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Breast Cancer Metastases to Bone: Role of the Microenvironment

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1. Introduction

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 act as 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, promotes 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 TGF β , 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.

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- Physiological regulation of breast and bone
 - Breast Growth and Development
 - Interactions of normal breast tissue with bone
 - Breast carcinoma
 - Bone Microenvironment

- Bone structure and composition
- Bone functions
- Osteoclasts, osteoblasts and osteocytes: origins, differentiation, function, physiology, pathology
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3. Physiological regulation of breast and bone

3.1 Breast growth and development

Interactions of normal breast tissue with bone

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 (1), 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] (2). These hormones exhibit their effects through three main target tissues – the intestines, kidneys and bone (3). 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 (4). 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 (3). The regulation of calcium homeostasis by the lactating mammary gland may be of critical importance, since nursing humans secrete 300-400 mg of calcium into milk each day (5). The hormonal balance changes again during lactation, with still-reduced PTH levels, but normalized calcitonin and 1,25(OH)D, and increased PTHrP (2). During this time, increased prolactin concentrations allow for the release of breast milk, and also act to enhance bone turnover (6,7). Suckling stimulates prolactin secretion and inhibits GnRH production, both of which reduce estradiol levels, leading to bone resorption (8). 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 (9). Lactation-induced fragility fractures have been reported as a result, but are not common (10). Of interest, other important molecular mediators for the developing of lactating mammary gland are receptor activator of nuclear factor κ B (RANK) and its ligand RANKL, which are better known for their key role in regulating the formation of osteoclasts. Expression of RANKL in the 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 complete inability to develop a lactating mammary gland (11). Thus, normal breast tissue can interact with bone through a system of hormonal regulators that are important during lactation, and it expresses molecular machinery that employs the same mediators to perform locally distinct functions (Figure 1).

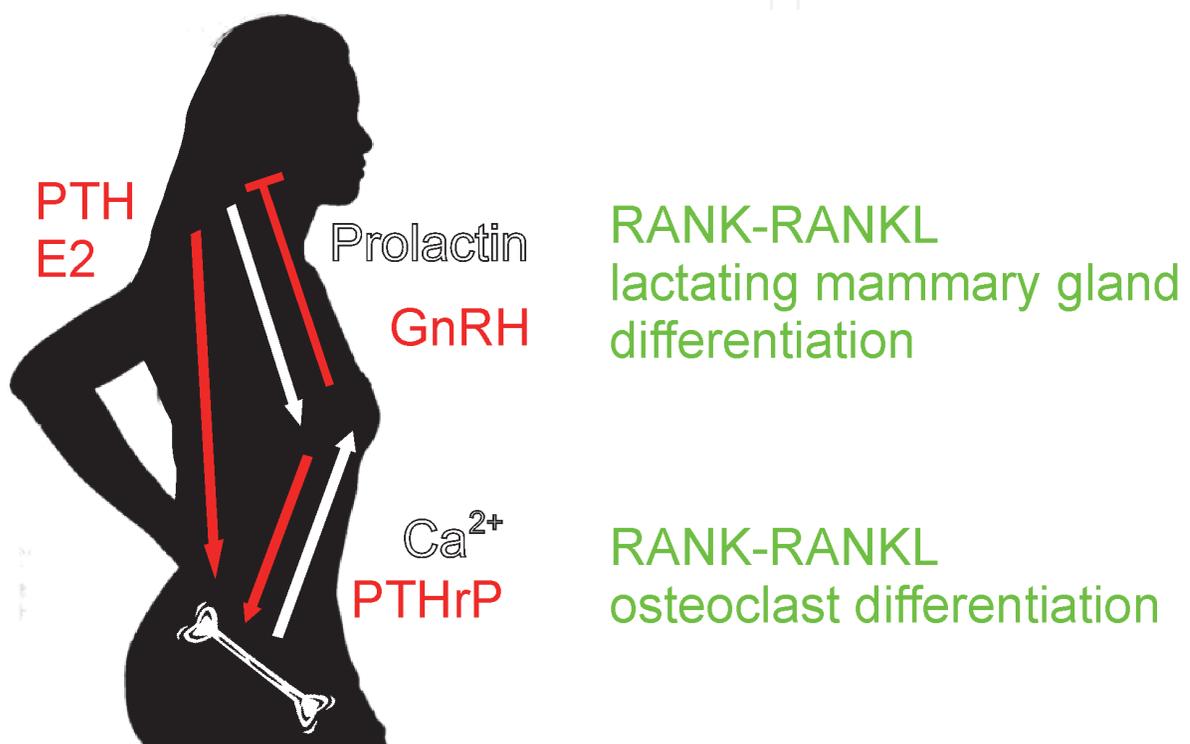


Fig. 1. Physiological interactions between the functions of breast and bone. Lactation involves secretion of large amounts of calcium. Bone is a key participant in calcium homeostasis. PTH is reduced during lactation while PTHrP production by the breast tissue is increased. Suckling stimulates prolactin 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.

