Chapter 8

Influence of the Bone Microenvironment on Breast Cancer Metastasis to Bone

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Abstract: Cancer patients do not generally die as a direct consequence of the primary tumour, but due to the formation of secondary tumours – metastases – that arise during tumour progression. Bone metastases are a common complication in patients with advanced breast and prostate cancer. Once established, bone metastases cause intractable pain, hypocalcaemia, spinal cord compression and bone frailty. The mechanisms regulating site-specific metastasis are not well understood despite being the focus of research for over a century. However, it is becoming clear that the microenvironment at the secondary tumour site contributes to metastatic progression by regulating the properties of metastatic cells. The stromal microenvironment provides an opportunistic niche in which circulating tumour cells can evade the immune system and be refractory to conventional therapies. A better understanding of tumour-stroma interactions may identify critical factors regulating metastatic progression and lead to the development of stromal therapies for breast and other malignancies. Here, the evidence implicating stromal factors in the metastasis of breast tumours to bone will be reviewed.

Key words: Bone metastasis, breast cancer, animal model, microenvironment, TGF-β, stromal therapy

1. METASTATIC PROGRESSION

Metastasis is a dynamic process consisting of a series of interrelated events, each involving interactions between the tumour cell and the tissue specific microenvironment of the host. Each stage needs to be completed to produce a secondary tumour. A primary tumour cannot grow beyond $1mm^3$ without an adequate blood supply (1). In hypoxic conditions, tumour cells hijack normal growth processes by inducing the expression of several cytokines, pro-angiogenic factors and growth factors within the microenvironment (2, 3). Having established a vascular network, tumour cells invade the surrounding stroma and intravasate into the circulation. This requires the recruitment of active proteolytic enzymes including matrix metalloproteinases (MMPs), the serine proteases urokinase (uPA) and tissue plasminogen activator (tPA), plasmin and thrombin into the invading front of the tumour (4, 5). Most proteases are actually expressed by the host and are activated by the presence of tumour cells (6). Proteolytic degradation of the extracellular matrix (ECM) enhances tumour progression by releasing entrapped growth factors and revealing cryptic adhesive binding sites. Integrins can bind to these adhesive sites and activate intracellular signalling cascades that promote cellular division, motility and invasion (7, 8). Whilst in circulation, tumour cells must survive anoikis and vasculature turbulence, prior to arresting in the capillary bed of a distant organ. The tumour cells must then extravasate from the circulation and successfully colonize the secondary organ, an event

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that requires the cell to survive and re-initiate proliferative activity. If these pre-metastatic lesions successfully evade the immune response of the host and attract a new blood supply, they will establish as clinically relevant metastatic lesions. Further, growing metastases can shed tumour cells into the circulation and initiate the development of other metastatic lesions, a phenomenon known as metastasis of metastases (9, 10).

Metastasis is a highly inefficient process. A tumour cell that is incapable of completing any of one of these events will fail to produce a clinically relevant lesion. Hence, each step of the metastatic process is a potential therapeutic target, with some steps being more rate limiting than others (11). For instance, studies using *in vivo* video microscopy of tumour cells have shown that 80% of melanoma cells arrest in the liver after injection into the superior mesenteric vein but only a fraction (0.02%) of them form overt metastases (12). Similarly, RT-PCR based assays for tumour cell markers have been utilized to detect micrometastases in the bone marrow of 25-70% of patients with common malignancies, including those tumour types that do not generally form bone metastases (reviewed in (13)). Collectively, these data suggest that although metastatic spread to specific sites such as bone is relatively efficient, it is the ability of the tumour cell to survive, proliferate and establish in the secondary site that is rate limiting. As with earlier steps in metastasis, factors within the microenvironment of the secondary site play a dominant role in subsequent growth of the metastatic nodule (11).

It is known that different tumour types have tissue specific metastatic patterns. Breast cancer cells commonly metastasize to the lymph nodes, lung, liver and bone. Such specific spread of tumour cells was originally observed by Steven Paget in 1889, who coined the “seed and the soil” hypothesis (14). This hypothesis stated that for breast tumour cells (the “seed”) to spread to distant tissues (the “soils”) the microenvironment of the organ must be congenial to their growth. More recent studies have shown that bone complications arising from breast cancer occur in approximately 70% of patients (15). Current hypotheses point to the function, structural composition and stromal-tumour cell interactions within bone that aid colonization by metastatic cells (13, 16, 17).

2. REGULATION OF NORMAL BONE REMODELLING

Bone is a dynamic organ, undergoing constant remodelling involving active destruction and re-synthesis of the bone matrix. Within normal adult bone, homeostatic mechanisms maintain the balance between the bone forming osteoblasts and the bone resorbing osteoclasts (Figure 1).

