Breast cancer at bone metastatic sites: recent discoveries and treatment targets

Osama Hussein · Svetlana V. Komarova

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Abstract Breast carcinoma is the most common cancer of women. Bones are often involved with breast carcinoma metastases with the resulting morbidity and reduced quality of life. Breast cancer cells arriving at bone tissues mount supportive microenvironment by recruiting and modulating the activity of several host tissue cell types including the specialized bone cells osteoblasts and osteoclasts. Pathologically activated osteoclasts produce osteolytic lesions associated with bone pain, pathological fractures, cord compression and other complications of metastatic breast carcinoma at bone. Over the last decade there has been enormous growth of knowledge in the field of osteoclasts biology both in the physiological state and in the tumor microenvironment. This knowledge allowed the development and implementation of several targeted therapeutics that expanded the armamentarium of the oncologists dealing with the metastases-associated osteolytic disease. While the interactions of cancer cells with resident bone cells at the established metastatic gross lesions are well-studied, the preclinical events that underlie the progression of disseminated tumor cells into micrometastases and then into clinically-overt macrometastases are just starting to be uncovered. In this review, we discuss the established information and the most recent discoveries in the pathogen-

esis of osteolytic metastases of breast cancer, as well as the corresponding investigational drugs that have been introduced into clinical development.

Keywords Bone metastases · Breast cancer · Osteoclast · Osteoblast · Metastatic niche · Drug development

Abbreviations

BCC	Breast Carcinoma Cells
BIM	Bcl-2-interacting mediator of cell death
CSC	Cancer Stem Cells
DTC	Disseminated Tumor Cells
EMT	Epithelial-Mesenchymal-Transition
ER	Estrogen Receptors
GPNMB	Glycoprotein Non-Metastatic B
HER2	Human Epidermal growth factor Receptor 2
HSC	Hematopoietic Stem Cells
ITAM	Immunoreceptor Tyrosine-based Activation Motifs
MBCC	Metastatic Breast Carcinoma Cells
MCSF	Macrophage Colony Stimulating Factor
MSC	Mesenchymal Stem Cells
mTOR	mammalian Target Of Rapamycin
NBP	Nitrogen-containing Bisphosphonates
NFAT	Nuclear Factor of Activated T cell
OPG	Osteoprotegerin
PTHrP	Parathyroid Hormone related Protein
RANKL	Receptor Activator of Nuclear factor κ B Ligand
SRE	Skeletal-Related Events
SRS	Src Responsive Signature
TRAIL	TNF-Related Apoptosis Inducing Ligand
ZA	Zoledronic acid

O. Hussein · S. V. Komarova
Faculty of Dentistry, McGill University,
Montreal, Quebec H3A 1A4, Canada

S. V. Komarova (✉)
740 Dr. Penfield Ave, 2201,
Montreal, Quebec, Canada H3A 1A4
e-mail: svetlana.komarova@mcgill.ca

Present Address:

O. Hussein
Faculty of Medicine, Mansoura University,
Mansoura 35516, Egypt

Breast cancer is the most common cancer of women. Globally, it accounted for 1.15 million incident cases in

2002, while 2.7 million new cases are projected for 2030 (Ferlay et al. 2010). Screening programs and the introduction of adjuvant therapies affected a decline of about 24% in death related to breast cancer in several developed countries (Berry et al. 2005). However, metastatic cases are still universally incurable.

Bone is the most common organ affected by distant relapse of breast cancer. Around 70% of metastatic patients will have lesions at bone (Hess et al. 2006). The skeleton and the lungs bear the major burden of the disease at autopsy studies (Cho and Choi 1980; Cifuentes and Pickren 1979; Lee 1983; Weiss 1992). Even though, breast carcinoma with metastases confined to bone is generally regarded as an indolent disease, bone secondaries usually present with bone pain and may be complicated with skeletal related events (SREs) such as pathological fractures, cord compression, bone marrow infiltration, or hypercalcemia of malignancy (Chiedozi 1988; Leone et al. 1988; Sherry et al. 1986a, b; Solomayer et al. 2000).

Breast carcinoma cells reach bones through blood spread. Invading metastatic cells landing at bone tissues induce a complex host reaction that alters the local microenvironment in favor of the growth and expansion of the secondary tumor deposits. Understanding the molecular interactions between the invading tumor cells and resident host cells at the metastatic sites allowed the invention of several biological therapies, which show promising results in the clinic. Other therapies are in pre-clinical development. In this review, we discuss the recent discoveries in the biology of tumor-host interactions at the metastatic bone sites and the status of the most promising investigational treatment strategies.

Predictors of occurrence of bone metastases

Investigators have been looking for the risk factors that can predict relapse at bone sites. Breast cancers are divided into distinctive molecular patterns according to their transcriptional profile. Relatively good-prognosis cancers show a gene expression reminiscent of a luminal or normal-like breast pattern, including positive expression of estrogen receptor (ER). More aggressive cancers usually demonstrate a basal-like phenotype or one that is characterized by the expression of human epidermal growth factor receptor 2 (HER2) (Perou et al. 2000; Sorlie et al. 2001; Sorlie et al. 2003). Smid et al. (Smid et al. 2008) found that around two-thirds of bone relapses occurred in luminal (ER+) cases while only 7% belonged to basal tumors. This incidence however roughly corresponds to the general prevalence of these tumor subtypes.

