Breast cancer cells affect bone cell differentiation and the bone microenvironment

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List of Abbreviations

AA – Ascorbic Acid
ADP – Adenosine Di-phosphate
ALP – Alkaline Phosphatase
AMP – Adenosine Monophosphate
ATF4 – Activating Transcription Factor-4
ATP – Adenosine Triphosphate
BMP – Bone Morphogenetic Protein
CTGF – Connective Tissue Growth Factor
DAPT - N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester
DKK-1 – Dickkopf-1
DMP1 – Dentin Matrix Acidic Phosphoprotein-1
FGF – Fibroblast Growth Factor
GnRH – Gonadotropin-releasing Hormone
GPNMB - Glycoprotein non-metastatic melanoma protein B
HSC – Haematopoietic stem cell
IGF – Insulin-like Growth Factor
MCP-1 – Monocyte Chemotactic Protein-1
M-CSF – Macrophage Colony Stimulating Factor
MMP9 – Matrix Metalloproteinase-9
MSC – Mesenchymal Stem Cell
mTOR – Mammalian Target of Rapamycin
NFATc1 - Nuclear factor of activated T-cells, cytoplasmic 1
OPG - Osteoprotegerin
PDGF – Platelet-Derived Growth Factor
PHEX - Phosphate Regulating Endopeptidase Homolog, X-linked
PTH – Parathyroid Hormone
PTHrP – Parathyroid Hormone-Related Protein
RANK - Receptor Activator of Nuclear Factor Kappa-B
RANKL - Receptor Activator of Nuclear Factor Kappa-B Ligand
SDF-1 – Stromal-Derived Factor-1
SRE – Skeletal Related Event
TGF-β – Transforming Growth Factor- β
TRAF6 - TNF Receptor Associated Factor-6
TRAP – Tartrate-Resistant Acid Phosphatase
VEGF – Vascular Endothelial Growth Factor

Abstract

Breast carcinoma is the most commonly diagnosed cancer among women worldwide, with approximately 1 in 7 expected to be affected during her lifetime. The spread of breast cancer to secondary sites is generally incurable. Bone is the preferred site of metastasis, where the development of a secondary tumour causes severe osteolysis, hypercalcemia and a considerable pain burden. However, how breast cancer cells establish supportive interactions with bone cells is not well understood. We have examined the effects of factors released from MDA-MB-231 and 4T1 breast cancer cells on the differentiation of C57BL6 mouse bone marrow cells. Treatment with cancer-derived factors resulted in a sustained 40–60% decrease in osteoblast differentiation markers, and induced an osteoclastogenic change in the ratio of receptor activator of NF-κB ligand (RANKL) to osteoprotegerin (OPG). Importantly, exposure of bone cells to breast cancer-derived factors stimulated the subsequent attachment of cancer cells to immature osteoblasts. Inhibition of γ-secretase using pharmacological inhibitors DAPT and Compound E completely reversed cancer-induced osteoclastogenesis as well as cancer-induced enhancement of cancer cell attachment, identifying γ-secretase activity as a key mediator of these effects. We next evaluated the effects of breast cancer cells on the energy metabolism of bone cells. Treatment of bone marrow cells with conditioned medium from 4T1 breast cancer cells resulted in an increase in glucose consumption by bone cells, higher mitochondrial transmembrane potential, and a 2.3-fold rise in cellular ATP content. In addition, breast cancer derived factors stimulated the expression of mRNA and protein levels of metabolic sensor, AMP-regulated protein kinase (AMPK). To assess if such change in cell bioenergetics may have consequences for cell differentiation and activity, we used defined models of osteoclastogenesis, and increased precursor metabolic activity by providing excess energy substrates. We have found that an increase in mitochondrial transmembrane potential and cellular ATP levels during osteoclastogenesis resulted in the formation of larger osteoclasts that

demonstrate higher resorptive activity. Thus, we have uncovered that osteoblasts act as a critical intermediate of premetastatic signalling by breast cancer cells, and pinpointed γ-secretase as a robust target for developing therapeutics potentially capable of reducing both the homing and progression of cancer metastases to bone. In addition, we have discovered heightened energetics in bone cells exposed to breast cancer cell-released factors, which may contribute to the formation of larger, more active osteoclasts. Modification of the AMPK pathway may prove an important therapeutic target for breast cancer metastasis to bone.

Résumé

Le cancer du sein est le cancer plus diagnostiqué chez les femmes. On estime qu'environ une femme sur sept en sera affectée. La diffusion du cancer du sein aux emplacements secondaires est généralement incurable. L'os est l'emplacement préféré de la métastase, où le développement d'une tumeur secondaire cause de l'osteolyse, de l'hypercalcemie, et une douleur considérable. Cependant, comment les cellules de cancer du sein établissent des interactions supportifs avec des cellules d'os n'est pas bien compris. Nous avons examiné les effets des facteurs libérés des cellules du cancer du sein MDA-MB-231 et 4T1 sur la différentiation des cellules de moelle de la souris C57BL6. Le traitement avec des facteurs cancer-dérivés a produit une diminution de 40-60% des marqueurs de différentiation d'osteoblast, comparé au traitement par l'acide ascorbique, et a induit un changement osteoclastogenique dans le rapport du RANKL/osteoprotegerin. L'exposition des cellules d'os à des facteurs dérivés du cancer du sein a ensuite stimulé l'attachement des cellules cancéreuses aux osteoblasts non mûrs. L'inhibition du γ-secretase utilisant les inhibiteurs pharmacologiques DAPT et le Compound E a complètement inversé l'osteoclastogenise cancer-induit aussi bien que le perfectionnement cancer-induit de l'attachement de cellules cancéreuses, identifiant l'activité de le γ-secretase comme étant le médiateur principal de ces effets. Nous avons ensuite évalué les effets des cellules cancereuse sur le métabolisme énergétique des cellules d'os. Le traitement des cellules de moelle avec le medium conditionné des cellules du cancer du sein 4T1 a eu comme conséquence une augmentation des mitochondries à haut -potentiel de membrane, une augmentation de 2.3 fois le contenu cellulaire de triphosphate d'adénosine, et une consommation plus rapide du glucose. Ce changement de l'énergétique a été accompagné d'une stimulation d'AMPK dans la protéine et l'ADN messagère. Pour évaluer les effets du statut de haute énergie dans les osteoclasts, nous avons élevé l'énergique des osteoclasts avec du pyruvate de sodium. Cette addition a causée une croissance des osteoclasts, avec

des plus grands nucleus, et la résorption de plus de substrat. Ainsi, nous avons découvert l'osteoblast comme étant un intermédiaire clé à la signalisation prémetastatique par des cellules du cancer du sein. Nous avons aussi indiqué le γ-secretase comme cible robuste pour le developpement de thérapeutique potentiellement capable de réduire l'autoguidage et la progression des métastases de cancer à l'os. Additonellement, nous avons découvert l'énergétique intensifiée chez les cellules d'os exposées aux facteurs cellule-libérés par le cancer du sein, qui mène à une osteoclastogenesise plus active et plus importante. La modification de la voie d'AMPK peut s'avérer être une cible thérapeutique importante pour que la métastase de cancer du sein aux os.

Originality and contributions

Materials presented in this thesis represent original contributions to knowledge.

Authors' contributions to the chapters are described below:

Chapter 2: Literature Review

JEF performed the literature search and analysis, and prepared the manuscript. SVK participated in discussions and edited the chapter.

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Chapter 4: Breast cancer cells affect the differentiation of bone cells

JEF performed all experiments, analysis and preparation of the chapter.

SVK conceived the study, participated in experimental design and data analysis, and edited the chapter.

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Chapter 5: Role of bone cells in supporting the homing of breast cancer cells to bone

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The continuation of the study, shown in Figures 5.4-5.6, was presented as a poster at the American Society for Bone and Mineral Research Annual Meeting 2010, and the 2010 Conference on Human Cell Transformation, and the abstracts were published in the following:

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Fong JE, Hussein O, Komarova SV. The Role of Gamma-secretase Mediated Cleavage of Notch and Amyloid Precursor Protein in Breast Cancer Cell Attachment to Osteoblasts. J Bone Miner Res (Suppl 1), 2010.

Chapter 6: The effect of breast cancer cells on bioenergetics state of bone cells

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Dr. Kerstin Tiedemann performed the AMPK immunoblots in Figure 6.4 E.

Dr. Damien Le Nihouannen conceived the osteoclast differentiation, study and performed the osteoclast differentiation assays in Figure 6.4 A, B, and performed data analysis.

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Data regarding osteoclast differentiation under oxidative condition discussed in this chapter are part of the manuscript Le Nihouannen D, Barralet JE, **Fong JE**, Komarova SV. Ascorbic acid accelerates osteoclast formation and death. Bone. 2010 May;46(5):1336-43. (JEF performed experiments, analysis and preparation of the manuscript).

Chapter 1

Introduction and Aim of the Work

Breast carcinoma is the most commonly diagnosed cancer among women, and when caught early breast cancer can be treatable. However, once it has progressed into advanced stages, the dissemination of cancer to distant sites is incurable. Bones are the most common sites of hematogenous metastases (1), where osteoclastic bone resorption is stimulated, leading to the destruction of bone. Bone metastasis is associated with significant morbidity due to the disruption of bone architecture and mineral homeostasis, which leads to hypercalcemia, pathological fractures, and a considerable pain burden.

Bone is a dynamic tissue that provides support and protection for organs and maintains body mineral homeostasis. It is maintained by the cooperative actions of osteoclasts that resorb old or damaged bone and osteoblasts that subsequently form new bone in this place. These cells work in concert to build bones, maintain mechanically sound bone tissue by replacing it on average every 10 years, and repair bones in the incidence of trauma. Osteoclasts are cells of hematopoietic origin that resorb bone by lowering the extracellular pH to dissolve hydroxyapatite crystals and release proteolytic enzymes, such as cathepsin K and matrix metalloproteinase-9 (MMP-9), to digest the organic matrix (2). Osteoblasts, derived from mesenchymal stem cells, secrete the extracellular matrix and regulate its subsequent mineralization. High bone turnover has been found to correlate to poor prognosis in patients with bone metastases (3), making bone homeostasis an essential part of understanding cancer progression.

During breast cancer metastasis to bone, tumour cells home to bone marrow, likely targeting the hematopoietic stem cell niche, and stimulate osteoclasts, which mediate osteolysis required for tumour expansion. The development of an osteolytic lesion depends highly on the differentiation of osteoclasts and their

subsequent resorptive activity. Although osteoblasts contribute to the regulation of the hematopoietic stem cell niche and control osteoclastogenesis through production of proresorptive cytokine RANKL, their role in cancer metastases to bone is not fully understood.

The 1st aim of this study was to examine the role of osteoblasts in breast cancer metastasis to bone.

The establishment of breast cancer cells into secondary tumours in the bone depends on the successful interaction between breast cancer cells and bone cells. Recent evidence has led to the idea that the bone marrow supports a pre-metastatic niche - a site that receives signals from the primary tumour mass before dissemination, and changes the landscape of the target tissue to be conducive to tumour growth. This can be accomplished through several different strategies. Breast cancer cells express higher levels of CXCR4 compared to normal breast tissue (4), and its ligand SDF-1 is strongly expressed in lung, liver, bone marrow and lymph nodes, the primary sites of secondary breast tumours. This has led to the identification of the role of the SDF-1/CXCR4 in promoting the migration of breast cancer cells to bone (5). To facilitate their establishment in the bone microenvironment, bone marrow derived hematopoietic stem cells that express VEGF form clumps of cells and have been implicated in the creation of an environment more receptive to breast cancer cells (6,7). Once the breast cancer cells have arrived at the secondary site, they develop new strategies for their establishment there. Proteins mediating cell interactions with extracellular matrix, such as integrins (8), and Annexin II (9,10) have been implicated in breast cancer progression. Osteoblasts are an integral part of the haematopoietic stem cell niche, but if and how they are involved in the pre-metastatic niche is unknown.

The 2nd aim of this study was to identify osteoblast-breast cancer cell interactions and examine their potential mediators.

The altered metabolism in cancer cells has long been studied for its paradoxical nature. Cancer cells have high metabolic rates, yet use the less efficient means of ATP generation, glycolysis followed by lactic acid fermentation, even in the presence of oxygen. This is hypothesized to be due to mitochondrial mutations or the cancer cells' adaptation to a hypoxic environment (11). Energy metabolism also changes from the primary site to the secondary site (12), and is not only confined to the cancer cells themselves, as the antioxidant levels in blood plasma have been shown to be lowered when there is cancer in a distant site (13). More recently, cancer-associated fibroblasts were shown to increase their metabolic substrate output in order to feed cancer cells (14), suggesting that bioenergetics of neighbouring cells may also be affected by cancer.

The robust demands of energy placed on osteoclasts and osteoblasts require considerable energy production to both resorb bone and form new bone. The energy metabolism of osteoblasts increases through their differentiation (11). In osteoclasts, a heightened energy state also coincides with differentiation (15-17). We hypothesized that the alteration of bioenergetic state of the bone microenvironment may affect the differentiation and function of bone cells, and used a well-defined model of osteoclastogenesis to test this hypothesis.

The 3rd aim of this study was to identify the changes in energy metabolism in bone cells exposed to factors released from breast cancer cells, and to examine potential consequences of such changes.

Study Objectives

1. To investigate how breast cancer cells affect osteoblast differentiation, and function, as well as the regulation of osteoclastogenesis by osteoblasts.
2. To characterize the interactions between breast cancer cells and osteoblasts that contribute to the establishment of breast cancer cells in the bone microenvironment.
3. To assess the changes in energetic status of bone cells that have been exposed to factors released by breast cancer cells.

Chapter 2

Literature Review

Breast cancer metastases to bone: role of the microenvironment

Abstract

Bone is the preferred site for breast cancer metastasis, which leads to altered mineral metabolism, disruption of bone architecture, and considerable pain burden. Prior to homing to the bone, the primary breast tumour releases soluble factors that lead to the creation of a pre-metastatic niche in the bone, which then serves to attract and maintain invading breast cancer cells. Breast cancer cells actively influence resident bone cells, altering both the action of and cross-talk between bone forming osteoblasts and bone-destroying osteoclasts. Breast cancer cells inhibit osteoblast differentiation and prevent them from creating and mineralizing new bone. Immature osteoblasts are part of a hematopoietic stem cell niche and provide an attachment site for breast cancer cells. Breast cancer cells also produce factors, such as parathyroid hormone-related protein (PTHrP), which induce osteoblasts to stimulate the production of the pro-resorptive cytokine RANKL and to inhibit the production of RANKL inhibitor, OPG. RANKL, together with other osteoclastogenic factors released from breast cancer cells, promote the fusion and differentiation of osteoclasts, resulting in bone destruction. As a result of bone resorption, growth factors stored in the bone matrix, such as TGFβ, are released and can further stimulate the proliferation and survival of tumour cells. Thus, the complex interactions between breast cancer cells and the bone microenvironment underlie the homing of the breast cancer to bone and the subsequent progression of osteolytic lesions. Current therapeutics against bone metastases aim to prevent osteoclastic bone resorption by blocking osteoclast differentiation or stimulating their apoptosis. The osteoblast provides a valuable potential target, as a source of osteoclastic differentiation factors, and a platform for cancer cell attachment. Recent results from basic and clinical

research provide new targets to prevent the interactions between breast cancer cells and the bone microenvironment at different stages of the metastatic cascade.

I. Physiological regulation of breast and bone

Breast Growth and Development

The interactions of normal breast tissue with bone arise during childbearing and breastfeeding. A normal human fetus needs approximately 30 g of calcium to mineralize its skeleton during gestation (6,18), that leads to significant changes in calcium homeostasis during pregnancy, including adjustments in levels of parathyroid hormone (PTH), calcitonin and 1,25 dihydroxy-vitamin D [1,25[OH]D] (19). These hormones exhibit their effects through three main target tissues – intestines, kidneys and bone (20). Parathyroid hormone related peptide (PTHrP) is a hormone closely related to PTH, but which is produced by local tissues, such as breast, and is important for its differentiation (21). In addition to its role in local tissue development, PTHrP can substitute for PTH in the tissues expressing their common receptor, and thus participate in calcium homeostasis by elevating 1,25(OH)D and suppressing PTH, regulating placental calcium transport, and affecting bone resorption in the maternal skeleton (20). The regulation of calcium homeostasis by lactating mammary gland is likely of critical importance, since nursing humans secrete 300-400 mg of calcium into milk each day (22). The hormonal balance changes again during lactation, with still reduced PTH levels, but normalized calcitonin and 1,25(OH)D, and increased PTHrP (19). During this time, increased prolactin concentrations allow for the release of breast milk, and also act to enhance bone turnover (23,24). Suckling stimulates prolactin secretion and inhibits gonadotropin-releasing hormone (GnRH) production, both of which reduce estradiol levels, leading to bone resorption (25). Bone resorption has been shown to increase during lactation, and bone formation to decrease, resulting in a loss of 5-10% of trabecular mineral content per month (26). Lactation-induced fragility fractures have been reported as a result, but are not common (27). Of interest, other important molecular mediators for the developing of lactating mammary gland are the receptor activator of nuclear factor κB (RANK) and its ligand RANKL, which are better known for their key role in regulating formation of osteoclasts. Expression of RANKL in mammary

epithelium is induced by hormones increased during pregnancy, such as prolactin, progesterone, and PTHrP, and mice lacking RANKL or RANK cannot form lobuloalveolar mammary gland structures, resulting in the complete inability to develop a lactating mammary gland (28). Thus, normal breast tissue can interact with bone through a system of hormonal regulators important during lactation, and it expresses molecular machinery that employs same mediators to perform locally distinct functions (Figure 2.1).

Breast carcinomas may arise from the inner lining of the milk ducts or from the lobules, known, respectively, as ductal carcinomas or lobular carcinomas (29). Once a tumour exceeds 1-2 mm in diameter, it requires extensive vascularization in order to survive (30), but the speed of cancer growth often exceeds its capability to form normal vascular organization. Poor angiogenesis results in an under-vascularized microenvironment, which leads to hypoxia, acidic pH and nutrient depletion in the tumour (31). Some cancer cells may develop the ability to detach from the primary tumour and invade other areas to form secondary tumours, in a process called metastasis. Breast cancer cells favour regional lymph nodes as well as the liver, lungs, brain and bone as sites of metastasis (4). The metastatic process occurs in a complex series of interrelated steps. An epithelial-to-mesenchymal-transition (EMT) may occur whereby epithelial breast cancer cells take on a mesenchymal phenotype of reduced attachment to neighbouring cells and increased migratory capabilities (32). This may assist in their intravasation process, where the cell breaks through the epithelium into a blood vessel (33). From here, the cell migrates to a distant site, driven by chemotaxis and the communication between the cancer cell and a secondary site where it aims to establish (6,34,35). Instead of combating cancer cells, tumour-associated macrophages and T-cells may assist in the survival and dissemination of cancer cells by mitigating the immune response and promoting cancer progression (36,37). When the cell has reached its destination, it will then undergo extravasation to exit the blood vessel and establish in a new tissue (38). Bone is a

preferred site for breast cancer metastases, therefore specific interactions are likely to establish between breast cancer cells and bone cells.

Bone Microenvironment

Bone is a dynamic tissue that provides support and protection for organs and maintains body mineral homeostasis. All 213 bones are constantly remodelled by the coordinated action of specialized bone cells—osteoclasts that destroy bone and osteoblasts that build bone. Bone remodelling contributes to the many functions that bones provide, and occurs at different rates in different areas. Higher rates of bone turnover are observed in trabecular bone compared to cortical bone (39), and at bone sites adjacent to actively hematopoietic bone marrow in the axial skeleton, where bone metastases also commonly occur (40). High bone turnover has been found to correlate to poor prognosis in patients with bone metastases (41), and prostate cancer cells have been shown to preferentially metastasize to sites of active bone turnover (42), making bone homeostasis an essential part of understanding cancer progression.

Structure

The adult skeleton is composed of 80% solid and dense cortical bone, surrounding the remaining 20% trabecular bone, a network of plates and rods through the bone marrow (43). Bone is composed of an organic phase of extracellular matrix containing collagen-1 triple-helical chains and non-collagenous proteins, and mineral phase of hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$]. Osteogenesis occurs by two distinct mechanisms – endochondral ossification, and intramembranous bone formation. Endochondral ossification occurs in most bones of mesodermal origin that form the axial skeleton, including long bones, skull, ribs and vertebrae, and involves the formation of initial mineralized cartilage template, which is first degraded by osteoclasts and then replaced with bone matrix by osteoblasts (44,45). Intramembranous ossification occurs in the flat bones and the mandible, maxilla and clavicle, where an ossification centre is created when mesenchymal stem cells condense, and directly differentiate into bone-forming osteoblasts (46).

Functions

The mechanical functions of bone are probably their best recognized. Bones protect internal organs from damage and support the structure of the body. Bones provide anchorage for muscles, ligaments and tendons to allow movement in three-dimensional space. Hearing is also attributed to the mechanics of bones, with several of the body's smallest bones involved in the transmission of sound in the ear. Bone is the body's major reservoir of calcium, storing approximately 99% of it in the bone's mineral phase. Plasma calcium levels are strictly regulated in the range of 2.2-2.6 mmol/L total calcium. Such control is achieved by regulating calcium exchange with the environment through the kidney and intestine, and, in the absence or insufficiency of environmental sources, by regulating calcium exchange between plasma and bone through osteoblastic bone formation and osteoclastic bone destruction (47). The coordination of calcium fluxes is achieved through complex hormonal regulation. Parathyroid hormone and 1,25 dihydroxy-vitamin D act to increase calcium by stimulating calcium reabsorption from the kidneys and small intestine, respectively, and both act by enhancing the mobilization of calcium from bone through resorption (48). Calcitonin acts to reduce blood calcium by suppressing renal calcium reabsorption and inhibiting the mobilization from bone by preventing bone resorption (49). The combined work of these systems ensures that hypo- or hyper-calcemia is corrected, and ingested calcium is stored or eliminated as waste.

Bone tissue also interacts with other functionally diverse systems in the body. The endosteal surface of the medullary cavity of bones houses the haematopoietic stem cell niche, the specific location where blood stem cells best differentiate. Osteoblasts are well known to support the haematopoietic stem cell niche directly (50), and haematopoietic cells in turn regulate osteogenesis (51). Adipocyte-derived leptin regulates both appetite and bone mass accrual (52), and osteoblast-derived osteocalcin affects insulin secretion and sensitivity, as well as energy expenditure (53,54). It has most recently been shown that the skeleton regulates

male fertility through osteocalcin (55), extending the breadth of bone's influence into reproduction as well.

Bone Cells

The three cell types critical to bone's structure and function are the bone-resorbing osteoclast, the bone forming osteoblast, and the mechanosensory osteocyte. These cells work in concert to build bones, maintain mechanically sound bone tissue by replacing it on average every 10 years, and repair bones in the incidence of trauma.