Osteoblasts arise from mesenchymal osteoprogenitor cells (reviewed in (18)). During development, these cells secrete a complex mixture of growth factors and ECM proteins into the surrounding bone microenvironment (bone matrix) before they either apoptose or terminally differentiate into osteocytes (the cellular component of hardened bone). The majority of bone matrix protein consists of type I collagen fibres (85-90%), which provide structural support for the mineralisation of bone (19). The remaining 10-15% consists of proteoglycans, γ-carboxylated (gla) proteins, cell adhesive proteins and growth factors. A large number of adhesive proteins found in bone contain RGD (Arg-Gly-Asp) motifs (17); examples of these are type I collagen, bone sialoprotein, fibronectin, laminin-10, osteopontin, thrombospondin and vitronectin. The RGD motif is a well-characterized binding site for several adhesion receptors of the integrin family and, depending on substrate- receptor context, can regulate cellular motility, invasion and growth (20). Osteoblasts also secrete growth factors into the bone matrix, including transforming growth factor-β (TGF-β), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), interleukins, platelet derived growth factor (PDGF) and bone morphogenic proteins (BMPs) (21), (22).

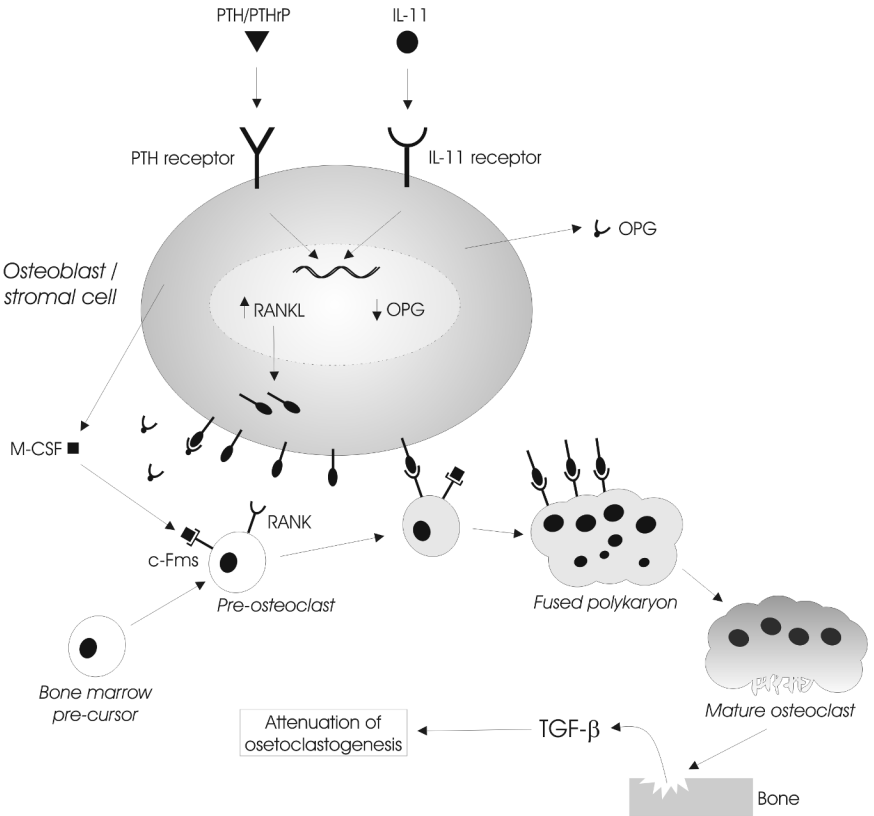

Figure 1. A model of normal bone remodelling. Bone is continually being remodelled, a process that requires interactions of bone forming osteoblasts with bone resorbing osteoclasts. Osteoclastogenesis occurs as a result of the interaction of bone marrow precursor cells, osteoblasts and bone marrow stromal cells with a multitude of growth factors, hormones and cytokines that alter the expression of key osteoclastogeneic factors RANK, RANKL, OPG and M-CSF. Active osteoclasts resorb bone and subsequently release growth factors including TGF-β, which attenuates osteoclastogenesis by increasing osteoblast proliferation and decreasing osteoclast activity.

These growth factors remain latent in the bone matrix but can be released and activated upon proteolytic degradation of the bone. FGF, PDGF, TGF-β and several BMPs have been reported to enhance the differentiation and growth of osteoblasts (reviewed in (23)). Thus, release of these factors from the bone matrix provides a feedback mechanism to promote bone formation and attenuate bone resorption.

