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Osteomimicry: how tumor cells try to deceive the bone

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1. ABSTRACT

Bone metastases are complications of multiple myeloma and solid tumors, including breast and prostate carcinomas. Several reports have demonstrated that the preference to metastasize to bone by tumor cells is not a casual but an addressed event, which relies on specific interactions among tumor cells, bone marrow microenvironment and bone cells. One of the features that gives tumor cells more chances to survive and proliferate into the bone tissue is osteomimicry, that is the ability to acquire a bone cell phenotype, especially osteoblast-like. As clearly demonstrated, prostate and breast cancer cells try to resemble osteoblasts by expressing bone matrix proteins, the specific marker alkaline phosphatase, and molecules regulating the osteoblast/osteoclast cross-talk. Based on this evidence it is crucial to dissect in more detail the molecular mechanisms underlying the osteomimetic properties of cancer cells and identify new therapeutic targets eventually leading to a better and prolonged life expectation for patients with bone metastases.

2. THE METASTASIS PROCESS

Metastasis is a process whereby tumor cells spread from their site of origin, that is the primary tumor, to a distant site in the body, using the blood and/or the lymphatic system as route for dissemination. In order to metastasize, a tumor cell must trigger a complex cascade of linked sequential events, each of them controlled by a specific molecular pathway (1,2) (Figure 1). In particular, the required steps imply:

1. Epithelial to Mesenchymal Transition (EMT). EMT is a process by which tumor cells lose their epithelial phenotype to acquire mesenchymal features. Although this is a physiologic phenomenon occurring during embryogenesis, tumor cells rely on it to become metastatic. EMT is the result of the accomplishment of several processes including: dissolution of adherence junctions, loss of cell polarity, loss of epithelial markers and gain of mesenchymal features, morphological changes towards a spindle-like cell morphology, eventually leading to a motile phenotype (3,4).

