

Bone Microenvironment and Role of Rank-Rankl-Opg in Breast Cancer Metastasis in Bone

Abstract

Bone metastasis affects more than 70% of advanced breast cancer patients and is the leading cause of breast cancer-related deaths, incapable of being controlled, still very unpredictable. As a consequence, this has led to increased mortality and worsen quality of life in these patients by causing severe complications such as excruciating bone pain, hypercalcemia and spinal cord compression, pathological fractures also known as skeletal related events (SRE). Vast majority of breast cancer patients with relapses in bone are asymptomatic during the course of the disease, hence making it difficult to diagnosis and detect the spread of the breast cancer cells to the bone prior to appearance of bone pain complains issued by this category of patients.

Due to increased frequency of bone metastases in breast cancer during the recent years, understanding cross-communications between tumor cells and bone cells accompanied by the action of various growth factors, proinvasive cytokines, chemokines released upon bone destruction are essential to help improve the development of new effective therapeutic interventions. The pathogenesis of bone metastases in breast cancer depends on the bidirectional tight interaction among breast cancer cells and various stromal cells also being identified as the "vicious cycle", which modulates the bone niche resulting in continuous activation of bone resorption process. One of the major important pathways involved in the development and progression of bone metastases in breast cancer is RANK-RANKL-OPG cascade, where a positive shift towards RANKL-RANK axis favors proliferation, activation and survival of osteoclasts, thus promoting tumorigenesis and metastasis in the bone. In this context, disturbance of RANKL-RANK interplay should be an effective method to prevent the survival and growth of the breast cancer cell in the bone microenvironment.

Keywords: Osteocyte; Osteoblast; Osteocyte; RANKL; RANK; OPG; Bone metastasis; Breast cancer

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Fabjola Sejdinaj and Hongwei Zhang*

Department of General Surgery, Zhongshan Hospital, Fudan University, China

***Corresponding author:** Hongwei Zhang, Department of General surgery, Zhongshan hospital, Fudan University, Fenglin road 180, Xujiahui, Shanghai, China, Tel: (0086)13681973106; Email: zhang.hongwei@zs-hospital.sh.cn

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Abbreviation: Ocs: Osteoclasts; IGF: Insulin Growth Factors; TGF- β : Transforming Growth Factor- β ; FGF: Fibroblast Growth Factors; OBs: Osteoblasts; BMU: Basic Multicellular Unit; SRE: Skeletal Related Events; IL-6: Interleukin-6

Introduction

Bone is a frequent site for distant metastasis and more prevalent in breast cancer patients. In advanced breast cancer, more than two thirds of patients will develop high rates of relapse in bone [1,2]. During the bone metastatic phase of breast cancer disease, nearly 50% of patients will be affected by skeletal related events (SRE) including pathologic fracture, bone surgery, spinal cord compression, and palliative radiation therapy to bone, and hypercalcemia of malignancy [3]. Bone-targeted therapies such as denosumab or potent biophosphonates are currently a standard approach in treatment of patients with bone metastases reducing skeletal morbidity from metastatic cancer [4,5]; nonetheless their capacity to neither prevent formation of bone metastasis nor improve overall survival in these patients is still controversial and these medical therapies are at best considered palliative. In order to improve the treatment of bone metastatic disease and

increase disease free survival rates, a full-scale comprehension of multiple interactions among breast cancer cells and bone microenvironment have generated considerable recent research interest .

Bone Microenvironment and Homeostasis Disturbance

Bone micro environmental features

Bone preserves the integrity and stability of skeletal system, acts as a protective shield for hematopoietic marrow, as well as serves as a storage site for calcium, phosphate [6] and many growth factors such as insulin growth factors (IGF), transforming growth factor- β (TGF- β), fibroblast growth factors (FGF) and other cytokines, insuring its role as vital organ in maintaining a healthy body. Originally bone homeostasis was thought to be preserved by a constant forward-feedback mechanism, also known as remodeling process among two main cell-types stored in that environment: osteoclasts (OCs) and osteoblasts(OBs), but much study in the recent years have radically redefined this concept. Nowadays bone remodeling process is carried out by the deep crosstalk interaction between components of the basic

multicellular unit (BMU), a compact anatomical cohort formed by osteoblasts (OBs), bone lining cells, osteocytes and osteoclasts (OCs) [7,8].