Osteoclasts are differentiated cells arising from the monocyte-macrophage lineage. The primary role of the osteoclast is to resorb bone. Activated osteoclasts are recruited to the bone surface and attach through interactions with the $\alpha_v\beta_3$ integrin receptor (24). This interaction is crucial in the bone remodelling process as β3 integrin knockout mice develop osteosclerosis due to the lack of functional osteoclasts (25). Osteoclasts acidify the local microenvironment at the bone-osteoclast interface ("resorption zone") and secrete several proteases such as MMPs and cathepsins B, L, K and S, which are used to degrade components of the ECM. The most abundant protease expressed by osteoclasts is cathepsin K which targets type I collagen (26, 27). Whilst cathepsin K seems to be the prevalent protease in solubilisation of the bone matrix, several MMPs have also been implicated in the proteolysis of bone (reviewed in (28)). Interestingly, osteoclast secreted MMPs – MMP-9, MMP-10, MMP-12 and MMP-14 – do not contribute significantly to bone degradation, whilst MMP-13, an osteoblast secreted MMP with collagenase activity, can be recruited into the resorption zone and degrade bone (29, 30). In addition to bone proteolysis, several MMPs have

been implicated in the regulation of osteoclast signalling, migration and invasion (28).

Osteoclastogenesis, the development of mature osteoclasts, is a process that is tightly regulated through a complex network of cytokines and receptor interactions within the bone stroma (Figure 1). In particular, stromal expression of macrophage colony-stimulating factor (M-CSF) and the receptor activator of NFκB ligand (RANKL) are necessary and sufficient to induce osteoclastogenesis *in vivo* and *in vitro* (31). M-CSF, through binding to its receptor c-Fms, acts as a survival factor for osteoclast precursor cells allowing them to respond to inducers of osteoclastogenesis. Expression of membrane bound RANKL is induced in stromal cells and osteoblasts by various stimuli, including parathyroid hormone (PTH), PTH related protein (PTHrP), calcitriol, tumour necrosis factor-α (TNF-α), glucocorticoids, prostaglandin E_2 (PGE_2), interleukin-1 (IL-1), interleukin-11 (IL-11), thyroid hormone, fibroblast growth factor-2 (FGF-2) and insulin like growth factor-1 (IGF-1) (32). Binding of RANKL to its membrane receptor RANK on osteoclast precursors activates inhibitor of NFκB kinase (IKK), c-Jun N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK) and Src signalling pathways that cooperate to induce the differentiation of haematopoietic progenitors into mature osteoclasts (31). Mice with homozygous deletions in either RANKL or RANK have no functional osteoclasts and develop severe osteopetrosis (33, 34), demonstrating the critical importance of the RANKL/RANK interaction in osteoclastogenesis.

Osteoprotegerin (OPG), a member of the tumour necrosis factor receptor superfamily, is secreted by osteoblasts and other bone stromal cells and suppresses osteoclastogenesis by competing with RANK for RANKL binding (35, 36). Consistent with this, OPG deficient mice exhibit decreased bone density due to increased osteoclast activity (37). The regulation of RANKL and OPG are intertwined as evidenced by factors such as IL-11, PTHrP and PGE_2 that increase RANKL but suppress OPG expression (32). Conversely, active TGF-β released during osteolysis stimulates osteoblastogenesis and attenuates osteoclastogenesis by increasing OPG and suppressing RANKL expression (38, 39).

Any perturbation of the delicate balance between osteoblast mediated bone formation and osteoclast mediated bone resorption is likely to impinge on normal bone turnover, resulting in enhanced bone degradation or formation. Tumour cells homing to bone cause an imbalance in osteoblast-osteoclast regulation to promote their survival and proliferation in this organ.

3. MODELLING THE PROCESS OF BREAST CANCER METASTASIS TO BONE

The development of improved animal models of metastasis has increased our understanding of the molecular mechanisms that regulate the colonization of breast cancer cells in bone. An excellent review on the current models of tumour metastasis to bone is available (40). In 1988, a mouse model was described in which melanoma established in bone following the inoculation of cells into the arterial circulation of immunocompromised mice (41). Since its conception, this model has been extensively used and has provided much insight in the mechanisms of metastatic colonization of bone by several tumour lines including breast (42-46). A further advance came from the development of a syngeneic mouse model that can spontaneously metastasize to bone following inoculation into the mammary fat pad (43, 47). In this model, the 4T1.2 tumour line produces spontaneous lung and osteolytic bone lesions following the inoculation of as few as 1000 cells into the mammary gland (Figure 2). The model is invaluable for studies of metastatic progression as it mimics both early and late stages of human breast cancer metastasis to bone. We are currently utilizing cDNA array profiling of this model and functional analysis to identify genes that are associated with metastatic progression. The model allows the contribution of both the stromal and tumour cell compartments in bone to be assessed.