Osteoclasts: The destruction of bone, both physiological in the case of morphogenesis and replacing old or damaged bone, and pathological in the case of osteolytic diseases such as osteoporosis, breast cancer metastasis to bone and rheumatoid arthritis, occurs through the activity of the osteoclast. Osteoclasts are cells of hematopoietic origin. The key molecular mediators of osteoclast formation from monocytic precursors are macrophage colony-stimulating factor (M-CSF) acting through its receptor c-fms, and RANKL which binds to its receptor RANK (56-58). Osteoprotegerin (OPG) is the high affinity decoy receptor for RANKL and is able to prevent osteoclast differentiation by inhibiting RANK-RANKL interactions (59). RANKL binding to RANK in the presence of M-CSF induces the recruitment of adaptor molecules including TRAF6 by RANK (60), resulting in activation of transcription factor NFκB. One of the early targets of NFκB is another transcription factor essential for osteoclastogenesis, nuclear factor of activated T-cells c1 (NFATc1), which later undergoes auto-amplification with the assistance of an activator protein-1 complex containing c-Fos (61-63). NFATc1 nuclear localization is regulated by calcium signalling, which also activates calmodulin-dependent kinase, critical for further osteoclast differentiation (64). These events lead to the expression of osteoclast-specific genes including tartrate-resistant acid phosphatase (TRAP), cathepsin K, and β3 integrin (65), which are important for the degradation of bone tissue. Osteoclasts resorb bone by creating a unique microenvironment localized between this cell

and bone tissue. Osteoclasts first recognize and bind to the bone matrix with integrin receptors β1 that bind collagen, fibronectin and laminin, and αvβ3 that binds osteopontin and bone sialoprotein (66). This border forms a sealing zone over the area of bone to be resorbed, and the polarization of osteoclasts results in formation of a ruffled border between the osteoclast and matrix (67). Targeted secretion of H^+ ions through the ruffled border H^+ ATPase, accompanied by movement of Cl^- through chloride channels, acidifies the sealed space to a pH of approximately 4.5 (68,69), resulting in dissolution of mineral phase of bone, and proteolytic enzymes cathepsin K and matrix metalloproteinase-9 (MMP-9) are released and activated to digest the organic matrix (2).

Osteoblasts: Osteoblasts are differentiated from the mesenchymal stem cells (MSC) that can also give rise to progenitors of myoblasts, adypocytes and chondrocytes (70). Commitment of MSC to become osteoprogenitors results in upregulation of receptors for hormones, cytokines and growth factors, including PTH, prostaglandin, interleukin-11, insulin-like growth factor-1 and transforming growth factor-β (71). Next, osteoprogenitor cells differentiate into preosteoblasts, cells that exhibit limited proliferation and start to express extracellular matrix proteins, such as collagen type I, bone sialoprotein and osteopontin. Preosteoblasts are also active in the production of pro-resorptive cytokine RANKL (72). Finally, mature osteoblasts do not proliferate, but actively produce and secrete collagen type I, bone sialoprotein and osteopontin as well as osteocalcin. In addition, mature osteoblasts switch to produce RANKL inhibitor, OPG (72). Osteoblastogenesis is driven by the downstream activities of Wingless-ints (Wnt) singling, the closely associated Hedgehog signalling pathway (Sonic Hedgehog, Indian Hedgehog) and bone morphogenetic proteins (BMPs), which determine where mesenchymal stem cells condense during embryonic patterning and cross-talk to induce osteoblast differentiation (73,74). Another developmentally important pathway, Notch signalling, has been shown to negatively regulate osteoblast differentiation (75-77). Important signalling events during osteoblast differentiation include the activation of the runt-related

transcription factor 2 (Runx2) transcription factor, which regulates the expression of the zinc finger-containing transcription factor Osterix (78). Osterix interacts with nuclear factor for activated T cells c2 (NFATc2), and in collaboration they control the transcription of osteoblastic target genes osteocalcin, osteopontin, osteonectin and collagen-1 (79,80). Transcription factor ATF4 is also essential for osteoblast maturation, and controls both gene transcription of osteocalcin and collagen-1 protein synthesis (81). Osteoblasts anchor to newly formed bone matrix by cadherin-11 and N-cadherin, and secrete type 1 collagen and non-collagenous matrix proteins (71). The osteoblasts then regulate the subsequent mineralization of extracellular matrix (82-84).

Osteocytes: Osteocytes are the most populous in bone and account for over 95% of all cells in the skeleton, covering 94% of all bone surface (85). Osteocytes are differentiated from osteoblasts embedded in the bone matrix. During differentiation, the osteocyte cell body size decreases, and the number of long dendrite-like cell processes increases and extends, connecting the cell with other osteocytes (85,86). Osteocyte-specific genes are activated, including phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX), matrix extracellular phosphoglycoprotein (MEPE), dentin matrix protein 1 (DMP1), and fibroblast growth factor-23 (FGF23) (87,88). Osteocyte networks in the bone tissue were implicated in regulating the maintenance and mineralization of bone tissue (85,89), through expression of sclerostin, a negative regulator of bone formation (90), as well as in sensing mechanical load in part through sheer stress generated by interstitial fluid moving through the lacuno-canalicular network (91). It has also been suggested that osteocytes participate in mineral homeostasis by resorbing the lacunae walls in which they are embedded (92-94).

Changes in the energy states of osteoblasts have been reported during differentiation, with the energetic demands resulting in several-fold increases of respiration, ATP production and mitochondrial activity (11). Healthy cells have a

high ATP to ADP ratio, and minor disruptions in ATP production result in an arrest of the cell cycle or even apoptosis of the cell (95). To adjust the ATP production to changing cellular demands, metabolic sensors, such as AMP-activated protein kinase (AMPK) are employed. When a cell is unable to meet its energetic demands, AMP concentrations are increased and AMPK is stimulated. AMPK in turn affects multiple downstream targets to decreases the cell's metabolic expenditure while simultabeously improving energy production by inducing mitochondrial biogenesis and fatty acid oxidation (96). The counterpart to AMPK is mTOR, which regulates protein synthesis through S6K1 and 4E-BP1 phosphorylation and controls the cell's cytoskeletal organization. As such, mTOR is suppressed when nutrients are limited.

Communication between bone cells during normal bone remodelling

Osteoblasts, osteoclasts and osteocytes must work in concert to maintain bone homeostasis (Figure 2.2). In normal bone physiology, the osteoclast will resorb worn or damaged bone, and then the osteoblast will form new bone in its place. The best studied example of the crosstalk between bone cells involves the RANK-RANKL-OPG triangle, where osteoblasts and osteocytes produce RANKL to promote osteoclast differentiation and survival, and OPG to prevent it, while osteoclasts express RANK, allowing them to respond to these regulatory cues. Many hormonal regulators of bone remodelling, such as PTH and estrogen, were demonstrated to act through changing the ratio of RANKL and OPG expression by osteoblasts (97). Interestingly, production of RANKL and OPG by osteoblasts is also regulated by their developmental stage, with immature osteoblasts producing more RANKL and mature osteoblasts produce more OPG (72). Osteocytes also, at least in part, affect osteoclastogenesis through production of RANKL, which is induced in mechanically-stimulated osteocytes (98). Osteoclasts are in turn able to influence osteoblast activity. The concept of osteoclast-mediated osteoblastogenesis arose from the finding that 97% of new bone formation occurs in resorption pits (99). Several studies where osteoclasts

have been genetically altered to have impaired function demonstrated diminished bone formation (100), and studies have begun to find mediators of this reversal coupling. Cardiotrophin-1 is among the first identified, and is expressed by osteoclasts and increases osteoblast activity (101). Sphingosine-1-phosphate has been shown to act earlier and induce osteoblast precursor recruitment and subsequent mature cell survival (102). Ephrin-B2/EphB4 bidirectional signalling between osteoclasts and osteoblasts, has also been identified as a key mediator of contact-dependent communication. Forward signalling by ephrin-B2 on osteoclasts to EphB4 on osteoblasts activates bone formation, whereas reverse signalling from EphB4 on osteoblasts binding to ephrin-B2 on osteoclasts inhibits osteoclastogenesis (103). Since the ability for bone cells to communicate is essential for the maintenance of bone homeostasis, it can be anticipated that disruptions in these the complex networks would lead to profound consequences. Indeed, the RANKL/OPG ratio represents one of the key mediators of pathological bone destruction (104).

II. Homing of breast cancer cells to bone

Creation of the pre-metastatic niche

Recent evidence has led to the idea that the bone marrow supports a pre-metastatic niche - a site that receives signals from the primary tumour mass before dissemination, and changes the landscape of the target tissue to be conducive to tumour growth. It has been shown in mice treated with medium conditioned by tumour cells of different origin, that the potential of subsequently injected cancer cells to home to different organs can be altered (7). In particular, in bone, bone marrow derived hematopoietic stem cells have been implicated in mediating the establishment of pre-metastatic niche (6,7). Molecular mediators such as vascular endothelial growth factor (VEGF) receptor 1 (VEGFR1) and integrin α4β1 have been implicated in this process. VEGFR1 positive haematopoietic progenitor cells are recruited to sites of future metastasis (7). VEGF receptors are expressed by

breast cancer cells as well as osteoclasts and osteoclast precursors, and VEGF expression correlates to increased tumour size and grade in humans (105).

One of the hallmarks of cancer is the stimulated glycolysis and use of lactic fermentation for ATP generation, even in aerobic conditions (106). The Warburg Effect explains that the predominant means of energy production by cancer cells is by glycolysis followed by lactic acid fermentation in the cytosol, unusual since most normal cells use comparatively low rate of glycolysis followed by oxidative phosphorylation in the mitrochondria (107). Fermentation of glucose to lactate occurs in cancer cells even if there is sufficient oxygen to support mitochondrial oxidative phosphorylation. This metabolic activity is similar to that in early embryonic cells, which suggests that cancer cells use a more primitive metabolic activity. It has been suggested that this may be due to mitochondrial DNA mutations, nuclear DNA mutations, oncogenic transformation or simply due to the heterogeneity of the tumour microenvironment, where the different parts of the population may use one or both forms of ATP synthesis (107). Additionally, it has also been suggested that breast cancer cells alter the metabolism of surrounding cells to produce more energy substrates for the growth of tumour cells, termed the "Reverse-Warburg effect" (14). The energy metabolism of bone cells changes through differentiation (11), therefore external influences altering the energetic state may affect differentiation.

Migration of breast cancer cells to bone

Breast cancer cells express receptors that direct their movement towards fertile sites where they may establish into secondary tumours. These proteins are generally expressed in normal cells, and are often involved in developmental pathways. Several chemokines have been suggested to be released from the bone microenvironment, implicating chemoattraction through G-protein-coupled chemokine receptors in driving the movement of tumour cells towards bone (108). Interactions between stromal-derived factor-1 (SDF-1) and CXCR4 are essential for the correct localization of lymphocytes and haematopoietic cells in

physiological states. Breast cancer cells express higher levels of CXCR4 compared to normal breast tissue (4), and SDF-1 is strongly expressed in lung, liver, bone marrow and lymph nodes, the common sites of secondary breast tumours, leading to the identification of the role of the SDF-1/CXCR4 in promoting breast cancer metastasis to bone (5). In addition to directional migration, chemokines have been shown to promote cancer cell survival, proliferation, and adhesion (109). In keeping, the inhibition of CXCR4 limited breast cancer metastases in mice (110), and the overexpression of CXCR4 indicates poor prognosis in both human and murine breast cancer (109,111). Another chemokine implicated in metastases of breast cancer cells expressing high levels of CCR7, is CCL21 that is expressed highly in metastatic sites, such as lymph nodes (4). Since haematopoietic stem cells (HSCs) use these chemokine and receptor interactions to home to the HSC niche in the bone marrow, it has been suggested that cancer cells use this same mechanism to parasitize these microenvironments and harvest the resources of HSCs (112). Another pertinent means of cancer cell migration towards bone relies on the cancer cell expression of RANK (113), which mediates directional migration of breast, melanoma and prostate cancer cells towards RANKL, produced in bone by osteoblasts (114,115).

Breast cancer cells may also stimulate the action of matrix metalloproteinases that support cancer cell migration and invasion. The murine orthologue of Glycogen Nonmetastatic Melanoma Protein B (GPNMB) is called osteoactivin and has been identified as a key modulator of osteolysis. Its forced expression leads to increased tumour grade and enhanced bone metastasis by upregulated MMP3 through ERK signalling (116,117). Furthermore, GPNMB was identified as a poor prognostic marker in patients with breast cancer (118). Most recently, this group has identified ADAM10 as a sheddase that releases osteoactivin from the cell, which induces endothelial cell migration and subsequent angiogenesis (119). ADAMTS1 and matrix metalloproteinase-1 (MMP1) are also tumour-derived metalloproteinases able to degrade the matrix. The stimulated action of these enzymes by breast cancer cells enhances osteoclast differentiation by suppressing

OPG expression, and their expression in human samples correlates to a greater incidence of bone metastases (120).

Attachment proteins between breast cancer cells and the bone

Cancer cells express or induce the expression of adhesion molecules that may facilitate their interactions with the bone microenvironment. The best studied family of proteins that bind cancer cells to bone cells are integrins, heterodimeric transmembrane glycoproteins whose α and β subunits combine to form 24 known combinations with unique specificity for binding, signalling and regulatory mechanisms (121). Integrins have been demonstrated to be involved in several stages of cancer dissemination, with highly metastatic cancer cells displaying a different integrin profile than cells from the primary tumour (122). Several integrins have been shown to interact with extracellular matrix proteins during bone metastasis, with the most important being αvβ3, a receptor for osteopontin, fibronectin and vitronectin (123). Adhesion molecules engaged between breast cancer cells and bone cells may overlap with those that bind haematopoietic stem cells (HSC) to osteoblasts. HSC preferentially home to areas with more fibronectin (7). Breast cancer cells can also attach to fibronectin in an integrin-dependent manner (124). The interaction of cancer cells with fibronectin increases the production of matrix metalloproteinase-2 from fibroblasts to facilitate invasion (125). Another molecule involved the adhesion of HSC to the endosteal niche is annexin II (112). By serving as an anchor for SDF-1/CXCL12, it has been shown to regulate the homing of HSC as well as prostate cancer cells to the HSC niche (126,127). Blocking annexin II or its receptor limited the localization of prostate cancer cells to osteoblasts and endothelial cells (128). In keeping, the inhibition of the SDF-1/CXCL12 and annexin II signalling was shown to inhibit breast cancer progression (9,10). Bone matrix proteins, such as bone sialoprotein (BSP) or osteopontin (OPN) have been shown to exhibit a potential to regulate the attachment of breast cancer cells to bone (129). Early reports have argued that BSP inhibits breast cancer cell binding to bone cells (130). However, breast cancer cells have been shown to express both BSP and OPN, and to upregulate

BSP expression in pre-osteoblasts through BMP signalling; and OPN was found localized between cancer cells and bone cells at sites of metastasis (131,132). Moreover, the expression of BSP has been found to correlate with bone metastasis development (133), and OPN expression and serum concentrations have been shown to be poor prognosis markers in breast cancer patients (134,135). As osteopontin is also a mediator of the hematopoietic stem cell niche, directing migration and acting as an adhesion molecule to HSC via β1 integrin (136), it represents a potentially valuable therapeutic target in bone metastases.

Osteomimicry

Osteomimicry describes the phenomenon where osteotropic cancer cells express proteins and receptors found on bone cells and the bone matrix. It was speculated that such measures allow cancer cells to evade the immune system and/or establish in the bone microenvironment (137,138). These proteins include but are not limited to osteocalcin, osteopontin, alkaline phosphatase and Runx2 (139). Osteoblast transcription factor Runx2 is ectopically expressed by breast cancer cells and stimulates their proliferation, motility, and invasion through increased MMP9 expression from both cancer cells and osteoblasts (140,141). Runx2 has also been shown to regulate TGFβ-influenced PTHrP levels, as well as upregulate Indian hedgehog (142). Breast cancer cells express Hedgehog ligands that activate osteopontin expression in osteoclasts, promoting osteoclast maturation and resorptive activity through upregulated Cathepsin K and MMP9 (143,144). Of interest, expression of anti-resorptive OPG has been demonstrated to correlate with increased bone-specific homing and colonization potential in breast cancer cells (137), and to promote cancer cell survival (145,146). Osteoclastic integrin αvβ3 (69), has been shown to be upregulated in metastatic versus primary tumour cells, and has been identified as a critical mediator of breast cancer metastasis to bone (124,147). It is unclear whether cells from the primary tumour display osteomimetic features that allow their metastasis to bone, or whether secondary tumour cells established in the bone marrow and matrix receive environmental factors that give them their osteomimetic features. Regardless, the ability of

cancer cells to produce many of these factors has been beneficial to thrive in the bone microenvironment.

III. Establishing of a metastatic tumour in the bone microenvironment

Interactions of breast cancer cells with osteoblasts

Inhibition of osteoblasts by breast cancer cells

Breast cancer metastasis to bone is associated with reduction in bone formation markers in patients with bone metastases (148). In vitro, breast cancer cells have been shown to produce soluble factors able to inhibit osteoblast differentiation (35,149), the effect that may be mediated at least in part by the dysregulation of Notch and Wnt developmental signalling pathways. Notch signalling is essential in embryogenesis but has distinct roles in bone homeostasis, regulating the proliferation of immature osteoblasts (150) and suppressing osteoblast differentiation (76,77). Upregulated Notch signalling in breast cancer, through ligand Jagged-1, has been shown to correlate with increased bone metastases (151). Wnt signalling is also a highly conserved developmental pathway, well studied in bone and essential for osteoblast and osteoclast differentiation, as well as for the production of pro-resorptive cytokine RANKL and anti-resorptive OPG (152). Wnt inhibitor DKK-1 has been shown to be upregulated in diseases associated with bone destruction, such as osteoarthritis (153), myeloma (154), and potentially in Paget's disease (155). Blocking DKK-1 in a breast cancer metastasis model has also been shown to reverse breast cancer-mediated suppression of osteoblast differentiation and reinstate OPG expression (156). Breast cancer cells have also been shown to induce osteoblast apoptosis, through increased Bax/Bcl-2 ratio and caspase expression in osteoblasts (157,158). In addition to preventing formation of new bone, breast cancer-induced inhibition of osteoblast differentiation likely indirectly contributes to the change in production of cytokines regulating osteoclast formation and function.

Contribution of osteoblasts to the creation of an osteolytic environment

The formation of an osteoclast-supportive microenvironment is critical for the successful establishment of osteolytic lesion during breast cancer metastasis to bone. It has been previously shown that an increase in the ratio between a pro-resorptive RANKL and anti-resorptive OPG is a key change induced by breast cancer cells (reviewed in (159,160)). Since osteoblasts are the primary source of both pro-resorptive and anti-resorptive cytokines, they represent a critical target for cancer-derived factors. Osteoblast production of RANKL is stimulated by tumour-derived PTHrP, Il-8 , Il-6 and Monocyte Chemoattractant Protein (MCP-1) (reviewed in (161)). Another osteoblast-produced osteoclastogenic factor, MCSF, has also been implicated in breast cancer metastases to bone (162).

Role of osteoblasts in supporting breast cancer cells

An emerging area of interest is the role of osteoblasts in supporting the haematopoietic stem cell niche and how cancer cells parasitize this relationship. Haematopoiesis occurs on the endosteal surface of the bone marrow, where haematopoietic stem cells (HSCs) are maintained by the supporting cells, including osteoblasts. The main functions of the interaction between these cell types are *i*) the maintenance of HSC quiescence through osteoblast-derived osteopontin, and *ii*) modification to expand the progenitor population through Notch signalling (50,136). Several osteoblast-expressed receptors, cytokines and growth factors have been found to regulate the haematopoietic stem cell niche (163,164), including PTH/PTHrP receptors and BMPs acting to expand the osteoblast population, and Notch ligand Jagged-1 to expand the population of HSCs (50,165). Cancer cells disseminated from the primary tumour may also lay dormant for long periods of time before being activated to form metastases (166), so it is plausible that cancer cells harvest resources from the HSCs niche to maintain their survival and to induce expansion at the right environmental cues.

Interactions of breast cancer cells with osteoclasts

Stimulation of osteoclasts by breast cancer cells

Breast cancer cells have been found to produce many factors capable of simulating osteoclastogenesis, both by inducing RANKL expression by osteoblasts and stromal cells, and by producing osteoclastogenic factors themselves. PTHrP was one of the first factors identified to be secreted by breast cancer cells and to promote osteolysis through the stimulation of RANKL by stromal cells (167). Although the expression of PTHrP in primary tumours has been associated with a lower incidence of bone metastasis (168,169), it was shown that increased PTHrP expression by cancer cells present in the bone metastatic lesion positively correlates with increasing osteoclast activity and subsequent osteolysis (169), suggesting that the expression pattern of the cancer cells can change during metastasis, and implicating local factors, such as TGFβ derived from osteoclastic bone resorption in affecting metastasizing breast cancer cells. Osteoclastogenesis may also be stimulated by IL-8 secreted from breast cancer cells and acting both directly on osteoclasts and through osteoblastic RANKL signalling (170,171). Although the mechanisms of IL-8 action are not fully understood, the expression of IL-8 correlated with a higher incidence of bone metastasis in mice *in vivo* (172).

It has also been shown that during differentiation osteoclast precursors may acquire sensitivity to cancer-derived factors that are ineffective in inducing osteoclast formation from naive monocytes (3). Several signalling pathways in osteoclast precursors have been implicated in these effects, including calcium signalling, NFATc1 activation and MAPKs, ERK1/2 and p38 (3,173). These effects can be relevant to the propensity of cancer cells to metastasize to bone sites undergoing acting bone remodelling, and thus containing increased numbers of RANKL-primed osteoclast precursors. At such sites, breast cancer cells can promote further osteoclast formation, and can affect the survival of mature osteoclasts increasing their resorptive capacity. In this regard, M-CSF secreted from breast cancer cells, was shown to be responsible for the delayed apoptosis in osteoclasts (161,174). Anti-apoptotic effects of breast cancer-derived factors

included PLCγ-mediated suppression of pro-apoptotic protein BIM, and M-CSF-mediated inhibition of caspase cleavage (161).

Role of osteoclasts in supporting breast cancer cells

During osteoclastic resorption, the bone matrix components, including many growth factors stored in the bone, such as TGFβ, bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), and platelet-derived growth factors (PDGF) are released into extracellular space, where they are free to act on surrounding cells, including metastasizing cancer cells (175). Matrix released- TGFβ activated by osteoclastic resorption (176), is one of the most commonly studied matrix-derived growth factors, which was shown to stimulate cancer cell growth, modify cell invasion, and affect immune regulation (177,178). Considerable research has linked increased TGF-β in the microenvironment to the progression of metastasis, with TGFβ altering both the growth and phenotype of breast cancer cells (179), and increasing their expression of connective tissue growth factor (CTGF), CXCL11 and PTHrP (180) via Smad and MAPK signalling in breast cancer cells (167,181,182). PTHrP increases VEGF production, leading to stimulated osteoclastogenesis through the ERK1/2 and p38 signalling pathways (183). TGFβ also acts on other cells present in the bone microenvironment, such as osteoclasts themselves by sensitizing them to other breast cancer derived factors (3), through the ERK1/2, p38 and c-Jun-NH_2 kinase signalling pathways (173,184). In keeping with a key role of TGFβ in bone metastases, pharmacological inhibition of TGFβ signalling through the TβRI kinase inhibitor SD-208 resulted in decreased bone metastasis and tumour burden, and improved bone quality (185). The self-accelerating cycle of osteoclast stimulation by breast cancer cells, resulting in release of matrix growth factors due to osteoclastic resorption, leading to further stimulation of breast cancer cells and further increase in osteoclastic resorption was coined the name of "vicious cycle" (186), underlying the strong rationale for the use of anti-resorptive drugs for the treatment of cancer metastases to bone.

IV. Therapeutic targets in the bone microenvironment

The bone microenvironment presents multiple targets for developing therapeutic treatments targeting the homing of breast cancer cells to bone, as well as progression of bone metastatic lesions (Figure 2.3). Molecular mediators of critical events underlying the stimulation of bone resorption and inhibition of bone formation, as well as tumour supportive environmental changes and cellular targets have been explored for their benefits in treatment of osteolytic bone metastases.

Since its discovery, the RANKL pathway has been considered to be of important therapeutic value given its role in osteoclastogenesis mediating osteolysis and subsequently discovered breast cancer cell migration, underlying pre-metastatic homing. The fully human monoclonal antibody against RANKL, Denosumab, was approved for major North American and European markets in 2010 for the prevention of osteoporosis and skeletal related events in patients with bone metastases from solid tumours. Compared to the most potent osteoclast-targeting drug in the market, bisphosphonate zoledronic acid, Denosumab treatment delayed the occurrence of the first skeletal related event (SRE), and provided a greater reduction in bone turnover markers in breast cancer patients (187). In non-metastatic breast cancer patients additionally receiving adjuvant aromatase inhibitors, which block the synthesis of estrogen, bone mineral density gains were greater with Denosumab treatment (188). Bisphosphonate-resistant patients with bone metastases from breast or prostate cancer also benefitted from Denosumab treatment, with most having normalized serum markers of bone resorption after 13 weeks of treatment (189). Although Denosumab proves an effective treatment option, long-term use and toxicity data remains unknown.