Breast carcinoma

Breast carcinomas may arise from the inner lining of the milk ducts or from the lobules, known, respectively, as ductal carcinomas or lobular carcinomas (12). Once a tumour exceeds 1-2 mm in diameter, it requires extensive vascularization in order to survive (13), 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 (14). 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 (15). 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 (16). This may assist in their intravasation process, where the cell breaks through the epithelium into a blood vessel (17). From here, the cell migrates to a distant site, which is driven by chemotaxis and the communication between the cancer cell and a secondary site where it aims to establish (18-20). Instead of combating cancer cells, tumor-associated macrophages and T-cells may assist in the survival and dissemination of cancer cells by mitigating the immune response and promoting cancer progression (21,22). When the cell has reached its destination, it will then undergo extravasation to exit the blood vessel and establish in a new tissue (23). Bone is a preferred site for breast cancer metastases, therefore specific interactions are likely to establish between breast cancer cells and bone cells.

3.2 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 (24), and at bone sites adjacent to actively hematopoietic bone marrow in the axial skeleton, where bone metastases also commonly occur (25). High bone turnover has been found to correlate to poor prognosis in patients with bone metastases (26), and prostate cancer cells have been shown to preferentially metastasize to sites of active bone turnover (27), 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 (28). 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 $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$. 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 (29,30). 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 (31).

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 regulation 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 (32). The coordination of calcium fluxes is achieved through complex hormonal regulation. Parathyroid hormone and 1,25 dihydroxy-vitamin D act to increase calcium by increasing calcium reabsorption from the kidneys and small intestine, respectively, and both act by enhancing the mobilization of calcium from bone through resorption (33). Calcitonin acts to reduce blood calcium by suppressing renal calcium reabsorption and inhibiting the mobilization from bone by preventing bone resorption (34). 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 (35), and haematopoietic cells in turn regulate osteogenesis (36). Adipocyte-derived leptin regulates both appetite and bone mass accrual (37), and osteoblast-derived osteocalcin affects insulin secretion and sensitivity, as well as energy expenditure (38,39). It has most recently been shown that the skeleton regulates male fertility through osteocalcin (40), 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 (41-43). Osteoprotegerin (OPG) is the high affinity decoy receptor for RANKL and is able to prevent osteoclast differentiation by inhibiting RANK-RANKL interactions (44). RANKL binding to RANK in the presence of M-CSF induces the recruitment of adaptor molecules including TRAF6 by RANK (45), resulting in the 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 (46-48). NFATc1 nuclear localization is regulated by

calcium signalling, which also activates calmodulin-dependent kinase, critical for further osteoclast differentiation (49). These events lead to the expression of osteoclast-specific genes including tartrate-resistant acid phosphatase (TRAP), cathepsin K, and α_3 integrin (50), 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 β_1 that bind collagen, fibronectin and laminin, and $\alpha_v\beta_3$ that binds osteopontin and bone sialoprotein (51). This border forms a sealing zone over the area of bone to be resorbed, and the polarization of osteoclasts results in the formation of a ruffled border between the osteoclast and matrix (52). 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 (53,54), resulting in dissolution of the mineral phase of bone, and proteolytic enzymes cathepsin K and matrix metalloproteinase-9 (MMP-9) are released and activated to digest the organic matrix (55).

Osteoblasts: Osteoblasts are differentiated from the mesenchymal stem cells (MSC) that can also give rise to progenitors of myoblasts, adipocytes and chondrocytes (56). Commitment of MSC to become osteoprogenitors results in the upregulation of receptors for hormones, cytokines and growth factors, including PTH, prostaglandin, interleukin-11, insulin-like growth factor-1 and transforming growth factor- β (57). 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 (58). 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 the RANKL inhibitor, OPG (58). Osteoblastogenesis commitment is driven by the downstream activities of Wnt signalling, 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 (59,60). Another developmentally important pathway, Notch signalling, has been shown to negatively regulate osteoblast differentiation (61-63). 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 (64). Osterix interacts with nuclear factor of activated T cells 2 (NFATc2), and in collaboration, controls the transcription of osteoblastic target genes osteocalcin, osteopontin, osteonectin and collagen-1 (65,66). Osteoblasts anchor to newly formed bone matrix by cadherin-11 and N-cadherin, and secrete type 1 collagen and non-collagenous matrix proteins (57). The osteoblasts then regulate the subsequent mineralization of extracellular matrix (67-69).

