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THEME | *Tissue Remodeling: From Regeneration to Fibrosis*

Thrombospondins in bone remodeling and metastatic bone disease

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Carminati L, Taraboletti G. Thrombospondins in bone remodeling and metastatic bone disease. *Am J Physiol Cell Physiol* 319: C980–C990, 2020. First published September 16, 2020; doi:10.1152/ajpcell.00383.2020.—Thrombospondins (TSPs) are a family of five multimeric matricellular proteins. Through a wide range of interactions, TSPs play pleiotropic roles in embryogenesis and in tissue remodeling in adult physiology as well as in pathological conditions, including cancer development and metastasis. TSPs are active in bone remodeling, the process of bone resorption (osteolysis) and deposition (osteogenesis) that maintains bone homeostasis. TSPs are particularly involved in aberrant bone remodeling, including osteolytic and osteoblastic skeletal cancer metastasis, frequent in advanced cancers such as breast and prostate carcinoma. TSPs are major players in the bone metastasis microenvironment, where they finely tune the cross talk between tumor cells and bone resident cells in the metastatic niche. Each TSP family member has different effects on the differentiation and activity of bone cells—including the bone-degrading osteoclasts and the bone-forming osteoblasts—with different outcomes on the development and growth of osteolytic and osteoblastic metastases. Here, we overview the involvement of TSP family members in the bone tissue microenvironment, focusing on their activity on osteoclasts and osteoblasts in bone remodeling, and present the evidence to date of their roles in bone metastasis establishment and growth.

bone metastasis; metastatic niche; osteoclasts/osteoblasts; TSP-1; TSP-2; tumor microenvironment

INTRODUCTION

Bone is the third most frequent site of metastasis, after lung and liver. The skeleton is the most common metastatic site for breast (especially the estrogen receptor-positive subtype) and prostate cancers, occurring in more than 70% of patients with advanced stage disease (24, 26). Bone metastases increase morbidity, due to skeletal-related events, including bone pain, pathological fractures, spinal cord injury, and tumor-induced hypercalcemia, which significantly worsen patients' quality of life and survival (52, 125).

The bone microenvironment is a fertile "soil" for cancer cells, because of its unique matrix composition, mineral content, hypoxia, and enrichment in cytokines and growth factors. This microenvironment comprises bone extracellular matrix (ECM), bone marrow stroma, blood vessels, resident bone cells (of the osteoclast and osteoblast lineages), immune cells, endothelial and hematopoietic cells, and bone-marrow-derived mesenchymal stem cells (MSCs), which can all participate in the metastatic process (26, 129).

Physiological Bone Remodeling

The adult skeleton is maintained throughout life by a tightly balanced process of bone remodeling, in which bone resident cells sustain a continuous removal (resorption) and replacement (deposition) of bone matrix. Less than 25% of the endosteal surface is actively involved in bone remodeling, which takes place in distinct areas of bone-degrading osteoclasts and bone-forming osteoblasts (26, 103).

Osteoclasts are multinucleated cells responsible for bone resorption. They derive from hematopoietic precursors of the myeloid lineage, through a multistep process involving proliferation, differentiation, fusion, and activation. Osteoclastogenesis is mainly controlled by the receptor activator of nuclear factor-κβ ligand (RANKL), produced mostly by osteoblasts and osteocytes (66), interacting with its transmembrane receptor RANK, expressed by osteoclast precursors (26, 109).

The osteoblast lineage, derived from bone marrow MSCs (5, 11), comprises cell types at different stages of differentiation: osteoprogenitors and active bone-forming osteoblasts that, once the bone deposition activity is completed, can undergo apoptosis or become either osteocytes, if embedded in the deposited bone matrix, or quiescent bone lining cells, if remaining on the endosteal bone surface. Osteoblasts also participate in the control of bone resorption, as they produce factors that regulate osteoclastogenesis, particularly RANKL and the soluble decoy receptor osteoprotegerin (OPG), which prevents RANK/RANKL signaling by scavenging RANKL (13).

Bone Metastasis

Bone metastasis formation is a multistep process, including *1*) colonization of bone marrow by circulating cancer cells; *2*) dormancy, as tumor cells adapt to the new environment and

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engage bone niches where they reside in a dormant state for long or short periods; *3*) reactivation of tumor cell proliferation; and *4*) development and growth of metastasis, which impair the bone remodeling balance and bone structure. When cancer cells reach the bone microenvironment, an interaction with the bone cells supports the development of bone metastasis at different levels.

Tumor cells can be maintained in a quiescent, dormant state even for years by the engagement of specific niches in the bone (126). Disseminated tumor cells use a number of receptors and adhesion molecules to compete with hematopoietic stem cells (HSCs) for the endosteal niches, which contain cells of the osteoblast lineage—mainly bone lining cells—actively supporting cell survival and controlling dormancy (93). Disseminated tumor cells, particularly breast cancer cells, have also been found associated with the vasculature in a perivascular niche, where endothelial cells support tumor cell dormancy (40). Once tumor cells are reactivated, by changes in the local niche microenvironment, the progression to overt metastasis is supported by continuous molecular cross talk between bone resident cells and tumor cells, which impairs the bone homeostasis leading to abnormal osteolysis or bone deposition.

