First, the metastatic cascade starts with angiogenesis at the primary tumor site. This not only provides nutrient for the tumor, but also recruits tumor-supporting cells and escape routes for the cells that have broken off from the primary tumor. The breakaway of the tumor cell typically involves an epithelial-mesenchymal transition in which the tumor cells loses E-cadherin-mediated intercellular adhesion through transcription factors such as Slug, Snail, Twist, zinc finger E-box binding homeobox 1 (ZEB1), and ZEB2 (Smith and Kang, 2013). Nevertheless, it is not the exclusive mechanism for cancer dissemination, as shown in in vivo studies with primary breast (Fischer et al., 2015) and prostate (Zheng et al., 2015) cancers. The tumor cells may then undergo local invasion, where they cleave basement membrane through proteolysis, and migration. The migration of cancer cells may be in the form of multicellular or single-cellular, which can be in a mesenchymal or amoeboid form (Friedl and Wolf, 2003). The entrance of cancer cells into the stroma also triggers inflammation, recruiting tumor-supporting cells such as the tumor-associated macrophages, and thereby enhances the aggressiveness of the tumor. The cancer cells may then intravasate and travel through the blood of lymphatic vessels, although metastasis to the bone via lymphatic vessels was not observed (Edwards et al., 2008). After tumor cells enter the circulation, only 0.001-0.02% of tumor cells manage to form a metastatic tumor (Weilbaecher, Guise and McCauley, 2011) since the cells have to survive the haemodynamic stress, evade the immune system, and grow in a new microenvironment. As it is only after the tumor cells arrive in the vessels of bone marrow that they may sense signals from bone cells, this thesis will be focused on the activity of cancer cells in the bone microenvironment (discussed below).
2.2.2 Arrival at the bone

2.2.2.1 Normal bone anatomy

To understand the behaviour of cancer cells in bone, normal bone anatomy has to be discussed. A healthy adult has 213 bones, categorized into 4 types: 1) flat bones that protect internal organs such as skull, 2) long bones that support weights such as femur, 3) short bones such as ankles, and 4) irregular bones such the vertebrae. The bone matrix is composed of 50-70% mineral, 20-40% organic matrix, 5-10% water, and <3% lipids (Clarke, 2008). The major mineral content in bone is hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$, while 95% of organic matrix in bone is type I collagen (Gejvall, 1974). In a healthy lamellar bone, the collagen fibrils are densely packed in alternating orientations, similar to plywood. This makes lamellar bone significantly stronger than non-organized woven bone, which is produced during infant growth, tissue repair, or pathological conditions. Finally, 90-95% of cells in the bone is osteocytes, which are embedded in the empty spaces in bone matrix called the lacunae.

Overall, bones are also classified into 2 compartments: 1) cortical bone, which is dense and solid and surrounds the bone marrow, and 2) trabecular bone, which is a honeycomb-like structure with bone rods interspersed in bone marrow (Figure 2-1). Cortical bones are consisted of structures called osteons or Harversian systems, which are concentric layers of lamellar bone with lacunae. The centers of osteons are Harversian canals, which contain nerve fibres and 1 or 2 capillaries of 8 µm in diameter. A Harversian system is typically 400 µm long and 200 µm wide, and human Harversian canals range from 30 to 170 µm in diameter (Hillier and Bell, 2007). On the other hand, the trabecular rods or plates are consisted of packets, which are semilunar bones with lacunae. Each packet is approximately 35 µm in thickness, and each trabecular rod or plate may be 50-400 µm thick. The outer surface of the cortical bone is surrounded by periosteum, a fibrous connective tissue containing blood vessels and nerve fibres and is attached to the bone by thick collagenous fibres. The inner surface of cortical bones and surface lining trabecular bones are covered by the endosteum, a membranous structure containing blood vessels (Clarke, 2008). The adult skeleton is composed of 80% cortical bone and 20% trabecular bone. More specifically, vertebrae are 25% cortical bone and 75% trabecular bone; while long bones are composed of diaphysis, a hollow shaft that is 95% cortical bone, and metaphyses and epiphyses that are both trabecular bones surrounded by thin shells of cortical bones.
2.2.2.2 Bone as a fertile soil for metastasis

Bone is a common site of metastasis found in breast cancer patients. It has long been recognized that different types of primary cancers have distinct metastatic patterns. Two opposing series have been proposed to explain the metastatic specificity of cancer. Edwin’s theory suggested that this tissue tropism is based solely on anatomy and that cancer cells metastasize to where they pass by first. Paget’s seed and soil theory, on the other hand, hypothesized that it is the preferred secondary organs that provides the fertile “soil” to attract the tumor cell “seeds”. Both theories have limitations as tumors rarely metastasize to the contralateral tissue while the two microenvironments would be identical, and breast cancer rarely metastasizes to the heart clinically while they pass by the heart during circulation (Flier, Underhill and Zetter, 1990). Therefore, in reality, it is most likely a mixture of the two theories that explain the metastatic specificity.

Being a frequent site for breast cancer metastasis, bone is indeed a fertile soil for tumor growth. First of all, bone is richly vascularized, greatly increasing the chance of circulating tumor cells
entering the bone microenvironment. In general, bones that are more vascularized, such as the vertebrae, ribs, and the ends of long bones, are more common sites for metastasis (Bussard, Gay and Mastro, 2008). Furthermore, the large sinusoidal blood vessels in bone marrow, where metastasis often occurs, are several hundred microns wide (Mazo and von Andrian, 1999) and within microns of bone. The slow blood flow that results from these large sinusoids give metastasizing cancer cells more time to interact with the endothelium for extravasation. The endothelial cells that line these sinusoids are also leakier to allow movement of hematopoietic cells, which also makes it easier for cancer cells to extravasate (Itkin et al., 2016). Many non-collagenous proteins in the bone, such as osteopontin and vitronectin, interact with cancer cells’ adhesion molecules to facilitate adhesion. Furthermore, the physical properties of the bone, such as hypoxia and high calcium concentrations, are also favourable for tumor cells (Zheng et al., 2013). As well, the bone extracellular matrix is a rich source of growth factors that support tumor growth and migration, such as transforming growth factor beta (TGF-β), insulin-like growth factor (IGF), fibroblast growth factors (FGF), platelet-derived growth factor (PDGF), and bone morphogenetic protein (BMP) (Kingsley et al., 2007).

Many cells of the bone also facilitate the bone tropism of cancer cells (Buenrostro et al., 2016) because breast cancer cells that are metastatic to the bone are capable of expressing genes typically specific to cells in the bone. A majority of previous studies on bone metastasis have focused on the bone-forming osteoblasts and bone-resorbing osteoclasts. Metastasizing cancer cells can interact with osteoblasts and osteoclasts and disrupt the balance of bone remodeling. This not only weakens the bone, but also accelerates the release of growth factors from the bone, leading to a feedback loop that promotes tumor growth. In addition, cancer-associated fibroblasts are frequently studied in terms of metastasis, although more often in the site of primary tumors. They have been shown to promote cancer invasion, angiogenesis, and matrix stiffening, which facilitate tumor cell motility (Calvo et al., 2013). Bone marrow mesenchymal stromal cells have also been shown to facilitate cancer cell survival and chemoresistance (Bergfeld, Blavier and DeClerck, 2014). As well, bone hosts a rich population of immune cells. While natural killer cells, T cells, and B cells are important players in the defense against tumor cells, macrophages seem to have a biphasic role. Although classically activated macrophages, or M1 macrophages that are stimulated by factors such as interferon-gamma, are capable of killing cancer cells, alternatively activated macrophages, or M2 macrophages that are stimulated by factors such as
interleukin-4, can promote tumor growth (Biswas, 2006) and migration (Li et al., 2017). As well, myeloid-derived suppressor cells, including immature macrophages, have been shown to support tumor cells through immunosuppression, remodeling of the pre-metastatic niche, and angiogenesis (Marvel and Gabrilovich, 2015). Furthermore, the bone is highly innervated and vascularized. Clinical studies have demonstrated the link between chronic stress and cancer mortality (Chida et al., 2008). Platelets have also been shown to aggregate with tumor cells to shield them from shear stress and immune cells. Finally, the endothelial cells, which line the blood vessels that the circulating tumor cells are travelling in, not only serve as an important barrier, but also actively interact with the cancer cells (Orr et al., 2000; Chavez-MacGregor, 2005; Mai et al., 2014). This will be discussed in detail in Section 2.2.3 below.

2.2.3 Extravasation and interaction with the endothelium

Once the metastasizing cancer cells arrive at the blood vessels of the secondary site, the circulating tumor cells may arrest in two ways before extravasation: 1) physical arrest when the cells arrive at small capillaries or when they form multicellular clumps, or 2) adhesion onto the endothelium (Orr et al., 2000). While most extravasation occurs at small capillaries (Stoletov et al., 2010), the later mechanism is more likely in the case of bone metastases since the most common sites for metastasis in the bone typically involve large sinusoidal blood vessels (Bussard, Gay and Mastro, 2008). The blood flow in the bone marrow is also lower than in other organs, facilitating adhesion (Mazo and von Andrian, 1999). The process of circulating tumor cells rolling, adhering, and extravasating is often likened to that of leukocytes. Selectins are responsible for the initial rolling of leukocytes on the endothelium before a more stable adhesion. Similarly, E-selectin expression by endothelial cells is inducible by inflammatory cytokines and have been shown to be important in metastasis (Li and Feng, 2011). Cancer cells have been shown to express a number of ligands for E-selectin, such as the transmembrane receptor mucin-1 (Regimbald et al., 1996). N-cadherin is another endothelial-expressed receptor that had been suggested to mediate the rolling of breast (Strell et al., 2007) and other cancer cells (Qi et al., 2005) on the endothelium. Next, a more stable adhesion is formed between rolling cancer cells and endothelial cells, mediated by cancer cell expression of integrins, CD44, and mucin-1 (Kobayashi, Boelte and Lin, 2007). Integrins are heterodimers with α- and β-subunits that bind to the extracellular matrix or endothelial receptors, such as intercellular adhesion molecules 1 (ICAM-1) or vascular CAM-1 (VCAM-1) (Askari et al., 2009). CD44 had been implicated in the
adhesion of breast cancer cells to the bone marrow endothelial cells (Draffin et al., 2004).

Finally, mucin-1 binds to endothelial ICAM-1 and this interaction had been shown to be essential for extravasation (Rahn et al., 2005). Interestingly, cancer cell adhesion onto the endothelium seem to be organ-specific and may be affected by the origin of the endothelial cells (Netland and Zetter, 1984).

After the cancer cells adhere onto the endothelium, they may extravasate in two ways: 1) paracellular extravasation, where they squeeze between endothelial cells, and 2) transcellular extravasation, where they migrate through the endothelial body, although it is controversial whether the later occur in vivo (Reymond, D’Água and Ridley, 2013). The trans-endothelial migration cascade has been quantified in vitro (Fan and Fu, 2016). It was demonstrated that 98% of trans-endothelial migration was in the paracellular form. Tumor cells adhered on the endothelium were seen to degrade the endothelial glycocalyx and extend protrusions to disrupt endothelial junction proteins, with the most important one being vascular endothelial cadherin (VE-cadherin). This facilitates extravasation before endothelial cells recover. This process requires morphological changes of cancer cells. Therefore, RHO-family GTPases, regulators of the cytoskeleton, play a key role. Cancer cells may transmigrate in amoeboid, when cells appear rounded, or mesenchymal, when cells appear elongated, form (Hecht et al., 2015). Finally, the cancer cells that have transmigrated across the endothelium may invade the basement membrane of the blood vessels and eventually the bone with matrix metalloproteinases (MMPs) (Deryugina and Quigley, 2006; Conrad et al., 2018).

Several cells other than endothelial cells and cancer cells may affect this process. One of the most studied chemokines in promoting cancer cell migration, adhesion, and extravasation is the CXC-chemokine ligand 12 (CXCL12) (Furusato et al., 2010), which may be secreted by stromal cells and osteocytes (Leucht et al., 2013). Platelets have also been shown to form multicellular aggregates with cancer cells. This not only increases the size and the chance of being arrested in small capillaries, but also provides platelet-derived P-selectins that have been shown to promote metastasis (Coupland, Chong and Parish, 2012). Leukocytes recruited by the tumor-secreted cytokines or by these tumor-associated platelets also facilitate cancer extravasation by producing cytokines such as interleukin-8 (Dong et al., 2005). Interleukin-8 may then in turn recruit neutrophils that further support cancer extravasation by binding to both the tumor cells and the
endothelial cells (Strell et al., 2007). As well, monocytes can secrete vascular endothelial growth factor (VEGF) that increase endothelial permeability and, therefore, trans-endothelial migration.

In addition to being a passive barrier, endothelial cells are also capable of signaling to cancer cells (Orr et al., 2000). For example, endothelial cells may secrete endothelial growth factor (EGF)-like growth factors and hepatocyte growth factors (HGF) that increase integrin expression in the cancer cells to further enhance adhesion (Sato et al., 1996). Endothelial interleukin-1 also increases cancer cell motility (Orr et al., 1988). As well, conditioned medium from endothelial cells has been shown to affect the chemotaxis of cancer cells through monocyte chemotactic protein 1 (Wakabayashi, Cavanaugh and Nicolson, 1995). Furthermore, endothelial cells secrete a variety of factors that support tumor growth and survival, including FGF, PDGF, IGF-1, epidermal growth factor (EGF), and interleukin-6 (Rak, St Croix and Kerbel, 1995). On the other hand, cancer cells are often capable of promoting angiogenesis. In addition to providing nutrients through angiogenesis, endothelial cells that are stimulated with angiogenic factors have been shown to awaken the dormant cancer cells by reducing the secretion of thrombospondin-1 (Ghajar et al., 2013). Angiogenesis may also accelerate bone remodeling that it is tightly coupled with. This will then in turn accelerate bone turnover and feeds into the vicious cycle of bone metastasis.

2.3 Secondary Tumor in the Bone Disrupts Bone Remodeling

2.3.1 Balanced bone remodeling

Bone is a rigid, yet active, organ that is constantly undergoing remodeling. Approximately 10% of healthy human adult bone is renewed annually (Feng and McDonald, 2011). In fact, remodeling is essential for the bone to maintain structural integrity by removing old bones and replacing them with new bones. Bone remodeling occurs within a specialized functional unit termed the basic multicellular unit (BMU) that includes the bone-resorbing osteoclasts, bone-forming osteoblasts, and vessels that supply nutrients and osteoclast and osteoblast precursors (Figure 2-2) (Sims and Martin, 2014). The process of bone remodeling is separated into 4 phases: 1) activation, 2) resorption, 3) reversal, and 4) formation. Bone remodeling may be activated by signaling from osteocytes, fractures or damage to the bone, and cytokines such as IGF, tumor necrosis factor (TNF)-α, parathyroid hormone (PTH), and IL-6. This initiation signals the detachment of bone-lining cells and the recruitment of osteoclast precursors to begin
the resorption phase. The process of osteoclast-mediated resorption forms Howship’s lacuna in trabecular bones and cutting cones in cortical bones (Figure 2-2). This phase lasts about 2 weeks before osteoclasts undergo apoptosis. The reversal phase is marked by the coupling of bone resorption and formation (Kular et al., 2012), when debris are removed by macrophage-like cells and osteoblast precursors are being recruited by osteoclast-secreted factors or factors released from the bone during resorption. Finally, the osteoblast-mediated formation phase lasts about 4 months, before the osteoblasts undergo apoptosis (approximately 50-70% of osteoblasts (Lynch et al., 1998)), become bone lining cells, or become osteocytes embedded in the bone they have built (Manolagas, 2000).

- Figure 2-2: The basic multicellular unit during bone remodeling. Replicated from Sims and Martin (2014).

2.3.1.1 Osteoclasts

Osteoclasts are giant multinucleated cells that can be up to 100 µm in diameter and typically contain 10-20 nuclei (Roodman, 1996). The number of osteoclasts is low in healthy bones, with
less than 1 osteoclast per mm$^2$ (Meunier et al., 1980). On endosteal surfaces within the
Harversian system or on the periosteal surface beneath the periosteum, osteoclasts are present in
the motile state, where they are flattened, non-polarized, and contain protrusions called
lamellipodia. Upon migration from the bone marrow to the resorptive site, the osteoclasts enter
the resorptive state and became polarized (Kular et al., 2012).

Osteoclasts are differentiated from hematopoietic stem cells with a number of steps and
contributing factors. In the bone marrow, osteoclasts precursors arise from hematopoietic stem
cells of the myeloid lineage. Macrophage colony-stimulating factor (M-CSF) is essential for the
proliferation and survival of the precursors (Wiktor-Jedrzejczak et al., 1990). It is produced by
osteocytes, osteoblasts, and stromal cells and bind to its receptor, c-Fms, on early osteoclast
precursors (Sherr, Roussel and Rettenmier, 1988). This binding signals through multiple
pathways, including the Grb2-ERK1/2 pathway and the Src-P13K-Akt pathway, to activate
transcription factors such as c-Fos and PU.1 (Ross, 2006), and also stimulates the expression of
receptor activator of nuclear factor kappa (RANK). RANK ligand (RANKL) binding to
RANK is essential for the fusion of osteoclast precursors into multinucleated immature
osteoclasts that express key osteoclast markers such as tartrate resistant acid phosphatase
(TRAP) (Kong et al., 1999). RANKL is produced mainly by osteocytes, osteoblasts, and stromal
cells (Nakashima and Takayanagi, 2011). The binding of RANKL to RANK triggers the
intracellular binding of TNF receptor associated factor 6 (TRAF6) to RANK, which then leads to
the phosphorylation of IκB kinase kinase (IKKK) that then phosphorylates IκB kinase (IKK) that
phosphorylates IκB. This targets IκB for degradation and releases nuclear factor kappa B (NF-
κB) for nuclear translocation. The NF-κB-initiated transcription of osteoclastic genes also
requires co-stimulation with the immunoreceptor tyrosine-based activation motif (ITAM)
pathway that occurs through receptors such as the osteoclast associated receptor (OSCAR) and
the triggering receptors expressed on myeloid cells (TREM-2). The signaling cascade includes
the activation of phospholipase C γ (PLCγ) and subsequent intracellular calcium release, which
then leads to the nuclear translocation of nuclear factor of activated T-cells cytoplasmic 1
(NATc1) through calmodulin kinase (CaMK). The binding of NF-κB and NATc1 initiates the
transcription of osteoclastic genes such as TRAP, cathepsin K, calcitonin receptor, and integrin
β3. Importantly, osteocytes and osteoblasts also secrete osteoprotegrin (OPG), a decoy receptor
for RANKL, that antagonizes osteoclastogenesis. Therefore, osteoclast differentiation is
governed by the RANKL to OPG ratio. The fusion of osteoclast precursors is mediated by several molecules, such as integrins, c-src, E-cadherin, dendritic cell-specific transmembrane protein (DC-Stamp), a disintegrin and metalloproteinase (ADAM) family proteins, the macrophage fusion receptor (MFR), and the V0 subunit d2 of the V-H+-ATPase (Maurizi and Rucci, 2018).

A mature osteoclast is multi-nucleated and polarized with distinct membrane domains. The sealing zone is bone-facing and contain adhesion structures called podosomes. The integrin of this region, α2β1, αvβ3, and αvβ5, anchor osteoclast to bone surface, which is essential for resorption. Beside the sealing zone is the ruffled border with fusion zones and uptake zones. This area is characterized by multiple membrane expansions and contains ion transporters that release chloride ions and protons and uptake resorbed materials. The release of protons through Vacuolar-ATPase (V-ATPase) enables acidification of the area to be resorbed and allows the dissolution of the inorganic matrix. In addition, lysosomes also in the ruffled border membrane release enzymes such as capthepsin K and matrix metalloproteinase 9 (MMP-9) to digest organic components of the bone. Finally, the basolateral membrane is in contact with the vasculature and releases digested matrix into the blood stream (Maurizi and Rucci, 2018).

