



Harnessing the versatile role of OPG in bone oncology: counterbalancing RANKL and TRAIL signaling and beyond

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Abstract

More than 2 decades ago, the discovery of osteoprotegerin (OPG) as inhibitor of the receptor of activator of nuclear factor K β (RANK) ligand (RANKL) revolutionized our understanding of bone biology and oncology. Besides acting as decoy receptor for RANKL, OPG acts as decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). OPG, RANKL, and TRAIL are ubiquitously expressed, stimulating per se pivotal signaling cascades implicated in cancer. In the context of cancer cell–bone cell interactions, cancer cells skew the OPG/RANKL/RANK (RANKL cognate receptor) balance towards bone destruction and tumor growth through favoring the RANKL/RANK interface, circumventing OPG. Numerous preclinical and clinical studies demonstrate the dual role of OPG in cancer: antitumor and tumor-promoting. OPG potentially conveys an antitumor signal through inhibiting the tumor-promoting RANKL signaling—both the osteoclast-dependent and the osteoclast-independent—and the tumor-promoting TRAIL signaling. On the other hand, the presumed tumor-promoting functions of OPG are: (i) abrogation of TRAIL-induced apoptosis of cancer cells; (ii) abrogation of RANKL-induced antitumor immunity; and (iii) stimulation of oncogenic and prometastatic signaling cascades downstream of the interaction of OPG with diverse proteins. The present review dissects the role of OPG in bone oncology. It presents the available preclinical and clinical data sustaining the dual role of OPG in cancer and focuses on the imbalanced RANKL/RANK/OPG interplay in the landmark “vicious cycle” of skeletal metastatic disease, osteosarcoma, and multiple myeloma. Finally, current challenges and future perspectives in exploiting OPG signaling in bone oncology therapeutics are discussed.

Keywords OPG · RANKL · TRAIL · Metastasis · Bone · Osteosarcoma · Multiple myeloma

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Introduction

In the context of the nomination of the inflammatory milieu as an “enabling characteristic” fostering the acquisition of “hallmarks of cancer” [1], cytokines—a fundamental feature of tumor microenvironment—have been conceived as orchestrators of cancer [2]. Osteoprotegerin (OPG) is a member of tumor necrosis factor (TNF) receptor superfamily, produced mainly by osteoblasts, assigned to ensure bone homeostasis. It acts as a decoy receptor for receptor activator of nuclear factor κ B ligand (RANKL), disrupting the RANKL-induced bone resorption [3–7], and for TNF-related apoptosis inducing ligand (TRAIL)—the protagonist of tumor immune surveillance [8]. Implicit in cancer biology, OPG binds myriad proteins beyond RANKL and TRAIL, transducing diverse signals [9]. Consequently, the discovery of OPG and RANKL in 1997 [4–7] revolutionized the notion, conceived in the mid 1970’s, that osteoclasts

are implicated in bone oncology and are modulated by an authentic bone cell—the osteoblast [10–13].

An in-depth understanding of the biology of bone oncology is a prerequisite for the refinement of current therapeutic strategies. Of paramount importance is the mitigation of skeletal related events (SREs)—bone pain, hypercalcemia, pathologic fractures, and neurologic complications—encountered in both primary and metastatic bone tumors, subverting the survival and the quality of life of patients [14]. The treatment of primary bone tumors, mainly of osteosarcoma and of giant cell tumor of bone, poses many challenges [15, 16]. Moreover, the compendium of osteotropic primary tumors has been enriched since the first report of bone metastases related with breast cancer [17], designating skeleton as the most common metastatic site. A high proportion of breast and prostate cancer patients—70%—harbor bone metastases, while thyroid, kidney, and bronchus carcinomas colonize the bone with an incidence hovering at 30–40% [14]. The present review provides a comprehensive overview on the role of OPG in bone oncology, which extends beyond the inhibition of RANKL and TRAIL, involving several signaling cascades. It focuses on the interplay among OPG, RANKL, and TRAIL that governs the initiation and progression of cancer, being implicated in both primary and metastatic bone disease. The rationale for exploration of the therapeutic implementation of OPG is discussed with emphasis on current and future perspectives.

OPG: a decoy receptor for RANKL and TRAIL

OPG owes its discovery to its function as a decoy receptor for RANKL, inhibiting RANKL—induced bone resorption, a property reflected in its name derived from the latin words *os* (bone) and *protegere* (to protect). The TNFRSF11B gene encodes the OPG protein comprising 401 amino acids (aa), 21 aa of which constitute a signal peptide. Cleavage of the signal peptide generates a 380 aa soluble glycoprotein of 55 kDa. The latter is homodimerized to form the dimeric OPG of 110 kDa [18]. OPG is predominantly expressed by bone marrow stromal cells, detected also in hematopoietic, immune, and vascular system [9, 19]. It exerts a multitude of functions, binding to myriad partners, such as syndecan-1, glycosaminoglycans (GAGs), proteoglycans, and Factor VIII von Willebrand Factor complex. Beyond being the cornerstone of bone biology, OPG is also implicated in cancer, metabolism, cardiovascular system, and rheumatic diseases [9, 19].