The concept of molecular classification of cancer according to gene signatures was introduced to increase

the accuracy of traditional clinico-pathological prognostic markers of breast cancer, aiming at tailoring adjuvant therapy to personalized patient risk of relapse. The scientists at the Netherland Cancer Institute used microarray technology to identify 70-gene expression signature that correlated with early relapse in young breast cancer patients (van 't Veer et al. 2002). This signature was later validated in a cohort of 295 consecutive patients (van de Vijver et al. 2002). A 67-gene signature was also described that predicted early metastases in node-negative cases (Foekens et al. 2006; Wang et al. 2005). Kang et al. used gene expression profiling to identify 50-genes associated specifically with bone metastases in a model of human breast cancer cell line xenografts. The signature genes included the chemokine receptor CXCR4, the metalloproteinase MMP-1, the cytokines CTGF and IL-11 as collaborative genes that mediate the bone metastatic program (Kang et al. 2003). The same group validated the bone metastases signature in a cohort of clinical specimens with available follow-up data (Minn et al. 2005). Although this 50-gene bone signature did not distinguish tumors that relapsed in bone when the analysis was performed on all specimens available (Minn et al. 2005), it distinguished between tumors relapsed to bone and those relapsed to lungs. The importance of some genes, such as CTGF and IL-11 was confirmed, although some genes identified in the animal model, such as MMP-1, lost their significance in the clinical samples (Minn et al. 2005). Smid et al. (Smid et al. 2006) analyzed samples from 107 node-negative breast cancer patients who experienced relapse. They identified a 31-gene list that predicted relapse to bone with 100% sensitivity, 50% specificity and 79.3% positive predictive value. Their signature agreed with Kang et al. gene set in highlighting the up-regulation of fibroblast growth factor pathway as a predictor of bone relapse. Later, Klein et al. (Klein et al. 2009) confirmed the correlation of bone relapse with cysteine-rich intestinal protein 1 (CRIP1) gene up-regulation reported by Smid et al. Although the data from these studies highlighted certain mechanistic pathways that operate to induce the development of breast cancer metastases at osseous sites, there is relatively little concordance between gene signatures reported by different groups to predict bone relapse in clinical cases. Thus, the long-sought signature that would predict bone relapse with enough accuracy to justify adjuvant bone-targeted treatment is yet to be identified.

The pathogenesis of bone metastases

Normal bone structure depends on the fine balance between two active cellular elements of bone tissues—the bone-forming osteoblasts and the bone-resorbing osteoclasts.

Osteolytic metastases depend entirely on the activation of osteoclasts; the sole bone-resorbing cells. Osteoclasts are formed by fusion of precursors of myeloid origin (Vaananen and Laitala-Leinonen 2008). Hematopoietic stem cells are diverted to myeloid lineage under the effect of the transcription factor PU.1 and PU.1^{-/-} mice lack both osteoclasts and macrophages (Tondravi et al. 1997). During osteoclastogenesis, three main signaling axes have been identified through: *i*) Macrophage Colony Stimulating Factor (MCSF), *ii*) Receptor Activator of Nuclear Factor κ B Ligand and *iii*) Immunoreceptor Tyrosine-based Activation Motifs (ITAMs).

MCSF induces commitment of myeloid cells to osteoclastic lineage. Binding of MCSF to its cognate receptor c-fms on the surface of hematopoietic cells leads to the up-regulation of the transcription factor c-fos responsible for diversion of myeloid cells to osteoclastic rather than macrophage phenotype. Mice lacking MCSF (op/op) (Kodama et al. 1991; Wiktor-Jedrzejczak et al. 1990), c-fms (Dai et al. 2002) or c-fos (Wang et al. 1992) are devoid of osteoclasts and are osteopetrotic.

Committed osteoclast precursors express the Receptor Activator of Nuclear Factor κ B (RANK) on their surface that allows them to respond to its respective ligand. RANK ligand (RANKL) is expressed on the surfaces of osteoblasts, which also secrete Osteoprotegerin (OPG), the decoy receptor that sequesters RANKL and blocks its action. RANKL is the master osteoclastogenic mediator, which controls formation and function of mature osteoclasts (Blair and Athanasou 2004; Boyce and Xing 2008; Knowles and Athanasou 2009; Matsuo and Irie 2008; Wada et al. 2006). Binding of RANKL to its receptor on the surface of osteoclasts recruits the TNF Receptor Associated Factor 6 (TRAF6). Phosphorylation of TRAF6 leads to the activation and ultimately nuclear translocation of the p50 or p52 subunits of the transcription factor NF κ B, responsible for fusion of pre-osteoclasts to form mature osteoclasts. RANKL signaling also induces the expression of the Nuclear Factor of Activated T cells c1 (NFATc1), the master transcription factor ultimately responsible for the osteoclastic phenotype (Negishi-Koga and Takayanagi 2009). The deficiency of either RANKL (Kong et al. 1999) or its receptor RANK (Dougall et al. 1999) in mice leads to severe osteopetrosis due to the absence of osteoclasts. Similar phenotype is observed in mice lacking TRAF6 (Naito et al. 1999), both p50 and p52 of NF κ B (Iotsova et al. 1997), or NFATc1 (Aliprantis et al. 2008).

Further induction of NFATc1 is promoted by the phosphorylation of the ITAMs within the receptor-associated adaptor molecules DNAX-activating protein (DAP)₁₂ and Fc receptor common gamma subunit (FcR γ). Activation by ITAM leads to amplification of NFATc1 expression, and induces its phospholipase C γ (PLC γ)/

calcium/calcineurin-dependent nuclear translocation, both of which are essential for osteoclast formation (Koga et al. 2004; Mocsai et al. 2004). Thus, signaling events essential for induction of osteoclast formation include MCSF/c-fms signal which up-regulates the transcription factor c-fos, the RANKL/RANK signal which up-regulates the transcription factors NF κ B and NFATc1 and the immunoreceptor signal down-stream of yet unknown ligand, which induces calcium signaling and activates the transcription factor NFATc1.

