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New therapeutic targets for cancer bone metastases

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Abstract

Bone metastases are dejected consequences of many types of tumors including breast, prostate, lung, kidney and thyroid cancers. This complicated process begins with the successful tumor cell epithelial–mesenchymal transition, escape from the original site, and penetration into circulation. The homing of tumor cells to the bone depends on both tumor-intrinsic traits and various molecules supplied by the bone metastatic niche. The colonization and growth of cancer cells in the osseous environment, which awaken their dormancy to form micro- and macro-metastasis, involve an intricate interaction between the circulating tumor cells and local bone cells including osteoclasts, osteoblasts, adipocytes and macrophages. In this review, we discuss the most recent advances in the identification of new molecules and novel mechanisms during each step of bone metastasis that may serve as promising therapeutic targets.

Keywords

Tumor intrinsic factors; bone metastatic niche; bone microenvironment; macrometastases; osteoclast; osteoblast

Bone Metastasis

More than 600,000 cases of cancer bone metastases are diagnosed every year in the United States [1]. Bone is the third most common site for metastatic disease (after lung and liver) and more prevalent in adult patients (>40 years of age) [2]. Notorious for causing severe pain, bone metastases are also the major reasons for pathologic fracture, life-threatening hypercalcemia, spinal cord compression, immobility and ultimate mortality in patients affiliated with advanced cancers in the breast, prostate, lung, kidney, thyroid, as well as hematologic malignancies like myeloma [3].

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A lot of progress has been made in the medical management of bone metastases including surgery approaches and radiation therapy, as well as targeted medical therapy. However, these strategies are at best only palliative and do not improve overall patient survival [4]. The challenges to identify more effective and specific molecularly targeted therapy to prevent and cure bone metastases as well as enhance the quality of life in these patients remain daunting.

Cancer bone metastasis refers to the ability of cancer cells to leave a primary tumor, travel through circulation, and form a secondary tumor in the distant bone tissue. It comprises five steps: i) escape from the primary tumor; ii) invade into lymphatic and/or blood vessels; iii) survive and travel in the circulation; iv) land on the bone; and v) finally flourish in the new bone environment [5, 6] (**Figure 1**). Every step of cancer bone metastases is tightly regulated by tumor cells, with the cooperation and assistance from non-cancerous cells (**Figure 1**). In this review, potential new therapeutic targets in every step of the cancer metastases discovered in the past two years will be discussed.

Escape from the original site

Epithelial to mesenchymal transition (EMT)

Tumor progression is often facilitated by both intrinsic genetic changes and alterations in the local environment. Although still under debate, increasing evidence suggests that activation of the EMT process is essential to allow carcinoma cells to undergo fundamental cytoskeleton reorganization to lose cohesiveness for single cell migration and escape [7]. Numerous molecules have been reported to be involved in this process. The classical marker of EMT is Cadherin switching, in which E-Cadherin is lost and N-Cadherin is gained [8]. Transcription factors that regulate junctional proteins such as E-Cadherin, β -catenin [9] and integrin [10], along with microRNAs and alternative splicing have been reported to play important roles in EMT [11].

EMT is commonly thought to promote general metastases; however, Smith *et al.* proposed an interesting hypothesis that EMT activators that also stimulate the expression of RANKL, a major cytokine for the differentiation of the bone resorbing osteoclasts from their myeloid progenitors, could drive the tumor cells specifically to the bone. They found that the pro-EMT factor Snail could usher prostate cancer cells to bone and stimulate tumor-bone vicious cycle through calcium and RANKL signaling [12]. Croset and colleagues also reported TWIST1, a well-known EMT-inducing transcription factor, as a contributor to breast cancer bone metastases through up-regulating the expression of miR-10b, a proinvasive factor [13]. By comparing immunohistochemical expression of EMT markers (i.e. E-cadherin, Vimentin, NF- κ B, Notch-1, ZEB1 and PDGF-D) in prostate cancer specimens (primary and bone metastasis) containing both tumor and adjacent normal tissues, Sethi *et al.* identified Notch-1 as a signature gene for the acquisition of EMT in prostate cancer and its bone metastases [11]. With the advances in profiling methodologies, we expect to see more bone-metastasis-specific targets to be identified during the EMT process, the significance of which awaits functional validations using various molecular genetic and pharmacological approaches.

Hypoxia

Low oxygen level (hypoxia) also increases the malignant behavior of cancer cells, predominantly via hypoxia-inducible factors (HIFs) [14]. Independent of established prognostic parameters, intratumoral hypoxia serves as an adverse indicator for patient prognosis [15]. The activated HIFs induce the expression of pluripotency-associated transcription factors (Oct-3/4, Nanog and Sox-2) [16], glycolysis- and EMT-associated molecules [17, 18], as well as angiogenic factors such as vascular endothelial growth factor (VEGF) [19]. Consequently, targeting the HIF signaling network and the altered metabolic pathways represent a promising strategy to improve the efficacy of current therapies against aggressive and metastatic cancers.

C-X-C chemokine receptor type 4 (CXCR4), osteopontin (OPN), *IL-6* and *IL-8* are hypoxia/HIF-inducible genes in the bone-specific metastatic gene signature in some cell types [20]. In breast cancer cells, HIF-1 α and HIF-2 α stimulate EMT by potentiating Notch signaling to upregulate SNAIL1 and SLUG - two transcription repressors of E-cadherin [21, 22, 23]. Inhibiting HIF-1 activity significantly suppresses breast cancer metastases to bone in animal models, establishing HIF-1 as a promising therapeutic target [24]. Hypoxia also stabilizes Growth arrest-specific 6 (GAS6) /AXL receptor tyrosine kinase (Axl) signaling in metastatic prostate cancer [25]. Interestingly, transcutaneous CO₂ application not only decreases HIF-1 α and increases apoptosis, but also suppresses pulmonary metastases in highly metastatic osteosarcoma cells, suggesting that reoxygenation via a novel transcutaneous CO₂ treatment could be a therapeutic breakthrough for metastasis suppression in osteosarcoma patients [26].