DKK-1 was identified as a key mediator of myeloma-induced inhibition of bone formation, and was demonstrated to play an important role in breast cancer induced inhibition of osteoblastogenesis. Neutralizing anti-DKK-1 antibodies have demonstrated significant benefits in preclinical studies in mouse models of

myeloma-induced bone disease, resulting in increased osteoblast numbers, reduced osteoclast numbers and increased bone volume, and stimulating interest in further development of this approach (190). Bortezomib, a proteasome inhibitor that among other proteins affects DKK-1 and BIM (pro-apoptotic protein that mediates osteoclast apoptosis) (191,192), was shown to inhibit osteoclastogenesis (193) and has been successful in combating the osteolytic effects of multiple myeloma (194), making it an attractive candidate for the prevention and treatment of breast cancer-induced osteolysis.

VEGF represents an interesting target potentially affecting breast cancer cell homing, development of pre-metastatic niche and new vasculature formation. Many anti-VEGF therapies exist to prevent vascularization of tumours and inhibit their growth (195). There have been several hindrances in the progress of this therapy due to drug resistance and toxicity (196), and the increased incidence of osteonecrosis of the jaw in combined bisphosphonate-antiangiogenic agent therapy (197). Notwithstanding, the use of VEGF-A monoclonal antibody Bevacizumab in combination with chemotherapy has proven beneficial in reducing breast cancer growth (198) and osteolysis (199). Other targets based on the in vitro and in vivo studies, such as TGFβ, GPNMB, and CXCR4 are being explored in preclinical and clinical studies, providing the basis for the next generation of treatments.

Osteoclasts are commonly targeted therapeutically for osteolytic disease, with one of the most widely used drugs being bisphosphonates. Analogs of mineralization-inhibiting pyrophosphate (200), bisphosphonates are a class of synthetic compounds composed of two phosphate groups covalently linked to carbon with a P-C-P backbone and side groups that vary their properties and pharmacokinetics. Bisphosphonates attach selectively to bone and induce osteoclast apoptosis when they are ingested during resorption. In osteoporosis studies, all bisphosphonates given daily have been shown to reduce osteoporotic vertebral fracture rates by 40-50% (201), and zoledronic acid and risedronate have been shown to significantly

reduce non-vertebral fracture risk in pivotal trials (202). Bisphosphonates are widely used in prevention and treatment of breast cancer metastases to bone, resulting in delay and reduction in skeletal related events (203). In addition to their effects on osteoclasts, bisphosphonates have been shown to inhibit tumour growth, induce tumour cell apoptosis, and stimulate the immune response against tumour cells (204). However, some patients do not tolerate bisphosphonates well, and low but significant incidences of osteonecrosis of the jaw have been observed in patients that have undergone dental extraction procedures while treated with bisphosphonates (205). In addition, significant proportion of patients failed to normalize bone resorptive indices in response to bisphosphonate treatment (189), demonstrating the need for new therapeutic approaches.

V. Conclusion

Breast cancer is the most commonly diagnosed cancer in women, which may lead to bone metastasis resulting in altered mineral homeostasis, the disruption of bone microarchitecture, pain and pathological fractures. Recent studies have demonstrated that breast cancer cells start affecting bone microenvironment prior to their dissemination from the primary tumour by secreting circulating soluble factors that prepare bone for the future arrival of metastasizing cancer cells, the process that likely involves mediators of hematopoietic stem cell niche. Multiple mediators of directional migration of breast cancer cells have been identified, as well as mediators of breast cancer cells anti-osteoblastic and pro-osteoclastic actions. Breast cancer-stimulated RANKL, M-CSF, PTHrP, TGFβ, GPNMB, Runx2 and CXCR4 remain among the most critical mediators of cancer-induced osteoclastic bone resorption. Yet, they are not the whole picture, and new players are being identified, providing more complex and comprehensive description of the events leading from the formation of primary tumour to the establishment of progressive osteolytic bone lesions. However, while considering the multitude of molecular mediators, it is important to remember the heterogeneity of breast cancer disease in patients, suggesting that treatments targeting different molecular mediators should develop in parallel with the testing capabilities able to implicate

a particular mediator in disease progression in a specific patient. An alternative approach is to target the processes and cellular targets similarly altered through different molecular mediators. An example of such an approach is the clinical success of bisphosphonates, which broadly target osteoclast formation and activity. Nevertheless, both approaches need to be developed to provide clinicians with the set of tools for broad preventive measures, as well as for targeted personalized medicine for non-responsive or atypical cases.

FIGURE 2.1

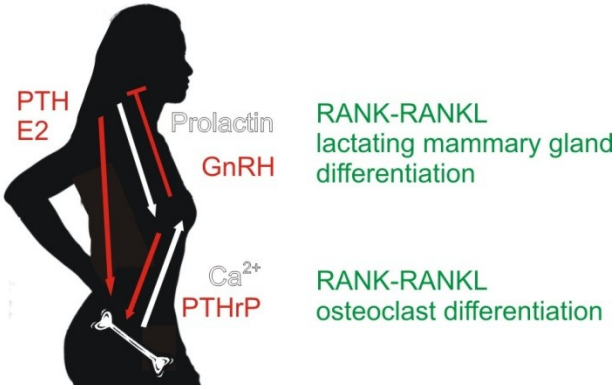

Figure 2.1: Physiological interactions between the functions of breast and bone.

Lactation involves the secretion of large amounts of calcium. Bone is a key participant in calcium homeostasis. PTH is reduced during lactation while PTHrP production by the breast tissue is increased. Suckling stimulates prolactin secretion and inhibits GnRH production, both of which reduce estradiol levels, leading to bone resorption. Prolactin and PTHrP induce breast expression of RANKL, necessary for normal lactating mammary gland function. In the bone tissue, osteoblast-produced RANKL is key regulator of osteoclastogenesis.

FIGURE 2.2

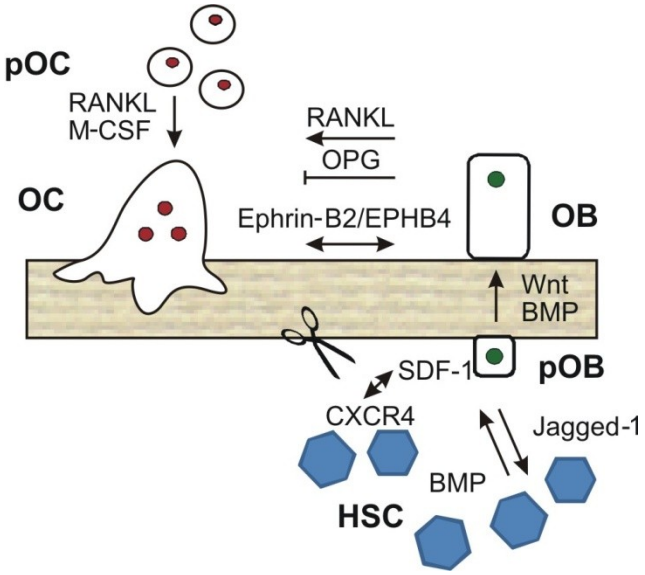

Figure 2.2: Cell-cell interactions in the bone microenvironment.

Osteoclast differentiation from monocytic precursors is induced by M-CSF and RANKL produced by osteoblastic cells. Osteoblasts are derived from mesenchymal stem cells through Wnt and BMP signalling pathways. Osteoblasts and osteoclasts communicate through osteoblast-derived RANKL/OPG and bidirectional Ephrin-B2/EphB4 signalling. Haematopoietic stem cells (HSC) support osteoblasts in the HSC niche through BMPs, while osteoblasts support HSCs through upregulated Notch signalling through Jagged-1. Osteoclasts cleave SDF-1 to mobilize HSCs from the endosteal niche.

FIGURE 2.3

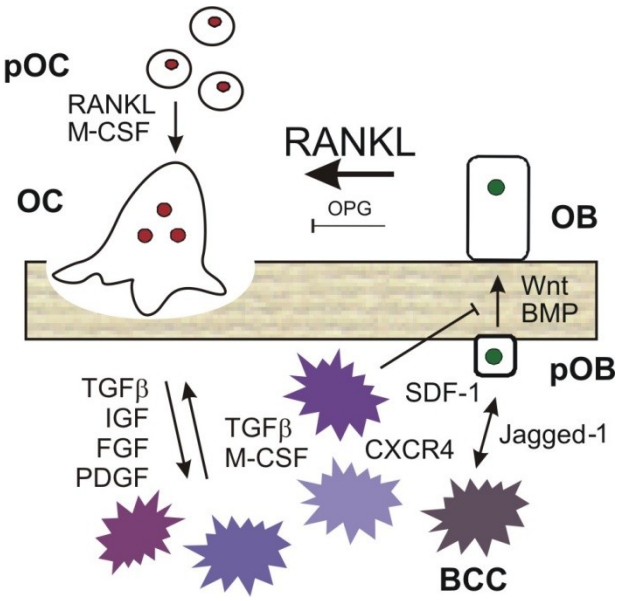

Figure 2.3: Breast cancer cells alter normal bone homeostasis.

Breast cancer cells maintain osteoblasts in an immature state and stimulate RANKL production by osteoblasts, while inhibiting OPG. Breast cancer cells stimulate osteoclastogenesis directly through TGFβ and M-CSF. Increased bone resorption by activated osteoclasts releases matrix-derived growth factors TGFβ, IGF, FGF, PDGF, which act back on breast cancer cells to stimulate their growth and survival.

Chapter 3

Materials and Methods

Test Compounds

L-Ascorbic acid (AA, Sigma, A5960) was freshly prepared and added to the medium on the day of medium change. LiCl (Sigma, L0505) was diluted in water, SB 216763 (Tocris Bioscience, 1616), SB 431542 (Tocris Bioscience, 1614), DAPT (Calbiochem, 565770), Compound E (CE; Calbiochem, 565790) were diluted in dimethyl sulfoxide, which was used as a vehicle (0.1%) in corresponding experiments. All inhibitors were present during the whole culture period. OPG (Sigma, 08137), and pan-specific TGFβ antibody produced in rabbit (R&D Systems, AB-100-NA) were incubated with MDAMB- 231 conditioned medium (CM) for 10 min before adding to cultures.

Cancer and Bone Marrow Cell Cultures

The MDA-MB-231 and MCF7 human breast carcinoma cell line, 4T1 murine mammary carcinoma cell line, and MCF10A human mammary epithelial cells were kindly provided by Dr. P. Siegel (McGill University) and cultured as described previously (3). The MC3T3-E1 mouse preosteoblastic cell line was kindly provided by Dr. M. T. Kaartinen (McGill University). Cells were cultured to 50% confluence, except for MCF10A, which was cultured to 100% confluence, in T-75 tissue culture flasks. Conditioned medium was collected after 48 h of incubation, centrifuged at 2000 rpm for 5 min, aliquoted, and stored at -80 °C. All animal studies were performed in accordance with the McGill University guidelines established by the Canadian Council on Animal Care. Mice (C57BL6/J, male, 6 weeks old) were purchased from Charles River. Mouse-derived bone marrow cells were collected from mouse tibia and femora under aseptic conditions as described previously (206). Bone marrow cells were plated at a density of 2.5×10^6 cells/cm^2 and cultured in minimal essential medium

supplemented with 1% penicillin-streptomycin (Wisent, 450-201-EL) and 10% fetal bovine serum (Hyclone, SH 30396-03). One day after plating, 50 µg/ml AA was added to induce osteoblast differentiation, and CM from the indicated cells (10%) was added to experimental cultures. All cultures were supplemented with fresh medium every other day. On the indicated days, samples were fixed with 10% formalin and stained for alkaline phosphatase (ALP; Fast Red, Sigma, F4381), tartrate-resistant acid phosphatase (TRAP; Sigma, 387A) and analyzed using BioQuant software. Mineralization was assessed using Von Kossa staining (Sigma, S6506).

RAW 264.7 monocyte cell culture

RAW 264.7 cells (ATCC) were cultured in 25 cm2 tissue culture flasks in DMEM supplemented with 1% penicillin-streptomycin and 10% FBS. To generate osteoclasts, RAW 264.7 cells were plated at 10^4 cells/cm^2. On day 1 and 3, medium was changed and RANKL (50 ng/ml) was added. RAW 264.7 cells were cultured with the following reagents: α-MEM (310-022-CL), DMEM (319-020-CL), pyruvate (600-110-EL), L-glutamine (609-065-EL), penicillin/streptomycin (450-201-EL), trypsin/ethylenediamine tetraacetic acid (T/E, 325-042-EL), from Wisent Inc. L-ascorbic acid (AA, A5960) D-Glucose (Glu, G7528), and Rapamycin (PHZ1233) were from Sigma-Aldrich Co. Dorsomorphin 2HCL (3093) and AICAR (2840) were from TOCRIS bioscience. Recombinant human M-CSF (300-25) was from Peprotech Inc. Recombinant glutathione S-transferase-soluble RANKL was purified from the clones kindly provided by Dr. M.F. Manolson (University of Toronto).

RAW 264.7 cells were grown for 5 days in the presence of RANKL (50 ng/mL) and the indicated additions. For replating, first, osteoclastogenesis was confirmed visually by light microscope, cells were washed with cold PBS for 5 minutes to remove monocytes, and then trypsinized. Cells were then centrifuged, resuspended in DMEM, and replated with the indicated additions for 24 hours before fixation and staining.

In vivo study

All animal studies were performed in accordance with the McGill University guidelines established by the Canadian Council on Animal Care. Six weeks old C57BL6/J mice (Charles River) received 0.75 g/kg/day of pyruvate solution, 0.5 g/kg /day of glucose solution (Wisent, 609-036-EL) or sterile saline by daily intraperitoneal injections for 6 days. In healthy animals, such injections are known to lead to a short-term, 20-40 min increase in blood levels of glucose (54) or pyruvate (207). On the day 7, 24 hours after the last injection, blood samples were collected and the long bones were isolated. Six-hour fasted blood levels of glucose and pyruvate were evaluated using an Accu-chek Aviva glucometer and EnzyChromTM Pyruvate Assay Kit (BioAssay Systems, EPYR-100). Steady-state pyruvate levels were similar in all mice, glucose levels were decreased by 28.1 ± 7.5% in pyruvate-treated mice (data not shown). Bone samples were embedded in paraffin and 5 μm sections were stained for tartrate-resistant acid phosphatase (TRAP). Osteoclast analysis in bone sections was conducted using Osteomeasure software (Osteometrics Inc, Atlanta GA).

Cell Proliferation Assays

Bone marrow cells were plated in 96-well flat-bottom plates at a density of 2.5 x 10^6/cm2 and cultured as described. Proliferation assay was performed after 9 days of culture using the BrdU CHEMICON Cell Proliferation Assay kit (Millipore, 2750) according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader (Beckman Coulter AD340), with a higher optical density indicating a higher BrdU concentration in the sample.

RNA Isolation and RT-PCR

Total RNA was isolated from primary cultures using the RNeasy mini kit and QIAshredder columns (Qiagen, 74104 and 79654). For real-time PCR, 2_g of total RNA was reverse transcribed using a cDNA archive kit (Applied Biosystems, 74322171). Real-time PCR was performed using 7500 Applied

Biosystems instrument, with TaqMan Universal PCR Master Mix (Applied Biosystems, 4304437) and the following TaqMan gene expression assays: *TRAP* (Mm00475698_m1), *MMP-9* (Mm00600163_m1), *cathepsin K* (Mm00484036_ m1), *osterix* (Mm00504574_m1), *collagen- 1a1* (Mm00801666_g1), β-*actin* (Mm00607939_s1), *RANKL* (Mm00441908_m1), *OPG* (Mm01205928 _m1), *Hey1* (Mm00468865_m1), and *Hes1* (Mm 01342805_m1). Real-time PCR for *Runx2*, *Delta1*, *JAG2*, *Cyclin A*, *Cyclin D1*, *p53*, *GAPDH,* and *AMPK* isoforms was performed using SYBR Green Universal PCR Master Mix (Applied Biosystems, 4367659) and the following primers:

Runx2 forward, TGGCTTGGGTTTCAGGTTAG, and reverse,TCGGTTTCTTAGGGTCTTGGA;

Delta1 forward, TTGGGCTTCTCTGGCTTCAAC, and reverse, CCACACACTTGGCACCGTTAG;

JAG2 forward, CAAGTTCTGTGACGAGTGTGTCCC, and reverse, TTGCCCAAGTAGCCATCTGG;

Cyclin A forward, CTGCCTTCCACTTAGCTCTC, and reverse, GAGGTAGGTCTGGTGAAGGT;

Cyclin D1 forward, CAGAAGTGCGAAGAGGAGGTC, and reverse, TCATCTTAGAGGCCACGAACAT;

p53 forward, CACAGCGTGGTGGTACCTTA, and reverse, GCACAAACACGAACCTCAAA;

GAPDH forward, TTCCGTGTTCCTACCCCCAA, and reverse, GATGCCTGCTTCACCACCTT;

PRKaa forward 5'- AGAGGGCCGCAATAAAAGAT -3', and reverse 5'- TGTTGTACAGGCAGCTGAGG -3';

PRKaa2 forward 5'- TGGCTGCCTTCTTATGCTTT -3', and reverse 5'- GCTTTGAAACGGCTTCTCAC -3';

PRKab1 forward 5'- TCCGATGTGTCTGAGCTGTC -3', and reverse 5'- CAGTGCTGGGTCACAAGAGA -3';

PRKab2 forward 5'- GTGATGTGACGTGGAAGTGG -3', and reverse 5'- GCAAGAACTTGGCTTTGAGG -3';

PRKag1 forward 5'- TCGGTCCCACTACTTTGAGG -3', and reverse 5'- GATGTCAGACAGCGAAACGA -3';
PRKag2 forward 5'- GCCTTATGTCCAAACGCAAT -3', and reverse 5'- AGCGCTTAGAGGCATCACAT -3',
PRKag3 forward 5'- CCACGAGAGCCTAGGTGAAG -3' and reverse, 5'- TTCCAAGATCCTTTCGTTGG -3'.

Immunofluorescence and Apoptosis Assay

Cells plated on glass coverslips were fixed with 10% formalin and immunostained as described previously (208). We used monoclonal antibodies for β-catenin (Cell Signalling, 9587), NICD (Santa Cruz Biotechnology, sc-6014), Amyloid Precursor Protein (APP; Sigma A8717) and Amyloid beta (Aβ; Médimabs MM-015-5C-FS). Staining was completed with biotinylated goat anti-mouse IgG (Invitrogen, A10519) and Alexa Fluor 488-conjugated streptavidin (Invitrogen, S11223). Nuclei were counterstained using DAPI dihydrochloride (Invitrogen, D1306). Ten random images/experimental condition were collected in each experiment, each image containing 8–25 precursors. Cell counts were performed by counting DAPI-labeled nuclei. Nuclear fluorescence intensity was evaluated using Volocity software, by first circling DAPI-labeled nuclei and then assessing the average fluorescence of the protein of interest within that area. For evaluation of apoptosis, nuclear morphology was examined and rated positive for apoptosis if it exhibited nuclear condensation and a loss of membrane integrity. The rate of apoptosis was estimated as a proportion of cells demonstrating nuclear fragmentation from the total number of cells analyzed. In addition, the fluorescent tagged annexin-V was used to detect apoptotic cells (Santa Cruz Biotechnology, sc-4252-AK). Live cultures were rinsed with PBS and incubated with the FITC-annexin in the supplied incubation buffer for 15 min at room temperature. Cultures were then fixed in 10% formalin, stained with DAPI, and immediately examined. 182–487 cells/experimental condition were scored.

Immunoblotting

For protein isolation, cells were treated with lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 2mM EDTA, and protease inhibitor mixture. Nuclear extraction was conducted using lysis buffers, first of 10 mM Tris, pH 8, 1.5 mM MgCl2, 5 mM KCl, 0.5 mM DTT, 0.1 M PMSF, 0.5% Nonidet P-40, and second of 20mM Tris, pH 8, 25% glycerol, 1.5 mM MgCl2, 0.5 mM DTT, 0.1 M PMSF, 0.2 mM EDTA, and 0.4 mM NaCl. Immunoblotting was performed as described previously (26) using anti-RANKL (Santa Cruz Biotechnology, sc-52950), anti-NICD (Santa Cruz Biotechnology, sc-6014), Amyloid Precursor Protein (APP; Sigma A8717) p-4E-BP1 (1:1000, 9451, Cell Signalling), 4E-BP1 (1:1000, 9452, Cell Signalling), p-S6K (1:1000, 9234, Cell Signalling), or AMPKγ1 (1:1000, 4187, Cell Signalling) followed by horseradish peroxidase-conjugated secondary antibodies (Jackson Laboratories, 705-065-003) and chemiluminescent substrate (Supersignal West Pico; Pierce, 34080). Blots were reprobed with β-tubulin antibody (Sigma- Aldrich, T9026) as a loading control. For mTOR blotting, mTOR antibody (1:200, 2972, Cell Signalling) was used and the lysis buffer contained 0.3% CHAPS instead of 1% triton to preserve the integrity of the mTOR complexes.

Cell Attachment Assay

Bone marrow cultures were treated as indicated for 9 days. MDA-MB-231 cells were loaded with Cell Tracker Green (5 μM; Invitrogen, C2925) in serum-free DMEM for 1 h, washed, incubated in serum-free DMEM for an additional 1 h, washed, trypsinized, centrifuged, resuspended in serum-free DMEM at a cell density of 4 x 10^4/ml, and applied to bone cell cultures. After 40 min of incubation, cultures were washed three times with serum-free DMEM and fixed with 10% formalin for 10 min before imaging.

Mitochondrial activity

RAW 264.7 cells were plated on 10 mm diameter glass coverslips. Mitochondrial activity was assessed using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; CBIC2(3), Invitrogen™, T-

3168) as described previously (209). In each experiment, images of at least 9 fields were collected, the background fluorescence in red and green channel was subtracted from the images, then all positively stained particles were selected and filtered to remove unrelated very small and very large items, and the ratio of the average fluorescence intensity of the red channel to the average fluorescence intensity of the green channel was identified.

Measurements of lactate, pH, ATP

C57 mouse bone marrow cells were plated in 6-well plates and supplemented with fresh medium containing indicated additions on day 1 and 3. On day 3 and 5, pH of the medium was measured with an electronic pH meter (Accumet® Basic AB15, Fisher scientific). On day 5, cultures were extracted in 1% Triton X-100 in HEPES buffer, pH 7.4, and then sonicated and centrifuged at 10,000 *g* for 3 min. Supernatants were stored at −80°C until ATP analysis was performed. Supernatants of samples were processed for lactate determination using EnzyChromTM L-lactate assay kit (BioAssay Systems, ECLC-100) and microplate reader (Tecan's Infinite® F200, Tecan US Inc). ATP levels were measured by the luciferin-luciferase method using an ATP Determination Kit (Invitrogen™, A22066) and a luminometer (Femtomaster FB12, Zylux Corp.). ATP concentrations were normalized to the protein content measured using a Quant-iT™ protein assay kit (Invitrogen).

Resorption Assay

Dentin pit resorption assay by bone marrow-derived osteoclasts was performed as described previously (210). For calcium phosphate resoroption assay, differentiated RAW 264.7 osteoclasts cells were replated on mineral-coated Osteoassay Plates (Corning Inc. 3988) as described above in *RAW 264.7 monocyte cell culture*, and grown with the indicated additions for another 5 days. At the end of culture, plates were washed with diluted bleach to remove cells, and the plates were air-dried. Resorption pits were counted and planar area of each pit

was measured by using a Nikon ECLIPSE TS100 microscope coupled to a Nikon ELWD 0.3 T1-SNCP camera and PixeLINK Capture SE image-analysis software.

