The Role of Bone Microenvironment, Vitamin D and Calcium

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Abstract

Starting first from Paget's "seed and soil" to the latest hypothesis about metastatic process involving the concept of a premetastatic niche, a large amount of data suggested the idea that metastatization is a multistep coordinated process with a high degree of efficiency. A specific subpopulation of cells with tumor-initiating and migratory capacity can selectively migrate toward sites that are able to promote survival, and/or proliferation of metastatic tumor cells through a microenvironment modification. Bone plays a pivotal role in this process, acting not only as a preferential site for cancer cells' homing and proliferation, due to a complex interplay between different cellular phenotypes such as osteoblasts and osteoclasts, but also as a source of bone marrow precursors that are able to facilitate the metastatic process in extra-skeletal disease. Moreover, bone microenvironment has the unique capacity to retain cancer stem cells in a quiescent status, acting as a reservoir that is able to cause a metastatic spread also many years after the resection of the primary tumor. To add a further level of complexity, these mechanisms are strictly regulated through the signalling through several soluble factors including PTH, vitamin D or calcium concentration. Understanding this complexity represents a major challenge in anti-cancer research and a mandatory step towards the development of new drugs potentially able not only to reduce the consequences

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of bone lesions but also to target the metastatization process from the "bone pre-neoplastic niche" to "visceral pre-neoplastic niches".

Contents

1 Bone Premetastatic Niche

Paget's "seed and soil" theory postulated that cancer cells "colonize" the organs whose microenvironment is advantageous. Starting from this theory, we can say that metastasis is a remarkably efficient, multistep process. The determinants of 'successful metastatic growth in a given organ are poorly understood, but there is substantial evidence to suggest that tumor cells and host tissue both play important roles in metastasis. This hypothesis started from a new model of metastasis formation. This 'early metastasis model' suggests that tumor cells leave the primary site much earlier in tumourigenesis (Pardal et al. 2003). The model is based on experimental data about cancer stem-like cells (CSC), a population of cells, within tumor mass, able to navigate in the bloodstream and to localize new metastatic sites (Pardal et al. 2003; Al-Hajj et al. 2003).

Populations of cells with tumor-initiating capacity have been shown to exist in human acute myeloid leukaemia (Lapidot et al. 1994; Bonnet and Dick 1997) and in several solid malignancies, such as breast (Al-Hajj et al. 2003) and colon cancer (O'Brien et al. 2007). Accordingly, "premetastatic niches" can be defined as a localized microenvironment that is being formed in metastatic target organs, prior to the arrival of metastatic tumor cells. Moreover, premetastatic niches consist of a collection of specific proteins and Bone Marrow Derived Cells (BMDCs). In fact, during the early development of primary tumors, neovascularization is guaranteed by VEGF-receptor 1+ (VEGFR1+), hematopoietic progenitor cells (HPC) which support the recruitment and incorporation of bone marrow-derived VEGF receptor 2+ (VEGFR2+) endothelial progenitors cells (EPC). These bone marrow-derived cells are also able to form clusters of cells in the tissue parenchyma at common sites of metastasis before actual tumor cell seeding. At these sites, bone marrow-derived cells express VEGFR1, CD11b, c-kit and other markers of their progenitor cell status within the tissue parenchyma of the premetastatic niche. Then, in response to the primary tumor chemokine secretion and other events, the VEGFR1+ HPCs proliferate and circulate in the bloodstream, but also preferentially localize to areas of increased fibronectin, newly synthesized by resident fibroblasts

and fibroblast-like cells. The VEGFR1+ HPCs express integrin VLA-4 (or $\alpha 4\beta 1$), allowing them to adhere specifically to the newly synthesized fibronectin for the initiation of cellular cluster formation. Other mediators such as metalloprotease 9 (MMP-9) allow extravasion of VEGFR1+ HPC. The VEGFR1+ HPCs, along with fibronectin and associated stromal cells, cause modifications of the local microenvironment, which leads to the activation of other integrins and chemokines such as SDF-1. SDF-1 itself promotes attachment, survival and growth of tumor cells.

Therefore, premetastatic niches are thought to be fertile regions of tissue that facilitate the invasion, survival and/or proliferation of metastatic tumor cells, providing a highly novel mechanism for the promotion of metastasis (Kaplan et al. 2005). Furthermore, some tissues are more receptive to a given metastasizing tumor cell type, which can explain the tendency of tumor cells to metastasize to some organs more often than other organs in a way that cannot be explained by differences in blood flow. In addition to target organ-specific growth of metastases, metastatic tumor foci seem to grow preferentially in specific areas of some tissues. So, we can postulate that cross-talking between bone microenvironment and cancer cells facilitates bone tropism of cancer cells. Moreover, there is some evidence that a specific subpopulation of cancer cells forming primary tumor sites can circulate as stem cells do.

1.1 Starting From the Primary Tumor Site: The Cancer Stem Cells

1.1.1 Cancer Stem Cell Characteristics

Stem cells are defined by their ability for self-renewal, differentiation into adult tissue and migration. Cancer stem-like cells are defined as cells capable of giving rise to a new tumor, and are thought to be the root cause of cancer. While still controversial, the Cancer Stem-like Cell (CSC) hypothesis may be directly relevant to metastatic theory, as such cells are good candidates for the acquisition of migratory capabilities and propagation of heterogeneous tumor cell populations at distant sites. Cancer stem-like cells may show some similarities to normal stem cells, but there appear to be differences too. While normal stem cells are present in tissues in relatively low numbers, the proportion of cells with specific CSC surface markers residing in a given tumor seems to vary greatly, up to 24.5% in colon cancer or 12–60% in breast cancer. In addition, putative breast cancer stem-like cells expressing CD44 (an adhesion molecule that binds hyaluronate) and lacking CD24 (an adhesion molecule that binds P-selectin) have been shown to switch to a more differentiated CD24-positive phenotype in distant metastases with loss of CD44, which can even progress to initiate further metastases (Shipitsin et al. 2007). As tumors progress through clonal selection of more malignant and less differentiated cells, differentiation between putative highly successful cancer stem-like cells and the rest of the tumor cells is difficult (Shipitsin and Polyak 2008). Another CSCs surface marker is CD133, which has been shown to be expressed in several solid malignancies such as

Table 1 Signaling pathway involved in stem-cells renewal

Pathway	Normal function	Function in tumorigenesis
WNT	WNT are secreted proteins that bind Frizzled receptors, causing a chain of reactions with final induction of cell proliferation	It activates the overexpression of target genes promoting cancer cell proliferation
PTEN	It plays an important role in self-renewal and activation of hematopoietic stem cells	It forms a complex signaling network and maintains the cancer stem cell population
NOTCH	It plays a role in the normal development of many tissues and cell types through diverse effects on cell fate decision, stem cell renewal, differentiation, survival and proliferation	Its upregulation is involved in tumor metastatization
SHH	It promotes osteoblast differentiation in multipotent mesenchymal cells by upregulating the expression and function of RUNX2	Abnormal activation of the pathway leads to development of disease through transformation of adult stem cells into cancer stem cells

intestine, brain, lung and prostate (Sing 2003; Collins et al. 2005; Shmelkov et al. 2008; Bertolini et al. 2009). However, it was demonstrated that CD133 is expressed in differentiated epithelium cells. Moreover, both CD133-positive and CD133-negative metastatic cells were able to start tumorigenesis. These findings raised the question of whether the CD133-negative cells have represented a largely non-epithelial population of stromal and inflammatory cells.