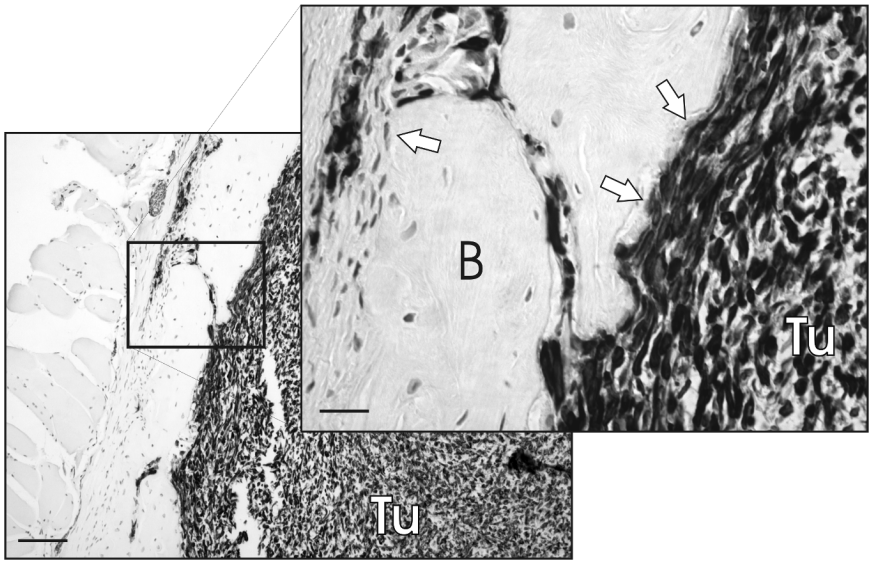

Figure 2. Cytokeratin 18 expression identifies bone metastases following growth of the 4T1.2 primary tumour in the mammary fat pad. These bone lesions are highly osteolytic, as indicated by the presence of osteoclasts (arrows;TRAP positive cells identified on an adjacent section) and the fracturing of the cortical bone. B = cortical bone, Tu = tumour cells. Scale bar; 100μm or 20μm (inset).

4. CHEMOKINE MEDIATED TUMOUR CELL HOMING TO BONE

The selectivity of metastatic cells for certain tissues is dictated, in part, by the spectrum of surface receptor molecules expressed on the cancer cell and by the presence of complementary ligands at the secondary site. When disseminating tumour cells arrive in bone they arrest in the small endothelium-lined venous sinusoids. These sinusoids are fenestrated and lack a basement membrane, making them highly permeable and permissive for the removal of metabolic waste or cellular migration into the organ. In fact, Muller et al. (2001) proposed that the metastatic dissemination of breast tumour cells is akin to the normal trafficking of leukocytes from the bloodstream into and out of target organs – a process that is critically regulated by chemokines and their receptors. Metastatic breast cancer cells, malignant tumours and metastatic nodules express high levels of the chemokine receptors CXCR4 and CCR7 compared to normal mammary epithelium. The ligands for these receptors, SDF-1α (CXCR4) and 6Ckine (CCR7), are expressed abundantly in tissues to which breast cancer metastasizes most avidly, namely lung, lymph node, liver and bone. The role of these receptors in breast tumour homing to bone is supported by the observation that neutralizing antibodies directed against CXCR4 inhibit *in vitro* migration of MDA-MB-231 cells towards a chemotactic gradient of SDF-1α. Similarly, neutralizing antibodies to CXCR4 inhibit experimental and spontaneous lung metastases derived from MDA-MB-231 tumours in SCID mice (48). Although the role of CXCR4/SDF-1α in metastasis to bone was not analysed, other studies have shown that SDF-1α signalling through CXCR4 stimulates transendothelial migration of prostate cancer cells (49). Collectively, these results implicate a potential 'homing' mechanism for the attraction of metastatic breast tumours to bone.

5. INTEGRIN MEDIATED TUMOUR CELL ADHESION IN BONE

Attachment to the vasculature and subsequent extravasation from the blood stream requires integrin-mediated tumour cell adhesion to

endothelial ECM components. Integrins are membrane bound receptors that function as heterodimers of α and β subunits. Combinations of α and β subunits confer substrate and signalling specificity (50) and several pairings including $\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$ and $\alpha_v\beta_3$ have been extensively studied in breast cancer metastasis (see reviews (51-53)). The $\alpha_v\beta_3$ integrin is of particular interest, as it is frequently upregulated during metastatic progression and is a receptor for several ECM proteins commonly found in bone including fibronectin, osteopontin, bone sialoprotein and vitronectin (8, 50). Engagement of integrin $\alpha_v\beta_3$ to fibronectin or vitronectin *in vitro* modulates several intracellular signalling pathways involving Rho GTPases, FAK, Src and PKC, leading to cytoskeletal changes and enhanced motility (54-56). Clinically, high $\alpha_v\beta_3$ integrin expression in primary breast cancers is correlated with a greater metastatic potential and the development of skeletal metastases (57). Further evidence for the role of tumour-associated $\alpha_v\beta_3$ integrin in metastasis comes from studies demonstrating that MDA-MB-435 cells selected for high levels of activated $\alpha_v\beta_3$ integrin display an enhanced ability to form spontaneous bone and lung metastases following orthotopic injection in mice (58). Integrin $\alpha_v\beta_3$ may play multiple roles during the metastatic spread of breast tumours to bone and appears to be required for the interaction of tumour cells with platelets and subsequent aggregation in thrombi and arrest in distant capillaries (58, 59). It has been demonstrated that $\alpha_v\beta_3$ integrin can recruit and activate local MMPs (MMP-2 and MMP-9, for instance) thereby facilitating ECM degradation and cellular migration (60, 61) and potentially enhancing the extravasation of tumour cells into bone.