### 2.3.1.2 Osteoblasts

The coupling of bone resorption by osteoclasts and formation by osteoblasts is essential for balanced bone remodeling. This can occur in several ways (Sims and Martin, 2014). Osteoclasts can stimulate osteoblastogenesis via secreted factors such as HGF, PDGF, sphingosine-1-phosphate (S1P), afamin, and collagen triple helix repeat containing 1 (CTHRC1) or membrane-bound protein ephrin B2. The release of factors from the resorbed bone matrix, such as TGF-β, FGF, IGF, PDGF, and BMP, may also attract osteoblast precursors. Osteoblasts originate from mesenchymal stem cells that first form preosteoblasts, which express alkaline phosphatase but do not produce mineralized tissue. TGF-β stimulates the maintenance and proliferation of osteoprogenitor cells as well as osteoblastogenesis through MAPKs-Smad2/3 signaling. BMP also activates osteogenic gene transcription through the Smads pathway. Mature osteoblasts are cuboidal and secrete type I collagen on the bone surface. They also deposit non-collagenous proteins such as osteocalcin and alkaline phosphatase, creating osteoid that will later be mineralized with calcium phosphate.
The main signaling pathway involved in bone formation is the canonical Wnt pathway. It is initiated by the binding of Wnt ligands to a complex containing frizzled receptor and co-receptor low-density lipoprotein receptor related protein 5 (LRP5) or LRP6. This leads to the activation of disheveled that inhibits the glycogen synthase kinase (GSK)-3β, which, in the absence of Wnt signalling, phosphorylates β-catenin and targets it for ubiquitination and degradation. Therefore, the binding of Wnt to LRP5/6 and frizzled receptors prevents β-catenin degradation and allows the translocation of β-catenin into the nucleus, where it may activate gene transcription mediated by lymphoid enhancer factor (LEF) and T-cell factor (TCF). A major downstream protein of this pathway is runt-related transcription factor 2 (RUNX2), which is required for osteoblastogenesis. This pathway can be regulated by many factors. For example, sclerostin and Dickoff-1 (Dkk-1) bind to LRP5/6 to prevent the binding of Wnt to LRP5/6 and frizzled receptors.

2.3.1.3 Endothelial Cells

The blood vessels of the basic multicellular units are essential for providing nutrients and precursors, as well as removing calcium and waste. Therefore, angiogenesis is tightly coupled to bone remodeling. Osteoblasts produce VEGF that stimulate angiogenesis and attract osteoclasts. Blood vessels deliver oxygen to the bone remodeling site. Oxygen is not only fundamental to cells, but also required for prolyl-4-hydroxylase, an enzyme for collagen production. Endothelial cells activated with cytokines have also been shown to promote osteoclast recruitment (Cheung, Simmons and You, 2012), differentiation, and activity (Collin-Osdoby et al., 2001).

2.3.1.4 Osteocytes

Osteocytes are terminally differentiated osteoblasts that became embedded in the bone matrix they produced. They account for 95% of cells in the bone (Dallas, Prideaux and Bonewald, 2013) and reside in empty spaces in the bone called the lacuna (15-20 μm in diameter) that are connected to each other and to the bone surface through channels called the canaliculi (250-300 nm in diameter). Fluid within this lacunar-canalicular system provides nutrient to the osteocytes. Tracer studies had demonstrated the rapid transport of molecules smaller than 7 nm or 43 kDa from the circulation to the lacunar-canalicular system (Fritton and Weinbaum, 2009; Price et al., 2011). The cutoff may be due to the existence of osteocyte glycocalyx between the osteocyte cell body and the lacunar-canalicular wall. In addition to soluble factors, dendrites of neighbouring osteocytes are connected by gap junctions that also allow direct transfer of molecules less than 1
kDa. This functional network of interconnected osteocytes is capable of orchestrating the bone remodeling balance by producing factors such as M-CSF, RANKL and OPG to affect osteoclastogenesis, and sclerostin and Dkk1 to affect osteoblastogenesis.

The differentiation of osteocytes from osteoblasts is not well understood but is hypothesized to be guided by the mechanical properties and the mineralization of the osteoid or the hypoxic environment (Irie et al., 2008; Mullen et al., 2013). This process involves several morphological and genetic changes (Bonewald, 2011). The first critical steps are depolarization and the formation of dendrites since osteocytes are highly dendritic, with approximately 40 dendrites per cell in humans (Beno et al., 2006). This is an invasive process that involves cleavage of collagen with MMPs such as MT1-MMP. Osteoblasts trapped within osteoid first extends dendrites towards the mineralizing front, then towards the vascular space. This morphological change is controlled by the E11 protein (also called podoplanin, OTS-8, gp38, or PA2.25), which is important for cytoskeletal function. The distribution of actin-binding proteins, such as fimbrin, villin, filamin, α-actinin, and spectrin, and cytoskeletal rearrangement involving macrophage capping protein (CapG), Cdc42, CD44, and destrin, are also important for the transition of osteoblast to osteocyte. Other markers for this transition include: reduced alkaline phosphatase and type I collagen, increased casein kinase II and osteocalcin, and expression of phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX), matrix extracellular phosphoglycoprotein (MEPE), dentin matrix protein 1 (DMP-1), fibroblast growth factor 23 (FGF-23), oxygen-regulated protein 150 (ORP150), and sclerostin. Interestingly, the distribution of osteocytes in the bone matrix is even, suggesting that osteocytes sense the environment before embedding.

Osteocytes may remain viable for decades in bones with a low turnover rate. However, osteocyte apoptosis may happen at damaged sites in the bone. Proapoptotic molecules are increased at the sites of the microcrack, while antiapoptotic molecules are expressed 1-2 mm from the crack (Verborgt et al., 2002). Apoptotic osteocytes have been shown to release apoptotic bodies expressing RANKL (Kogianni, Mann and Noble, 2008) and other factors (Al-Dujaili et al., 2011) to promote osteoclastogenesis. Furthermore, apoptotic osteocytes can also stimulate ICAM-1 expression on endothelial cells, promoting osteoclast recruitment (Cheung, Simmons and You, 2012).
2.3.2 Vicious cycle of bone metastasis

As cancer cells arrive at the bone microenvironment, they are capable of disrupting the well-maintained balance between bone formation and bone resorption. This causes lesions with either excessive bone resorption, in the case of osteolytic lesions, or excessive bone formation, in the case of osteosclerotic lesions. Most bone metastases will cause a mixture of both lesion types. Breast cancer causes predominantly osteolytic lesions. In either type of lesions, the disrupted bone remodeling balance causes accelerated bone turnovers, which, in turn, releases factors from the bone matrix that support cancer cell growth and survival. This establishes the vicious cycle of bone metastasis where both the tumor and lesion size increase.

2.3.2.1 Osteolytic lesions

Osteolytic lesions significantly increase the fragility of the bone, causing the bone to break with little or no trauma. The molecular mechanisms of osteolytic lesions have been studied extensively (Kingsley et al., 2007). Metastasized cancer cells can cause osteolysis in many different ways. A major factor mediating osteolytic lesions is the parathyroid hormone-related protein (PTHrP). It is secreted by tumor cells and binds to the PTH receptor type 1 (PPR) on osteoblasts, osteocytes, and tumor cells. Not only can it act in an autocrine manner to stimulate tumor proliferation and resistance to apoptosis, but it can also act in a paracrine way to stimulate osteocytes and osteoblasts to secret RANKL, increasing osteoclastogenesis. Several tumor-expressed interleukins are also involved in this increased osteolysis. Autocrine stimulation of IL-6 in cancer cells increases their proliferation and expression of PTHrP and RANKL (Grivennikov and Karin, 2008). It also upregulates Dkk-1, which, in turn, reduces osteoblastogenesis through the Wnt pathway, as described in Section 2.3.1.1.2. In addition, it stimulates the expression of cyclooxygenase-2 (COX-2) in osteoblasts. This increases the secretion of prostaglandin (PGE-2), which can upregulate RANKL production by osteoblasts and RANK expression in osteoclasts (Steeve et al., 2004). Furthermore, cancer cells express a variety of interleukins (ILs). IL-6 can signal to endothelial cells to increase the recruitment of osteoclast precursors (Cheung, Simmons and You, 2012). IL-6 and IL-11 can also stimulate osteoclastogenesis independent of RANKL (Kudo et al., 2003). In addition, IL-1 (Jimi et al., 1999) and IL-8 stimulates monocytes to undergo osteoclastogenesis, and IL-8 stimulates osteoblasts to express RANKL (Bendre et al., 2003). Other factors involved include Jagged 1, GL12, hypoxia-inducible factor (HIF)-1α, and MMPs. In autocrine fashions, Jagged 1 can
upregulate IL-6 production (Sethi et al., 2011), while GL12 induces PTHrP (Sterling et al., 2006), in breast cancer cells. HIF-1α, induced by the hypoxic environment in bone, contributes by inhibiting osteoblastogenesis while promoting osteoclastogenesis (Hiraga et al., 2007). Furthermore, MMPs from tumor cells not only degrade collagen and facilitate invasion, but also suppress OPG and thereby increases osteoclastogenesis.

The above factors explain the promotion of bone resorption by cancer cells. On the other hand, factors released from the bone matrix during bone resorption also feed back to promote tumor growth. One of the most important ones is TGF-β (Korpal et al., 2009), which increases tumor cells’ production of PTHrP and establishes a mechanism for the vicious cycle (Yin et al., 1999). TGF-β can also induce VEGF and C-X-C chemokine receptor type 4 (CXCR4) expression in cancer cells, promoting angiogenesis and chemotaxis towards CXCL12 (Dunn et al., 2009), a chemokine secreted by stromal cells and osteocytes. IGFs are also stored in the bone matrix and increases PTHrP expression. In addition, bone is an abundant source of calcium that can bind to calcium-sensing receptors (CASR) on breast cancer cells to promote growth and PTHrP secretion (Sanders et al., 2000). Some proteins in the bone matrix also promote metastatic tumor growth, such as osteopontin (OPN) (Shevde et al., 2010) and bone sialoprotein (BSP) (Bellahcène et al., 2008). Finally, cytokines from the increased osteoclast population can support cancer cell growth and survival (Krzeszinski et al., 2017).

2.3.2.2 Osteosclerostic lesions

In the cases of osteosclerostic lesions, where there is excessive bone formation, the bone is harder but more prone to fracture due to the abnormal structure. There are a couple of molecules mediating this process. For example, the BB isoform of PDGF secreted by tumor cells can promote the migration and proliferation of osteoblasts (Yi et al., 2002). Tumor-produced endothelin-1 also stimulates bone formation (Yin et al., 2003). IGF released from the bone matrix during cancer cell invasion are capable of stimulating bone formation and decreasing collagen degradation (Hock, Centrella and Canalis, 1988). As bone formation is tightly coupled with bone resorption, the release rate of growth factors from the bone is still increased in osteosclerostic lesions, feeding back into tumor growth. Osteoblasts can also produce chemokines, specifically CXCL12, to attract cancer cells, and HIF to promote tumor growth (Devignes et al., 2018).
2.4 Current Treatment

2.4.1 Breast cancer treatment

The 5-year survival rate for primary breast cancer has improved greatly and is now more than 90% (Steeg, 2016). Standard ways to treat primary breast cancer have been established (Senkus et al., 2015). Breast cancer is typically diagnosed with imaging including mammography (low-energy X-rays), ultrasound, and magnetic resonance imaging (MRI). Core needle biopsy should then be used for pathological diagnosis. Tumors are categorized into subtypes based on cell markers for oestrogen receptor (ER), human epidermal growth factor 2 receptor (HER2), and progesterone receptor (PgR): Luminal A (ER-positive, HER2-negative), Luminal B (ER-positive, HER2-negative or -positive), non-luminal (HER2-overexpression, ER and PgR absent), and basal-like (ER and PgR absent, HER2-negative). The commonly used cell-line MDA-MB-231 is triple-negative (negative for ER, PgR, and HER2), the subtype with the worst patient outcomes (Chavez, Garimella and Lipkowitz, 2011). Treatments may then be determined based on subtypes, tumor location and size, and the general health of the patients. Surgery and radiation therapy are done to remove the primary tumor. Endocrine therapy such as tamoxifen and aromatase inhibitor should be used to treat all luminal cancers. Chemotherapy such as Taxol is used for triple-negative and non-luminal cancers, and Trastuzumab may be used in combination to target HER2 in non-luminal cancers.

2.4.2 Antiresorptive drugs

Although bone metastasis is incurable, therapies targeting bone remodeling are often prescribed to improve patients’ quality of life (Fontanella et al., 2015). Bisphosphonates are effective treatment to reduce skeletal-related events such as pain and fracture. They are stable derivatives of inorganic phosphonate where 2 phosphate groups are linked by esterification. They bind to hydroxyapatite crystals and, therefore, where the bone turnover rate is high. They target osteoclasts by inducing apoptosis in osteoclasts and inhibiting osteoclastogenesis. Bisphosphonates include clodronate, pamidronate, ibandronate, and zoledronic acid. Another common treatment is denosumab, a human IgG2 antibody against RANKL, that has been shown in clinical trials to be more efficient than bisphosphonates (Stopeck et al., 2010). New antibodies targeting osteoblasts such as romozumab against sclerostin are also being explored.


2.4.3 Exercise

In addition to prescribed treatments, exercise is often suggested for patients with breast cancer and has been shown to improve quality of life (McNeely, 2006; Adamsen et al., 2009) and survival (Holmes, 2005; Hamer and Warner, 2017). The recruitment of natural killer cells (Pedersen et al., 2016) and the stimulation of the anti-tumor effect of macrophages (Goh et al., 2012) are potential mechanisms for this observation. However, it had been thought previously that exercise may increase the risk of fracture in the fragile bones of patients with bone metastasis. Yet, studies in patients with prostate cancer bone metastasis had shown that this is not the case (Galvão et al., 2011; Cormie et al., 2013; Zopf et al., 2017). A recent clinical review further demonstrated that not only does exercise not increase fracture risk, but it can in fact be beneficial for patients suffering from bone metastasis (Sheill et al., 2018). An in vivo study also showed that tibial loading can reduce tumor and lesion size after the injection of breast cancer cells into the bone (Lynch et al., 2013). All these studies suggest an exciting potential for prescribing bone-loading exercise in patients with bone metastasis.

2.5 Mechanical Loading Regulates Bone Remodeling via Osteocytes

As described in section 2.3.1, in a normal bone without metastasized cancer cells, the balance between bone formation and resorption is maintained during remodeling through coupling and regulated by osteocytes. Importantly, Wolff’s law identified that bone adapts to the load it bears (Wolff, 1986). Disuse of the bone leads to a decline in bone mass, while dynamic loading increases bone mass (Lanyon and Rubin, 1984).

2.5.1 Bone mechanical loading

There are several ways loading on the bone can be sensed by cells in the bone (Figure 2-3) (Chen et al., 2010). Since the lacunar-canalicular system of the bone matrix is filled with fluid, mechanical loading on the bone produces a pressure gradient that drives interstitial fluid flow. This fluid flow in the bone matrix had been clearly demonstrated through an in vivo real-time imaging study using tracers (Price et al., 2011). Models have predicted the shear stress of this bone fluid flow to be 0.8-3 Pa on osteocytes residing in the lacunar-canalicular system during physiological activities, such as walking (Weinbaum, Cowin and Zeng, 1994). This level of shear stress had been shown to elicit response in osteocytes to signal to bone remodeling cells
(Bakker et al., 2001; You et al., 2008; Hoey, Kelly and Jacobs, 2011; Middleton, Al-dujaili, et al., 2017). Bone mechanical loading also causes hydrostatic pressure in the lacunar-canalicular system. Under dynamic loading, the hydrostatic compression would be cyclic. Oscillatory loading of 0 to 1000 microstrain at 1 Hz was estimated to induce hydrostatic pressure of 5 MPa around osteocytes in the lacunar-canalicular system (Gardinier et al., 2010), which is enough to stimulate osteocytes (Liu et al., 2010). In addition, during a physiological level of loading, strain ranges from 0.04% to 0.3%, but typically under 0.1% (Lanyon and Rubin, 1984). Although this is below the stimulatory level of strain for osteocytes and osteoblasts (You et al., 2000), strain may be amplified locally around osteocyte processes (L. You et al., 2001; Han et al., 2004), at the lacunae (Nicolella et al., 2006), and in bone remodeling units (McNamara et al., 2006). Therefore, fluid shear stress is the most well-understood mechanism for mechanically stimulating the osteocytes during bone loading, but cyclic pressure and strain may also contribute.

Figure 2-3: Different mechanisms through which cells in the bone may be stimulated when the bone is mechanically loaded. Figure replicated from Chen et al. (2010).
Outside the lacunar-canalicular system, the mechano-sensing mechanisms are less understood. A study has demonstrated using an *ex vivo* model that osteocytes (identified to be cells 40 µm below the periosteal surface) were more mechanosensitive than cells at the bone surface (Jing *et al.*, 2014). Osteoprogenitor cells in the bone marrow may sense the fluid flow from bone deformation (Qin *et al.*, 2003), although, outside the lacunar-canalicular system, they are unlikely to experience high shear stress (Bonewald and Johnson, 2008). For osteoprogenitors that reside in soft tissues and bone matrix that have not yet been mineralized, strain may be the major mechanical stimulus. Mechanical strain, fluid flow, and hydrostatic pressure have all been shown to stimulate osteoprogenitors (Chen *et al.*, 2010).

### 2.5.2 Osteocytes as bone mechanosensors

Since osteocytes make up more than 90% of the cells in the bone, are located in the lacunar-canalicular system that are capable of translating bone mechanical loading, and are more mechano-sensitive than osteoblasts (Lu *et al.*, 2012), they are likely to be the major mechanosensors in the bone. Evidently, mice with absence of osteocytes not only had more fragile bone, but also were resistant to disuse-induced bone loss (Tatsumi *et al.*, 2007).

Osteocytes can sense mechanical stimulation through a variety of mechanisms. Integrins expressed by osteocytes, such as the highly expressed αvβ3 integrin, have been shown to be required for bone’s adaptation to mechanical stimulation (Phillips *et al.*, 2008). They tether the cytoskeletons of osteocyte processes to the canalicular wall and may be involved in osteocytes’ sensing of bone fluid flow as flow creates drag on pericellular glycocalyx (L. You *et al.*, 2001). Fluid flow has also been shown to increase osteocytes’ expression of connexin 43 (Cx43) (Gluhak-Heinrich *et al.*, 2006), subunits of connexons, which form gap junction that allow direct cell-cell communication between neighbouring osteocytes and with osteoblasts (Guo *et al.*, 2006; Yellowley *et al.*, 2010). Integrins and Cx43 have been shown to interact and mediate the opening of hemichannels in response to fluid flow (Batra *et al.*, 2012). Ion channels may be also a potential candidate as osteocytes express gadolinium-sensitive cation channels that are sensitive to stretch and flow (Rawlinson, Pitsillides and Lanyon, 1996), as well as nifedipine-sensitive (L-type voltage-dependent) channels and a, b, and g units of epithelium sodium channels that respond to strain (Mikuni-Takagaki, 1999). Finally, primary cilia, which are deflected with fluid
flow (Malone et al., 2007), and the associated polycystin 1 and 2 (PC1/PC2) mechanosensory complex are present on osteocytes (Xiao et al., 2006) and may mediate mechanotransduction.