Confocal microscopy

Osteoclasts were generated from RAW 264.7 cells on glass coverslips. Fixed cells were incubated with the fluorescent lipophilic membrane probe DiI (5 µl/ml, Vybrant® DiI, Invitrogen™, V-22885), Alexa 488-conjugated Phalloidin (Invitrogen A12379), and DAPI stain (Invitrogen D1306), and visualized with confocal microscope (LSM510, Carl Zeiss Inc). Images of at least 20 fields/condition were used to evaluate osteoclast height.

Statistical Analysis

Data are presented as representative images, representative experiments, as means ± S.E.M., with *n* indicating the number of independent experiments, or as means ± S.D., with *n* indicating the number of replicates. Differences were assessed by Student's *t* test and accepted as statistically significant at $p < 0.05$.

Chapter 4

Breast cancer cells affect the differentiation of bone cells

Introduction

Bone is one of the most common sites for distant metastases from breast cancer (40). Once bone metastases have occurred, they cannot be cured and the patient five-year survival rate falls from 95% to 20% (211). Bone metastasis is associated with significant morbidity due to the disruption of bone architecture and mineral homeostasis, which leads to hypercalcemia, pathological fractures and considerable pain burden.

In order to home to and grow in the bone, cancer cells need to establish successful interactions with the bone microenvironment. Bone is a dynamic tissue that provides support and protection for organs, and maintains body mineral homeostasis. Bone is constantly remodeled by a coordinated action of specialized bone cells: osteoclasts that destroy bone and osteoblasts that build bone (212). Osteoclasts are cells of hematopoietic origin that resorb bone by lowering the extracellular pH to dissolve hydroxyapatite crystals and release proteolytic enzymes, such as cathepsin K and matrix metalloproteinase-9 (MMP-9), to digest the organic matrix (2). Osteoblasts are derived from mesenchymal stem cells and secrete the extracellular matrix which later mineralizes to form bone. Major pathways controlling osteoblast differentiation include Wnt/β-catenin, Notch and TGFβ signalling (76,150,213). The formation of osteoclasts is regulated by cells of osteoblastic lineage, which produce the pro-resorptive cytokine, RANKL, as well as its negative regulator, soluble decoy receptor osteoprotegerin (OPG).

It has previously been shown that breast cancer cells inhibit osteoblast differentiation and induce osteoblast apoptosis (149,157,214). Osteoblasts are central for the osteolytic effects of breast cancer cells, which do not secrete RANKL themselves (215), but produce factors such as PTHrP (216) that stimulate osteoblasts to produce RANKL, while inhibiting production of OPG (217-219). In turn, RANKL stimulates osteoclast formation often leading to

catastrophic bone destruction (220,221). In addition, breast cancer-derived factors were previously shown to directly induce osteoclastogenesis from late osteoclast precursors (3,173). During bone resorption, growth factors trapped in the bone matrix, such as TGFβ and IGF, are released and act back on the tumour cells to stimulate their growth (222,223). Several cytokines have been implicated in the progression of cancer metastasis, with TGFβ (224) and Wnt signalling inhibitor DKK-1 (156) being of considerable importance in the metastatic process. In addition, Notch signalling, has been implicated in the control of hematopoietic stem cell niche (225,226), as well as in cancer development (227), suggesting that it can also play a role in formation of pre-metastatic niche. Notch signalling is initiated by ligand binding, which induces γ -secretase-mediated release of the Notch intracellular domain (NICD), which translocates to the cell nucleus and alters gene expression (227).

In this chapter, I describe the studies examining the effect of breast carcinoma cells on the bone marrow cultures that retain potential for differentiation into both osteoblasts and osteoclasts, thus better representing the complex bone microenvironment. The objective of this part of the study was to identify how soluble factors released by breast cancer cells may affect the differentiation of bone cells.

Results

Breast cancer cells inhibit differentiation of osteoblasts and stimulate differentiation of osteoclasts.

We examined the effects of soluble factors produced by human metastatic breast cancer cells MDA-MB-231, human breast cancer cells MCF7, or mouse metastatic breast cancer cells 4T1 on the differentiation of osteoblasts and osteoclasts from precursors derived from mouse bone marrow. Bone marrow cells were treated with AA in the presence or absence of medium conditioned by MDA-MB-231 cells, MCF7 cells, or 4T1 cells for 9 days, and examined for the expression of osteoblast differentiation marker ALP (Fig 4.1A, top) and osteoclast

differentiation marker TRAP (Fig 4.1A, bottom). Medium conditioned by the MCF10a human breast epithelial cell line and MC3T3 mouse pre-osteoblastic cell line were used as controls. Cultures treated with ascorbic acid (AA) displayed robust ALP staining in osteoblastic nodules. In contrast, in cultures treated with AA in the presence of MDA-MB-231, 4T1 or MCF7 CM, the ALP-positive area was significantly reduced (Fig. 4.1B, left), and the staining exhibited punctuated pattern localized in small clusters (Fig. 4.1A, Fig. 4.2A). Cultures treated with AA alone rarely contained visible osteoclasts. In contrast, treatment with MDA-MB-231 CM, 4T1 or MCF7 CM induced the formation of 4-9 large multinucleated osteoclasts/cm^2. Since the numbers of visible osteoclasts were relatively low, we examined changes in area covered by TRAP-positive cells (Fig. 4.1A, B right) and found a significant increase in TRAP-positive area in MDA-MB-231 CM-treated cultures. Close investigation confirmed the identity of large, multinucleated TRAP-positive osteoclasts, typically located under a layer of osteoblastic cells (Figure 4.3A). Both the inhibition of osteoblast differentiation and stimulation of osteoclast formation by MDA-MB-231 CM were sustained over 6-15 days of culture (Fig. 4.2B, 4.3B). Addition of MCF10a or MC3T3 CM did not affect osteoblast or osteoclast differentiation (Fig. 4.1B).

We next assessed if the functional activity of osteoblasts and osteoclasts reflects their observed differentiation status. When AA-treated cultures were provided with a source of inorganic phosphate to induce mineralization, they developed mineralized nodules easily identified by Von Kossa staining (Fig. 4.2C, left). Addition of MDA-MB-231 CM to these cultures prevented mineralization (Fig. 4.2C, right) and induced significant and sustained decrease in expression of osteoblast differentiation markers, Collagen-1, Osterix, and Runx2 (Fig. 4.2D), confirming the inhibition of osteoblast differentiation by soluble factors produced by breast cancer cells. It is conceivable that the effect of breast cancer cells on osteoblast may be due to induction of cell death rather than inhibition of differentiation. We have found that the average cell density was not significantly different in cultures treated with AA alone or a combination of AA and MDA-

MB-231 CM (Fig. 4.2E). Nevertheless, cultures treated with AA and MDA-MB-231 CM exhibited a significant increase in nuclear fragmentation and loss of membrane integrity compared to cells treated with AA alone (Fig 4.2F). In addition, cultures treated with MDA-MB-231 CM demonstrated an increase in the number of cells positive for early apoptosis marker, annexin-5 (from 6 ± 4% in control cultures, to 14 ± 2% in MDA-MB-231 CM-treated cultures, n = 2 independent experiments). Cell proliferation, as assessed using a BrdU incorporation assay, demonstrated a trend towards higher proliferation in cultures treated with AA and MDA-MB-231 CM compared to those treated with AA alone (Fig 4.2G, left). To confirm this, we analyzed the gene expression of cell proliferation markers Cyclin A, Cyclin D1 and p53 and found that treatment with 4T1 CM led to significant increases in each of their expression (Fig. 4.2G, right). Thus, although MDA-MB-231 CM induced higher apoptosis rates in bone cells, proliferation was increased and the overall result was only a relatively small change in the total numbers of cells in culture.

To assess osteoclast functional activity, bone marrow cells were plated on dentin slices and treated for 9 days with either AA alone or a combination of AA and MDA-MB-231 CM. Whereas cultures treated with AA alone did not exhibit osteoclastic resorption (Fig. 4.3C, left), numerous resorption pits were identified in cultures treated with AA and MDA-MB-231 CM (Fig. 4.3C, right). We quantified the gene expression of osteoclast differentiation markers cathepsin K, TRAP and MMP9 by real-time PCR. Treatment with MDA-MB-231 CM induced significant and marked increase in expression of TRAP and especially MMP9, whereas expression of cathepsin K was increased but did not reach a change of statistical significance (Fig. 4.3D).

It has been studied that different tumors may produce some amounts of RANKL (228), however it is known that the MDA-MB-231 cell line does not (229). Since the cultures were not treated with exogenous osteoclastogenic factors, we assessed if breast cancer-derived factors affect the expression of RANKL and

OPG by osteoblasts. We have found that MDA-MB-231 CM induced a 3-fold increase in RANKL expression level and a 30% decrease in OPG expression level (Fig. 4.4A). Immunoblotting further confirmed a significant increase in the protein levels of RANKL in MDA-MB-231 CM-treated cultures (Fig. 4.4B).

The roles of Notch and Wnt signalling in the inhibition of osteoblast differentiation have been firmly established (213,230), and the TGFβ, Wnt and Notch signalling pathways have been shown to be affected by breast cancer cells. Soluble factors released by breast cancer cells create aberrant signalling through the TGFβ pathway (178), inhibit Wnt singaling (156) and stimulate Notch signalling (231). We assessed the roles of these pathways in osteoblastic cultures exposed to breast cancer-derived factors by using two modulators of the TGFβ signalling pathway, a glycogen synthase kinase (GSK3β) inhibitor and lithium chloride (LiCl) to reinstate Wnt signalling and two pharmacological inhibitors of Notch signalling. We treated bone marrow cells for 9 days with AA alone or with AA and MDA-MB-231 CM (10%) together with anti-TβR1, anti-TGFβ, GSK inhibitors LiCl or SB216763, or γ-secretase inhibitors DAPT or CE, and examined the expression of osteoblast marker ALP and osteoclast marker TRAP. Antagonizing the TGFβ pathway, which was previously suggested as a mediator of antiosteoblastic effects of breast cancer cells (149), was ineffective in preventing MDA-MB-231 CM-induced osteoblast inhibition (Fig. 4.5A). We next evaluated whether blocking osteoclast formation could reinstate osteogenesis. The addition of exogenous OPG blocked osteoclast formation but further stimulated the inhibition of osteoblast differentiation (Fig. 4.5B). To reinstate Wnt signalling, GSK inhibitors were applied but were unable to rescue osteoblast differentiation (Fig. 4.5C). Moreover, treatment with SB216763 induced an additional increase in osteoclast numbers (Fig. 4.5F). Although inhibitors of γ-secretase partially rescued MDA-MB-231 CM-induced osteoblast inhibition (Fig. 4.5D), their effect was relatively minor, and it was not observed in cultures treated with 4T1 and MCF7 CM (Fig. 4.5E). However, both γ-secretase inhibitors completely

prevented the stimulation of osteoclast formation by breast cancer-derived factors (Fig. 4.5F).

Because Notch inhibition was able to partially rescue MDA-MB-231 CM-affected osteoblast differentiation and fully rescue osteoclast differentiation, we assessed the status of this signalling pathway in osteoblastic cultures exposed to breast cancer-derived factors. Localization of the cleaved NICD was examined by immunofluorescence. Cultures treated only with AA exhibited little nuclear staining (Fig. 4.6A, left), whereas treatment with MDA-MB-231 CM resulted in the appearance of nuclear staining of NICD (Fig. 4.6A, middle). Quantification of the intensity of nuclear staining for NICD demonstrated significant increase in nuclear localization of the NICD in MDA-MB-231 CM-treated cultures compared to cultures treated with AA alone (Fig. 4.6A, right). The increase in nuclear NICD was confirmed by immunoblotting (Fig. 4.6B). NICD's direct transcriptional targets Hey-1 and Hes-1, and Notch ligand Jag-2 exhibited a trend towards higher expression in cultures treated with breast cancer derived factors, whereas Notch ligand Delta-1 was significantly higher expressed in cultures treated with breast cancer derived factors (Fig. 4.6C). Wnt and Notch signalling are known to cross-talk; therefore we assessed the activation of β-catenin using immunofluorescence. MDA-MB-231 CM-treated cultures exhibited significantly less nuclear intensity for β-catenin compared to control cultures (Fig. 4.6D), confirming some inhibition of Wnt signalling was due to breast cancer-derived factors.

Discussion

This study demonstrates that soluble factors produced by breast cancer cells inhibit osteoblast differentiation while stimulating osteoclast differentiation. We have identified γ-secretase as a critical mediator of breast cancer-induced stimulation of osteoclastogenesis and a potential partial mediator of breast cancer-induced inhibition of osteoblastogenesis. Pharmacological inhibition of γ-secretase thus provides a potential therapeutic target to reduce the progression of cancer metastases to bone.

Because of the usually osteolytic nature of breast cancer metastases in bone, the effects of breast cancer cells on osteoclasts have been studied extensively; however, much less attention has been given to the interactions of breast cancer cells with osteoblasts. Using osteoblastic cell lines, primary calvarial osteoblasts, or co-cultures of separately isolated osteoblastic and osteoclastic cells, it has been shown that breast cancer cells inhibit osteoblast differentiation (149,156,166), induce osteoblast apoptosis (149,157), and increase the production of pro-osteoclastic factors by osteoblasts (218,219). To model the cell types and interactions potentially encountered by tumour cells in the bone microenvironment more accurately, we have developed a unique culture system that allows for monitoring the differentiation of osteoblasts and the osteoblast-dependent differentiation of osteoclasts directly from bone marrow cells. This model has allowed us to investigate complex interactions among osteoblasts, osteoclasts, and cancer cells. We have shown that although breast cancer cells induce osteoblast apoptosis, this effect may be countered by cell proliferation, resulting in the maintenance of a sufficient pool of osteoblastic cells acting as a source of proresorptive factors. We have confirmed that in the presence of breast cancer cells, immature osteoblasts up-regulated the production of RANKL and down-regulated the production of OPG. This can be due to the direct effect of breast cancer cells on osteoblasts, or it can be a consequence of the osteoblast differentiation status since it has previously been shown that immature osteoblasts produce more RANKL and less OPG compared with more mature cells (72,232-234).

To assess the potential mediators of the effect of breast cancer cells on osteoblast and osteoclast precursors, we considered the involvement of pathways known to affect osteoblast and osteoclast differentiation, including Wnt, TGFβ, and Notch. Wnt signalling is an essential pathway in osteogenesis, which has been shown to play a critical role in myeloma bone metastases (153) and was shown to be altered in the breast cancer bone metastases model (156). TGFβ is well known to play an

important role in promoting tumour progression specifically in models of MDA-MB-231 breast cancer metastasis (167,235-237) and has been suggested to mediate the inhibitory effects of breast cancer cells on osteoblasts (149). Notch signalling has recently been identified as a key mediator of bone formation (150) and a mediator of osteosarcoma (226). Notch signalling is also implicated in the regulation of osteoclastogenesis (238,239). We have shown that treatments normalizing Wnt signalling or antagonizing TGFβ signalling did not interfere with the effects produced by the breast cancer-derived factors on bone cells. In contrast, inhibition of γ-secretase, a key enzyme mediating Notch signalling, resulted in the full reversal of breast cancer-induced osteoclastogenesis. Although the inhibition of Notch signalling also resulted in a partial rescue of MDA-MB-231-induced inhibition of osteoblast differentiation, this effect was relatively minor and not reproduced when different breast cancer cells, MCF7 and 4T1, were employed. The ineffectiveness of inhibitors of γ-secretase in fully rescuing the osteoblastic phenotype suggests that RANKL/OPG expression by osteoblasts may be regulated independently from differentiation. These data may also suggest an important role for osteoblast-independent interactions, such as the direct effects of breast cancer cells on osteoclast differentiation (3,173).

Notch signalling has been previously demonstrated to play important roles in breast cancer progression. Approximately half of breast cancers express low levels of Notch inhibitor Numb (240), which when highly produced, can suppress breast cancer (241). High level co-expression of Notch ligand Jagged-1 and receptor NOTCH1 have been shown to correlate with poor survival in breast cancer patients (242), making Notch signalling a potentially important target in the progression of breast cancer. Gamma secretase inhibitors block Notch signalling and have been successful in reducing the breast cancer stem-like cell population (243), triggering apoptosis in breast cancer cells (244), and increasing sensitivity to chemotherapeutic agents (245). For the first time, we have shown that these agents may also protect the bone from the osteolytic effects of breast cancer cells.

Alongside this study, the laboratory of Dr. Peter Siegel has identified matricellular protein CCN3 as a novel factor that is highly expressed in both mouse and human bone-metastatic breast cancer cells. CCN3 overexpression enhances the ability of weakly bone metastatic breast cancer cells to colonize and grow in the bone. Since CCN3 has been shown to interact with Notch signalling (246), we have assessed the role of CCN3 in mediating the differentiation of both osteoblasts and osteoclasts. Human recombinant rCCN3 impaired AA-induced osteoblast differentiation in primary bone marrow concentrations, with a 19% decrease at 300 ng/mL and 44% decrease at 600 ng/mL (247). We next evaluated whether CCN3 could alter the RANKL/OPG ratio to favour osteoclastogenesis. In AA-treated bone marrow cultures, the addition of 600 ng/mL of CCN3 was able to stimulate RANKL and inhibit OPG, similar to the effects of a complete breast cancer CM on these factors. Thus, CCN3 was identified to be an important factor derived from breast cancer cells that contributes to bone metastases, and a strong candidate for future inhibition studies and potentially targeted therapeutics.

Our study suggests the critical role of γ-secretase in the establishment of successful interactions between breast cancer cells and bone cells, which are potentially mediated by CCN3. Complete reversal of a key prometastatic event, cancer-induced enhancement of osteoclastogenesis, by the inhibition of γ-secretase provides evidence for the use of γ-secretase inhibitors to treat the progression of cancer metastases to bone.

The data presented in this chapter have been published as part of the following manuscripts:

Fong JE, Le Nihouannen D, Komarova SV. Tumour-supportive and osteoclastogenic changes induced by breast cancer-derived factors are reversed by inhibition of {gamma}-secretase. *J Biol Chem.* 2010 Oct 8;285(41):31427-34.

Ouellet V, Tiedemann K, Mourskaia A, **Fong JE**, Tran-Thanh D, Amir E, Clemons M, Perbal B, Komarova SV, Siegel PM. CCN3 Impairs Osteoblast and Stimulates Osteoclast Differentiation to Favor Breast Cancer Metastasis to Bone. *Am J Pathol.* 2011 May;178(5):2377-88.

FIGURE 4.1

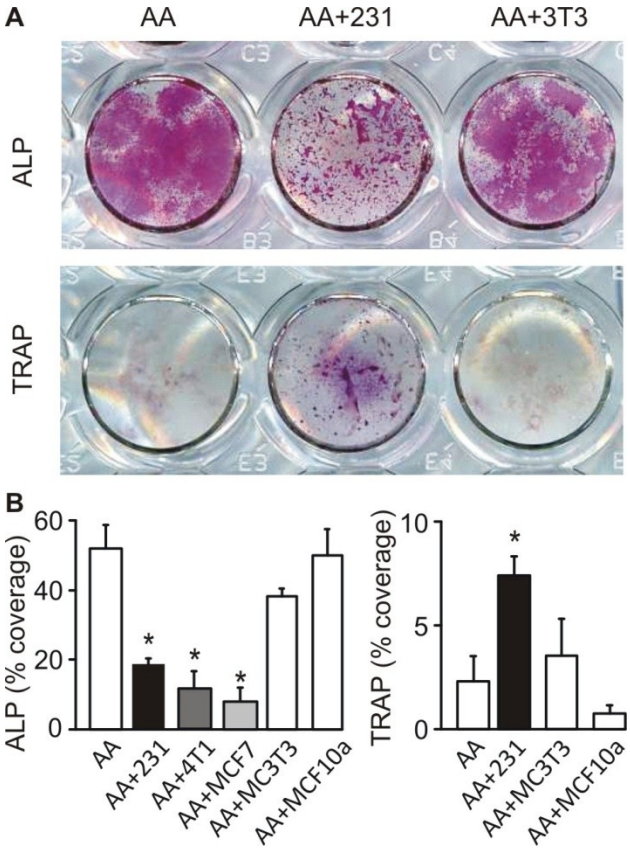

Figure 4.1: Breast cancer cells inhibit osteoblasts and stimulate osteoclasts. Mouse bone marrow cells were grown for 3–15 days with AA (50 μg/ml) without additions (open bars) or in the presence of MDA-MB-231, 4T1, or MCF7 CM (10%, shaded bars) or controls MC3T3-E1 CM (10%) and MCF10A CM (10%). A, representative images of cultures treated with AA only (AA, left), with AA and MDA-MB-231 CM (AA+231, center), or with AA and MC3T3-E1 CM (AA+3T3, right), fixed on day 6–9, and stained for ALP (red, upper) or TRAP (purple, lower). Scanned are the wells of a 24-well plate. B, average area covered on day 9 by ALP-positive cells (left) and on day 6 by TRAP-positive cells (right). Treatment with MDA-MB-231, 4T1, or MCF7 CM significantly reduced ALP-positive osteoblast staining (left). Treatment with MDA-MB-231 CM significantly increased TRAP-positive osteoclast staining (right).

Supplementation of cultures with AA and conditioned medium from MC3T3 or MCF10A did not produce significantly different results from treatment with AA alone. Data are means ± S.E. (error bars), n = 2–6 independent experiments, $p < 0.05$.

FIGURE 4.2

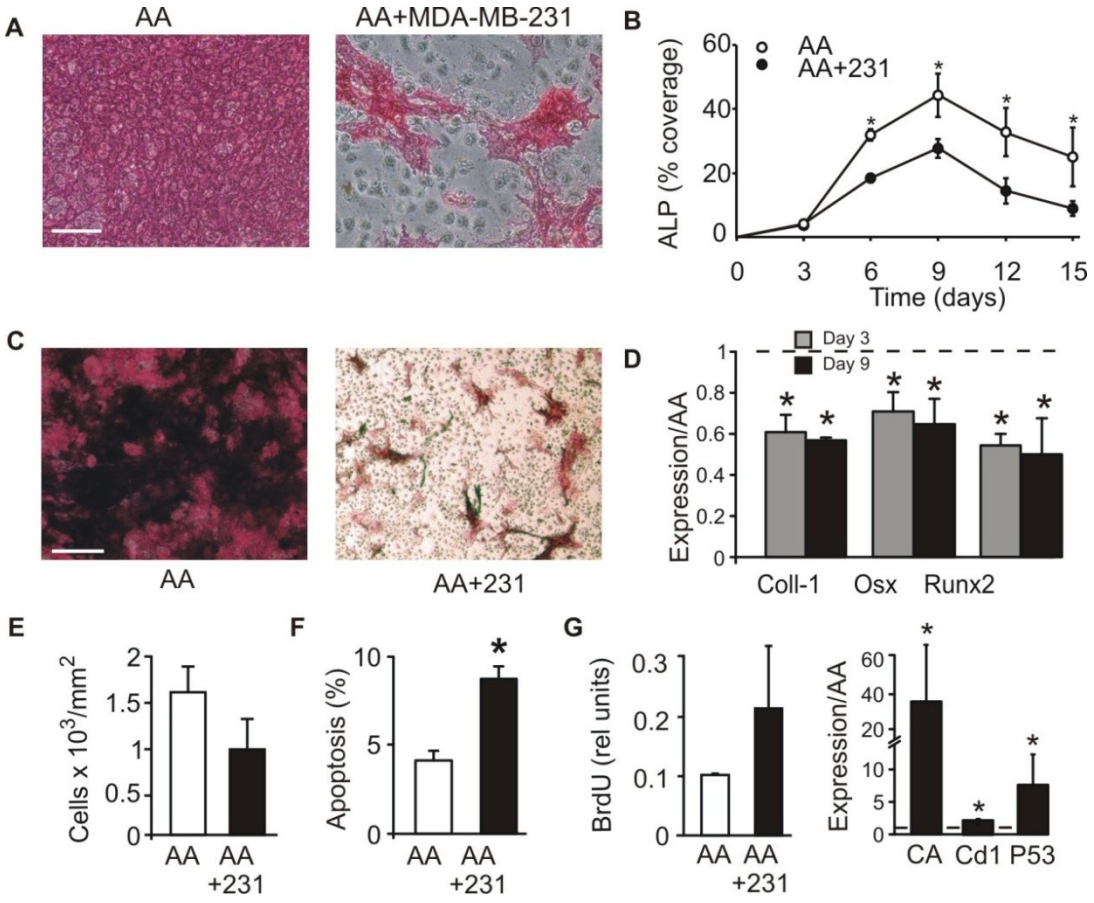

Figure 4.2: Breast cancer cells maintain osteoblasts in an immature, proliferative state.

