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Review

Genes involved in breast cancer metastasis to bone

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Abstract. Metastasis to bone occurs frequently in advanced breast cancer and is accompanied by debilitating skeletal complications. Current treatments are palliative and new therapies that specifically prevent the spread of breast cancer to bone are urgently required. While our understanding of interactions between breast cancer cells and bone cells has greatly improved, we still know little about the molecular determinants that regulate specific homing of breast cancer cells to the bone. In this review, we focus on genes that have been implicated in migration and adhesion of breast cancer cells to bone, as well as genes that promote tumor cell proliferation in the bone microenvironment. In addition, the review discusses new technologies, including better animal models, that will further assist with the identification of the molecular determinants of bone metastasis and will guide the development of new therapies.

Key words. Breast cancer; metastasis; bone resorption; gene; new therapy.

The clinical problem

Metastasis to bone is a common and painful consequence of advanced breast cancer. Bone is the most prevalent site of first distant relapse of breast cancer, with approximately 70% of patients with advanced breast cancer suffering from bone metastases [1, 2]. The majority of bone metastases from breast cancer are osteolytic. Resorption of bone in these metastases leads to complications including osteoporosis, hypercalcemia, spinal cord compression and fractures of the long bones. Quality of life becomes an issue for these patients, especially since the median survival after diagnosis of bone disease is considerably longer (20–30 months) than in patients who are first diagnosed with soft tissue metastases (3–5 months) [2]. The morbidity associated with bone metastasis and the long clinical course of the disease in patients with bone complications highlight the need for effective therapies.

Once bone disease has developed, current therapies are rarely curative. Palliative radiotherapy and surgery are occasionally used to reduce the size of tumor deposits in bone. Chemotherapy is usually not successful, due to drug resistance that commonly develops during progression of the disease. Bisphosphonates have been used for the past two decades to treat hypercalcemia and skeletal metastases. These drugs reduce the frequency and severity of skeletal complications by preventing bone resorption, although survival is generally not improved in patients with advanced breast cancer [3, 4]. Bisphosphonates incorporate into the bone matrix, rendering it more resistant to resorption by osteoclasts. In addition, they inhibit the formation and osteolytic activity of osteoclasts and induce apoptosis in osteoclasts [5, 6]. Bisphosphonates also exert pro-apoptotic and anti-adhesive effects on tumor cells in vitro; however, the importance of these effects in vivo has yet to be determined [reviewed in ref. 7].

New treatments that specifically prevent or reduce metastasis of breast cancer to bone are urgently required. Development of such therapies requires a better understand-

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ing of the molecular mechanisms responsible for the specific homing and proliferation of breast cancer cells in bone. However, research in this field has been hampered by a lack of physiologically relevant models. Most studies to date have made use of the cell line MDA-MB-231 that was derived from a pleural effusion in a breast cancer patient [8]. MDA-MB-231 cells form lung metastases after intravenous injection. After injection into the left ventricle of the heart, MDA-MB-231 cells proliferate in bone, but rarely form non-osseous metastases [9, 10]. The MDA-MB-231 intracardiac model has been informative for investigating the later stages of metastasis, including proliferation of breast cancer cells in bone. However, the poor rate of metastasis to non-osseous sites has made it difficult to verify the specificity of the effects of genes on metastasis to bone. An orthotopic model of breast cancer metastasis to bone and soft tissue sites that has been developed will greatly facilitate research in this field [11]. In this syngeneic Balb/c mouse model, bone and soft tissue metastases arise spontaneously after mammary fat pad injection of the tumor cell line 4T1.2. Independent cell lines derived from the same primary tumour as 4T1.2 exhibit different metastatic phenotypes making this a powerful model for investigating site-specific metastasis.

The ability to metastasize is the single most important life-threatening characteristic of a malignant breast tumor. Metastasis is a multistage process that begins with the acquisition of a motile and invasive phenotype by primary tumor cells. Intravasation of local capillaries and lymph ducts is followed by circulation around the body and arrest by adhesion to the vessel wall. Extravasation from the vessel allows infiltration and proliferation in specific secondary tissues [12].

Why is bone such an attractive site for breast cancer cells? Bone is a highly vascularized organ that provides a fertile environment for colonization by tumor cells. However, it has long been recognized that metastasis is not a random process and that different types of tumors preferentially metastasize to specific sites. The principle of specific interaction between breast cancer cells and the bone microenvironment was first postulated by Paget in 1889. After studying autopsy records of 735 breast cancer patients, he recognized that 'in cancer of the breast the bones suffer in a special way, which cannot be explained by any theory of embolism alone' [13]. Other tumor types, including renal, lung, thyroid and prostate cancer, also demonstrate a predilection for growth in bone [14], while some, including stomach and ovarian cancer, rarely metastasize to bone. Thus, cells of different lineages differ in their ability to home to bone, to proliferate in the bone environment and to interact with stromal components of bone.