Bone metastases from breast, lung, and renal cancer and myeloma have mainly an osteolytic phenotype, resulting from the aberrant activation of osteoclasts. A "vicious cycle" is established, where tumor cells produce cytokines and growth factors, including tumor necrosis factor-alpha (TNF-α) and jagged and parathyroid hormone-related protein (PTHrP), which activate osteoclasts either directly or indirectly, by stimulating osteoblast production of osteoclastogenic factors such as RANKL. Osteoclast-mediated bone resorption releases growth factors, which in turn promote tumor cell survival and proliferation, creating an amplifying loop that drives metastasis growth (26).

Prostate cancer, in contrast, develops mostly osteoblastic bone metastasis, driven by increased osteoblast differentiation and aberrant bone deposition. Tumor cells secrete a variety of factors, including fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs), to stimulate osteoblast differentiation and activity (127). The distinction between the two phenotypes of bone metastasis is not always sharp, because some tumors, especially breast, gastrointestinal, and squamous cancers, develop mixed metastasis, with both osteolytic and osteoblastic lesions.

Treatment options for patients with metastatic bone disease mainly aim at prevention and palliation of skeletal-related events, together with standard tumor-specific therapies to reduce tumor burden, but they are often more palliative than curative (31).

An increased understanding of the mechanisms underlying the impairment of bone remodeling in bone metastasis led to the development of bone-targeting agents, especially antiresorptive drugs, including bisphosphonates (zoledronic acid, ibandronate, and clodronate) and anti-RANKL antibodies (denosumab), and radiopharmaceuticals, efficient in relieving bone metastasis-related pain (31). However, although these strategies ensure some improvement in the quality of life and delay bone metastasis growth, bone metastases are in most cases still incurable. Nonetheless, these approaches do indicate that targeting the tumor-bone microenvironment interaction is a promising direction for new therapeutic approaches to control dormancy and bone metastasis.

Thrombospondins

Thrombospondins (TSPs) are a family of five secreted multimeric modular matricellular glycoproteins, expressed with specific spatiotemporal patterns in embryonic and adult tissues, and are involved in several processes, including angiogenesis, wound healing, cell proliferation and migration, and connective tissue organization (2). TSP-1, the first member to be identified as a component of platelets, is a major endogenous inhibitor of angiogenesis and a key player in the tumor microenvironment (72, 74). Structurally, the trimeric TSP-1 and TSP-2 are more closely related to each other than the pentameric TSP 3–5. TSP-1/2 domains include the globular *N*-terminus, von Willebrand factor/procollagen domain, type I repeats (also called thrombospondin repeats, TSRs), type II repeats (EGF-like), type III repeats (calcium-binding domain), and the globular C-terminus. TSP-3, TSP-4, and TSP -5 (COMP) do not contain TSR.

The multidomain structure allows TSPs to interact with a variety of ligands, including cellular receptors, other extracellular matrix proteins, growth factors, cytokines, and proteases, leading to a wide range of activities in physiological and pathological conditions, including cancer and metastasis (2, 96). TSP-1 and TSP-2, belonging to subgroup A of the TSP family, have comparable functional properties: antiangiogenic activity (2, 80, 100), inhibition of nitric oxide (NO)-dependent signaling (56), inhibition of proinvasive matrix metalloproteinase (MMP) activity (9), modulation of collagen fibrillogenesis (99, 101), and ability to organize the ECM (17, 99). Despite their significant structural homology, TSP-1 and TSP-2 differ in their gene expression regulation and also in some activities. A major difference between the two TSPs is the ability to activate transforming growth factor-beta (TGF-β). Both TSP-1 and TSP-2 can bind latent TGF-β, but only TSP-1 has the sequence required to activate it, whereas TSP-2 acts as a competitive antagonist of this activation (90).

The pentameric TSP-3, TSP-4, and COMP—subgroup B—have different functional properties from subgroup A, especially with regard to angiogenesis. Little is known about TSP-3's role in tissue homeostasis and disease. TSP-4, differently from TSP-1 and TSP-2, mediates upregulation of angiogenesis (88) and reduces fibrosis and collagen production (106). COMP is mostly involved in stabilizing the ECM, especially in cartilage, with roles in chondrocyte proliferation, collagen secretion, and fibrillogenesis (94).

A number of TSP molecular ligands, including integrins, TGF-β, jagged/notch, CD47, CD36, vascular endothelial growth factor (VEGF), FGF-2, and collagenous and noncollagenous components of the ECM, are involved in the regulation of bone remodeling through effects on osteogenesis and osteoclastogenesis, as well as in the formation and organization of important components of the bone microenvironment such as the ECM and blood vessels. These complex interaction networks are also involved in bone metastasis (46), where TSPs, derived from tumor cells as well as bone-resident cells, such as osteoblasts and endothelial and immune cells, can modulate not only bone remodeling but also other aspects of tumor and microenvironment biology, such as angiogenesis, invasion, ECM organization, and immunity.

This review focuses on the role of thrombospondins in bone remodeling associated with skeletal metastasis, to gain a fuller

understanding of their involvement in the vicious cycle that drives bone metastasis, offering new cues for therapeutic approaches.

TSP-1

Expression in Bone

TSP-1 is widely expressed early in the development of mouse and human embryos, localized in several tissues, including bones (54, 97, 115). In the adult, TSP-1 expression is generally more restricted. In the adult skeleton, it is present in long bones as a component of the mineralized matrix, where it is synthesized and deposited by osteoblasts during bone formation (18, 23, 97, 119).