Upon sensing mechanical stimulations, osteocytes can translate this signaling into a multitude of downstream biochemical effectors (Chen et al., 2010), with two major ones being: 1) the reduction of the secreted RANKL to OPG ratio in response to flow, inhibiting osteoclastogenesis (You et al., 2008), and 2) the upregulation of sclerostin in response to disuse, inhibiting bone formation (Lin et al., 2009). An early event that occurs in mechanically stimulated osteocytes is the increase in intracellular calcium, most likely mediated by the T-type voltage-sensitive calcium channels as they are more common than the L-type in osteocytes (Thompson et al., 2011; Lu et al., 2012). Within minutes after, the release of nitric oxide (McGarry, Klein-Nulend and Prendergast, 2005), adenosine triphosphate (ATP), and PGE-2 (McGarry, Klein-Nulend and Prendergast, 2005; Ponik, Triplett and Pavalko, 2007) is also increased. Nitric oxide has been shown to inhibit bone resorption and increase bone formation (Klein-Nulend et al., 1995), and potentially reduce osteocyte apoptosis (Tan et al., 2008). PGE-2 is likely released through the Cx43 hemichannels. PGE-2 can stimulate in an autocrine way to increase Cx43 expression, and in a paracrine way to increase both bone formation (Forwood, 2009) and bone resorption (Nefussi and Baron, 1985). ATPs are also released through the Cx43 hemichannels. The deletion of the P2X<sub>7</sub> nucleotide receptor, an ATP-gated ion channel, significantly reduces the anabolic response of the bone to mechanical loading (Li et al., 2005). Blocking P2X<sub>7</sub> receptor in osteocytes in vitro also showed suppression of PGE-2 release. Furthermore, osteocytes stimulated with fluid flow reduce the production, but increase the secretion, of osteopontin (Ponik, Triplett and Pavalko, 2007), a factor that mediates the bone’s adaptation to disuse (Ishijima et al., 2002). Osteocytic markers, such as E11, PHEX, MEPE and DMP1, are also upregulated in response to mechanical loading. As well, fluid flow protects osteocytes against apoptosis (Cheung et al., 2011) and promotes dendrite elongation (Zhang et al., 2006).

2.5.3 Potential role of osteocytes in the mechanical regulation of bone metastasis

Osteocytes account for a majority of cells in the bone and are important orchestrators of the bone remodeling balance. Yet, while extensive work had been put into studying the interaction of tumor cells with bone-forming osteoblasts and bone-resorbing osteoclasts (Section 2.3.2), until very recently, the role osteocytes play in bone metastasis was unknown. The simplest interaction
to be studied would be the direct effect of non-stimulated osteocytes on cancer cells. Cui et al. (2016) collected conditioned medium (CM) from monolayers of MLO-Y4 osteocytes and applied the CM to breast cancer cells (MDA-MB-231, MCF-7, and ZR-75.1), prostate cancer cells (PC3 and DU145), and PZ-HPV-7 prostate non-cancerous epithelial cells. They found that CM from osteocytes 1) stimulated proliferation of MDA-MB-231, MCF-7, PC3, and DU145 cells, 2) promoted wound-healing migration of PC3 cells, but inhibited that of PZ-HPV-7, MCF-7, and ZR-75.1 cells, and 3) promoted Transwell migration of MDA-MB-231 cells. Chen et al. (2018) also investigated this direct interaction using spheroids of MDA-MB-231 breast cancer cells and MLO-A5 osteocytes. They observed that tumor spheroids are more compact when they are cultured with osteocyte spheroids or in CM from osteocyte spheroids. They further showed that this compaction may be due to osteocytes’ expression of collagen, biglycan, and osteonectin, and that these factors downregulates the transcription factor Snail in cancer cells to reverse the epithelial-to-mesenchymal transition (EMT).

A follow-up study by Liu et al. (2018) employed a similar spheroid system, but examined this interaction under stimulation of two drugs: Fluphenazine (FP) and Trifluoperazine (TFP). FP and TFP are both modulators of dopaminergic signaling, which has been suggested as a potential target for preventing the growth and bone metastasis of breast cancer (Minami et al., 2017). They showed that FP and TFP suppressed tumor growth and bone loss by reducing proliferation and migration of breast cancer cells, downregulating differentiation and function of osteoclasts, and increasing OPG expression by osteoblasts. Importantly, they showed that Snail was downregulated in 4T1.2 breast cancer cells by FP or TFP, and further reduced by simultaneous application of FP or TFP with CM from osteocyte spheroids. Another interesting study of the effect of drugs on osteocytes in the context of bone metastasis was provided by Zhou et al. (2015). They showed that CM from MLO-Y4 osteocytes treated with alendronate, a bisphosphonate antiresorptive drug, inhibited Transwell migration of MDA-MB-231 breast cancer cells. They then demonstrated that this is due to the activation of purinergic P2X receptor by ATP, whose release by osteocytes is increased upon bisphosphonate stimulation due to the increase of Cx43 hemichannels (Zhou et al., 2016). Yet, they also found that when ATP is supplied at higher concentrations (200 µM), ATP is hydrolyzed to ADP and this leads to an increase in breast cancer cell migration and growth. Furthermore, it was shown that ATPγS, a
non-hydrolyzable ATP analogue, significantly reduces tumor growth and migration in vitro and in vivo.

As osteocytes are located in the lacunar-canalicular system that efficiently translates mechanical loading on the bone to stimulatory fluid flow, it became of interest to study osteocytes’ effect on cancer cells under mechanical stimulation. Therefore, Zhou et al. (2016) further investigated the ATP-mediated regulation of osteocytes on cancer cells with the addition of fluid flow. They stimulated MLO-Y4 osteocytes with gravity-driven fluid flow using a peristaltic pump at a shear stress of 16 dyne/cm² (1.6 Pa) before collecting CM at 2 and 16 hours. They showed that, similar to the bisphosphonate-stimulated osteocytes, CM from flow-stimulated osteocytes inhibited the migration of MDA-MB-231 breast cancer cells due to the increase in Cx43 hemichannels on osteocytes and the subsequent increase in ATP release. Additionally, Sottnik et al. (2015) applied pressure to MLO-Y4 osteocytes at the level exerted by tumor growth in the bone (peak average of 37.84 mmHg 16 days after injection of DU145 prostate cancer cells into tibia). CM from these pressure-stimulated osteocytes increased the viability, migration, and invasion of DU145, LNCaP, and PC3 prostate cancer cells. Using neutralizing antibodies, they showed that these effects are due to osteocytes’ expression of the (C-C motif) ligand 5 (CCL5) cytokine and MMPs.

In addition to osteocytes and bone remodelling, cancer cells and other components of the tumor microenvironment can also be mechanically regulated, as summarized in our review paper (Ma, Middleton, et al., 2018) and described in detail in a recent review (Huang, Segall and Wu, 2017). Constriction and stiffness have been shown to increase migration of cancer cells. Flow was also shown to increase tumor cells’ migration but reduce their extravasation, probably due to the enhanced endothelial integrity under flow. Although the direct effect of biophysical forces on tumor cells is highly important, it may not play a major role in the bone marrow because vessels in the bone marrow have slower blood flow, are of larger size compared to tumor cells, and result in lower wall shear stress (Table 2-1). The cancer cells are expected to experience significant mechanical stimulation once in the bone matrix. Nevertheless, the focus of this thesis is during early metastasis before secondary tumors establish in the bone. Therefore, the aim of this project is to investigate how flow-stimulated osteocytes affect the cancer cells and their interaction with other cells in the bone marrow microenvironment.
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<td>Diameter (um)</td>
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<td>Blood Flow Velocity (mm/s)</td>
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Table 2-1: Vessel parameters. aValues from Popel and Johnson (2005). bValues from Mazo and von Andrian (1999). cValues calculated with the equation $\tau_w = \gamma \cdot \mu$, where $\mu$ (dynamic viscosity) is estimated to be 1.32 mPa·s (Pedersen et al., 2014).
Chapter 3

3 Hypothesis and Objectives

3.1 Rationale and Focus

Given the essential role osteocytes play as major bone mechano-sensors in regulating bone remodeling (Section 2.5.2), it is likely that osteocytes mediate the potential mechanical regulation of bone metastasis. We focused on breast cancer bone metastasis because the prevalence of bone metastasis in patients with advanced breast cancer has resulted in a significant increase in patient morbidity and mortality (Section 2.1). In particular, we examined the steps in the metastatic cascade after the cancer cells arrive in the bone microenvironment, but before the establishment of a secondary tumor (Sections 2.2.2 and 2.2.3). This is likely the earliest step that metastasizing cancer cells are subjected to signaling from osteocytes and may allow us to investigate the preventative effect of osteocytes on bone metastasis. Since osteoclasts play a major role in the vicious cycle of bone metastasis (Section 2.3.2) and respond to signaling from osteocytes (Sections 2.3.1 and 2.5.2), we explored whether osteoclasts mediate the signaling between osteocytes and breast cancer cells. In addition to osteoclasts, endothelial cells also play a major role in early metastasis (Section 2.2.3) and respond to osteocyte signaling (Sections 2.3.1). Because endothelial cells are located, in large numbers, between osteocytes of the bone matrix and cancer cells in the blood vessels during early metastasis, this thesis placed emphasis on the endothelial-mediated signaling of osteocytes on breast cancer cells.

To summarize, this thesis placed focus on investigating the effect of osteocytes’ response to oscillatory fluid flow on breast cancer cells’ bone metastatic potential through 1) direct signaling to the cancer cells, and indirect signaling mediated by 2) osteoclasts and 3) endothelial cells.

3.2 Hypothesis

Oscillatory fluid flow activates a multitude of signaling pathways in osteocytes that stimulate downstream effector cells such as osteoblasts, osteoclasts, and endothelial cells (Section 2.5.2). As bone-metastatic cancer cells often behave similarly to mesenchymal stem cells that give rise to osteoblasts or haematopoietic stem cells that give rise to osteoclasts (Section 2.3.2), they are expected to respond to a variety of signals secreted by flow-stimulated osteocytes. Additionally,
since osteoclasts and endothelial cells respond to signals from osteocytes (Section 2.3.1) and interact with cancer cells (Sections 2.3.2 and 2.2.3), they are likely to mediate the effect of flow-stimulated osteocytes on breast cancer cells.

### 3.3 Objectives

To investigate the hypothesis, this research was split up into three objectives, with one chapter dedicated to present the research results for each objective.

**Objective 1 (Chapter 4):** To examine whether factors secreted by osteocytes upon stimulation with oscillatory fluid flow can affect cancer cell activities.

**Objective 2 (Chapter 5):** To examine whether osteoclasts and endothelial cells mediate the effect of flow-stimulated osteocytes on breast cancer cell apoptosis and migration.

**Objective 3 (Chapter 6):** To investigate the impact of flow-stimulated osteocytes on the interaction between endothelial cells and breast cancer cells.
Chapter 4

4 Direct Effect of Flow-Stimulated Osteocytes on Cancer Cells

4.1 Introduction

Bone metastases, the spread of cancers from their primary sites to bone, are common and severe complications of cancers. It is estimated that 65-75% of patients with advanced breast and prostate cancer develop bone metastasis (Coleman, 2001). Potential symptoms of bone metastasis include pathological bone fractures, pain, hypercalcaemia, and nerve-compression syndromes (Roodman, 2004). Even more devastatingly, cancers are usually incurable once they have metastasized to bone (Weilbaecher, Guise and McCauley, 2011), greatly compromising the survival rate and the living standards of the cancer patients. It is estimated that 350,000 people in the United States die annually from bone metastases (Mundy, 2002).

Bone metastasis is a multi-step process (Chambers, Groom and MacDonald, 2002). First, angiogenesis has to occur to bring blood vessel and hence nutrient supply to the primary tumor. This then provides an escape route for spontaneously broken off cancer cells to intravasate into the blood vessels. These cancer cells then need to survive and travel in the circulation until they adhere or get physically arrested in capillaries that are of smaller sizes than the cancer cells. Here, chemokines from the bone may signal for cancer cells to extravasate and invade the bones. Metastatic cancer cells that preferentially migrate to the bone may express genes normally restricted to cells in the bone (Chen, Sosnoski and Mastro, 2010), allowing them to home to the bone in a mechanism similar to that of hematopoietic stem cells (Weilbaecher, Guise and McCauley, 2011).

A common intervention prescribed to improve the quality of life in patients with cancer is exercise (McNeely, 2006). Interestingly, a recent clinical review demonstrated the benefits of exercise for patients with bone metastases (Sheill et al., 2018). In addition, in an in vivo study, tibial loading was shown to reduce bone-metastatic tumor growth and lesion after the injection of breast cancer cells into the bone (Lynch et al., 2013). These studies suggest an inhibitory role of mechanical regulation on bone metastasis. Osteocytes are the major mechanosensors in the bone. They are terminally differentiated osteoblasts that reside in the empty spaces in the bone called
the lacuna that are interconnected to each other and to the bone marrow space via canals called the canaliculi. This lacunar-canicular system is filled with interstitial fluid that has been shown to produce a significant flow when the bone is mechanically loaded (Price et al., 2011). This fluid flow then exerts shear stress on the cells in the network at a frequency of around 1 Hz and a peak shear stress of 0.8-3 Pa (Weinbaum, Cowin and Zeng, 1994). In vitro experiments have shown that this level of shear stress is sufficient to elicit cellular responses from osteocytes (You et al., 2008; Chen et al., 2010; Middleton, Al-dujaili, et al., 2017). As these factors have been shown to reach endothelial cells and osteoclast progenitors in the bone marrows (Cheung, Simmons and You, 2012; Prasadam et al., 2014), the factors secreted by osteocytes should be able to reach and affect the metastasizing cancer cells in the blood vessels. Therefore, we hypothesized that soluble factors secreted by osteocytes in response to oscillatory fluid flow can affect cancer cell behaviour. In this chapter, we examined the impact of conditioned medium (CM) from flow-stimulated osteocytes on a variety of cancer cell activities. Some of the results presented in this chapter was published in the article “Mechanical regulation of breast cancer migration and apoptosis via direct and indirect osteocyte signaling” (Ma, Lam, et al., 2018).

4.2 Methods

4.2.1 Cell cultures

MLO-Y4 cells (gift of Dr. Bonewald, Indiana University, Indianapolis, IN, USA) were used as in vitro models for osteocytes. They were isolated from long bones of transgenic mice with the SV40 large T-antigen oncogene expressed under the osteocalcin promoter. When grown on collagen-coated surfaces, they have properties very similar to primary osteocytes in vivo, with a dendritic morphology and low alkaline phosphatase (ALP; an osteoblast marker) and high osteocalcin expression (Kato et al., 2010). They were cultured in alpha-Modified Essential Medium (α-MEM; catalog number 12571071; Gibco, Carlsbad, CA), supplemented with 2.5% fetal bovine serum (FBS; 12483-020; Gibco) for phenotype expression, 2.5% calf serum (CS; 16010-159; Gibco) for proliferation, and 1% penicillin and streptomycin (P/S; 15140122; Gibco) on petri dishes or glass slides coated with 0.15 mg/mL type-I rat tail collagen (354236; Corning Life Science, Lowell, CA).

The less-mechanosensitive MC3T3-E1 osteoblasts were used as controls (Klein-Nulend et al., 1995; Lu et al., 2012). They were cultured in α-MEM, supplemented with 10% FBS and 1% P/S
on petri dishes or glass slides coated with type-I rat tail collagen. They behave similarly to primary osteoblasts by exhibiting high ALP activity and having the capability to deposit hydroxyapatite (Sudo et al., 1983).

Highly metastatic MDA-MB-231 breast cancer cells and PC3 prostate cancer cells (ATCC, Manassas, VA) were used as models for breast and prostate cancer cells. Although they are human cancer cells, they have been widely used in animal models for bone metastasis (Yoneda et al., 2001; Pécheur et al., 2002; Kang et al., 2003; Darash-Yahana et al., 2004; Canon et al., 2008; Ganapathy et al., 2010; Rath Stern et al., 2012; Hibberd, Cossigny and Quan, 2013; Lynch et al., 2013). They were cultured in Kaighn's Modification of Ham's F-12 Media (21127022; Gibco), supplemented with 10% FBS and 1% P/S in filtered-cap flasks. Less migratory MCF-7 cells (HTB-22; ATCC) that are positive for estrogen and progesterone receptors were used as a control. They were cultured in Minimum Essential Medium Eagle (M4655; Sigma-Aldrich, St. Louis, MO), supplemented with 10% FBS and 1% P/S. Although they are weakly invasive on their own (Thompson et al., 1992), their migratory potential had been shown to be stimulated by factors such as IL-1β (Pérez-Yépez et al., 2012) and reduced by factors such as OPG (Jones et al., 2006).

4.2.2 Oscillatory fluid flow

2 hours of oscillatory fluid flow at 1 Hz with a peak shear stress of 1 Pa was applied to MLO-Y4 osteocytes and MC3T3-E1 osteoblasts. A peak shear stress of 1 Pa approximately simulates the level of fluid flow in the lacunar-canalicular network during physiological exercises, such as walking (Weinbaum, Cowin and Zeng, 1994). A 2-hour exposure is selected based on a previous experiment showing that this exposure period maximally impacted MLO-Y4 secretion of RANKL and OPG, important regulators of osteoclastogenesis (Kim et al., 2006), similar to osteocytes in vivo.

The application of oscillatory fluid flow was done with parallel-plate flow chambers (Figure 4-1), with detailed protocols published in a book chapter I co-authored (Middleton, Ma and You, 2017). Cells were seeded on collagen-coated glass slides 48 hours prior to experiment to ensure 80% confluency at the time of fluid flow stimulation. Flow chambers were assembled with glass slides seeded with cells inserted, placed in a humidified incubator, and connected to syringes on a linear actuator through tubing. The linear actuator generates an oscillatory fluid flow with a
peak flow rate of 27 mL/min, which yields a laminar parabolic flow in the flow chambers with a peak sinusoidal wall shear stress of 1 Pa at 1 Hz. Height of fluid flow in the flow chamber is kept low (width to height ratio of over 20) to ensure that more than 85% of cells experience homogenous shear stress.

Figure 4-1: Flow chamber (A) schematic, (B) parts, and (C) assembled. MLO-Y4 osteocytes were seeded on the glass slides and embedded into the flow chambers. The flow chamber is connected at the inlet to a syringe on the linear actuator via a tubing to generate oscillatory fluid flow.

After 2 hours of fluid flow stimulation, the glass slides were removed from the flow chambers and placed in 10 mL regular growth media. After 24 hours incubation, medium was extracted (conditioned medium; CM). Cells on glass slides were detached with trypsin and counted for normalization of protein to cell number. MLO-Y4 cells seeded on glass slides, but not placed in flow chambers, were used as controls for some experiments to account for potential variation between different experimental batches.
4.2.3 Transwell migration

Cancer cells were stained with cell tracker green (C2925; Life Technologies, Carlsbad, CA) on the day of experiment. 1200 μL of CM was added per well in a 12-well plate. Transwells with an 8 μm pore-size semi-permeable membrane (665638; Greiner Bio-One, Cassina de Pecchi, Italy) were then placed in the wells. 200 μL of 400,000 cells/mL cell suspension was added to Transwells and allowed to migrate for 6 hours. Cells were then fixed with 10% neutral buffered formalin (HT501128; Sigma-Aldrich), and the top of the Transwells was scrapped with cotton swabs to remove cells that did not migrate. Cells that remained on the Transwells were imaged (5 images per Transwell) and quantified with ImageJ (NIH, Bethesda, MD).

4.2.4 Apoptosis, proliferation, and viability

Cancer cells were cultured in 96-well plates in 50% regular growth media and 50% MLO-Y4 CM for 48 hours. Apoptosis of these cells was then visualized by staining with 5% APOPercentage dye (A1000; Biocolor, Carrickfergus, UK) for 1 hour. The APOPercentage dye binds to phosphatidyl ethanolamine on the outside of cell membrane and is taken in when cell membrane asymmetry is lost in cells committed to apoptosis. 2 images were taken per well and the number of all cells and APOPercentage-stained cells were counted. Bromodeoxyuridine (BrdU; 10280879001; Sigma-Aldrich), which are incorporated into newly synthesized DNAs in place of thymidine, was also added to the cells to quantify proliferation. Cells were then stained with eBioscience™ Fixable Viability Dye eFluor 450 (65-0863-14; Thermo Fisher Scientific, Waltham, MA, USA), fixed with ethanol, had DNA denatured, and stained with anti-BrdU fluorescein (11202693001; Sigma-Aldrich). The percentage of proliferating and viable cells was quantified using a flow cytometer (Beckman Coulter FC500).