OBs's bone absorption and OCs's bone resorption activity achieves a precisely balanced interaction which maintains the strength and vitality of bone mass also known as "coupling" [9,10] an extensive process not only referring to the BMU interrelationships but also impacted by the action of many factors such as sphingosine-1-phosphate, semaphorins, ephrins, interleukin-6 (IL-6) family cytokines and marrow-derived factors [11]. OCs firm attachment to the underlying bone matrix activates a chain of digestive mechanisms through the release of proteolytic enzyme, cathepsin K and hydrochloric acid onto the bone [11,12], which disintegrates the trabeculae and cortical bone filled cavities, followed by OBs covering these gaps with new bone matrix termed osteoid. The mechanism underlying constant bone renewal is a compact safe grounded system that still attracts widespread interest among scientists, but a more in-depth understanding of processes underlying OCs formation and function can unwind some of its potential applications.

Bone cells

Osteoclasts

OCs differentiate from myeloid lineage of hematopoietic mononuclear precursor cells and their function is extended beyond bone resorption, also including maintenance of hematopoietic niche [13], induction of angiogenesis via platelet-derived growth factor-BB (PDGF-BB) [14], bone formation activity [15] and safekeeping of endocrine function of bone through production of osteocalcin [16], a bone matrix protein. The importance of osteoclast-mediated bone formation has been demonstrated by investigating and finally recognizing the term clastokines, also known as osteoclast derived coupling factors, (Tartrate resistant acid phosphatase (TRAcP), S1p, BMP6, Wnt10b, hepatocyte growth factor (HGF), collagen triple helix repeat-containing protein 1 (CTHRC1), PDGF BB) [17], a particular set of substances derived from osteoclasts which have the capacity to specifically induce osteogenesis. As Drissi H & A Sanjay [18] wondered in their brief paper many questions have started to intrigue scientists regarding clastokines, among them the most decisive ones include: whether all osteoclast-mediated factors possess the same equal ability to induce bone formation? Can indeed clastokines be considered a supplementary route to the action exhibited by the bone matrix released factors? This needs further comprehensive investigation to achieve a more profound knowledge regarding the tightly interaction between osteoclast and osteoblast leading to dissimilar degrees of bone remodeling in different parts of the bone!

Osteoclast precursors (OCPs) are allured to the blood circulation by a bioactive sphingolipid, sphingosine-1 phosphate (S1P) secreted by red blood cells and platelet which adjust the ability of OCPs to move spontaneously and actively from bone marrow into the bloodstream and later on differentiate into osteoclasts [19]. OCs formation and activation is a perplex process initiated primarily by receptor activator of NF- κ B ligand (RANKL), a crucial

cytokine, commonly found on the surface of osteoblasts, osteocytes, and other cells such as T and B lymphocytes, which after binding to its receptor activator of nuclear factor κ B (RANK) induces osteoclastogenesis through NF- κ B signaling cascade [19-22]. Another essential signaling molecule required for differentiation of osteoclast progenitors into precursors is macrophage-colony stimulating factor (M-CSF), an integral osteoclast differentiation factor, which after binding to its receptor, c-fms, mediates OPC's proliferation and differentiation through MAP kinases and extracellular signal-regulated (ERKs) kinases [23].

Osteoblasts

OBs, cuboidal cells comprising 4-6% of all bone cells, commonly known as the cells which absorb bone descend from the pluripotent mesenchymal stem cells (MSC) [12,24]. Osteoblastogenesis, as a very specific multistep process, is driven by the expression of several osteoblastic-specific transcription factors including: Runt-related transcription factor 2 (Runx2), Distal-less homeobox 5 (Dlx5), Osterix (Osx) and synthesis of Wingless (Wnt), lipoprotein receptor-related protein 5 and 6 (Lrp5/6), and bone morphogenetic proteins (BMPs), all required for the commitment of MSC to mature osteoblasts [23,25]. Runx2 has been identified as the master gene responsible for osteoblast differentiation, as indicated by absence of bone tissue or osteoblasts in Runx2-deficient mice [26]. Osteoblasts express high levels of Runx2 in vivo and in vitro. Additionally Runx2 has been demonstrated to be up-regulated by *BMPS via Smads* [27], indicating a very intricate relationship among various transcription factors and genes responsible for osteoblast differentiation, therefore further research is required to assess these ties and their clinical importance.