Bone Remodeling

TSP-1 has been assigned multiple roles in bone homeostasis and remodeling, through its activity in the differentiation and activation of bone resident cells, organization of the bone microenvironment, and modulation of bone matrix properties. TSP-1-deficient mice were first described to present only slight spinal deformation and mild growth plate cartilage disorganization (73, 95), indicating functional compensation mechanisms between TSP-1 and other factors. Further studies conducted by Amend et al. (6) highlighted that TSP-1 deficiency led to increased trabecular and cortical bone volume, associated with osteoclast functional deficit and decreased bone resorption, indicating an important role of TSP-1 in physiological bone remodeling/homeostasis. Furthermore, TSP-1 deficiency, given the ability of TSP-1 to affect collagen fibrillogenesis (99), results in reduced collagen cross linking and altered ECM organization in bone (101).

Osteoblast differentiation and bone deposition. TSP-1 affects osteoblast precursor proliferation and osteogenic differentiation mainly through its ability to activate latent TGF-β [reviewed in (89)]. TGF-β is a major player in the regulation of bone remodeling, where it stimulates osteoprogenitor proliferation and maturation, while inhibiting late osteoblast differentiation and bone deposition (15, 59). Via TGF-β activation, TSP-1 stimulates proliferation of MSCs (10) and inhibits their in vitro differentiation into osteoblasts (8).

TSP-1 is highly expressed by MSCs during early osteogenesis, when TGF-β stimulates committed osteoblast progenitor recruitment and proliferation. In contrast, TSP-1 expression and TGF-β activity decrease during osteoblast differentiation (8), in line with their role as negative regulators of osteoblast differentiation and bone deposition. Thus, TSP-1 dose-dependently inhibited mineralization by mouse osteoblastic MC3T3-E1 cells, whereas TSP-1 knockdown in these cells increased the expression of osteoblast markers and accelerated bone deposition (117), confirming the inhibitory effect of TSP-1 on osteoblast differentiation. Although the ability of TSP-1 to influence osteoblast differentiation through latent TGF-β activation has been deeply studied in vitro, the only evidence of this mechanism in vivo derives from a study in a myeloma model (78). The possible role of a TSP-1-dependent modulation of TGF-β bioavailability in physiological regulation of bone remodeling in vivo requires further investigation.

Osteoclastogenesis and bone resorption. TSP-1 regulates bone homeostasis by not only preventing osteoblast-dependent bone deposition but also promoting bone-resorbing osteoclasts, as TSP-1 receptors—especially CD47, CD36, and β3 integrins—are involved in osteoclast signaling (35, 64, 82, 118). Carron and colleagues demonstrated that platelet-derived TSP-1 enhanced bone matrix resorption by osteoclasts in vitro (20) and identified the CD36-binding sequence as the site responsible for the resorption-promoting effect, in line with the evidence of CD36 expression in models of bone tumors characterized by aberrant osteoclast activity (19).

TSP1-binding partners CD47 and CD36 are important in stimulating cell fusion, a key step in the formation of osteoclasts from myeloid progenitors (41, 51). The direct involvement of TSP-1 binding to CD47 in stimulating cell fusion during osteoclastogenesis has been demonstrated in a myeloma model, in which dendritic cells cocultured with tumor cells upregulated TSP-1 and transdifferentiated into bone-resorbing osteoclasts (68).

Numerous studies have reported that differentiation of myeloid progenitors into osteoclasts is sensitive to nitric oxide (NO) signaling (14, 63, 128), which is negatively regulated by TSP-1 binding to CD47 or CD36 (55, 57). Amend et al. (6) found that TSP-1-null mice had increased cortical and trabecular bone volume, consistent with decreased osteoclastogenesis and bone resorption, and that overexpression of inducible nitric oxide synthase (iNOS) and aberrant NO signaling were partly responsible for the decrease in osteoclast function, confirming that TSP-1 promotes osteoclastogenesis by inhibiting iNOS. This is in line with the increased iNOS expression, reduced osteoclastogenesis, and consequent diminished bone loss in osteolytic metastasis in CD47-null mice, confirming the role of TSP-1/CD47-induced NO inhibition in osteoclastogenesis (118).

This has been further confirmed in models of osteoclast differentiation in vitro, driven by osteoblasts or CSF-1/RANKL stimulation, in which osteoclastogenesis was inhibited by neutralizing TSP-1 (67). In agreement with the finding that neutralizing TSP-1 antibodies prevented osteoclastogenesis only in the early phases of osteoclast differentiation (6), expression studies confirmed that TSP-1 signaling through CD47 and CD36 occurs mostly during early osteoclast precursor differentiation, whereas expression levels of these molecules are markedly lower in fully formed osteoclasts (67).

TGF-β is another potential mediator of the stimulating effect of TSP-1 in osteoclastogenesis and bone resorption. Although its role in osteoclast differentiation or activity has not been completely clarified, TGF-β has been described as promoting bone catabolism through enhancement of RANKL production, stimulation of osteoclast progenitor commitment, and inhibition of osteoclast apoptosis (62). Furthermore, TGF-β signaling is activated in mature osteoclasts involved in tumor-associated osteolysis (39). A role for TSP1-dependent activation of TGF-β as a mechanism of osteoclastogenesis stimulation by TSP-1 is supported by the low osteoclast number in vivo after treatment with an antagonist of TSP1-mediated TGF-β activation (78).