6. TYPES OF BONE METASTASES

Bone metastases can be categorized into three distinct phenotypes: osteolytic (bone resorbing), osteoblastic (bone forming) and mixed lesions containing elements of both (62). In patients with advanced breast cancer, the majority of bone lesions are osteolytic, while approximately 15-30% are osteoblastic, and 5% have mixed lesions (62, 63). In contrast, patients with advanced prostate cancer generally develop osteoblastic lesions. These phenotypes reflect the perturbation of normal bone remodelling processes by the presence of tumour cells. Interestingly, secondary bone formation is observed in osteolytic lesions formed by breast tumour cells and some bone resorption occurs in osteoblastic metastases (62, 64). This suggests that the pathology of each type of lesion is not static, rather, the observed phenotype in each metastatic lesion results from a shift in the dynamic equilibrium of normal bone remodelling.

7. OSTEOLYTIC BONE METASTASIS

Breast tumour lines such as MDA-MB-231, MDA-MB-435 and 4T1.2 are responsive to growth factors found in bone and promote their release from bone by activating osteolytic mechanisms. The 'vicious cycle' theory describes the special predilection of breast tumours to metastasize to bone (65) by proposing that dual paracrine feedback mechanisms operate between the tumour and bone stromal cells, leading to the uncoupling of osteoblast-osteoclast signalling, resorption of bone and amplification of metastatic tumour growth (Figure 3).

Although tumour cells have been implicated in the direct resorption of bone *in vitro* (66), the majority of bone degradation *in vivo* is mediated by activated osteoclasts (67). Osteoclasts regulate the activity of several proteolytic factors, including cathepsins, uPA and MMPs, which degrade the bone matrix and release and activate several growth factors from mineralised bone such as TGF-β (68, 69). TGF-β has been shown to elicit diverse responses in bone including cellular proliferation, ECM deposition, protease production, angiogenesis and suppression of immune surveillance (70-72). Although TGF-β inhibits the proliferation of normal mammary epithelium and delays the development of primary breast tumours (73, 74), it appears to promote the establishment of epithelial tumour cells in bone. Experimental MDA-MB-231 metastasis to bone is reduced when cells are made insensitive to the action of TGF-β by transfection of a dominant-negative TGF-β type II receptor (TβRII) (75). Furthermore, metastases derived from this TGF-β

insensitive cell line are less osteolytic and fewer activated osteoclasts are observed compared to the parental cell line. Transfection of a constitutively active TGF-β type I receptor in these cells restored the osteolytic phenotype.

Further reports have shown that TGF-β leads to altered gene expression in breast cancer cells by activating SMAD and p38 MAPK pathways (76). Several TGF-β responsive genes have been implicated in the development of osteolytic metastases. These include TNF-α, PTHrP, IL-11 and IL-6 (77-79). As described above, PTHrP and IL-11 stimulate osteoclastogenesis in normal bone by elevating RANKL and suppressing OPG expression in osteoblasts. This leads to the release of matrix-associated growth factors that further enhance osteoclastogenesis and tumour cell growth (Figure 3).

PTHrP is expressed in 50-70% of primary breast carcinomas (80, 81) but its expression is markedly elevated in bone metastases (82). Whilst PTHrP expression in the primary tumour is associated with improved survival and reduced metastasis (81), in bone it has the potential to be induced in tumour cells by the bone microenvironment and thereby promote tumour growth in bone. This has been demonstrated in an experimental bone metastasis assay using MDA-MB-231 cells (44). Furthermore, increased expression of PTHrP in MCF7 cells (which were weakly osteolytic in this study), promoted experimental bone metastatic lesions with an enhanced osteolytic phenotype (83). The use of neutralizing PTHrP antibodies for the treatment of osteolytic bone disease is currently under clinical investigation (67).

IL-6 and IL-11 are multifunctional cytokines that can enhance osteoclastogenesis and bone resorption in bone organ cultures (84), through mechanisms that increase RANKL/RANK signalling and inhibit osteoblast calcification (79, 84). Although IL-6 and IL-11 bind to separate receptors, both cytokines transduce signals through the gp130 receptor. Signalling through the gp130 receptor in osteoblasts is critical for the induction of osteoclastogenesis, as neutralizing antibodies to this receptor inhibit the formation of active osteoclasts in bone organ cultures (85). The expression of IL-11 is upregulated in tumour cells upon TGF-β stimulation and in osteoblasts upon PTHrP or TGF-β stimulation (77, 79). IL-11 also acts in an autocrine manner to induce PGE_2 expression in osteoblasts (77). PGE_2 potentiates osteoclast activation by further increasing RANKL expression, while suppressing the inhibitory factors OPG and granulocyte-macrophage colony stimulating factor (GM-CSF) in stromal cells (77, 86).