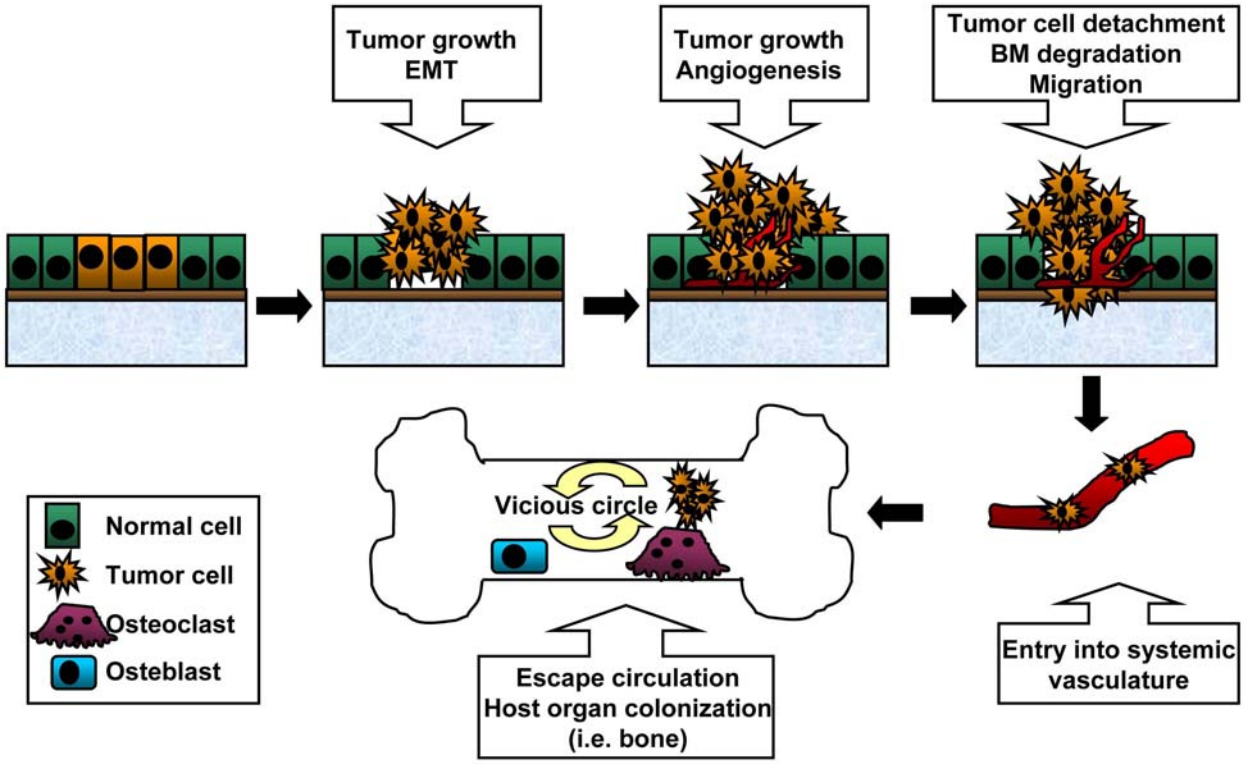

Figure 1. Schematic representation of the metastatic process. Transformed cells growing in the primary site undergo epithelial to mesenchymal transition (EMT) thus acquiring features potentially favoring their detachment and motility. Moreover, tumor cells trigger angiogenesis in order to self-feed and degrade the basement membrane to enter the systemic vasculature or the lymphatic bed. This allows them to join a distant organ (i.e. the bone) that they could colonize if favorable conditions for grow are found. (BM = Basement Membrane).

2. Local tumor growth in the primary site and induction of angiogenesis in order to self-feed.

3. Tumor cell detachment from the primary site. This process requires a cytoskeletal remodeling which, along with a reduction of cell-cell and cell-extracellular matrix interactions, leads to an increased motility and ability to migrate towards the neighboring tissues or the basement membrane.

4. Secretion of proteolytic enzymes (i.e. metalloproteinases) in order to disrupt the basement membrane and migrate through it.

5. Entry into the systemic vasculature or the lymphatic system, by penetrating the basement membrane surrounding a blood or lymph vessel and the layer of endothelial cells lining it.

6. Arrest in a distant capillary bed, escape the circulation and proliferate into the host site.

Furthermore, during their journey in the bloodstream, tumor cells must be able to escape host immune response and resist to physical circulatory forces. Indeed, clinical and experimental evidence indicates that metastasis is a poor efficient process, given that less the 0.1% of tumor cells can generate secondary tumors (5,6).

If we take into account only these steps of the metastatic cascade, which are shared by all types of cancers, we should assume that tumor cell spreading in a target organ is a casual and not an addressed event. This of course is not true and many questions remain to be answered in order to explain why a tumor cell prefers to colonize an organ instead of another. Of note, the first vascular bed encountered by tumor cells, in which they can be trapped, is in the lung, which is one of the most common sites of metastasis. In contrast, other tumors, such as breast and prostate, show a striking preference to invade bone tissue. Batson's anatomical studies (7) showed that venous blood from breasts and pelvis flows not only into the *venae cavae* but also into a vertebral-venous plexus that extends from the pelvis throughout the epidural and perivertebral veins. This route could, at least in part, explain the tendency of breast and prostate cancers to produce metastases in the axial skeleton. In addition to the hemodynamic theory, the "seed and soil" theory proposed by Paget (8) emphasizes the importance of the host milieu in the selectivity of tumor cells to metastasize a target organ. This process requires interactions among the tumor cells (seed), the circulatory system and the bone microenvironment (soil). Moreover, bone is also a storage for calcium and growth factors, including TGFbeta

Table 1. List of the osteomimetic proteins expressed by prostate and breast cancer cells

Factor	References
Bone matrix proteins	
BSP II (Bone Sialoprotein II)	15,18,19, 20,21, 62, 64, 65
OC (Osteocalcin)	10,15,40,58,
OPN (Osteopontin)	15,17,18,19,66,67,86
ONC (Osteonectin)	68
ALP (Alkaline Phosphatase)	85
BMPs (Bone Morphogenetic Proteins)	74,75,76
RUNX2 (Runt-related transcription factor 2)	70,71,85
Osteoclast-regulating factors	
OPG (Osteoprotegerin)	26,27
PTHrP (Parathyroid Hormone related peptide)	30,31,32,33,46,
M-CSF (Macrophage-Colony Stimulating Factor)	34
RANKL (Receptor Activator of NF-kappaB Ligand)	34

(Transforming Growth Factor beta), IGF-I and II (Insulin-like Growth Factor I and II), FGFs (Fibroblast Growth Factors), PDGF (Platelet-Derived Growth Factor), BMPs (Bone Morphogenetic Proteins), which are released and activated during bone resorption, providing the fertile ground in which tumor cells can grow (9).