Osteocytes: While each cell type is essential for the maintenance of bone homeostasis, osteocytes are the most populous and account for over 95% of all cells in the skeleton, covering 94% of all bone surface (70). 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 they extend, connecting the cell with other osteocytes (70,71). 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) (72,73). Osteocyte networks in the bone tissue are implicated in regulating the maintenance and mineralization of bone tissue (70,74), through expression of sclerostin, a negative regulator of bone formation (75), as well as in sensing mechanical load in part through shear stress generated by interstitial fluid moving through the lacuno-canalicular network (76). It has also been suggested that osteocytes participate in mineral homeostasis by resorbing the lacunar walls in which they are embedded (77-79).

Communication between bone cells during normal bone remodelling

Osteoblasts, osteoclasts and osteocytes must work in concert to maintain bone homeostasis (Figure 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 (80). 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, (58). Osteocytes also, at least in part, affect osteoclastogenesis through production of RANKL, which is induced in mechanically-stimulated osteocytes (81). 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 (82). Several studies where osteoclasts have been genetically altered to have impaired function demonstrated diminished bone formation (83), 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 (84). Sphingosine-1-phosphate has been shown to act earlier and induce osteoblast precursor recruitment and subsequent mature cell survival (85). Ephrin-B2/EphB4 bidirectional signaling 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 (86). 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 (87).

4. Homing of breast cancer cells to bone

4.1 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, the potential to home to different organs of subsequently injected cancer cells can be altered (88). In

particular, in bone, bone marrow derived hematopoietic stem cells have been implicated in mediating the establishment of pre-metastatic niche (19,88). Molecular mediators such as vascular endothelial growth factor (VEGF) receptor 1 (VEGFR1) and integrin $\alpha 4\beta 1$ have been implicated in this process. VEGFR1 positive haematopoietic progenitor cells are recruited to sites of future metastasis (88). 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 (89). In addition, we have shown that breast cancer cells secrete factors that support the subsequent attachment of breast cancer cells acting at least in part through γ -secretase-mediated Notch signalling (20).

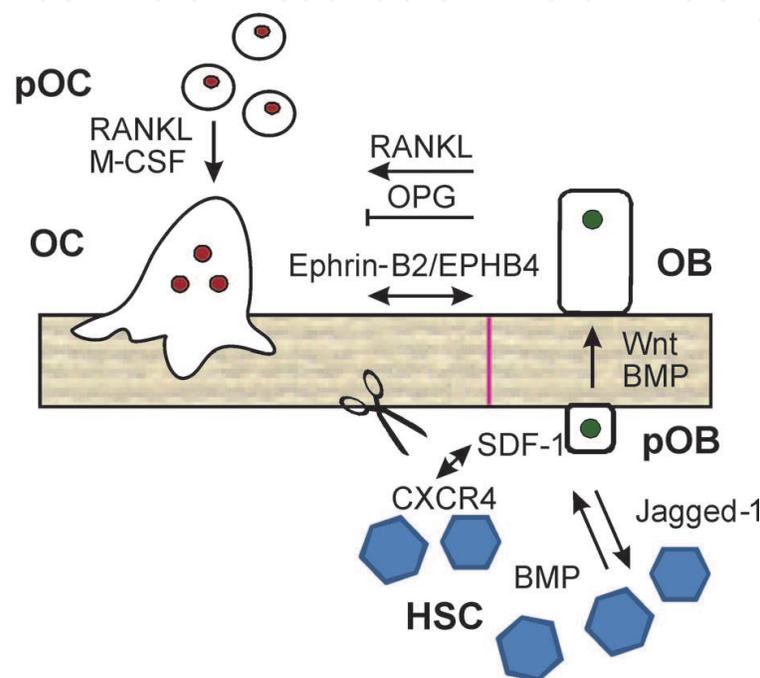


Fig. 2. Cell-cell interactions in the bone microenvironment. Osteoclast differentiation from monocytic precursors is induced by M-CSF, RANKL produced by osteoblastic cells. Osteoblasts are derived from mesenchymal stem cells through Wnt and BMP signalling pathways. Osteoblasts and osteoclasts communicate 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.