A recent study utilized microarray profiling to identify genes that are causal to the establishment of breast tumours in bone (46). Several genes relating to bone colonization efficiency were identified by expression profiling of parental MDA-MB-231 cells and bone metastatic variants isolated from bone after intracardiac inoculation. Among the genes identified were osteopontin (OPN), CXCR4, IL-11 and connective tissue growth factor (CTGF), all of which were expressed at higher levels in the bone metastatic variants. Although expression of any one of these four genes in parental MDA-MB-231 cells produced little, if any change in metastatic potential, co-expression of two or more enhanced the ability of the cells to colonize bone. Using chromatin immunoprecipitation assays, they demonstrated that induction of IL-11 and CTGF expression resulted from activation of the TGF-β/Smad signalling pathway in the tumour cells. Consistent with this, the expression of IL-11 and CTGF could be induced by treatment of MDA-MB-231 cells with TGF-β (46).

Genes identified in this study may constitute novel therapeutic targets for metastatic bone disease. CTGF is an extracellular matrix protein that has been implicated in bone remodelling and angiogenesis (reviewed in (87)). By binding to cytokines in the bone matrix, CTGF can modulate cellular signalling. CTGF binds to both BMP4 (a known inducer of bone formation) and TGF-β and antagonizes the former but promotes the signalling of the latter (88). Through suppression of BMP4 and induction of TGF-β signalling, CTGF could potentially be involved in the vicious cycle of osteolytic bone metastases. Collectively, these studies show that successful bone metastasis requires the coordinated action of multiple paracrine pathways, in which TGF-β signalling plays a central role by altering the bone microenvironment and promoting the growth of the metastatic lesion.