RANKL is the official nomenclature for a 316 aa protein, member of TNF family, encoded by TNFSF11 gene, initially nominated as OPG ligand (OPGL) [4, 5]. RANKL is indispensable for differentiation of osteoclasts in the presence of macrophage colony stimulating factor (M-CSF) and

orchestrates the osteoclasts-mediated bone resorption. Interestingly, research in the field of immunology had unraveled RANKL, prior to its discovery via OPG. RANKL is expressed on T cells and interacts with its receptor, RANK, on mature dendritic cells (DCs) in order to regulate the survival of the latter [20, 21]. Several isoforms of RANKL have been recognized [22]. The expression of RANKL in a wide spectrum of tissues, including lymph nodes, thymus, mammary gland, lung, spleen and bone marrow, highlights its pleiotropic effect [23]. RANKL exerts a pivotal role in immune system through mechanisms highly divergent [24]. RANKL signaling curtails the antitumor immunity, stimulating the mTEC-mediated self-tolerance towards cancer specific antigens and the proliferation of T regulatory cells (T regs). On the other hand, RANKL enhances the antitumor immunity through stimulating memory cytotoxic T-lymphocyte responses toward tumor-associated antigens and promoting the lymph nodes genesis, the antigen presentation, the survival of DCs and monocytes/macrophages [24].

RANKL exerts its functions mainly via its cognate receptor RANK, a type I homotrimeric transmembrane protein encoded by TNFSF11A gene, initially identified on osteoclast precursors (OCPs), mature osteoclasts, and DCs [6, 19–21]. Crucial adaptor molecules downstream of the interaction RANKL/RANK, such as TNF receptor associated factor 6 (TRAF6), stimulate seven signaling cascades, activating NFkB, cjun/activator protein 1 (AP-1), c-Myc, calcineurin/nuclear factor of activated T cells [NFAT]c1 (NFATc1), Src, mitogen-activated protein kinase (MAPK)/p38, and extracellular signal-regulated kinase (ERK) [19, 23]. Consequently, a plethora of signals additionally to differentiation and activation of osteoclasts are transduced.

OPG acts also as a decoy receptor for TRAIL, a type II transmembrane protein, identified as a member of TNF family, nominated initially as APO2L. Produced by T cells and natural killers (NK) cells, TRAIL is a crucial player of tumor immune surveillance, stimulating the extrinsic pathway of apoptosis in cancer cells expressing TRAILR1 (DR4) and TRAILR2 (DR5), leaving normal cells unaffected [25–28]. Interestingly, a TRAIL-elicited inhibition of cancer cell migration has been reported, mediated through MAPK Activating Death Domain protein (MADD) and chemokine receptor 7 (CXCR7) in breast cancer cells [29].

Lessons from clinical studies concerning the role of OPG in cancer

A potential tumor-promoting and prometastatic role of OPG was reported as early as in 2006 in a rich repertoire of tumors. This role was based on *in vitro* (breast, prostate, gastric cancer) and *in vivo* (breast, prostate, gastric, bladder cancer) data, as well as on clinical data concerning serum

OPG concentrations (breast, prostate, bladder cancer) and OPG expression on malignant tissue (breast, prostate, gastric cancer) [30]. Likewise, mRNA expression of OPG in human colorectal cancer tissues has been significantly correlated with aggressive tumor behavior [31].

In a recent clinical study evaluating serum OPG levels in melanoma patients, the serum OPG levels proved to be statistically significantly higher in the melanoma group (median = 355.2 pg/ml) compared to the healthy group (median = 234.1 pg/ml) ($p < 0.0001$). The researchers stratified the melanoma patients into four groups according to Breslow score, which describes the melanoma thickness (depth) assessed by a pathologist. Comparing OPG levels in the healthy group with OPG levels at each Breslow score stage, no statistically significant difference in the levels of OPG at first Breslow score (median = 287.6 pg/ml) compared to healthy group (median = 234.1 pg/ml) ($p = 0.0239$ pg/ml) was observed. Statistically significant elevation of OPG levels in comparison to healthy group was demonstrated starting from the second Breslow score stage as follows: (i) Breslow score 1–2 mm: OPG median = 323.9 pg/ml, $p < 0.0001$; (ii) Breslow score 2–4 mm: OPG median = 398.0 pg/ml, $p = 0.0004$; (iii) Breslow score > 4 mm: median = 500.7 pg/ml, $p < 0.0001$. Comparing the OPG levels in group of positive and negative sentinel node metastases, no statistically significant change was observed. Considering that the Breslow score stage is a good indicator of the risk of metastasis reflecting spread beyond the skin, it is reasonable to hypothesize that OPG levels could have a clinical utility for melanoma patients, but this remains to be proved. In this study there was no significant correlation of OPG with sentinel lymph node positivity; therefore, OPG was not anticipated to correlate significantly with disease stage according to American Joint Committee on Cancer (AJCC)/tumor size, lymph nodes affected, metastases (TNM) staging system. Most importantly, in this study there was no analysis of direct correlation of OPG levels with overall survival and response to therapy. Consequently, although OPG was suggested as a promising biomarker for early stages of melanoma, it could not be designated either as a prognostic or a predictive biomarker; this perspective warrants further investigation [32].

Notably, an antitumor role of OPG is indicated by other clinical studies [33, 34]. According to Tromsø study, which recruited 6279 subjects from general population without prior cancer, OPG serum levels correlated with reduced risk of incident cancer at all sites and breast cancer [33]. Moreover, Parker et al. demonstrated a positive relation of tissue OPG expression with favorable clinicopathological characteristics of breast cancer, in particular small tumor size, absence of lymph node metastases, and low Ki-67 [34].

To interpret the discrepant clinical evidence, it is mandatory to dissect the direct and indirect role of OPG in cancer.