Induction of osteoclast resorptive function also depends on specific signaling pathways. Mature osteoclasts express $\alpha_v\beta_3$ integrins that bind the fibronectin in the organic bone matrix (Nakamura et al. 2007). Integrin signaling induces c-Src-mediated changes in cytoskeletal organization that lead to formation of active actin ring and a tight sealing zone between osteoclast cytoplasmic membrane and the matrix surface (Horne et al. 2005; Miyazaki et al. 2006). Sealing zone is a pre-requisite for effective secretion of the resorptive mediators such as proton ions (H⁺), cathepsin K and MMP-9 that dissolve the bone matrix (Lakkakorpi and Vaananen 1996; Mulari et al. 2003; Nakamura et al. 2003; Ory et al. 2008; Saltel et al. 2008; Takahashi et al. 2007; Vaananen et al. 1988; Vaananen et al. 2000; Zaidi et al. 1993). Mice lacking β_3 integrins or c-Src are osteopetrotic due to the presence of dysfunctional osteoclasts that fail to resorb bone (McHugh et al. 2000; Soriano et al. 1991). Metastatic cancer cells landing at bony sites exploit osteoclasts by inducing one or more of these pathways to elicit the pathological osteoclast formation and activity and excessive bone resorption.

The journey from breast to bone

The metastatic process is classically viewed as a sequential multi-step program (Hanahan and Weinberg 2000). Tumor cells at the primary site acquire the properties allowing them to invade the surrounding stroma and vasculature and gain access to the blood stream (i), to survive in the circulation (ii), to home at specific secondary site(s), and survive at these secondary site (iii) which may or may not entail a variable period of dormancy state (iv) and to establish secondary growth at the metastatic sites (v). Steps from (i) to (iv) constitute the pre-clinical stages of the metastatic process, while the last of these five steps is associated with clinically overt metastatic disease (Fig. 1).

The pre-clinical stages of the metastatic process

- i. *Invasion at the primary site*: Migrating cancer cells execute a complex program known as Epithelial-to-Mesenchymal Transition (EMT). This program entails

Fig. 1 Schematics demonstrating the interactions between invading tumor cells and bone cells during breast cancer metastasis to bone. Red arrows indicate the effects of tumor cells on bone cells; blue arrows depict the reciprocal effects of bone cells on tumor cells

down-regulation of the homophilic adhesion molecule E-cadherin allowing cancer cells to lose the cohesive properties characteristic of epithelium. EMT also confers cancer cells the motile phenotype necessary for these cells to invade the surrounding stroma and blood vessels (Creighton et al. 2010; Hugo et al. 2007; Tomaskovic-Crook et al. 2009; Trimboli et al. 2008; Willipinski-Stapelfeldt et al. 2005). Associated with these changes is the secretion of various proteolytic enzymes that help opening the path of cancer cells through the tissues (McMahon and Kwaan 2008). Loss of cohesiveness, acquisition of motility and proteolytic enzyme secretion pave the way for cancer cells to escape from primary site to the blood stream.

- ii. *Survival in the blood stream:* The majority of cancer cells liberated to the circulation fail to induce secondary deposits and probably succumb to apoptosis. The surviving minority however find their way to their preferable soil at specific secondary organs (Crocker and Allan 2008).
- iii. *Homing:* Arrival of cancer cells to secondary organs is far from being a random process. Different types of cancers, tumors from different patients suffering from the same cancer and even different sub-clones of the same tumor have specific proclivity to home and thrive at specific organ(s). Homing of a particular cancer cell to its preferable secondary organ is facilitated by the directional movement of cancer cells towards a gradient of a soluble mediator (chemotaxis) and/or the active binding to a surface-bound molecule (haptotaxis). Two chemo-attractant interactions are particularly relevant to guiding breast cancer cells (BCCs) to home specifically at bone. One of these interactions is the migration of breast cancer cells expressing RANK (Mori et al. 2009; Santini et al. 2010) towards bone rich in its ligand (RANKL). Jones et al. have identified RANKL as an important factor that directed the migration of RANK-bearing carcinoma and melanoma cells (Jones et al. 2006). Similarly,

prostate cancer cells have been shown to exhibit a chemotactic response to RANKL (Armstrong et al. 2008). The other molecule established to guide breast cancer cells expressing chemokine receptor CXCR4 (Hassan et al. 2008, 2010, 2009; Muller et al. 2001) to bone is Stromal-Derived Factor 1 (SDF-1) also known as CXCL12. RANKL and SDF-1 are highly expressed on the surface of osteoblasts residing at the highly vascular endosteal surface of the bone metaphyses (which were also demonstrated to be the first landing site for circulating BCCs (Phadke et al. 2006)), suggesting an important role of osteoblasts in guiding the BCCs to the bone marrow. In keeping, CXCR4 expression in early breast cancer correlated with the risk of relapse in bone (Andre et al. 2009). In metastatic patients, CXCR4 was higher in bony metastatic lesions compared to visceral ones (Cabioglu et al. 2009). Hassan et al. reported that high serum levels of SDF-1, which dump the chemotactic gradient guiding CXCR4-bearing cells to secondary sites, was associated with decreased risk of distant metastases in breast cancer patients (Hassan et al. 2008).