As mentioned above, osteoblasts possess the ability to interfere with bone degradation as the main contributors of RANKL expression followed by embedded osteocytes and stromal cells. Even though many studies discovered that osteocytes indeed synthesize high quantity of RANKL, nonetheless their assistance in osteoclast formation is lesser than that of osteoblasts due to the presence of RANKL as a soluble molecule transferred to OCPs through cytoplasmic projections of osteocytes [28]. On the other hand, Osteoblastic RANKL is primarily located in lysosomes and only the interaction with RANK beads can initiate the adequate stimulatory-dependent signals to dispense these cytokines to the cell surface and subsequently instigate osteoclastogenesis [29].

Osteocytes

Osteocytes are the preeminent type of cell seen in the bone, representing more the 90% of the total bone cells and besides that, are the cells which have the longest lifespan within the bone matrix, up to 25 years [30,31]. Osteocytes are derived from MSC lineage through osteoblast differentiation, when at the end of osteoblastogenesis, a subdivision of osteoblasts undergoes noticeable morphological and ultra structural changes and becomes embedded into the bone matrix as newly transformed osteocytes. This process includes 4 distinctive phases: osteoid-osteocyte or type I preosteocyte, preosteocyte or type II preosteocyte, young osteocyte or type III preosteocyte

and mature osteocytes [32]. During the osteocytogenesis, precursor osteoblast round appearance gradually is changed and reduced in size into a stellar shaped cell and numerous cytoplasm projections (up to 50 per each cell in normal human bone) start to emerge from each osteocyte body influenced by the expression of membrane-associated protein E11/gp38 (podoplanin) [33]. The number of organelles gradually declines, whereas the nucleus-to-cytoplasm ratio inclines. As the mineralization of osteoid proceeds, specifically in type III preosteocyte, the endoplasmic reticulum and Golgi apparatus are significantly reduced compared to type I and II preosteocytes, thus resulting in the decrease of protein synthesis and secretion [7,34].

Osteocytes extend their cytoplasmic projections within the canaliculi of the lacuna, forming the lacunar-canalicular system, where they establish a virtual network of functional innercommunications, not only with other neighbouring osteocytes, but also with other bone cells such as: osteoblasts, osteoclast and bone lining cells [35]. These direct cell-to cell interactions are achieved by two main routes:

1. Gap junctions formed by connexin 43 Cx43 (abundant) but connexin 46 is also present, which facilitate the intercellular transport of small signaling molecules such as prostaglandins and nitric oxide [36].
2. Interstitial fluid which fills the space between osteocytes dendrites and canaliculi and transports osteocytes' products to their target site (proteins ranging from 70Kda-7nm in diameter) [37].

Sclerostin, the product of SOST gene, is selectively expressed by matrix encased mature osteocytes and represent the key factor throughout osteocytes settle their role as a main orchestrator in bone remodeling [38]. Sclerostin inhibits bone formation through means of counteracting the connical wingless (Wnt) signaling by binding to a Wnt co-receptor family, the lipoprotein receptor-related proteins (LRP5/6), even at some extent with LRP4, thus restricting the interaction between Wnts and their co-receptor Frizzled (Frz) [39]. Human conditions related to lack of Sost gene expression in humans (Van Buchem's disease) [40] or decreased in the function of Sost gene (sclerosteosis) [41,42] are two types of skeletal sclerosis diseases which have a hyperactive Wnt signaling, hence corresponding to a high bone mass and increased bone strength. The above findings have generated a growing interest regarding the effect of sclerostin in the regulation of bone formation, as a new potential blockage axis to be exploited for the further treatment of osteoporosis and osteoporosis-related comorbidities that requires a more personalized approach [42,43].

Moreover osteocytes exhibit another important function that of being the essential coordinators of the bone adaption process during consistent mechanical stress. They display an extremely sensitive side towards stimuli caused by the fluid shear stress or hydrostatic changes, thereby representing the primary mechanosensory cells in the bone [44,45].

Bone lining cells

Bone lining cells form a monolayer covering the bone surface in a quiescent state where neither bone destruction nor osteogenesis occurs [46]. Bone lining cells are considered to be very important elements in the activity of BMU, nevertheless their functions aren't entirely understood.