The direct role of OPG in cancer is considered tumor-promoting—especially protumorigenic, prometastatic, and proangiogenic. The indirect role of OPG is defined by the distinct RANKL and TRAIL signaling that abrogates, given that OPG acts as decoy receptor for RANKL and TRAIL, which exert per se both tumor-promoting and antitumor effects. In other terms, OPG plays an indirect antitumor role through inhibition of tumor-promoting RANKL and TRAIL signaling, while it plays an indirect tumor-promoting role when abrogating the antitumor RANKL and TRAIL signaling.

The protumorigenic/prometastatic effect of OPG

A plethora of experimental and preclinical data provide the mechanistic underpinnings of the protumorigenic role of OPG. The landmark study of Goswami et al. shed more light into this role. In this study, administration of human recombinant OPG was shown to drive normal primary human mammary epithelial cells (HMEC) spheres towards a malignant phenotype. The OPG-mediated activation of various kinases, such as Aurora-A, Bub1, and BubR1, was demonstrated to induce aneuploidy in HMEC spheres. The reported pro-survival and pro-proliferative effect of OPG on HMEC spheres was ascribed to activation of AKT, glycogen synthase kinase 3b (GSK3b), p44/42, p65, and b-catenin. Moreover, the OPG-mediated upregulation of cluster of differentiation (CD) 24 (CD24) in HMEC spheres was incriminated in the OPG-induced enhancement of cancer cell proliferation, invasion, and migration [35]. This study demonstrated for the first time the immunohistochemical OPG expression in the invasive and the inflammatory breast cancer [35]. Recently, it was demonstrated that addition of human recombinant OPG (500 pg/ml) in sphere cultures of breast cancer cells SUM149PT, SUM1315MO2, inflammatory breast cancer tissue from patients, and primary human mammary epithelial cells (HMEC) spheres resulted in amplification of the DNA copy numbers of well-established oncogenes—serine-threonine kinase (AKT1), Aurora-A kinase (AURK1), epidermal growth factor receptor (EGFR), Myc, Serine/threonine-protein kinase (PAK1), cyclin dependent kinase 4 (CDK4), erb-b2 receptor tyrosine kinase 2 (ERBB2)—while downregulated tumor suppressive genes—cyclin-dependent kinase inhibitor 2A (CDKN2A), phosphatase and tensin homolog (PTEN), DNA topoisomerase II alpha (TOP2A), and retinoblastoma (RB) 1 (RB1) [36]. The protumorigenic and prometastatic role of OPG has been currently revolutionized by a mass spectrometry analysis in inflammatory and aggressive breast cancer cells, unveiling several proteins-partners of OPG which orchestrate the initiation and progression of cancer [36]. Moreover

Weichhaus et al. observed reduced expression of proteases cathepsin D and Matrix Metalloproteinase-2 following OPG knockdown in a chick embryo model, suggesting that the prometastatic effect of OPG may be ascribed to regulation of protease expression and invasion [37]. From a different viewpoint, Heymann et al. speculated that OPG expressed on endothelial and immune cells at metastatic lymph nodes of human medullary thyroid carcinoma, detected by immunohistochemistry, acts as a chemoattractant for cancer cells, guiding the migration thereof [38]. Holen et al. underscored the anti-apoptotic role of OPG, demonstrating that OPG expression in breast cancer cells MDA-MB 436/231 inhibits TRAIL-mediated apoptosis, providing cancer cells with a survival advantage [39]. Benslimane-Ahmim et al. demonstrated that the OPG-induced expression of stromal cell-derived factor (SDF) 1 (SDF-1) by endothelial colony forming cells (ECFCs) guides the migration of MNNG-HOS human osteosarcoma cells and DU145 prostate cancer cells [40].

Recent studies designate OPG as a pivotal player of the inflammation-induced breast cancer progression *in vitro* and *ex vivo*. The interplay of OPG with fatty acid synthase (FASN)—a multifaceted enzyme critical for lipid biosynthesis—and with cyclooxygenase-2 (COX2)—a critical player in inflammatory component of tumor microenvironment—has been implicated in the inflammatory cascades in highly invasive inflammatory breast cancer, inducing survival kinase signaling, essential for breast cancer progression [41]. *In vitro* and *ex vivo* data reported by Chung et al. indicated OPG as mediator of the prometastatic signaling of interleukin (IL) 1 β (IL-1 β) in breast cancer. In fact, an autocrine loop was revealed in which OPG secretion was controlled by IL-1 β via p38- and p42/44-mediated pathways and induced *per se* both the IL-1 β expression and the IL-1 β —mediated breast cancer cell invasion and matrix metalloproteinase (MMP) 3 upregulation [42].

To the best of our knowledge, so far, there are no straightforward analyses with statistically significant results addressing the protumorigenic/prometastatic effects of OPG described in model systems in the settings of human cancer. Moreover, it is acknowledged that the expression of molecules does not equate to function. More studies to resolve these issues are needed.

The proangiogenic effect of OPG

Since the first report of the role of OPG in the survival of endothelial cells and the formation of cord-like structures *in vitro* in humans [43], immense research has sustained the proangiogenic OPG signaling. The data of proteomics are consistent, showing that OPG pretreatment modifies human endothelial colony-forming cells (ECFCs) protein

expression via down-regulating 23 spots and upregulating 6 spots, reflecting proteins regulating cell motility, adhesion, signal transduction, and apoptosis [44].