Cancer invasion

Degradation of extracellular matrix (ECM)

Invasion of carcinoma cells requires degradation of ECM, which forms the structural framework for most tissues and is composed of fibrous proteins (such as collagens, elastins, fibronectins and laminins) and proteoglycans (such as chondroitin sulphate, heparan sulphate, keratan sulphate and hyaluronic acid) [27]. Several genes related to the ECM have elevated expression in highly metastatic tumors [28]. Transforming growth factor-beta (TGF- β) plays a critical but complicated role in not only the synthesis but also the degradation of ECM [29]. Various types of proteinases are implicated in ECM degradation, but the major enzymes are considered to be matrix metalloproteinases (MMPs), which are Zn²⁺-endopeptidases that cleave the constituents of the ECM. MMP-2 and MMP-9 are the predominant MMPs responsible for ECM protein degradation thus play key roles in tumor development, growth and metastasis [30]. miR-29c has recently been reported to suppress lung cancer cell adhesion to ECM and metastases by targeting integrin β 1 and MMP2, and thus represents a novel therapeutic target for lung cancer metastasis [31].

Angiogenesis

After ECM degradation by MMPs, endothelial cells are attracted by the angiogenic stimuli produced by the tumor cells to migrate into the perivascular space and form new blood vessels [32]. This is a highly regulated process that involves essential signaling pathways

such as VEGF, VEGF receptors, anti-angiogenic factors (e.g. thrombospondin-1), pro-angiogenic factors (e.g. HIFs), Notch and several ECM proteins [33]. Angiogenesis, regarded as a prerequisite for cancer metastases, has been studied extensively. FDA approved bevacizumab, a monoclonal antibody against VEGF-A, was the first commercially available angiogenesis inhibitor that has been clinically used to treat metastatic colorectal, lung, breast and renal cancers [34]. Cabozantinib, a dual inhibitor of VEGFR2 and receptor tyrosine kinase MET, has exhibited beneficial effects on radiographically evident bone metastases [35, 36]. Researchers have also generated other inhibitors for VEGFRs, including sunitinib, sorafenib and cediranib [37], as well as aflibercept - a small recombinant protein that acts as a decoy receptor for VEGFs [38]. However, none of these drugs have been proven to afford a survival advantage. This suggests that angiogenesis inhibitors may require co-administration of other therapies or dual-pathway blockade to achieve clinical gains [36].

Intravasation and Extravasation

Blood vessels formed by tumor-induced-local-angiogenesis are generally leaky, with weak cell-cell junctions, through which cancer cells can enter vasculature [39]. By comparing peripheral blood plasma in patients with breast cancer bone metastases to healthy volunteers, Martinez and colleagues found that the plasma from patients can induce trans-endothelial migration of MCF-7 cells (a human breast adenocarcinoma cell line). These findings indicate that there are circulating factors in these patients that may promote intravasation, angiogenesis, survival and EMT of circulating tumor cells [40].

Genes mediating specialized cancer cell extravasation required for bone metastases have also been identified. A comparison of the expression profiles in bone-metastatic human breast cancer sublines with the parental cell line identified several mediators of bone metastases, in particular CXCR4, IL-11, OPN and MMP1, the combination of which are sufficient to enhance osteolytic metastases when overexpressed in the parental cell line [41]. Subsequently, several other profiling and microarray studies have been published using different types of tumor cells, revealing a number of other potentially important bone metastasis targets that require further functional characterization in future research [42, 43, 44, 45].

Survival in circulation

After intravasation, a few tumor cells are able to survive in the circulation through the expression of various genes, cooperating with other cell types, and at the same time evading immune system recognition to establish metastases at distant sites upon extravasating from the vessels. Priming by platelets and surface shielding from natural killer (NK) cells are thought to be the major approaches that circulating tumor cells (CTCs) utilize to survive and develop into metastasis [46]. Experimental models of breast cancer and melanoma show that platelets enhance metastasis [47]. Interestingly, ATP released from platelets is found to be required for platelet-dependent tumor cell transmigration; moreover, ATP-induced tumor cell extravasation and metastasis depends on endothelial purinergic P2Y₂ receptor [48]. It has also been shown that platelet-derived TGF β and direct platelet-tumor cell contacts synergistically activate the TGF β /Smad and NF- κ B pathways in cancer cells, enabling the tumor cells for metastasis by transitioning to an invasive mesenchymal-like phenotype [49].

Tumor intrinsic factors are also reported to assist tumor survival. Prostate cancer cells evade NK cell immunity by secreting core 2 beta-1,6-N-acetylglucosaminyltransferase (C2GnT), a key enzyme in the formation of core 2 branched [50]. CTCs have recently attracted lots of attention due to their potential as an early prognostic marker for the clinical management of metastatic breast cancer patients [51, 52]. Furthermore, analysis of patient-derived CTCs for therapeutic gene targets could possibly lead to the development of personalized treatment regimens to prevent metastasis [53].

Homing to the bone

Tumor cells, bone cells as well as tumor-bone interactions all have been shown to contribute to the colonization of cancer cells in bone.

Factors secreted from tumor cells

Different types of cancers exhibit different tropism to develop metastases in different organs. Factors secreted by tumor cells can alter the microenvironment at distant organ sites, generating pre-metastatic niches for subsequent metastatic cancer cell colonization. There is ample evidence showing that chemokines and their receptors play a key role in organ-specific cancer metastasis. The most studied is the CXCR4 and its ligand stromal-derived-factor-1 (SDF-1). SDF-1, produced by several bone marrow cell types including osteoblast, is known to be important in hematopoietic stem cell homing to the bone marrow. CXCR4, expressed by multiple bone-metastatic tumors including breast, prostate and multiple myeloma, controls the metastatic destination of cancer cells by directing them to organs where SDF-1 is highly produced such as bone marrow [54]. This makes disruption of the CXCR4/SDF-1 axis an attractive therapeutic strategy to prevent tumor cell spreading to bone. CTCE-9908, a peptide analog of SDF-1 and competitive inhibitor of CXCR4, reduces the size of metastatic lesions derived from MDA-MB-231 human breast cancer cells, following injection into the left cardiac ventricle of nude mice [55]. Plerixafor/AMD3100, a small-molecule CXCR4 antagonist, also delays metastatic outgrowth of orthotopically transplanted 4T1 mouse breast cancer cells [56]. However, within the bone microenvironment, SDF-1 and CXCR4 appear to have conflicting roles. Genetic disruption of CXCR4 in the hematopoietic cells turns to enhance osteoclast activity and thereby stimulates tumor cell growth in bone [57]. In short, the effects of the CXCR4/SDF-1 axis on tumor cell growth within the bone are not yet fully defined. Furthermore, there are theoretical risks that blockade of this chemokine axis could impair many important physiological processes that include stem cell homing, angiogenesis and immune cell trafficking [58]. As such, caution should be taken when designing therapeutic strategies targeting CXCR4/SDF-1 to specifically block the tumor cells.