This review will consider genes that are involved in the steps leading to bone metastasis. Table 1 lists genes that are important in breast cancer metastasis. Genes highlighted in bold have been implicated in bone metastasis and will be discussed here. The first part of the review focuses on genes that have been implicated in homing of breast cancer cells to bone. The next part concentrates on genes that are involved in interactions between breast cancer cells and bone cells. In particular, the role of bone resorption, and the possible role of osteoclast-independent osteolysis in the development of bone metastases will be discussed. Finally, the review considers new techniques, including better animal models, which together with array technologies will greatly assist with the identification of the molecular determinants of bone metastasis.

Table 1. Genes involved in breast cancer metastasis.

Stage of metastasis	Gene	Ref.
Chemotaxis	**chemokine receptor CXCR4**	41
	osteonectin	46
Invasion and adhesion	**$\alpha v\beta 3$ integrin**	54
	autocrine motility factor	103
	bone sialoprotein	104
	cathepsin D	105
	cortactin	20
	galectin-3	106
	matrix metalloproteinases	28, 90
	osteopontin	107
	urokinase system	31
	semaphorin	108
Growth in bone	**insulin-like growth factor-1**	109
	interleukin-11	87
	parathyroid hormone-related protein	82, 83
	transforming growth factor-β	66
Metastasis suppressors	breast cancer metastasis suppressor 1 (BRMS1)	110
	E-cadherin	10, 15
	Kai1	111
	KiSS-1	112
	Nm23	113
	tissue inhibitors of metalloproteinases	21

Genes implicated in metastasis to bone are in bold and will be discussed in more detail in the review. The RefSeq/LocusLink designation for these genes is available from http://www.ncbi.nlm.nih.gov/LocusLink.

Homing determinants

Breast cancer cells express a number of genes that may act as homing determinants to facilitate their migration to bone. These include genes that promote invasion and allow extravasation from capillaries within bone marrow, genes that confer responsiveness to chemotactic cues and genes that allow adhesion to the bone extracellular matrix.

Invasion and motility

Acquisition of an invasive phenotype is an essential step in metastasis. Metastatic cells lose expression of genes such as E-cadherin that mediate adhesive interactions at the primary site and hinder extravasation from the vasculature [15]. In contrast, metastatic cells gain expression of integrins that enhance adhesive interactions at the secondary site. Metastatic cells become motile by modifying their cytoskeleton to form invadopodia and lamellipodia by expression of genes such as cortactin [16, 17]. In addition, secretion of proteases including matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA) allows degradation of the basement membrane and connective tissue, and access to the secondary site [18, 19].

Each of the genes described above has been implicated in metastasis to bone using the MDA-MB-231 intracardiac model. Thus, overexpression of cortactin in MDA-MB-231 cells has been shown to promote osteolytic lesions, but not metastasis to other sites, after intracardiac injection in nude mice [20]. In contrast, expression of a dominant negative cortactin mutant decreased skeletal tumor burden in this model. Similarly, restoring E-cadherin expression, overexpressing the MMP inhibitor TIMP-2, or disrupting the function of the UPA receptor (uPAR) in MDA-MB-231 cells all reduce the frequency and size of osteolytic lesions and diminish skeletal tumor burden in nude mice after intracardiac injection compared to parental MDA-MB-231 cells [10, 21, 22]. However, the studies do not demonstrate that these genes specifically influence metastasis to bone. Since E-cadherin, cortactin, MMPs and uPA are likely to have a general role in metastasis, demonstrating a bone-specific effect using the MDA-MB-231 model that rarely metastasizes to non-osseous tissue is difficult. Further work with the 4T1.2 orthotopic model that spontaneously metastasizes to both bone and soft tissue will be useful to delineate the roles of these genes in metastasis to bone. This will complement other studies, described below, that have investigated the role of these genes in metastasis.

E-cadherin

E-cadherin is a transmembrane protein involved in cell-cell adhesion. Loss of E-cadherin expression has been linked to many types of cancer, including breast cancer where invasive and metastatic cancers express reduced levels of E-cadherin [15, 23]. In addition to reducing skeletal tumor burden, there is some evidence that overexpression of E-cadherin in the MDA-MB-231 intracardiac model also results in decreased metastasis to non-osseous sites [10]. E-cadherin expression does not alter anchorage-dependent growth, suggesting that reduced metastasis is not due to impaired cell growth or decreased tumorigenicity [10]. In this model, E-cadherin-mediated adhesion appears to hinder extravasation of tumor cells from the vascular system to both bone and soft tissue. While this study implicates E-cadherin as an important metastasis suppressor, E-cadherin is unlikely to play a specific role in metastasis to bone.