1.1.2 The Acquisition of Self-Renewal and Migration Ability (The Epithelial Mesenchymal Transition)

Many signaling pathways involved in stem-cells renewal cause neoplastic proliferation and migration when dysregulated by mutations. The most studied pathways are WNT, sonic hedgehog (SHH), NOTCH and PTEN.

The features of these pathways in normal and neoplastic cells are summarized in Table 1.

WNT Pathway

WNTs are secreted proteins which bind Frizzled receptors. This link activates Dishevelled (DSH) which disrupts the complex of glycogen synthase kinase 3β (GSK3β), Casein Kynase 1 (CK1), axin and adenomatosis poliposis coli (APC). This disruption inhibits interaction with APC-β-catenin and allows β-catenin to accumulate and translocate to the nucleus, binding LEF\TCF transcription factors and activating the expression of target genes promoting cellular proliferation. Mutations of this pathway have been implicated in many types of cancers (Polakis

1999; Zhu and Watt 1999). Expression of stabilized β-catenin promotes the self-renewal of many types of stem cells. WNT signaling activates the same pathway in colorectal cancer cells (Chenn and Walsh 2002). Mutations that activate WNT signaling cause the hyperproliferation of crypt progenitors, generating benign polyps (Powell et al. 1992). So, tumorigenesis in the intestinal epithelium seems to be caused by the hyper-self-renewal of intestinal-crypt stem cells, followed by the accumulation of additional mutations (Kinzler and Vogelstein 1996).

PTEN Pathway

PTEN plays an important role not only in self-renewal and activation of hematopoietic stem cells but also in the prevention of leukemogenesis (Zhang et al. 2006). It is quite likely that PTEN also plays an important role in breast cancer stem cells by negatively regulating PI3K/mTOR/STAT3 signaling. Some in vitro studies showed that overexpression of PTEN decreased cancer cell tumorigenicity (Cheney et al. 1998; Zhou et al. 2007). PTEN/PI3K/mTOR/STAT3 signaling forms a complex signaling network that is able to maintain the cancer stem cell population within the whole cell population (Zhou et al. 2007).

Notch Signaling System

Notch plays a key role in the normal development of many tissues and cell types through diverse effects on stem cell renewal, cellular differentiation, survival and proliferation (Artavanis-Tsakonas et al. 1999). The Notch signaling system includes Notch ligands (Jagged 1), receptors, negative and positive modifiers and Notch target transcription factors. One of the most important functions of the Notch pathway is expansion of the hematopoietic stem cell compartment during bone development, and participation in osteoblast differentiation (Nobta et al. 2005). Moreover, this signaling system is aberrantly activated in a variety of human cancers, including T-cell acute lymphoblastic leukemia and carcinomas of the lung, colorectum, prostate and the breast (Radtke and Raj 2003; Kunnimalaiyaan and Chen 2007; Proweller et al. 2006). Notch upregulation is involved in tumor metastatization, and its inhibition impairs tumor spreading (Hughes 2009). Osteoblasts within the bone marrow are identified as the niche for supporting long-term hematopoietic stem cells, providing the Notch ligand Jagged1 and other factors under regulation by bone morphogenetic protein (BMP) and PTH/PTHrP signaling (Calvi et al. 2003). Increasing evidence suggests that the osteoblast niche inhibits drug-induced apoptosis and confers de novo drug resistance in myeloma cells (Nefedova et al. 2004). This type of paracrine Notch signaling in metastatic cancer cells could explain their predisposition to bone metastasis. Moreover, cross-talk occurs between TGF-β and the Notch pathway. TGF-β increases the expression of Hes-1, a direct target of Notch, in several cell types (Blokzijl et al. 2003). TGF-β induces the interaction of the intracellular domain of Notch1 with Smad3. TGF-β-induced EMT is blocked by RNA silencing of the Notch target gene Hey-1 and the Notch ligand Jagged1, and by chemical inactivation of Notch (Zavadil et al. 2004).

SHH Pathway

Sonic hedgehog (SHH) ligands have an autocrine and paracrine action. When SHH reaches its target cell, it binds to the Patched-1 (PTCH1) and −2 (PTCH2) receptors. These in turn relieve the Smoothened (SMO) inhibition, leading to activation of the GLI (GLI 1, GLI 2, GLI 3) transcription factors. Abnormal activation of the pathway probably results in early carcinogenesis through transformation of adult stem cells into cancer stem cells (Dahmane et al. 1997; Ruiz i Altaba et al. 2002). In vitro models showed that cancer cells overexpressing SHH upregulated the expression of SHH-responsive target genes GLI1 and PTCH1 in pre-osteoblasts cells, leading to the induction of early phase osteoblast differentiation. Cancer cells that metastasize to bone are in close physical contact with bone stromal cells including bone cells and their osteoblast progenitors, fibroblasts, hematopoetic cells and multipotent mesenchymal stem cells, and the SHH pathway induces bone modification in order to create the conditions for the premetastatic niche. In fact, downstream mechanism through which SHH-signaling induces osteoblast differentiation is not fully understood. A recent study has demonstrated that SHH promotes osteoblast differentiation in multipotent mesenchymal cells by upregulating the expression and function of RUNX2 (Shimoyama et al. 2007; Spinella-Jaegle et al. 2001). Other data suggest that in cells that are already committed to the osteoblast lineage and express endogenous levels of RUNX2, the induction of osteoblast differentiation by SHH occurs through a mechanism that does not require further transcriptional upregulation of RUNX2 (Zunich et al. 2009).

These and other pathways enable cancer cells to acquire stem cell-like characteristics. Furthermore, the acquisition of mesenchymal markers such as fibronectin, and progressive loss of E-cadherin in tumor cells with nuclear β-catenin accumulation, suggest that they have undergone an epithelial–mesenchymal transition (EMT) or transdifferentiation. This process of EMT can be described by the chronological sequence of five morphogenetic events:

1. Disassembly of tight junctions, which results in the redistribution of Zonula Occludens (ZO) proteins, claudins and occludins.
2. Disruption of the polarity complex.
3. Initiation of cytoskeleton reorganization (through actin reorganization).
4. Metalloprotease upregulation.
5. Increased deposition of extracellular matrix proteins.

In vitro and in vivo experiments showed that EMT is also promoted by Tumor Growth Factor β (TGF-β) (Miettinen et al. 1994; Piek et al. 1999; Derynck et al. 2001; Hugo et al. 2007). In fact, its related proteins cause transcription of different mesenchymal genes and repression of epithelial genes (Xu et al. 2009). These phenotypic changes finally promote cell mobility and migration to the premetastatic niche (bone microenvironment), and eventually cellular differentiation into distinct cell types (acquisition of the osteomimetic phenotype, osteomimicry). EMT is initiated by external signals, the extracellular matrix and soluble factors such as those of the TGF-β superfamily. These signaling pathways are thought to control the invasive behavior of solid cancers.