Bone Metastasis

TSP-1 expression and engagement in bone metastasis apparently varies with the type of bone lesion—osteolytic or osteoblastic—in line with its opposing activities of osteoclast stimulation and osteogenesis inhibition. Prostate cancer mostly develops osteoblastic bone metastasis, with tumor cells secreting factors that increase osteoblast activity. Proteomic analysis of serum from

patients with prostate cancer, comparing patients with and without bone metastasis, identified TSP-1 among a group of proteins downregulated in patients with osteoblastic bone metastasis (123). By contrast, proteomic profiling of the metastatic microenvironment in a xenograft breast cancer model, which develops osteolytic bone metastasis, identified differential expressions of ECM components in tissue-specific metastatic niches, revealing TSP-1 as a markedly upregulated protein in the bone niche (48). TSP-1 is overexpressed and acts as a major regulator of latent TGF-β activation in the myeloma bone marrow microenvironment, where it plays an important role in promoting osteolytic bone metastasis. The specific antagonism of TSP1-dependent TGF-β activation, through a peptide based on the TSP1-binding sequence of TGF-β, reduced myeloma tumor burden and osteolytic bone disease (78).

The potential role of TSP-1 in the bone metastasis microenvironment is ascribed not only to its activity in osteoblast and osteoclast differentiation and functions but can involve also other functions in tumor and host cells, such as angiogenesis, cell attachment, motility and invasiveness, and the deposition and organization of the extracellular matrix.

Fuhrman-Luck and colleagues (37) identified TSP-1 as a preferential substrate of the kallikrein-related peptidase 4 (KLK4), a serine protease overexpressed in prostate cancer that promotes tumor cell migration and proliferation and is positively correlated with prostate cancer bone metastasis. Human osteoblast-derived TSP-1 was established as a direct target of KLK4, which released two fragments covering the *N*-terminal domain of TSP-1, which promotes angiogenesis and lacks the ability to activate TGF-β, likely favoring metastasis growth and bone deposition at the site of cancer metastasis (37).

Another important mechanism by which TSP-1 regulates bone metastasis, independently from its effects on bone remodeling, is the enhancement of tumor cell dormancy. Disseminated tumor cells in the bone marrow interact with bone resident cells, which create a protected, dormant niche that keeps the tumor cells in a quiescent—but vital—state. Ghaiar et al. (40) found that in breast cancer models, endothelial cells in perivascular niches sustain the dormancy of disseminated tumor cells via TSP-1. Dormant perivascular niches were associated with a stable microvasculature, where TSP-1 was highly expressed, whereas actively growing tumor clusters were found surrounding the sprouting neovasculature, characterized by loss of TSP-1 expression (40). The ability of TSP-1 to regulate tumor cell proliferation has been confirmed in vitro, where TSP-1 reduced the proliferation of invasive ductal breast carcinoma cells by inducing cell cycle arrest, by activating the IFN-γ/indoleamine 2,3-dioxygenase 1 (IDO1)/TSP-1 axis in the microvasculature-associated niche (77).

TSP-2

Expression in Bone

In the embryo, TSP-2 expression is more restricted than TSP-1 and is mainly found in areas of vasculogenesis and in developing cartilage and bone (71, 115). This pattern is consistent with its role in connective tissues, as confirmed by the phenotype of mice that lack TSP-2, which present connective tissue abnormalities, anomalous collagen fibrillogenesis, and increased bone density in long bones (70). In adult bones, TSP-2 is constitutively expressed by MSCs and osteoblasts in the periosteum (43, 102, 130). TSP-2 expression markedly increases after bone injury (114). Using a TSP-2 reporter mouse model, Zondervan and colleagues (130) reported that TSP-2 expression has a spatiotemporal and context-specific pattern in skeletal fractures, increasing during early fracture healing and decreasing when MSCs differentiate into chondrocytes.

Bone Remodeling

TSP-2 has complex contextual activity in bone remodeling, regulating not only osteoblasts and osteoclasts but also the bone matrix composition. Similar to TSP-1, TSP-2 deficiency also results in reduced LOX function and cross-linked collagen, thus altering bone matrix quality (101).