The proangiogenic role of OPG appears to be exerted through numerous sophisticated mechanisms, involving the kinases ERK and protein kinase B (AKT), as well as inhibition of RANKL, which inhibits angiogenesis, according to the study of Mc Conigle et al. in the rat aortic ring model of angiogenesis [45]. Moreover, OPG has been shown to elicit cytoskeletal reorganization and to activate FAK, Src, and ERK signaling in human dermal microvascular endothelial cells (HuDMECs) *in vitro* [46]. The *in vitro* binding of OPG with thrombospondin (TSP-1) in Weibel–Palade bodies (WPB) of human vascular endothelial cells has pointed to the OPG-mediated inhibition of the antiangiogenic function of TSP-1 [47].

A signaling pathway initiated by the $\alpha(v)\beta3$ integrin has been shown *in vitro* and *in vivo* to activate NF κ B, leading to upregulation of OPG, which in turn acts as a survival factor for endothelial cells [48]. The recruitment of endothelial cells by cancer cells has been demonstrated *in vitro* to orchestrate the $\alpha v\beta3$ -mediated increase of OPG in HuDMECs [49]. In bone, osteopontin has been demonstrated *in vitro* to induce the $\alpha v\beta3$ integrin-mediated OPG expression, providing a survival advantage to rat aortic endothelial cells during bone resorption [50]. Moreover, a proangiogenic effect of OPG exerted by fibroblast growth factor (FGF)-2 has also been observed *in vivo* in human ECFCs [51].

To the best of our knowledge, so far, there are no straightforward analyses with statistically significant results addressing the proangiogenic effects of OPG described in model systems in the settings of human cancer. Moreover, it is acknowledged that expression of molecules does not equate to function. More studies to resolve these issues are needed.

The RANKL-induced signaling transduction in cancer

The RANKL-induced signaling plays a key role in fundamental pathways of “osteimmunology” (the integration of immunity into bone biology), osteoclastogenesis, carcinogenesis, and cancer progression, which cross-talk to define the fate of cancer cells [52]. Expressed both by cancer cells *per se* and by tumor microenvironment, RANKL appears to exert a tumor-promoting—protumorigenic and prometastatic—role in a wide array of malignancies, including breast, prostate, colorectal, lung, bladder, and gastric cancer [reviewed in 52].

The most convincing evidence supporting the protumorigenic role of RANKL is derived from the investigation of the progesterin-driven mammary carcinogenesis in a mouse model, demonstrating that both the genetic [53] and the

pharmacological [54] interception of RANKL halted the carcinogenic effect of the synergy of medroxyprogesterone acetate with 7, 12 dimethylbenzanthracene.

The signaling pathways activated downstream of RANKL/RANK interaction are implicated in epithelial mesenchymal transition (EMT), neo-angiogenesis, cancer cell migration, and invasion [52, 55–59]. RANKL expressed on T regs infiltrating breast cancer has been involved in generation of pulmonary metastasis [60]. Furthermore, RANKL has been considered to orchestrate the migration of cancer cells, acting as either a chemoattractant or a “soil” factor [61].

The leucine rich repeat containing G protein-coupled receptor 4 (LGR4) is a recently recognized receptor interacting with RANKL, inhibiting osteoclast differentiation and bone resorption. Recent data implicate the RANKL/LGR4 interface in cancer cells proliferation [62].

However, the prognostic value of RANKL expression in cancer is further complicated by the increasingly reported antitumor aspect of RANKL signaling [34, 63–65]. For instance, Park et al. designated immunohistochemical RANKL expression in breast cancer tissues as independent predictor of improved skeletal-disease free survival in breast cancer patients [34]. Bhatia et al. observed that absence of RANKL immunohistochemical expression on breast cancer tissues correlated with a metastatic breast cancer phenotype. The authors postulated that decrease or absence of RANKL expression on cancer cells may allow the interaction of RANK expressed on cancer cells with RANKL expressed on normal osteoblasts and stromal cells of the bone, guiding osteotropism, thereby orchestrating a metastatic phenotype [63]. Further, decreased RANKL transcript levels in breast cancer samples in comparison with normal tissue has been associated with increased risk of local recurrence, bone metastases or disease-related death in breast cancer [64]. Interestingly, an antitumor RANK-c isoform inhibiting RANK signaling has been recently discovered by Papanastasiou et al. in breast cancer. This isoform was upregulated in breast cancer cells lines and was inversely associated with tumor grade and proliferation index [65].

The TRAIL-induced signaling transduction in cancer

Despite the established role of TRAIL in initiation of apoptosis of cancer cells [25–28], the latter may acquire resistance to TRAIL-induced apoptotic signaling through a plethora of mechanisms, involving the non-signaling TRAIL receptors TRAILR3, TRAILR4, and OPG [27]. Non-canonical TRAIL-induced cascades, downstream of TRAIL/TRAILR interaction, have been demonstrated to involve pivotal kinases, such as JNK, p38, ERK, protein

kinase C (PKC), and PI3K/AKT, resulting in a dual signal transduction: tumor-promoting and antitumor. Cancer cells that escape from TRAIL-induced tumor immune surveillance have been postulated to highjack, through mechanisms as yet unidentified, the aforementioned pathways and divert them towards pro-survival, pro-proliferative, and promigratory signal transduction [25–28].