1.1.3 Tumor-Associated Macrophages

Solid tumors are not only composed of malignant cells, but they are also complex organ-like structures comprising many cell types, including a wide variety of migratory hematopoietic and resident stromal cells. Migration of these cell types into tumors has been interpreted as evidence for an immunological response of the host against any growing tumor. However, it is now acknowledged that tumors are largely recognized as self and lack efficient antigens. Instead, they appear to have been selected to escape the host immune system, to prevent rejection and facilitate tumor growth and spreading. This led to the proposal that infiltrates of hematopoietic cells have a causal role in carcinogenesis. Clinical data collected from a wide range of solid tumors underscore these findings, showing high densities of leukocytic infiltrations—mostly macrophages—correlating with a poor prognosis. Tumor-Associated Macrophages (TAM) originate from circulating monocytes and are activated macrophages of the polarized type II (M2 macrophages or activated macrophages), mainly induced by IL-4, IL-10, IL-13 and corticosteroids. Differential cytokine and chemokine production, and coordinated temporal and spatial activities of these cells in the tumor stroma are key features of polarized macrophages, which promote tumor angiogenesis and growth (Pollard 2009). These data suggest the new hypothesis that tumors can modify the behavior of macrophages from a potentially hostile antitumor phenotype to one that promotes malignancy. But what is the precise nature and function of these tumor-promoting macrophages? Can they participate in the building of a premetastatic niche? Increasing data support the identifying of a specific subpopulation of macrophages which:

1. Express the endothelial-cell marker TIE2 receptor (also known as TEK); their importance is shown by their ablation that blocks angiogenesis in xenograft tumors (De Palma et al. 2007).
2. Secrete VEGF through the HIF pathway (Murdoch et al. 2008).
3. Facilitate tumor cell motility; moreover, intravasation of tumor cells also occurs next to clusters of macrophages on the vessel surface (Wyckoff 2007).
4. Secrete MMP leading to ECM degradation (Hagemann 2005).
5. Secrete TNF, and activates the wNT-β-catenin pathway (Pukrop et al. 2006).

Moreover, TAM produce TGF-β which is involved in the process of EMT (Mantovani et al. 2006; Pollard 2009; Kagan and Li 2003). By doing so, macrophages may participate in the building of a premetastatic niche. M2 macrophages are known as differentiated cells in response to parasitic infection, allergic conditions and during tissue repair. IL-13 and IL-4 are the most important cytokines supporting the process of EMT. In the tumor microenvironment however, macrophages develop in the presence of growth factors such as CSF1, or in response to molecules that signal through nuclear factor-κB (NF-κB), becoming non-immunogenic and trophic (Pollard 2009). Overall, these data suggest macrophages to substantially participate in the building of the premetastatic niche.

1.1.4 Lysyl Oxidase

Lysyl Oxidase (LOX) is produced by fibrogenic cells. The main activity of LOX is thought to be oxidation of specific lysine residues of collagen (Kagan and Li 2003). Increased expression of LOX has been found in metastatic and/or invasive breast cancer cell lines and is associated with higher stages of disease in patients with renal cell carcinoma (Kirschmann et al. 2002). Hypoxic tumor cells often have increased LOX expression and secretion, enabling cell movement to more oxygenated and nutrient-rich areas. Then, LOX increases cell invasion and migration through regulation of cell–matrix adhesion. In addition, matrix remodeling by invasive tumor cells provides a more ideal soil for any succeeding tumor cells, building a kind of "highway to metastasis". LOX may be involved in tumor interactions with the cell–matrix required for intravasation and extravasation. Then, LOX is required for the formation of a mature ExtraCellular Matrix (ECM) at the secondary site, allowing tumor cell survival and possibly BMDC recruitment (Erler and Giaccia 2006). LOX secreted by hypoxic tumor cells accumulates at premetastatic sites, crosslinks collagen IV in the basement membrane and is essential for myeloid cell recruitment. Finally, tumor cells adhere to crosslinked collagen IV and produce matrix metalloproteinase-2, which cleaves collagen, enhancing the invasion and recruitment of BMDCs and metastasizing tumor cells (Erler et al. 2009).

1.2 The Long Way to the Bone: The Importance of Chemokines

Chemokines are small chemoattractant cytokines that bind to specific G-protein-coupled transmembrane receptors present on the plasma membranes of target cells. These molecules can guide circulating cancer cells to the bone.

1.2.1 CXCR4\SDF-1 Pathway

The chemokine receptor CXCR4 (or CD184) is an alpha-chemokine receptor for stromal-derived-factor-1 (SDF-1 or CXCL12) alpha or beta. SDF-1 and CXCR4 are a relatively 'monogamous' ligand-receptor pair (in contrast to other chemokines that bind several chemokine receptors in a more 'promiscuous' manner) (Arya et al. 2007). In the normal bone marrow, SDF-1 is produced by osteoblasts, fibroblasts and endothelial cells. Parathyroid hormone (PTH), PDGF (platelet-derived growth factor), interleukin-1 (IL-1), vascular endothelial growth factor (VEGF) and tumor necrosis factor alpha (TNF-α all induce SDF-1 production by osteoblasts (Jung et al. 2006). SDF-1 is important in hematopoietic stem cell homing to the bone marrow and in hematopoietic stem cell quiescence. It has been demonstrated that by blocking the CXCR4 receptor, hematopoietic stem cells mobilize into the bloodstream as peripheral blood stem cells. Moreover, it has been demonstrated that SDF-1 production can induce all of the following:

1. Osteoclast precursor recruitment by promoting chemotaxis, proteinase activity and collagen transmigration (Yu et al. 2003a, b).

2. Angiogenesis by recruiting endothelial progenitor cells (EPC) from the bone marrow via CXCR4-dependent mechanisms (Zheng et al. 2007).
3. Lymphocyte chemotaxis (Bleul et al. 1996; Ma et al. 1998).

There are many experimental data concerning the importance of the CXCR4\SDF-1 pathway for neovascularisation and metastatic spreading to SDF-1-expressing tissues, especially the bones:

1. Cancer cells in some organs such as lung, liver and bone produce large quantities of SDF-1 (Muller et al. 2001).
2. SDF-1 mRNA expression is observed in the metaphysis of the long bones, near the endosteal surfaces covered by osteoblastics (Sun et al. 2005). In an experimental animal model of breast cancer bone metastasis, it has been demonstrated that single tumor cells are homing to the metaphyses of the long bones after systemic inoculation. Furthermore, they were mostly located in close proximity to osteoblasts and lining cells (Phadke et al. 2006).
3. A gradient of chemokine expression was found between peripheral blood and bone marrow, with an increased expression of SDF-1 in the bone marrow along with lower concentrations of SDF-1 in serum, while increased expression of CXCR4 is found in peripheral blood with lower expression in bone marrow. This gradient promotes cancer cell migration into the bone marrow and prevents further trafficking of cancer cells (Hofbauer et al. 2008).
4. There is some evidence that activation of the SDF-1/CXCR4 pathway not only regulates migration and homing of cancer cells to the bone but also regulates adhesion, invasion and cytoskeletal rearrangement of cancer cells (Gerritsen et al. 2002).
5. Blocking CXCR4 with antibodies reduces the formation of experimental bone metastases induced by CXCR4-expressing breast or prostate cancer cells (Liang et al. 2004).

1.2.2 RANK\RANK-L Pathway

The RANK\RANK-L pathway is involved in tumor-induced osteoclastogenesis and osteolysis. RANK-L is physiologically produced by osteoblasts and stimulates osteoclast precursor recruitment and maturation. Then, mature osteoclasts cause bone reabsorption (Lacey et al. 1998). Normal glandular epithelial cells express RANK, and the RANK–RANKL pathway is involved in normal development of lactating mammary glands (Fata et al. 2000). It has been demonstrated that RANK expression facilitates cancer cell migration into the bones. Recent in vivo studies demonstrated the association between RANK expression and cancer cell osteotropism (Jones et al. 2006). In fact, it has been found that RANK is expressed by solid tumors, with a high concordance of the expression profile between bone metastases and corresponding primary tumors (Santini et al. 2010a, b). Moreover, RANK is clearly associated with early bone metastasis formation in breast cancer (Santini et al. 2010a, b) and, consistent with those data, it has been demonstrated that osteoprotegerin (OPG), a natural RANK-inhibitor, blocks cancer cell osteotropism (Dougall and Chaisson 2006).