Besides CXCR4, C-X-C motif chemokine 7 (CXCR7) has also been uncovered as a second SDF-1 receptor, which exerts similar function as CXCR4 in tumor development [59]. Azab *et al* found that inhibition of CXCR7 delayed tumor progression in multiple myeloma by specifically abrogating the trafficking of angiogenic mononuclear cells rather than through direct effects on tumors, demonstrating a novel role of CXCR7 in the recruitment of angiogenic mononuclear cells to areas of multiple myeloma in the bone marrow niche [60].

Similarly, C-X-C motif chemokine 10 (CXCL10) has been reported to facilitate the trafficking of C-X-C chemokine receptor type 3 (CXCR3)-expressing cancer cells to bone, which augments its own production, enhances tumor growth and at the same time promotes osteoclastic differentiation, indicating that the CXCL10/CXCR3 axis may be another attractive therapeutic target for osteolytic metastasis [61].

Additional secretory proteins from metastatic tumor cells have been discovered to function as autocrine or paracrine signals that mediate cross-talks among different cell types to facilitate tumor cell colonization in bone. Several cytokine products of breast cancers, such as parathyroid hormone-related protein (PTHrP) [62], IL-11 [63] and IL-8 [64], have been shown to modify host osseous environment to promote osteoclast formation and bone colonization. More recently, adrenomedullin, a small peptide secreted by breast cancer cells and increased by hypoxia, has been found to potentiate osteolytic responses to metastatic breast cancer cells. Overexpression of adrenomedullin in breast tumor cells accelerated osteolytic bone metastases development, increased osteoclast activity and decreased survival; whereas a small molecule adrenomedullin antagonist dramatically suppressed both tumor burden in bone and osteoclast activity [65]. Insulin-like growth factor binding protein 2 (IGFBP2) secreted from metastatic breast cancer cells recruits endothelia by modulating insulin-like growth factor 1 (IGF1) activation of the IGF type-I receptor on endothelial cells; whereas c-Mer tyrosine kinase (MERTK) receptor cleaved from metastatic cells promotes endothelial recruitment by competitively antagonizing the binding of its ligand GAS6 to endothelial MERTK receptors. Interestingly, microRNA-126, targeting both IGFBP2 and MERTK, suppresses metastatic endothelial recruitment, metastatic angiogenesis and breast cancer metastatic bone colonization [66]. A set of microRNAs, including miR-126 and miR-135, were lost in the breast cancer cells with high bone metastatic potential. Restoration of their expressions in cancer cells significantly inhibits tumorigenesis and bone metastatic colonization [67].

Three genes, encoding the cytokine IL-1 β , the chemokine CXCL6 (GCP-2), and the protease inhibitor elafin (PI3), are positively associated with the occurrence of skeletal metastases in animal models inoculated with human prostate cancer cells. Further studies showed that IL-1 β can promote skeletal colonization of prostate cancer cells [68]. By performing mass spectrometry on conditioned media from isolated PCa-118b prostate tumor cells, which came from an osteoblastic bone lesion generated by a patient-derived xenograft, Lee and colleagues identified 26 secretory proteins that may regulate prostate cancer bone colonization, including TGF- β 2, growth differentiation factor 15 (GDF15), fibroblast growth factor 3 (FGF3), fibroblast growth factor 19 (FGF19), C-X-C motif chemokine 1 (CXCL1), galectins and β 2-microglobulin [69].

In lung cancer bone metastases, inhibition of collagen receptor discoidin domain receptor-1 (DDR1) reduces cell survival, homing, and bone colonization [70]. Receptor of activated protein C (EPCR) promotes tumor cell survival and is negatively correlated with the clinical outcome in lung adenocarcinoma (AC) patients. Genetic silencing or antibody blockade of EPCR leads to strong abrogation of bone metastasis [71]. Simultaneous silencing of histone deacetylase 4 (HDAC4), paired-like homeodomain1 (PITX1) and roundabout axon guidance receptor homolog 1 (ROBO1) decreases bone metastatic ability of lung adenocarcinoma

[72]. Hyperactive canonical WNT/TCF signaling through LEF1 and HOXB9 enhances the competence of lung adenocarcinoma to metastasize to the bone and brain [73]. In addition, overexpression of miR-335 in small cell lung cancer (SCLC) down-regulates IGF1 receptor and RANKL, leading to reduced bone metastases [74].

In melanomas, exosomes derived from highly metastatic tumors have been shown to promote the mobilization of bone marrow cells through upregulation of pro-inflammatory molecules at pre-metastatic sites via the receptor tyrosine kinase MET. Moreover, these exosomes also induce vascular leakiness at pre-metastatic sites and reprogram bone marrow progenitors toward a pro-vasculogenic phenotype. Consequently, the production and transfer of these exosomes increase the incidence and burden of metastatic diseases [75].

Hematopoietic stem cell (HSC) niche

The concept of the HSC niche was first proposed by Ray Schofield in 1978 [76]. It is the microenvironment where HSCs are thought to reside in the bone marrow, which has been proposed to comprise several cell types such as vascular endothelium and endosteal osteoblasts [77, 78]. Components of the HSC niche have also been indicated as fertile ground for the development of bone metastases. Some evidence supports the hypothesis that bone metastatic cancer cells disseminate in the same manner as how HSCs home to bone marrow such as via the CXCR4/SDF1 axis [79, 80].

A prostate cancer model suggests that HSCs co-localize with prostate tumor cells at the endosteal bone surfaces *in vivo* and *in vitro*, indicating a niche competition [81]. If tumor cells target HSC niche, then it is rational to hypothesize that bone metastasis can be altered by manipulating the size of the niche. Indeed, niche expansion by parathyroid hormone (PTH) treatment resulted in significantly more tumor cells in the bone marrow [82]. Although the detailed mechanisms still remain unclear, several factors have been implicated in how tumor cells hijack the HSC niche. Binding to the niche osteoclasts induces the expression of TRAF family member-associated NF-kappa-B activator (TANK) binding kinase 1 (TBK1) in prostate tumor cells PC3 and C4-2B. An increased TBK1 level enhances tumor cell survival and drug resistance in the bone microenvironment by inhibiting mTOR function [83]. Gas6, a ligand for AXL and MER, is produced by the HSC niche. Gas6 can stimulate the survival of prostate tumor cells by inducing tumor dormancy through AXL [84]. Consistent with this notion, the Gas6/MER interaction also protects metastatic leukemia cells from chemotherapy-induced apoptosis [85].