4.2.5 Adhesion to collagen

Multi-well plates were coated with 0.15 mg/mL type-I rat tail collagen (354236; Corning Life Science). Cell tracker green-stained cancer cells were then added to the wells and incubated for 30 minutes in MLO-Y4 CM. The multi-well plates were then shaken for 1 minute and rinsed with phosphate-buffered saline solution (PBS). Cells that remained adhered were imaged (5 images per well) and quantified using imagej.
4.2.6 Protein quantification and blocking

Vascular endothelial growth factor (VEGF) concentration in MLO-Y4 CM was quantified with the Mouse VEGF Quantikine ELISA Kit (MMV00; R&D Systems, Minneapolis, MN). Each CM sample counts as 1 biological replicate and includes 3 technical replicates. All values were normalized to the numbers of MLO-Y4 cells on the slides when MLO-Y4 CM were collected. VEGF-neutralizing antibodies (goat antibody that binds mouse VEGF\textsubscript{164} isoform and had been shown to neutralize endothelial proliferation; AF-493-NA; R&D systems) was added to MLO-Y4 CM for blocking experiments.

4.2.7 Statistics

In some cases, average from an individual experiment is treated as 1 data-point in statistical analysis because this approximates the data as normally distributed (central limit theorem). The \( n \) number in these cases are the numbers of experiments with at least 4 biological replicates in each experiment. Student’s t-test (2-tail, paired) was used to test significance between flow and no-flow groups. P-values were reported for all tests, but a result is only considered significant when the p-value is lower than 0.05.

4.3 Results

4.3.1 Breast cancer cell activities

Since migration is essential for metastasis and cancer cells have been shown to home to the bone following chemokines secreted by cells in the bone, we first assayed cancer cell migration. The migration of highly metastatic MDA-MB-231 breast cancer cells towards MLO-Y4 CM was 45% higher when the MLO-Y4 osteocyte-like cells were stimulated with oscillatory fluid flow (1 Pa peak shear stress, 1 Hz, 2 hours). Meanwhile, the less-migratory MCF-7 breast cancer cells did not migrate significantly towards MLO-Y4 CM, either when the MLO-Y4 cells were stimulated to flow or not (Figure 4-2). Therefore, as MCF-7 cells would not reach osteocytes due to lack of migration, other activities in MLO-Y4 CM was not examined for MCF-7 cells.

Interestingly, CM from flow-stimulated MC3T3-E1 osteoblasts had no effect on MDA-MB-231 cell migration (Figure 4-3), although the MDA-MB-231 cells are just as attracted to the CM from static MC3T3-E1 cells as to the CM from static MLO-Y4.
Figure 4-2: Breast cancer cell migration towards osteocyte CM. (A) Number of MDA-MB-231 and MCF-7 breast cancer cells that migrated across Transwell towards CM from MLO-Y4 osteocytes, normalized to controls (MDA-MB-231 cell migration towards CM from MLO-Y4 cells not placed in flow chambers; shown as grey line). Data is presented as mean ± standard deviation, n = 3 experiments with 14 samples for MDA-MB-231, n = 2 experiments with 12 samples for MCF-7. (B) Sample paired images of cell tracker green-stained MDA-MB-231 and MCF-7 cell migration towards no-flow and flow MLO-Y4 CM. Scale bar = 200 µm.

Figure 4-3: Breast cancer cell migration towards osteoblast CM. Number of MDA-MB-231 breast cancer cells that migrated across Transwell towards CM from static and flow-stimulated MC3T3-E1 osteoblasts, normalized to controls (MDA-MB-231 migration towards CM from MLO-Y4 cells not placed in flow chambers). Data is presented as mean ± standard deviation, n = 4.

MDA-MB-231 breast cancer cells were then cultured in 50% MLO-Y4 CM and 50% MDA-MB-231 growth media for 48 hours and tested for their apoptosis. With the APOPercentage stain, it was shown that the apoptosis of MDA-MB-231 cells dropped by 12% when they were cultured in the CM from flow-stimulated MLO-Y4 osteocytes (Figure 4-4).
Figure 4-4: Breast cancer cell apoptosis in osteocyte CM. Percentage of apoptotic MDA-MB-231 breast cancer cells in CM from static and flow-stimulated MLO-Y4 osteocytes, normalized to controls (MDA-MB-231 cell apoptosis in CM from MLO-Y4 cells not placed in flow chambers). Data is presented as mean ± standard deviation, n = 3 experiments with 14 samples. Sample paired images of MDA-MB-231 cells stained with APOPercentage after culturing in no-flow and flow MLO-Y4 CM. Arrows indicate sample apoptotic cells in pink. Scale bar = 200 µm.

No difference was observed for proliferation and viability of MDA-MB-231 breast cancer cells conditioned in CM from static and flow-simulated osteocytes (Figure 4-5). However, the viabilities of MDA-MB-231 cells were greater than 97% in all samples. Further investigation with serum-starvation may be needed to reduce the viability baseline for better differentiation.

Figure 4-5: Breast cancer cell growth in osteocyte CM. Percentage of (A) proliferating and (B) viable MDA-MB-231 cells in MLO-Y4 CM. Data is presented as mean ± standard deviation, n = 12 for (A), n = 4 for (B).

In addition to migration and survival, adhesion to the secondary site is another important early step of metastasis. Since collagen accounts for 95% of organic components in bone, we measured cancer cell adhesion on collagen-coated surfaces. Nevertheless, no difference was observed in the adhesion of MDA-MB-231 breast cancer cells to collagen-coated surfaces in MLO-Y4 CM (Figure 4-6).
4.3.2 Osteocyte-secreted VEGF and breast cancer cell migration

The major observation from Section 4.3.1 was the increase in cancer cell migration towards CM from flow-stimulated osteocytes. VEGF is secreted by osteocytes (Cheung et al., 2011) and increases MDA-MB-231 breast cancer cells’ migration, invasion, and adhesion on endothelial monolayers (Lee et al., 2003). To investigate VEGF’s contribution in our observations, we first verified that flow-stimulated MLO-Y4 osteocytes secreted 48% more VEGF (Figure 4-7A). Next, we investigated whether the increased VEGF production is responsible for MDA-MB-231 breast cancer cells’ preferential migration towards CM from flow-stimulated MLO-Y4 osteocytes. It was shown that the application of VEGF-neutralizing antibodies abolished the differences in MDA-MB-231 cell migration towards MLO-Y4 CM, validating the effect of osteocyte-secreted VEGF on breast cancer cell migration (Figure 4-7B).

4.3.3 Prostate cancer cell activities

In addition to breast cancer, prostate cancer is another primary cancer that frequently metastasizes to the bone. We repeated selected experiments using the highly metastatic PC3 prostate cancer cells and demonstrated similar results. Specifically, we saw that the migration of PC3 cells towards MLO-Y4 CM was increased by 30% when the MLO-Y4 osteocytes were stimulated with flow, while CM from flow-stimulated MC3T3-E1 osteoblasts had no effect. This difference in migration was also blocked by the application of anti-VEGF antibodies. PC3 cell apoptosis in CM from flow-simulated MLO-Y4 osteocytes was also reduced by 11% (Figure 4-8).
Figure 4-7: VEGF and breast cancer cell migration towards osteocyte CM. (A) Concentration of VEGF in CM from MLO-Y4 osteocytes, normalized to MLO-Y4 cell numbers. Data is presented as mean ± standard deviation, n = 3 experiments with 21 samples. (B) MDA-MB-231 cell migration across Transwell towards MLO-Y4 CM with 0.015 µg/mL anti-VEGF neutralizing antibodies, normalized to controls (MDA-MB-231 migration towards CM from MLO-Y4 cells not placed in flow chambers without antibodies). Data is presented as mean ± standard deviation, n = 5.

Figure 4-8: Prostate cancer cell migration and apoptosis in osteocyte CM. (A) Number of PC3 prostate cancer cells that migrated across Transwell towards CM from static or flow-stimulated MLO-Y4 osteocytes or MC3T3-E1 osteoblasts with or without anti-VEGF neutralizing antibodies, normalized to controls (average PC3 cell migration towards CM from static MLO-Y4 cells in each experiment). Data is presented as mean ± standard deviation, n = 5. (B) Percentage of apoptotic PC3 prostate cancer cells in CM from static and flow-stimulated MLO-Y4 osteocytes, normalized to controls (average PC3 cell apoptosis in CM from static MLO-Y4 cells in each experiment). Data is presented as mean ± standard deviation, n = 8.
4.4 Discussion

In this chapter, we investigated the potential impact of factors secreted by osteocytes in response to oscillatory fluid flow on cancer cell activities. We observed that applying CM from flow-stimulated MLO-Y4 osteocyte-like cells directly to the highly metastatic MDA-MB-231 breast cancer cells and PC3 prostate cancer cells increased the migration (Figures 4-2 and 4-8) while reducing the apoptosis (Figures 4-4 and 4-8) of cancer cells. Meanwhile, CM from flow-stimulated osteocytes had no impact on breast cancer cells’ proliferation, viability, and adhesion to collagen surfaces.

There are many factors secreted by flow-stimulated osteocytes that may have a direct impact on cancer cell migration. For example, flow-stimulated osteocytes’ decreased RANKL (You et al., 2008) and increased adenosine triphosphate (ATP) (Zhou et al., 2016) secretion could inhibit cancer cell migration (Jones et al., 2006; Zhou et al., 2015); whereas their increased vascular endothelial growth factor (VEGF) (Cheung et al., 2011) and osteopontin (Terai et al., 1999) secretion could increase cancer cell migration (Bachelder, Wendt and Mercurio, 2002; Standal, Borset and Sundan, 2004). Here, we demonstrated that CM from osteocytes exposed to oscillatory fluid flow increased cancer cells’ migration due to the increased VEGF secretion (Figure 4-7). VEGF may act by upregulating cancer cells’ expression of C-X-C chemokine receptor type 4 (CXCR4) (Bachelder, Wendt and Mercurio, 2002), a receptor that is often implicated in bone metastasis (Furusato et al., 2010) because it increases cancer cell migration, that bind to stromal cell-derived factor 1 (SDF-1; also known as CXCL12) in MLO-Y4 CM. In addition, a recent study showed that CXCL12 is secreted by osteocytes in vivo and that CXCL12 production is increased when osteocytes are mechanically stimulated (Cabahug-Zuckerman et al., 2018). This is likely to further strengthen the migration of cancer cells towards CM from flow-stimulated osteocytes.

Interestingly, we also demonstrated that flow-stimulated MC3T3-E1 osteoblasts do not seem to have an impact on cancer cell migration (Figures 4-3 and 4-8). Although MC3T3-E1 osteoblast were isolated from cranial bones whose mechanisms for mechano-sensing are less understood (Pearson and Lieberman, 2004), they have been shown in various experiments to respond to oscillatory (J. You et al., 2001; Ponik, Triplett and Pavalko, 2007), pulsatile (McGarry, Klein-Nulend and Prendergast, 2005), and laminar (Genetos et al., 2004) fluid flow. Meanwhile, this
response of osteoblasts to mechanical stimulation had also been shown to be less sensitive than osteocytes (Klein-Nulend et al., 1995; Lu et al., 2012). Our study not only supports this observation, but also validates that the observed effect is specific to MLO-Y4 osteocytes and not a general response to mechanical stimulation.

Similarly, it should be noted that the non-metastatic breast cancer cell-line MCF-7 did not migrate towards CM from osteocytes or osteoclasts under the same experimental condition as the highly metastatic MDA-MB-231 cells. This confirms a previous study that had shown the weakly invasive and migratory property of MCF-7 cells both in vitro and in vivo (Thompson et al., 1992). More importantly, this confirms that CM from osteocytes specifically attracts MDA-MB-231 cells, which have been shown to metastasize to the bone in vivo (Hibberd, Cossigny and Quan, 2013). Since MCF-7 cells did not migrate towards osteocyte CM, their apoptosis was not studied.

Our observation aligns with the study by Sottnik et al. (2013), which showed that pressure, another form of mechanical stimulation, supports prostate cancer cells viability and migration. On the other hand, it seem to contradict the study by Zhou et al. (2016), which demonstrated that osteocytes’ increased connexin hemichannels expression and subsequent adenosine triphosphate (ATP) release when stimulated with uni-directional flow can inhibit breast cancer cell migration. However, there are significant differences on the experimental design between this study and our study. First of all, we apply oscillatory fluid flow, which more closely simulates bone fluid flow (Weinbaum, Cowin and Zeng, 1994) and has been shown to affect osteocyte signaling in a different way from other flow profiles (Jacobs et al., 1998), instead of uni-directional flow. Secondly, we incubated osteocytes for 24 hours post-flow before CM collection. As ATP molecules are degraded fast once outside of cells (Fitz, 2007), the ATPs secreted in response to flow may have been broken down during the 24-hour incubation.

In addition, we observed a decrease in the apoptosis of MDA-MB-231 breast cancer cells and PC3 prostate cancer cells when they were cultured in CM from flow-stimulated MLO-Y4 osteocytes (Figures 4-4 and 4-8). Although the magnitude of change is small, it is still in contrast to the in vivo observation by Lynch et al. (2013). They saw that tibial loading reduced tumor growth in the bone after MDA-MB-231 cells were injected into mice tibia, while we observed an overall pro-metastatic potential of mechanically stimulated osteocytes. Although we believe the
regulation of bone mechanical loading on bone metastasis is mediated by osteocytes as they are the largest population of cells in the bone and are highly mechano-sensitive, the direct signaling from osteocytes to cancer cells may not be the major pathway. In a physiological environment, there are many other cells in the bone marrow blood vessels, where the metastasizing cancer cells are, that also respond to factors secreted by osteocytes. As osteocytes in the bone matrix are not in an ideal location to signal directly to the cancer cells in the blood vessels, it is likely that these other cells mediate the signaling between the two cell populations.

Therefore, the limitations of this study include the lack of other cells and real-time signaling. Another major limitation of this study comes from the use of cell-lines. MLO-Y4 cells are the most well-understood osteocyte cell-line (Dallas, Prideaux and Bonewald, 2013). However, unlike osteocytes in vivo, they have a low expression of sclerostin, an important factor that mediate flow-stimulated osteocytes’ effect on bone formation by osteoblasts. Therefore, there may also be other unidentified differences between the immortalized cells and primary osteocytes. Unfortunately, primary osteocytes are difficult to obtain and cannot proliferate as they are terminally differentiated (Rath Stern et al., 2012). Nevertheless, this also means that the MLO-Y4 mice osteocytes would be from a different species than the cancer cells as we wish to study human cancer cells. Fortunately, the cancer cell-lines chosen here (MDA-MB-231 and PC3) are very well-established cell-lines for in vivo mice metastasis models (Yoneda et al., 2001; Pécheur et al., 2002; Kang et al., 2003; Darash-Yahana et al., 2004; Canon et al., 2008; Ganapathy et al., 2010; Rath Stern et al., 2012; Hibberd, Cossigny and Quan, 2013; Lynch et al., 2013), and their interactions with mice bone cells have been characterized extensively. Using these commonly used cell-lines allows us to better compare our results to previous studies.

4.5 Conclusion

The direct signaling from osteocytes stimulated with oscillatory fluid flow to cancer cells seem to be pro-metastatic. Secreted factors from flow-stimulated osteocytes increased the migration and reduced the apoptosis of breast and prostate cancer cells. However, the direct communication between osteocytes and cancer cells may not be the major pathway since this contradicts a previous in vivo study that demonstrated an attenuation of an established bone metastasis by mechanical loading. The effects of flow-stimulated osteocyte signaling on cancer cells mediated by other cell types are discussed in Chapters 5 and 6.
Chapter 5

5 Osteoclast- and Endothelial-Mediated Effect of Flow-Stimulated Osteocytes on Breast Cancer Cells

5.1 Introduction

Breast cancer bone metastasis, the migration of primary breast cancer cells to the bone, occurs in 65-75% of patients with advanced breast cancer (Coleman, 2006), and significantly increases the morbidity and mortality of patients. The metastasized tumor cells are capable of disrupting the bone remodelling balance (Chen, Sosnoski and Mastro, 2010), causing bone lesions and leads to symptoms such as bone fractures and pain. It is also incurable once a secondary tumor is established in the bone, resulting in a 5-year survival rate of only 20% (Green, Murray and Hortobagyi, 2000) that has not been significantly improved in the past 10 years (Steeg, 2016).

Since extravasation and secondary tumor growth requires complex interactions with other cells in the secondary site, there is a distinct preference in metastasis sites for different cancers (Chambers, Groom and MacDonald, 2002). As breast cancers most frequently metastasize to the bone, researchers have studied the interaction between breast cancer cells and multiple cells types in the bone. In particular, a vicious cycle has been identified between breast cancer cells and osteoclasts in which both tumor and lesions in the bone increase (Kingsley et al., 2007). The cycle starts with breast cancer cells upregulating the recruitment and differentiation of osteoclasts by signaling directly to osteoclasts (Mancino et al., 2001) and through osteoblasts (Thomas et al., 1999). The recruited osteoclasts can then support the cancer cells both by direct signaling (Krzeszinski et al., 2017) and by releasing growth factors from the bone matrix (Weilbaecher, Guise and McCauley, 2011). Therefore, many current treatments target osteoclasts. For example, denosumab blocks receptor activator of nuclear factor-kB ligand (RANKL), an essential factor for osteoclastogenesis (Yasuda et al., 1998), and has been demonstrated to successfully reduce bone turnover in patients with breast cancer bone metastasis (Stopeck et al., 2010).

However, osteocytes, an important regulator of bone remodeling (Chen et al., 2010), have only recently been studied for their potential role in regulating bone metastasis (Zhou et al., 2015, 2016; Cui, Evans and Jiang, 2016). Osteocytes are the most abundant cells in the bone
(Bonewald, 2011). They reside in lacunae in the bone that are interconnected by canaliculi. When the bone is mechanically loaded, such as during exercise, oscillatory fluid flow is generated in this lacunar-canalicular system (Price et al., 2011). Osteocytes are known to signal to other cells in the bone when stimulated with a physiological levels of fluid shear stress (0.5-3 Pa (Weinbaum, Cowin and Zeng, 1994)) (Chen et al., 2010). Specifically, bone resorption by osteoclasts is downregulated (You et al., 2008), while bone formation by osteoblasts is upregulated (Robling et al., 2008). This demonstrates the capability of osteocytes to regulate the bone-remodeling balance and break the vicious cycle of bone metastasis.

Furthermore, mechanically stimulated osteocytes can signal to endothelial cells and affect angiogenesis (Cheung et al., 2011) as well as their expression of intercellular adhesion molecule 1 (ICAM-1) by lowering interleukin 6 (IL-6) secretion (Cheung, Simmons and You, 2012). This, consequently, have a potential regulatory impact on cancer cell extravasation. In fact, since endothelial cells will be in greater abundance and at a closer distance to the metastasizing breast cancer cells in blood vessels than osteoclasts and osteocytes (Kopp et al., 2005), this signaling pathway may have more impact before the vicious cycle is established.

Therefore, we hypothesize that mechanical loading may regulate processes in bone metastases via osteocyte signaling to osteoclasts and endothelial cells. Mechanically stimulated osteocytes could affect cancer cell extravasation as well as signaling to osteoclasts to alter their interaction with metastasizing cells. Although numerous studies have been performed to investigate the role of osteoclasts (Weilbaecher, Guise and McCauley, 2011) and endothelial cells (Chouaib et al., 2010) on bone metastasis, studies involving osteocytes, the major mechanosensors in the bone that regulate both osteoclasts (You et al., 2008) and endothelial cells (Cheung et al., 2011; Cheung, Simmons and You, 2012), remains limited. This chapter aims to investigate the effect of osteocytes’ response to oscillatory fluid flow on breast cancer migration and apoptosis through indirect signaling mediated by osteoclasts and endothelial cells. Understanding the role of mechanically stimulated osteocytes in bone metastasis could eventually lead to future studies on optimizing exercise regimen that not only improve patients’ quality of life (McNeely, 2006), but also lower their risk for bone metastasis. Contents of this chapter, along with some reasons from Chapter 4, have been published in the article “Mechanical regulation of breast cancer migration and apoptosis via direct and indirect osteocyte signaling” (Ma, Lam, et al., 2018).
5.2 Methods

5.2.1 Cell cultures

Highly metastatic MDA-MB-231 human breast cancer cells (catalog number HTB-26; ATCC, Manassas, VA) were cultured in Kaighn's modification of Ham's F-12 media (21127022; Gibco, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) (12483-020; Gibco) and 1% penicillin-streptomycin (P/S) (15140122; Gibco). Non-metastatic MCF-7 cells (HTB-22; ATCC) were used as a control. They were cultured in Minimum Essential Medium Eagle (M4655; Sigma-Aldrich, St. Louis, MO), supplemented with 10% FBS (12483-020; Gibco) and 1% P/S. MLO-Y4 osteocyte-like cells (a gift from Dr. Bonewald, Indiana University, Indianapolis, IN, USA) were cultured in α-Modified Essential Medium (12571071; Gibco), supplemented with 5% FBS (12483-020; Gibco), 5% calf serum (16010-159; Gibco), and 1% P/S on petri dishes or glass slides coated with 0.15 mg/mL type-I rat tail collagen (354236; Corning Life Science, Lowell, CA). RAW264.7 osteoclast precursors (TIB-71; ATCC) were cultured in Dulbecco's Modified Eagle Medium (D5671; Sigma-Aldrich) supplemented with 10% FBS (SH30071.03; HyClone, Logan, UT), 2% L-Glutamine (25030081; Gibco), and 1% P/S. Human umbilical vein endothelial cells (HUVECs) (a gift from Dr. Young and Dr. Simmons, University of Toronto, Toronto, ON, Canada) were cultured in endothelial cell growth media (CC-3162; Lonza, Basel, Switzerland). All cells were maintained at 37°C and 5% CO₂ in a humidified incubator.