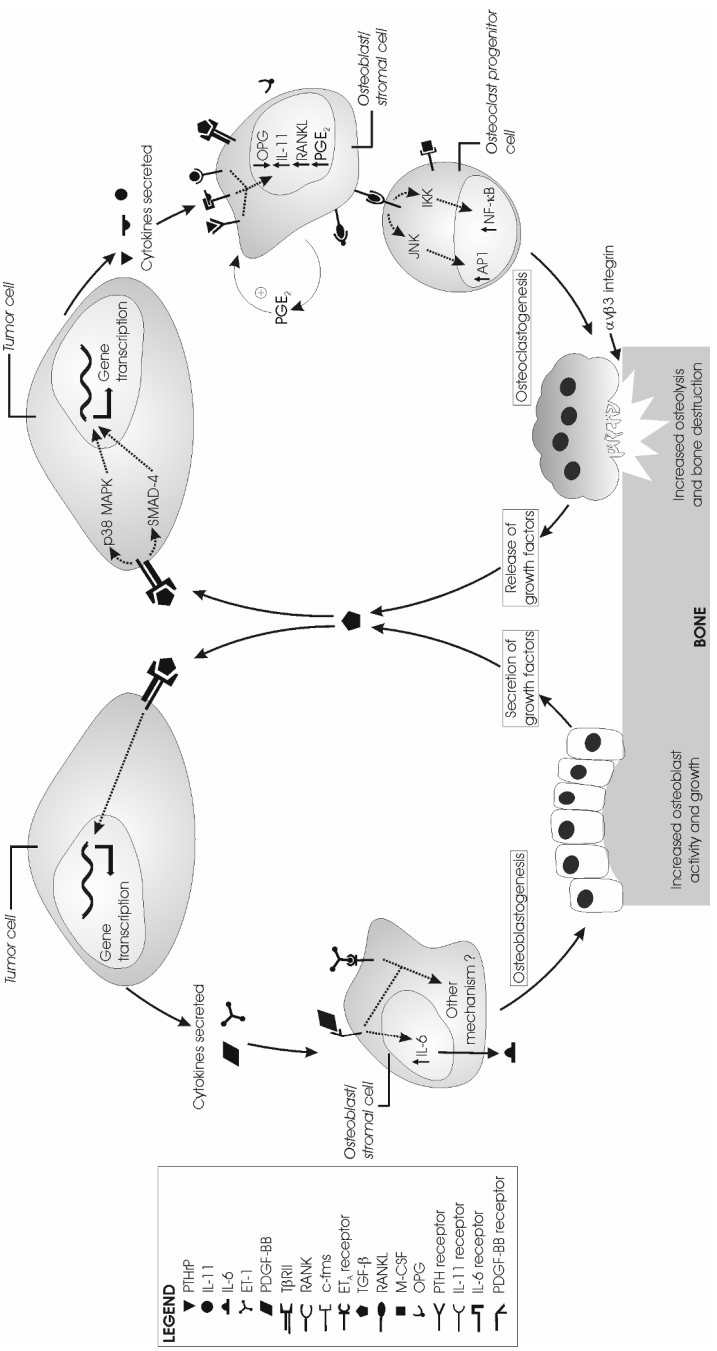

Figure 3. Interactions between tumour cells and the bone microenvironment. Breast tumour cells disrupt the homeostatic mechanisms that regulate normal bone remodelling, leading to morphological changes in bone structure and enhanced release of bone derived cytokines that aid the growth and establishment of the tumour cell. Lesions can be osteoblastic, leading to increased bone deposition (left side of diagram) or osteolytic, leading to bone loss (right side of diagram).

8. OSTEOBLASTIC METASTASIS

Osteoblastic metastases are less common in breast cancer but are well documented in metastatic prostate cancer. The mechanisms responsible for the formation of osteoblastic metastases in both types of cancers are poorly understood. Recently, this area of research has been strengthened by the development of new models of breast cancer with associated osteoblastic metastases.

MCF-7 cells expressing the *Neu* oncogene (MCF-7/*Neu*) produce overt osteoblastic bone metastases (with ectopic sites of active osteolysis) and high plasma levels of PDGF-BB after arterial inoculation of cells into nude mice (64). PDGF-BB is a potent osteotrophic factor expressed by osteoblasts, osteoclasts and aggregated platelets (89). Introduction of antisense DNA or neutralizing antibodies reduced PDGF-BB levels *in vivo* and subsequently decreased metastatic burden in bone. Furthermore, overexpression of PDGF-BB in osteolytic MDA-MB-231 cells resulted in the formation of mixed osteolytic/osteoblastic lesions *in vivo*. Comparisons of PDGF-BB transfected MDA-MB-231 cells and control cells showed no difference in PTHrP levels, which may account for the observed mixed phenotype. Interestingly, PDGF-BB can induce IL-6 expression in osteoblasts (90) and can directly activate osteoclasts *in vitro* (91), which could potentially explain the partial-osteolytic nature of the MCF-7/*Neu* cells. A mechanism for the formation of osteoblastic metastases by PDGF-BB expressing tumour cells has not been elucidated *in vivo*, however bone stromal cells express PDGF-BB receptors and signalling through these receptors could disrupt osteoblast-osteoclast homeostasis in favour of enhanced bone growth.

Three breast tumour lines (MCF-7, T47D and ZR-75-1) have been reported recently to produce osteoblastic metastases after intracardiac inoculation in *nude* mice (92). In these tumour lines, endothelin-1 (ET-1) was found to be the secreted factor that was causal for the formation of osteoblastic metastases. Endothelin-1 regulates angiogenesis, osteoblast proliferation and activity *in vitro* and is elevated in the serum of patients with advanced prostate cancer (93-95). ET-1 can act via two receptors, ET_A or ET_B, which are expressed in bone stromal cells. *In vivo* studies in the model described by Yin et al., demonstrated that blockade of the ET_A receptor prevented the ability of ZR-75-1 but not MDA-MB-231 tumour cells to colonize bone (92).

Several bone-derived growth factors including IL-1β, TNF-α and TGF-β can increase ET-1 in PC3 cells *in vitro* (96). Hence, it is plausible that the 'vicious cycle' theory proposed for the mechanism of osteolytic bone destruction by breast cancer cells may also hold true for the establishment of osteoblastic bone lesions. In this case, ET-1 stimulates osteoblast activity, which enriches the local microenvironment with growth factors that induce tumour growth and subsequent expression of ET-1 (Figure 3). A dual role for TGF-β for the establishment of either osteolytic or osteoblastic metastases would be most intriguing, however a causal role for TGF-β in the formation of ET-1 induced osteoblastic breast cancer metastases remains to be established.

9. BONE STROMAL THERAPY FOR METASTATIC BREAST CANCER

The interaction between breast tumour cells and the host stroma is critical for the successful formation of bone lesions. Since the events that lead to bone resorption and to the release of factors from bone can contribute to survival and establishment of tumour cells in bone, therapies designed to target the mechanisms of osteoclastogenesis may prove to be effective. Over the last decade, several therapeutic approaches that target stromal-tumour interactions including proteolytic inhibitors, inhibitors of osteoclastogenesis and compounds that disrupt the action of breast tumours in bone have been developed (Table 1).

Table 4 . Bone stroma targeted compounds currently in use or under investigation as inhibitors of bone metastases.