3. BONE AS PREFERENTIAL SITE OF METASTASIS

Bone represents the preferential site for metastasis in advanced breast and prostate cancer patients, with an incidence up to 70 % (9). Even with a lesser incidence (30-40 %) also carcinomas of the thyroid, kidney and bronchus commonly metastasize the bone (10), while the incidence is < 10 % for tumors of the gastrointestinal tract (10). Bone metastasis development is characterized by high morbidity caused by pain, hypercalcemia, pathologic fractures, and spinal cord and nerve root compression. Nevertheless, in many patients the metastatic bone disease leads to a more favourable prognosis if compared with visceral metastases (11), which encourages identifying specific treatments able to slow the progression of the disease and improve the quality of life.

Skeletal metastases are the result of interactions among tumor cells, endothelial cells, bone marrow environment and bone cells (12). According to their clinical and histological features, they can be classified in osteolytic, osteosclerotic (or osteoblastic) and mixed. Bone metastases of breast cancer (BrCa) patients are predominantly osteolytic, while only 15-20% of them develops osteoblastic lesions. In contrast, the nature of bone metastases in prostate cancer (PrCa) is preferentially osteoblastic, with a 70-80 % frequency (13). However, a substantial body of evidence showed that osteoclast activation is required not only in osteolytic but also in osteosclerotic metastases, and that the bone resorption phase is a pre-requisite for the subsequent deposition of bone (14).

4. OSTEOMIMICRY

The term osteomimicry identifies the ability of tumor cells that preferentially metastasize to bone, to acquire features normally expressed by bone cells, with particular regards to the osteoblast phenotype (Table 1). This theory, first applied for PrCa cells (15) can be also extended to other tumors that preferentially metastasize to bone, such as breast and lung carcinomas.

As clearly demonstrated by a number of reports, PrCa and BrCa cells "try" to resemble osteoblasts by expressing bone matrix proteins such as osteocalcin (OC) (16) osteopontin (OPN) (17,18) and its receptor CD44, bone sialoprotein II (BSP II) (18-21) and osteonectin (ONC) (15). Other osteoblast-related factors expressed by tumor cells are alkaline phosphatase (ALP) and the key osteoblast-specific transcription factor Runx2 (Runt related transcription factor 2) (22). Moreover, bone homing metastatic human PrCa cells of the LNCaP line, C4-2B, were found to mineralize *in vitro* under appropriate conditions (22).

As suggested by this evidence, osteomimicry relies onto the assumption that the acquisition of osteomimetic properties by tumor cells enhances their survival and proliferation in the bone microenvironment.

OPG (Osteoprotegerin), PTHrP (ParaThyroid Hormone-related Peptide), M-CSF (Macrophage Colony Stimulating Factor) and RANKL (Receptor Activator of NF-kappaB Ligand), which are secreted by osteoblasts and play pivotal roles in the regulation of osteoclastogenesis and bone resorption, were also found to be expressed by PrCa cells (23-25). OPG acts also as survival factor by protecting cancer cells from TRAIL-mediated apoptosis (26) and its expression in BrCa cell lines was directly correlated with bone specific homing and colonization (27).

Starting from this evidence, an open question that remains to be answered is when a tumor cell acquires osteomimetic properties. Two hypothesis have been formulated: i) some tumor cells in the primary site express an osteotropic phenotype which eventually allow them to metastasize to bone after dissemination or ii) tumor cells join the bone and some of them, under the stimulation of local factors, acquire the ability to express a bone-like phenotype and consequently to survive and proliferate in this organ.

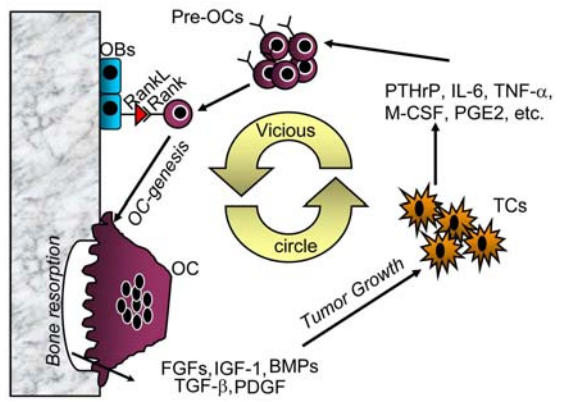

Figure 2. Schematic illustration of the vicious circle. Tumor cells intruding the bone marrow produce factors, such as PTHrP, IL-6, TNFα, M-CSF, PGE2 etc., which stimulate osteoclastogenesis either directly or inducing RANKL expression by osteoblasts (PTHrP). Increased osteoclast formation leads to an increase of bone resorption, with a consequent release of growth factors stored in the bone matrix, such as BMPs, PDGF, TGFbeta, IGF-I, FGFs that enhance tumor growth, thus perpetuating the vicious circle (OBs = osteoblasts; pre-OCs = *pre-osteoclast; OC = Osteoclast; TCs = tumor cells).*