Osteoblast differentiation and bone deposition. TSP-2's effects on osteoblast proliferation and differentiation are highly context dependent and have been widely studied in TSP-2-null mouse models. These mice have a unique phenotype, with connective tissue abnormalities, increased endocortical bone thickness and density in long bones, and reduced collagen fibrillogenesis in bone matrix (70, 79). The increased bone deposition is consistent with a larger number of endosteal osteoblasts, derived from an enlarged pool of marrow-derived osteoprogenitors (42). This is due to the enhanced proliferation of MSCs in TSP-2-null mice, since TSP-2 acts as an autocrine inhibitor of bone marrow stromal cell proliferation (43). Parallel to the high proliferation rate, TSP-2-null MSCs in culture showed delayed onset of terminal osteoblastic differentiation and mineralization, with delay in the expression of osteoblast-associated genes, such as osteocalcin and type I collagen. Exogenous TSP-2 restored early mineralization (43), suggesting that TSP-2 positively regulates osteoblast differentiation and bone deposition. Alford and colleagues (3) further demonstrated that osteoblasts, differentiated from TSP-2-null MSCs, deposited less type I collagen. The interaction between type I collagen and integrins on the surface of osteoprogenitors is required for terminal osteoblast differentiation, and the formation of collagenous ECM is essential for mineralization. Thus, TSP-2 may stimulate osteogenesis and bone matrix mineralization by promoting the formation of a collagen-rich matrix (3). This is consistent with the finding that knockdown of TSP-2 in MC3T3-E1 preosteoblasts reduced mineralization and altered the organization of the collagenous extracellular matrix (4). The positive regulation of osteoblast differentiation by TSP-2 during fracture healing or other perturbations of bone homeostasis has been extensively studied by Hankenson's group (46). In TSP-2-null mice, the fracture callus showed increased vascularity and bone formation, but less cartilage than in wild-type mice, due to a shift in differentiation fate toward osteoblasts rather than chondrocytes in the absence of TSP-2 (60, 114), hence leading to accelerated fracture healing (85).

TSP-2 therefore plays a contextual time-dependent role in osteogenesis, because it acts in an autocrine manner to limit expansion of the progenitor pool, while promoting osteoblast differentiation and bone deposition.

Osteoclastogenesis and bone resorption. In contrast to the widely described role of TSP-2 in osteoblastogenesis, its involvement in osteoclastogenesis has been studied less.

The increased bone deposition in TSP-2-null mice (70) might suggest that TSP-2 controls bone resorption. However, Hankenson and colleagues (42) demonstrated that the increased bone

deposition in these mice was the result of a preferential increase in endosteal bone deposition rather than reduced osteoclast activity, as these mice were capable of bone resorption. Although physiological osteoclastogenesis and bone resorption were equal in TSP-2-null and wild-type mice, an increased osteoclast activity was observed in conditions of bone homeostasis perturbations. In a model of bone loss secondary to estrogen depletion in ovariectomized TSP-2-null mice, the absence of TSP-2 had a protective effect that resulted from more osteogenesis as well as less bone resorption (45). This is consistent with the lower osteoclast number and reduced osteolytic bone metastasis upon TSP-2 knockdown in a lung cancer model (121), confirming the potential role of TSP-2 as a positive regulator of osteoclastogenesis. In agreement with this, TSP-2 was expressed more frequently than TSP-1 in human samples of osteoclastoma, characterized by excessive osteoclastogenesis (18).

In vitro, TSP-2 promoted osteoclast differentiation of RAW 264.7 by enhancing RANKL signaling, via inhibition of miR-486–3p expression and activation of the transcription factor nuclear factor of activated T cells 1 (NFATc1) (121), involved in the control of osteoclast formation, maturation, and activity. TSP-2 also altered the RANKL:OPG ratio in osteoblasts, increasing RANKL expression compared with OPG, thus adding a further mechanism of osteoclast stimulation (121).

Bone Metastasis

There are few studies of the specific involvement of TSP-2 in bone metastasis development, generally reporting different effects depending on the tumor type, and in most cases unrelated to activity in bone remodeling.

In a proteomic analysis of a human breast cancer, TSP-2 was among the proteins downregulated in bone metastasis, compared with the primary tumor (32), suggesting its role in contrasting skeletal metastasis. However, TSP-2 promoted bone metastasis from a human prostate cancer model (22). In this case, TSP-2 expression was higher in prostate-bone metastasis than in the primary tumor and was positively correlated to activation of the proinvasive matrix metalloproteinase MMP-2. TSP-2 enhanced the migration and invasion of prostate cancer cells in vitro by inducing the activation of MMP-2, which was mediated by downregulation of miR-376c expression upon TSP-2 binding to its receptors CD36 and integrin $\alpha_v\beta_3$. The involvement of TSP-2/MMP-2 axis in bone metastasis development was further confirmed in vivo, where TSP-2 knockdown led to significant reduction of metastatic tumor growth and osteolytic area (22). Interestingly, the MMP-2-activating effect of TSP-2 observed in prostate cancer bone metastasis is opposite to the described role of TSP-2 in fibroblasts, where it reduces MMP-2 levels by promoting its clearance (124), further demonstrating the complex and highly contextual roles of TSP-2.

Singh and colleagues (105) recently reported that growth of breast cancer bone metastasis was boosted in aged mice compared with in young mice, with downregulation in the bone secretome of a mixture of factors, defined as quiescence-promoting molecules, which included TSP-2. These factors were highly expressed in the bone marrow of young mice, mainly by pericytes, and were further upregulated by radiation or chemotherapy. Although stimulation of quiescence by TSP-2 was not directly demonstrated, this finding does suggest a role of TSP-2 in promoting tumor cell quiescence in the bone and indicates that pericyte-derived TSP-2 might act similarly to endothelial cell-derived TSP-1 in promoting dormancy of tumor cells in the bone niche: this possibility calls for further investigation.

TSP-3, TSP-4, AND COMP

Expression in Bone

Group B, pentameric thrombospondins are detected in bones and cartilage in embryos and adults, with different expression patterns [reviewed in (2, 46)]. The expression and role of COMP have been widely studied, mainly in the cartilage, whereas TSP-3 and TSP-4 are less characterized.