Moreover, HSCs-derived bone morphogenic proteins (BMPs), such as BMP2 and BMP6, can promote osteoblast differentiation [86]. Osteoblasts in turn maintain the endosteal HSC niche, creating a positive feedback loop between HSCs and osteoblasts, which leave an open opportunity for prostate cancer cells to alter the regulation of normal bone formation by HSCs through BMPs signaling pathway [87]. BMPs are also involved in both HSC quiescence and tumor cell dormancy, therefore representing promising candidates for therapeutic targeting of bone metastases [88, 89].

Factors secreted from bone cells

One of the most interesting features of mammalian skeleton is its ability to continuously remodel and renew itself. During this process of degrading and reforming bones, abundant factors are secreted from bone cells and bone matrix into the osseous environment.

TGF- β is one of the most abundant cytokines released from stromal cells. It stimulates tumor cell expression of PTHrP, which activates osteoclasts [90]. Osteoclasts, in turn, resorb bone and release TGF- β from bone matrix to promote tumor cell proliferation and survival, forming a “vicious cycle”. Inhibition of TGF- β with small molecules or neutralizing antibodies can reduce osteolytic lesion and tumor burden in tibial injection mouse models for both breast cancer and melanoma [91, 92]. Interestingly, Li *et al* suggested that loss of stromal TGF- β responsiveness in the fibroblast instead promoted bone metastasis of prostate cancer cells, which might be mediated by increased expression of cytokines CXCL1 and CXCL16 and thus augmented prostate cancer cell adhesion to the bone matrix [93].

High serum levels of IGFs are linked to high risk for breast, prostate and colorectal cancer. Studies in animal models and in humans have established critical roles for IGFs in skeletal growth and development [94]. As for bone metastases, neutralizing antibody against IGF type I receptor (IGF-IR), but not other bone-secreted factors such as TGF- β , fibroblast growth factors (FGFs), and platelet-derived growth factors (PDGFs), blocks breast cancer bone anchorage. This supports a unique and important role of bone-derived IGF-I in the crosstalk between the bone microenvironment and the cancer cells [95].

FGFs are a family of widely expressed polypeptide ligands that regulate a broad spectrum of physiological processes via fibroblast growth factor receptors (FGFRs). FGF signaling has been shown to mediate a positive feedback loop between prostate cancer cells and bone cells. Augmented expression of FGF ligands and FGF receptors activates multiple mechanisms to promote prostate tumorigenesis and metastasis [96]. Two small-molecule tyrosine kinase inhibitors that can block FGFR function, dovitinib and nintedanib, are currently in clinical trials for metastatic castration-resistant prostate cancer as well as metastatic renal cell carcinoma (RCC) [96, 97]. The antitumor and anti-bone-metastasis activity of dovitinib has been shown to be mediated by FGFR1 inhibition in osteoblasts rather than cancer cells, hence improving bone quality and blocking cancer-bone interaction. This may account for clinical observations of dovitinib effects such as reductions in lesion size and intensity on bone scans, lymph node size, and tumor-specific symptoms without proportional declines in serum prostate-specific antigen level [98]. These studies suggest that FGFR inhibition may represent a promising new therapeutic strategy to treat advanced cancers and bone metastasis.

Beyond these well studied “classical” bone secreted molecules, several new factors have been recently identified as key partners-in-crime for tumor bone metastasis. PDGF is secreted by bone stroma. Functional disruption of PDGF receptor (PDGFR) can alter heterotypic tumor–stromal and tumor–matrix interactions in lung cancer, thereby preventing efficient engagement required for bone homing and osseous colonization [99]. In addition, CCL5 secreted from bone marrow MSCs can promote the migration and invasion of

hepatocellular carcinoma cells (HCCs), thus representing a potentially important factor in HCC bone metastases [100].

Adhesion of tumor cells to bone cells

Adhesion between tumor cells and bone marrow stromal cells is critical for cancer colonization in distant bone tissues. Integrins are a family of trans-membrane receptors that function as the bridges for cell-cell and cell-ECM interactions, thus playing major role in this adhesion process. So far, $\alpha\text{v}\beta 3$ integrin has been the focus of most studies. Various *in vivo* and *in vitro* experiments suggest that $\alpha\text{v}\beta 3$ integrin increases the potential of human and murine breast cancer cells to form bone metastases [101, 102, 103]. ATN-161, a fibronectin-derived peptide known to interfere with integrin $\alpha\text{v}\beta 1$ and $\alpha\text{v}\beta 3$ binding, has completed phase I and II clinical trials [104]. In a preclinical animal model using intra-cardiac injection of MDA-MB-231 human breast cancer cells in nude mice, ATN-161 treatment resulted in a remarkable reduction in the incidence and number of bone metastases, supporting a critical role for these integrin heterodimers in cancer osteotropism [105].

Cadherins are another family of cell adhesion molecules that have been implicated in the colonization of cancer cells to distant organs. Cadherin-11 was originally identified in mouse osteoblasts and known to regulate bone development [106]. Later on, it was found that the level of cadherin-11 expression increased when primary prostate cancer is developed into metastatic disease, especially in bone [107, 108]. The same phenotype was also observed in breast cancer patients, indicating that cadherin-11 is specifically associated with bone metastasis of multiple cancers [109]. Cadherin-11 is proposed to mediate the binding of tumor cells to osteoblasts and thereby promoting cancer cell colonization in bone [107, 108].

Recently it was reported that heterotypic adherent junctions (hAJs) that involves cancer-derived E-cadherin and osteogenic N-cadherin plays a key role in mediating the interaction between cancer cells and osteogenic cells *in vitro* and *in vivo* [110]. Interestingly, treatment with trans-2-phenylcyclopropylamine hydrochloride (PCPA), an inhibitor of the chromatin-modifying protein lysine demethylase 1 (LSD1), induced the expression of E-cadherin and other epithelial markers. PCPA treatment markedly suppressed the migration and invasion of triple-negative breast cancer cells and attenuated bone metastases without affecting lung infiltration, when MDA-MB-231 cells were injected directly in the blood circulation of nude mice [111]. There may be several possible explanations for these seemingly contrasting observations. First, cancer cell E-cadherin may play different roles in the pre- and post-metastatic site, in which it suppresses EMT in the primary tumor but facilitate bone adhesion and colonization in the bone metastatic niche. Second, other epithelial markers up-regulated by PCPA may play different and perhaps more dominant roles compared to E-cadherin, driving the anti-metastatic outcome.