4.1. Osteomimicry in osteolytic bone metastases

It has been well established that the destruction of bone in osteolytic metastases is mediated by the osteoclasts rather than by the tumor cells (9,28). Indeed, tumor cells that colonize the bone produce factors that directly or indirectly induce the formation of osteoclasts. Destruction of bone matrix by osteoclasts results, in turn, in the release of tumor-seeking factors therein stored, such as TGFbeta, IGFs, FGFs, PDGF and BMPs, that further stimulate cancer expansion (9,28,29). This mutual stimulation between tumor cells and the bone microenvironment results in a vicious circle that progressively increases both bone destruction and tumor burden (Figure 2).

The ability of BrCa cells to secrete into the colonized bone microenvironment osteoclastogenic factors normally produced by osteoblasts, contribute to foster the vicious circle. Among them, the PTHrP, which is produced by tumor cells under the action of TGFbeta (30), elicits the expression of RANKL by bone marrow stromal cells. Retrospective studies on breast cancer bone metastases revealed that 90% of them express PTHrP, while in primary tumors and in non-bone metastasis sites, this incidence drops to 60% and 17%, respectively (31-33). BrCa cells also produce M-CSF (34), PGE2 (Prostaglandin E2) and several pro-inflammatory cytokines such as IL-1 (Interleukin-1), IL-6 (35), TNFalpha (Tumour Necrosis Factor Alpha), GM-CSF (Granulocyte Macrophage-Colony Stimulating Factor) and IL-8, which stimulate osteoclast formation and enhance their bone-resorption activity (9,28,36). This latter cytokine can also act on osteoblasts by inducing RANKL expression (37).

A work from Kang *et al.* (38) identified by global gene profiling analysis a subset of osteotropic genes which are up-regulated in MDA-MB-231 human BrCa cells metastasizing exclusively in the bone. These include CTGF (Connective Tissue Growth Factor), which is an osteolytic and angiogenic factor, CXCR4 (Chemokine Receptor 4), associated with bone marrow-homing and extravasation, IL-11, having an osteoclastogenic role (55006) and MMP-1 (Metalloproteinase 1), the latter associated with proteolysis and invasion. These genes have little effects on metastatic activity when overexpressed individually. However, combining overexpression of as few as three of them produces a level of metastatic activity close to that of the highly aggressive cells that express the entire bone metastasis gene set (38).

A recent contribution to the osteomimicry theory comes from Bellahcène and colleagues (40). In particular, their transcriptome analysis on the MDA-MB-231 cell line and its more osteotropic variant BO2 (41) revealed a subset of significantly regulated genes which are associated with osteoblast differentiation. Among them, fibroblast growth factor 13 (FGF13), known as an osteogenic factor homologous to FGF2, was the most up-regulated. Consistently, other up-regulated genes were CTGF, endhotelin 1, osteonectin and IL-8, whose expression by human breast cancer cells correlates with bone metastases *in vivo*. Among the down-regulated genes, S100A4 (S100 calcium binding protein A4) and noggin are two negative regulators of osteoblast differentiation (42).

4.2. Osteomimicry in osteoblastic bone metastases

Prostate carcinoma is unique in that bone metastases have osteosclerotic features, although underlying osteolytic areas can be also observed (43). As for osteolytic lesions, osteoblastic metastasis development is the result of a vicious circle in which PrCa cells interact with both osteoclasts and osteoblasts in a complex interplay (23). Indeed, there is some evidence suggesting that metastatic PrCa cells also stimulate osteoclast activity. Urinary secretion of the N-terminal telopeptide of type I collagen, and free deoxypyridinoline and hydroxyproline, which are markers of bone resorption, are elevated in PrCa patients with bone metastases (44), along with high serum levels of IL-6, (45). Like BrCa, PrCa cells have been shown to consistently express PTHrP (46) and RANKL (47). Furthermore, inhibiting RANKL activity with a recombinant soluble form of RANK was shown to inhibit the progression of PrCa growth in bone (48). One of the mechanisms that trigger PrCa cell bone colonization is the expression of the alpha2 beta1 integrin, which facilitates their anchoring to type I collagen in the bone matrix (49). Expression of this integrin is also stimulated by TGFbeta, normally stored in bone matrix and released after bone resorption (15). Once attached, tumor cells are promoted to growth by local factors, such as IGF I and II, bFGF, PDGF and EGF, which are considered common mitogens for both osteoblasts and PrCa cells (50).