1.2.3 The Integrin System

The study by Kaplan et al. (2005) showed that VLA4 or anti-VEGFR1 antibodies inhibit the proliferation and binding affinity of tumor cells to VEGFR1-positive HPCs, demonstrating their direct role in adhesion and growth of tumor cells. Moreover, other integrins are critical for cancer cell homing in other target organs. In fact, breast cancer cells expressing $\alpha v\beta 3$-integrin and prostate cancer cells expressing $\alpha v\beta 2$-integrin, which bind many bone matrix components, have a higher propensity for spreading to the bones (Pecheur et al. 2002). Additionally, proto-oncogenic tyrosine kinase c-SRC is similarly involved in the integrin's pathway, and has an important role in cancer cell osteotropism. SRC binds to activated RANK, thereby recruiting TRAF6 and Grb2-associated binder 2 (Gab2), followed by phosphorylation of $I\kappa B\alpha$ and JNK, which ultimately leads to activation of the transcription factors NF-κB and AP-1.

There are clinical and experimental data suggesting that SRC expression:

1. Increases the survival of tumor cells in the bone microenvironment, leading to the establishment of bone metastases (latent phase of bone metastasis).
2. Increases cell mobility (Zhang et al. 2009).
3. Increases osteotropism of tumor cells (Rucci et al. 2006; Boyce et al. 2003).

Moreover, expression of SRC is involved in bone marrow seeding and sustains the outgrowth of indolent cancer cells in the bone marrow microenvironment. Activated SRC plays a critical role in the initiation and maintenance of a tumor cell response to bone-derived factors CXCL12/SDF and TRAIL. Latency status is maintained until overt progression to the phase of osteolytic outgrowth.

1.2.4 The BMP Receptor Axis

Bone morphogenetic proteins (BMP) are members of the TGF-β (transforming growth factor-β) family. So far, three BMP receptors have been characterized: BMP-R Ia, Ib and II (van Dijke et al. 1994). It has been shown that prostate, breast and lung cancer cells express BMP-2 mRNA and its protein. Moreover, BMP receptors are expressed in prostate cancer cell lines (Schwalbe et al. 2003). The most important functions of BMP-2 in cancer cells are:

1. Promotion of cancer cell migration to the bones (Ite et al. 1997).
2. Modulation of cancer cell migration through the integrins axis.

In fact, breast cancer cell lines upregulate bone sialoprotein (BSP) expression in pre-osteoblasts (Bunyaratavej et al. 2000), and in vivo inhibition of BMP in osteoinductive prostate cancer cells inhibits the osteoblastic response in bone (Schwaninger et al. 2007). Moreover, BMP has been shown to reverse TGF-β-induced EMT by decreasing vimentin expression and increasing E-cadherin expression in breast cancer cells and in normal mouse mammary epithelial cells (Zeisberg et al. 2003; Valcourt et al. 2005).

The role of the cited chemokine network is summarized in Table 2.

Table 2 The importance of chemokines

Pathway	Normal function	Function in tumorigenesis
CXCR4/SDF-1	Osteoclastic precursor recruitment, endothelial progenitor cell recruitment, lymphocyte chemotaxis	It is linked to the neovascularisation and metastatic spreading to tissues releasing the ligands such as bone
RANK/RANKL	Osteoclast precursor recruitment and maturation	It is involved in tumor-induced osteoclastogenesis and osteolysis
Integrin system (avb3 e avb2)	Integrins are expressed by normal osteoclasts and interact with components of the bone matrix, contributing to bone resorption	Tumor cells expressing these integrins, which bind many bone matrix components, have higher incidence of bone metastases
BMPs/BMP	Bone morphogenetic proteins (BMP) are a group of growth factors able to induce the formation of bone and cartilage. They have an important role during embryonic development and on early skeletal formation	It has two important functions: promotion of migration of cancer cells to the bone, and modulation of migration of cancer cells through the integrin axis
VCAM-1/ICAM-1	Allows the adhesion and transmigration of hematopoietic and lymphoid cells	The cancer cells migrate through the vasculature using a process of attachment–detachment through a cell adhesion mechanism mediated by these molecules
OPN	Promotes the adherence of osteoclasts and hematopoietic stem cells to the bone matrix	Is involved in bone metastatic spread of breast, prostate and lung cancer, and OPN expression confers migratory abiliy and invasive phenotype in human mammary cells
Endothelin-1	Stimulates mitogenesis in osteoblasts	Induces expression and activation of the tumor proteases that degrade the tissue matrix to permit local invasion and formation of metastases

1.2.5 Other Adhesion Molecules

Osteopontin

Osteopontin (OPN) is a glycophosphoprotein and is one of the major components of non-collagenous bone matrix. OPN is expressed in osteoblasts and osteocyctes (bone-forming cells) as well as osteoclasts (bone-resorbing cells).

Osteopontin promotes the adherence of osteoclasts and hematopoietic stem cells to the bone matrix (Asou et al. 2001). Hypocalcemia and hypophosphatemia both stimulate kidney proximal tubule cells to produce calcitriol (1α,25-dihydroxyvitamin D3) that stimulates OPN gene translation via the VDRE (vitamin D response element) in the OPN promoter region (Prince and Butler 1987; Yucha and Guthrie 2003). OPN expression is also regulated by exposure of cells to various factors and metabolic settings, including tumor necrosis factor α, infterleukin-1β, angiotensin II, transforming growth factor β (TGFβ) and parathyroid hormone (PTH), hyperglycemia and hypoxia (Noda and Rodan 1989; Hullinger et al. 2001). Finally, it has been shown that OPN is involved in bone metastatic spread of breast, prostate and lung cancer cells (Wai and Kuo 2004), and OPN expression confers migratory ability and invasive phenotype in human mammary cells (Tuck et al. 2003).

Sinusoidal Endothelial Cell Adhesion Molecules

Cancer cells usually settle in bone metaphysis that is rich in sinusoidal microvasculature, rather than bone diaphysis. This is mainly caused by:

1. Hemodynamic properties of the sinusoidal vascular bed, with 90% of the blood circulating through metaphyseal sinusoids.
2. Characteristics of the sinusoidal endothelia, allowing adhesion and transmigration of hematopoietic and lymphoid cells.

In a model studying the kinetics of metastatic breast cancer cell trafficking in bone, it has been shown that the majority of cancer cells tended to settle in the endosteal marrow, rather than in the centrum of the marrow and that primary tumor cells most often locate in close proximity to osteoblasts and bone lining cells.

However, migration to the sinusoids of the bone marrow is not sufficient to ensure colonization by cancer cells. Moreover, cancer cells migrate through the vasculature using a process of attachment–detachment through cell adhesion mediated by several adhesion molecules such as E-selectin, N-cadherin, intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) (Makuch et al. 2006). VCAM-1, a member of the immunoglobulin family of cell adhesion molecules, has ICAM-1 and VLA-4 as its main receptors, with the latter being constitutively activated in osteoclasts and are also found on many cancer cells. Inflammatory cytokines produced by osteoblasts in the presence of breast cancer cells may cause endothelial activation, expression of adhesion molecules and cancer cell invasion (Glinsky et al. 2001).