Protein glycosylation has also been suggested to modulate bone metastases. β -galactoside α -2,3-sialyltransferase (ST3GAL6), an important factor in selectin ligand synthesis, was found highly expressed in multiple myeloma cells and patients. Selectins are a family of cell adhesion molecules that are composed of single chain transmembrane glycoproteins.

Knockdown of ST3GAL6 attenuated multiple myeloma cell adhesion and migration *in vitro*, and reduced the ability of these cells to home to the bone marrow *in vivo*, resulting in a reduction in tumor burden and a prolonged survival in an xenograft mouse model [112]. In an adhesion assay using a flow device, P-selectin was found to induce strong adherence to cells expressing CXCR4 such as cancer cells, especially when SDF-1 is co-expressed on the surface of bone marrow stromal cells. This indicates a potential synergistic effect of P-selectin and SDF-1 in promoting cancer cell adhesion and bone colonization [113].

CD44 is an adhesion molecule that binds to ECM and primarily to hyaluronan (HA). It is recognized as a potential cancer stem cell marker, has also been implicated to promote bone metastases by enhancing tumorigenicity, cell motility, and HA production in a breast cancer xenograft model. As such, CD44–HA interaction has been suggested as a potential novel target for therapeutic intervention for bone metastases [114].

Bone microenvironment and macrometastases

Bone provides a unique environment in which multiple cell types co-reside and interact, including hematopoietic cells such as osteoclasts and immune cells, mesenchymal cells such as osteoblasts, osteocytes and adipocytes, as well as cells forming the vasculature such as endothelial cells and pericytes. After successfully landing on bone, tumor cells start to mingle with the local bone cells and coerce them to create a hospitable environment for cancer cells to survive and thrive, using several different approaches and molecules.

Cancer cells influence bone cells mainly in two ways. Most often, the cancer cells manipulate the osteoclast lineage to increase osteoclast differentiation and activity. When this osteoclastic bone resorption supersedes osteoblastic bone formation, osteolytic metastatic lesions occur in which the excessive bone degradation leaves “cavities” in the mineralized tissue where tumor cells reside as often seen in X-ray images (**Figure 2A**). Sometimes, the cancer cells also release substances to manipulate the osteoblast lineage to increase osteoblast differentiation and new bone deposition. When this osteoblastic bone formation supersedes osteoclastic bone resorption, osteoblastic metastatic lesions similar to sclerosis occur in which the excessive bone growth leaves “bulges” in the mineralized tissue where tumor cells reside, which are also discernible in X-ray images (**Figure 2B**). Although there is more bone deposition in these osteoblastic lesions, their structure is abnormal, leading to weak bones and fractures. The distinction between these two types of lesions is not absolute. Many patients with bone metastases have both osteolytic and osteoblastic lesions, and individual metastatic lesion can contain both osteolytic and osteoblastic components (**Table 1**). Mechanistically, osteoclasts have been shown to play a pro-metastatic role in both types of lesions.

Osteoclasts

In osteolytic bone metastases, tumor cells enhance osteoclast formation by continually secreting pro-osteoclastogenic factors including PTHrP, RANKL, interleukins, prostaglandin E, tumor necrosis factor (TNF) and macrophage colony stimulating factor (MCSF). Conversely, osteoclastic bone resorption releases growth factors, such as TGF β , IGFs, PDGFs and BMPs, from the bone matrix to enhance tumor growth. This “tumor-

osteoclast cooperation” is the major foundation of the “vicious cycle” hypothesis [115]. Clinically, anti-resorptive drugs have been proven to be effective to slow down the process of cancer bone metastases.

Bisphosphonates are a group of pyrophosphate analogs that preferentially bind to calcium, which is mainly stored in bone in vertebrates. When attached to bone, bisphosphonates are ingested by osteoclasts and kill the osteoclasts [116]. Moreover, partially beneficial from their anti-angiogenesis effects, bisphosphonates exert anticancer properties as well [117, 118, 119, 120, 121]. Thus, bisphosphonate therapy is currently a standard of care for patients with malignant bone disease. This osteoclast poisoning tactic has been used to restrict skeletal metastases for many tumor types, including prostate, breast, lung and multiple myeloma [122]. Denosumab, a fully human monoclonal antibody against RANKL, has also been approved for the treatment of osteoporosis and for delaying skeletal-related events in patients with bone metastases secondary to breast or prostate cancer [123].

TGF- β is a major bone responder to tumor signals. Overproduction of active TGF- β by osteoclastic bone resorption in the skeletal metastases setting not only increases tumor cell invasiveness and angiogenesis, but also induces immunosuppression. Thus blocking the TGF- β signaling pathway to interrupt the vicious cycle between cancer and bone offers a promising therapeutic approach [124]. TGF- β -targeting neutralizing antibodies (1D11, Fresolimumab), tyrosine kinase inhibitors (LY2109761, LY2157299) and soluble decoy receptor proteins have been developed and tested in preclinical studies as well as clinical trials on their efficacy of ameliorating cancer bone metastases [125, 126].

Sarcoma (Src) is an oncogene encoding a tyrosine kinase that promotes cellular proliferation, differentiation, motility and survival. It is also highly expressed in osteoclasts and essential for the organization of osteoclast cytoskeleton [127, 128]. Moreover, Src inhibits RUNX2 target genes, thus negatively impact osteoblast differentiation [129]. Due to the multiple roles of Src in osteoclasts, osteoblasts and tumor cells, targeting Src has been the center of investigation. Dasatinib (a Src inhibitor) and saracatinib (a dual inhibitor of Src and Abl) are currently evaluated in multiple clinical trials for their efficacy in skeletal and metastatic diseases [130, 131].