The role of TRAIL in the context of bone oncology is underexplored and rather dual: both antitumor and tumor-promoting. TRAIL has been shown in vivo in an animal model to reduce tumor incidence and growth and prevent tumor-related osteolysis, increasing significantly animals’ survival [66]. This effect consolidated the in vitro antitumor role of TRAIL observed previously in Ewing’s sarcoma and osteosarcoma cell lines. On the other hand, TRAILR1 has been suggested as a novel circulating tumor cells (CTC) marker for giant cell tumor of bone (GCT). The subpopulation of circulating tumor stem-like cells (CTSC) that expressed TRAILR1 showed increased potential for tumor formation in vitro and in vivo, increased resistance to denosumab (monoclonal antibody against RANKL), more frequent detection in circulation, and increased induction of tumorigenesis after serial adoptive transplantation in comparison with GCT cells non expressing TRAILR1 [67].

Given the complex role of TRAIL in cancer, the OPG-induced inhibition of TRAIL signaling extends beyond being a cancer cell strategy against TRAIL-mediated apoptosis [68]. Moreover, it is conceivable that the clinical outcome of the interplay OPG/RANKL/TRAIL is defined by a dynamic equilibrium among RANKL, TRAIL, and OPG [68].

Could the OPG/RANKL/TRAIL interplay fertilize the bone to be a “soil” for metastasis?

Basic principles of bone homing and colonization

Intense research over the last decades has revealed the steps of the metastatic process: metastatic “seeding”, generation of pre-metastatic niches, dormancy and reactivation of disseminated tumor cells (DTC). The creation of an immunosuppressive landscape is a prerequisite for the entire process [69]. Despite the great body of theories that have been postulated to interpret the development of metastases, an outstanding question is why some cancer types predilect to metastasize to bone. One of the most challenging issues as regards bone homing and colonization is to decipher the interaction of bone microenvironment with the genetic profile of cancer cells that is specific for bone tropism.

Though not completely elucidated, the generation of bone metastases has been conceived as a stepwise process. Epithelial–mesenchymal transition (EMT) endows cancer

cells with an invasive phenotype that enables detachment from primary tumor, intravasation, survival in the blood stream evading immune surveillance and sheer stress of the blood flow, extravasation, homing to bone, and colonization of bone. Some of the bone colonizing cells adapt to bone microenvironment and grow immediately competing with hematopoietic stem cells (HSC) for the endosteal niche, while others enter a dormancy state. Reactivation of DTC gives rise to bone metastases [70].

Refining the prevailing “seed and soil” hypothesis of Paget, Sleeman et al. provided a comprehensive insight into the metastatic process, suggesting the “stromal progression hypothesis”. The authors postulated that the interplay between the “genetic signature” of cancer cells and the inflammatory tumor stroma, governs not only the early stages of carcinogenesis in primary sites but also the metastatic process resulting in creation of metastatic niches [71]. In that respect, the process of bone homing and colonization is determined by the cross-talk between DTC and bone microenvironment. This cross-talk is facilitated by the unique characteristics of bone microenvironment, principally: (i) the high vascularity of red marrow; (ii) the direct cancer cell–mesenchymal stromal cells interactions; (iii) the secretion of growth factors, angiogenic factors, and bone-resorbing factors from a multitude of cells in bone marrow (BM) [72].

Fundamental cells of BM are considered orchestrators of bone homing and colonization: (i) the bone mesenchymal stem cells (BMSCs), which constitute multipotent cells capable of differentiating into osteoblasts, chondrocytes, and adipocytes; (ii) the pericytes, (iii) the fibroblasts; and (iv) the osteoblasts.

Recently, bone marrow adipocytes (BMAs) have been brought into spotlight as crucial determinants of the predilection sites of bone metastasis, being abundant near the endosteal surface of diaphysis and the trabecular bone of epiphysis and metaphysis [73]. In 2015, the innovative study of Scheller et al. suggested an explanation for the proclivity of bone metastases for regions such as proximal femur, hip, and lumbar spine. The authors demonstrated *in vivo* that the “regulated bone marrow adipocytes” (rBMAs), which prevail in these sites, provide cancer cells with a survival advantage in challenging conditions, contrary to the stable “constitutive bone marrow adipocytes” [74]. BMA (especially the rBMA) play a tumor-promoting role through supplying free fat acids as a fuel for cancer cells, increasing leptin/adiponectin ratio, upregulating IL-6, and inducing the differentiation and activation of osteoclasts [73]. *In vitro* experiments have demonstrated that the parathyroid hormone related protein (PTHrP)-induced expression of thermogenic genes, such as uncoupling protein 1 (UCP1) and iodothyronine deiodinase 2 (Dio2), stimulates lipolysis in mature adipocytes, thereby providing cancer cells with

energy [75]. This effect may contribute to the prometastatic effect of PTHrP for bone, but not visceral, metastases that has been demonstrated *in vivo* [76].

The molecular mechanisms underlying bone metastases constitute an evolving field of research, as extensively discussed elsewhere [77–81]. The present review highlights the evidence that sustains the implication of the RANKL/RANK/OPG axis in bone metastases. RANKL/RANK/OPG axis is expressed on cancer cells and in components of tumor as well as bone microenvironment.

The RANKL/RANK/OPG axis in bone homing and colonization

Bone is a metabolically active organ governed by a continuous remodeling cycle, finely balanced between bone formation and resorption; therefore, it is conceivable that “osteomimicry”, i.e. the acquisition from cancer cells of a bone cell–predominantly an osteoblast-like—phenotype, is mandatory for bone colonization [82].