Mouse bone marrow cells were grown for 3-15 days with AA (50 nM) without additions or in the presence of MDA-MB-231 CM (10%), fixed at different times and stained for ALP (red). A, Representative images of osteoblastic cells in cultures treated with AA only (AA, left), or with AA and MDA-MB-231 CM (AA+231, right). Scale bar is 20 μm. B, Average area covered by ALP-positive cells is significantly reduced in MDA-MB-231 CM-treated cultures at day 6-15. Data are means ± SEM, n = 4 independent experiments, $p < 0.05$. *C*, Bone marrow cells were grown for 12 days with AA (50 μg/ml) and β-glycerophosphate (10 mm) in the absence (*left*) or presence of MDA-MB-231 CM

(10%, *right*). The cultures were fixed and stained for ALP (*red*) and mineralized deposits (*black*). *Scale bar* is 100 μm. D, Bone marrow cells were grown for 3–9 days with AA (50 μg/ml) in the absence or presence of MDA-MB-231 CM (10%, *left*) or 4T1 CM (10%, *right*). Expression of *Collagen-1* (*Coll-1*), *osterix* (*Osx*), and *Runx2* was analyzed on day 3 (*gray*) or 9 (*black*). Data are means ± S.E. (*error bars*), normalized to expression of β-*actin* (*left*) or *GAPDH* (*right*), and presented relative to levels observed in AA only samples (*dashed line*), n = 3–5 independent experiments, $p < 0.05$. *E*, bone marrow cells were grown for 9 days with AA (50 μg/ml) in the absence or presence of MDA-MB-231 CM (10%). The parallel samples were fixed, stained with DAPI nuclear stain, and the cell density was estimated (*left*). F, The rate of apoptosis was estimated as a proportion of cells demonstrating nuclear fragmentation from the total number of cells analyzed (*center*). G, Cell proliferation was measured by BrdU incorporation (*left*). Expression of *Cyclin A* (*CA*), *Cyclin D1* (*CD1*), and *p53* was analyzed on day 9 (right). Data are means ± S.E., n = 3–5 independent experiments, $p < 0.05$.

FIGURE 4.3

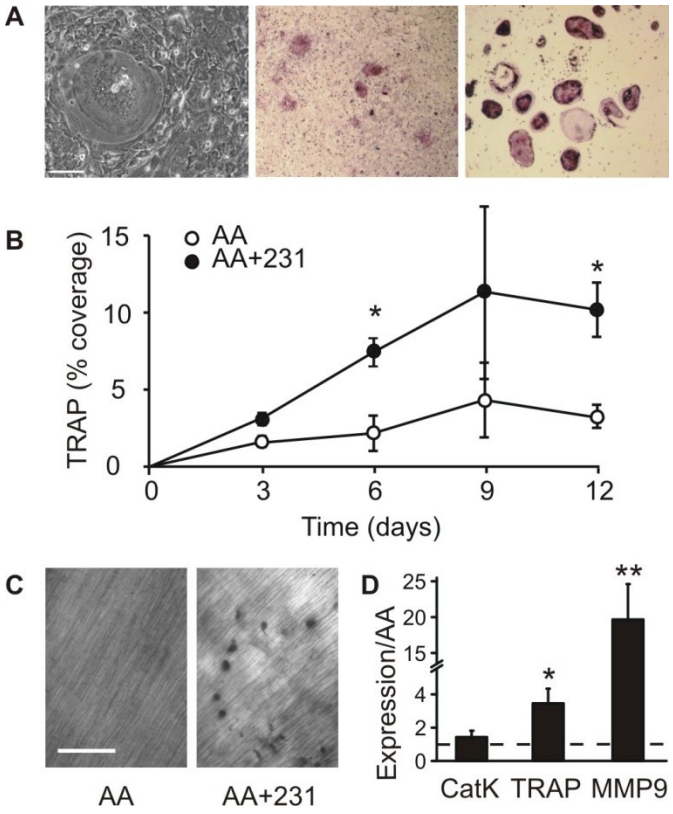

Figure 4.3: Breast cancer cells induce the differentiation of functional osteoclasts.

Mouse bone marrow cells were grown for 3-15 days with AA (50 nM) without additions or in the presence of MDA-MB-231 CM (10%), fixed at different times and stained for TRAP (purple). A, Representative images demonstrating an osteoclast that breached through a layer of osteoblasts (left), TRAP-positive condensations, which are likely osteoclasts growing under a monolayer of osteoblasts (middle), and multinucleated, TRAP-positive osteoclasts evident after the monolayer of osteoblasts has been lifted (right). Scale bar is 20 μm. B, Average area covered by TRAP-positive cells is significantly increased in MDA-MB-231 CM-treated cultures at day 9-12. Data are means ± SEM, n = 4 independent experiments, $p < 0.05$. C, bone marrow cells were grown for 9 days on dentin slices with AA (50 μg/ml) in the absence (left) or presence of MDA-

MB-231 CM (10%, right), then the cells were removed, and dentin was stained with toluidine blue to reveal resorption pits. Scale bars represent 100 μm. D, expression of Cathepsin K (Cat K), TRAP, and MMP-9 were analyzed on day 9. Data are means ± S.E., normalized to expression of β-actin, and presented relative to levels observed in AA only samples (dashed line), n = 4–6 independent experiments, $p < 0.05$.

FIGURE 4.4

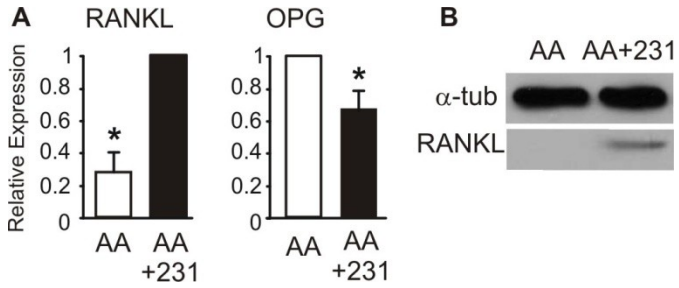

Figure 4.4: Breast cancer cells induce osteoclastogenic change in RANKL/OPG expression.

Bone marrow cells were grown for 9 days with AA (50 μg/ml) in the absence (AA, open bars) or presence of MDA-MB-231 CM (10%, AA+231, filled bars). A, expression of RANKL and OPG normalized to expression of β-actin and presented relative to levels observed in cells grown with AA+231 for RANKL and AA only for OPG. Data are means ± S.E. (error bars), n = 5 independent experiments, $p < 0.05$. B, RANKL protein level assessed by immunoblotting in whole cell lysates. Shown is a representative immunoblot with α-tubulin as a loading control.

FIGURE 4.5

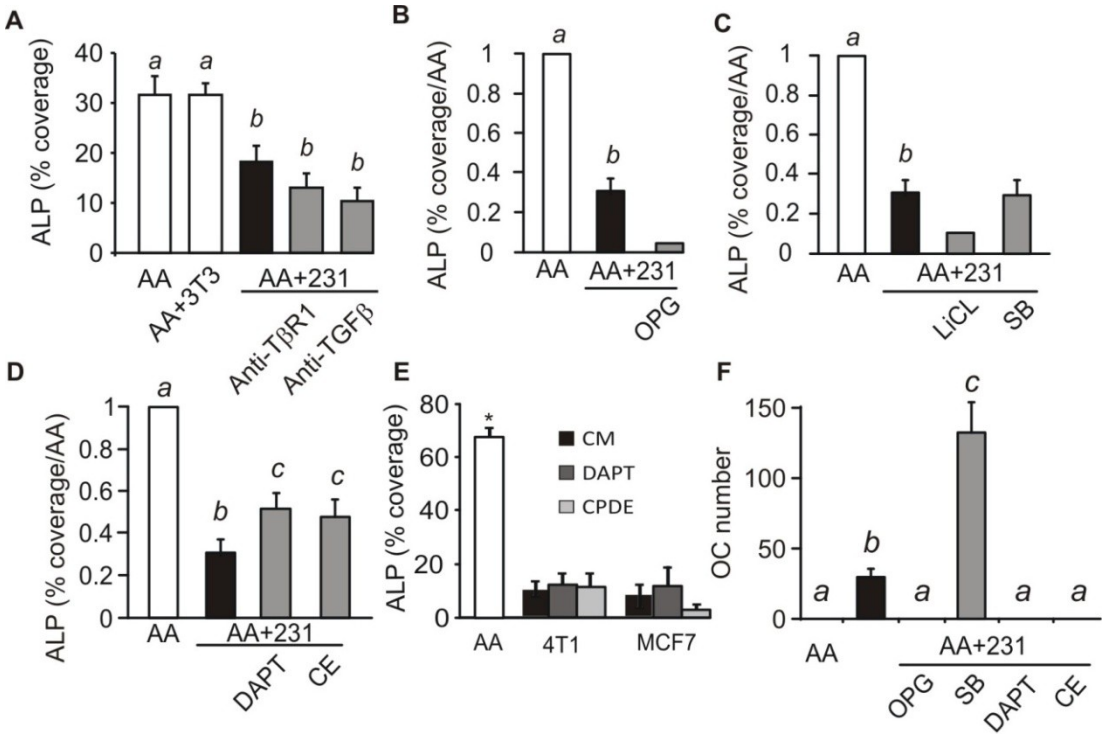

Figure 4.5: Normalization of TGFβ or Wnt signalling, and impaired osteoclastogenesis does not rescue the osteoblast-inhibitory effects of breast cancer cells, but normalization of Notch signalling rescues osteoclastogenic stimulation.

A, Mouse bone marrow cells were grown for 6 days with AA (50 nM) in the absence (AA, open bars) or presence of MDA-MB-231 CM (10%), combined with vehicle (AA+231, black bars), TGFβ neutralizing antibody (anti-TGFβ, 50 nM), or TGFβ type I receptor inhibitor (anti-TβRI, SB431542, 10 μM). The parallel samples were fixed, stained for ALP and the area covered by ALP-positive cells was assessed. Data are means ± SEM, n = 5 independent experiments, different letters indicate significant difference at $p < 0.05$. B-D, bone marrow cells were grown for 9 days with AA (50 μg/ml), MDA-MB-231 CM (10%), and the following inhibitors (gray bars): B, OPG (500 ng/ml), C, LiCl (10 mM), SB216763 (SB, 10 μM), D, DAPT (100 nM), or CE (100 nM). The parallel

samples were fixed and stained for ALP or TRAP. Area covered by ALP-positive cells was normalized to the samples grown with AA only. E, Mouse bone marrow cells were grown for 6 days with AA (50 nM) in the absence (AA, open bars) or presence of 4T1 or MCF7 CM (10%, black bars), combined with DAPT (100 nM, dark gray bars), or Compound E (CE, 100 nM; light gray bars). The parallel samples were fixed, stained for ALP and the area covered by ALP-positive cells was assessed. Data are means ± SEM, n = 3-5 independent experiments, asterisk indicates significant difference at $p < 0.05$. F, number of TRAP-positive osteoclastic cells was counted in the same experiments.

FIGURE 4.6

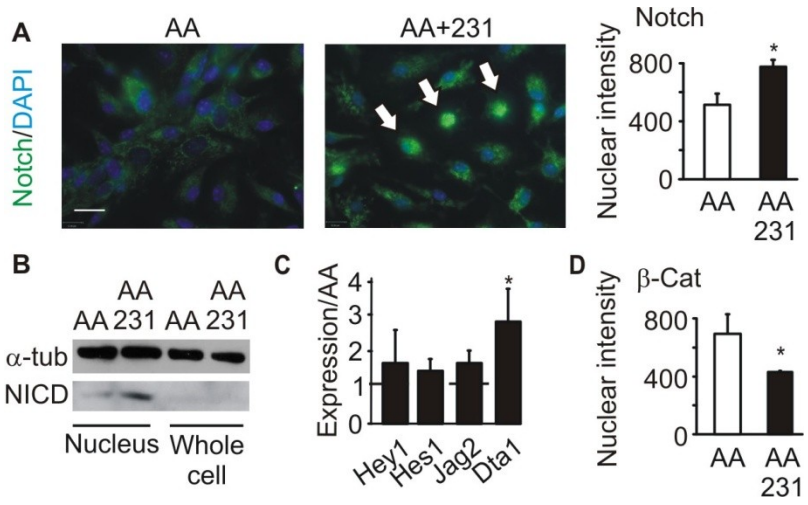

Figure 4.6: The Notch signalling pathway is stimulated in bone cells by breast cancer-derived factors.

Bone marrow cells were grown for 9 days with AA (50 μg/ml) in the absence (AA, open bars) or presence of MDA-MB-231 CM (10%, AA+231, black bars). A, NICD localization was assessed by immunofluorescence (green), and nuclei were stained using DAPI (blue). Left and center, representative images of negative (left) and positive (center) nuclear staining for NICD are shown. Scale bar is 20 μm. Right, nuclear intensity of NICD is quantified. Data are means ± S.E. (error bars), n = 3 independent experiments, $p < 0.05$. B, NICD level was assessed by immunoblotting in nuclear extracts and whole cell lysates. Shown is a representative immunoblot with α-tubulin as a loading control. C, expression of the transcriptional targets of the NICD, Hey-1 and Hes-1, and Notch ligands Jag-2 and Delta1 (Dta1) was analyzed on day 9. Data are means ± S.E., normalized to expression of β-actin for Hey-1 and Hes-1 or GAPDH for Jag-2 and Delta1 and presented relative to levels observed in cells grown with AA only (dashed line), n = 3 independent experiments. D, nuclear intensity of β-catenin (β-Cat) is shown. Data are means ± S.E., n = 3 independent experiments, $p < 0.05$.

Chapter 5

Role of bone cells in supporting the homing of breast cancer cells to bone

Introduction

In the previous chapter, we have identified that breast cancer cells inhibit osteoblast differentiation and stimulate osteoclast differentiation to favour an osteolytic environment where growth factors released from the bone matrix can feed back to cancer cells to promote their growth and survival. In addition, osteoblast maturation was shown to be inhibited, resulting in the production of large amounts of RANKL by immature osteoblasts, which were unable to mineralize a matrix. These changes in bone cells are produced by soluble factors secreted by breast cancer cells, and thus may occur prior to the arrival of breast cancer cells to bone. Therefore, we next examined if the exposure of bone cells to breast cancer-derived factors may affect their subsequent direct interactions with breast cancer cells themselves.

The presence of a developing tumour has been suggested to alter the microenvironment of distant sites even before the tumour cells arrive, thus forming a "pre-metastatic niche" that facilitates homing of tumour cells and development of metastatic lesions (6,7,248). It has been shown that in mice injected with medium conditioned by tumour cells of a different origin, the potential of subsequently injected cancer cells to home to different organs of can be altered (7). Breast cancer cells express receptors that direct their movement towards fertile sites where they may establish into secondary tumours. In addition to directional migration, chemokines have been shown to promote cancer cell survival, proliferation, and adhesion (109). Interactions between stromal-derived factor-1 (SDF-1) and its receptor CXCR4, for example, are essential for the correct localization of haematopoietic cells in physiological states. With regard to the bone, tumour cells have been suggested to simulate the behavior of

hematopoietic stem cells (249), allowing them to harvest resources from the hematopoietic stem cell niche to establish neoplasms (250).

Cancer cells can also express or induce the expression of adhesion molecules that may facilitate their interactions with the bone microenvironment. In the previous chapter, we identified the role of γ-secretase in rescuing the osteoclastogenic effects of breast cancer cells on bone cell differentiation. Besides Notch, γ-secretase may also act on amyloid precursor protein (APP), a membrane protein best known for its cleavage into amyloid beta, a component of the plaques found in Alzheimer's disease patients (251). A mouse model of Alzheimer's disease called Tg2576, containing a human APP695 transgene with the double mutation K670N/M671L, shows decreased bone volume, surface area and thickness compared to wildtype (252). Further analyzed, this same study shows that osteoprogenitors express APP and γ-secretase, and secrete Aβ, and will adhere to amyloid plaques on bone matrix in vitro. Therefore, in addition to known osteoblast differentiation initiator Wnt, we examined both Notch and Aβ in the interaction of breast cancer cells with bone cells (Figure 5.3).

The objective of this part of the study was to assess if exposure to breast cancer factors, potentially arriving from a distant primary site, may affect subsequent direct interactions between bone cells and breast cancer cells.

Results

Because bone cells have been shown to be critical players in mediating stem cell attachment to the hematopoietic bone marrow niche (50,165,253), we investigated how exposure to soluble factors produced by breast cancer cells may affect the direct interaction of breast cancer cells with osteoblasts. Bone marrow cultures were treated for 9 days with AA alone or with a combination of AA and MDA-MB-231 CM, and then MDA-MB-231 cells labelled with Cell Tracker Green were incubated for 40 min on top of bone cells. Although some breast cancer cells adhered to mature osteoblasts in cultures treated with AA alone (Fig. 5.1A, left),

markedly more breast cancer cells attached to immature osteoblast precursors in AA and MDA-MB-231 CM-treated cultures (Fig. 5.1A, right). Quantification confirmed that the treatment with CM from MDA-MB-231, 4T1 or MCF7 cells significantly increased subsequent attachment of breast cancer cell to bone cells, while treatment with CM of normal breast cells, MCF10a did not affect the subsequent attachment of these cells (Fig. 5.1B).

We analyzed whether the stimulation of osteoclastogenesis by breast cancer cells was involved in their subsequent attachment to osteoclasts. Image analysis revealed that breast cancer cells did not attach to osteoclasts (Fig. 5.2A, lower). Moreover, treatment of MDA-MB 231 CM-exposed bone marrow cultures with OPG completely blocked osteoclast formation in these cultures (Fig. 4.5F) but did not interfere with cancer factor-induced breast cancer cell attachment to immature osteoblasts (Fig. 5.2B).

Because pharmacological inhibitors of Notch and Wnt signalling were suggested to mediate interactions between osteoblasts and hematopoietic stem cells, we next assessed if these pathways were involved in the attachment of breast cancer cells to bone cells (Fig. 5.3). We performed the attachment assay on bone marrow cells treated for 9 days with AA alone or with AA and MDA-MB-231 CM (10%) together with glycogen synthase kinase (GSK) inhibitors LiCl or SB216763, or γ-secretase inhibitors DAPT or CE, and examined the attachment of breast cancer cells to bone cells. Treatment with GSK inhibitors LiCl or SB216763 did not interfere with cancer factor-induced breast cancer cell attachment to bone cells (Fig. 5.4A). In contrast, treatment with γ-secretase inhibitor DAPT or CE fully reversed the MDA-MB-231 CM-induced breast cancer cell attachment to osteoblasts (Fig. 5.4A). Similarly, attachment of 4T1 or MCF7 breast cancer cells was abolished by treatment with γ-secretase inhibitors DAPT or CE (Fig. 5.4B). In the absence of conditioned medium, neither DAPT nor CE affected cancer cell attachment to bone cells (Fig. 5.4A).

Since the inhibition of γ-secretase prevented attachment of breast cancer cells to bone cells, we next investigated the involvement of one of the targets of γ-secretase activity, amyloid precursor protein (APP). We have found that the exposure of osteoblastic cultures to breast cancer factors resulted in decrease in the high molecular weight APP, as assessed by immunoblotting (Fig. 5.5A). We identified APP in non-permeabilized cultures by immunofluorescence and found positive staining for APP in AA-treated bone marrow cultures, which appeared less intensely stained in APP in CM-treated cultures (Fig. 5.5B).

The cleavage of APP into amyloid beta was next assessed by immunofluorescence. Osteoblastic cultures treated with AA exhibited some staining of Aβ (Fig. 5.6A, left). Exposure to 4T1 CM increased the intensity of fluorescence (Fig. 5.6A, middle). This effect of breast cancer-derived factors was inhibited by treatment with γ-secretase inhibitor DAPT (Fig. 5.6A, right). Interestingly, in CM-treated cultures, the staining often formed clumped aggregates of Aβ staining (Fig. 5.6B, left), reminiscent of amyloid plaques found in the Alzheimer's brain (254). Quantification of these plaques revealed significantly more aggregates in CM-treated cells than in cells treated with AA alone, or cells treated with 4T1 CM in the presence of DAPT (Fig. 5.6B, right).

We next assessed if any of the γ-secretase products colocalize to the bone cells exhibiting direct attachment to breast cancer cells. We performed the attachment assay, this time with breast cancer cells labeled red and the protein of interest in green, to demonstrate the colocalization of breast cancer cells with the different γ-secretase products (Fig. 5.7A). We have found that there was no difference in immunofluorescence staining for Notch Intracellular Domain or APP between osteoblasts in direct interactions with breast cancer cells and those not attached to breast cancer cells. However, we found that areas of osteoblasts where cancer cells attached exhibited a significantly lower intensity of Aβ than osteoblasts which did not support breast cancer cell attachment (Fig. 5.7B).

Discussion

This study demonstrates that soluble factors produced by breast cancer cells support the subsequent attachment of breast cancer cells to immature osteoblasts. We have identified γ-secretase as a critical mediator of these effects. Pharmacological inhibition of γ-secretase completely reversed the enhancement of cancer cell attachment, providing a potential therapeutic target capable of reducing the homing of cancer metastases to bone.

These data combined with the findings from the previous chapter have shown that the inhibition of osteoblastogenesis together with a stimulation of osteoclastogenesis by the soluble factors produced by breast cancer cells induced a significant shift in the bone microenvironment toward (i) a more supportive environment for the homing of arriving cancer cells and (ii) a more osteolytic milieu for the further growth of tumours at the bone site. The ability of breast cancer cells to modify the distant microenvironment of the bone tissue is consistent with the notion of a premetastatic niche (7). The involvement of osteoblasts in the maintenance of the hematopoietic stem cell niche is long recognized (50,165,253). It has been speculated that in the metastatic niche tumour cells take advantage of the stem cell habitat in the bone marrow (255), thus equating hematopoietic stem cell niche with the metastatic niche. We have shown that not only can osteoblasts directly support the attachment of breast cancer cells, but also that breast cancer cells can augment this property of the osteoblasts while acting distantly. These findings are consistent with the role of osteoblasts as a part of a premetastatic niche permitting the attraction of tumour cells and their incorporation into the niche. Cancer cells attached preferentially to immature osteoblasts, suggesting that the increase in cancer cell attachment is likely a combination of direct effect of breast cancer cells on osteoblasts and indirect consequence of osteoblast differentiation status. Breast cancer cells never attached to osteoclasts, and inhibition of osteoclast formation using OPG did not prevent a breast cancer factor-induced increase in breast cancer cell attachment to

osteoblasts. However, osteoclasts still appear to play a role in the effects of breast cancer cells. The inhibition of a breast cancer factor-induced increase in breast cancer attachment to osteoblasts using γ-secretase inhibitors correlated with a strong inhibition of osteoclast formation, but the stimulation of osteoblastogenesis was not required, suggesting the possible supportive action of osteoclasts in breast cancer attachment. Thus, we demonstrated that soluble factors produced by breast cancer introduce changes in osteoblasts and osteoclasts consistent with the establishment of a pre-metastatic niche.