4.2 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 tumors. 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 (90). 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 (15), and SDF-1 is strongly expressed in lung, liver, bone marrow and lymph nodes, the primary sites of secondary breast tumours, leading to the identification of the role of the SDF-1/CXCR4 in promoting breast cancer metastasis to bone (91). In addition to directional migration, chemokines have been shown to promote cancer cell survival, proliferation, and adhesion (92). In keeping, the inhibition of CXCR4 limited breast cancer metastases in mice (93), and the overexpression of CXCR4 indicates poor prognosis in both human and murine breast cancer (92,94). Another chemokine implicated in metastases of breast cancer cells expressing high levels of CCL21, is CCR7 that is expressed highly in metastatic sites, such as lymph nodes (15). 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 (95). Another pertinent means of cancer cell migration towards bone relies on the cancer cell expression of RANK (96), which mediates directional migration of breast, melanoma and prostate cancer cells towards RANKL, produced in bone by osteoblasts (97,98).

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 signaling (99,100). Furthermore, GPNMB was identified as a poor prognostic marker in patients with breast cancer (101). Most recently, this group has identified ADAM10 as a sheddase that releases osteoactivin from the cell, which induces endothelial cell migration and subsequent angiogenesis (102). ADAMTS1 and 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 (103).

4.3 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, signaling and regulatory mechanisms (104). 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 (105). Several integrins have been shown to interact with extracellular matrix proteins during bone metastasis, with the most important being $\alpha v \beta 3$, a receptor for osteopontin, fibronectin and vitronectin (106). 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 (88). Breast cancer cells can attach to fibronectin, in an integrin-dependent manner (107). The interaction of cancer cells with fibronectin increases the production of matrix metalloproteinase-2 from fibroblasts to facilitate invasion (108). Another molecule involved the adhesion of HSC to the endosteal niche is annexin II (95). 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 (109,110).

Blocking annexin II or its receptor limited the localization of prostate cancer cells to osteoblasts and endothelial cells (111). In keeping, the inhibition of the SDF-1/CXCL12 and annexin II signaling was shown to inhibit breast cancer progression (112,113). 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 (114). Early reports have argued that BSP inhibits breast cancer cell binding to bone cells (115). 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 (116,117). Moreover, the expression of BSP has been found to correlate with bone metastasis development (118), and OPN expression and serum concentrations have been shown to be poor prognosis markers in breast cancer patients (119,120). As osteopontin is also a mediator of the hematopoietic stem cell niche, directing migration and acting as an adhesion molecule to HSC via $\beta 1$ integrin (121), it represents a potentially valuable therapeutic target against bone metastases.

4.4 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 (122,123). These proteins include but are not limited to osteocalcin, osteopontin, alkaline phosphatase and Runx2 (124). 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 (125,126). Runx2 has also been shown to regulate TGF β -influenced PTHrP levels, as well as upregulate Indian hedgehog (127). Breast cancer cells express Hedgehog ligands that activate osteopontin expression in osteoclasts, promoting osteoclast maturation and resorptive activity through upregulated Cathepsin K and MMP9 (128,129). Of interest, expression of anti-resorptive OPG has been demonstrated to correlate with increased bone-specific homing and colonization potential in breast cancer cells (122), and to promote cancer cell survival (130,131). Osteoclastic integrin $\alpha v \beta 3$ (54), 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 (107,132). 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.