Homeostasis Disturbance

A more in depth understanding of the intricate interactions between tumor cells and bone cells, two major players in the bone niche, may shed more light into the mechanism required for the development of so called "vicious cycle" during bone metastasis. A "vicious cycle" represents a tumor-osteoblast-osteoclast network which under the influence of cancer-induced factors provides a homing soil for disseminated cancer cells to proliferate into osteolytic, osteoblastic or mixed bone lesions.

Bone metastasis refers to the dissemination of the malignant tumor cells from the primary site into the distant bone where a secondary tumor will aggressively colonize and thrive in the bone microenvironment. Foremost breast cancer tumor cells need to grow strong, enhance their malignancy and expand within the primary site microenvironment aided initially by epithelial to mesenchymal transition (EMT) [47], and hypoxia [48,49], thereby gaining the ability to break free from the local site and infiltrate the proper vessels to navigate into the bloodstream. EMT promotes carcinogenesis through transformation of epithelial cells into motile and invasive mesenchymal cells followed by loss of epithelial cells markers such as CK or EpCAM and in reverse the expression of transcriptional factors characteristic for mesenchymal cells is enhanced such as Vimentin, Fibronectin, TWIST, Akt2, Snail, Slug, PI3Kalpha, ZEB1, ZEB2, FoxC2 and others [50,51]. In breast cancer cells, two downstream signaling pathways were found to modulate the gateway of cancer cells from the primary tumor and their translocation into the bone: TWIST1 stimulates EMT via expression of metastatic inducer miR-10b and hypoxia-inducible factor 1 α (HIF-1 α and HIF-2 α) which illicit a positive feedback to SNAIL1 and SLUG via NOTCH activation of EMT resulting in cadherin shifting, loss of E-cadherin, overexpression of N-cadherin and cytoskeletal alterations (e.g., expression of vimentin) [48, 52, 53].

As soon as the migrating cancer cells undergo a drastic alternation and detach from the primary site, they are known as circulating tumor cells (CTCs), a novel promising insight into relapses of primary carcinomas to the distant sites, whilst malignant cells which extravagate from the circulation and colonize the bone are called disseminated tumor cells (DTCs) [54, 55]. Presence and persistent detection of CTCs in bloodstream followed by DTCs in bone marrow may indicate an alteration in the phenotype of these malignant cells resulting in an increased resistance towards selected adjuvant therapy, therefore CTCs are considered as an early negative prognostic factor for the clinical management of metastatic breast cancer patients [56-58]. Malignant tumor cells upon populating the bone undergo a rapid mechanism of constructive adaption under the influence

of various growth cytokines released by the bone matrix, which promote cancer cells differentiation and proliferation [59], hence providing a fertile soil for cancer cells to flourish in the bone microenvironment. It has been suggested that stromal cells, descendants of mesenchymal cells especially via vascular cell adhesion molecule1 (VCAM-1) mediated interactions with breast cancer cells signal these malignant cell to further enhance their aggressiveness and proliferate in the bone habitat. Another important proinvasive factor is TGF- β , liberated upon bone degradation, extensively contribute to the growth and prosperity of bone metastasis especially in breast cancer by upregulating the production of parathyroid hormone-related peptide (PTHrP), resulting in an enhanced activity of bone resorption process, accelerating even more the "vicious" cycle [60]. Moreover TGF- β and Notch signaling release of IL-6 stimulated by tumor-mediated Jagged1 are believed to act as of conjugated pathway and accentuate the strong tendency of metastatic breast cancer for spreading to the bone [61], thus providing a challenging area to be explored that may lead to improved diagnosis and development of new therapeutic options for bone metastasis. Under these circumstances humanized, Jagged1-blocking antibodies have generated promising results in preclinical trials.

To sum up, the whole process of the bone metastasis in breast cancer can be divided into five coordinated and progressive stages: 1) Detachment from the primary tumor; 2) Intravasation of the bloodstream ; 3) Surviving in the circulation and being motile within it; 4) Colonizing of the bone and 5) Prospering in the bone [63]. In each of these steps, this process initiated by invasive malignant tumor cells is incessant and aggressive.