Although the ability of these osteoclast inhibitor drugs in controlling bone metastases deterioration is encouraging, significant challenge still lies ahead. First, the side effects of currently approved drugs, such as osteonecrosis of the jaw (ONJ), hypocalcemia and renal toxicity [132, 133, 134], are hard to ignore; second and more importantly, in fact none of these drugs can actually cure bone metastases or increase long-term survival, begging for better pharmacological strategies. Hence, researchers continue to investigate tumor influence on osteoclasts in the search for new and better therapeutic targets.

Identification of microRNAs (miRNAs) that play key functions in bone-specific metastasis is a major breakthrough. Ell and colleagues reported that miR-141 and miR-219 can inhibit osteoclast differentiation and experimental bone metastases in a xenograft mouse model. Moreover, miR-16 and miR-378 may have diagnostic potentials as circulating biomarkers

for osteolytic bone metastases [135]. These findings highlight miRNAs as exciting new strategies for the diagnosis and treatment of cancer skeletal metastasis.

Similarly, our lab has uncovered miR-34a as a potent suppressor of osteoclast differentiation, bone resorption and skeletal metastasis, which functions through inhibiting the homeobox transcription factor Tgif2 (TGF β inducible factor 2) [136]. Osteoclastic miR-34a over-expressing transgenic mice exhibit decreased osteoclastogenesis and bone resorption, higher bone mass, leading to resistance to osteoporosis and bone metastases of breast cancer and melanoma. Conversely, miR-34a knockout mice display enhanced osteoclastogenesis and bone resorption, leading to lower bone mass and exacerbated cancer bone metastasis. Pharmacologically, systemic delivery of miR-34a mimetic via chitosan-based nanoparticles effectively targets the circulating osteoclast progenitors to impede the development of osteoporosis and cancer metastasis to the bone. These findings reveal miR-34a as a potential therapeutic strategy to confer skeletal protection and ameliorate bone metastasis of cancers [136]. Moreover, miR-34a was originally identified as a p53 target that is frequently deleted in several types of human cancer [137], and pharmacological miR-34a treatment in mouse models exhibited efficacy to suppress tumor growth and lung metastasis via the inhibition of CD44 [138]. Therefore, miR-34a therapy may confer multiple benefits at both pre- and post-metastatic sites in the combat of cancer malignancy.

Interestingly, recent studies reveal that certain miRNAs can be released from cancer cells within microvesicles or exosomes, which can then be taken in by other local cells. For example, when overexpressed in bone metastatic lung cancer cells, miR-192 can be transferred to endothelial cells to inhibit angiogenesis; and systemic delivery of miR-192 can decrease osteolytic lesions in a mouse model [139]. Hence, both cell-intrinsic and exosome-delivered miRNAs represent potential powerful therapeutic targets to attenuate osseous metastases of cancers.

Osteoblasts

Osteoblasts produce and deposit growth factors into the bone matrix, such as TGF- β and IGFs, which are inactive until released and activated by osteoclastic bone resorption in osteolytic metastases [140]. In addition to turning off osteoclast functions, it is proposed that another strategy to ameliorate the bone loss is to turn on osteoblast functions [141]. Proteasome inhibitors are reported to promote bone formation by stimulating progenitor proliferation and osteoblast differentiation. Bortezomib is the first FDA approved therapeutic proteasome inhibitor, which demonstrates efficacy in the treatment of both multiple myeloma and lymphomas in humans [142]. Interestingly, bortezomib has also been shown to inhibit breast cancer osteolytic metastasis in preclinical models [141]. Future clinical studies are required to assess the therapeutic benefits of bortezomib on bone metastasis of solid tumors in patients.

In the osteoblastic bone metastases, there is also a vicious cycle between tumor cells and osteoblasts. Tumor cells produce osteoblast-stimulating factors such as BMPs, FGFs and PDGF. Tumor cells also activate endothelin-1 (ET-1), which down-regulate Dkk1, a negative regulator of osteoblastogenesis [143]. The activated osteoblasts produce factors including interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), VEGF and

macrophage inflammatory protein-2 (MIP-2), which in turn assist cancer cell colonization and amplification upon arrival in the bone microenvironment. Particularly, endothelins and their receptors are emerging plausible targets for osteoblastic bone metastasis. Inhibition of ET-1 is reported to have dual suppressive effects on both tumor cells and osteoblasts. ET-1 promotes cancer cell proliferation and invasion via up-regulating MMPs, inhibiting apoptosis and enhancing VEGF-induced angiogenesis [144]. ET-1 also increases osteoblast differentiation by reducing Dkk1. Both ET-1 neutralizing antibodies and ET-A receptor antagonists (ABT-627) were under clinical evaluation [145, 146]. The role of osteoblasts in skeletal metastasis is less well defined - whether they are pro-metastatic or anti-metastatic may depend on multiple factors such as the type of cancer cells and whether the lesion is more osteolytic or osteoblastic.

Adipocytes

Adipocytes are another important component in the bone marrow environment. The number of marrow adipocytes increases greatly with aging and obesity, which are also risk factors for the development of metastatic cancers. Marrow adipocytes secrete hormones, cytokines and fatty acids that may have profound effects on hematopoietic niches, neighboring bone cells and inflammation, hence shaping the local milieu, hematopoiesis and skeletal homeostasis [147]. It has been suggested that tumor cells can also be attracted to marrow adipocytes and affected by adipokines. The interaction between these two cell types may create a chronic inflammatory environment that is pro-tumorigenic, as well as the translocation of adipocyte-stored lipids to the metastatic tumor cells, causing an increased motility in tumor cells [147, 148, 149].

Marrow adipocytes may promote tumor growth in bone in an FABP4-dependent manner. FABP4 is a cytosolic lipid chaperone that is predominantly expressed in adipocytes, macrophages and endothelial cells. Adipocyte FABP4 is transcriptionally regulated by insulin and fatty acids, the latter of which function as agonists for the nuclear receptor peroxisome proliferator associated receptor γ (PPAR γ) [150]. Herroon *et al* propose that FABP4 inhibition can block lipid trafficking between marrow adipocytes and cancer cells, thus subsiding the formation of tumor metastasis in bone and marrow. These studies reveal adipocyte FABP4 as a potential driving mechanism for skeletal metastasis [151]. Clearly, marrow adipocytes impact cancer bone metastases; however, little is known about how exactly marrow fat cells modulate tumor colonization and macrometastases in the skeleton. Detailed genetic and molecular studies are required in the future to delineate the underlying mechanism and elucidate new therapeutic targets for the fat-bone-cancer connection.