5. Establishing of a metastatic tumour in the bone microenvironment

5.1 Interactions of breast cancer cells with osteoblasts

Inhibition of osteoblasts by breast cancer cells

Breast cancer metastasis to bone is associated with a reduction in bone formation markers in patients with bone metastases (133). In vitro, breast cancer cells have been shown to produce soluble factors able to inhibit osteoblast differentiation (20,134), 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 (135) and suppressing osteoblast differentiation (62,63). Upregulated Notch signalling in breast cancer, through ligand Jagged-1, has been shown to correlate with increased bone metastases (136), and breast cancer cells have been shown to induce Jagged-1 expression and upregulate Notch signalling by osteoblasts (20). Wnt signaling 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 (137). Wnt inhibitor DKK-1 has been shown to be upregulated in diseases associated with bone destruction, such as osteoarthritis (138), myeloma (139), and potentially in Paget's disease (140). 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 (141). Breast cancer cells have also been shown to induce osteoblast apoptosis, through increased Bax/Bcl-2 ratio and caspase expression in osteoblasts (142,143). In addition to preventing the 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 an 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 (144,145)). 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 (146)). Moreover, under the influence of breast cancer cells, undifferentiated osteoblasts express higher levels of RANKL and lower OPG, resulting in an increase in osteoblast-mediated osteoclastogenesis (20), an effect that was reversed when osteoblastic cultures were treated with the inhibitors of γ -secretase - an enzyme implicated in Notch signalling (20,136). One of the mediators of these changes was shown to be the tumour-overexpressed CCN3, that can inhibit osteoblast differentiation and shift the RANKL/OPG ratio to favour osteolysis (147). Another osteoblast-produced osteoclastogenic factor, MCSF, has also been implicated in breast cancer metastases to bone (148).

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 signaling (35,121). Several osteoblast-expressed receptors, cytokines and growth factors have been found to regulate an haematopoietic stem cell niche (149,150), including PTH/PTHrP receptors and BMPs acting to expand the osteoblast population, and Notch ligand Jagged-1 to expand the population of HSCs (35,151). Cancer cells disseminated from the primary tumour may also lay dormant for long periods of time before being

activated to form metastases (152), 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.

5.2 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 (153). Although the expression of PTHrP in primary tumours has been associated with a lower incidence of bone metastasis (154,155), 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 (155), suggesting that the expression pattern of the cancer cells can change during metastasis, and implicating local factors, such as TGF β 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 (156,157). 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* (158).

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 (159). Several signalling pathways in osteoclast precursors have been implicated in these effects, including calcium signalling, NFATc1 activation and MAPKs ERK1/2 and p38 (159,160). Tumour-produced CCN3 was demonstrated to stimulate osteoclast formation from RANKL-primed osteoclast precursors (147). These effects can be relevant to the propensity of cancer cells to metastasize to bone sites undergoing active 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 (146,161). Anti-apoptotic effects of breast cancer-derived factors included PLC γ -mediated suppression of pro-apoptotic protein BIM, and M-CSF-mediated inhibition of caspase cleavage (146).

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 TGF β , BMPs, 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 (162). Matrix released- TGF β activated by osteoclastic resorption (163), 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 (164,165). Considerable research has linked increased TGF- β in the microenvironment to the progression of metastasis, with TGF β

altering both the growth and phenotype of breast cancer cells (166), and increasing their expression of CTGF, CXCL11 and PTHrP (167) via Smad and MAPK signalling in breast cancer cells (153,168,169). PTHrP increases VEGF production, leading to stimulated osteoclastogenesis through the ERK1/2 and p38 signalling pathways (170). TGF β also acts on other cells present in the bone microenvironment, such as osteoclasts themselves by sensitizing them to other breast cancer derived factors (159), through the ERK1/2, p38 and c-Jun-NH₂ kinase signalling pathways (160,171). In keeping with a key role of TGF β in bone metastases, pharmacological inhibition of TGF β signalling through the T β RI kinase inhibitor SD-208 resulted in decreased bone metastasis and tumour burden, and improved bone quality (172). 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” (173), underlying the strong rationale for the use of anti-resorptive drugs for the treatment of cancer metastases to bone.

6. 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 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. 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 further delayed the occurrence of the first skeletal related event (SRE), and provided a further reduction in bone turnover markers in breast cancer patients (174). In non-metastatic breast cancer patients additionally receiving adjuvant aromatase inhibitors, bone mineral density gains were greater with Denosumab treatment (175). 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 (176). 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 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 (177). Bortezomib, a proteasome inhibitor that among other proteins affects DKK-1 and BIM (a pro-apoptotic protein that mediates osteoclast apoptosis) (178,179), was shown to inhibit

osteoclastogenesis (180) and has been successful in combating the osteolytic effects of multiple myeloma (181), making it an attractive candidate for the prevention and treatment of breast cancer-induced osteolysis.