- iv. *Dormancy:* Prolonged survival of the cancer cells at the secondary sites prior to appearance of clinically detectable metastases is a common phenomenon in breast cancer. Secondary tumors particularly in bone appear after a variable period of disease-free interval that may be as long as several years or even decades. Dormancy state is not well understood since it contrasts with the notion of inevitable exponential growth of cancer. Two models have been proposed to explain the mechanisms that allow BCCs to remain dormant at the metastatic sites. These two mechanisms are the micro-metastatic dormancy (a) and the single-cell dormancy (b) models (Fig. 2).

Fig. 2 Different models of breast cancer dormancy: In the single-cell model (left), cells detached from indolent breast carcinomas lodge at the sites of future metastases and remain quiescent in a state of prolonged survival. In the micro-metastasis model (right), cells detached from aggressive carcinomas lodge at the sites of future metastases and form microscopic tumor foci that have a balanced state of proliferation and apoptosis

- a) *In the micro-metastasis model*, a microscopic tumor remains short of attaining a clinically detectable size through a maintained balance of its proliferation and apoptosis rates. Escape of such microscopic tumors into progressive growth may be induced by an angiogenic, immunologic, hormonal or other microenvironmental switches. Multiple evidences point to the cell-to-matrix signaling as a putative proliferation switch for BCCs microscopic lesions at bony secondary sites. Binding of $\alpha_5\beta_1$ integrin to its extracellular ligands (fibronectin and to a lesser extent to collagen-1 fragment) induces cell proliferation and cell motility through induction of ERK and FAK pathways respectively (Aguirre-Ghiso et al. 2001; Barkan et al. 2010). Fibroblastic growth factor-2 (FGF-2) signaling on the other hand keeps the cell in a quiescent immotile non-proliferative state (Barrios and Wieder 2009; Korah et al. 2004; Najmi et al. 2005) characterized by the dominance of AKT over ERK and Rho-C over Rho-A (Barrios and Wieder 2009; Chatterjee and van Golen 2010; Danen et al. 2002).
- b) *In the single-cell dormancy model*, single scattered cells at the future metastatic organ linger into a prolonged period of cell cycle arrest and remain viable through tonic induction of anti-apoptotic survival signals. This model of arrested apoptosis contrasts with the micro-metastatic model where both proliferation and apoptosis are active. In bone, BCCs may gain a survival advantage through blocking of the receptors for TNF-Related Apoptosis Inducing Ligand (TRAIL). Two survival mechanisms that inhibit TRAIL signaling have been described and may be of relevance to the microenvironment at the bony tissues. TRAIL receptors in BCCs can be blocked by OPG (Fisher et al. 2006; Holen et al. 2005; Rachner et al. 2009; Schubert et al. 2008). Neville-Webb et al. demonstrated that bone marrow stromal cells isolated from breast cancer patients secrete enough OPG to inhibit BCCs apoptosis in vitro (Neville-Webb et al. 2004). More recently, another survival pathway was identified that counteracts the apoptotic TRAIL signaling and is mediated through stimulation of Src; a tyrosin-specific kinase involved in breast cancer progression and metastasis (Zhang et al. 2009).

The apparent dichotomy of the two models of dormancy raises the question of which model represents breast cancer dormancy at bone. While there is no definite answer to this question at the present time, we suggest that the answer lies

in the well-known heterogeneity of the disease. Indolent breast cancers may fit into the dormant single-cell model while the more aggressive cases fit into the micro-metastatic model. In fact, recent studies corroborate this hypothesis. Zhang et al. reported that up-regulation of Src signaling in breast cancer specimens correlated with hormone-receptor (ER+) status and specifically with late-onset bone relapse (Zhang et al. 2009). Src was shown to induce prolonged survival of a slowly growing breast cancer cell line in the host bone marrow and induced the survival of these cells in bone metastases by counteracting TRAIL signaling. The authors also demonstrated the involvement of SDF-1/CXCR4 in mediating BCC survival, which depended on Src and Akt signaling (Zhang et al. 2009). These data linked Src-mediated anti-apoptotic mechanisms to survival of single BCCs in bone marrow and to late onset bony metastases.

Another indication of the distinctive dormancy mechanisms of indolent versus aggressive breast cancer comes from studies of cell lines in vitro. Barkan et al. (Barkan et al. 2008) compared the growth characteristics of the ER+ breast cancer cell line MCF7 with MDA-MB-231 cell line, which represent the aggressive basal type of the disease. In three-dimensional culture, MDA-MB-231 proliferated readily while MCF7 remained in a state of cell-cycle arrest, potentially linking the dormancy state associated with arrested proliferation with the less aggressive disease phenotype.

Cancer metastases and the stem cell concept

Since bone metastases are classically associated with late onset relapse and indolent metastatic disease, it is convenient to address the dormant single-cell model and the related concept of cancer stem cells in some detail. The concept of a migrating cancer cell that reaches the bone marrow, remain dormant for prolonged period of time and then resurges as a secondary tumor has always been appreciated by scientists and was later directly demonstrated with the description of circulating tumor cells (CTC) and disseminating tumor cells (DTC) that were retrieved from the blood and bone marrow respectively of apparently disease-free breast cancer survivors (Bidard et al. 2008; Slade and Coombes 2007; Tjensvoll et al. 2010). DTCs have been shown beyond doubt to correlate with the risk of subsequent tumor relapse (Bidard et al. 2008; Slade and Coombes 2007; Tjensvoll et al. 2010). Investigators tried to explain how DTC survive in the bone marrow, remains viable and later induce the formation of gross metastases. The fact that human bone marrow contains the niche for the adult hematopoietic stem cells (HSC) inspired the theory of

tumor dormancy maintained by a cancer stem cell (CSC) population.