5.2.2 Oscillatory fluid flow on osteocytes

MLO-Y4 osteocyte-like cells were cultured on collagen-coated glass slides until 80% confluency, then parallel-plate flow chambers (Jacobs et al., 1998) were used to apply oscillatory fluid flow (peak shear stress of 1 Pa; 1 Hz; 2 hours) as described in Chapter 4. Static MLO-Y4 osteocytes were also placed in flow chambers, but not stimulated with flow. Cells were then placed in 10 mL MLO-Y4 growth medium and incubated for 24 hours. Conditioned medium (CM), containing soluble cytokines secreted by MLO-Y4 cells, was then extracted (MLO-Y4 CM). A peak fluid shear stress of 1 Pa was selected because osteocytes residing in the lacuna are estimated to experience shear stress of 0.5-3 Pa physiologically (Weinbaum, Cowin and Zeng, 1994), and 1 Pa approximately simulates mechanical loading during a mild exercise. The timings of 2-hour flow and 24-hour post-flow incubation were chosen based on studies that demonstrated MLO-Y4 CM collected after these durations regulates osteoclastogenesis (You et al., 2008).
5.2.3 Osteoclastogenesis

RAW264.7 osteoclast precursors were cultured in 50% growth media and 50% MLO-Y4 CM in 12-well plates. 10 ng/mL RANKL (462-TR-010; R&D Systems, Minneapolis, MN) was added to the MLO-Y4 CM because MLO-Y4 CM can regulate osteoclastogenesis (You et al., 2008), but is not enough to stimulate osteoclastogenesis alone (Zhao et al., 2002). Medium was changed daily after day 3. At day 6, medium was replaced with 3 mL of RAW264.7 growth media to ensure the collected CM did not contain factors secreted by MLO-Y4 cells. At day 7, medium was collected (RAW264.7 CM) and cells were fixed and stained for tartrate-resistant acid phosphatases (TRAP) using a leukocyte acid phosphatase kit (387A; Sigma-Aldrich). Osteoclasts were identified as TRAP-positive cells containing three or more nuclei (Hsu et al., 1999). The total number of osteoclasts in a well was quantified.

5.2.4 Transwell migration

Breast cancer cells (MDA-MB-231 or MCF-7 cells) were stained with cell tracker green (C2925; Life Technologies, Carlsbad, CA) on the day of experiment. 1200 µL of CM was added per well in a 12-well plate. Transwells with an 8 µm pore-size semi-permeable membrane (665638; Greiner Bio-One, Cassina de Pecchi, Italy) were then placed in the wells. 200 µL of 400,000 cells/mL cell suspension was added to Transwells and allowed to migrate for 6 hours. Cells were then fixed with 10% neutral buffered formalin (HT501128; Sigma-Aldrich), and the top of the Transwells was scrapped with cotton swabs to remove cells that did not migrate. Migrated cells were imaged (5 images per well) and quantified with ImageJ (NIH, Bethesda, MD).

5.2.5 Trans-endothelial migration

HUVECs were seeded on Transwells coated with 0.15 mg/mL type-I rat tail collagen, a technique that is commonly used in cancer extravasation studies (Gupta et al., 2007; Ghislin et al., 2012; Rodrigues-Ferreira et al., 2012) and has been shown to maintain the polarization of endothelial cells (Georgieva et al., 2011). 1200 µL of MLO-Y4 CM was added to the wells and 200 µL of cell tracker green-stained breast cancer cell suspension (400,000 cells/mL) was added to the Transwells. After 18 hours, cells were fixed 10% neutral buffered formalin, and the cells that have not migrated were scrapped off with a cotton swab. 5 images were taken per well and number of migrated cells was quantified using imagej. An extra Transwell seeded with HUVEC was stained with anti-VE-Cadherin antibody (ab33168; abcam, Cambridge, UK) for each set of
experiment to confirm that an intact layer was formed before performing the trans-endothelial migration. Blocking experiments were performed similarly to previously described (Cheung et al., 2012). Briefly, 1 μg/mL mouse anti-human ICAM-1 neutralizing antibody (BBA3; R&D Systems) was added to endothelial cells in MLO-Y4 CM for 1 hour prior to the addition of breast cancer cells, and 2 μg/mL rat anti-mouse IL-6 neutralizing antibody (MAB406; R&D Systems) was added to MLO-Y4 CM 1 hour prior to addition to endothelial cells.

5.2.6 Apoptosis

MDA-MB-231 cells were cultured in 50% regular growth media and 50% RAW264.7 CM in 96-well plates for 48 hours. Apoptosis of these cells was then visualized by staining with 5% APOPercentage dye (A1000; Biocolor, Carrickfergus, UK) for 1 hour. For assaying apoptosis of MDA-MB-231 cells in CM from HUVECs conditioned in MLO-Y4 CM, HUVECs were first cultured in 96-well plates until 100% confluency was achieved. 100 μL of MLO-Y4 CM and 100 μL of HUVEC growth medium was then added to the HUVECs. After 24 hours, medium was removed and HUVECs were cultured in 200 μL of HUVEC growth medium for 12 hours. Finally, 100 μL of the medium (HUVEC CM) was collected and added to MDA-MB-231 cells. Apoptosis of these MDA-MB-231 cells was imaged (2 per well) and quantified in 48 hours.

5.2.7 Statistics

Each type of experiment was performed at least 3 times with each experiment containing at least 4 samples (biological replicates), paired between the flow-osteocyte groups, where MLO-Y4 osteocytes were stimulated with flow, and the static-osteocyte groups, where MLO-Y4 osteocytes were placed in flow chambers but not stimulated with flow. The order within the pair was randomized to avoid time-dependent variations and this order is maintained throughout sequential conditioning and experiments. Results were normalized to controls (results from MDA-MB-231 breast cancer cells in CM when the MLO-Y4 osteocytes were seeded on glass slides but not placed in flow chambers) to account for potential differences in experimental conditions. Average from an individual experiment is treated as 1 data-point in statistical analysis because this approximates the data as normally distributed (central limit theorem). Student’s t-test (2-tail, paired) was used to test significance between static- and flow-osteocyte groups (significance was taken at α = 0.05).
5.3 Results

5.3.1 Osteoclast-mediated regulation

Since studies have shown that osteocytes signal to osteoclasts (You et al., 2008) and that osteoclasts affect cancer cell activities (Krzeszinski et al., 2017), it is important to investigate whether osteoclasts mediate the signaling of flow-stimulated osteocytes on breast cancer cells. First, using TRAP-stain, we confirmed that 44% fewer osteoclasts differentiated when RAW264.7 cells were cultured in CM from flow-stimulated MLO-Y4 osteocytes (Figure 5-1).

Figure 5-1: Osteoclastogenesis in osteocyte CM. (A) Number of differentiated osteoclasts, normalized to controls (RAW264.7 osteoclastogenesis in CM from MLO-Y4 cells not placed in flow chambers). Data is presented as mean ± standard deviation, n = 3 experiments with 19 samples. (B) Sample paired images of differentiated osteoclasts after culturing in no-flow and flow MLO-Y4 CM. Osteoclasts are identified as TRAP-positive (purple-stained) multi-nucleated cells, indicated by arrows. Scale bar = 100 µm.

Subsequently, breast cancer cell migration towards CM from RAW264.7 cells cultured in MLO-Y4 CM was assayed. Migration of highly metastatic MDA-MB-231 cells towards RAW264.7 CM was 47% less when the MLO-Y4 cells were stimulated with flow, while the non-metastatic MCF-7 cells did not migrate towards RAW264.7 CM (Figure 5-2). Therefore, the apoptosis of MCF-7 cells in RAW264.7 CM was not quantified as they will not migrate towards osteoclasts.
Figure 5-2: Breast cancer cell migration towards CM from osteoclasts cultured in osteocyte CM. (A) Number of MDA-MB-231 and MCF-7 cells that migrated through Transwell towards RAW264.7 CM, normalized to controls (MDA-MB-231 migration when MLO-Y4 cells were not placed in flow chambers). Data is presented as mean ± standard deviation, n = 3 experiments with 20 samples for MDA-MB-231, n = 2 experiments with 12 samples for MCF-7. (B) Sample paired images of migration. Scale bar = 200 µm.

Apoptosis rate of MDA-MB-231 cells in RAW264.7 CM was shown to be 55% higher when MLO-Y4 cells were stimulated with flow (Figure 5-3).

Figure 5-3: Breast cancer cell apoptosis in osteoclast CM. Number of apoptotic MDA-MB-231 cells in CM from RAW264.7 cells cultured in MLO-Y4 CM, normalized to controls (MDA-MB-231 apoptosis when MLO-Y4 cells were not placed in flow chambers). Data is presented as mean ± standard deviation, n = 3 experiments with 14 samples. (D) Sample paired images of MDA-MB-231 cells stained with APOPercentage. Arrows indicate apoptotic cells in pink. Scale bar = 200 µm.

5.3.2 Endothelial-mediated regulation

Although osteoclasts are potent mediators of flow-stimulated osteocytes’ signaling to breast cancer cells, there are a relatively small number of osteoclasts at the bone surface (Kular et al., 2012) before the bone metastasis vicious cycle is established. On the other hand, endothelial
cells, the first barrier encountered by metastasizing cancer cells, are present in greater number and in close proximity to the metastasizing cancer cells in blood vessels (Kopp et al., 2005). They play a major role in metastasis (Chouaib et al., 2010) and are regulated by osteocytes, as shown by our previous studies (Cheung et al., 2011; Cheung, Simmons and You, 2012). Therefore, we here examined whether endothelial cells mediated osteocytes’ effect on metastasis. The trans-endothelial migration of highly metastatic MDA-MB-231 cells towards MLO-Y4 CM was reduced by 34% when MLO-Y4 cells were stimulated with flow (Figure 5-4). An extra Transwell seeded with HUVEC was stained for VE-cadherin to confirm that an intact endothelial layer was achieved for each set of experiment. Since the non-metastatic MCF-7 cells did not migrate towards MLO-Y4 CM without the presence of endothelial cells, their trans-endothelial migration was not examined.

![Figure 5-4](image)

**Figure 5-4: Breast cancer cell trans-endothelial migration towards osteocyte CM.** (A) Number of MDA-MB-231 cells migrated across a layer of HUVECs on a Transwell towards MLO-Y4 CM, normalized to controls (MDA-MB-231 cell trans-endothelial migration towards CM from MLO-Y4 cells not placed in flow chambers). Data is presented as mean ± standard deviation, n = 4 experiments (23 samples) for no-antibody, n = 4 experiments (18 samples) for ICAM-1 antibody, n = 3 experiments (12 samples) for IL-6 antibody. (B) Sample image of an intact layer of HUVECs on Transwells stained for VE-cadherin. Scale bar = 200μm. (C) Sample paired images of cell tracker green-stained MDA-MB-231 cell trans-endothelial migration towards no-flow and flow MLO-Y4 CM, with and without ICAM-1 and IL-6 neutralizing antibodies. Scale bar = 200 μm.
As endothelium is the first barrier encountered by breast cancer cells circulating in blood vessels during metastasis, we also investigated the factors responsible for the effect we observed on trans-endothelial migration. Because mechanically stimulated MLO-Y4 cells are less apoptotic (Cheung et al., 2011) and secrete less IL-6 (Cheung, Simmons and You, 2012), MLO-Y4 CM from these cells reduces the expression of ICAM-1 by endothelial cells (Cheung, Simmons and You, 2012), and subsequent MDA-MB-231 cancer cell extravasation (Nizamutdinova et al., 2008). Here we administered anti-IL-6 and anti-ICAM-1 neutralizing antibodies to investigate the mechanism behind observed difference in trans-endothelial migration. Application of anti-ICAM-1 and anti-IL-6 (Figure 5-4) antibodies not only significantly reduced the overall trans-endothelial migration of MDA-MB-231 cells, but also abolished the increased trans-endothelial migration towards MLO-Y4 CM from static MLO-Y4 osteocytes.

Furthermore, the apoptosis rate of MDA-MB-231 cells in HUVEC CM was shown to be 29% less when MLO-Y4 cells were stimulated with flow (Figure 5-5).

**Figure 5-5:** Breast cancer cell apoptosis in CM from endothelial cells cultured in osteocyte CM. (A) Apoptosis of MDA-MB-231 cells in CM from HUVECs cultured in MLO-Y4 CM, normalized to controls (MDA-MB-231 apoptosis in CM from HUVECs cultured in CM from MLO-Y4 cells not placed in flow chambers). Data is presented as mean ± standard deviation, n = 3 experiments (16 samples). (B) Sample paired images of MDA-MB-231 cells stained with APOPercentage after culturing in HUVEC CM when MLO-Y4 cells were static and stimulated with flow. Arrows indicate sample apoptotic cells in pink. Scale bar = 200 μm.

### 5.4 Discussion

In this study, we investigated whether osteoclasts and endothelial cells mediate the effect of mechanically stimulated osteocytes on breast cancer cells. We observed that, indeed, signals from osteocytes exposed to oscillatory fluid flow (1 Pa peak shear stress, 1 Hz, 2 hours) can have potent regulatory effect on cancer cells. Specifically, as summarized in Figure 5-6, direct and cell-mediated signaling from flow-stimulated osteocytes seem to have opposite effects on breast
cancer migration and apoptosis. Applying CM from flow-stimulated MLO-Y4 osteocyte-like cells directly to metastatic MDA-MB-231 breast cancer cells increased migration while reducing apoptosis of breast cancer cells (Chapter 4). However, CM from RAW264.7 osteoclasts cultured in CM from flow-stimulated osteocytes reduced breast cancer cells’ migration (Figure 5-2) while increasing their apoptosis (Figure 5-3). As well, CM from flow-stimulated osteocytes reduced the trans-endothelial migration of breast cancer cells (Figure 5-4). CM from HUVECs cultured in CM from flow-stimulated osteocytes also increased breast cancer cell apoptosis (Figure 5-5). These suggest an anti-metastatic potential of flow-stimulated osteocytes mediated by osteoclasts and endothelial cells.

**Figure 5-6: Summary of direct and indirect signaling from flow-stimulated osteocytes on breast cancer cell migration and apoptosis.** Applying CM from flow-stimulated osteocytes directly to breast cancer cells seem to be pro-metastatic, by increasing breast cancer cell migration and reducing apoptosis (Chapter 4). However, incorporating osteoclasts and endothelial cells suggests an anti-metastatic potential of flow-stimulated osteocytes, by reducing breast cancer cell migration (Figure 5-2) and extravasation (Figure 5-4), while increasing their apoptosis (Figures 5-3 and 5-5).

As previously mentioned, although osteocytes are the major population of cells in the bone, they reside within the bone matrix, and thus other cells that are located closer to metastasizing cancer cells in the blood vessels may mediate the signaling from osteocytes. In particular, osteoclasts, identified as a major player in the vicious cycle of bone metastasis, interact closely with cancer cells (Krzeszinski *et al.*, 2017) and osteocytes (You *et al.*, 2008). They are located in the bone remodeling units that are close to the sinusoid blood vessels (Sims and Martin, 2014), which has a slow blood flow and, therefore, is a common site for breast cancer cells to adhere and extravasate (Mastro, Gay and Welch, 2003). Consequently, their proximity and known
interaction with cancer cells and osteocytes make them a potential candidate for mediating of osteocytes’ effect on cancer cells.

In this study, we confirmed that flow-stimulated osteocytes reduce osteoclastogenesis (Figure 5-1). We also demonstrated that CM from these osteoclasts reduced migration (Figure 5-2) and increased apoptosis (Figure 5-3) of MDA-MB-231 breast cancer cells, while the MCF-7 breast cancer cells again was not stimulated to migrate. This suggests an anti-tumor potential of mechanical loading that aligns well with a previous in vivo study showing a reduction in tumor size when mice with breast cancer cells injected into their tibia are subjected to tibial loading (Lynch et al., 2013). Since we have verified that flow-stimulated osteocytes reduce osteoclastogenesis, we hypothesized that this observation is a result of osteoclasts secreting various chemoattractants for breast cancer cells. This is supported by the Krzeszinski et al. study, which recently identified arachidonic acid as the osteoclast-secreted lipid cytokine responsible for attracting breast cancer cells (Krzeszinski et al., 2017). The osteoclast-secreted factors that recruit osteoblasts for bone remodeling balance (Sims and Martin, 2014) could potentially contribute as well since breast cancer cells are known to express signaling factors and receptors usually unique to bone cells (Awolaran, Brooks and Lavender, 2016). For example, sphingosine-1-phosphate (S1P) is secreted by osteoclasts (Ryu et al., 2006) and is known to support cancer cell migration and growth (Sarkar et al., 2005). However, more investigation would be needed to identify the osteoclast-secreted factors responsible for our observation.

On the other hand, osteoblasts have also been shown to regulate metastasizing breast cancer cells directly and indirectly through osteoclasts (Logothetis and Lin, 2005). A study demonstrated osteoblasts’ potential to modulate the high bone turnover rate caused by metastatic breast cancer cells (Krawetz et al., 2009). In vivo, mechanically stimulated osteocytes reduce sclerostin secretion (Robling et al., 2008), which in turn increase osteoblastic bone formation through BMP and Wnt signaling pathways (Winkler, 2003; Lin et al., 2009) as well as reduce osteoclastic bone resorption through RANKL-dependent pathway (Wijenayaka et al., 2011). This may help to restore the altered bone turnover rate in bone metastasis. Interestingly, breast cancer cells can also express sclerostin to reduce osteoblastic bone formation (Mendoza-Villanueva, Zeef and Shore, 2011). Although the role of sclerostin and osteoblasts in bone metastasis is highly interesting, very little is known regarding how osteocyte-derived sclerostin affects bone metastasis. Unfortunately, our model could not investigate this due to the low sclerostin
expression by MLO-Y4 cells. New osteocyte cell-lines that have high expression of sclerostin, such as the OCY454 cells (Spatz et al., 2015), may be used for further investigation.