Cortactin

Gene amplification at the 11q31 chromosomal band and the consequent overexpression of cortactin (EMS1) is frequently associated with breast cancer [24]. Cortactin activates the Arp2/3 complex, a central regulator of actin dynamics [25]. This interaction facilitates actin cytoskeleton reorganization leading to the formation of lamellipodia and membrane ruffles [16]. Anti-cortactin antibodies block matrix degradation at invadopodia in MDA-MB-231 cells [17]. Interestingly, cells overexpressing cortactin demonstrate enhanced adhesion to bone endothelial cells compared with parental cells [20]. These studies underline the importance of cortactin in cell invasion and suggest cortactin may be involved in vascular adhesion in bone metastasis. Further work using new models of bone metastasis will help to elucidate the role of cortactin in bone metastasis.

Matrix metalloproteinases

MMPs comprise a family of over 20 members of zinc-dependent extracellular or membrane-bound proteinases. MMPs function in tissue remodeling and repair by cleaving a range of extracellular matrix proteins [26, 27]. Expression of MMPs by stromal cells is often increased in response to the presence of a tumor or other pathological condition. In addition, tumors can express high levels of one or more MMP family members. MMPs are involved in many stages of tumor progression from expansion of the primary tumor to initiating and maintaining local and distal invasion [19]. Thus, synthetic MMP inhibitors reduce primary tumor growth and local invasion, and decrease skeletal tumor burden in the MDA-MB-231 intracardiac model [28–30]. MMP activity is regulated at a transcriptional level, post-translationally by protease cleavage and by endogenous MMP inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) [27]. MDA-MB-231 cells overexpressing TIMP2 exhibit a decreased ability to invade endothelial cell monolayers, indicating that MMPs are likely to facilitate cell extravasation from the marrow sinus [21] but, again, further work is needed to demonstrate a specific role in metastasis to bone.

The urokinase system

uPA is a secreted serine protease that has various biological roles related to extracellular proteolysis. It converts the inactive zymogen plasminogen into the active serine protease plasmin, which cleaves extracellular matrix components including laminin, fibronectin and collagen [18]. Via interactions with integrins, uPA also regulates

cell adhesion and proliferation [31]. Through these actions, uPA promotes tumor cell migration and invasion. Thus, patients with elevated uPA levels in breast cancer tissue have a poor prognosis [32] and overexpression of uPAR in a rat breast cancer cell line leads to increased primary tumor growth and metastatic progression in vivo [33].

Recently, several studies have addressed the role of the urokinase pathway in the development of osseous breast cancer metastases. The presence of tumor cells expressing uPA in bone marrow has been shown to have prognostic relevance in breast cancer patients, correlating with a significantly shorter metastasis-free interval (36 months) compared to patients who were uPA negative (44.5 months) [34]. However, uPA expression in primary breast tumors appears not to be predictive of metastastic outcome. In a study of 144 patients, there was no significant difference in uPA activity in tumors from patients who were disease free post-operatively, compared with those who went on to develop soft tissue or bone metastases [35]. This suggests that uPA may not be important specifically for invasion to bone but may promote growth of tumor cells that are already in bone. Indeed, uPA is mitogenic for a number of cell types including osteoblasts and prevents apoptosis of MDA-MB-231 cells [36, 37]. These actions of uPA may promote breast cancer progression and contribute to bone metastasis.

Levels of uPA, uPAR and plasminogen activator inhibitor type-1 (PAI-1) have been profiled in human breast carcinomas and their bone metastases, using in situ hybridization [38]. Both uPA and uPAR levels were elevated in malignant cells compared to normal breast epithelium. uPA was expressed in similar amounts in non-invasive and invasive tumors and in bone metastases, whereas uPAR mRNA levels were increased in the latter two tissue types. Thus, uPAR but not uPA appears to correlate with metastatic disease, but not specifically with bone metastases [38].

In addition to mediating proteolytic actions, uPAR is present at the cell surface in functional complexes with integrins where it regulates cell interactions with the extracellular matrix [39]. Exogenous administration or endogenous expression of a peptide that interferes with formation of uPAR/β1 integrin complexes on MDA-MB-231 breast tumor cells inhibits tumor burden in bone following intracardiac injection [22]. This work shows that uPAR/integrin complexes are involved in tumor progression. Further work will clarify whether adhesive and proteolytic events mediated by uPAR signaling play a role in metastasis to bone.

Chemoattraction

In contrast to normal breast epithelial cells, breast tumor cells frequently express proteins that in hemopoietic cells have been shown to function as homing factors [40, 41]. This has led to the hypothesis that these molecules may mediate homing of breast cancer cells to specific sites, including bone.