Although osteomimicry is not completely understood, it clearly entails the presence of an “osteoblast-like gene signature” in osteotropic cancer cells, which encompasses several bone related genes (BRG) encoding proteins—hallmark of osteoblasts [83]. Bone metastatic breast cancer tissues have been shown to highly co-express a constellation of BRG that encode: (i) bone matrix proteins (e.g. asporin [ASPN], cartilage oligomeric matrix protein [COMP], secreted protein acidic and cysteine rich [SPARC]); (ii) extracellular matrix-degrading enzymes (e.g. matrix metalloproteinases MMPs, metalloendopeptidases [ADAMs], urokinase-type plasminogen activator [PLAU/uPA]); (iii) tissue inhibitors of metalloproteinases [TIMPs]; (iv) osteoblast cadherin [OB-cadherin/CDH11]; (v) osteoblast transcription factor (e.g. Runt-related transcription factor 2 [Runx2]); and (vi) cytokines (e.g. Stem Cell Growth Factor 1 [SCGFI]). Runx2 was shown *in vitro* to mediate the cancer-associated fibroblasts (CAF)/BMP2-induced co-expression of BRG in breast cancer cells through direct and indirect regulation of their transcription. The BRG expression was demonstrated *in vitro* to be implicated in chemotaxis, adhesion, anchorage-independent growth and proliferation of breast cancer cells colonizing the bone microenvironment [84]. The Wnt and the RANKL/RANK/OPG pathways are integrated in the osteoblast-like osteotropic profile of bone colonizing cancer cells. The Wnt signaling acts autocrinally to trigger cancer cell proliferation and osteotropism and paracrinally to favor osteoblast differentiation.

The *in vivo* study of Chu et al. revealed that RANKL expressed by prostate cancer cells in the setting of osteomimicry contributed to establishment of a pre-metastatic niche stimulating a “feed forward loop”. Especially, RANK/RANKL signaling induced the reprogramming of cancer cell

and a prometastatic signal transduction through activation of crucial transcription factors, such as c-Myc/Max and AP4. The latter were shown to regulate the expression of genes implicated in EMT (Twist1, Slug, Zeb1, Zeb2), stem cell characteristics (Sox2, Myc, Oct3/4, and Nanog), neuroendocrine differentiation (Sox 9, HIF-1 α and FoxA2), and osteoblast-like phenotype (c-Myc/Max, Sox2, Sox9, HIF1 α and Runx2) [85].

It is known that signaling cascades required for EMT, invasion, motility, adaptation to bone microenvironment, and reactivation of dormant cancer cells involve multitude molecules, such as cell adhesion molecules, proteolytic enzymes (e.g. MMPs, cathepsins, CD26/DPPIV and uPA), chemokine receptors, integrins, and kinases indispensable for pro-proliferative and pro-survival signals (e.g. ERK1/2, p38, FAK/AKT), most of which being regulated by or interacting with RANKL/RANK/OPG axis [69, 86]. For instance, RANKL has been shown to favor EMT in mammary epithelial cells, in head and neck squamous carcinoma, in endometrial cancer, and in prostate cancer cells [57, 59, 87, 88]. An interesting mechanism through which RANKL induces EMT has been demonstrated *in vitro* in breast cancer cells, implicating increase of expression of vimentin, N-cadherin, Snail, and Twist and decrease of E-cadherin expression, as well as activation of NF- κ B [89].

Moreover, bone homing and colonization appears to recapitulate a cross-talk between tumor and immune cells in which RANKL prevails. In particular, RANKL—expressed not only in osteoblasts but also in T and B cells—interfaces with its receptors on DCs, monocytes, macrophages, and tumor cells to create a “bridge” between immune system and bone remodeling [78]. Convincing evidence sustains this hypothesis. DCs, also known as antigen-presenting cells (APC), play a key role in regulation of cytotoxic T cell immune response activation [90]. *In vitro* and *in vivo* studies have shown that the interaction of RANK expressed on DCs with RANKL overexpressed on activated T cells fosters the survival of T cells, thereby priming T cells. FOXP3⁺ T regs (CD4⁺ T cells), which are well established immune suppressors, have been demonstrated as a major source of RANKL implicated in homing of cancer cells to bone [59]. Th17 cells, another crucial subset of CD4⁺ T cells, have been postulated to reinforce the osteoclastic activity and thus the osteolytic bone disease via the RANKL pathway [90].

Consolidating the hypothesis that the local chemokine milieu in bone microenvironment attracts preferentially certain cancer cells, Jones et al., in 2006, provided *in vitro* and *in vivo* evidence concerning the role of RANKL as a “soil” factor, which stimulates cytoskeletal changes, facilitating the migration of several human epithelial RANK expressing cancer cells. Interestingly, this study showed that RANKL could foster the migration of normal, non-transformed, cells as well [91].

RANKL and M-CSF—both indispensable for osteoclasts differentiation and activation—have been shown to be released from BMA, stimulating the BMA-mediated induction of osteolysis [73]. Interestingly, *in vivo* data indicate that this function is a characteristic of BMA at a preadipocyte stage [92, 93] and it is not observed in white adipose tissue [93]. Takeshita et al. working on a mouse model showed that the early adipogenic transcription factors C/EBP β and C/EBP δ , but not the late factor peroxisome proliferator-activated receptor γ , elicit the transcription of RANKL gene through binding to RANKL promoter [92]. Notably, early loss of parathyroid hormone 1 receptor (PTH1R) signaling in BMSCs in mice was shown *in vivo* to result in uncontrolled differentiation of these cells to BMA capable of producing high levels of RANKL, inducing bone resorption [93].