Macrophages

Macrophages, derived from myeloid progenitors, are also an important component of bone marrow. Genetic dissection suggests that cathepsin K (CTSK) from macrophages, in addition to osteoclasts, plays a key role in promoting prostate cancer progression in bone [152]. Macrophages are classified as two main subsets that can be distinguished by their regulation of inflammation, with M1 macrophages generally promote inflammation, whereas M2 macrophages typically suppress inflammations and assist tissue repair [153]. When associated with tumors, M1 macrophages have been proposed to be anti-tumorigenic by

releasing reactive oxygen species, nitrogen intermediates and inflammatory cytokines to kill cancer cells; while M2 macrophages are generally considered pro-tumorigenic by releasing a variety of growth factors such as FGFs and VEGF to promote tumor growth and invasion [154]. Rac2, a small GTPase, that facilitates macrophage M1 to M2 polarization, was reported to promote tumor metastases [155].

Hiraoka *et al.* used clodronate-packaged liposomes (Cl2MDP-LIP) to deplete monocytes and macrophages, in addition to osteoclasts, to broadly assess the effects of these cells on cancer metastasis in a metastatic lung cancer cell cardiac injection mouse model. They found that targeting both macrophage and osteoclast by Cl2MDP-LIP treatment showed a more pronounced reduction in the number and size of bone metastatic lesions compared to the sole osteoclast-targeting agent reveromycin A [156]. Another study showed that anti-CD115 antibody can deplete tumor associated macrophages, leading to decreased osteolytic bone lesions in nude mice transplanted with MDA-MB-231 human breast cancer cells via cardiac injection [157].

By contrast, elevated expression of hyaluronan synthase 2 (HAS2) in metastatic tumor cells was shown to be critical for the interaction between tumor cells and macrophages in bone, resulting in an increased secretion of PDGFs from macrophages, which then activated stromal cells and promoted cancer cells self-renewal. Thus, HAS2 inhibitors were proposed as potential anti-metastatic agents that disrupt a macrophage-tumor paracrine growth factor loop within this microenvironment [158].

The contribution of macrophages to tumor growth, progression and metastasis has been the subject of intense investigation in the past few years. However, there is little direct evidence that clearly demonstrates a suppressive effect of macrophages on bone metastases, mainly because most strategies for depletion and/or inhibition of macrophages, as well as for manipulating macrophage intrinsic factors, also target osteoclasts at the same time.

Concluding remarks

Cancer bone metastasis is a highly complicated process that involves not only tumor cells themselves but also multiple other cell types at both pre-metastatic and post-metastatic sites. Potential molecular markers have been identified in the early stages of metastases to diagnose or predict skeletal metastatic events in advanced cancers (**Table 2**). For the subsequent steps of cancer homing and macrometastases in bone, accumulating evidence has revealed an intricate network by which tumor cells interact with many local bone cells to form a vicious cycle and orchestrate a successful skeletal metastasis (**Table 2**).

This cancer-bone feed forward loop provides ample opportunities for therapeutic targeting to prevent and treat bone metastasis of advanced cancers. Since most current regimens only attenuate the progression of existing bone metastasis and do not confer long-term survival, a therapeutic strategy that can act on early stages will be highly desirable to inhibit EMT, eradicate cancer stem cells, stop the dissemination of circulating tumor cells, as well as prevent bone colonization and micrometastasis formation. The major goals and challenges for future investigation reside in the following aspects: i) early biomarkers, ideally circulating factors, for the prediction and diagnosis of metastatic disease need to be

identified and clinically validated; ii) genetic or humoral factors that allow the assessment of cancer cell dormancy need to be explored; iii) better characterization of the mechanisms by which osteoclasts, osteoblasts, marrow adipocytes and macrophages influence bone metastasis of various cancers is required; iv) therapeutic approaches that can simultaneously target multiple components in multiple steps during cancer bone metastasis are needed to more effectively prevent and eliminate cancer malignancy; v) pharmacological agents that specifically target the mechanisms employed by the tumor cells and their interaction with niche cells, without overtly interfering normal physiological functions, will promise less side effects and an improved quality of life for the patients; and finally vi) new and alternative experimental animal models need to be established, such as genetic models that can recapitulate the entire process of cancer bone metastasis, as well as patient-derived cancer cell xenograft models that can predict patient-specific propensity of bone metastasis as well as patient-specific efficacy of potential treatments (**Box 1**). As increasing number of molecular targets and mechanisms being discovered by the researchers, greater understandings of the etiology of cancer bone metastases are leading towards innovations for not only effective and safe agents to treat but also efficient diagnostic approaches to predict and finally prevent cancer bone metastases.

Box 1

Experimental models for bone metastasis imaging

The development of experimental animal models for the imaging and quantification of cancer bone metastasis has significantly facilitated our research endeavor. These are mainly transplantation mouse models using luciferase-labeled bone-metastasis-prone cell lines, such as MDA-MB-231 human breast cancer cell sub-lines [159], 4T1.2 mouse mammary tumor cell line [160], B16-F10 mouse malignant melanoma cell line [161] and TSU-Pr1-B1/B2 human bladder cancer cell line [162]. These cell lines can develop bone metastasis when injected intracardiacally and in some cases orthotopically. Recently, GFP-labeled bone metastasis-prone cell lines, such as human lung cancer H460 cells [163] and human prostate cancer PC-3 cells [164], have been generated to image bone metastasis when a single highly fluorescent subcutaneously growing tumor is transplanted by surgical orthotopic implantation. In addition, GFP-labeled mouse melanoma B16 cell line and human melanoma LOX cell line [165], as well as *in vivo* selected highly metastatic MDA-MB-435-GFP human breast cancer cells [166] can also develop bone metastasis. Interestingly, PC3-GFP cells have been used to show that fluorescence-guided surgery significantly reduced recurrence of experimental prostate cancer bone metastasis compared with bright-light surgery; and the combination of fluorescence-guided surgery and zoledronic acid increased disease-free survival over bright-light surgery and zoledronic acid [167]. Challenges for future investigation will be to establish genetic animal models that recapitulate the entire process of cancer bone metastasis, as well as patient-derived cancer cell xenograft models that can predict patient-specific propensity of bone metastasis as well as patient-specific efficacy of potential treatments.