CXCR4 chemokine receptor

Chemokines are small cytokine-like proteins that elicit directional cell migration and activate signaling pathways that regulate cytoskeletal rearrangement and adhesion [42, 43]. Chemokines are critical for the development and homing of hemopoietic cells to specific organs including bone marrow [43]. There is evidence that similar mechanisms may be involved in homing of breast cancer cells to specific secondary sites. Upregulated expression of the chemokine receptor CXCR4 was recently reported on human breast cancer cell lines, malignant breast tumors and metastases, compared to normal mammary epithelial cells [41]. High levels of mRNA encoding the CXCR4 ligand, CXCL12/SDF-1α, are present in lymph node, lung, liver and bone marrow, which are common sites of breast cancer metastasis. In contrast, organs that are rarely targets of breast cancer metastasis, including kidney and small intestine, express low levels of CXCL12 mRNA.

Consistent with a role in metastasis, the chemokine CXCL12 increases pseudopodia formation, directional migration and invasion in MDA-MB-231 cells, and these events can be blocked by neutralizing anti-CXCR4 antibody [41]. Furthermore, neutralizing anti-CXCR4 antibodies inhibit primary tumor growth in the mammary gland, as well as metastasis to lung and lymph nodes after intravenous injection of MDA-MB-231 cells. Since CXCL12/CXCR4 interactions have been implicated in the homing and repopulation of human stem cells into the bone marrow of SCID mice [44], a similar mechanism may be involved in metastasis of breast cancer cells to bone. However, this remains to be investigated, since the study described above did not use models that are suitable for studying bone metastasis. If expression of CXCR4 by breast cancer cells is shown to mediate homing to bone, this receptor or its ligand may be good targets for future therapies against tumor progression and metastasis. However, examining the consequences of inhibiting CXCR4 on normal physiology, including immune function, will be important.

Osteonectin

Osteonectin (SPARC/BM-40) has been proposed to be a chemoattractive agent that can induce homing of breast cancer cells to bone. Osteonectin is one of the most abundant non-collagenous matrix proteins in bone. It regulates cellular interactions with the extracellular environment by controlling turnover and assembly of the extracellular matrix, and by modulation of growth factor activity and cellular morphology [45]. MDA-MB-231 breast cancer

cells exhibit increased chemotaxis and invasion in vitro in response to osteonectin, but not to other bone-derived proteins including bone morphogenic protein-4 [46]. Treatment with exogenous osteonectin downregulates the MMP inhibitor TIMP2 and stimulates MMP-2 activity in MDA-MB-231 cells, providing a potential link with the enhanced invasive ability of breast cancer cells induced by osteonectin [47].

Whether chemoattraction by osteonectin is responsible for homing of breast cancer cells to bone in vivo remains to be determined. The osteonectin receptor has not been identified. Osteonectin is not expressed in normal mammary tissue or benign breast lesions [48, 49]. Similarly, several breast cancer cell lines, including MDA-MB-231 cells, do not express osteonectin [50] and therefore have the potential to respond to an osteonectin chemoattractive gradient. However, in contrast to MDA-MB-231 cells, both in situ and invasive breast carcinoma lesions show strong expression of osteonectin [49, 50], suggesting that these cells may not be responsive to chemoattraction by osteonectin. While osteonectin may contribute to an invasive phenotype in breast cancer cells through activation of MMPs, further work is required to determine if the presence of osteonectin in bone provides a homing stimulus for breast cancer cells in vivo.

Adhesion

The ability to attach to extracellular matrix molecules within bone is required for breast cancer cells to gain a foothold. Expression of $\alpha v\beta 3$ integrin by breast cancer cells may be important for adhesion within the bone microenvironment.

$\alpha v\beta 3$ integrin

Integrins are cell surface heterodimeric glycoproteins that mediate cellular interactions with the extracellular matrix. $\alpha v\beta 3$ integrin is abundantly expressed by osteoclasts and is important in osteoclast-mediated bone resorption. It is required for migration of osteoclasts and for maintenance of the osteoclast sealing zone during bone resorption [51]. Expression of $\alpha v\beta 3$ integrin has been observed in normal breast epithelial cells, primary human breast tumors, invasive breast cancer lines and bone metastases [52–55]. $\alpha v\beta 3$ integrin binds the tripeptide Arg-Gly-Asp (RGD) that is present in extracellular matrix proteins found in bone, including vitronectin, osteopontin and bone sialoprotein, and mediates binding of breast cancer cells to trabecular bone [56]. In addition, $\alpha v\beta 3$ integrin regulates migration of breast cancer cells and possibly invasion [57–59]. Consequently, a role for $\alpha v\beta 3$ integrin has been postulated in specific homing of breast cancer cells by mediating adhesion and migration of breast cancer cells to the bone extracellular matrix.