The precise role of OPG in the context of bone metastatic niche remains controversial. It is postulated that the anti-osteoclastogenic effect of OPG may contradict bone colonization; yet, the direct and indirect tumor-promoting effect of OPG could be consistent with bone colonization. More studies are needed to clarify this issue. Whether OPG interacts with EMT is underexplored. The possibility of a role of OPG in EMT either directly or indirectly through abrogating RANKL/RANK interaction is an appealing hypothesis that merits future investigation.

Finally, it should be mentioned that cancer cells are devoid of bone resorption properties, but they may fuse with OCPs or bone marrow-derived cells to create mature osteoclasts. In fact, genes-landmark of osteoclasts, such as cathepsin K, are included in the “osteomimicry gene signature”.

Imbalance in the OPG/RANKL/RANK ratio as an orchestrator of cancer cell–bone cell interactions

The interrelationship of cancer cells with bone microenvironment spurs a “vicious cycle” wherein cancer cells activate the osteoclasts to degrade the bone, releasing growth factors stored in bone matrix, which in turn foster the growth of tumor. This “vicious cycle” is a hallmark of bone metastases of solid tumors, of primary lesions of bone tumors such as osteosarcoma, and of bone disease related to multiple myeloma (MM). Considering that the balance among OPG, RANKL and RANK is credited with maintenance of skeletal integrity, it appears rational that the imbalance thereof is fundamental in bone oncology [94].

Indeed, it has been postulated that cancer cells hijack the host equilibrium among OPG, RANKL and RANK, favoring bone destruction via upregulation of RANKL in concert with either downregulation of OPG or rarely upregulation—but to a lesser extent than RANK—of OPG [95]. In the context

of a cross-talk between cancer cells, bone microenvironment and tumor microenvironment, cancer cells recruit bone stromal cells—fibroblasts, endothelial cells, immune cells, and osteoblasts—via secretion of osteoclastogenic cytokines and growth factors, including IL-1, IL-6, IL-8, IL-11, TNF- α , PTHrP, that act in a paracrine, autocrine and endocrine way to initiate a “vicious cycle”. The tumor-induced M-CSF and RANKL expressed on osteoblasts, mesenchymal stem cells (MSCs), and immune cells interact with c-fms and RANK on OCPs, in order to induce the differentiation and activation of osteoclasts. Accordingly, the osteoclasts-driven resorption of bone matrix releases growth factors, including transforming growth factor beta (TGF- β), insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), and FGF, which enhance the tumor proliferation, perpetuating the “vicious cycle”. In response to the stimulative effect of tumor microenvironment, cancer cells per se produce RANKL, indicating a reciprocal dialogue between tumor and bone microenvironment. Moreover, osteoclasts nurture the “vicious cycle” via producing protumorigenic and prometastatic factors that foster the growth of bone tumors and metastatic lesions. In parallel, activated T cells in tumor microenvironment contribute to production of RANKL [94, 96]. Intriguingly, prolactin has been shown to transduce osteoclastogenic signaling, mainly via sonic hedgehog (SHH), that in turn activates ERK, reinforcing the “vicious cycle” [96]. As noted earlier, in osteosarcoma, this “vicious cycle” has been shown in vivo to be inhibited by TRAIL, reducing both osteolysis and tumor growth [66].

More intricate is rendered the signaling in cancer-induced osteoblastic, and mixed—both osteolytic and osteoblastic—lesions, emphasizing the multifaceted role of TGF- β [69]. Beyond TGF- β , a plethora of tumor-derived pro-osteoblastic growth factors and cytokines released through the osteolytic process favor the osteoblastic function, amplifying the osteoblastic lesions.

The clinical relevance of OPG in the “vicious cycle” recapitulates the interrelation of its tumor-promoting role and its antitumor role. While OPG signaling per se harbors an inherent tumor-promoting potential, the OPG-mediated abrogation of RANKL—and TRAIL-induced signaling could be rendered both tumor-promoting and antitumor. Table 1 depicts the in vitro and in vivo evidence sustaining the tumor-promoting role of OPG. It is highlighted that the majority of preclinical and clinical data building on the role of OPG in cancer are derived from studies outside the bone, necessitating further evaluation in the setting of bone oncology. Moreover, most studies pursuing skeletal metastatic disease focus on breast and prostate cancer; thus, it is mandatory to further address bone metastases in the context of other cancer types.

The RANKL/OPG axis in the context of the “vicious cycle” of the interplay among cancer cells, tumor

microenvironment, and bone microenvironment is illustrated in Fig. 1.

The role of RANK/RANKL/OPG axis in osteosarcoma

Osteosarcoma is the most prevalent malignant pediatric primary bone tumor and the second most frequent cause of cancer-related death in pediatric population [97]. It usually affects the metaphysis of long bones and is diagnosed in the second decade of life [98]. The five-year survival rate is estimated 65% for localized disease, declining at 20% in case of lung metastases at initial diagnosis.

In 2007, Mori et al. reported for the first time the expression of RANK in osteosarcoma in vitro in human osteosarcoma cell lines and ex vivo in pathological tissues, showing also that the interaction RANKL/RANK phosphorylates the kinases ERK1/2, p38, and inhibitor of nuclear factor- κ B (I κ B) [99]. Osteosarcoma, originated from osteoblast cell lineage, could provide the archetype of the deleterious “vicious cycle” orchestrated by RANKL/RANK interaction [97], resulting in mixed, osteolytic and osteoblastic, lesions.