Endothelin 1 and ET Receptors

Endothelin-1 (ET-1) has been detected in osteocytes, osteoblasts, osteoclasts and vascular endothelial cells (Sasaki and Hong 1993). Endothelin-1 stimulates mitogenesis in osteoblasts (Stern et al. 1995). Moreover, ET-1 enhances the effect of other osteoblast-stimulatory factors, such as BMP-7, to induce bone formation (Nelson et al. 1995; Kitten et al. 1997).

Additionally, ET-1 stimulates the expression of osteopontin and osteocalcin in rat osteosarcoma cells (Shioide and Noda 1993), and mineralization of the bone matrix also depends on the ET-1 receptor pathway. Other studies of ET-1 null mice showed that ET-1 may regulate proliferation and migration of osteogenic cells rather than modulating the expression of bone matrix proteins (Kitano et al. 1998).

Nevertheless, the role of the ET-1 pathway in bone metastasis is not clear. Malignant tumors of the breast and prostate are typically associated with osteoblastic bone metastases, and both tumors express ET-1 and its receptors. Importantly, paracrine effects of ET-1 on bone cells may provide a favorable growth environment for tumor cells in bone. Endothelin-1 is found in normal prostate epithelium, throughout the entire gland. Addition of exogenous ET-1 increases the proliferation of prostate cancer cells and enhances the mitogenic effects of IGF-1, IGF-2, PDGF, epidermal growth factor (EGF) and FGF-2 on cancer cells. Moreover, ET-1 concentrations were significantly higher in men with advanced, hormone-refractory prostate cancer with established metastases to the bones as compared to patients with early-stage disease (Nelson et al. 1995). Increased ET-1 production has been described in prostate cancer cells through contact with bone (Chiao et al. 2000). Tumor-derived ET-1 stimulates new bone formation via ETA receptors on the surface of osteoblasts. Subsequently, growth factors produced by osteoblasts are incorporated into the new bone matrix as well as the local microenvironment. Interestingly, ET-1 mediates vasoconstriction of distal blood vessels, but not of those vessels directly supplying the tumor bed. This 'vascular steal phenomenon' improves local blood supply to the tumor and thereby improves oxygenation of tumor cells. Activation of endothelial cells by ET-1 stimulates the production of vascular endothelial growth factor (VEGF) by increasing the levels of hypoxia-inducible factor-1α (HIF-1α). Endothelin-1, acting through ETA, also induces the expression and activation of tumor-associated proteases, matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA) (Spinella et al. 2003). Metalloproteinases degrade the local tissue matrix to permit local tumor invasion and formation of metastases. Urokinase plasminogen activator in turn converts plasminogen into plasmin, which degrades the tumor stroma, allowing the tumor to invade the surrounding tissue and prepare metastatic spread. Urokinase plasminogen activator might also activate MMP. Taken together, these factors have the potential to stimulate tumor growth as well as further increase tumor production of ET-1 (Guise et al. 2003).

1.3 Modification of the Bone Microenvironment: Premetastatic Niche Formation

Many data suggest the hypothesis that growth of macrometastases starts from the interaction between the target organ (e.g. bone) and the primary tumor that builds the premetastatic niche. In fact, studies in breast cancer demonstrated that although about 30% of patients may have micrometastatic disease in their bone marrow at

the time of presentation, only 50% of these patients develop overt bone metastatic disease after 5 years. Furthermore, many patients with breast and prostate cancer do not develop bone metastases until many years after the surgical removal of the primary cancer. The Paget's "seed and soil theory" hypothesizes that metastatic cells can enter into the bone marrow and remain in a quiescent status for many years. However, molecular mechanisms and signaling pathways that promote the switch to a premetastatic bone microenvironment are not well understood. We can postulate that cancer stem cells, like physiologic haematopoietic stem cells, can establish a relationship with bone marrow stroma in order to mantain their survival. Physiologically, two fundamental niches exist in the bone marrow:

1. *Endosteal Niche*. Stem cells are closely associated with spindle-shaped N-cadherin positive osteoblasts (SNO). Moreover, these osteoblasts are involved in the maintenance of stem cell quiescence. The same osteoblasts might also bind cancer stem cells, thereby maintaining their dormancy (Yin et al. 2006).
2. *Vascular Niche*. More differentiated cells are generally located in central parts of the bone marrow.

It is well understood that circulating cancer cells arrive in the endosteal niche and are kept in a dormancy status via the link with SNO and the release of inhibitory molecules by stromal cells (such as fibronectin). RANK\RANK-L is also involved in cancer cell reactivation via osteoclast activation and the consequent release of growth factors such as TGF-β, BMPs, PTH-related protein (Oh et al. 2004). These factors are typically involved in the process of epithelial–mesenchymal transition, and likely activate dormant metastatic cancer cells. Activated osteoclasts may directly induce hematopoietic stem cells, and probably metastatic cancer cells, to separate from the endosteal niche through increasing proteolytic activity of MMP-9 and cathepsin K, which clive and inactivate SDF-1, osteopontin and other niche factors (Kollett et al. 2006). Moreover, stromal cells secrete inactive metalloprotease-2, which is activated by cancer cells increasing their migratory capacity (Harada and Rodan 2003). At the time of bone marrow colonization, when pathologic bone lesions are still not evident, cancer cells acquire a bone-like phenotype, and this process is known as osteomimicry (Knerr et al. 2004).

1.4 Osteomimicry

We still do not know whether cancer cells already possess the osteomimetic phenotype when they detach from their primary tumor, or whether these characteristics are acquired when they colonize the bone niche. There is some evidence though that at least some cancer cells do need a biological signature to invade osseous structures (Ramaswamy et al. 2003).

As shown in Table 3, many molecules are produced from cancer cells invading bone marrow.

Table 3 Molecules produced from cancer cells invading the bone marrow (osteomimicry)

Molecules	Function when expressed by normal cells	Function when expressed by cancer cells
PTHrP	Homolog of PTH that has a direct action on the PTH receptor, stimulating bone resorption and renal tubular calcium resorption	It causes bone microenvironment modification that facilitates the establishment of circulating cancer cells, the release of bone-derived growth factors and the formation of the premetastatic bone niche
RANKL/OPG/TRAIL	RANKL induces osteoclast activation, OPG binds RANKL thys inhibiting osteoclast development, TRAIL is an anticancer cytokine which binds OPG	The RANK expression status determines the predominant migration into bone, where RANKL is abundantly expressed
VEGF	Induces angiogenesis	It drives bone marrow-derived cells to neoangiogenetic sites in the tumor
C-KIT/SCF		Both ligand (SCF) and receptor (C-KIT) are expressed by cancer cells for intraosseous development, indicating the existence of an autocrine loop
IGF system	Stimulates osteoblast differentiation, increases bone matrix apposition and decreases collagen degradation	It increases prostate cancer cell proliferation and chemotaxis
RUNX	Essential for osteoblastic differentiation and skeletal morphogenesis	It promotes transcription of genes involved in the acquisition of migration and invasiveness
Calcium sensing receptor	Implicated in the regulation of ion and water transport, proliferation, differentiation and apoptosis	Its activation leads to increased PTHrP secretion which drives osteolysis by osteoclasts leading to release of growth factors and calcium from the bone matrix and further stimulation of tumoral cell proliferation