$\alpha v\beta 3$ integrin regulates a cell death suppression signal in breast cancer cells. Adhesion to osteopontin through $\alpha v\beta 3$ integrin blocks apoptosis in cells sensitive to phorbol esters [60]. Anti-$\alpha v\beta 3$ antibodies and RGD-containing peptides, but not anti-$\alpha v\beta 5$ antibodies, inhibit survival. By allowing adhesion to bone matrix proteins, $\alpha v\beta 3$ may provide a survival advantage to breast cancer cells that have metastasized to the bone.

Recent work suggests that the activation state of $\alpha v\beta 3$ integrin is an important determinant of metastasis. Overexpression of constitutively active $\alpha v\beta 3$ integrin in the highly metastatic human breast cancer cell line MDA-MB-435 enhances lung metastasis after tailvein injection into SCID mice, compared to cells expressing inactive $\alpha v\beta 3$ or not expressing $\alpha v\beta 3$ [61]. The role of activated $\alpha v\beta 3$ integrin in metastasis to bone has yet to be investigated.

Interactions between breast cancer cells and bone cells

Under normal physiological conditions, bone undergoes constant remodeling. New bone is laid down by stromal cells called osteoblasts. To balance the production of new bone, osteoblasts stimulate the fusion and maturation of osteoclast precursors to generate bone-resorbing osteoclasts**.** Osteoclasts secrete proteases and acids that dissolve bone matrix and resorb calcified bone. The tightly regulated interaction between osteoblasts and osteoclasts ensures the maintenance of bone integrity.

The recently identified RANKL cytokine signaling system is important in osteoclast-osteoblast interactions. Receptor activator of NF-κB ligand (RANKL) is a member of the tumor necrosis factor (TNF) family of cytokines and is expressed by osteoblasts (fig. 1) [62]. RANKL binds its receptor RANK on osteoclast precursors and induces their maturation into multinucleated bone-resorbing osteoclasts. Interactions between RANKL and RANK are inhibited by the soluble decoy receptor osteoprotegerin (OPG) [63, 64]. OPG is produced by osteoblastic stromal cells and acts locally to neutralize RANKL, thereby inhibiting activation of osteoclasts. Thus, osteoblasts regulate osteoclast activity by production of both activating and inhibitory proteins.

In bone metastasis, interactions between breast cancer cells and bone cells set up what has been termed the vicious cycle, resulting in increased osteoclast-mediated bone resorption [65]. Bone contains a diversity of growth factors including insulin-like growth factors, transforming growth factor-β (TGF-β), bone morphogenic proteins and fibroblast growth factors sequestered in the bone matrix. These are released into the bone microenvironment by osteoclast-mediated bone resorption. TGF-β, one of the most abundant of the bone-derived factors, stimulates

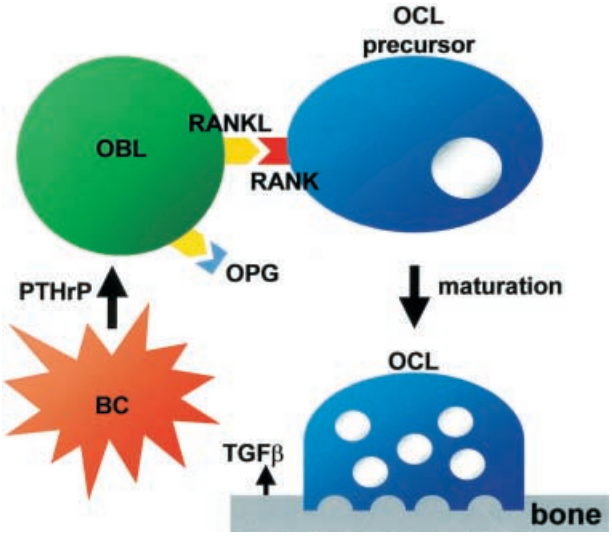

Figure 1. Breast cancer cells interact with bone cells. Osteoblasts (OBL) regulate osteoclast (OCL) maturation and activity by the alternate expression of RANKL, an osteoclast-activating factor, or OPG, an inhibitory decoy receptor. Breast cancer cells (BC) respond to release of bone-derived factors such as transforming growth factor-β (TGFβ) by producing parathyroid hormone-related protein (PTHrP). PTHrP enhances RANKL expression and decreases OPG expression by osteoblasts, leading to enhanced osteoclast activity.