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Highlights

- We highlight newly identified molecular targets
- We discuss their molecular modes of action
- We summarize their preclinical and clinical validation
- We propose the major goals and challenges for future investigation

Figure 1. Multiple steps in cancer bone metastasis and the major regulatory factors in the tumor-bone microenvironment

The cellular basis of cancer progression includes tumor cell local invasion, intravasation, survival in the circulation, extravasation, colonization and thriving in the bone. In the primary site, tumor cells undergo EMT, ECM degradation and angiogenesis to invade vascular or lymphatic circulation (A). After intravasation, a few tumor cells can survive from NK cells attack with the assistance of platelets (B). Through the communication between tumor intrinsic factors and factors in the osseous environment, cancer cells extravasate to the bone and marrow. Interactions between tumor cells and bone cells mediated by adhesion molecules are critical for colonization (C). After landing, tumor cells start to interact with local cells, including osteoclasts, osteoblasts, adipocytes and macrophages, in order to thrive in bone and form micro- and macro-metastasis (D).

A. Osteolytic lesion **B. Osteoblastic lesion**

Figure 2. Illustration of osteolytic and osteoblastic bone metastatic lesions

Cancer causes predominantly two types of bone metastatic lesions based on the radiological appearance, with bone destructions observed in osteolytic bone metastatic lesions (A), while bone forming phenotypes are observed in osteoblastic bone metastatic lesions (B).

Table 1

Different bone lesions in cancer bone metastases

Osteolytic	Osteoblastic	Mixed
Renal cell carcinoma	Prostate cancer	Breast cancer
Melanoma	Carcinoid	Gastrointestinal cancers
Multiple myeloma	Small cell lung cancer	Squamous cancers
Non-small cell lung cancer		
Thyroid cancer		

Table 2

Molecular targets and current drugs for cancer bone metastases

Molecular targets	Mechanisms	Agents/Drugs	References
Escape of cancer cells from the original site			
Snail	Stimulate EMT and tumor-bone vicious cycle		[12]
TWIST1	Promote breast cancer bone metastases through mir-10b		[13]
Notch1	Signature gene for the acquisition of EMT in prostate cancer bone metastases		[11]
HIF1	Induced by hypoxia to stimulate EMT	Transcutaneous CO ₂ treatment	[24-26]
Invasion of cancer cells			
MMP2/MMP9	Cleave the constituents of the ECM		[30]
miR-29c	Suppress lung cancer cell adhesion to ECM and metastases		[31]
VEGF receptors	Essential for angiogenesis	Bevacizumab	[34]
		Cabozantinib	[35,36]
		Sunitinib, sorafenib and cediranib	[37]
		Aflibercept	[38]
P2Y2	Important for ATP-induced tumor cell extravasation		[48]
C2GnT	Protect cancer cells from NK cell immunity		[50]
Homing to the bone			
<i>Tumor cell secreted factors</i>			
CXCR4/SDF-1 axis	Attract cancer cells to HSC niche	CTCE-9908	[54,55]
		Plerixafor/AMD3100	[56]
CXCR7	Promote tumor progression by recruiting angiogenic mononuclear cells		[60]
CXCL10/CXCR3 axis	Enhances tumor growth and osteoclast differentiation		[61]
Adrenomedullin	Increase osteoclast activity		[65]
IGFBP2, MERTK	Recruit endothelia	microRNA-126	[66]
TGF- β 2, GDF15, FGF3, FGF19, CXCL1, galectins and β 2-microglobulin	Highly expressed in the conditioned medium from isolated osteoblastic bone lesion cancer cells		[69]
DDR1	Associate with cancer cell survival and homing on bone		[70]
EPCR	Promote cancer cell survival		[71]
HDAC4, PITX1, ROBO1	Promote lung adenocarcinoma bone metastases		[72]
Canonical WNT/TCF	Enhance both bone and brain metastases		[73]
<i>HSC niche</i>			
PTH	Expand HSC niche		[82]
TBK1	Inhibits mTOR function and enhances cancer cell survival		[83]
Gas6	Induces cancer cell dormancy		[84,85]
BMP2, 6	Promote osteoblast differentiation, maintain HSC niche and regulate cancer cell dormancy		[86-89]

Molecular targets	Mechanisms	Agents/Drugs	References
<i>Bone cell secreted factors</i>			
IGFs	Modulate both bone microenvironment and cancer cells		[94,95]
FGFs	Mediate a positive feedback loop between cancer cells and bone cells	Dovitinib Nintedanib	[96-98]
PDGFR	Associate with cancer bone homing and osseous colonization		[99]
CCL5	Promote cancer cell migration and invasion		[100]
<i>Adhesion of tumor cells to bone cells</i>			
$\alpha v \beta 3$ integrin	Promote cancer bone colonization	ATN-161	[101-105]
cadherin-11	Mediate binding of cancer cells to osteoblasts		[106-109]
hAJs	Mediate the interaction between cancer cells and osteogenic cells		[110]
ST3GAL6	Formulate single chain transmembrane glycoproteins, participates in cancer cell adhesion and migration		[112]
P-selectin	Induce strong adherence to cells expressing CXCR4		[113]
CD44	Bind to ECM and primarily to HA		[114]
Bone microenvironment and macrometastases			
<i>Osteoclast</i>			
Calcium/RANKL/ TGF β	Main factors of tumor-osteoclast vicious cycle	Bisphosphonates Denosumab 1D11, Fresolimumab LY2109761, LY2157299	[116-122] [123] [124-126]
Src	Promote osteoclast and inhibit osteoblast	Dasatinib saracatinib	[127-131]
miR-16, miR-378	Potential circulating biomarkers for osteolytic bone metastases		[135]
miR-34a	Inhibit osteoclast differentiation by suppressing Tgif2, and impede cancer progression at both pre- and post- metastatic sites as a p53 target and a CD44 inhibitor		[136-138]
miR-192	Inhibit angiogenesis and decrease osteolytic lesions		[139]
<i>Osteoblast</i>			
Proteasome inhibitors	Promote bone formation	Bortezomib	[141,142]
ET-1	Promote cancer cell invasion and osteoblast differentiation	ABT-627	[143-146]
<i>Adipocyte</i>			
FABP4	Regulate lipid trafficking between marrow adipocytes and cancer cells		[150,151]
<i>Macrophages</i>			
Rac2	facilitate macrophage M1 to M2 polarization		[155]
HAS2	Increase secretion of PDGFs from macrophages		[158]