Although the mechanisms underlying osteoblastic metastasis have not been completely elucidated, several factors, described below, are involved

and, interestingly, some of them play crucial roles in osteoblast differentiation and function.

4.2.1 Urokinase-type plasminogen activator (uPA)

uPA is a serine protease known to have a pivotal role in tumor progression (22,51), and transfection of tumor cells with an anti-sense DNA to u-PA significantly reduced the ability to metastasize (9). Several line of evidence demonstrate also a role for uPA in the PrCa cells homing in bone (52). uPA is able *per se* to stimulate osteoblast mitogenesis either directly or by increasing the bioavailability of IGFs and TGFbeta, via degradation of IGFBPs and latent TGFbeta, respectively (53,54). Besides uPA, PrCa cells secretes hK2 (human kallikrein 2) which in turn activates single chain uPA.

4.2.2. Endothelin 1 (ET-1)

Although known as a vasoconstrictor, ET-1 can also regulate bone homeostasis by stimulating proliferation of osteoblasts (55). Moreover, a role for ET-1 in the development of osteoblastic metastasis has been supported by recent reports (56,57), and clinical data showed a significant increase of serum ET-1 levels in patients with osteoblastic metastasis from PrCa (58). By secreting ET-1, PrCa cells may promote their own growth directly, as well as *via* stimulation of osteoblasts, which produce growth factors, like IGF-I and PDGF, that further promote tumor growth. In an *in vivo* animal model of osteoblastic metastasis, treatment with a selective ET-1A-receptor antagonist decreased both osteoblastic metastasis as well as the tumor burden (56). Finally, Atrasentan, an ET-1 receptor A antagonist, was found to improve quality of life and bone pain in PrCa patients (58).

4.2.3. Beta2-Microglobulin (Beta2-M)

Beta2-M is involved in the presentation and stabilization of MHC I antigen on the cell surface; however a role of this protein in cancer and bone metastasis has recently been identified. In PrCa, Beta2-M protein levels seem to correlate with a more malignant phenotype (59). Beta2-M is also a mitogen capable of increasing growth of human osteoblasts (60) and of PrCa cells (61). Moreover, by a c-AMP/PKA-dependent mechanism it enhances the synthesis and deposition of the bone matrix proteins OC and BSP in PrCa cells (62). Finally, Beta2-M promotes EMT in prostate, renal and lung tumor cells (63).

4.3. Key molecules of tumor osteomimicry

4.3.1. Bone matrix proteins

The expression of OC and BSP seems to confer the ability to PrCa and BrCa cells to grow and survive in the bone microenvironment by favoring their adhesion to the extracellular matrix through integrin receptors (64,65). OC can also act as a chemoattractant for the recruitment of osteoblasts and osteoclasts, contributing to the dynamics of bone turnover, which is a pre-requisite for the onset and the development of bone metastasis. OPN is a bone matrix protein having a role in the process of metastasis, as their levels are increased in metastatic tumors (66). As far as its role in bone metastases is concerned, Khodavirdi *et al.*, showed that OPN overexpression in PrCa cells increased their proliferation and invasion (67). Finally, ONC has been found to increase survival, proliferation and invasion of PrCa cells (68).

4.3.2. Runt-related transcription factor 2 (Runx2)

Runx2 is the principal transcription factor regulating osteoblast differentiation, thus playing a prominent role in skeletal development (69). It is also expressed in normal mammary gland, while its overexpression is strongly associated to metastasis. Indeed, metastatic BrCa cells express high levels of Runx2 along with some genes regulated by this transcription factor, such as BSP, OPN and collagenase-3, which confer them an osteoblast-like phenotype (70). It has been recently demonstrated that transfection of human BrCa cell lines with a dominant negative Runx2 variant significantly decreased their ability to develop *in vivo* osteolytic metastases and inhibited the expression of the osteoclastogenic cytokines TNFalpha, RANKL and IL6 in bone marrow stromal cells (70,71).