Target	Mechanism	Stage	Ref.
Bisphosphonates	Bone resorption; tumor cell growth and reduced bone pain	In clinical use	102
Osteoprotegerin	Prevents RANKL binding to RANK to activate osteoclasts	Phase I	106
RANKL antibody	Prevents RANKL binding to RANK to activate osteoclasts	Phase I	104
PTHrP antibody	Neutralizing PTHrP effects	Phase III	67
Vitamin-D analogues	Inhibition of PTHrP expression	Phase III	67
MMP inhibitors	Inhibition of proteolysis	Phase III	113
integrin beta3 inhibitors	Osteoclast adhesion, tumor migration	preclinical	108

Bisphosphonates, based on their affinity for calcium ions, have a high avidity for mineralised bone (97). When released by osteolysis, bisphosphonates are readily absorbed by the osteoclast, resulting in altered metabolism and induction of apoptosis (98). In Phase III clinical trials zoledronic acid, a nitrogen containing bisphosphonate, has shown efficacy in reducing skeletal morbidity in patients with bone metastatic tumours (99, 100). Zoledronic acid inhibits the formation of mature osteoclasts by preventing the fusion of osteoclast precursors, most likely through the disruption of RANKL/RANK signalling (98, 101). It also displays direct anti-tumour effects by inducing apoptosis, inhibiting breast cancer cell invasion and reducing angiogenesis *in vitro* (102).

Reduced tumour-induced bone destruction can also be achieved by disruption of RANKL/RANK signalling through the administration of monoclonal antibodies to RANKL, recombinant OPG or RANK-Fc fusion proteins. These proteins compete with RANK for RANKL binding, effectively reducing osteoclastic bone resorption in several pre-clinical models (103-105). Initial clinical trials of recombinant OPG administration in patients with advanced breast cancer have produced promising results. The agent is well tolerated and suppresses bone resorption to a similar extent as pamidronate (106). Although shown to be an effective suppressor of tumour induced osteolysis in experimental models of myeloma (ARH-77 cells) and prostate cancer (LNCap cells) (105, 107), RANK-Fc is yet to be tested in models of breast cancer.

Tumour expressed integrin receptors that interact with components of the bone extracellular matrix offer another therapeutic target. Using β_3 knockout mice Bakewell et al., demonstrated that β_3 integrin is crucial for tumour cell adhesion to platelets and entry into the bone marrow and suggest that drugs designed to target platelet $\alpha_{II}\beta_3$ integrin may be a promising antimetastatic therapy (108). The $\alpha_v\beta_3$ integrin or downstream components of its signalling pathway are also attractive targets, since $\alpha_v\beta_3$ integrin expression is not widespread but is elevated in bone metastatic tumours, activated osteoclasts and angiogenic vessels (17). Soluble collagen type I fragments effectively inhibit the adhesion of tumour cells to bone (109). Similarly, neutralizing antibodies to α_2, α_3, α_5, α_V, β_1, β_3, and β_5 integrin subunits inhibit the *in vitro* adhesion of breast tumour cells to bone matrix (109, 110). Furthermore, neutralizing antibodies to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins disrupt tumour angiogenesis, migration and proliferation of breast cancer cells (8, 111). Specific peptide inhibitors to $\alpha_v\beta_3$ integrin prevent osteoclast mediated bone resorption *in vitro* and bone loss in an *in vivo* model of osteoporosis (112). Collectively, these studies support the use of integrin inhibitors to block the development of osteolytic lesions, however this strategy is yet to be tested in metastasis-associated bone resorption *in vivo*.

The recent development of pre-clinical models of osteoblastic metastasis have already made an impact on therapeutic interventions that target the bone stroma (92). As described previously, blocking the interaction of the osteoblastic factor such as ET-1 through the use of drugs targeting the ET_A receptor, reduced osteopetrosis and metastatic burden. Interestingly, targeting of the ET_B receptor did not inhibit osteoblastic bone metastases in this model, suggesting that the action of ET-1 to induce osteoblastic bone lesions is specific to the ET_A receptor.

In summary, the successful establishment of metastatic bone tumours requires complementary interactions between the tumour cells and the local microenvironment. Bone is a rich source of several stimulatory factors that are released after proteolysis of the bone matrix. In particular, TGF-β appears to play a critical role in the establishment of a vicious cycle of tumour growth in bone (Figure 3). Current therapeutics aim to inhibit the known interactions between the bone stroma and tumour cells that induce osteotrophic bone lesions. To date, only a handful of genes that drive tumour growth in bone have been identified. Studies on the TGF-β signalling axis in breast cancer metastasis clearly demonstrate how a locally produced cytokine can aid in the establishment of bone metastases. However, it is likely that several other cytokines including BMPs, interleukins and IGFs are also important but their role in breast cancer metastasis to bone will require further investigation. Implementation of gene expression profiling on clinically relevant models of breast cancer metastasis to bone will aid in the identification of novel genes required for the formation of bone metastatic lesions. These may prove to be relevant therapeutic targets and lead to the development of improved treatments for metastatic breast cancer.

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