TSP-3 expression has been detected in the developing skeleton and in adult bone, particularly in the early proliferative zone of the growth plate of long bones, where it is expressed by chondrocytes (54). TSP-4 is mainly expressed in the articular cartilage but has also been detected in bone, restricted to the osteoblast lineage (61, 116). TSP-4 is specifically expressed by articular chondrocytes, whereas, in contrast to other TSPs, it is not detected in the growth plate (61).

TSP-5, also known as the cartilage oligomeric matrix protein (COMP), was initially considered cartilage specific but was subsequently found in other tissues, such as ligaments, tendons, synovium, and bone, where it is expressed by osteoblasts from the initial stages of osteogenesis (30).

Bone Remodeling

Evidence of the role of TSP-3 and COMP, compared with TSP-1, in bone remodeling and development, comes from studies by Posey and colleagues (95) who analyzed the phenotype of single and combinatorial TSP knockout mice. Deficiency in any one of these TSPs led to modest alterations in murine skeletal development, specifically associated with decreased organization of chondrocytes in the bone growth plate, although the lack of TSP-1 slightly impaired chondrocyte arrangement. However, none of the TSP knockout mice showed any significant impairment of the final development of long bones, despite the abnormal growth plate organization. Given the significant structural homology and the similar expression patterns of TSP-3 and COMP, the two proteins might play compensatory roles. However, this possibility was ruled out by the finding that loss of both TSP-3 and COMP did not affect chondrocyte organization more than the loss of individual proteins, despite a mild decrease in long bone length in double-null mice (95).

The TSP-3-null phenotype observed by Posey et al. confirmed the results reported by Hankenson's group (44), studying the skeletal phenotype of TSP-3-null mice. Compared with the wild-type, TSP-3-null mice had normal prenatal skeletal patterns but altered postnatal bone development, although most of the abnormalities were transient and not detected in adult, 15-wk-old mice. TSP-3-null mice had different geometric and biomechanical properties, with accelerated endochondral ossification of the femoral head, as trabecular bone proximal to the growth plate developed earlier than in wild-type mice. These findings provided evidence for a role of TSP-3 in regulating bone development, especially at the level of endochondral ossification during postnatal skeleton maturation, although the mechanism is currently unknown (44).

By interacting with several other ECM proteins, such as collagens, proteoglycans, fibronectin, and cell surface receptors, including integrins and CD47 (21, 111), COMP is essential for maintaining cartilage matrix organization and homeostasis and in stimulating chondrocyte proliferation [reviewed in (1)]. Although COMP-deficient mice had apparently normal skeletal development (110), growth plate organization in long bones was impaired (95). Autosomal dominant mutations in the COMP gene do in fact cause massive retention of the misfolded protein in the endoplasmic reticulum of chondrocytes, where COMP interacts with other ECM proteins, such as collagens, leading to cell stress and death of chondrocytes in the growth plate (50, 84). This affects cartilage development and causes skeletal dysplasias, as multiple epiphyseal dysplasia and pseudoachondroplasia (PSACH) (16, 49, 94).

Although the role of COMP in the skeleton is mostly limited to chondrocytes, there is some evidence of its involvement in bone remodeling, particularly in osteoblast differentiation. COMP has multiple binding sites for TGF-β superfamily members, including TGF-β1 (47) and bone morphogenetic proteins (BMP-2, BMP-4, and BMP-7) (58), which play a role in bone development by inducing bone formation (1). The COMP/BMP-2 interaction enhanced BMP-2-induced osteogenesis of the CDC12 murine precursor cell line as well as primary human mesenchymal stem cells in vitro, findings that were further confirmed by improved osteogenesis in vivo.

COMP's involvement in osteoblast differentiation has been confirmed in MT-COMP mice, bearing the most common mutation of COMP and recapitulating the PSACH phenotype, which display altered bone structure and properties, correlated with dysregulation of the adipogenesis/osteogenesis balance (25). The retention of mutated COMP into chondrocytes results in an inflammatory microenvironment, where miR-223 is upregulated and represses the differentiation of bone marrow MSCs toward osteoblasts, while favoring adipogenesis, resulting in shorter long bones with less bone density and cortical thickness (25).

Compared with TSP-3 and COMP, TSP-4 is the least characterized TSP family member in the skeleton. Analysis of TSP-4-null mice revealed that its deficiency did not affect skeletal development, bone structure, or bone density (36), suggesting that it is not a key modulator of bone formation and remodeling. However, TSP-4-deficient mice had a significant, but transient, reduction in articular cartilage thickness in long bones, reflecting a protective role of TSP-4 in articular cartilage integrity (61).

Bone Metastasis

Little is known about the roles of TSP-3, TSP-4, and COMP in bone metastasis.

TSP-3 was overexpressed, and correlated with metastasis and worse overall survival after chemotherapy, in patients with osteosarcoma, a very aggressive tumor with a tendency to invade the surrounding tissue (27). This suggests that it has a role in the tumor bone microenvironment, although to date its involvement in bone metastasis has not been investigated.

TSP-4 has been reported as overexpressed in the stroma of several solid tumors, such as breast, prostate, and gastric cancers (69, 75, 81). There is currently no evidence of its involvement in bone metastasis, with the exception of a proteomic study that found TSP-4 and TSP-2 to be downregulated in breast cancer bone metastasis (32).