Normal adult stem cells are pluri-potent undifferentiated cells that constitute a reservoir for generation of mature complex tissues at the time of physiological needs. It was proposed that cancers originate from Cancer Stem Cells (CSCs), while metastases arise from Metastases Cancer Stem Cells (MCSCs) lodged at the future metastatic sites. Al-Hajj et al. (Al-Hajj et al. 2003) described a fraction of BCCs characterized by $CD^{44+}/CD^{24-/low}$ expression that exhibited an augmented capacity of forming invasive tumors from heterogeneous cell population. In contrast to the rest of the tumor population, as few as 200 cells of this fraction formed palpable masses when transplanted in SCID mice. $CD^{44+}/CD^{24-/low}$ cells were also found to represent the majority of DTCs in the bone marrow of breast cancer patients (Balic et al. 2006), and $CD^{44+}/CD^{24-/low}$ cells content of the primary tumor was shown to correlate with subsequent development of osseous metastases (Abraham et al. 2005). It is plausible to assume that dormant BCCs at the bone marrow may reside in specific cellular niches that may help them to maintain their quiescent survival. It is also convenient to assume that such a CSC niche may have some resemblance to the better-characterized HSC niche at the bone marrow. There are at least two distinct niches that harbor HSC at the bone marrow. One niche is formed by osteoblasts (Calvi et al. 2003) and/or mesenchymal stem cells (MSCs) (Mendez-Ferrer et al. 2010) at the endosteal surface of bone and generally keeps HSCs at quiescent state. This niche retains HSCs in part through CXCR4/SDF-1 mediated adhesion between HSCs and osteoblasts/MSCs (Mishima et al. 2010; Van Overstraeten-Schlogel et al. 2006). The other niche is formed mainly by pericytes around blood vessels (Kiel and Morrison 2008) and generally favors expansion and mobilization of HSCs at the time of need. It is possible that BCCs exploit a parallel niche system to colonize the bone marrow. Cancer cells may remain dormant in an osteoblastic/MSCs niche and later cascade into the circulation through interactions with blood vessels. Indeed, there are indications to support this claim. Our group demonstrated in vitro that breast cancer cells produce soluble mediators that facilitate the selective attachment of the breast cancer cells to immature osteoblasts and identified γ -secretase-dependent signaling as a critical mediator of this process (Fong et al. 2010). Phadke et al. followed GFP-tagged human breast cancer cell line after intra-cardiac injection in athymic mice, and demonstrated that cancer cells lodged at the endosteal surface in close proximity to osteoblasts and remained as single-cell foci for 72 h post-injection (Phadke et al. 2006). This study provided direct evidence supporting the presence of a bone marrow metastatic niche for mammary carcinoma that is supported by endosteal osteoblasts. As mentioned above,

BCCs often express RANK and CXCR4 on their surfaces. These two receptors target the directional migration of BCCs to bone where their respective ligands are highly expressed on the surface of osteoblasts. Having adhered to their osteoblastic niches, BCCs may gain survival advantage through Src mediated signaling down-stream of CXCR4 (Zhang et al. 2009). Mature osteoblasts also secrete increased amounts of OPG which can inhibit TRAIL receptors to maintain survival of BCCs (Neville-Webbe et al. 2004). Thus, osteoblasts at the endosteal side of the bone marrow are rational constituent of the niche for BCCs in the single-cell dormancy pattern.

The role of osteoclasts in driving the osteolysis associated with clinically overt bony metastases has been confirmed beyond doubt (Akhtari et al. 2008; Bendre et al. 2003a, b; Berenson et al. 2006; Casimiro et al. 2009; Chirgwin and Guise 2000; Clines and Guise 2005; Gaten et al. 2010; Guise et al. 2006; Kakonen and Mundy 2003; Kozlow and Guise 2005; Lipton 2006; Siclari et al. 2006). Less evident is the role of osteoclasts in the pre-clinical stages of bone metastases. The current paradigm of HSC niche suggests that osteoblasts maintain HSC at bone marrow while osteoclasts favor mobilization of vascular progenitors towards blood stream (Aicher et al. 2008; Brouard et al. 2010; Cho et al. 2010; Kollet et al. 2007, 2006). Do osteoclasts help mobilize dormant cancer cells and initiate cancer spread? Currently this question has no definite answer. On one hand, there are evidences that osteoclasts can suppress proliferation of leukemia cells at least in vitro (Yokota et al. 2010). On the other hand, prostate cancer cells have been shown to preferentially metastasize to sites of bone turnover containing active osteoclasts (Schneider et al. 2005), which suggests that osteoclasts may contribute to cancer cell homing.

Taken together, an emerging understanding of the pathogenesis of the pre-clinical stages of breast cancer metastases at bone includes the following events. Cancer cells are directed to migrate to bony sites by a chemotactic gradient of RANKL and/or SDF-1. Homing BCCs lodge in an osteoblastic niche where they gain survival advantage probably mediated through Src inhibition of TRAIL apoptotic signaling. Remains to be solved is the nature of the stimuli that later switch-on the macroscopic tumor growth and whether osteoclastic activation may be involved in this event.