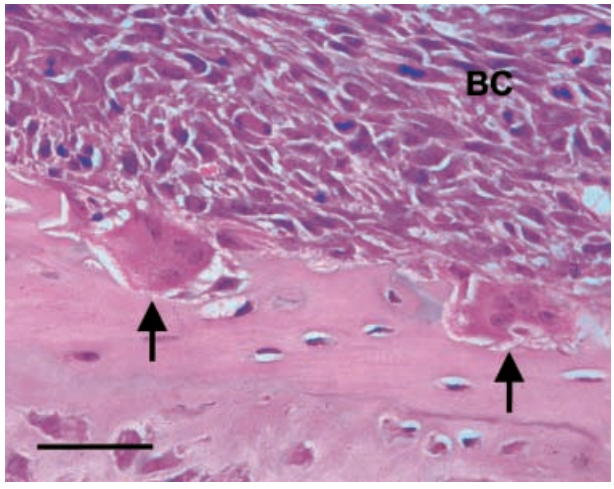

Figure 2. Hematoxylin and eosin-stained section through bone showing breast cancer cells (BC) adjacent to resorbing osteoclasts (arrows). Scale bar: 40 µm. The section is taken from an orthotopic mouse model of breast cancer metastasis.

breast cancer cells to produce the potent osteoclast-stimulating factor parathyroid hormone-related protein (PTHrP) [66]. This induces further activation of osteoclasts and increases bone resorption (fig. 1).

Breast cancer cells disturb the balance between osteoblasts and osteoclasts by interfering with RANKL signaling [67]. In co-cultures of osteoclast precursors, osteoblasts and breast cancer cells, overexpression of PTHrP by the tumor cells enhances RANKL mRNA expression and decreases OPG mRNA expression in osteoblasts [67]. This increases production of active osteoclasts, leading to further osteoclast-mediated bone resorption and release of growth factors. Whether breast cancer cells can directly activate osteoclasts is uncertain. In a study of 18 samples, primary breast tumors were shown to express RANK and OPG, but not RANKL [67], suggesting that direct activation is not likely. However, investigation of gene expression profiles of breast cancer cells that are growing in the bone microenvironment will be necessary to conclude whether they can act as surrogate osteoblasts and directly activate osteoclasts.

Bone resorption accompanies osseous metastatic disease and is responsible for the skeletal complications (fig. 2). However, is bone resorption *essential* for proliferation of breast cancer cells and development of bone metastases? Experiments that alter bone resorption in the MDA-MB-231 intracardiac model indicate that bone resorption and the occurrence of metastases are intimately linked. For example, inhibiting osteoclast activation by treatment with recombinant OPG reduces skeletal tumor burden in nude mice after intracardiac injection of MDA-MB-231 cells [68]. Overexpression of PTHrP in the same model increases the number of osteolytic lesions [69]. In contrast, treatment with anti-PTHrP antibodies results in decreased bone resorption due to a reduction in osteoclast numbers and is accompanied by reduced tumor burden in bone [69]. Similarly, inhibiting endogenous PTHrP production by MDA-MB-231 cells by overexpression of a dominant negative TGF-β receptor that inhibits TGF-β signaling reduces tumor burden in the bones of nude mice after intracardiac injection [66, 69]. Restoration of TGF-β signaling increases PTHrP production by the tumor cells and enhances bone metastasis in vivo.

Bone resorption may be important for releasing from the bone matrix cytokines that promote proliferation of breast cancer cells. In particular, insulin-like growth factor-1 (IGF-1) is a likely candidate. It is an abundant protein in the bone matrix and stimulates the proliferation of normal and transformed breast epithelial cells [70]. Overexpression of a dominant negative IGF type I receptor that impairs responsiveness to IGF-1 reduces skeletal tumor burden in the MDA-MB-231 intracardiac model, indicating that the ability to respond to IGF-1 promotes bone metastasis [71]. Other cytokines present in the bone microenvironment, including TGF-β and interleukin-11 (IL-11), are less likely to stimulate growth of tumor cells. IL-11 inhibits proliferation of both breast cancer cell lines and solid tumors [72, 73]. TGF-β inhibits proliferation of primary human mammary epithelial cells and loss of sensitivity to its effects has been associated with tumorigenesis [74, 75].

While bone resorption is consistently observed with bone metastasis and could stimulate tumor cell proliferation

through release of growth factors, a causal role has yet to be shown. Experiments to determine if bone metastases can form in osteopetrotic animal models where bone resorption is defective would provide a more definitive answer. Several osteopetrotic mouse models including mice deficient for β3 integrin or RANK may be useful for such experiments [76].

Parathyroid hormone-related protein

PTHrP was identified as the causal agent in humoral hypercalcemia of malignancy [77]. It is a potent osteoclast-stimulating factor that elevates blood and renal calcium levels by promoting osteoclast-mediated bone resorption [78]. PTHrP promotes branching morphogenesis in the developing breast and is produced during lactation [79]. Seventy percent of primary breast tumors express PTHrP [80–82]. In addition, an immunohistochemical study documented PTHrP expression in 12 of 13 breast cancer-derived bone metastases, compared with 3 of 18 metastases to non-osseous sites [83]. These observations implicated PTHrP as important in resorption associated with bone metastasis from breast cancer.