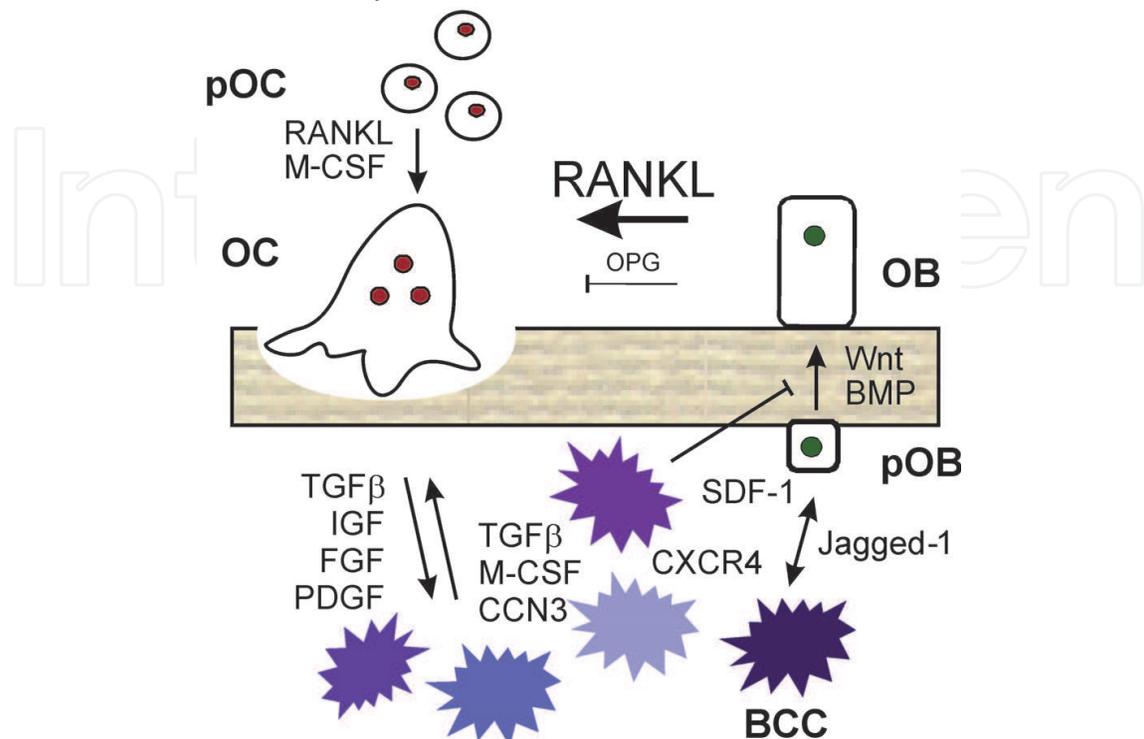


Fig. 3. Breast cancer cells alter normal bone homeostasis. Breast cancer cells maintain osteoblasts in an immature state and stimulate RANKL production by osteoblasts, while inhibiting OPG. Breast cancer cells stimulate osteoclastogenesis directly through TGF β , M-CSF and CCN3 production. Increased bone resorption by activated osteoclasts releases matrix-derived growth factors TGF β , IGF, FGF, PDGF, which act back on breast cancer cells to stimulate their growth and survival.

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 (182). There have been several hindrances in the progress of this therapy due to drug resistance and toxicity (183), and the increased incidence of osteonecrosis of the jaw in combined bisphosphonate-antiangiogenic agent therapy (184). Notwithstanding, the use of VEGF-A monoclonal antibody Bevacizumab in combination with chemotherapy has proven beneficial in reducing breast cancer growth (185) and osteolysis (186). Other targets based on the *in vitro* and *in vivo* studies, such as TGF β , 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 (187), 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% (188), and zoledronic acid and risedronate have been shown to significantly reduce non-vertebral fracture risk in pivotal trials (189). Bisphosphonates are widely used in prevention and treatment of breast cancer metastases to bone, resulting in delay and reduction in skeletal related events (190). 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 (191). 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 (192). In addition, significant proportion of patients failed to normalize bone resorptive indices in response to bisphosphonate treatment (176), demonstrating the need for new therapeutic approaches.

7. 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 the 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, a process that likely involves mediators of the 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, TGF β , 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 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.

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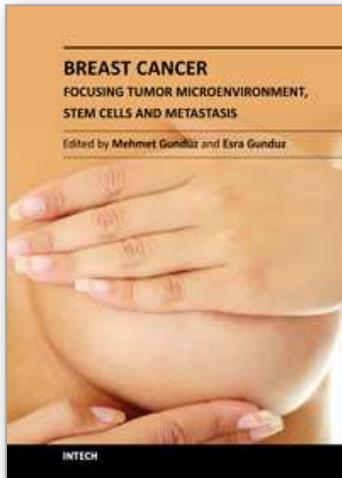
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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed characteristics of breast cancer cell, role of microenvironment, stem cells and metastasis for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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