The clinically overt metastatic stage

Osteoclasts have a pivotal role in the pathology of the clinically overt osseous lesions of breast carcinoma. The clinical appearance of osseous metastases is associated with increased proliferation of cancer cells, which succeed to mount a supportive microenvironmental reaction. BCCs

induce intense osteoclastic response which perpetuates the growth of cancer cells at the involved bone tissues through resorption of bone matrix and the release of growth factors stored in that matrix. This reaction potentially derives more cancer growth at the metastatic site in what came to be known the “vicious cycle of bone metastases” and has been exhaustively reviewed in the literature (Akhtari et al. 2008; Bendre et al. 2003a, b; Berenson et al. 2006; Casimiro et al. 2009; Chirgwin and Guise 2000; Clezardin and Teti 2007; Clines and Guise 2005, 2008; Gatten et al. 2010; Guise et al. 2006; Kakonen and Mundy 2003; Kozlow and Guise 2005; Lipton 2006; Mundy 1997; Reddi et al. 2003; Siclari et al. 2006; Wang et al. 2006). BCCs induce increased osteoclasts formation, survival and function through direct and indirect mechanisms. Cancer cells derived mediators can act directly on osteoclasts or can act on osteoblasts to modulate the expression pattern of osteoclast regulatory molecules.

i. Direct osteoclastogenic effects of BCCs

Breast cancer cells of different clones were found to produce molecules capable of supporting and inducing osteoclastogenesis. Interleukin-8 (IL-8) was identified as one such molecule produced by bone metastatic subclone of breast carcinoma cells MDA-MB-231 (Bendre et al. 2005). IL-8 was shown to directly induce osteoclastogenesis (Bendre et al. 2003a, b), and its expression was found to correlate with bone metastases in vivo (Bendre et al. 2002). Breast cancer cells can also secrete MCSF that directly promotes osteoclasts formation (Mancino et al. 2001) and survival (Gallet et al. 2006, 2004; Hussein et al. 2010). Mancino et al. generated osteoclasts from the bone marrow derived cell line UAMS-33 by supplying soluble RANKL and MCSF. Osteoclasts also developed in the same system when supplied with RANKL and MDA-MB-231 cells as a source of MCSF (Mancino et al. 2001). Gallet et al. showed that MDA-MB-231-derived MCSF is also responsible for inhibition of apoptosis of mature osteoclasts (Gallet et al. 2006, 2004). We recently confirmed the anti-apoptotic effect induced by MDA-MB-231 on mature osteoclasts and we have identified MCSF as a partial mediator of these effects and the pro-apoptotic protein BIM as an osteoclastic target for down-regulation by breast cancer-derived factors (Hussein et al. 2010). These findings suggest that BIM-targeted therapies such as the proteasome inhibitor Bortezomib, which is known to inhibit human osteoclastogenesis (von Metzler et al. 2007), may be interesting candidates for further investigation in the setting of tumor-associated osteolytic disease. Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) has also been shown to induce osteoclasts formation and promote cancer-mediated osteolysis in animal models of metastatic breast cancer (Dai et al. 2010; Park et al. 2007).

Our group has also found that during osteoclastogenesis, osteoclast precursors acquire the ability to strongly respond to factors produced by highly proliferating breast cancer cells (Guo et al. 2008; Tiedemann et al. 2009). We have found that breast cancer-derived factors can substitute for RANKL in inducing intracellular calcium signaling and nuclear translocation of NFATc1, resulting in robust osteoclastogenesis in RANKL-primed osteoclast precursors in vitro (Guo et al. 2008). In addition, breast cancer-derived factors induced unique signaling profile in differentiating pre-osteoclasts and mature osteoclasts, which was distinct from normal RANKL-induced differentiation and survival signals (Hussein et al. 2010; Tiedemann et al. 2009), suggesting the involvement of pathological mediators of osteoclastogenesis.

ii. Role of osteoblasts-mediated osteoclastogenesis in BCC establishment in bone

Physiologically, osteoclastogenesis is regulated by osteoblasts, producing both pro- and anti-resorptive mediators. This property of osteoblasts has been used by the invading cancer cells to their advantage. Parathyroid hormone related protein (PTHrP) (Guise et al. 1996; Kitazawa and Kitazawa 2002; Yin et al. 1999), IL-8 (Bendre et al. 2003a, b; Kinder et al. 2008a, b; Singh et al. 2006), IL-6 and Monocyte Chemoattractant Protein (MCP-1) (Chen et al. 2009; Kinder et al. 2008a, b) are the BCCs-derived mediators that act on osteoblasts to induce the expression of the osteoclastogenic mediators such as RANKL (Berenson et al. 2006; Fong et al. 2010; Kozlow and Guise 2005) and Prostaglandin E2 (PGE2) (Chen et al. 2009; Li et al. 2008; Morgan et al. 2004). The resulting increased osteoclasts population at the tumor-bone interface secrete proteolytic enzymes such as Cathepsin K (Guo et al. 2008; Le Gall et al. 2007) and Cathepsin G (Wilson et al. 2008, 2009) that resorb the bone matrix and release stored growth factors such as IGF and TGF- β . The latter induces further PTHrP release from BCCs (Chirgwin and Guise 2000; Kozlow and Guise 2005; Yin et al. 1999) setting on the vicious cycle of bone metastases.