To assess the potential mediators of the effect of breast cancer cells on osteoblast and osteoclast precursors, we considered the involvement of signalling molecules known to affect osteoblast and osteoclast differentiation, including Wnt, and Notch. We have shown that normalizing Wnt signalling did not interfere with the effects produced by the breast cancer-derived factors on bone cells. In contrast, inhibition of γ-secretase, a key enzyme mediating Notch signalling, resulted in the full reversal of breast cancer-induced enhancement of cancer cell attachment. Because the targets of γ-secretase are not limited to Notch signalling, we have examined the potential role of amyloid precursor protein in the interactions between breast cancer cells and bone cells. Activation of γ-secretase cleaves amyloid precursor protein into amyloid beta protein. In keeping, full-size amyloid precursor protein was reduced with CM-treatment and using immunofluorescence we identified significant increase in amyloid beta aggregates in these cultures. While we did not find changes in fluorescence intensity of either Notch or APP in areas with or without breast cancer cells attached, areas where cancer cells attached exhibited a significantly lower intensity of Aβ than where cells did not attach. While γ-secretase plays a role in the attachment of breast cancer cells to bone cells, it does not appear to be mediated by its cleavage products, the NICD or Aβ. Since γ-secretase is not a specific enzyme, it is plausible that other substrates of γ-secretase are involved in mediating the attachment of breast cancer cells to bone cells. EphrinB2 is another known substrate of γ-secretase and its interaction with osteoclast-derived Ephb4 is involved in the differentiation of

osteoblasts (103,256). This interaction has also been shown to enhance SDF-1-induced signalling and chemotaxis required for extracellular matrix-dependent endothelial cell clustering (257), making it another potential avenue for breast cancer-mediated attachment to bone cells.

The inhibitors of γ-secretase are widely used in Alzheimer's disease patients, with the goal of attenuating amyloid precursor protein cleavage into β-amyloid plaques. However, the use of γ-secretase inhibitors in cancers has recently been of interest for their anti-proliferative effects in glioblastoma, gastric, prostate and breast cancers (258-260). Additionally, γ-secretase inhibition has been shown to attenuate angiogenesis in models of glioblastoma and human lung adenocarcinoma tumours, providing further evidence of the drug's benefits in the treatment of cancers (261). Small molecule inhibitor of γ-secretase, RO4929097, binds to gamma secretase and blocks the activation of the Notch receptor, and is currently in Phase I clinical trials treating breast cancer patients, with studies led by the MD Anderson Cancer Center and National Institutes of Health.

Thus, our study suggests the critical role of γ-secretase in the homing and establishment of osteolytic bone metastases from breast cancer. Complete reversal of key prometastatic events such as cancer-induced enhancement of cancer cell attachment by the inhibition of γ-secretase provides a robust therapeutic target and a rationale for the use of these drugs, potentially capable of reducing both the homing and progression of cancer metastases to bone.

This work was published as part of the manuscript Fong JE, Le Nihouannen D, Komarova SV. Tumour-supportive and osteoclastogenic changes induced by breast cancer-derived factors are reversed by inhibition of {gamma}-secretase. J Biol Chem. 2010 Oct 8;285(41):31427-34.

The continuation of the study shown in Figures 5.4-5.6 was presented as a poster at the American Society for Bone and Mineral Research Annual Meeting 2010, and the 2010 Conference on Human Cell Transformation, and the abstracts were published in the following:

Fong JE, Hussein O, Komarova SV. The Role of Gamma-secretase Mediated Cleavage of Notch and Amyloid Precursor Protein in Breast Cancer Cell Attachment to Osteoblasts. In, "The 2010 Conference on Human Cell Transformation" by Springer Science, NY (in press).

Fong JE, Hussein O, Komarova SV. The Role of Gamma-secretase Mediated Cleavage of Notch and Amyloid Precursor Protein in Breast Cancer Cell Attachment to Osteoblasts. J Bone Miner Res (Suppl 1), 2010.

FIGURE 5.1

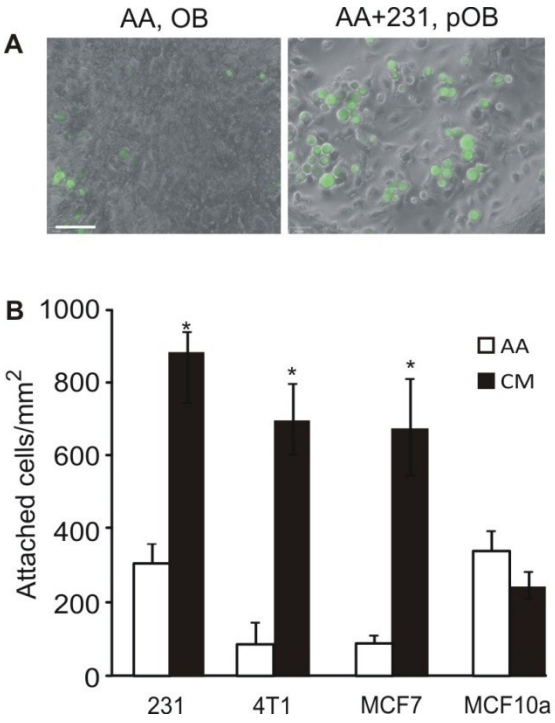

Figure 5.1: Exposure to breast cancer-derived factors enhances subsequent breast cancer cell attachment to immature osteoblasts.

Bone marrow cells were grown for 9 days with AA (50 μg/ml) in the absence (AA, open bars) or presence of MDA-MB-231, 4T1, MCF7, or MCF10A CM (10%). The MDA-MB-231 cells were labeled with Cell Tracker Green and added to bone marrow cultures for 40 min, and then the cultures were washed to remove nonattached cells, fixed, and analyzed. A, representative images demonstrate attachment of breast cancer cells (green) to mature osteoblasts (OB) in cultures treated with AA only (left); to immature osteoblast precursors (pOB) in cultures treated with AA and MDA-MB-231 CM (right). Scale bar is 20 μm. B, significantly more breast cancer cells attached to bone marrow cultures treated with AA and MDA-MB-231, 4T1 or MCF7 breast cancer cell CM compared with cultures treated with AA alone or CM from breast epithelial cell line MCF10a. Data are means ± S.E. (error bars), n = 2–6 independent experiments; asterisks indicate significant difference at $p < 0.05$.

FIGURE 5.2

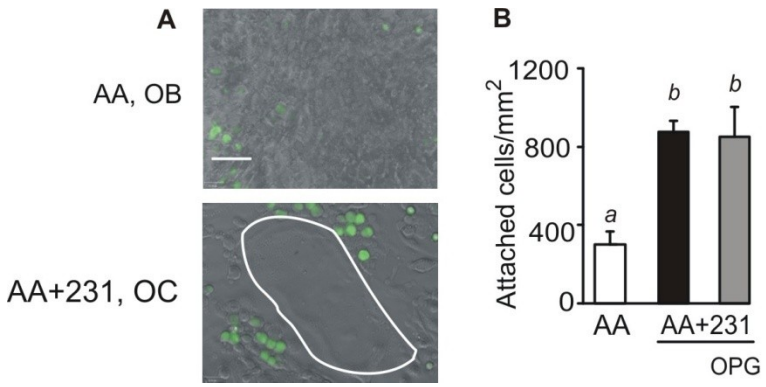

Figure 5.2: Inhibition of osteoclastogenesis cannot reverse breast cancer cell attachment to bone cells.

Bone marrow cells were grown for 9 days with AA (50 μg/ml) in the absence (AA, open bars) or presence of MDA-MB-231 CM (10%), combined with OPG (500 ng/ml). The MDA-MB-231 cells were labeled with Cell Tracker Green and added to bone marrow cultures for 40 min, and then the cultures were washed to remove nonattached cells, fixed, and analyzed. A, representative images demonstrate attachment of breast cancer cells (green) to mature osteoblasts (OB) in cultures treated with AA only (top); or to osteoclasts (OC, white outline) in cultures treated with AA and MDA-MB-231 CM (bottom). Scale bar is 20 μm. B, significantly more breast cancer cells attached to bone marrow cultures treated with AA and MDA-MB-231 CM compared with cultures treated with AA alone. OPG was ineffective at preventing this effect. Data are means ± S.E. (error bars), n = 2–6 independent experiments; different letters indicate significant difference at $p < 0.05$.

FIGURE 5.3

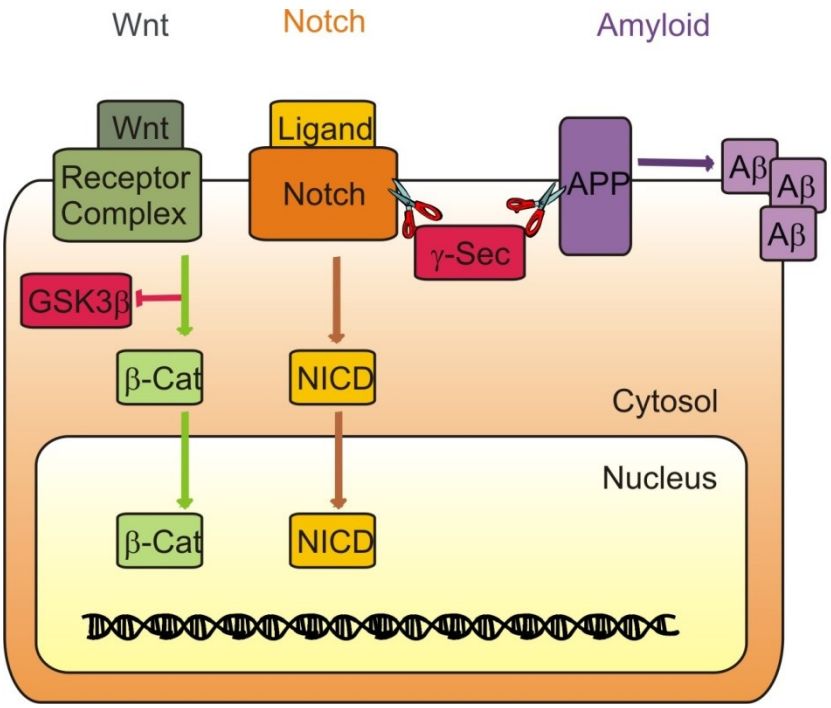

Figure 5.3: Wnt, Notch and Amyloid signalling as potential mediators of the effects of breast cancer cells on bone cells.

In the canonical Wnt signalling pathway, when the Wnt molecule binds the receptor complex, composed of LRP5/6, Frizzled and the Frizzled co-receptors, GSK3β is displaced, allowing the stabilization of β-catenin and its translocation to the nucleus, where it will induce transcription of target genes.
Notch signalling occurs when a Notch ligand binds to the extracellular domain of 1 of 4 Notch receptors and induces the proteolytic cleavage and release of the intracellular domain (NICD) by γ-secretase. The NICD then enters the nucleus where it modifies gene expression.
Amyloid precursor protein is also cleaved by γ-secretase, and it releases amyloid-β (Aβ) proteins, which form extracellular Aβ plaques.

FIGURE 5.4

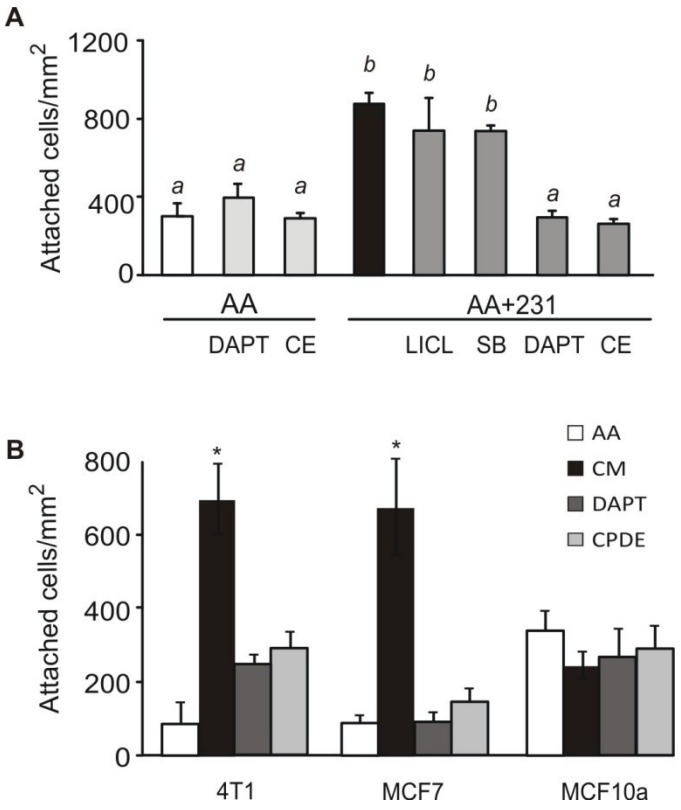

Figure 5.4: Inhibition of Notch signalling, but not the normalization of Wnt signalling or inhibition of osteoclastogenesis, reverses breast cancer cell attachment to bone cells.

Bone marrow cells were grown for 9 days with AA (50 μg/ml) in the absence (AA, open bars) or presence of 10% conditioned medium from MDA-MB-231, 4T1 or MCF10a, combined with vehicle (AA+231, black bars) or the following inhibitors (gray bars): LiCl (10 mM), SB216763 (SB, 10 μm), DAPT (100 nM), or Compound E (CE, 100 nM). For attachment assay, the same cells as were used for CM treatment were labeled with Cell Tracker Green and added to bone marrow cultures for 40 min, and then the cultures were washed to remove non-attached cells, fixed, and analyzed. A, significantly more breast cancer cells attached to bone marrow cultures treated with AA and MDA-MB-231 CM

compared with cultures treated with AA alone. Inhibitors of γ-secretase DAPT and CE prevented this effect of MDA-MB-231 CM, whereas glycogen synthase kinase inhibitors were ineffective. Data are means ± S.E. (error bars), n = 2–6 independent experiments; different letters indicate significant difference at $p < 0.05$. B, Treatment with 4T1 or MCF7 CM significantly increased attachment of these cells to bone marrow cultures, which was inhibited by γ-secretase inhibitors. Data are means ± S.E., n = 3 independent experiments, $p < 0.05$.

FIGURE 5.5

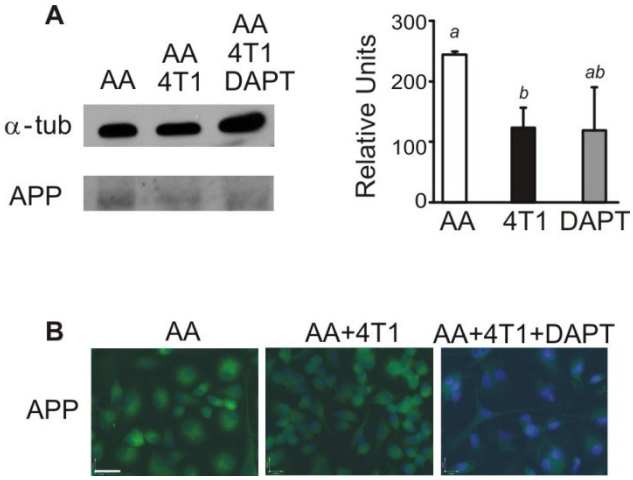

Figure 5.5: Breast cancer cells reduce the levels of amyloid precursor protein in bone marrow cells.

Bone marrow cells were grown for 9 days with AA (50 μg/ml) in the absence (AA, open bars) or presence of 4T1 CM (10%, 4T1, filled bars) and with γ secretase inhibitor DAPT (100 nM, grey bars). A, left, Immunoblot of amyloid precursor protein (APP) with α-tubulin as a loading control. A, right, Quantification of blots was conducted using Image J. Presented are APP protein levels relative to levels of α-tubulin. Data are means ± S.E. n = 3 independent experiments, $p < 0.05$, different letters indicate different significance. B, APP localization was assessed by immunofluorescence (green), and nuclei were stained using DAPI (blue). Scale bar is 20 μm.

FIGURE 5.6

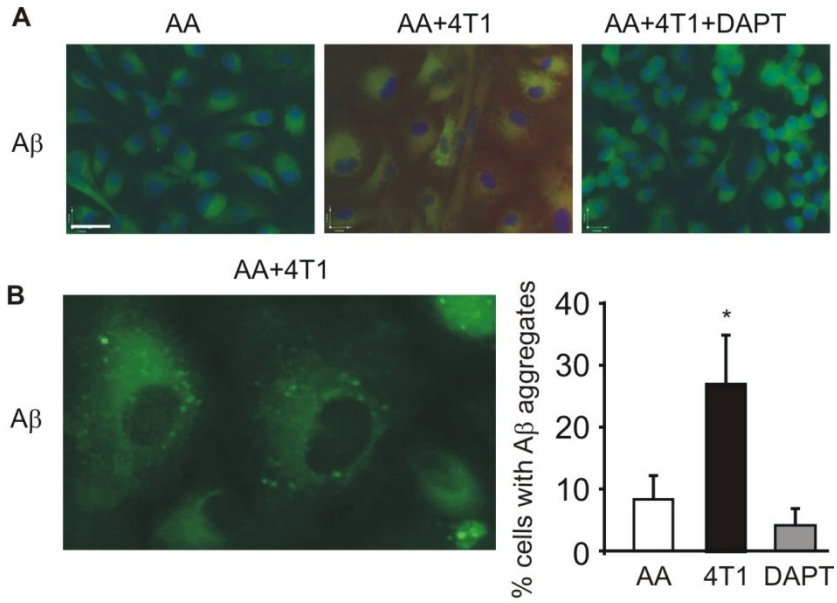

Figure 5.6: Breast cancer cells increase aggregates of γ-secretase product beta-amyloid in bone marrow cells.

Bone marrow cells were grown for 9 days with AA (50 μg/ml) in the absence (AA, open bars) or presence of 4T1 CM (10%, AA+4T1, filled bars) and with γ secretase inhibitor DAPT (100 nM, grey bar). A, Representative images of the immunofluorescence localization of beta amyloid (green), and nuclei stained for DAPI (blue). B, left, representative image of the beta-amyloid aggregates found in AA + 4T1-treated cultures. B, right, quantification of the percentage of cells with beta amyloid aggregates, assessed visually in 9-44 cells in 5 different areas per condition. Data are means ± S.D. n = 3 independent experiments, $p < 0.05$. Scale bar is 20 μm.

FIGURE 5.7

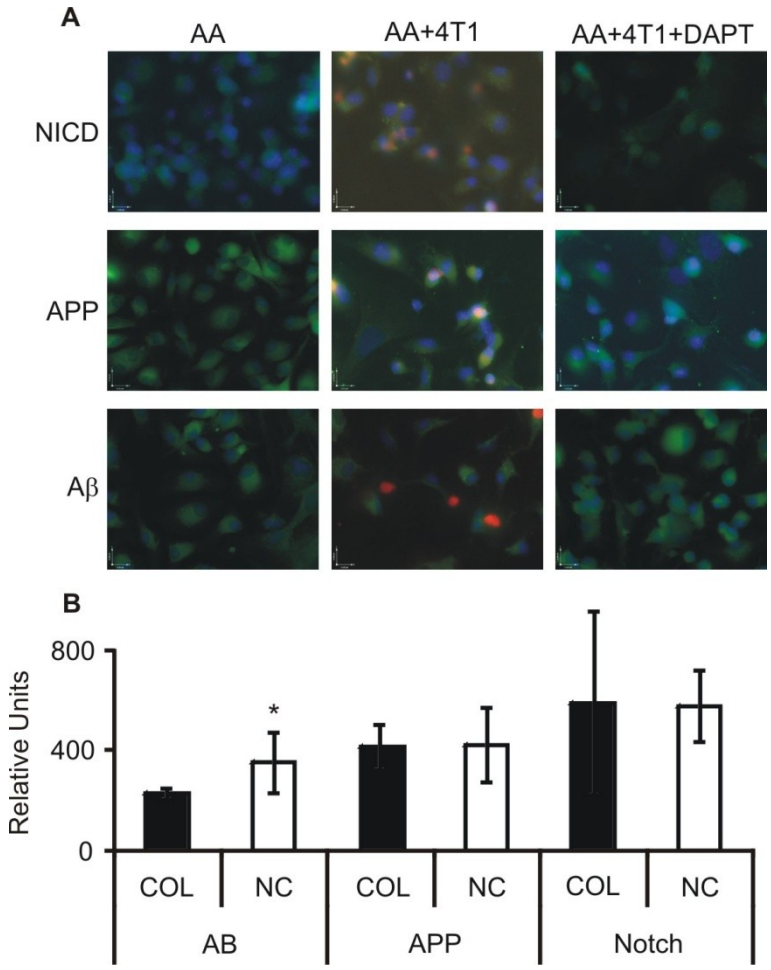

Figure 5.7: Co-localization of γ-secretase products with breast cancer cell attachment to bone cells.

Bone marrow cells were grown for 9 days with AA (50 μg/ml) in the absence (AA, open bars) or presence of 4T1 CM (10%, AA+231, filled bars) and with γ secretase inhibitor DAPT (100 nM). A, Attachment of breast cancer cells (red) to bone cells stained green for NICD, APP or Aβ, and nuclei stained blue for DAPI. B, Quantification of the intensity of NICD, APP or Aβ staining (green) in areas where breast cancer cells have attached (colocalization; COL), or where there are no breast cancer cells attached (NC). Data are means ± S.D., n = 3 independent experiments, $p < 0.05$. Scale bar is 20 μm.

Chapter 6

The effect of breast cancer cells on bioenergetics state of bone cells

Introduction

The metabolism of a cell encompasses the chemical reactions that allow it to live. Metabolism depends on the molecular unit of intercellular energy transfer, adenosine triphosphate (ATP), which is generated in the cell through glycolysis and aerobic respiration. ATP is formed when glucose is first converted to pyruvate in the cell cytosol, and then, in the presence of oxygen which acts as an electron acceptor, aerobic respiration occurs in the mitochondria, where pyruvate and other substrates are converted into carbon dioxide and water to yield 36 molecules of ATP. In the absence of oxygen, pyruvate is reduced to lactate in the cell cytosol, yielding 2 molecules of ATP.

The generation of ATP must be regulated in order to provide sufficient ATP for cellular processes, which often vary through the functional life of a cell. One of the hallmarks of cancer is the use of anaerobic glycolysis for ATP generation, even in aerobic conditions, termed the Warburg Effect (106). The Warburg Effect is unusual since highly proliferative cancer cells switch to a relatively ineffective process of ATP production, even if there is sufficient oxygen to support mitochondrial oxidative phosphorylation. This metabolic activity is similar to that in early embryonic cells, which suggests that cancer cells use a more primitive metabolic activity. The advantage is unclear, however it has been suggested that this may be due to mitochondrial DNA mutations, nuclear DNA mutations, oncogenic transformation or due to the influence of the tumour microenvironment, where the heterogeneous population may use both forms of ATP synthesis (107).

Healthy cells have a high ATP to ADP ratio, and minor disruptions in ATP production result in an arrest of the cell cycle or even apoptosis of the cell (95).

To adjust the ATP production to changing cellular demands, metabolic sensors, such as AMP-activated protein kinase (AMPK) are employed. When a cell is unable to meet its energetic demands, AMP concentrations are increased and AMPK is stimulated. AMPK in turn affects multiple downstream targets to decrease the cell's metabolic expenditure while simultaneously improving energy production by inducing mitochondrial biogenesis and fatty acid oxidation (96). The counterpart to AMPK is mTOR, which regulates protein synthesis through S6K1 and 4E-BP1 phosphorylation and controls the cell's cytoskeletal organization. As such, mTOR is suppressed when nutrients are limited.

Both bone resorption by osteoclasts and bone formation by osteoblasts demand large amounts of energy, resulting in adaptation changes occurring during cell differentiation and leading to improved ATP production (11,262). On the other hand, changes in metabolism may alter cell functional capacity. Diabetes mellitus is characterized by high blood glucose, due to dysfunctional insulin secretion or function (263). Complications extend to the bone, and diabetic patients experience changed bone mineral density, increased fractures, and delayed fracture repair (264-266).