Although osteoclasts on the bone remodeling surface are in the vicinity of, and interact with, both osteocytes ad metastasizing cancer cells, there are relatively few osteoclasts in normal bone before the bone metastasis vicious cycle is established (Kular et al., 2012). Therefore, we shifted our focus to endothelial cells. Endothelial cells are present in the greatest number and closest proximity to breast cancer cells in bone marrow sinusoids (Kopp et al., 2005), a common site for cancer cells to adhere and extravasate due to reduced blood flow (Mastro, Gay and Welch, 2003). Furthermore, extravasation across endothelial cells into the bone is an essential step in bone metastasis; and flow-stimulated osteocytes are capable of regulating endothelial cells (Cheung et al., 2011; Cheung, Simmons and You, 2012). Our study showed that, on top of reducing osteoclasts’ support for cancer cells, CM from flow-stimulated osteocytes also reduced the trans-endothelial migration of breast cancer cells (Figure 5-4). CM from endothelial cells conditioned in CM from flow-stimulated osteocytes increased breast cancer cell apoptosis as well (Figure 5-5). These further strengthen the anti-metastatic potential of bone mechanical loading. There are several factors in CM from flow-stimulated osteocytes that may affect endothelial permeability, which would in turn affect cancer cell extravasation. For example, the increase in prostaglandin E2 (PGE-2) production by mechanically loaded osteocytes (Zhang et al., 2015) may result in reduced endothelial permeability (Birukova et al., 2007). The reduced apoptosis of flow-stimulated osteocytes (Cheung et al., 2011) also leads to lower IL-6 secretion by osteocytes and lower ICAM-1 expression on endothelial cells (Cheung, Simmons and You, 2012). This will then reduce the adhesion and trans-endothelial migration of MDA-MB-231 cells (Nizamutdinova et al., 2008). Here we showed that the application of anti-IL-6 and anti-ICAM-1 antibodies significantly reduced the trans-endothelial migration of MDA-MB-231 cells and abolished the difference in MLO-Y4 CM from static and flow-stimulated MLO-Y4 cells (Fig 4A). This demonstrates the important regulatory role of mechanically stimulated osteocytes on breast cancer extravasation through osteocyte-secreted IL-6 and endothelial-expressed ICAM-1.

Same as the studies in Chapter 4, there are again a few limitations in this study due to the use of cell-lines. MLO-Y4 osteocytes and RAW264.7 osteoclast precursors used in this study are of mice origins, while MDA-MB-231 and MCF-7 breast cancer cells of human origins. However, both human breast cancer cell types are very well-established and have been studied extensively
in mice models. Osteoclasts differentiated from RAW264.7 cells have also been used in numerous studies with MDA-MB-231 cells (Guo et al., 2008; Krzeszinski et al., 2017) or MLO-Y4 cells (You et al., 2008; Yao et al., 2017). In addition, as mentioned above, the low sclerostin expression by MLO-Y4 cells prevented us from studying the highly interesting role of sclerostin and osteoblasts in bone metastasis. However, although MLO-Y4 cells are not ideal, it is the most well-understood osteocyte cell-line (Dallas, Prideaux and Bonewald, 2013). The use of established cell-lines as opposed to primary cells allow for a better control of difference between experiments. Furthermore, primary osteocytes are difficult to obtain and proliferate in vitro as they are terminally differentiated. Therefore, although these established cell-lines come with limitations, using them granted us the capability to better control experimental variabilities and to focus on the factors of interest. The approach of sequential conditioning instead of a direct co-culture involving all cell populations or an in vivo system, although may be less physiological, also allowed us to isolate effects and investigate only the interaction between two cell types at a time. Similarly, although it would be interesting to observe the combined effect of osteoclasts and endothelial cells on breast cancer cells, it would be difficult to isolate factors secreted from distinct cell populations and to investigate mechanisms. In addition, as the number of osteoclasts is low before bone metastasis is established, the effect of osteoclasts may be over-represented in a system of combined CM or co-culture. Finally, the use of CM means only soluble factors could have an impact. This is more realistic since it is unlikely for the cancer cells travelling in the blood vessels to have direct contacts with osteocytes in the bone matrices or differentiated osteoclasts on the bone surfaces.

5.5 Conclusion

In summary, results in this chapter demonstrated the anti-metastatic potential of flow-stimulated osteocytes mediated by osteoclasts and endothelial cells. We saw that conditioned medium from osteoclast or endothelial cells conditioned in conditioned medium from flow-stimulated osteocytes reduced the migration and increased the apoptosis of breast cancer cells. Investigating the mechanisms of this phenomenon may provide potential drug targets that can be used in combination with exercise routines to improve patients’ quality of life while reducing their risk for bone metastasis.
Chapter 6

6 Effect of Flow-Stimulated Osteocytes on Endothelial-Breast Cancer Cell Interaction

6.1 Introduction

Recent advancements in cancer research have greatly improved treatments for breast cancer. The 5-year survival rate for localized breast cancer is now more than 90% (Steeg, 2016). However, bone metastasis, the spread of tumors to the bone, occurs in 65-75% of patients with advanced breast cancer (Coleman, 2006) and remains difficult to treat. It is a severe complication that significantly worsens the bone quality of patients, and has a 5-year survival rate of less than 15% (Svensson et al., 2017). Current therapies targeting bone metastasis, such as bisphosphonates and denosumab, may reduce pain and fracture, but do not cure bone metastasis or prolong survival (Fontanella et al., 2015). Therefore, it is essential to establish an effective strategy in preventing bone metastasis before the secondary tumor is developed in the bone.

Dynamic loading on the bone is known to improve bone quality (Rubin and Lanyon, 1984) and may mitigate bone metastasis. Although exercise is commonly suggested to breast cancer patients to improve their quality of life (McNeely, 2006), patients suffering from bone metastasis often hesitate to follow due to concerns of increasing fracture risks. Furthermore, since the incentive of improving quality of life is not strong, many patients with breast cancer may not be motivated enough to exercise. Yet, a recent clinical review revealed that exercise can in fact be beneficial for patients suffering from bone metastasis (Sheill et al., 2018); an in vivo study also showed that bone loading reduces the size of tumor and lesions created by the injection of breast cancer cells into the bone (Lynch et al., 2013). Additionally, as discussed in Chapter 5, our previous in vitro study demonstrated that mechanical stimulation of cells in the bone can reduce the metastatic potential of breast cancer cells (Ma, Lam, et al., 2018). In this study, we intend to further investigate the mechanism of the preventative effect of mechanically stimulated bone cells on breast cancer bone metastasis.

One reason that breast cancers commonly metastasize to the bone is the tumor cells’ ability to interact with cells in the bone (Gupta and Massagué, 2006) and disrupt the bone remodelling balance. This not only causes bone lesions and increases fracture risk and pain, but also
facilitates cancer cell migration and growth in the bone, thereby creating a vicious cycle between bone lesions and metastatic tumor growth. On the other hand, the bone remodelling balance is normally regulated by osteocytes, the major population of cells in the bone matrix (Bonewald, 2011). Specifically targeting osteocytes’ signalling to bone-resorbing osteoclasts reduced osteolysis during bone metastasis in vivo (Qiao et al., 2017). Importantly, the regulation of bone remodelling by osteocytes had also been shown to be mechanically stimulated (Chen et al., 2010; Dallas, Prideaux and Bonewald, 2013). Since the bone matrix is filled with interstitial fluid, an oscillatory fluid flow is created during bone-loading activities such as walking. A physiological level of shear stress of 0.8-3 Pa that results from this oscillatory fluid flow (Weinbaum, Cowin and Zeng, 1994) had been shown to elicit response in osteocytes to signal to bone remodelling cells (Bakker et al., 2001; You et al., 2008; Hoey, Kelly and Jacobs, 2011; Middleton, Al-dujaili, et al., 2017). Consequently, mechanically stimulated osteocytes may break the vicious cycle of bone metastasis and be responsible for the attenuation of bone metastasis by bone loading (Lynch et al., 2013). Indeed, our previous in vitro study demonstrated that flow-stimulated osteocytes can reduce migration and increase apoptosis of breast cancer cells through signalling to osteoclasts or endothelial cells (Ma, Lam, et al., 2018).

Endothelial cells are present in large number and close proximity to the metastasizing cancer cells in blood vessels before a secondary tumor is established in the bone. Since the bone is highly vascularized, endothelial cells are ideally located to communicate with both the osteocytes in the bone matrix (Prasadam et al., 2014) and the metastasizing cancer cells in the blood stream. Endothelial cells had been extensively studied in the context of bone metastasis. In addition to being a passive barrier for the metastasizing cancer cells (Ma, Middleton, et al., 2018), they also actively play a major role in interacting with the cancer cells (Orr et al., 2000; Chavez-MacGregor, 2005; Mai et al., 2014). Unfortunately, drugs targeting endothelial cells, such as vascular endothelial growth factor (VEGF) inhibitors, have performed poorly in clinical trials (Ebos and Kerbel, 2011). Therefore, more investigation is needed. Previous research has shown that endothelial cells can respond to factors secreted by osteocytes (Cheung et al., 2011; Prasadam et al., 2014). This suggests that endothelial cells may be able to relay signals from mechanically stimulated osteocytes to the breast cancer cells and downregulate metastasis.

Our preceding work demonstrated that the signalling from osteocytes stimulated with oscillatory fluid flow to endothelial cells and breast cancer cells strongly reduced the trans-endothelial
migration of breast cancer cells (Ma, Lam, et al., 2018). In this study, we aim to identify the mechanism underlying this observation. Extravasation involves multiple processes. Factors that alter endothelial cells’ permeability and adhesion molecule expression, and breast cancer cells’ ability to degrade the endothelial extracellular matrix had all been shown to affect extravasation (Reymond, D’Água and Ridley, 2013). Therefore, we investigated whether factors secreted by flow-stimulated osteocytes affect 1) endothelial permeability, 2) cancer cell adhesion onto the endothelial monolayer, and 3) cancer cells’ gene expression profile that may affect downstream invasiveness. This study presents the potential mechanisms of bone mechanical loading in regulating the metastatic potential of cancer cells; and aims to offer novel insights into the potential of exercise for patients with breast cancer in preventing bone metastasis. Contents of this chapter are published in the Journal of Cellular Biochemistry under the title “Mechanically stimulated osteocytes reduce the bone-metastatic potential of breast cancer cells in vitro by signalling through endothelial cells” (Ma, Xu, et al., 2018).

6.2 Methods

6.2.1 Cell cultures

MLO-Y4 osteocyte-like cells (Gift from Dr. Lynda Bonewald, Indiana University, Indianapolis, IN, USA) were cultured in α-Modified Eagle’s Medium (α-MEM) (catalog number 310-010-CL; Wisent, St-Bruno, QC, Canada), supplemented with 2.5% fetal bovine serum (FBS) (12483-020; Gibco, Carlsbad, CA, USA), 2.5% calf serum (CS) (16010-159; Gibco), and 1% penicillin-streptomycin (P/S) (15140122; Gibco), on petri dishes or glass slides coated with 0.15 mg/mL type-I rat tail collagen (354236; Coring Life Science, Lowell, CA, USA). Human umbilical vein endothelial cells (HUVECs) (Gift from Dr. Edmond Young, University of Toronto, Toronto, ON, Canada) were cultured in EndoMax medium (301-010-CL; Wisent) supplemented with 10% FBS and 1% P/S. Metastatic MDA-MB-231 human breast cancer cells (HTB-26; ATCC, Manassas, VA, USA) were cultured in Kaighn's modification of Ham's F-12 medium (21127022; Gibco), supplemented with 10% FBS and 1% P/S. MDA-MB-231/1833 cells (Gift from Dr. Arun Seth, Sunnybrook Research Institute, Toronto, ON, Canada), a subpopulation of MDA-MB-231 cells that had been selected in vivo to be specifically metastatic to the bone (Kang et al., 2003), were cultured in Dulbecco's MEM (319-005-CL; Wisent) supplemented with 10% CS and 1% P/S. All cells were maintained at 37°C and 5% CO₂ in a humidified incubator.
6.2.2 Oscillatory fluid flow on osteocytes

Conditioned medium (CM) was acquired from MLO-Y4 osteocyte-like cells that were stimulated with oscillatory fluid flow, as described previously (Ma, Lam, et al., 2018). Briefly, parallel-plate flow chambers (Jacobs et al., 1998) and a linear actuator were used to apply oscillatory fluid flow at 1 Hz and 1 Pa peak shear stress to MLO-Y4 cells at 80% confluency for 2 hours. MLO-Y4 cells in the static-osteocyte groups were placed in flow chambers, but not stimulated with flow; while the control MLO-Y4 cells were seeded on glass slides but not placed in flow chambers. MLO-Y4 cells were then incubated for 24 hours in 10 mL fresh medium before CM was obtained (osteocyte CM).

6.2.3 Endothelial permeability

HUVECs were grown to full confluency on Transwells with a 0.4 μm pore-size semi-permeable membrane (665641; Greiner Bio-One, Cassina de Pecchi, Italy) that were pre-coated with collagen then conditioned in osteocyte CM for 6 hours. 200 μL of 1mg/mL fluorescein isothiocyanate-dextran (40 kDa) (FD40; Sigma-Aldrich, St. Louis, MI, USA) was then added to the top of the Transwell and 500 μL phosphate buffered solution (PBS) was added to the bottom well. After 30 minutes, Transwells were removed and fluorescence was read for the bottom well.

6.2.4 Adhesion on endothelial monolayer

HUVECs were grown to full confluency in the collagen-coated channels of µ-Slide VI 0.4 (ibidi, Martinsried, Bavaria, Germany), then conditioned in osteocyte CM for 16 hours. MDA-MB-231 breast cancer cells stained with cell tracker green (C2925; Life Technologies, Carlsbad, CA, USA) were added to the channels with HUVECs at 800,000 cells/mL and allowed to adhere for 30 minutes. Oscillatory fluid flow was then applied to the channels with a linear actuator at 1 Hz and a 1 Pa peak shear stress for 30 minutes to wash away the loosely adhered MDA-MB-231 cells. The channels were then rinsed three times with PBS and the MDA-MB-231 cells that remained adhered were imaged and quantified with ImageJ (NIH, Bethesda, MD, USA). Blocking experiments were performed as described previously (Cheung, Simmons and You, 2012; Ma, Lam, et al., 2018) by adding 1 μg/mL mouse anti-human ICAM-1 antibody (BBA3; R&D Systems) to endothelial cells in osteocyte CM 1 hour prior to the addition of breast cancer cells.
6.2.5 RNA sequencing

HUVECs grown to 100% confluency in 6-well plates were conditioned in 50% osteocyte CM and 50% fresh HUVEC medium for 24 hours. The medium was then replaced with 100% fresh HUVEC medium to ensure that there was no leftover osteocyte-secreted factor, and medium was collected from the conditioned HUVECs (endothelial CM) after 12 hours. MDA-MB-231/1833 bone-metastatic breast cancer cells grown to full confluency in 6-well plates were subsequently conditioned in 50% endothelial CM and 50% fresh MDA-MB-231/1833 medium for 24 hours. RNA content was isolated from the conditioned MDA-MB-231/1833 cells with RNeasy Mini Kit (74104; Qiagen, Venlo, Limburg, Netherlands), treated with DNA-free™ Kit (AM1906; Life Technologies), and sent in for RNA-sequencing at The Donnelly Sequencing Centre (University of Toronto). The library was prepared with TruSeq Stranded mRNA Library Prep Kit (Illumina®) and sequenced with NextSeq500 (Illumina®; High Output, 75 Cycles, v2 Chemistry; R1: 85bp, IR1: 6bp, single read). This sequencing data has been deposited in the ArrayExpress database at EMBL-EBI under accession number E-MTAB-7176.

Read counts were obtained with RNA Express v1.1 on Illumina’s BaseSpace (https://basespace.illumina.com), which aligns RNA-sequencing reads to reference human genome hg38 (Lander et al., 2001) using STAR (Dobin et al., 2013). Differential gene expression was computed using DESeq2 v1.18.1 (Love, Huber and Anders, 2014) in R/Bioconductor (Team and others, 2013). Enriched gene ontology terms (Ashburner et al., 2000; The Gene Ontology Consortium, 2017) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (Kanehisa and Goto, 2000; Kanehisa et al., 2016, 2017) were computed on the significantly upregulated and downregulated genes (Hong et al., 2013) using GOseq v1.3 (Young et al., 2010) in R/Bioconductor with count bias accounted for. Revigo (Supek et al., 2011) was used to trim down the list of gene ontology terms (allowed similarity = 0.5 using SimRel). The heatmap was prepared with gplots 3.0.1 (Warnes et al., 2016) and KEGG pathway maps were prepared with pathview 1.18.2 (Luo and Brouwer, 2013) in R/Bioconductor.

Quantitative polymerase chain reaction (qPCR) was done on selected genes to validate key results from RNA sequencing. The RNA contents isolated from conditioned MDA-MB-231/1833 cells as described above were treated with DNase I (EN0521; Thermo Fisher Scientific, Waltham, MA, USA) and reverse-transcribed using SuperScript™ III RT (18080-044;
Invitrogen, Carlsbad, CA, USA). qPCR was performed with LightCycler® 480 SYBR Green I Master (04707516001; Roche, Mannheim, Baden-Württemberg, Germany) and gene-specific primers (listed in Table 1) purchased from Thermo Fisher Scientific.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence (5’-3’)</th>
<th>Reverse Sequence (3’-5’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>GAGGCGCTCATGTACCCTATGTAC</td>
<td>GTTCAGGGCGAGGACCATAAGAG</td>
</tr>
<tr>
<td>FZD-4</td>
<td>TGGGCCACGAGCTGCAGACG</td>
<td>TGAGCACACATGCAGGCTCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TTGCCATCAATGACCCCTTCA</td>
<td>CGCCCACTTGATTGGGA</td>
</tr>
</tbody>
</table>

Table 6-1: Primers used for qPCR.

6.2.6 Invasion assay

100 µL of 1 mg/mL Matrigel™ (354234; BD Biosciences, Bedford, MA, USA) was allowed to gel for 2 hours on 24-well plate Transwells with an 8 µm pore-size semi-permeable membrane (662638; Greiner Bio-One). MDA-MB-231/1833 bone-metastatic breast cancer cells conditioned in endothelial CM as described above were stained with cell tracker green. 40,000 stained cells were added on top of each Matrigel-coated Transwell and allowed to invade towards 20%-FBS MDA-MB-231/1833 medium in the bottom wells for 24 hours. Cells were then fixed with 10% neutral buffered formalin. Non-invaded cells were scrapped off with cotton swabs. Invaded cells were imaged and quantified with imageJ. Blocking experiments were performed by adding 1 µM MMP-9 inhibitor (ab142180; Abcam, Cambridge, Cambridgeshire, UK) to endothelial CM.

6.2.7 Statistics

Each type of experiment was performed at least 3 times with each experiment containing at least 3 samples paired between the flow-osteocyte groups, where MLO-Y4 osteocytes were stimulated with flow, and the static-osteocyte groups, where MLO-Y4 osteocytes were placed in flow chambers but not stimulated with flow. The paired samples were prepared as closely in time as possible, with the same reagents, and from the same dish of cells. The order within the pair was randomized to avoid time-dependent variations and this order is maintained throughout sequential conditioning and experiments. Functional assay results were normalized to controls (results using CM from MLO-Y4 osteocytes seeded on glass slides but not placed in flow chambers) for each set of experiment to account for potential differences in experimental conditions. Student’s t-test (2-tail, paired) was used to test significance between static- and flow-osteocyte groups (significance was taken at α = 0.05). For RNA-sequencing results, a gene is
considered differentially expressed when the adjusted p-value (corrected for multiple testing with the Benjamini-Hochberg method) calculated by DESeq2 is less than 0.05.

6.3 Results

6.3.1 Endothelial permeability

First, we investigated whether factors secreted by flow-stimulated osteocytes affect the permeability of endothelial monolayers. To do this, conditioned medium (CM) was collected from flow-stimulated or static MLO-Y4 osteocytes. This CM (osteocyte CM) was added to monolayers of human umbilical vein endothelial cells (HUVECs). After 6 hours, endothelial permeability of this monolayer was measured using FD40 (40 kDa fluorescent tracer). Results in Figure 6-1 showed that HUVECs conditioned in osteocyte CM from flow-stimulated MLO-Y4 osteocytes were 15% less permeable.

![Figure 6-1: Endothelial permeability in osteocyte CM. Permeability of HUVEC monolayers to 40 kDa fluorescent tracers (FD40) after 6-hour conditioning in CM from MLO-Y4 osteocytes that were static (but placed in flow chambers) or flow-stimulated, normalized to controls (CM from MLO-Y4 osteocytes not placed in flow chambers). Data is presented as paired data between static- and flow-osteocyte groups, n = 12 from 3 experiments.]

6.3.2 Cancer cell adhesion on endothelial monolayer

Next, we investigated whether soluble factors from flow-stimulated osteocytes change the endothelial monolayer’s potential to be adhered by breast cancer cells. HUVECs were conditioned in osteocyte CM from either static of flow-stimulated MLO-Y4 osteocytes for 16 hours. MDA-MB-231 breast cancer cells were then allowed to adhere for 30 minutes before flow was applied to wash away the loosely adhered cells. We observed that 18% more MDA-MB-231
cells remained adhered when the HUVECs were conditioned in CM from static MLO-Y4 osteocytes (Figure 6-2).