Role of RANK-RANKL-OPG Pathway in Breast Cancer Metastases to Bone

RANKL is a potent downstream mediator of bone-degrading osteoclast differentiation, activation and survival, is expressed by stromal cells of osteoblast lineage; binds with its natural receptor RANK on the surface of osteoclastic precursors cells stimulating OCPs differentiation and bone degradation by mature osteoclasts [20,64,65]. The RANKL/RANK pathway may directly stimulate breast cancer cells to preferentially migrate into bone, the must-have demand to initiate colonization of the bone and subsequently formation of osteolytic skeletal metastases [66-68]. Overexpression of RANK in a setting of tumoral and normal human mammary cells stimulates the expression of breast cancer stem in human mammary epithelial cells by rising the cohort of CD44 (+) CD24 (-) cells, inducing stemness and EMT [69]. EMT represents the pivotal step to activate extravasations and migration of malignant breast cells into the bone marrow in a metastatic setting. RANKL activates NF- κ B pathway [68] and stimulates the expression of Snail and TWIST, altering the breast cells morphology, increasing the expression levels of vimentin, N-cadherin, and down regulating the levels of E-cadherin [70]. In consequence, activation of RANK/RANKL pathway by inducing development of mammary stem cells and breast cancer promotes breast tumorigenesis, tumor growth, CTCs migration and metastasis in human breast epithelial cells. In addition, several studies reported elevated expression of RANKL as detected by immunohistochemistry in breast cancer patients that had metastasis to the bone compared to those breast cancer patients

without bone metastasis [71,72]. High expression RANK levels in primary breast tumors have been correlated with poor survival rates and higher risk of developing bone metastases [65].

Moreover RANK/RANKL interplay plays an essential role in the control of proliferation and differentiation of mammary epithelial cells during pregnancy [73]. Pregnancy further enhances the RANKL expression at transcriptional levels in both normal mammary gland and primary tumor [74]. Studied conducted on RANK/RANKL-null mice demonstrated severe impairment in ductal-side branching and alveologenesis due to decreased rates of proliferation and enhanced apoptosis of mammary epithelial cells which lead to the development of lactating defects [73]. Later on was discovered that in humans, RANKL expression is significantly 3 times higher between the 17th and 26th day of the menstrual cycle than in other days correlated with serum progesterone levels [73]. RANKL expression is also regulated by sex hormones, in particular progesterone, both located in luminal epithelial cells, which promotes mammary gland morphogenesis via proliferation of mammary epithelial cells [75]. This interaction includes two separate signaling pathways: firstly progesterone directly acts on progesterone-receptor (PR) cells via cyclin D1-dependent. Secondly PR-cells proliferate by a RANKL induced paracrine mechanism where RANKL-expressing cells initiate a signal transduction cascade, inducing changes in nearby PR-negative cells leading to proliferation of mammary epithelial PR-negative cells [76,77].

Whereas osteoprotegerin (OPG), abundantly expressed by osteoblast and vascular cells, acts as a decoy receptor for RANKL by counteracting the action of RANK, resulting in oppression of bone resorption, thus preventing bone osteolysis [78]. Furthermore, studies have shown that OPG is also expressed by breast tumor cells [68,79], so it appears its action is beyond bone remodeling and linked directly to breast cancer proliferation and progression. Research investigating OPG's effect on breast cancer demonstrated that OPG induces breast carcinogenesis via its specific feature as a "decoy" receptor in this case for TRAIL, blocking interaction between TRAIL and the Death Receptors, thereby preventing TRAIL-mediated apoptosis of breast cancer cells, exhibiting an anti-apoptotic action [74]. OPG also promotes endothelial cell growth and tube formation, exerting a positive effect on angiogenesis, thus increasing breast cancer cells survival rates [80]. While OPG has clearly the ability to induce breast cancer progression and promote metastasis into the bone, investigation into the mechanisms and overall effects outcome on breast cancer are of great concern to explore in potential future applications.

Many extensive studies already have concluded that RANK-RANKL-OPG axis is not only involved in the normal mammary physiology, but also promotes breast cancer cells proliferation via progesterone and mediates bone-disseminated breast cancer cells relapse in the bone niche. Even though denosumab, a monoclonal antibody against RANKL, has become the standard treatment in breast cancer patients affected by bone metastases, its benefits are at best aimed at improving quality of life and reducing the frequency of SREs in this cohort of patients. For this reason interference with this pathway will be beneficial in identifying novel therapeutic options intended at decreasing disease free survival and morbidity of breast cancer homing to the bone.

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