1.4.1 PTHRP

The parathyroid hormone-related protein is a homolog of PTH and has a direct action on PTH receptors, stimulating bone resorption and renal tubular calcium resorption (Yates et al. 1988). PTHRP is released by cancer cells of many solid tumors (Moseley et al. 1987; Burtis et al. 1987; Strewler et al. 1987), and contributes to metastatic spreading. It has been demonstrated that PTHRP is abundant in tumors with osteoclastic and osteoblastic bone metastases. In fact, breast carcinomas metastatic to the bones express PTHRP in >90% of the cases,

compared with only 17% of metastases to extraosseous sites (Southby et al. 1990; Grill et al. 1991; Powell et al. 1991; Vargas et al. 1992). Furthermore, growth factors such as TGF-β or IGF, which are abundant in mineralized bone matrix (Hauschka et al. 1986), are released and activated by osteoclastic bone resorption (Pfeilschifter and Mundy 1987) and may enhance PTHRP secretion from cancer cells (Zakalik et al. 1992; Merryman et al. 1994). Finally, other studies have shown that PTHRP, secreted by prostate cancer cells, stimulates ostoblastogenesis and osteoblast differentiation (Liao et al. 2008). Accordingly, it was postulated that PTHRP causes bone microenvironment modification in order to facilitate the establishment of circulating cancer cells, the release of bone-derived growth factors and the formation of the premetastatic bone niche.

1.4.2 RANKL\OPG\TRAIL

RANKL is produced by osteoblasts and stromal cells for inducing osteoclast activation and bone resorption. It was demonstrated that many types of solid tumors produce RANKL, both at the primary site and in metastatic bone lesions (Brown et al. 2001a, b; Chen et al. 2006; Sasaki et al. 2007). OPG is a soluble decoy-receptor of RANKL, which is expressed by various cell types, including osteoblasts and tumor-associated stromal cells. OPG binds RANKL and prevents RANK–RANKL association, inhibiting osteoclast development (Cross et al. 2006). It was observed that many tumor cell lines produce OPG (Holen et al. 2005; Holen et al. 2002). High serum levels of OPG were found in patients with advanced-stage prostate cancer (Brown et al. 2001a, b). Furthermore, inhibition of RANKL results in inhibition of malignant bone lesions and tumor growth in bone (Canon et al. 2008; Kostenuik et al. 2009), while OPG inhibits cancer-induced osteoclastogenesis (Zhang et al. 2001) and increases bone density. Moreover, OPG shares some sequence homology with Endothelin-1 (ET-1), and was shown to stimulate bone formation through ET-A receptor activation (Nelson et al. 1999). OPG also binds tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (Emery et al. 1998), the latter being an anticancer cytokine. Finally, based on high constitutive RANK expression in breast cancer cell lines, recent data actually indicate that RANK expression by cancer cells determines whether tumors predominantly migrate into bone, where the corresponding ligand RANKL is abundantly expressed (Jones et al. 2006). In murine animal models, the correlation between high expression of RANK and osteotropism has been demonstrated across different tumor types, including breast cancer and melanoma.

1.4.3 VEGF

Tumor induces angiogenesis through VEGF signaling (Ferrara 2009). Moreover, VEGF secretion attracts bone marrow-derived cells such as VEGFR1-positive HPC and VEGFR2-positive EPC to neoangiogenic sites in the tumor (Lyden et al. 1999). VEGF is closely associated with the early phases of bone remodeling and induces osteoblast chemotaxis and differentiation (Tombran-Tink and Barnstable 2004; Li et al. 2005). VEGF also upregulates RANK on endothelial cells, resulting

in the amplification of the angiogenic response in the presence of RANKL (Min et al. 2003). In effect, VEGF mediates both a direct and an indirect effect on bone growth by activating osteoblasts and promoting angiogenesis in metastatic sites with high concentrations of RANKL (Street et al. 2002). For these reasons, the localized production of VEGF, such as that observed in metastatic tissue, is likely to contribute to osteolysis and local tumor progression (Aldridge et al. 2005).

1.4.4 C-KIT\SCF

C-kit (or CD 117) is a tyrosine kinase receptor of the Stem Cell Factor (SCF). In vitro models demonstrated that prostate cancer cells in bone express high levels of c-kit, while prostate cancer cells from extraosseous sites are c-kit negative. Additionally, SCF was found to be overexpressed in bone metastases from prostate cancer (Wiesner et al. 2008). In vivo models showed that prostate cancer cells preferentially metastasize to regions of the bone marrow where SCF was expressed by stromal cells (BMS) interacting with c-kit expressed on the surface of bone marrow progenitor cells (Heissig et al. 2002). There is an association between coexpression of SCF and c-kit in prostate cancer cells and bone metastases, suggesting that both ligand and receptor should be expressed by prostate cancer cells for intraosseous development, also suggesting some autocrine loops. The increased expression of c-kit in bone metastases from prostate cancer might be the result of a selective dissemination and/or growth into the bone of c-kit-positive cells already present in primary tumors of the prostate, which are known to be heterogeneous (Patrawala et al. 2006). These c-kit-positive cancer cells could also represent cancer stem cells which were reported to be present in some metastatic lesions from human carcinomas (Kleeberger et al. 2007; Wiesner et al. 2008).

1.4.5 IGF

The Insulin-Like Growth Factors (IGF) comprise 3 receptors, 3 ligands and 6 binding proteins (IGFBP). IGF-I and IGF-II are known to induce osteoblast differentiation, increase bone matrix apposition and decrease collagen degradation (Koch et al. 2005). IGF are abundant in the bone microenvironment, and in vitro studies showed that they increase prostate cells proliferation and chemotaxis. Moreover, the IGF-I pathway is upregulated in prostate cancer cells localized in the bones (Ritchie et al. 1997a, b; Rubin et al. 2004). However, IGF-I is neither necessary nor sufficient for an adequate osteoblast response to prostate cancer metastases (Rubin et al. 2004).

In addition, tumor cells invading the bone express several transcription factors that are involved in the acquisition of the osteomimetic phenotype, among other RUNX.

1.4.6 RUNX

RUNX is a family of transcription factors (RUNX2 and RUNX1 and RUNX3). It was demonstrated that RUNX2 is essential for the differentiation of osteoblasts

and skeletal morphogenesis (Li et al. 2008). RUNX2 is overexpressed in metastatic breast cancers cells, promotes transcription of genes involved in the acquisition of migration and invasiveness, including MMP and VEGF among others. Importantly, inhibition of Runx2 function in metastatic breast cancer cells transplanted to bone results in prevention of tumorigenesis and osteolysis (Javed et al. 2005).

These data indicate that a multigenic program facilitates the acquisition of osteomimetic properties by certain cancer cells, improving their chance for survival, adaptation to the bone environment and the development of bone metastases.

1.4.7 The Role of the Calcium Sensing Receptor in Bone Marrow Homing

The calcium sensor (CaSR) is also expressed in several cell types in the kidney, osteoblasts, a variety of hematopoietic cells in the bone marrow, the gastrointestinal mucosa and squamous epithelial cells of the esophagus. At these sites, the CaSR has been implicated in the regulation of a number of cellular processes, such as ion and water transport, proliferation, differentiation and apoptosis. Moreover, human breast cancer cell lines express CaSR and its activation leads to increased PTHrP secretion from these cells. The secretion of PTHrP by tumoral cells drives osteoclast-mediated osteolysis, leading to the release of growth factors and calcium from the bone matrix, and further stimulation of cell proliferation (Coyle et al. 2006). Additionally, the loss of CaSR expression in the transition from normal colonic epithelial cells to malignant adenocarcinoma cells is associated with a low potential of colonic carcinomas to generate bone metastases (Rodland 2004).