PTHrP is one of the most extensively studied tumor-derived mediators that increase RANKL expression by osteoblasts. In one study of metastatic breast cancer cases, PTHrP was detected by immunohistochemistry in 92% of bony metastatic lesions, but in 17% of metastases to other organs (Powell et al. 1991). However, a prospective study of 526 breast cancer patients demonstrated that PTHrP expression at the primary tumor site correlated with better survival and even with fewer incidences of bone metastases, indicating that PTHrP at the primary tumor may delay tumor invasion (Henderson et al. 2006). The authors also examined 19 bone metastases specimens and their corresponding primary tumor lesions. Of the seven

PTHrP-negative primary tumors, six cases developed PTHrP-positive bony metastases (Henderson et al. 2006), suggesting that the tumor expression of PTHrP is driven by the local milieu at bone tissue. TGF β , which is abundant in bone matrix, augments the expression of PTHrP in breast cancer cells (Yin et al. 1999). The metastatic breast cancer cell line MDA-MB-231 grows readily and forms extensive osteolytic metastases when inoculated in nude mice. Treatment of mice with PTHrP blocking antibodies prior to MDA-MB-231 inoculation reduced both osteoclasts number and tumor area compared to controls (Guisse et al. 1996). The ability of MDA-MB-231 to form tumor growth was also significantly reduced in TGF $\beta^{-/-}$ mice relative to wild type animals (Mourskaia et al. 2009), which further underscore the importance of the host microenvironment in deriving bony metastases.

In addition to its increased ability to induce osteoclastogenesis, bone metastatic breast cancer cells have enhanced ability of secreting Matrix metalloproteases such as MMP1 (Kang et al. 2003) and MMP3 (Rose et al. 2007). Rose et al. demonstrated the importance of osteoactivin, the murine counterpart of Glycogen Nonmetastatic Melanoma Protein B (GPNMB) in inducing MMP3 expression by cancer cells at bony metastatic sites. In their model, forced expression of osteoactivin in the murine mammary carcinoma 4T1 cells enhanced their ability to form osteolytic metastases (Rose et al. 2007). They also reported the prognostic importance of GPNMB as an independent predictor of relapse in breast cancer patients (Rose et al. 2010).

Targeted therapy of bone metastases

Currently, the most promising results of targeted therapy for osseous lesions of MBC came from trials involving drugs that inhibit either osteoclasts function or formation (Bisphosphonates, RANKL inhibitor and Src inhibitors). The published and ongoing trials of targeted therapy of osseous metastases of breast cancer have been recently reviewed (Onishi et al. 2010; Rose and Siegel 2010). In the following paragraphs, we will briefly discuss the current status of the investigational targeted therapies that gain rationale from the above discussion of the disease pathogenesis (Fig. 3).

i. Bisphosphonates

Bisphosphonates bind the inorganic matrix of the bone with high affinity due to their structure of two phosphate groups linked to a carbon atom. Systemically administered bisphosphonates are mainly deposited in the bone or excreted by the kidney. Osteoclast activity solubilizes bisphosphonates, which then become internalized by osteoclasts. The first generation bisphosphonates, such as clodronate, induce

Fig. 3 Schematics of the main therapeutic targets explored for treatment of breast cancer metastases to bone. TCE 9908 is a CXCR4 inhibitor, Denosumab is a fully humanized monoclonal antibody against RANKL, Dasatinib is an inhibitor of multiple tyrosine kinases including the Src family, NBPs are nitrogen-containing bisphosphonates. The main intracellular signalling molecules in osteoclasts are represented by white ovals while transcription factors are represented by green ovals

osteoclast apoptosis, while nitrogen-containing bisphosphonates (NBPs) inhibit farnesyl transferase, which is essential for the post-translational modification of Ras, Rac and Rho small GTPases leading to inhibition of osteoclast activity (Russell 2007). Although non-nitrogenous bisphosphonates showed efficacy in the treatment of breast cancer (Diel et al. 2008), there has been a trend towards using NBPs for this disease.

Zoledronic acid (ZA) is the most potent NBP approved at present. In clinical trials, ZA reduced the incidence of SREs (Carteni et al. 2006; Facchini et al. 2007; Kohno et al. 2005; Lipton et al. 2007a, b; Rosen et al. 2003; Rosen et al. 2004) and improved survival of metastatic breast cancer patients with osseous lesions. Survival benefit of ZA in the metastatic setting was associated with its anti-resorptive effect (Lipton et al. 2007a, b). In the adjuvant setting, ZA decreased bone loss related to cancer therapy (Brufsky et al. 2008, 2007, 2009; Bundred et al. 2008; Gnani et al. 2008, 2009, 2007; Hershman et al. 2008, 2010) and improved the disease-free survival in premenopausal women under hormonal therapy (Gnani et al. 2009). Other pharmacological actions have been attributed to ZA that might contribute to its therapeutic benefit, including direct action on cancer cells (Denoyelle et al. 2003; Hiraga et al. 2004; Ottewill et al. 2008), anti-angiogenic action (Santini et al. 2007) and immunomodulatory action (Diel et al. 2007;

Kondo et al. 2008; Meraviglia et al. 2010). ZA treatment was shown to decrease DTCs population in early breast cancer patients, indicating its potential role in combating tumor dormancy and late relapse (Aft et al. 2010; Rack et al. 2010). The main side effects of ZA are renal toxicity, hypocalcemia (Zuradelli et al. 2009) and osteonecrosis of the jaw (Fehm et al. 2009; Kyrgidis et al. 2008).