Mammalian cell uptake of nutrients is regulated through growth factors, but cancer cells overcome this dependence on growth factors by acquiring mutations that change their own metabolism and that of other cells. Cancer cells will adapt to secondary tumour environmental conditions through changes in their energy metabolism (12), and have been shown to influence the metabolism of secondary areas as well, including changing oxidation and antioxidant levels in the blood and marrow plasma (13). However, it is not known if cancer cells may affect the bioenergetics of other cell types in areas of secondary tumour formation.

In this study, we examined the effect of proliferating breast carcinoma cells on the bioenergetic status of bone marrow cultures that retain potential for differentiation into both osteoblasts and osteoclasts. To examine if such changes in cell

bioenergetics may affect differentiation, we next artificially elevated the energy status of osteoclasts by supplementing the growth medium with metabolic substrates, and assessed if osteoclastogenesis is affected by cell bioenergetics.

Results

Breast cancer cells alter energy metabolism in bone cells

We examined the effects of soluble factors produced by the 4T1 mouse metastatic breast cancer cell line on the differentiation of osteoblasts from precursors derived from mouse bone marrow. Bone marrow cells were treated with AA in the presence or absence of medium conditioned by 4T1 cells for 5 days and examined for the expression of osteoblast differentiation marker alkaline phosphatase (ALP; Figure 6.1A, top). Cultures treated with AA displayed robust ALP staining in osteoblastic nodules. In contrast, in cultures treated with AA in the presence of 4T1 CM, the ALP-positive area was significantly reduced (Fig. 6.1B, top).

To assess if oxidative phosphorylation is affected by the presence of breast cancer cells, we examined the osteoblast mitochondrial activity using the live cell mitochondrial transmembrane potential ($\Delta\psi m$) indicator JC-1. At low $\Delta\psi m$, JC-1 forms monomers emitting green fluorescence, indicating membrane depolarization. At high $\Delta\psi m$, JC-1 aggregates, shifting to emit a red fluorescence (Figure 6.1A, bottom) and indicating a polarized mitochondrion. Treatment with both conditioned media significantly increased the area of mitochondria exhibiting high $\Delta\psi m$ (Fig. 6.1B), indicating increased oxidative phosphorylation in these cultures.

We next examined the effect of 4T1 CM on the rates of media acidification and glucose consumption in bone marrow cultures. Treatment of bone marrow cells with 4T1 CM induced significantly more media acidification than treatment with AA alone (Fig. 6.1C). Media glucose content was analyzed to determine if metabolic substrates were being depleted more rapidly in cultures treated with 4T1 CM. We have found that in 4T1 CM-treated cultures, the glucose

concentration in the end of the 48 h culture period was significantly lower than in the AA treated cultures, and was often undetectable (Fig. 6.1D). Moreover, 4T1 CM-treated cultures exhibited a 30% increase in lactate production, compared to cells treated with AA alone (Fig. 6.1E). Intracellular ATP concentration ([ATP]) was also evaluated and breast cancer-derived factors produced a 2.3-fold increase in [ATP] compared to AA-treatment (Fig. 6.1F). Thus, exposure to breast cancer-derived factors resulted in increased bioenergetics of bone cells characterized by higher levels of glucose consumption and lactate production, increase in mitochondrial transmembrane potential and increase in [ATP].

Exposure to breast cancer-derived factors modifies metabolic sensors in bone cells

We next assessed if bone cells metabolic sensors, AMPK and mTOR, are affected by the exposure to breast cancer-derived factors. We have found that the protein levels of both AMPKγ (an AMP-sensing isoform of AMPK) and mTOR are increased in bone cells exposed to 4T1 CM (Fig. 2A). We next evaluated the gene expression of the heterotrimeric AMPK subunits. The the α subunit is catalytic, while the β subunit are regulatory, and the γ senses the ATP:AMP ratio (267). We have found that exposure to breast cancer factors leads to changes in the subunit composition and decreased AMPKγ subunits (Fig. 2B), indicating a potential change in AMPK regulation in cancer factor-exposed osteoblasts.

Effect of energy state modulation on osteoclast differentiation

To assess if modulation of cellular energy state may have consequences on differentiation, we next attempted to model this effect in a defined model of osteoclast differentiation. We increased the availability of energy substrates by adding excess amounts of pyruvate (1 mM), and examined the resulting osteoclast energy state in RAW 264.7 cells. Mitochondrial activity in osteoclast precursors and mature osteoclasts was examined using live cell mitochondrial

transmembrane potential ($\Delta\psi m$) indicator JC-1 (Fig. 6.3A, B). Treatment with pyruvate increased $\Delta\psi m$ both in osteoclast precursors and, more dramatically, in mature osteoclasts (Fig. 6.3C). Moreover, treatment of RAW 264.7 cells with RANKL induced a significant 5-fold increase in [ATP], and addition of 1 mM pyruvate during osteoclastogenesis resulted in a further 3-fold increase in [ATP] (Fig. 6.3D). Treatment of RAW 264.7 with RANKL induced significant media acidification, which was dramatically increased in the presence of pyruvate (Fig. 6.3E). Lactate exhibited an overall trend to increase in the presence of RANKL and pyruvate, which did not reach statistical significance (Fig. 6.3F). Thus, the addition of moderate amounts of pyruvate during osteoclast differentiation resulted in increased mitochondrial transmembrane potential, intracellular [ATP], and media acidification, all reminiscent of the effects of breast cancer-derived factors on the energy states of bone cells.

We next evaluated the differentiation of osteoclasts in high energetic states (Fig. 6.4A). The addition of pyruvate significantly increased the number of osteoclasts formed in the presence of RANKL (Fig. 6.4B, left), the osteoclast size as estimated by cell planar surface area, (Fig. 6.4B, middle), and the number of nuclei per osteoclast (Fig. 6.4B, right). Moreover, pyruvate-treated osteoclasts created significantly larger resorption pits (Fig. 6.4C, D, left) and resorbed greater areas of mineralized substrate (Fig. 6.4D, right). Since AMPK is regulated by the AMP/ATP ratio, we next assessed its involvement in the pyruvate-induced osteoclastogenesis. Using immunoblotting, we have found that the addition of pyruvate leads to an increase in AMPKα1 and to a smaller extent, AMPKγ1 protein levels and in AMPKα1 phosphorylation (Fig. 6.4E). AMPK is activated in energy-deficient conditions, therefore we first assessed if activation of AMPK with AICAR will interfere with the effects of pyruvate. Treatment with AICAR did not affect the ability of pyruvate to increase osteoclast number (Fig. 6.4F, left), but decreased cell size (Fig. 6.4F, right). Dorsomorphin is an AMPK inhibitor; therefore we anticipated that treatment with dorsomorphin should mimic the effect of pyruvate. Inhibition of AMPK with low concentrations of

dorsomorphin in control cultures had a minor effect on cell number (Fig. 6.4F, left), but strongly increased osteoclast size (Fig. 6.4F, right).

Osteoclasts are known to significantly change shape during their transition from quiescent to actively resorptive state. Therefore, we next assessed if the measurement of osteoclast planar area is reflective of its size. We formed osteoclasts on control or fibronectin-coated glass, or on calcium phosphate substrates, fixed the samples, stained osteoclasts for nuclei with DAPI, actin with FITC-conjugated phalloidin, and membranes with DiI, and examined osteoclast nucleation, planar area and height using confocal microscopy (Fig. 6.5A). The average osteoclast height was similar in the absence or presence of pyruvate (Fig. 6.5B). Moreover, we have found that on non-resorbable glass and fibronectin-coated glass surfaces the height of the osteoclast does not correlate to its size or nucleation (Fig. 6.5C), while on resorbable calcium phosphate surface a significant correlation was observed between the height of the osteoclast and the number of nuclei in the cell (Fig. 6.5C). This data indicate that only on resorbable substrates is the osteoclast able to change its shape, while on non-resorbable surfaces osteoclasts remain flattened at a single nucleus height. Thus, on glass surfaces, the osteoclast planar area reflects its three-dimensional size.

Excess substrate modifies osteoclastogenesis in vivo

To confirm that an excess of energy substrates can affect osteoclastogenesis in vivo, we injected healthy mice with glucose (0.5 mg/kg/day), or pyruvate (0.75 mg/kg/day) for 7 days and examined multinucleated TRAP-positive osteoclasts formed in long bones (Fig. 6.6A). We evaluated areas in the proximal tibia, at least 50 μM distal to the growth plate. Treatment with pyruvate or glucose did not significantly increase osteoclast number (Fig. 6.6B), but treatment with pyruvate significantly increased the osteoclast surface adjacent to the bone surface (Fig. 6.6B). Thus, addition of 1-2 mM of pyruvate results in the formation of greater osteoclast surfaces both in vitro and in vivo.

Discussion

The changes in metabolic activity that occur in cancer cells have been studied for nearly a century, with Otto Heinrich Warburg first identifying the metabolic changes in tumours in 1923 (106). That publication was the first to identify that the predominant means of energy production by cancer cells is glycolysis followed by lactic acid fermentation in the cytosol. Since it was published, the energetics of cancer cells have been studied extensively, but their influence on the energetics of other cells in the tumour microenvironment has not been explored until now. We have shown that conditioned medium from proliferating breast cancer cells increases the energy state of bone cells, as apparent by their increased glucose consumption, mitochondrial transmembrane potential and intracellular levels of ATP. An increase in the protein levels of metabolic sensors AMPK and mTOR accompanies the heightened energy metabolism, likely indicating an adaptive response of bone cells. Moreover, we have shown that similar increases in bioenergetics during osteoclastogenesis, achieved by addition of excess metabolic substrates, can significantly augment osteoclast formation, resulting in formation of larger, more active osteoclasts both in vitro and in vivo.

The ability of cells to sense their own metabolic status allows them to decrease metabolic expenditure to improve energy production. Energy-sensing AMPK is most highly expressed in skeletal muscle, where it stimulates fatty acid oxidation and glucose uptake to maintain energy homeostasis (268). In bone cells, AMPK activation is known to stimulate bone formation (269), and inhibits RANKL production by osteoblasts (270). Its function in osteoclasts has not been studied explicitly, but our data suggests that inhibition of AMPK may stimulate osteoclastogenesis. Interestingly, cancer cells have been shown to induce angiogenesis through the stimulation of mTOR in the bone microenvironment (271). mTOR regulates protein synthesis and cytoskeletal organization (272,273). It has been shown to be important in osteoblastic bone formation (274), and osteoclast survival (275), but its effects on osteoclast differentiation are unknown.

Sensors AMPK and mTOR were found to be upregulated in cancer-treated osteoblasts. We have also found AMPK to be important for bioenergetic-modulated changes in osteoclastogenesis. We have found that metabolic sensors are involved in breast cancer factors-induced changes in bone bioenergetics, and can mediate subsequent adaptation of bone cell differentiation and function to changes in microenvironment.

The establishment of secondary tumours requires a microenvironment conducive to their growth. The bone is a hypoxic microenvironment which increases the growth of metastatic tumour cells that have adapted to growth in low oxygen environments (185). The resulting induction of HIF-1α and VEGF acts to increase vasculature in surrounding tissues. We have found that breast cancer-derived factors stimulate oxidative phosphorylation in bone cells. Such metabolic changes may have profound effects on the function and differentiation of bone cells. We have previously demonstrated that in the presence of oxidator ascorbic acid, osteoclast differentiation and death are significantly accelerated (209). In this study, we have shown that a heightened energy state during osteoclastogenesis results in the formation of larger osteoclasts with higher resorptive activity.

Because of the rapidly evolving nature of cancer cells, their metabolic adaptation to the surrounding environment allows them to live in hypoxic, acidic and nutrient-depleted conditions. Modification of tumour metabolism with drugs inhibiting glycolysis, or using cancer's augmented glycolytic activity as a drug delivery system is therefore a promising therapeutic strategy. Drugs like glycolysis inhibitors Lonidamine and 2-Deoxyglucose, toxic 3-bromopyruvate, and alkylating agent Glufofsamide, have been shown to induce potent cytotoxic effects on cancer cells (276). For the osteoclast, we have shown that changes in metabolism can lead to changes in osteoclast differentiation and function. Pharmacological modulation of cell energetics has been successful in the treatment of metabolic diseases like diabetes and polycystic ovary syndrome. More recently, AMPK inhibitor and diabetic drug Metformin has found a new

role in the prevention of cancer, as well as specifically breast cancer, in diabetic patients (277,278). The protective effects of inhibiting metabolic sensors extend to mTOR as well. Our group has recently shown that mTOR is upregulated in osteoclasts treated with factors released from breast cancer cells, and that mTOR inhibitor rapamycin may prevent bone metastases and subsequent osteolysis in experimental model of breast cancer metastasis to bone in immunocompetent mice (161). The therapeutic benefit of normalizing cellular energy metabolism is becoming more important, and Toronto's Princess Margaret and Mount Sinai Hospitals are currently recruiting early breast cancer patients for Metformin therapy. Our study suggests that such treatment may have additional benefits in the prevention and treatment of breast cancer metastases to bone.

Taken together, our study has shown that breast cancer cell-derived factors enhance bone cell energy metabolism. We have examined how increased energy metabolism affects the behaviour of cells and how these can change their differentiation. In a model of stimulated energy states in bone cells, we have shown that osteoclasts fuse to form bigger cells and resorb more bone. These studies are important for understanding the mechanisms underlying skeletal disorders associated with cancer or hyperglycemia, as well as for understanding the general relationship between homeostatic and functional cellular operations.

The osteoclast diferentiation work is part of the manuscript in preparation Le Nihouannen D*, **Fong JE***, Hussein O, Tiedemann K, Barralet JE, Komarova SV. Interactions between homeostatic and differentiation signalling during osteoclastogenesis (**Authors contributed equally to the study)*

Data regarding osteoclast differentiation under oxidative condition discussed in this chapter are part of the manuscript Le Nihouannen D, Barralet JE, **Fong JE**, Komarova SV. Ascorbic acid accelerates osteoclast formation and death. *Bone*. 2010 May;46(5):1336-43. (JEF performed experiments, analysis and participated in the preparation of the manuscript).

FIGURE 6.1

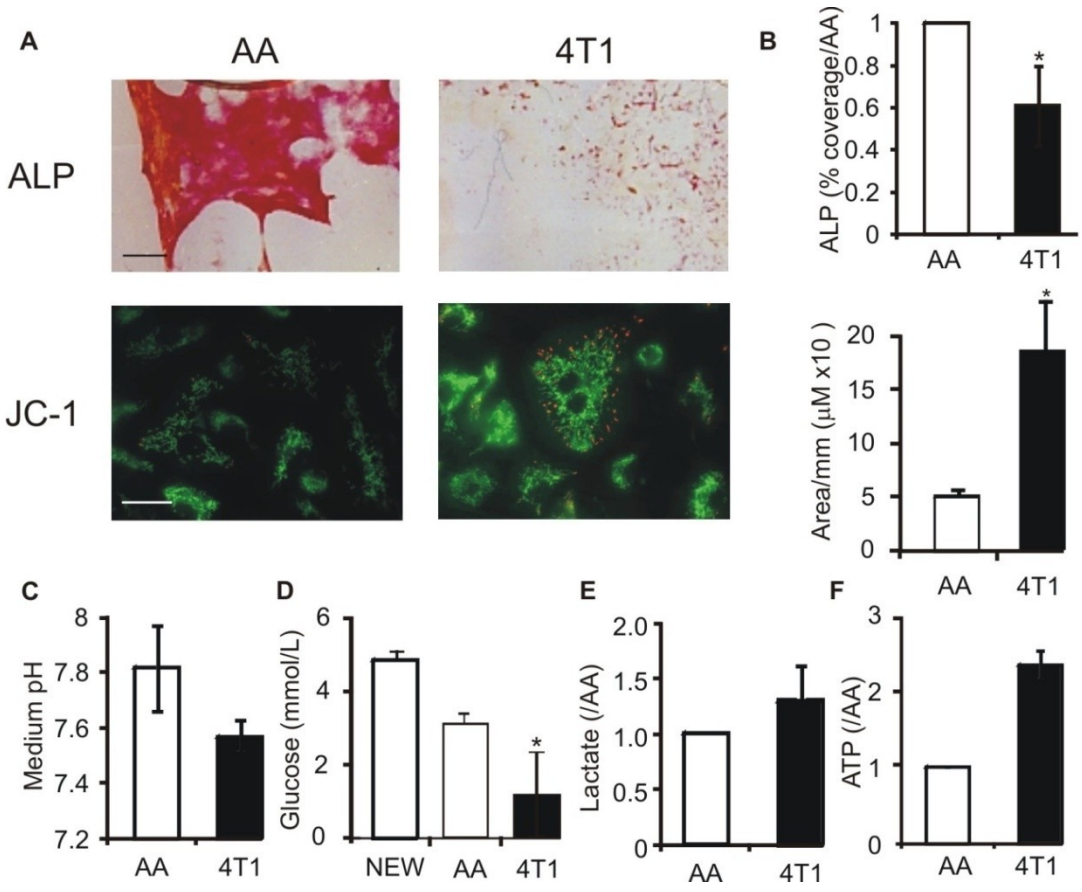

Figure 6.1: Breast cancer cells heighten the energy states of bone cells.

Bone marrow cells were grown for 6 days with AA (50 μg/ml) in the absence (AA, open bars) or presence of 4T1 CM (10%, AA+4T1, filled bars). Data are means ± S.E., with the number of independent experiments indicated; $p < 0.05$. A, representative images of cultures treated with AA only (AA; left), or with AA and 4T1 CM (4T1; right), fixed on day 6–9, and stained for ALP (red, upper) or mitochondrial transmembrane potential dye JC-1 (green for low membrane potential, red for high membrane potential; lower). Scale bar represents 2 mm for ALP images and 20 μM for JC-1 images. B, average area covered on day 6 by ALP-positive cells (upper) and the area of high-membrane potential mitochondria in bone cells (lower). Treatment with 4T1 CM significantly reduced ALP-positive osteoblast staining, while increasing the area of high membrane potential

mitochondria, n = 3 independent experiments. C, Media pH, n = 3. D, Media glucose concentration after 48 hours of culture; n = 4. E, Media lactate concentration, n = 3. F, Intracellular ATP concentration, n = 2-4.

FIGURE 6.2

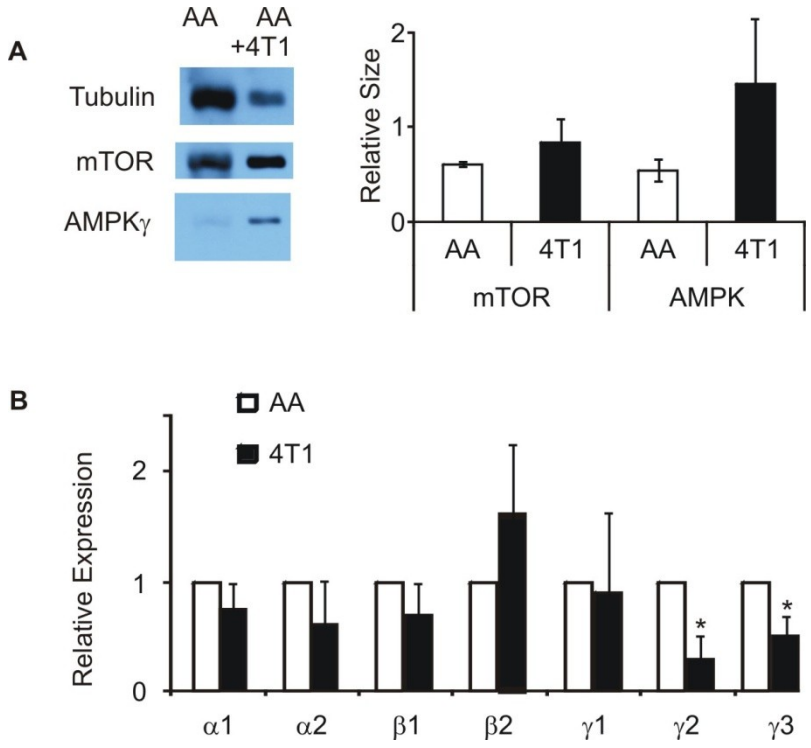

Figure 6.2: Metabolic sensors mTOR and AMPK are increased in breast cancer factors-treated bone cells.

Bone marrow cells were grown for 3 days with AA (50 μg/ml) in the absence (AA, open bars) or presence of 4T1 CM (10%, AA+4T1, filled bars). A, left, Immunoblot of AMPK and mTOR with α-tubulin as a loading control. A, right, Quantification of blots was conducted using Image J, and presented relative to levels of α-tubulin. B, expression of AMPK isoforms AMPKα1, AMPKα2, AMPKβ1, AMPKβ2, AMPKγ1, AMPKγ2, AMPKγ3, normalized and presented relative to levels observed in cells grown with AA only. n = 3 independent experiments, data are means ± SEM, $p < 0.05$ indicate significance assessed by paired t-test.

FIGURE 6.3

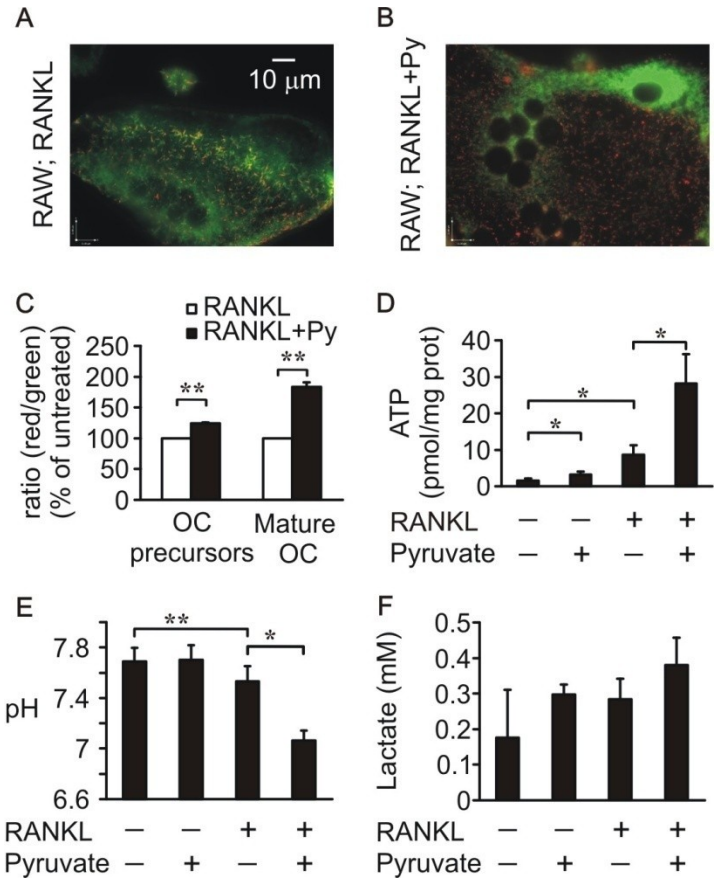

Figure 6.3: Pyruvate supplementation heightens the energy state of osteoclasts.

RAW 264.7 cell were cultured for 2 (OC precursors) to 4 (mature OC) days with or without RANKL and with or without pyruvate (1 mM). A, B, Representative images of mitochondria stained with the vital mitochondrial dye JC-1 in osteoclasts formed in the presence of RANKL alone (A) or RANKL and pyruvate (B). C, Mitochondrial transmembrane potential $\Delta\psi m$ (indicated by red to green intensity ratios of JC-1) normalized to the ratio obtained in cultures treated with RANKL only, n = 3 independent experiments. D, Intracellular ATP concentration, n = 4 independent experiments. E, Media pH, n = 3 independent experiments. F, Media lactate concentration, n = 3 independent experiments. Data are means ± SEM, $p < 0.05$ indicate significance assessed by paired t-test.