1.5 Role of Calcium, Vitamin D and PTH in the Premetastatic Niche

1.5.1 Vitamin D

Serum concentrations of calcium are closely regulated by a close interplay between 25(OH)D levels and PTH levels. The precision of this integrated control is such that in normal individuals, serum ionized calcium fluctuates by no more than 0.1 mg/dl in either direction from its physiological set-point throughout the day. Interestingly, PTH gene expression is not only regulated through serum calcium concentrations by the CaSR but also independently through vitamin D metabolites, principally 25(OH)D, regardless of both 1,25(OH)D and calcium. (Pepe et al. 2006). 25(OH)D and 1,25 (OH)D control PTH gene expression, CaSR and VDR gene expression, and the proliferation of parathyroid cells. Therefore, low serum calcium concentrations and/or low vitamin D levels increase PTH secretion, in turn increasing distal tubular calcium reabsorption, intestinal 1,25(OH)D-mediated calcium absorption and osteoclast-medicated bone resorption to normalize serum calcium concentrations.

1.5.2 Antineoplastic Effect of Vitamin D

Vitamin D depletion (and secondary hyperparathyroidism) has been reported as a very common condition worldwide both in men and women, with many implications for general health conditions (Holick and Chen 2008). Very recently, it has been documented that low serum vitamin D levels are similarly prevalent in young individuals (Adami et al. 2009; Crew et al. 2009). Biological and epidemiological data suggest vitamin D levels to influence cancer development (IARC Working Group Reports 2008), but data are not consistent. For breast and prostate cancer, case-control studies suggest an inverse association between serum 25(OH)D concentration and the prevalence of these diseases, but this finding was not confirmed by prospective studies that analyzed 25(OH)D years before the diagnosis of cancer (Yin and Grandi 2010; Trump et al. 2009; Tretli et al. 2009; Yin et al. 2009; Chlebowski et al. 2008; Lappe et al. 2007). This might indicate that low 25(OH)D concentrations in serum are rather a consequence of the malignant disease than causing cancer. Also, studies on intake/supplementation of vitamin D in breast or prostate cancer patients showed conflicting results (Trump et al. 2006; Flaig and Barqawi 2006; Chan and Beer 2008; Attia et al. 2008). Many molecular pathways mediate the anticancer effects of calcitriol. The active form of vitamin D,1,25(OH)D has been established as an antiproliferative and pro-differentiation agent. More recent work showed calcitriol to be a proapoptotic agent and an inhibitor of cell migration and angiogenesis, supporting its potential in cancer prevention and cure (Peterlik and Grant 2009; Matthews et al. 2010). Among the breast cancer cell lines that do respond to 1,25(OH)D, a range of phenotype alterations have been reported, emphasizing that the mechanistic basis for the differentiating effects of 1,25(OH)D in cellular systems of breast cancer is very complex. (Gocek and Studzinski 2009). An interesting link to differentiation in 1,25(OH)D-treated breast cancer cells is the fact that vitamin D receptor (VDR) and Estrogen Receptor (ER) pathways converge to regulate BRCA-1, thus controlling the balance between cellular differentiation and proliferation (Campbell et al. 2000). In the prostate cancer cell line LNCaP, 1,25(OH)D up-regulates the expression of the insuline-like factor binding protein 3 (IGFBP-3) that functions to inhibit cell proliferation and up-regulates the expression and activity of the androgen receptor (AR) and the AR-mediated androgenic differentiation (Gocek and Studzinski 2009). The receptor of vitamin D (VDR) has been described in many types of cancer cells, including tumors of the breast, prostate, colon, bladder, skin, pancreas, leukaemia and lymphoma cells (Bouilon et al. 2006). In Caucasians, polymorphisms of VDR (VDR FokI and BsmI) migh modulate the risk of malignant tumors of the breast, skin and prostate, and possibly affect cancer risk also at other sites (Raimondi et al. 2009).

1.5.3 Inhibition of NFkB Activation, Angiogenesis, Invasion and Metastasis

Angiogenic factors such as IL8 and VEGF are crucial for the promotion of the premetastatic niche, and are similarly important for continued tumor growth and

disease progression. NFkB plays a major role in the control of immune responses and inflammation, and promotes malignant behaviour by increasing the transcription of the antiapoptotic gene BCL-2, proteolytic enzymes such as matrix metalloproteinase 9 (MMP-9), urokinase-type plasminogen activator and angiogenic factors such as IL-8 and VEGF (Catz and Johnoston 2001). Calcitriol is known to directly modulate basal and cytokine-induced NFkB activity in many cells, including lymphocytes, monocytes, fibroblasts, osteoblasts and in cancer cells. In addition to the direct inhibition of NFkB, 1,25(OH)D indirectly inhibits NFkB-signaling by up-regulating the expression of other proteins that interfere with NFkB activation such as IGFBP-3 and Clusterin (CLU) (Folkman 1995). Early studies indicate that calcitriol is a potent inhibitor of tumor cell-induced angiogenesis by inhibiting VEGF-induced endothelial cell tube formation in vitro, decreasing tumor vascularization in mice and inhibiting angiogenesis through IL-8 in a NFkB-dependent manner (Bao et al. 2006). Furthermore, calcitriol directly inhibits the proliferation of endothelial cells (Chung et al. 2009). MMP in turn promote angiogenesis by mediating the degradation of the basement membrane of the vascular epithelium and the extracellular matrix. In human prostate cancer cells, calcitriol decreases the expression of MMP-9 by increasing the activity of TIMP-1 (tissue inhibitor of MMP-1) (Bao et al. 2006). Finally, it has been demonstrated that calcitriol reduces the invasive and metastatic potential of many malignant cells. In prostate cancer, calcitriol increase E-cadherin, a tumor suppressor gene, whose expression is inversely correlated with metastatic potential (Campbell et al. 1997).

1.5.4 Calcium

Inadequate calcium intake, low serum calcium (through CaSR) together with low vitamin D levels may directly or indirectly (through PTH) impact cell proliferation, differentiation and function. The CaSR, VDR and PTH-1R are expressed both in normal and malignant breast cells, and their expression is correlated with the occurrence of skeletal metastases. Interestingly, signaling pathways that are initiated via VDR and CaSR converge on the same downstream elements, e.g. the canonical Wnt pathway. Increasing extracellular calcium levels increases cellular differentiation in experimental models, decreases proliferation, induces apoptosis and down-modulates invasion, all of which seem to have tumor-protective effects (McGrath et al. 1984). Several studies have suggested an inverse association between dietary calcium intake and serum calcium levels with breast cancer risk in pre- and post-menopausal women (Almquist et al. 2007; Cui and Rohan 2006). However, the relationship between serum calcium concentrations and cancer risk is not consistent and complex, also because of the reciprocal effects of PTH and vitamin D levels. High serum calcium concentrations could indicate high bone turnover, which in turn suggests a bone microenvironment rich in chemotactic, adhesive and neoangiogenic factors that might promote the homing of cancer cells and the development of metastases (Schnieder et al. 2005). On the other hand, low serum calcium concentrations are generally associated with low 25(OH)D levels

and high PTH levels that both promote cancer progression and bone metastases. Very likely, calcium concentrations play a crucial role in cellular signaling in bones in general and in the premetastatic niche in particular. The bone microenvironment is enriched in calcium during osteoclast-mediated bone resorption, reaching high local concentrations up to 40 mmol/L. Serum calcium homeostasis is closely regulated by the calcium sensing receptor (CaSR) expressed on parathyroid cells, modulating PTH secretion. However, CaSR is also expressed at high levels in breast cancer cells from patients with bone metastases and in prostate cancer cells (Mihai 2008). Activation of the CaSR by high calcium concentrations induces PTHrP expression, but may also attract breast and prostate cancer cells in areas of increased bone remodeling, thereby facilitating migration of cancer cells into the bones. Furthermore, high concentrations of calcium enhanced proliferation of prostate cancer cell lines and the proliferative response is associated with CaSR overexpression (Casimiro et al. 2009). Notably, CaSR is essential for stem cell migration and the settlement of HSC in the endosteal niche, suggesting preferential localization of these cells expressing CaSR in close proximity to calcium-releasing osteoclasts (Adams 2005).