Because intercellular adhesion molecule 1 (ICAM-1) had been shown to be regulated by osteocyte CM (Cheung, Simmons and You, 2012) and is important for breast cancer cell adhesion to the endothelium (Regimbald et al., 1996), extravasation (Rahn et al., 2005), and metastasis in vivo (Nizamutdinova et al., 2008), we hypothesized that it may be a key factor for the observed difference. Indeed, after incubating the conditioned HUVECs with anti-ICAM-1 neutralizing antibody, the difference in adhesion strength was abolished between the static- and flow-osteocyte groups (Figure 6-2).

Figure 6-2: Breast cancer cell adhesion to endothelial monolayers conditioned in osteocyte CM. Adhesion strength of MDA-MB-231 breast cancer cells on endothelial monolayers of HUVECs after 16-hour conditioning in CM from MLO-Y4 osteocytes that were static (but placed in flow chambers) or flow-stimulated, normalized to controls (HUVECs conditioned in CM from MLO-Y4 osteocytes not placed in flow chambers), with and without anti-ICAM-1 neutralizing antibodies. Data is presented as paired data between static- and flow-osteocyte groups, n = 15 from 3 experiments for no-antibody, n = 11 from 3 experiments for anti-ICAM-1.

6.3.3 Gene expression

In addition to simply affecting endothelial cells, osteocytes may stimulate the endothelial cells to signal to the metastasizing breast cancer cells in blood vessels. Therefore, we used RNA sequencing to identify differential gene expression between MDA-MB-231/1833 bone-metastatic breast cancer cells conditioned in endothelial CM from HUVECs conditioned in CM from static and flow-stimulated MLO-Y4 osteocytes. We used the 1833 bone-metastatic subpopulation of MDA-MB-231 breast cancer cells (Kang et al., 2003) for this part of the study because we were
interested in genes that may be responsible for the tissue tropism. After sequencing and analysing 4 pairs of RNA sequencing data with DESeq2 in R/Bioconductor (Love, Huber and Anders, 2014), we identified a list of significantly differentially expressed genes (adjusted p-value < 0.05). Specifically, 15 genes (NEAT1, MMP9, TFPI2, NNMT, LTBP3, EFEMP2, SAA1, SHE, FZD4, JAG2, RALA, BCL6B, PTGS2, GIMAP8, and SLC9B2) were significantly downregulated and 3 genes (RPL28, C19orf53, DPM2) were significantly upregulated in MDA-MB-231/1833 bone-metastatic breast cancer cells when MLO-Y4 osteocytes were stimulated with flow (Figure 6-3). The top panel of Figure 3 shows the heat-map for genes that were found to have significant or a trend for (adjusted p-value < 0.1) differential expression by the cancer cells between static- and flow-osteocyte groups, along with their respective adjusted p-values. Red indicates a high expression of the gene by breast cancer cells, while blue indicates a low expression.

Bottom panel of Figure 3 shows the gene ontology terms that involve the significantly differentially expressed genes. Gene ontology (Young et al., 2010; The Gene Ontology Consortium, 2017) and KEGG pathways (Kanehisa and Goto, 2000; Kanehisa et al., 2016, 2017) are 2 large databases compiled from past research to describe genes involved in cellular processes. We used the 2 databases to predict differences in functions of cancer cells between static- and flow-osteocyte groups. GOSeq in R/Bioconductor (Young et al., 2010) was used to perform enrichment analysis where the list of 18 significantly differentially expressed genes were mapped onto gene ontology terms or KEGG pathways. With this, 35 gene ontology terms were found to be downregulated (adjusted p-value < 0.08), while none was upregulated, in MDA-MB-231/1833 bone-metastatic breast cancer cells when the MLO-Y4 osteocytes were stimulated with flow. Revigo (Supek et al., 2011) was subsequently used to combine similar gene ontology terms. In the bottom panel of Figure 3, blue squares represent gene ontology terms that are downregulated in the cancer cells when the osteocytes were stimulated with flow, shown under the corresponding genes. For example, the third row of the bottom panel in Figure 6-3 shows that locomotion of bone-metastatic breast cancer cells is reduced when the osteocytes were stimulated with flow due to the downregulation of MMP-9, SAA1, FZD4, JAG2, RALA, PTGS2, and SLC9B2. Additionally, part of the cancer KEGG pathway (hsa05200) that contains the genes downregulated by breast cancer cells when osteocytes were stimulated with flow (MMP9, FZD4, JAG2, RALA, and PTGS2; highlighted in blue) is shown in Figure 6-4.
Figure 6-3: Breast cancer cell gene expression in static- and flow-osteocyte groups. Differential gene expression between MDA-MB-231/1833 bone-metastatic breast cancer cells conditioned in endothelial CM from HUVECs conditioned in CM from MLO-Y4 osteocytes that were static (but placed in flow chambers) or flow-stimulated. Top panel shows the heat map (red: high expression; blue: low expression) of cancer cell gene expression with p-values between static- and flow-osteocyte groups (n = 4 paired samples), adjusted for multiple testing. Bottom panel shows the corresponding gene ontology terms that involve the significantly differentially expressed genes (blue: downregulated in the cancer cells of the flow-osteocyte group).
Figure 6-4: KEGG cancer pathway with genes downregulated by breast cancer cells in the flow-osteocyte group. Part of the KEGG cancer pathway map, where green rectangles indicate all the genes involved in the pathway, and gray rectangles indicate downstream functions. Blue rectangles indicate the genes that were significantly downregulated in MDA-MB-231/1833 bone-metastatic breast cancer cells when conditioned in endothelial CM from HUVECs conditioned in CM from MLO-Y4 osteocytes stimulated with flow, in comparison to results from samples with static osteocytes that were placed in flow chambers.
6.3.4 MMP-9 and FZD4 expression

Of the genes downregulated by MDA-MB-231/1833 bone-metastatic breast cancer cells when MLO-Y4 osteocytes were stimulated with flow, MMP-9 (matrix metallopeptidase 9) is of particular interest. It was the second most significantly differentially expressed gene from our RNA sequencing data, and is often implicated in metastasis for its importance in degrading the extracellular matrix (Deryugina and Quigley, 2006). It participates in several gene ontology terms and was the most significantly downregulated gene that is involved in the cancer KEGG pathway. SAA1 (serum amyloid A1) and FZD4 (frizzled 4), two other genes downregulated by cancer cells in the flow-osteocyte group, may also be of interest since serum amyloid A and interaction involving frizzled proteins had been shown to be upstream of matrix metalloproteinase production (Lee et al., 2005; Rabbani, Arakelian and Farookhi, 2013). Since FZD4 is also involved in the KEGG cancer pathway, we performed qPCR on both MMP-9 and FZD4 as two key genes to verify our RNA sequencing results. Consistent with RNA sequencing results, we showed that MDA-MB-231/1833 breast cancer cells lowered their expression of MMP-9 by 62%, and FZD4 by 61%, when conditioned in CM from HUVECs conditioned in CM from flow-stimulated MLO-Y4 osteocytes (Figure 6-5).

![Graph showing MMP-9 and FZD4 expression by breast cancer cells in static- and flow-osteocyte groups.](image)

**Figure 6-5:** MMP-9 and FZD4 expression by breast cancer cells in static- and flow-osteocyte groups. qPCR data showing MMP-9 and FZD-4 expression by MDA-MB-231/1833 bone-metastatic breast cancer cells conditioned in endothelial CM from HUVECs conditioned in CM from MLO-Y4 osteocytes that were static (but placed in flow chambers) or flow-stimulated, normalized to GAPDH expression. Data is presented as paired data between static- and flow-osteocyte groups, n = 11 from 3 experiments.
6.3.5 Invasion

Since MMP-9 is a proteinase important for degrading the extracellular matrix (Deryugina and Quigley, 2006), we then performed an invasion assay to investigate whether the downregulation of MMP-9 is relevant in terms of cellular function. Indeed, we found that the MDA-MB-231/1833 bone-metastatic breast cancer cells in the flow-osteocyte group, which are the cancer cells found to have lower 62% MMP-9 expression, had 47% less invasion across Matrigel-coated Transwells. This difference in invasion was abolished when MMP-9 inhibitor was applied (Figure 6-6), verifying the importance of MMP-9 in invasion.

Figure 6-6: Breast cancer cell invasion in static- and flow-osteocyte groups. Invasion through Matrigel-coated Transwells by MDA-MB-231/1833 bone-metastatic breast cancer cells after conditioning in endothelial CM from HUVECs conditioned in CM from MLO-Y4 osteocytes that were static (but placed in flow chambers) or flow-stimulated, normalized to controls (invasion of MDA-MB-231/1833 cells when MLO-Y4 cells were not placed in flow chambers). Data is presented as paired data between static- and flow-osteocyte groups, n = 18 from 3 experiments for no-inhibitor, n = 9 from 2 experiments for MMP-9 inhibitor.

6.4 Discussion

Bone mechanical loading has been shown to have downregulatory effects on bone metastasis (Lynch et al., 2013). Our previous in vitro study identified signalling from osteocytes through endothelial cells as a potential pathway for these observations (Ma, Lam, et al., 2018). In this study, we identified the elements in the extravasation cascade that are affected by molecules released from osteocytes stimulated with oscillatory fluid flow (2 hours at 1 Hz and a peak shear stress of 1 Pa). We found that flow-stimulated osteocytes reduced endothelial cells’ permeability...
and ability to be adhered by breast cancer cells, as well as strongly reduced the invasive potential of breast cancer cells through signalling mediated by endothelial cells (Figure 6-7). RNA sequencing was used to identify genes significantly altered in bone-metastatic breast cancer cells.

![Diagram](image)

**Figure 6-7: Summary of the effects of mechanically stimulated osteocytes on the interaction between endothelial cells and breast cancer cells to reduce the bone-metastatic potential.** When the osteocytes are stimulated with oscillatory fluid flow, the application of osteocyte CM to endothelial cells reduces their permeability (Figure 6-1) and adhesion by breast cancer cells (Figure 6-2). The application of CM from these conditioned endothelial cells to bone-metastatic breast cancer cells reduces their MMP-9 and FZD4 expression (Figures 6-3 and 6-5) and invasiveness (Figure 6-6).

We first observed that endothelial monolayers conditioned in CM from flow-stimulated osteocytes have a slightly reduced permeability to FD40 (40 kDa fluorescent tracer) (Figure 6-1). This may be due to the change in the expression of several factors secreted by osteocytes in response to flow. For example, the increased production of prostaglandin E2 (Zhang et al., 2015), nitric oxide (Zaman et al., 1999), adenosine triphosphate (Genetos et al., 2007), and sphingosine-1-phosphate (Dobrosak and Gooi, 2017) by flow-stimulated osteocytes may contribute in making the endothelial layer less permeable. These factors have been shown to maintain the endothelial layer by enhancing either the adheren junctions or the glycocalyx layer (Draiher et al., 1995; Gündüz et al., 2003; Birukova et al., 2007; Zhang et al., 2016). As cancer cell extravasation requires the degradation of glycocalyx and the separation of endothelial cells (Fan and Fu, 2016), the increased production of these factors will reduce extravasation. This aligns well with our previous study showing reduced extravasation towards CM from flow-stimulated osteocytes.
We then observed that the adhesion strength of breast cancer cells to endothelial monolayers is attenuated when the endothelial cells were incubated in CM from flow-stimulated osteocytes (Figure 6-2). Upregulation of E-selectin and ICAM-1 by endothelial cells had been shown to be responsible for increasing breast cancer cell adhesion to the endothelium (Li and Feng, 2011). However, the induced upregulation of selectins returns to basal levels after 6-12 hours (Rahn et al., 2005), which is shorter than the 16-hour conditioning of endothelial cells in our experiment. Therefore, we hypothesized that the difference we observed is due to the difference in ICAM-1 expression, and not selectin expression. Indeed, the difference in adhesion strength was abolished after blocking with anti-ICAM-1 neutralizing antibodies (Figure 6-2). Although adhesion was reduced in the flow-stimulated osteocyte group as well, adhesion was not completely abolished in either group. This suggests that ICAM-1 is responsible for the difference in breast cancer cell adhesion to endothelial monolayers between the static- and flow-osteocyte groups. ICAM-1 expression is inducible by shear stress and a variety of cytokines. Previous work had shown the regulation of endothelial ICAM-1 expression by osteocyte-secreted interleukin-6 (Cheung, Simmons and You, 2012), which may be responsible for our observed phenomenon.

We then performed a double-conditioning experiment to explore effects on breast cancer cells, where endothelial cells were first conditioned in CM from static or flow-stimulated osteocytes, and bone-metastatic breast cancer cells were then cultured in CM collected from these endothelial cells. Using CM instead of a direct co-culture with osteocytes and endothelial cells guarantees that only soluble factors will be involved. CM from osteocytes was also replaced with fresh medium 12 hours before the collection of CM from endothelial cells to ensure that the collected endothelial CM does not contain factors secreted by osteocytes. This not only narrows down the potential factors, but also better mimics the physiological environment since it is more likely for the osteocytes in the bone matrix to signal to the blood vessel via secreted factors. The MDA-MB-231/1833 bone-metastatic subpopulation of MDA-MB-231 breast cancer cells was used for this experiment because we were interested in potential changes in genes that allow for the bone-metastatic specificity.

With the double-conditioning experiment, we observed 18 significantly differentially expressed genes in the bone-metastatic breast cancer cells when the osteocytes were stimulated with oscillatory fluid flow in comparison to static-osteocyte groups (Figure 6-3). The adjusted p-
values calculated by DESeq2 were selected to rank the differentially expressed genes because the p-values consider both the fold change and the expression level of a gene and, therefore, excludes genes with low fold-changes as well as false-positives with large fold-changes but low expressions or high variations. The differentially expressed genes were then used to identify significantly affected gene ontology terms and KEGG pathways. Although no gene ontology terms were found to be significantly enriched after Benjamini-Hochberg correction, 35 gene ontology terms exhibited trends of being downregulated (p < 0.08) in breast cancer cells when osteocytes were stimulated with flow. This includes processes important in metastasis, such as cellular response and signalling (MMP9, SAA1, SHE, FZD4, JAG2, BCL6B, and PTGS2), locomotion (MMP9, SAA1, FZD4, JAG2, RALA, PTGS2, and SCL9B2), TGF-β production and activation (LTBP3, PTGS2, and EFEMP2), and the extracellular matrix (MMP9, TFPI2, LTBP3, and SAA1). Moreover, genes MMP9, FZD4, JAG2, RALA, and PTGS2, which were downregulated in bone-metastatic breast cancer cells when osteocytes were stimulated with flow, participate in the KEGG cancer pathway (Figure 6-4). Furthermore, although the most significantly downregulated gene, NEAT1, is not involved in any significantly enriched gene ontology terms or the KEGG cancer pathway, it is a nuclear long non-coding RNA that had been shown to be correlated with poor survival in patients with breast cancer (Choudhry et al., 2015).

We also examined the sequencing data further for variations. First, we verified that the expression of GAPDH (glyceraldehyde 3-phosphate dehydrogenase), the housekeeping gene used for normalization in qPCR, was constant across samples, with a fold change of 1.004 and an adjusted p-value of 1.000 between static- and flow-osteocyte groups. This also ensures that the observed differences in gene expressions were not due to variation in cell number. However, the heat-map shown in Figure 6-3 makes it apparent that breast cancer cells in sample 3 of the static-osteocyte group had an abnormally low expression for most genes. Therefore, we repeated differential gene expression analysis excluding the paired sample 3 from static- and flow-osteocyte groups to verify that the overall trend was not affected by this outlier. After doing this, we saw that 174 genes were downregulated, and only 1 gene (RPL28) was upregulated, in the bone-metastatic breast cancer cells when the osteocytes were stimulated with flow. Although p-values changed after the exclusion of sample 3, all the genes that were found to be downregulated in the cancer cells of the flow-osteocyte group remained to be so. Intriguingly, C19orf53 and DPM2 were no longer significantly upregulated after the removal of sample 3.
This is probably due to their abnormally high expression in sample 3 of the flow-osteocyte group.

Out of the 18 genes differentially expressed by bone-metastatic breast cancer cells when conditioned in CM from endothelial cells conditioned in CM from flow-stimulated osteocytes, the downregulation of MMP-9 was especially worth noting. MMP-9 is often implicated in metastasis as it plays a major role in extracellular matrix degradation and extravasation (Deryugina and Quigley, 2006). From Figure 6-3 and Figure 6-4, it is apparent that MMP-9 participates in many gene ontology terms, as well as the KEGG cancer pathway. Therefore, we performed qPCR on MMP-9 and FZD4, a potential upstream promoter of MMP-9 (Rabbani, Arakelian and Farookhi, 2013) that is also involved in the KEGG cancer pathway, and verified that they were significantly downregulated (Figure 6-5). Our invasion assay results also confirmed that cancer cells in conditions found to have lower MMP-9 expressions also had reduced invasion through Matrigel-coated Transwells (Figure 6-6). This is relevant since the invasiveness of breast cancer cells affects their invasion through the basement membrane of endothelial cells and the bone in vivo. Furthermore, the application of MMP-9 inhibitors abolished the difference in invasion, while invasion was not completely blocked. This suggests that signalling from flow-stimulated osteocytes through endothelial cells reduced the invasive potential of breast cancer cells by downregulating MMP-9, which may also involve the downregulation of potential upstream genes such as FZD4 (Rabbani, Arakelian and Farookhi, 2013) and SAA1 (Lee et al., 2005). In addition to these genes, PTGS2 (prostaglandin-endoperoxide synthase 2, also known as cyclooxygenase-2 or COX2), another significantly downregulated gene, plays a role in many biological processes, such as locomotion and TGF-β (transforming growth factor beta) production. It may be of interest as well since cell locomotion is essential for metastasis and TGF-β is often implicated in metastasis (Yin et al., 1999).

6.5 Conclusion

In conclusion, we observed that flow-stimulated osteocytes reduced the endothelial permeability and the adhesion of breast cancer cells on the endothelial monolayer. Interestingly, we showed that gene expression of breast cancer cells can be strongly regulated by signaling from flow-stimulated osteocyte through endothelial cells. Specifically, we demonstrated the reduction in invasiveness of bone-metastatic breast cancer cells by downregulating MMP-9 and FZD4, two
genes that play a major role in cancer and metastasis. This study further explored the
downregulation of bone-metastatic potential by mechanical loading on cells of the bone \textit{in vitro}
and demonstrated the potential of bone-loading exercise in preventing bone metastasis.
7 Conclusion

7.1 Summary of Findings

The research results are presented in Chapters 4-6. In Chapter 4, we showed that factors secreted by osteocytes stimulated with oscillatory fluid flow increased the migration and decreased the apoptosis of breast and prostate cancer cells. This work has been published in:

A) Peer-reviewed journal publication

B) Conference podium presentation

C) Conference poster presentation


In contrast, results in Chapter 5 demonstrated the anti-metastatic potential of flow-stimulated osteocytes on breast cancer cells. Specifically, flow-stimulated osteocyte reduced the differentiation of osteoclasts, and conditioned medium (CM) from these less differentiated osteoclasts reduced the migration and increased the apoptosis of breast cancer cells. CM from flow-stimulated osteocytes also reduced the trans-endothelial migration of breast cancer cells. As well, CM from endothelial cells conditioned in CM form flow-simulated osteocytes increased the apoptosis of cancer cells. This work has been published in:

A) Peer-reviewed journal publication


B) Conference podium presentation


C) Conference poster presentation

1. Ma, Y. H. V., Dalmia, S., Gao, P., Young, J., Liu, C., You, L. ‘Osteocytes’ Response to Mechanical Loading Supports Cancer Cell Growth and Migration,


Since endothelial cells comprised the first barrier cancer cells encounter and are subjected to signaling from osteocytes, in Chapter 6, we further investigated the effect of flow-stimulated osteocytes on the interaction between endothelial cells and breast cancer cells. Here, we demonstrated that CM from flow-stimulated osteocytes reduced the permeability of endothelial monolayers as well as the adhesion of breast cancer cells on the endothelial monolayers. Furthermore, CM from endothelial cells conditioned in CM from flow-stimulated osteocytes significantly altered the gene expression of breast cancer cells. In particular, the expressions of MMP-9 and FZD4, two genes that are involved in the KEGG cancer pathway, were significantly downregulated, leading to a significant reduction in the invasiveness of the breast cancer cells. This work has been published in:

A) Peer-reviewed journal publication

B) Conference podium presentation
1. (Accepted to preset in spotlight session). Ma, Y. H. V., Xu, L., Mei, X., You, L. ‘Mechanically stimulated osteocytes reduce the bone-metastatic potential of breast cancer cells in vitro by signalling through endothelial cells’ to be presented at the Orthopaedic Research Society Annual Meeting, Austin, TX, USA, 2019.