FIGURE 6.4

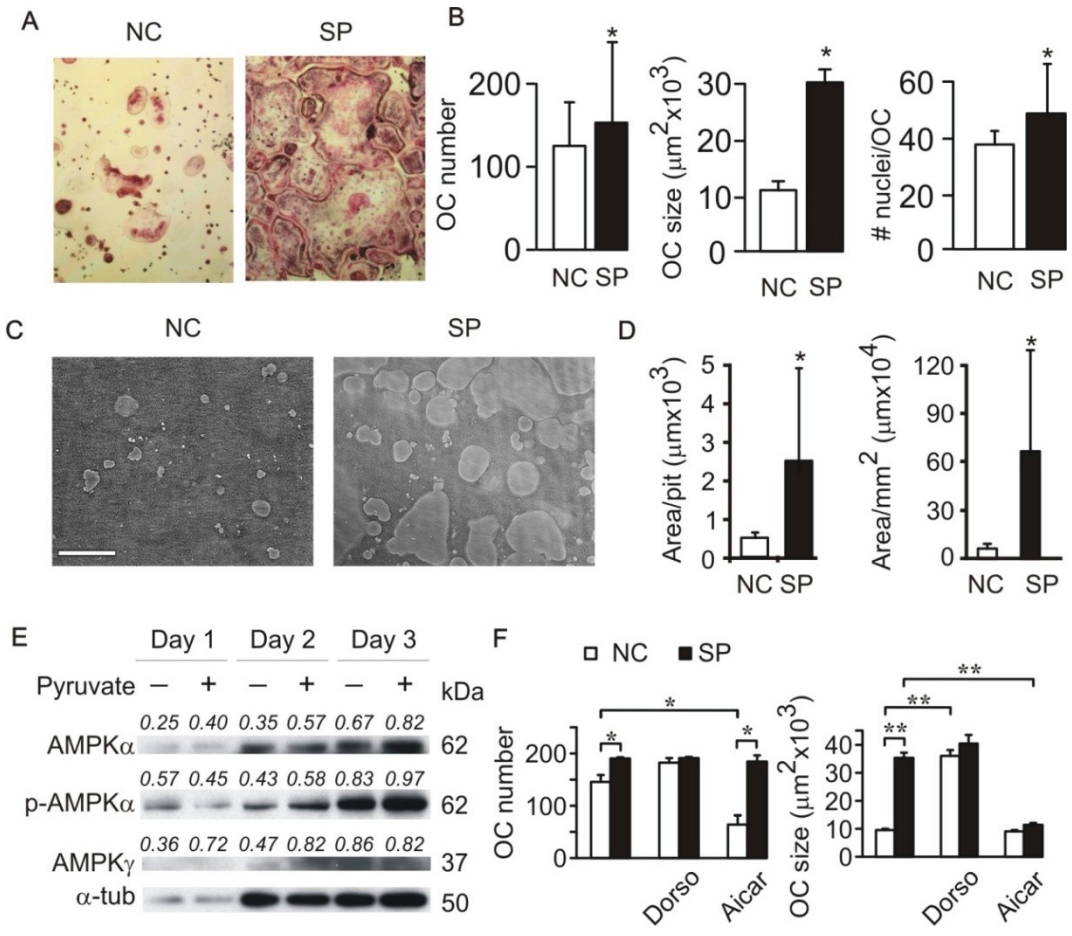

Figure 6.4: Pyruvate increases osteoclast differentiation and activity.

A-B) RAW 264.7 cells treated with AA (50 mg/ml), and RANKL (50 ng/m) for 4 days. A, Representative images of osteoclasts generated in negative control cultures (left, NC) or in - cultures treated with pyruvate (SP, 1 mM). B, Average number of osteoclasts (left) osteoclast surface area (middle) and number of nuclei per osteoclast (right) formed in control and pyruvate-treated cultures. Data are means ± SEM; n = 4 independent experiments, $p < 0.05$ assessed by paired t-test. C-D, RAW 264.7 cells were plated on a resorbable calcium phosphate substrate and cultured for 7 days with RANKL (50 ng/ml) without or with pyruvate (1 mM). C, Representative images of resorption pits in control cultures (left) and cultures treated with pyruvate (right). Scale bar represents 50μM. D, Average area

of a single resorption pit (left) and total resorption area per 1 mm^2 of substrate (right,) Data are means ± SD, n = 5 fields per well in n=4 wells of a 96-well plate, per condition. $p < 0.05$ indicate significance assessed by t-test. E, RAW 264.7 cells were treated for 3 days with RANKL (50 ng/ml), with or without pyruvate (1 mM), cell lysates were collected and phospho-AMPKα, AMPKα and AMPKγ were assessed by immunoblotting, with α-tubulin used as a loading control. The number above the blot indicates the ratio of protein levels relative to α-tubulin for AMPKα and AMPKγ or relative to total AMPKα for phospho- AMPKα. F) RAW 264.7 cells were treated for 4 days with RANKL, with or without pyruvate (1 mM), and in the absence or presence of AMPK inhibitor (dorsomorphine, Dorso, 0.5 μM) or AMPK stimulator (AICAR, 50 nM), and osteoclast number (left) and osteoclast size (right) were assessed. Data are means ± SEM, n = 3 independent experiments, $p < 0.05$ indicate significance assessed by paired t-test.

FIGURE 6.5

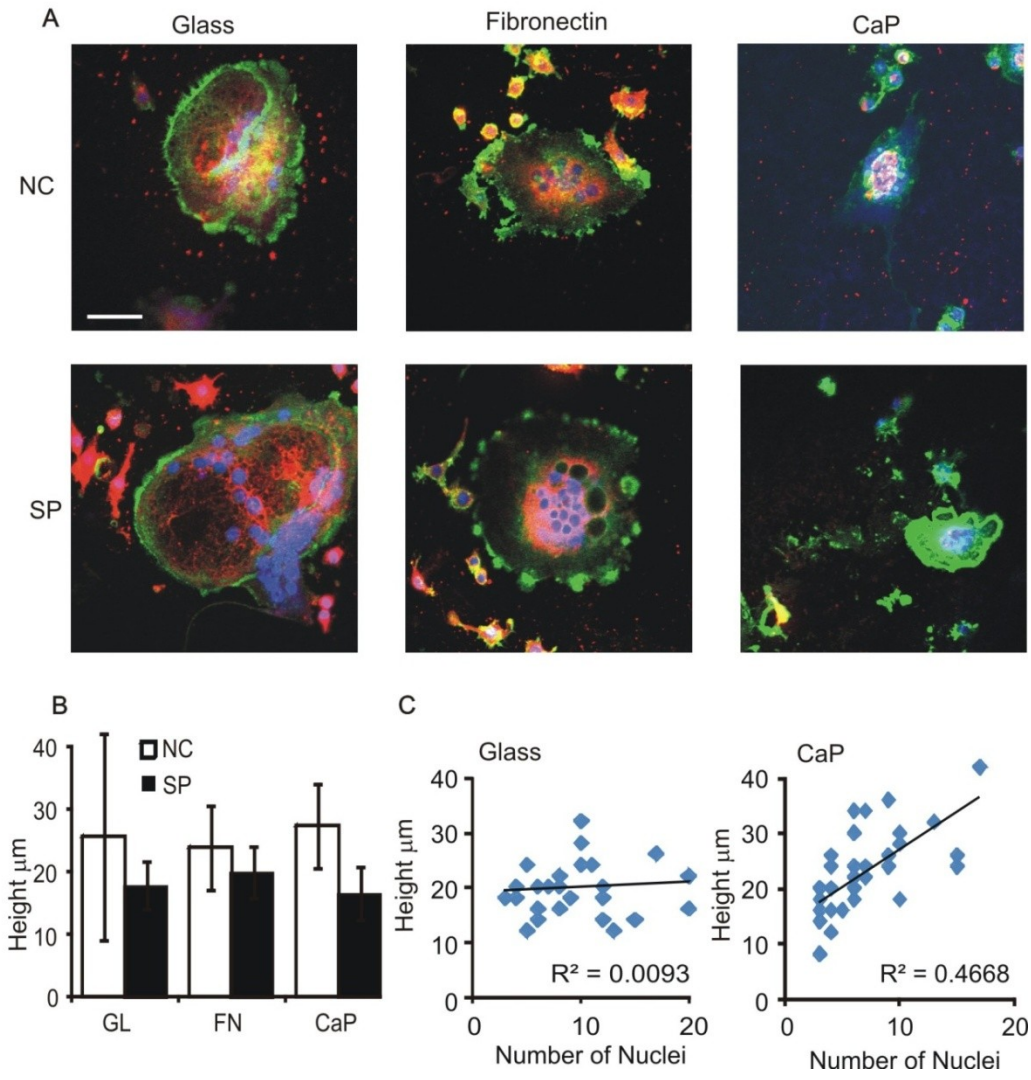

Figure 6.5: Osteoclast planar area is reflective of its size.

RAW264.7 cells were treated with RANKL (50 ng/mL) for 5 days and replated on glass coverslips uncoated (GL), coated with fibronectin (FN) or coated with calcium phosphate (CaP) for 24 hours before analysis. A, Osteoclasts were stained for actin (Alexa 488-conjugated Phalloidin; green), membrane (DiI; red) and nuclei (DAPI; blue) and visualized using confocal microscopy. Scale bar represents 50µM. B, Average osteoclast height in cultures maintained on

different substrates and in the absence or presence of pyruvate. Data are means ± SD, n=20 osteoclasts from 3 coverslips. C, the correlation between the number of nuclei and their height was assessed.

FIGURE 6.6

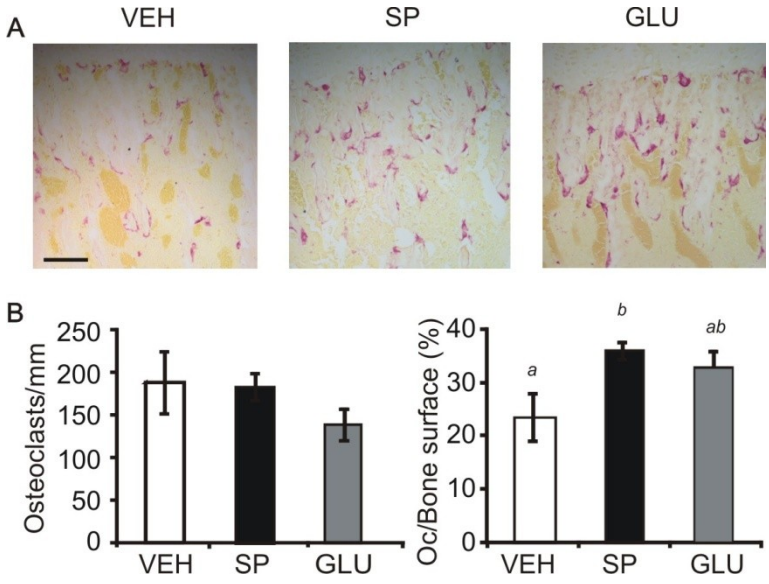

Figure 6.6: Pyruvate and glucose augment osteoclastogenesis in vivo.

Mice were injected for 7 days with pyruvate (0.75 g/kg/day) or glucose (0.5 g/kg/day). Osteoclast number and surface were assessed on paraffin-embedded sections from proximal tibiae. A, Representative images of osteoclasts in vehicle-treated bone (left), pyruvate treatment (middle), and glucose treatment (right). Scale bars represent 50 μM. B, left, Average number of osteoclasts. B, right, Average osteoclast surface per bone surface. Data are means ± SEM, n = 3 sections from 3 different animals, statistical significance was assessed by t-test, $p < 0.05$.

Chapter 7

Conclusions

The metastatic milieu consists of bone cells and breast cancer cells, and the interactions between them determine the progression of the osteolytic lesion. While there is considerable knowledge regarding the role of osteoclasts and osteoclastic bone resorption in breast cancer-mediated osteolysis, the role of osteoblasts is much less studied. Osteoblasts are the main source of pro-resorptive cytokine RANKL, and produce other osteoclastogenesis-related proteins like PTHrP and IL-6. It is known that when osteolysis occurs, the resorptive activity of osteoclasts outweighs bone formation by osteoblasts, indicating a dysregulation in their interactions. The goal of our study was to first identify how breast cancer cells and bone cells interact, by examining the differentiation and function of bone cells exposed to breast cancer-derived factors. It is known that breast cancer cells stimulate osteoblast-dependent osteoclast differentiation, and our lab has previously shown that breast cancer cells also directly induce osteoclastogenesis from late osteoclast precursors. We have shown that in addition to their effects on osteoclasts, breast cancer cells directly affect osteoblast differentiation, inhibiting their maturation and functionality in extracellular matrix mineralization, but allowing them to mature just enough to produce quantities of RANKL for robust osteoclastogenesis without producing enough OPG to counteract this effect. This may be mediated by the CCN3 protein, highly expressed in breast cancer. We next characterized the direct interactions between breast cancer cells and bone cells, and found that breast cancer cells release factors that facilitate their subsequent attachment to osteoblasts. Both the induction of osteoclast formation and subsequent attachment of breast cancer cells to immature osteoblasts were prevented by γ-secretase inhibitors DAPT and Compound E. In keeping, we identified the alterations in Notch signalling and amyloid precursor protein cleavage in bone cells treated with breast cancer-derived factors. Breast cancer

cells increased Notch signalling in bone cells, and stimulated the cleavage of APP into amyloid beta plaques, although neither appeared to be directly involved in the attachment of breast cancer cells to bone cells. We next identified that bone cells exposed to breast cancer-derived factors consumed more glucose, expressed higher mitochondrial membrane potential and produced more ATP. To assess if such bioenergetics changes can have consequences for cell differentiation and function, we modeled heightened energy state during osteoclast differentiation, in a defined model of osteoclastogenesis – RANKL-treated RAW 264.7 cells. We have found that increase in cell bioenergetics during osteoclastogenesis results in formation of bigger osteoclasts, which are also more active in resorption. Expression and activity of metabolic sensors, AMPK and mTOR, was affected by exposure of bone cells to breast cancer-derived factors, and was central for bioenergetics-induced changes in osteoclastogenesis. Thus, we have identified that breast cancer cells inhibit osteoblast differentiation and stimulate osteoblast-dependent osteoclast differentiation, and that these changes in bone cells facilitate subsequent attachment of breast cancer cells to immature osteoblasts. Importantly, we have found that activity of γ-secretase is central for both the osteoclastogenic effects of cancer cells, and the direct interactions between breast cancer cells and osteoblasts. In addition, we have found that exposure to soluble factors produced by breast cancer cells heighten the energy states of bone cells. Moreover, we have found that such change in bioenergetics may have functional consequences for osteoclastogenesis, and identified metabolic sensors, in particular AMPK, as mediators of such effects. Overall, our studies provide further rationale for the use of γ-secretase inhibitors and AMPK activators in preventing the progression of breast cancer metastases. Currently, γ-secretase inhibitor RO4929097 is in clinical trials alone or in combination with aromatase inhibitor Letrozole, for the treatment of invasive breast cancer. AMPK-activator Metformin, which has long been used for diabetes treatment, was recently discovered to protect patients against cancers. Based on these data, several clinical trials have begun to evaluate its potential for reduced breast cancer metabolism, growth and metastasis. Interestingly, it has recently been shown that γ-secretase inhibitor MRK-003 has

the potential to reduce Notch signalling and attenuate mTOR signalling and glucose uptake in a mouse model of breast cancer (279). In the future of breast cancer treatment, it will be essential to develop combinatorial therapies that attenuate tumour growth and prevent tumour cell dissemination to secondary sites.

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Appendix

Published articles co-authored by Jenna Fong during PhD training

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Ascorbic acid accelerates osteoclast formation and death

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ABSTRACT

Ascorbic acid (AA) plays a key role in bone formation. However controversy remains about the effect of AA on cells responsible for bone destruction, osteoclasts. We investigated the effect of AA on osteoclastogenesis using primary mouse bone marrow cultures and monocytic RAW 264.7 cells treated with osteoclastogenic factors RANKL and MCSF. Treatment with AA resulted in significant increase in osteoclast number, size and nucleation. To assess osteoclast oxidative stress level, a ratio of reduced (GSH) to oxidized (GSSG) glutathione and the total glutathione content (GSH_t) were evaluated. Osteoclast differentiation was associated with a decrease in GSH/GSSG and GSH_t. AA induced further decrease in both parameters, and resulted in significant production of H_2O_2, indicating its pro-oxidant action. At low concentration, H_2O_2 induced similar effects to AA, although less potently, and catalase partially inhibited AA-induced osteoclastogenesis. To assess the modification in osteoclast metabolism, the mitochondrial activity was evaluated using JC-1 and the ATP levels were assessed. Osteoclast formation was associated with the increase in mitochondrial activity and ATP concentration, which were further increased in the presence of AA. Importantly, the stimulatory effect of AA was only evident at early phase of osteoclastogenesis, whereas at the late stage AA significantly accelerated osteoclast death. Thus, during osteoclastogenesis AA acts as an oxidant, first stimulating osteoclast formation, but later limiting osteoclast lifespan. This duality of AA action allows reconciling the stimulatory action of AA on osteoclastogenesis observed *in vitro* with an overall attenuation of bone resorption in the presence of AA observed *in vivo*.

Introduction

Osteoclasts are the only cells in the body capable of destruction of mineralized tissues. Osteoclast function is important in the physiological processes of remodeling of fatigued bone, maintaining calcium homeostasis and allowing tooth eruption, as well as in contributing to diseases associated with abnormal bone and joint destruction, such as osteoporosis, osteo- and rheumatoid arthritis and osteolytic metastases [1–4]. Osteoclasts are multinucleated cells formed from hematopoietic precursors [5]. Key factors regulating osteoclast formation are the receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), which are produced by bone forming cells, osteoblasts [6]. Interestingly, it has recently been shown that osteoclast formation is associated with significant oxidative stress, resulting in a decrease in the cell redox status reflected by the ratio of reduced (GSH) to oxidized glutathione (GSSG) as well as total glutathione content (GSH_t) [7, 8]. Moreover, osteoclastogenesis itself leads to the production of reactive oxygen species (ROS), which has been suggested to partially mediate RANKL signaling [7,9]. In turn, moderate redox stress induced by ROS promotes osteoclast differentiation and enhances bone resorption [8,10,11], whereas severe redox stress suppresses osteoclastogenesis [8]. The importance of ROS in osteoclast regulation has also been confirmed *in vivo*, where Lean et al. have shown that treatment with catalase prevents ovariectomy-induced bone loss in mice [12]. Thus, redox status is critical in regulating osteoclast formation and function.

Ascorbic acid (AA) has long been known as an effective and abundant water-soluble antioxidant [13–16]. AA is the reduced and predominant form of vitamin C, an essential nutrient for humans and several other animals and insects that have lost the ability to synthesize vitamin C over evolution [17]. AA is an important cofactor in collagen hydroxylation, and the lack of AA results in scurvy which manifests in anemia, gingivitis, skin and mucosa hemorrhages, as well as bone abnormalities in affected children [18,19]. AA is an essential regulatory agent for differentiation of various cell types [20,21], including osteoblasts [22], where it is routinely used to induce osteoblast differentiation *in vitro*. In contrast, the effect of AA on osteoclastogenesis remains controversial. Several studies agree that osteoclastogenesis is promoted by AA in cultures containing both osteoclasts and osteoblasts [23,24], which is attributed to stimulation of RANKL expression by osteoblasts [24,25]. However, when AA is applied to osteoclast cultures in the absence of osteoblasts, some studies have demonstrated an inhibitory effect of AA on osteoclast differentiation [25,26], whereas others have shown a direct stimulatory role [27]. *In vivo*, AA leads to attenuation of osteoclastogenesis and preservation of bone mass [28,29].

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Tumor-supportive and Osteoclastogenic Changes Induced by Breast Cancer-derived Factors Are Reversed by Inhibition of γ-Secretase*

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Abstract

During breast cancer metastasis to bone, tumor cells home to bone marrow, likely targeting the stem cell niche, and stimulate osteoclasts, which mediate osteolysis required for tumor expansion. Although osteoblasts contribute to the regulation of the hematopoietic stem cell niche and control osteoclastogenesis through production of proresorptive cytokine RANKL (receptor activator of NF-κB ligand), their role in cancer metastases to bone is not fully understood. C57BL/6J mouse bone marrow cells were treated for 3-12 days with ascorbic acid (50 μg/ml) in the presence or absence of 10% medium conditioned by breast carcinoma cells MDA-MB-231, 4T1, or MCF7. Treatment with cancer-derived factors resulted in a sustained 40-60% decrease in osteoblast differentiation markers, compared with treatment with ascorbic acid alone, and induced an osteoclastogenic change in the RANKL/osteoprotegerin ratio. Importantly, exposure of bone cells to breast cancer-derived factors stimulated the subsequent attachment of cancer cells to immature osteoblasts. Inhibition of γ-secretase using pharmacological inhibitors DAPT and Compound E completely reversed cancer-induced osteoclastogenesis as well as cancer-induced enhancement of cancer cell attachment, identifying γ-secretase activity as a key mediator of these effects. Thus, we have uncovered osteoblasts as critical intermediary of premetastatic signaling by breast cancer cells and pinpointed γ-secretase as a robust target for developing therapeutics potentially capable of reducing both homing and progression of cancer metastases to bone.

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Tumorigenesis and Neoplastic Progression

CCN3 Impairs Osteoblast and Stimulates Osteoclast Differentiation to Favor Breast Cancer Metastasis to Bone

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Bone is a preferred site for breast cancer metastasis, causing pain, fractures, spinal cord compressions, and hypercalcemia, all of which can significantly diminish the patient's quality of life. We identified *CCN3* as a novel factor that is highly expressed in bone metastatic breast cancer cells from a xenograft mouse model and in bone metastatic lesions from patients with breast cancer. We demonstrate that *CCN3* overexpression enhances the ability of weakly bone metastatic breast cancer cells to colonize and grow in the bone without altering their growth in the mammary fat pad. We further demonstrated that human recombinant CCN3 inhibits osteoblast differentiation from primary bone marrow cultures, leading to a higher receptor activator of NF-κB ligand (RANKL)/osteoprotegerin (OPG) ratio. In conjunction with its ability to impair osteoblast differentiation, we uncovered a novel role for CCN3 in promoting osteoclast differentiation from RANKL-primed monocyte precursors. CCN3 exerts its pro-osteoclastogenic effects by promoting calcium oscillations and nuclear factor of activated T cells c1 (NFATc1) nuclear translocation. Together, these results demonstrate that CCN3 regulates the differentiation of bone resident cells to create a resorptive environment that promotes the formation of osteolytic breast cancer metastases. *(Am J Pathol 2011, 178:2377–2388; DOI: 10.1016/j.ajpath.2011.01.033)*

Bone is the preferred site for breast cancer metastasis.[1,2] Although patients with bone metastasis display better overall survival relative to patients with visceral metastases, their quality of life can be significantly diminished due to pain, fractures, spinal cord compressions, and hypercalcemia.[3] Given the pivotal role of the osteoclast in bone breakdown associated with osteolytic lesions, bisphosphonates (a class of drugs that inhibit osteoclast-dependent bone resorption) are routinely given to patients with breast cancer bone metastases.[4] However, significant research efforts are now focused on the identification and development of targeted therapeutics to further enhance the management of breast cancer metastasis.[5,6]

The CCN family derives its name from the three founding members: cysteine-rich protein 61 (*Cyr61/CCN1*), connective tissue growth factor (*CTGF/CCN2*), and nephroblastoma overexpressed (*NOV/CCN3*).[7,8] The family also includes Wnt-inducible secreted protein-1 (*WISP1/CCN4*), *WISP2/CCN5*, and *WISP3/CCN6*.[9] These matricellular proteins are grouped together based on the similarity of four modular domains, which include an insulin-like growth factor–binding domain, a von Willebrand factor type C domain (VWC), a thrombospondin type-1 repeat domain, and a cysteine-knot–containing C-terminal domain.[10–12] Although

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