Although bisphosphonates demonstrate promising results in cancer setting, their potency seems to be reduced relative to what is observed in osteoporosis (Levine 2006) or Paget's disease of bone (Josse et al. 2007). In this regard, we recently reported that the effect of NBP on osteoclasts was strongly affected by the presence of breast cancer cells. Soluble factors released from breast cancer cells prevented alendronate or pamidronate from inducing caspase-3 mediated apoptosis of mature osteoclasts. We identified the pro-apoptotic protein BIM as a target of down-regulation by cancer cells-derived mediators that was associated with osteoclasts resistance to spontaneous and bisphosphonate-induced apoptosis (Hussein et al. 2010).

ii. Denosumab

Denosumab is a fully humanized monoclonal antibody against RANKL. Denosumab demonstrated high efficacy in NBPs-naïve (Body et al. 2006; Lipton et al. 2007a, b, 2008) and NBPs-resistant (Fizazi et al. 2009) patients. Fizazi et al. described the results of a randomized trial from participating institutions recruiting patients with solid tumors (lung excluded) or melanoma who had experienced persistent elevated markers of bone resorption despite 8 weeks or more of IV treatment with NBPs. Normalization of resorption markers was achieved in 71% and 29% of the patients in the Denosumab arms and NBPs arm respectively. More patients in the NBPs arm developed first on-study SRE and adverse events were similar across study arms (Fizazi et al. 2009). Denosumab is a viable option for NBPs-resistant patients with bone lesions, however long-term toxicity data remain to be characterized.

iii. Src inhibitors

Src is a logical target for bone metastases prevention and treatment, since it is involved in tumor growth, osteoclasts function and resistance to endocrine therapy (Araujo and Logothetis 2009; Araujo and Logothetis 2010; Hiscox et al. 2010; Saad and Lipton 2010), as well as tumor cells survival and dormancy in bone marrow (Zhang et al. 2009). Dasatinib is the most widely used Src inhibitor in the clinic. It was shown to inhibit proliferation, migration and invasion of basal-like BCCs in vitro (Finn et al. 2007; Nautiyal et al. 2009; Pichot et al. 2009), osteoclasts formation and function (Araujo et al. 2009) and to enhance osteoblasts differentiation (Id Boufker et al. 2010; Lee et al. 2010) that may decrease RANKL/OPG ratio and decrease

the osteoclastogenic derive. Clinical trials of Src inhibitors in metastatic breast cancer are under way (Araujo and Logothetis 2009, 2010; Onishi et al. 2010; Saad and Lipton 2010).

iv. CXCR4 antagonists

In view of the strong evidences for CXCR4 involvement in tumor metastases, targeting CXCR4 remains a viable option for both adjuvant and palliative treatment settings. CTCE-9908, a peptide analogue of SDF-1 that competitively inhibits CXCR4, was demonstrated to decrease primary and metastatic tumor burden in mouse models of breast cancer (Hassan et al. 2010; Huang et al. 2009; Richert et al. 2009). The drug has been in Phase II clinical trial and publication of the results is awaited (Hotte et al. 2008).

v. Blocking the main players of the “vicious cycle”

The classic vicious cycle concept implies that bone metastases are driven by a positive feedback loop initiated by BCCs expressing PTHrP, which induce expression of RANKL by osteoblasts, thus stimulating osteoclastic resorption and release of TGF β from bone, which further stimulates tumor growth. Prospective studies however correlated PTHrP expression in primary tumor with better survival and even less bone metastases (Henderson et al. 2006). The explanation of this controversy is not known but the authors demonstrated that BCCs may change their PTHrP expression on arrival to bone. At the moment, we are not aware of an active trial of PTHrP antagonists in bony MBC. Similarly, the multi-facet effects of TGF- β in cancer progression delayed the clinical application of targeting strategies in breast cancer (Mourskaia et al. 2007; Rose and Siegel 2010; Yang and Moses 2008). In fact, abrogation of TGF- β response signaling was associated with poor outcome in several independent human data sets (Bierie et al. 2009).

Other targeted therapies

Cathepsin K inhibitors had a therapeutic benefit in animal models of metastatic breast cancer (Le Gall et al. 2007) but development in clinical phases was halted by the toxicity profile. Similarly, matrix metalloproteinase inhibitors were almost arrested in clinical trials due to dose-limiting toxicities (Pavlaki and Zucker 2003). The mechanistic role of connective tissue growth factor (CTGF) in osteolytic metastases of breast carcinoma was demonstrated in xenograft models (Kang et al. 2003; Shimo et al. 2006). Neutralizing antibody to CTGF decreased tumor associated osteolysis in a mouse model of bone metastases of human breast carcinoma (Shimo et al. 2006). Glembatumumab is a

fully humanized monoclonal antibody against the extracellular domain of GPNMB that has shown antitumor activity in phase I/II clinical trials (Naumovski and Junutula 2010).

Closing remarks

Since the discovery of RANKL as a master osteoclastogenic cytokine, the vicious cycle associated with bone metastases has been well-characterized. The potent osteoclast inhibitors ZA and Denosumab proved to be of valuable benefit in the treatment of osteolytic disease of breast cancer bone metastases. Nevertheless, these two drugs are expensive to a prohibitive degree in many societies. In addition, there is a need for second line therapies for the intolerant cases and for the non responders. While the vicious cycle is clearly portrayed, targeting the main molecular players often did not translate to the clinic because of the low therapeutic index in case of cathepsin K and MMPs inhibitors or because of their bidirectional role in the pathogenesis of the disease as in case of TGF- β and PTHrP. Therefore, continuous research into the pathogenesis of clinical stages of metastatic breast cancer is important to identify new therapeutic targets. Such studies, may lead to the development of new treatments, or may provide strong rationale for the use of established drugs in the bone metastatic setting. Importantly, the pre-clinical stages of bone metastases are much less understood compared to active metastases. Recent studies of the mechanisms involved in the journey of metastatic cells from the primary site to overt metastases renewed interests in molecular targets such as Src and CXCR4 and revived the hope in finding a curative treatment for breast cancer metastases.

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