C) Conference poster presentation

### 7.2 Limitations and Future Directions

Although this research offers exciting insights into the impact of bone mechanical loading on bone metastasis, there are several limitations associated with the experimental design. First, there are limitations involved in using cell-lines, as discussed in detail in the discussion sections of Chapters 4 and 5. In addition, the current design uses CM and lacks real-time signaling between different cell populations. This excludes many important factors that have a short half-life, such as ATP and nitric oxide. Furthermore, results from this research can only be extrapolated to bones that adapt to mechanical loading, where mechanically stimulated osteocytes have been shown to regulate the bone remodeling balance. Fortunately, many frequent sites of bone metastasis, such as spine, femur, and tibia, have been shown to adapt to mechanical stimulation. The other limitations may be overcome with potential future directions listed below.

#### 7.2.1 Mechanism for the anti-metastatic regulation by flow-stimulated osteocytes

In this thesis, we have demonstrated that signaling from flow-stimulated osteocytes through osteoclasts and endothelial cells reduces the bone-metastatic potential of breast cancer cells. The next immediate step would be investigating into the mechanisms involved. As endothelial cells are present in the greatest number and closest proximity to cancer cells in blood vessels in early stages of bone metastasis before a secondary tumor is established, we placed emphasis on the endothelial-mediated pathway. We have shown that factors endothelial cells secrete in response to signaling from flow-stimulated osteocytes alter the gene expression of breast cancer cells. Specifically, MMP-9, a gene known to play an important role in bone metastasis, is downregulated in breast cancer cells. More studies could be performed on identifying the factors secreted by flow-stimulated osteocytes and endothelial cells that are responsible for the observed reduction in MMP-9 expression. Sequential filtration based on size may be done on the CM to measure the size of the molecules mediating the effects. Blocking antibodies and supplemental proteins may be used for validation. In addition, IL-6 secretion by osteocytes have been shown to
alter the adhesion molecule expression of endothelial cells, which, in turn, affects the adhesion of
cancer cells onto the endothelium. This may be an important pathway in regulating bone
metastasis as adhesion is an essential step in early stage bone metastasis.

7.2.2 Microfluidics device

A variety of metastasis models had been developed on microfluidic devices, as summarized in
our recent review article (Ma, Middleton, et al., 2018). A microfluidics device had been
developed by an alumnus, Dr. Kevin Middleton, and applied by colleague Xueting Mei to study
the role of osteocytes in the mechanical regulation of bone metastasis. Preliminary results have
been published in conferences (Middleton, Shim, et al., 2017; Mei et al., 2018). Microfluidics
device has several advantages over my current experimental design. Instead of using CM
collected after loading, micro-channel device allows real-time cross-talk between multiple cell
populations, which better mimic the in vivo situation. Although the current device only includes
osteocytes, breast cancer cells, and endothelial cells, it could easily be expanded to include other
cell populations as well. The small number of cells required for micro-channel devices also
permits the use of primary osteocytes instead of osteocyte-like cell-lines since primary
osteocytes are difficult to harvest in large numbers or expand in vitro. As well, flow on
endothelial cells and cancer cells, although slow in the bone marrow vessels (Section 2.5.3),
could be incorporated. Furthermore, the micro-channel device can be used to better assay cancer
cell migration as opposed to the Transwell system due to its ability of real-time monitoring.
There is also a potential for this device to be adapted for a microfluidics platform that can
investigate osteocyte mechano-sensing in a high-throughput fashion, which is currently being
designed by another lab mate, Liangcheng Xu. Preliminary results for this high-throughput
microfluidics device had also been published in a conference abstract (Xu, Fuller-Thomson and
You, 2018).

7.2.3 Animal studies

After investigating the effect of osteocytes’ response to mechanical loading on cancer metastasis
in vitro, studies on animal models will also be conducted to confirm the pro- or anti-metastatic
effect that is observed. There had been one in vivo study that suggested mechanical loading can
relieve both tumor burden and bone lesion (Lynch et al., 2013). However, they directly injected
breast cancer cells into the tibia and looked at the effect of tibial compression on the established
tumor. Although the finding is highly interesting, this model does not simulate the behaviour of cancer cells prior to a developed bone metastasis, which is focus of this project. Therefore, an animal model studying the preventative effect of exercise on bone metastasis is still needed.

There are several ways that this could be done. Current methods to introduce cancer cells into animal models to study cancer cell metastasis into the bone include: 1) intravenous injection into tail vein, 2) intracardiac injection into left ventricles, 3) orthotopic injection into mammary fat pads, and 4) injection into a bone and observe cancer cell migration to other bones. The major limitation of tail vein and intracardiac injection lies in that not all cells in a heterogeneous cell-line, such as the MDA-MB-231 cells, will metastasize to the bone. Bone-metastatic sub-clones, such as the B02 sub-population of MDA-MB-231 cells (Garcia et al., 2008), had been developed based on in vivo passages, where cancer cells were injected into circulation, isolated from the bone, then injected and isolated again, until the sub-clone only metastasizes to the bone (Yoneda et al., 2001; Pécheur et al., 2002; Garcia et al., 2008). Other sub-clones, such as the 1833 subpopulation of MDA-MB-231 cells, had also been selected by individually injecting all the single-cell colonies cultured from a heterogenous cell population and isolating the ones that had metastasized to the bone (Kang et al., 2003; Ganapathy et al., 2010). Additionally, while bone metastasis did not seem to develop after the injection of human cancer cells into mammary pads (Kelly et al., 2005), injection of mouse tumor cells into mammary pads had successfully induced bone metastasis (Hiraga et al., 2003; Withana et al., 2012). However, using mouse cells might lead to discrepancy to in vitro findings using MDA-MB-231 cells, and may not accurately represent changes in human cancer cells. Finally, another potential is injecting human cancer cells to the human bone implanted and then observing the migration of these cancer cells to another implanted human bone (Yang et al., 2007; Benatar et al., 2012). Studying the effect of bone mechanical loading on cancer cell invasion in these models would offer better knowledge on the preventative effect of exercise on bone metastasis.

7.2.4 Potential for vibration therapy

Tailored exercise programs had been proven to be safe even in patients with bone metastasis and do not increase fracture risk or pain (Sheill et al., 2018). However, it may still be difficult for some patients to exercise as cancer and treatments often weaken patients. Recently, high frequency (20-90 Hz) low magnitude (<1 g) (HFLM) vibration has emerged as a novel
therapeutic strategy to promote healthy skeletal homeostasis and thus manage several diseases such as osteoporosis (Yang et al., 2009). Interestingly, our previous in vitro study found that, at a cellular level, osteocytes inhibited osteoclastogenesis in response to 1 hour of HFLM vibration at 0.3 g and 60 Hz (Lau et al., 2010). Therefore, HFLM vibration therapy can potentially be an alternative treatment method that allows patients to gain the benefits of improved bone quality from mechanical loading without exercise. This will be carried out by colleague Xueting Mei.

7.2.5 Cancer cell on osteocyte mechanosensitivity

This project only involves one-way signaling from osteocytes to cancer cells. As stated in Section 2.3, cancer cells have been shown to signal to osteoblasts and osteoclasts. Since osteocytes are terminally differentiated osteoblasts, it is highly likely that cancer cells will signal to osteocytes and may alter their ability to respond to mechanical loading. In fact, multiple myeloma can alter the transcriptome of osteocytes (Eisenberger et al., 2008), and melanoma disrupts collagen in the lacuna around osteocytes (Sekita et al., 2017). These may, in turn, affect the bones’ ability to adapt to mechanical loading and reduces the bone quality of patients. We had investigated briefly into this, although preliminary results suggest that CM from breast cancer cells did not induce any change in calcium signaling and RANKL production by osteocytes in response to mechanical stimulation. Interestingly, a recent study showed that CM from breast cancer cells inhibited the production of OPG by osteocytes in response to mechanical stimulation, although RANKL secretion was unaffected (Wang et al., 2018). This increases the overall RANKL to OPG ratio and suggests that breast cancer cells can induce osteoclastogenesis through osteocytes.

7.3 Conclusion

In conclusion, studies presented in this thesis strongly demonstrated the anti-metastatic potential of signaling from mechanically stimulated osteocytes mediated by osteoclasts and endothelial cells. In particular, Chapter 6 had identified various pathways in which mechanically stimulated osteocytes affect the interaction between endothelial cells and breast cancer cells to reduce the bone-metastatic potential.
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Appendices

A. Code (R/Bioconductor)

A.1 RNA Sequencing Analysis

A.1.1 DESeq2 analysis

```r
library(DESeq2)
library(gplots)
library(RColorBrewer)

# Read the counts from the downloaded data
seqdata <- read.csv("Counts.csv")
# Remove first column (gene names) from seqdata
countdata <- seqdata[,-(1:1)]
# Store Genes as rownames
rownames(countdata) <- seqdata[,1]
# Store Genes as colnames
colnames(countdata) <- c("1N", "2N", "3N", "4N", "1F", "2F", "3F", "4F")
remove(seqdata)
# Read information about samples
sampleinfo <- read.delim("SampleInfo.txt")
row.names(sampleinfo) <- sampleinfo$FileName
sampleinfo <- sampleinfo[,2:3]

##### Make design matrix #####
.dds <- DESeqDataSetFromMatrix(countData = countdata,
colData = sampleinfo,
design= ~ Flow)

# Minimal pre-filter to get rid of low counts
keep <- rowSums(counts(dds)) >= 10
.dds <- dds[keep,]
.dds$Flow <- relevel(dds$Flow, ref = "Static")
```
remove(keep)

# Run DESeq
dds <- DESeq(dds)
resultsNames(dds) # lists the coefficients

# Shrink log fold changes association with condition:
library(apeglm)
resLFC <- lfcShrink(dds, coef="Flow_Flow_vs_Static", type="apeglm")
results_LFC <- as.data.frame(resLFC@listData)ownames(results_LFC) <- rownames(resLFC)
remove(dds)

A.1.2 EdgeR analysis
library(edgeR)
library(gplots)

########## Pre-Processing ##########
# Calculate the Counts Per Million measure with EdgeR
 # Use countdata from DESeq2 analysis
 # Identify genes with at least 0.25 cpm in at least 3 samples
myCPM <- cpm(countdata)
thresh <- myCPM > 0.25
# Plot to check the cpm makes sense (counts ~10)
plot(myCPM[,1],countdata[,1],ylim=c(0,50),xlim=c(0,3))
abline(v=0.25)
# Plot looks good, subset the rows of countdata to keep the more highly expressed genes
keep <- rowSums(thresh) >= 3
counts.keep <- countdata[keep,]
myCPM.keep <- myCPM[keep,] #Counts per million for more highly expressed genes
remove(myCPM)
remove(keep)
remove(thresh)

# Convert to an edgeR object (two slots: counts, samples with library size and normalized factors)
dgeObj <- DGEList(counts.keep)

# Plot to check discrepancies
barplot(dgeObj$samples$lib.size, names=colnames(dgeObj), las=2)

# 3F larger library size than all other samples
# Boxplot looks good (log2 CPM)
logcounts <- cpm(dgeObj, log=TRUE)
boxplot(logcounts, xlab="", ylab="Log2 counts per million",las=2)

## Perform TMM normalisation
dgeObj <- calcNormFactors(dgeObj)
remove(counts.keep)

################# EdgeR #################

# Make design matrix
sampleType <- rep("N", ncol(dgeObj)) #N=no flow
sampleType[grep("F", colnames(dgeObj))] <- "F" #F=flow #'grep' is a string matching function.
design <- model.matrix(~sampleType)

##Estimate disperson
dgeObj <- estimateCommonDisp(dgeObj)
dgeObj <- estimateGLMTrendedDisp(dgeObj)
dgeObj <- estimateTagwiseDisp(dgeObj)
plotBCV(dgeObj)

# Fit the linear model
fit <- glmFit(dgeObj, design)
names(fit)
# Conduct likelihood ratio tests for N VS F

```r
lrt <- glmLRT(fit, coef=2)
topTags(lrt)
```

# Save

```r
DifGenes <- as.data.frame(topTags(lrt, n = Inf)) # 13688 entries
```

```r
DifGenes[,"logFC"] <- -DifGenes[,"logFC"]
```

```r
remove(dgeObj)
remove(lrt)
remove(fit)
remove(design)
remove(sampleType)
remove(myCPM.keep)
```

### A.1.3 Gene ontology and KEGG pathway analysis

```
# Access database
library(org.Hs.eg.db)
x <- org.Hs.egSYMBOL
# Get the gene symbol that are mapped to an entrez gene identifiers
mapped_genes <- mappedkeys(x)
# Convert to a list
xx <- as.list(x[mapped_genes])
eids <- "
ENTREZID2GENESYMBOL <- matrix(0,length(xx),2)
colnames(ENTREZID2GENESYMBOL) <- c('ENTREZ_IDS','GENE_SYMBOLS')
for(k in 1:length(xx)){
  if(k==1){
    eids <- unlist(xx[k])
    goids <- names(xx[k])
  }
  if(k>1){
    # code continues...
  }
}
```
eids <- c(eids, unlist(xx[k]))
goids <- c(goids, names(xx[k]))
}

dd <- ""
for(m in 1:length(unlist(xx[k]))){
  if(m==1)
    dd <- unlist(xx[k])[m]
  if(m>1)
    dd <- paste(dd, unlist(xx[k])[m], sep=';')
}
ENTREZID2GENESYMBOL[k,2] <- dd

ENTREZID2GENESYMBOL[,1] <- rownames(ENTREZID2GENESYMBOL) <- goids
idx <-
  unlist(sort(as.numeric(rownames(ENTREZID2GENESYMBOL)), index=TRUE)[2])
ENTREZID2GENESYMBOL <- ENTREZID2GENESYMBOL[idx,]

ENTREZID2GENESYMBOL <- as.data.frame(ENTREZID2GENESYMBOL)
remove(x)
remove(xx)
remove(dd)
remove(eids)
remove(goids)
remove(idx)
remove(k)
remove(m)
remove(mapped_genes)

################ Combine DESeq2-LFC with edgeR results ################

# DESeq2 result: results_LFC
# EdgeR result: DifGenes
results.combined <- cbind(results_LFC, DifGenes[, "logFC"])[match(rownames(results_LFC), r
ownames(DifGenes)))

names(results.combined)[6]<-"logFC.edger"

results.combined <- cbind(results.combined, DifGenes[, "logCPM"])[match(rownames(results_LFC), rownames(DifGenes))]

names(results.combined)[7]<-"logCPM.edger"

# Add entrez ID

results.combined <- cbind(results.combined, ENTREZID2GENESYMBOL[, "ENTREZ_IDS"][match(rownames(results.combined), ENTREZID2GENESYMBOL$GENE_SYMBOLS)])

names(results.combined)[8]<-"entrez"

results.combined <- cbind(results.combined, (rownames(results.combined)))

names(results.combined)[9]<-"symbols"

#Remove NA

results.combined <- results.combined[complete.cases(results.combined), ]
results.combined <- results.combined[order(results.combined[,"padj"]), ]

#Save file

write.table(results.combined, file="Results_Combined.csv")

remove(results_LFC)
remove(DifGenes)

# Similar results from DESeq2 VS EdgeR

###### goseq ######

library(goseq)

# Derive list of DE genes by filtering on DESeq2 padj

genes.DE <- (results.combined$padj < 0.05)

#length(genes.DE[genes.DE==TRUE]) gives 18 TRUEs
	names(genes.DE) <- rownames(results.combined)

# Adjusted for count bias # Slightly different results

countbias <- rowSums(countdata)[rowSums(countdata)!=0]
countbias <- countbias[names(genes.DE)]
pwf.counts <- nullp(genes.DE, "hg19", "geneSymbol", bias.data=countbias)
GO.counts <- goseq(pwf.counts,"hg19","geneSymbol",method="Sampling",repcnt=1000, use_g
enes_without_cat=TRUE) #Random sampling
enriched.GO.counts <- GO.counts[GO.counts$over_represented_pvalue<1,]
enriched.GO.counts <- cbind(enriched.GO.counts, p.adjust(enriched.GO.counts$over_represented_pvalue, method="BH"))
names(enriched.GO.counts)[8]<-"padj"
remove(countbias)

# View genes in a GO term
allGos <- stack(getgo(names(genes.down[genes.down==TRUE]), 'hg19', 'geneSymbol'))
# View(enriched.GO.counts[,cbind("category","term","padj")])
# subset(allGos, values %in% "GO:0031012")

########## goseq - downregulated ##########
# Derive list of down-regulated genes by filtering on DESeq2 padj
genous.down <- (results.combined$padj < 0.05 & results.combined$log2FoldChange < 0)
names(genes.down) <- rownames(results.combined)

# Adjusted for count bias # Slightly different results
countbias <- rowSums(countdata)[rowSums(countdata)!=0]
countbias <- countbias[names(genes.down)]
pwf.counts <- nullp(genes.down, "hg19", "geneSymbol", bias.data=countbias)
GO.counts <- goseq(pwf.counts, "hg19", "geneSymbol",method="Sampling",repcnt=1000, use_genes_without_cat=TRUE) #Random sampling
enriched.GO.counts <- GO.counts[GO.counts$over_represented_pvalue<1,] # Can use all
enriched.GO.counts <- cbind(enriched.GO.counts, p.adjust(enriched.GO.counts$over_represented_pvalue, method="BH"))
names(enriched.GO.counts)[8]<-"padj"
remove(countbias)

# View genes in a GO term
allGos <- stack(getgo(names(genes.down[genes.down==TRUE]), 'hg19', 'geneSymbol'))
# View(enriched.GO.counts[,cbind("category","term","padj")])
# subset(allGos, values %in% "GO:0031012")
# KEGG pathway analysis

KEGG <- goseq(pwf.counts,'hg19','geneSymbol',test.cats="KEGG")

KEGG.padj <- cbind(KEGG, p.adjust(KEGG$over_represented_pvalue, method="BH"))

remove(KEGG)

# Only 05200 is significant → cancer

############################ goseq - upregulated (none padj<0.1)############################

# No gene ontology terms with padj <0.1

remove(countbias.up)
remove(countbias)

A.1.4 Gene expression heat map

library(gplots)
library(RColorBrewer)

# Uses results from DESeq2, EdgeR, and combined results from gene ontology analysis

# Subset logcounts matrix

DE.genes <- results.combined[(results.combined$padj < 0.05),]

DE.cpm <- logcounts[rownames(DE.genes),]

mypalette <- brewer.pal(11,"RdYlBu")

morecols <- colorRampPalette(mypalette)

# Set up colour vector for celltype variable

col.cell <- c("purple","orange")[sampleinfo$Flow]

# Plot the heatmap

heatmap.2(DE.cpm, 
    col=rev(morecols(50)),
    trace="column",
    main="Differentially Expressed Genes",

ColSideColors=col.cell, scale="row",
Rowv=FALSE, Colv=FALSE)

# Save the heatmap in high resolution: Plots --> Export
remove(DE.genes)
remove(DE.cpm)
remove(mypalette)
remove(morecols)
remove(col.cell)
Copyright Acknowledgements


Figure 2-2 is reprinted from “Coupling the activities of bone formation and resorption: a multitude of signals within the basic multicellular unit” by Sims, N. A. and Martin, T. J., 2014. BoneKEy Reports, doi: 10.1038/bonekey.2013.215, Copyright [2018] by Nature Publishing Group.