1.5.5 PTH

PTH and Factors Involved in the Premetastatic Niche

Primary and secondary hyperparathyroidism is associated with a poor prognosis in patients with cancer (Schwartz 2008). PTH and PTHrP are immunologically distinct proteins that bind to the PTH-1R with equal affinity (Bryden et al. 2002). Many cancer cell types express PTHrP and its receptor PTH-1R, and PTHrP acts as an autocrine growth factor promoting proliferation, migration and disease progression (Deftos et al. 2005; Henderson et al. 2006). PTH could play a crucial role in promoting the homing of cancer cells in the bone environment, the persistence of the cancer stem cell niche, and the development of cancer metastases (Ritchie et al. 1997a, b). PTH and PTHrP both induce the activation of chemokines through PTH-1R, further inducing SDF-1 expression in the bone marrow. Major sources of SDF-1 in the marrow are cells of the osteoblastic lineage, mainly osteoblasts lining the bone endosteum (Ponomariov et al. 2000). The SDF-1/CXCR4 axis is known for regulating many aspects of stem cell functioning, including stem cell trafficking and development. It has been demonstrated that transgenic animals expressing constitutively active PTH/PTHrP receptors have increased numbers of hematopoietic stem cells recovered from the animals' bone marrow (Calvi et al. 2003). Interstingly, PTH increased the expression of SDF-1 in the local marrow environment in animal models, along with decreased SDF-1 in serum, creating a homing gradient for hematopoietic stem cells towards the bone marrow (Jung et al. 2006). There are many parallels between the metastasis of circulating carcinoma cells and the homing behavior of hematopoietic cells (Sun et al. 2003). Therefore, high circulating PTH levels in cancer patients could prime the marrow for metastatic spread by altering the SDF-1 axis. Physiologic bone

remodeling takes place in specialized vascular structures called bone remodeling compartments (BRC), and PTH induces high bone turnover with subsequent expansion of the BRC space (Eriksen et al. 2007) Angiogenesis is closely associated with bone turnover, and angiogenic factors such as VEGF and endothelin are important regulators of both osteoclast and osteoblast activity. In addition, it has recently been demonstrated that PTH induces osteoclast formation in cooperation with RANKL, osteoclast activity through KDR/Flk-1 and/or Flt-1 receptors expressed in mature osteoclast, and survival involving beta-3-integrin-mediated attachment of osteoclasts to the extracellular matrix (Nakagawa et al. 2000). PTH/PTHrp induces PKC, ERK, MAPK and p38, with the MAPK pathway ultimately resulting in VEGF gene expression in osteoblasts and in epithelial cells of normal rat renal tubules (Esbrit et al. 2000; Alonso et al. 2008). VEGF expression was specifically observed in PTH1R-positive cancer cells after invasion of the bone marrow, using in vivo and in vitro models (Isowa et al. 2010). Finally, VEGF has been reported to increase SDF-1 expression in endothelial cells and in several prostate cancer cell lines (Dai et al. 2004).

PTH and the Hematopoietic Stem Cell Niche

The endosteal surface is rich in vasculature with close approximation of osteoblasts and vessel walls. Within the bone marrow, cells of the osteoblast lineage have unequivocally been shown to constitute a niche for Hematopoietic Stem Cells (HSC) (Xie et al. 2009). Cells derived from osteoprogenitors provide distinct niches for hematopoietic cells. Terminally differentiated osteoblasts along the endosteal surface could serve as a niche for HSC in their most quiescent stage, whereas the stromal reticular cell fraction including osteoprogenitors located in the bone marrow induce proliferation and differentiation of hematopoietic cells (Wu et al. 2009). It is widely known that stem cells are usually in the quiescent state or G0 phase, and this prevents stem cells from entering into the cell cycle and undergoing differentiation. CXCL12/CXCR4-mediated chemokine signaling plays an essential role in maintaining the quiescent pool of HSC (Sugiyama et al. 2006). Osteopontin (OP) and Angiopoietin-1 expressed by osteoblasts interact with Tie-2 expressed in HSC, activating N-cadherin and integrin (Arai et al. 2004). These interactions enhance the adhesion between the hematopoietic stem-cell niche and the stem cell, contributing to the maintenance of HCS quiescence. The bone morphogenetic protein (BMP) signaling pathway, which acts through BMP receptor type IA expressed in osteoblast, controls the number of HSC by regulating the size of the niche and is involved in maintaining quiescence and supressing proliferation. Different signaling pathways such as Wnt/cathenin and Notch/Jagged 1 promote self-renewal, proliferation and differentiation of HSC (Iwasaki and Suda 2009). Therefore, osteoblasts clearly have a role in the establishment and mainteinance of the HSC niche that could be modulated through PTH and other molecular mechanisms that are incompletely defined so far. Osteoblasts are the main targets of PTH/PTHrP, and PTH/PTHrp also has some modulating potential on HSC via osteoblasts. PTH may increase the proportion of bone marrow-derived stromal cells (BMC) that commit to the osteoblastic lineage both in vitro and

in vivo, thus expanding the osteoblast pool. Furthermore, PTH induces osteoblasts to express BMP, SDF-1, VEGF, osteopontin and activate signaling pathways involved in the HSC niche. It has been demonstrated that PTH expands the HSC pool through activation of the PTH-1R on osteoblasts. The Jagged1/Notch signaling pathway is implicated in the control of stem cell self-renewal in several organs, and is necessary for PTH-dependent HCS expansion (Calvi 2006). PPR activation by the Notch ligand Jagged-1 in osteoblasts is associated with an increase in the number of HSC, and this increase can be stopped by administration of a secretase inhibitor. The same effects can be achieved by using exogenous PTH (Calvi 2006). Furthermore, PTH (as well as other stress conditions including inflammation, injury or chemotherapy) could result in a destabilization of HSC with induction of massive stem mobilization. Accordingly, osteoclast activity has been demonstrated to promote the proliferation and mobilization of hematopoietic progenitors from HCS. Bone resorbing osteoclasts secrete enzymes, including MMP-9 and cathepsin K that give them SDF-1 and osteopontin degradation capacity (Kollt et al. 2006). Therefore, PTH could impact both bone turnover and the bone marrow niche through modulation of osteoblast and osteoclast activity, resulting in improved engraftment both of HSC and cancer stem cells. It is notable that the majority of the signaling pathways involved in the interaction between normal stem cells and their niche are also involved in the interaction between cancer stem cells and their niche.

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