Several retrospective clinical studies show that expression of PTHrP in primary breast tumors correlates with increased bone metastasis, but not recurrence or reduced survival. [81, 84, 85]. Consequently, PTHrP has been suggested as a useful prognostic indicator of metastasis to bone. However, a recent prospective study questions the prognostic significance of PTHrP expression by primary breast tumors [82]. The study of 367 consecutively accrued breast cancer patients showed that PTHrP expression in the primary tumor associated independently with *improved* survival [82]. Consistent with previous studies, PTHrP was detected in 72% of primary tumors. In contrast to previous studies, patients with PTHrP-positive tumors were less likely to develop either bone or soft-tissue metastases than those with PTHrP-negative tumors, suggesting that PTHrP expression in the primary tumor correlates with a less invasive phenotype. Those patients who returned for follow-up bone surgery, however, presented with PTHrP-positive bone metastases, and included some patients who had had PTHrP-negative primary tumors. This study suggests that PTHrP expression is regulated by the bone microenvironment and may facilitate growth of breast cancer cells after they have metastasized to bone. Consistent with this concept, human breast cancer cells isolated from a metastatic bone lesion in an experimental metastasis model express higher levels of PTHrP than the parental population [86]. The finding that PTHrP is expressed by the majority of breast cancer-derived bone metastases, but by only a small proportion of metastases to non-osseous sites [83], is consistent with specific upregulation of PTHrP within the bone microenvironment. The conclusion drawn in that study was that PTHrP expression in the primary tumor favors metastasis to bone; however, PTHrP expression by the primary tumors was not examined to confirm this explanation. Furthermore, experiments using the 4T1.2 spontaneous orthotopic mouse model of breast cancer metastasis to bone [11] indicate that PTHrP expression by the primary tumor is not sufficient to induce metastasis to bone. Elevation of PTHrP expression in a cell line that is metastatic to lung was insufficient to induce metastasis to bone [R. Anderson et al., unpublished results]. While PTHrP is unlikely to be a useful prognostic indicator of metastatic outcome, these studies do support the central role of PTHrP in mediating interactions of breast cancer cells with the bone marrow stroma. Consequently, PTHrP may be an appropriate target for new therapies to reduce tumour-associated bone resorption.

Cytokines

The cytokines IL-6 and IL-11 promote osteolysis by stimulating osteoclast formation. Endogenous expression of IL-6 and IL-11 has been documented in invasive primary breast tumors [87]. No significant association has been found between IL-6 status and occurrence of bone metastasis. However, tumors expressing IL-11 mRNA have a significantly higher rate of bone metastases than IL-11 negative tumors [87]. IL-11 is likely to play an important role in promoting osteolysis at the site of bone disease, and expression in the primary tumor may be a useful predictive factor for the subsequent development of bone metastases.

MMPs – osteoclast-independent osteolysis?

The central role of osteoclast-mediated bone resorption in the development of skeletal metastasis is well documented. However, direct degradation of bone by human breast cancer cells has also been observed in vitro and may be mediated by MMPs [30, 88]. Osteoblasts and osteoclasts produce MMPs that regulate bone homeostasis [89]. Metastatic tumor cells present in bone produce MMPs capable of degrading bone matrix collagen [90]. Interestingly, while TGF-β enhances expression of MMP-1 and -9, and TIMP-1 and -2, by MDA-MB-231 cells in culture, it inhibits MMP expression in normal breast epithelial cells [75]. This suggests a potential mechanism whereby the release of TGF-β from the bone matrix during osteoclast-mediated osteolysis may alter expression of MMPs and their tissue inhibitors in bone-metastasizing cancer cells.

These observations suggest that production of MMPs by breast cancer cells may contribute to osteoclast-independent degradation of the bone matrix or connective tissue. This may be especially relevant in the advanced stages of metastatic disease when growth factors such as TGF-β have been released into the bone microenvironment

through resorption of bone. The development of therapies that inhibit tumor-associated bone degradation will need to address the potential role of MMPs in this process. If breast cancer cells are shown to mediate both osteoclast-dependent and -independent osteolysis, inhibition of both processes will be necessary to block the vicious cycle of bone destruction and tumor cell proliferation.

Development of new therapies

The most promising therapies for osteolytic metastases to date are aimed at inhibiting bone resorption. Direct interruption of the molecular interactions between RANKL and RANK using recombinant OPG has generated promising results in animal models, as described above [68]. In addition, a recent phase I clinical study in breast cancer patients with bone metastases showed that a single dose of a recombinant human OPG construct resulted in decreased urinary N-telopeptide (NTX), a marker of bone resorption, at levels comparable to those achieved by treatment with the bisphosphonate pamidronate [91].