COMP was expressed in breast (33), prostate (34), and colon (76) cancers. In patients with breast cancer, high levels of COMP in serum correlated with bone metastasis and reduced survival (92). The specific mechanism by which COMP could promote breast cancer bone metastasis is still not known,

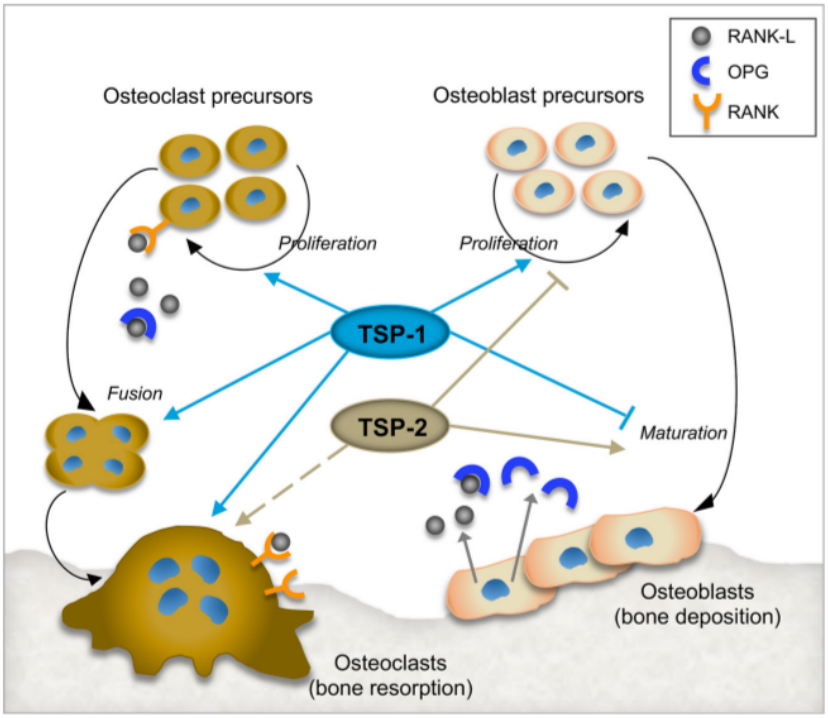

Fig. 1. TSP-1 and TSP-2 in bone remodeling. Osteoclast differentiation and bone resorption are positively regulated by TSP-1 (mainly through binding to CD47 and CD36 and inhibition of NO signaling, as detailed in the text) and TSP-2 (by inhibiting miR-486–3p or increasing the RANKL:OPG ratio in osteoblasts). TSP-2 acts on osteoclasts mainly in condition of bone homeostasis loss (*dashed line*). TSP-1 stimulates osteoblast precursor proliferation, mostly by binding and activating latent TGF-β, while inhibiting osteoblast differentiation and bone-forming activity. TSP-2 acts as an autocrine inhibitor of osteoprogenitor proliferation, while promoting osteoblast maturation and bone deposition. OPG, osteoprotegerin; RANK-L, receptor activator of nuclear factor-κβ ligand; TGF-β, transforming growth factor beta; TSP, thrombospondin.

although its ability to interact with cell receptors (CD47 and αvβ3 integrin) might contribute to the preferential homing and attachment of breast cancer cells to bone tissue (98).

CONCLUSIONS AND FUTURE DEVELOPMENTS

TSP family members, produced by bone-resident cells, especially osteoblasts, endothelial, and immune cells, as well as by metastatic tumor cells, show different patterns of expression (2, 46) in bone remodeling and bone metastasis, reflecting their different and contextual functional properties. The well-characterized TSP-1 and TSP-2 have some divergent effects on bone remodeling (Fig. 1). TSP-1 inhibits osteoblast differentiation, mainly by TGF-β activation, and promotes osteoclastogenesis and bone resorption. By contrast, TSP-2 inhibits the proliferation of osteoblast progenitors, while promoting osteoblast differentiation and bone deposition, and stimulating osteoclastogenesis, mostly when bone homeostasis is perturbated. The divergent and important roles of the two TSPs in bone remodeling are maintained in bone metastasis, where TSP-1 was upregulated in osteolytic bone metastasis (48) and downregulated in patients with osteoblastic metastases (123), whereas TSP-2 was downregulated in osteolytic bone lesions (32).

Table 1 summarizes known actions of TSP family members in bone metastasis, detailing their effects and, when identified, the underlying molecular mechanism and targets. Other activities of TSPs, described to be involved in the regulation of cancer metastasis to other organs, might also be relevant for bone metastasis and deserve further investigations.

Although this review focuses on aspects related to bone remodeling, TSP family members, besides their activity in bone cells, have the potential to affect metastasis by acting on tumor cells and other components of the tumor microenvironment as well. TSPs can directly regulate tumor cell functions related to the metastatic process, including cell adhesion, motility, invasion, and dormancy/proliferation. Examples of this are the ability of TSP-1 to induce tumor cell dormancy in the bone marrow perivascular niche (40) and that of TSP-2 to promote activation of the proinvasive protease MMP2 (22). As mentioned in the section "TSP-2", the opposite effect of TSP-2 on MMP-2—activation in bone metastatic prostate cancer cells and inhibition in fibroblasts—reflects the ability of TSPs to exert different roles depending on the cell type and the nature of the specific biological setting.