4.3.3. Bone Morphogenetic Proteins (BMPs)

BMPs are a large group of proteins, belonging to the TGFbeta family, involved in the regulation of bone formation (72,73). Recent evidence demonstrated an additional role of these proteins in PrCa and BrCa metastases to bone. As demonstrated by Dai *et al.* (74), PrCa cells and tumor tissues produce BMP-6, whose expression increases with aggressiveness. *In vivo* treatment of mice injected with LuCaP 23.1 PrCa cells with anti-BMP-6 antibody or with the BMP inhibitor noggin reduces intraosseous tumor size. Conversely, Feeley *et al.* (75) observed that over-expression of noggin in PC3 PrCa cells reduces *in vivo* osteolytic bone metastases.

BMP-7 is another member of the TGFbeta family involved in tumor progression, as it induces EMT of PC3 cells and has an anti-apoptotic effect in the LNCaP PrCa cell line and, remarkably, in its bone metastatic variant, C4-2B.

Interestingly, a recent work by Buijs *et al.* (76) found that decreased BMP7 expression in primary BrCa is associated with the development of bone metastases. Moreover, in mice subjected to intracardiac injection of MDA-MB-231 BrCa cells overexpressing BMP-7, a significant inhibition of formation and progression of osteolytic bone metastases was observed. Finally, MDA-MB-231 treatment with BMP-7 decreased the expression of the mesenchymal marker vimentin, thus suggesting a role of this protein in EMT (76).

4.3.4. Wnt signaling

Wnt proteins are a family of glycoproteins which play a crucial role in bone development (77). Wnt proteins bind two surface receptors: Frizzled (FZD) and low density Lipoprotein Receptor-related Protein-5/6 (LRP-5/6). This interaction results in the stabilization and accumulation in the cytoplasm of the beta-catenin, which then translocates to the nucleus and acts as a co-factor for the T-cell factor (TCF) family of transcription factors and Lymphoid Enhancer Factor (LEF) (78). Conversely, the activity of Wnt proteins is controlled by soluble extracellular

antagonists, including secreted FZD-related proteins (sFRP), Wnt inhibitory factor-1 (WIF-1) and Dickkopf (DKK) (79). It has been shown by several reports that wnt signaling increases bone mass by stimulating osteoblast differentiation, proliferation, survival and activity (80). Moreover wnt signaling indirectly inhibits osteoclast differentiation by increasing osteoblast expression of OPG, the decoy receptor for RANKL (81).

As far as the role of wnt signalling in cancer is concerned, after translocation into the nucleus the beta catenin can drive the expression of oncogenes, including c-myc, of cell cycle proteins and of matrix metalloproteinase-7. Moreover, Wnt-1 and beta-catenin have been observed to be overexpressed in PrCa cells of patients with advanced prostate cancer (82) and to have a specific role in the development of osteoblastic metastasis. As demonstrated by Hall and colleagues (83), inhibition of wnt activity by stable transfection of DKK-1 switched the mixed osteoblastic/osteolytic C4-2B prostate cancer cell to a highly osteolytic cell line. Conversely, increasing wnt activity through blocking DKK-1 in osteolytic PC3 PrCa cells promoted osteoblastic activity. These results provide evidence that wnt not only contribute to the osteoblastic phenotype of PrCa bone metastases but may also act as a molecular switch mediating the transition of the bone metastasis from an osteolytic to an osteoblastic feature.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

Bone represents the third most common site of metastases, which are preferentially induced by breast and prostate cancer cells. Although characterized by severe morbidity due to bone pain, nerve compression, pathologic fracture and hypercalcemia, patients with bone metastases have higher chances of a longer survival relative to those with visceral metastases (10). At present, the anti-resorptive agents nitrogen-containing bisphosphonates represent the most effective therapy in patients with bone metastases. Unfortunately, experimental studies in animals, as well as clinical trials in humans, have demonstrated that bisphosphonates do not significantly affect tumor burden, so they can relieve the morbidity, but are unable to improve survival. Moreover, recent reports documented high likelihood to develop osteonecrosis of the jaw, an adverse effect that may severely compromise the quality of life of patients (84). Based on these assumptions, it is crucial to know more deeply the molecular mechanisms underlying the homing and colonization of bone by tumor cells. Indeed, studies investigating the osteomimetic properties of cancer cells have shed light on these mechanisms and could allow to identify new therapeutic targets eventually leading to a better and prolonged life expectancy for patients.

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