Experiments with animal models have indicated that the inhibitory effect of OPG on tumor cell growth appears to be specific to bone metastases since exogenous OPG has no effect on subcutaneous primary tumor growth of prostate cancer cells [92] or on primary breast tumor growth and lung metastases that occur after growth in the mammary fat pad in mice [Anderson et al., unpublished observations]. Furthermore, the reduction in bone metastasis seen with OPG treatment in the MDA-MB-231 intracardiac model did not result in tumor redistribution to other sites [68], which is an important consideration for site-specific metastasis inhibitors. However, this may be attributed to the limited ability of MDA-MB-231 cells to colonize non-osseous sites. Studies in orthotopic models, such as 4T1.2, that also metastasizes to soft tissue will clarify this point.

Similar OPG levels were found in breast cancer patients with no evidence of bone or non-osseous metastatic disease compared with stage IV patients with bone involvement [93]. Thus, OPG expression in primary tumors is unlikely to be a useful prognostic marker of improved outcome or reduced bone metastasis. However, if OPG can be demonstrated to decrease skeletal tumor burden or improve quality of life in patients, it will become a novel and specific therapy to treat tumor-associated bone resorption.

The central role of PTHrP in bone resorption makes it an attractive candidate for new therapies to treat osteolytic bone disease. Humanized anti-PTHrP antibodies are being investigated [94]. Small-molecule inhibitors of PTHrP that reduce osteolysis and skeletal tumor burden in an experimental model of breast cancer metastasis have been identified and may form the basis for future therapies [95]. Therapies that target IL-11 may also be useful to suppress osteolytic bone disease [96]. These include cyclooxygenase inhibitors that target the prostaglandin E2-dependent mechanism of IL-11-mediated bone resorption.

Inhibition of MMP activity is an attractive clinical strategy due to the central role played by MMPs in tumour progression. However, results from phase III trials have shown little or no clinical efficacy [27]. Future trials may need to target patients with specific MMP expression profiles determined at an early stage in their disease [97]. Trials may also be more successful with the development of more potent inhibitors of MMP function. Antagonists of $\alpha v\beta 3$ integrin are being investigated for use in diseases including osteoporosis [98]. With a better understanding of the role of $\alpha v\beta 3$ integrin in bone metastasis, these drugs may be used to prevent adhesion of breast cancer cells to the bone or to reduce bone resorption by inhibiting osteoclast activity.

The future

Considerable advances have been made in identifying genes that facilitate various steps required for bone-specific metastasis, including homing and adhesion to bone, and genes that create and maintain an environment that supports proliferation of tumor cells in bone. Mouse models of metastasis have been invaluable for the identification of these genes. However, most models of breast cancer metastasis have been inadequate at delineating site-specific metastasis. They lack relevant pathophysiological pathways of metastasis since tumor cells are often injected subcutaneously or into the vascular system. While the MDA-MB-231 intracardiac model has been informative, more definitive results will come from new orthotopic models of breast cancer metastasis to bone such as the 4T1.2 model. Another orthotopic model of metastasis to bone may be developed from observations that bone disease develops in SCID mice 4 weeks after removal of primary mammary gland tumors derived from MDA-MB-435 human breast cancer cells [61]. This model would allow the metastatic process to bone to be investigated in vivo using a human cell line.

Models of spontaneous metastasis to bone will allow all stages of breast cancer metastasis to be investigated, from escape of the primary tumor cells to metastatic proliferation at multiple specific sites that mirror the human disease. In addition to confirming the role of previously identified candidates, the new models of metastasis provide a system for unbiased phenotypic or genetic screening for novel genes that influence bone metastasis. Introduction of cDNA libraries into cell lines used in the mouse metastasis models will allow unbiased identification of genes that alter the metastatic capacity of these

cells. cDNA microarray technology will be useful for identifying new diagnostic or prognostic markers of bone metastasis and targets for future therapies. cDNA microarray screening enables simultaneous evaluation of the expression profiles of thousands of genes. This technology has already been used to identify genes that correlate with specific metastatic phenotypes [99–101]. Informative experiments will include comparison of gene expression profiles of samples with specific metastatic phenotypes (for example, bone metastases versus soft-tissue metastases, or primary breast tumours with different metastatic outcomes) from both human samples and animal metastasis models.

In the same way that breast cancers differ in their genetic determinants of tumorgenicity (for example, mutations in Neu/ErbB2 or BRCA1), the dominant determinants for metastatic growth in bone are also likely to vary (for example, bone resorption stimulated by IL-11 or PTHrP). Most current candidate genes have only been investigated in the MDA-MB-231 intracardiac model. Validating these results in the new models of metastasis to bone and in human material will be important. This will be assisted by the development of tissue microarrays [102], which allow rapid screening of large numbers of human tumor samples on a scale not technically feasible by traditional histological methods.

These technologies will provide invaluable tools for characterizing the molecular basis of metastasis to bone. They will complement the current studies of candidate genes and should reveal the genes that are essential for bone metastasis. The results of this research will pave the way for the development of new therapies that will specifically target the steps involved in breast cancer metastasis to bone.

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