TSPs also affect other components of the metastatic niche, including blood vessels, ECM composition and organization, and immunity.

The tumor microenvironment is a highly integrated system in which different components are spatially and functionally interconnected and influence each other. For instance, RANKL, a major regulator of bone remodeling, also acts on endothelial cell proliferation and migration (65) and the angiogenic factor VEGF, which is negatively regulated by TSP-1, induces RANK expression in endothelial cells, further increasing the proangiogenic activity of RANKL (86). Furthermore, FGF signaling, inhibited by TSP-1 (80) and TSP-2 (100), is involved in not only angiogenesis but also osteoblastogenesis (108). In this context, the effects of the potent antiangiogenic ability of TSP-1 and TSP-2 or the reported proangiogenic activity of TSP-4 (87) in the interplay between angiogenesis and bone resorption during bone metastasis warrant further investigation. Moreover, given the links between vascular endothelial cells, angiogenic factors, and the immune system (29, 38), TSP activity in angiogenesis might also indirectly affect the tumor immune response.

Cells of the innate and adaptive immune response, particularly bone marrow macrophages and T-cells, are other major determinants of bone metastasis, not only for their activity in the immune response to tumors but also for their involvement in maintaining the HSC niche (83, 104), and osteoclastogenesis (113, 120, 122). Little is known about the role of TSPs in the immune compartment of bone metastasis, but their ability to bind factors that act on immunity, such as TGF-β and CD47—but also VEGF—indicates a potential role in the regulation of immunity in bone metastasis, a topic worthy of further investigation.

Finally, the behavior of cells in the tumor microenvironment is controlled by the composition and mechanical properties of the ECM. The known role of TSPs in the control of matrix deposition and organization (99, 112)—in particular the modulation of collagen fibrillogenesis by TSP-1, TSP-2, and COMP—is therefore another potential mechanism for the regulation of bone metastasis by TSPs. For instance, breast cancer cells have been demonstrated to alter their gene expression in response to

Table 1. *TSPs in bone metastasis*

TSPs	Cancer Type	Expression/Role in Bone Metastasis	Molecular Mechanism	Ref.
TSP-1	Myeloma	Promotes osteolytic bone metastasis	Activation of latent TGF-β	(78)
	Prostate cancer	Downregulated in the serum of patients with osteoblastic metastasis	*	(123)
	Prostate cancer	Promotes bone deposition and metastasis growth	Release of tumor-promoting TSP-1 fragments by KLK4	(37)
	Breast cancer	Upregulated in osteolytic bone metastasis	*	(48)
	Breast cancer	Enhancement of tumor cell dormancy in the perivascular niche	Tumor cell cycle arrest through an IFN-γ/IDO/TSP-1 axis	(77)
TSP-2	Prostate cancer	Promotes bone metastasis	Tumor cell invasion, through activation of MMP-2	(22)
	Breast cancer	Downregulated in bone metastasis	*	(32)
	Breast cancer	Downregulated in the bone of aged mice, prone to bone metastasis	Suggested role in promoting tumor cell quiescence in the bone	(105)
TSP-4	Breast cancer	Downregulated in bone metastasis	*	(32)
COMP	Breast cancer	High levels in serum correlate with bone metastasis	*	(92)

For each TSP, evidences of its expression and/or activity in bone metastasis, the tumor type, and the molecular mechanism involved are reported. COMP, cartilage oligomeric matrix protein; KLK4, kallikrein-related peptidase 4; TGF-β, transforming growth factor beta; TSP, thrombospondin. *Molecular mechanism has not been determined.

an increased stiffness of the bone matrix, with a transition to a bone-destructive phenotype (91, 107).

As already noted, TSPs can bind numerous factors, thanks to their multidomain structure, with different activities depending on which domains, ligands, and cell types are present and functional in a specific environment. In the bone metastatic niche, there is evidence that TSPs can be degraded by specific proteases, including KLK4, that release bioactive fragments (37). In keratinocytes, BMP-1 has been recently found to cleave TSP-1, potentiating TSP1-mediated activation of latent TGF-β (7). Since BMPs are widely expressed in the bone marrow and are involved in bone metastasis, this finding opens interesting perspectives for future studies to investigate proteolytic cleavage as a mechanism of regulation of TSP activity and final outcome in bone metastasis.

Another open question is whether antineoplastic therapies affect the expression of TSPs in the bone microenvironment and—vice versa—whether TSPs play a role in tumor response to therapies. TSP-1 was upregulated after low-dose, metronomic administration of chemotherapeutic drugs (12, 28) or after treatment with the bisphosphonate zoledronic acid (53), indicating a potential role for TSPs in microenvironmental changes induced by antineoplastic therapies in solid tumors as well as in bone metastasis.

In conclusion, TSPs emerge as important players in bone remodeling and possibly also in the microenvironment of bone metastasis, although further investigations are needed to fully understand their roles in skeletal metastasis. Clarifying the involvement of these matricellular proteins and their specific domains could provide insights into the molecular pathways regulating the bone metastatic niche and lay the basis for designing therapeutic approaches targeting the tumor microenvironment in bone metastasis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.C. prepared figures; L.C. and G.T. drafted manuscript; L.C. and G.T. edited and revised manuscript; L.C. and G.T. approved final version of manuscript.

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