Indeed, a great body of convincing evidence implicate RANK signaling in osteosarcoma. For instance, increased RANKL expression on cancer cells was observed in osteosarcoma-bearing mice in the study of Lamoureux et al. In this study, RANKL expression led to osteosarcoma related osteolysis, a process abrogated by OPG [100].

In 2010, Molyneux et al., working on a transgenic mouse model of osteosarcoma, showed that abundant expression of RANKL due to loss of the Protein Kinase CAMP-Dependent Type I Regulatory Subunit Alpha (Prkar1a) bone tumor suppressor gene enhanced tumorigenesis [97]. Furthermore, aberrant RANK expression and signaling has been noted in human [99] and mouse [101] osteosarcoma cell lines.

Prompted by this evidence, Beristain et al. selected RANK-positive mouse osteosarcoma and RANK-negative preosteoblastic MC3T3-E1 cells and subjected them to loss- and gain-of-RANK function analyses in order to explore the hypothesis that RANK, via an autocrine (homotypic) signaling cascade, incites tumorigenesis in cells of both epithelial and mesenchymal origin. Among a wide array of tumorigenic properties examined, RANK homotypic signaling proved to exert an insignificant effect on cell proliferation, while favoring cell motility and anchorage-independent growth of osteosarcoma cells and preosteoblasts. On the other hand, RANK signaling in non-tumorigenic mammary epithelial NMuMG cells evoked their proliferation and anchorage-independent growth, while motility was not affected. Furthermore, RANK signaling activated kinase ERK1/2, a step pivotal for the anchorage-independent survival and invasion of osteoblastic cells, as well as the

Table 1 The tumor-promoting role of OPG

Mechanism of OPG action	Effect of OPG
Activation of Aurora-A, Bub1, and BubR1 in HMEC spheres [35]	Induction of aneuploidy
Activation of AKT, GSK3b, p44/42, p65, and b-catenin in HMEC spheres [35]	Pro-survival and pro-proliferative effect on breast cancer cells
Upregulation of CD24 in HMEC spheres [35]	Enhancement of cancer cell invasion, and migration
Amplification of DNA copy numbers of oncogenes—AKT1, AURK1, EGFR, Myc, PAK1, CDK4, ERBB2—and downregulation of tumor suppressive genes—CDKN2A, PTEN, TOP2A, RB1—in sphere cultures of breast cancer cells SUM149PT, SUM1315MO2, inflammatory breast cancer patients' tissues, and HMEC spheres [36]	Initiation and progression of cancer
Binding several proteins-partners of OPG in inflammatory and aggressive breast cancer cells [36]	Promotion of initiation and progression of cancer
Induction of proteases cathepsin D and Matrix Metalloproteinase-2 in a chick embryo model [37]	Promotion of invasion and metastases
OPG on endothelial and immune cells at LNM of MTC patients acts as a chemoattractant for MTC cells, according to IHC study [38]	Development of LNM in human MTC
Inhibition of TRAIL-mediated apoptosis in breast cancer cells MDA-MB 436/231 [39]	Pro-survival effect on breast cancer cells
Induction of expression of SDF-1 by ECFCs [40]	Promotion of migration of MNNG-HOS human osteosarcoma cells and DU145 prostate cancer cells
Interaction with FASN and COX2 in SUM149PT and SUM1315MO2 cells [41]	Enhancement of inflammatory milieu and pro-survival effect on highly invasive inflammatory breast cancer cells
Induction of IL-1 β expression in breast cancer cells [42]	Promotion of breast cancer cell invasion
Activation of ERK and AKT in rat aortic ring model of angiogenesis [45] as well as FAK and Src in endothelial cells in vitro [46]	Promotion of angiogenesis
Inhibition of antiangiogenic function of TSP-1 in human vascular endothelial cells [47]	Promotion of angiogenesis
Inhibition of TRAIL-mediated apoptosis in breast cancer cells MDA-MB 436/231 [39]	Pro-survival effect on breast cancer cells

AKT (1) serine-threonine kinase (1), *AURK1* Aurora-A kinase 1, *CD24* cluster of differentiation 24, *CDK4* cyclin dependent kinase 4, *CDKN2A* cyclin-dependent kinase inhibitor 2A, *COX2* cyclooxygenase-2, *ECFCs* endothelial colony-forming cells, *EGFR* epidermal growth factor receptor, *ERBB2* erb-b2 receptor tyrosine kinase 2, *ERK* extracellular signal related kinase, *FAK* focal adhesion kinase, *FASN* fatty acid synthase, *GS3K- β* glycogen synthase kinase 3b, *HMEC* human mammary epithelial cells, *IHC* immunohistochemical, *IL-1 β* interleukin 1 β , *LNM* lymph node metastases, *MTC* medullary thyroid cancer, *OPG* osteoprotegerin, *PAK1* Serine/threonine-protein kinase, *PTEN* phosphatase and tensin homolog, *RANKL* receptor activator of NF κ B, *RANKL* RANK ligand, *RANKc* c RANK isoform, *RB1* retinoblastoma 1, *SDF 1* Stromal cell-derived factor 1, *TOP2A* DNA topoisomerase II alpha, *TRAIL* TNF related apoptosis inducing ligand, *TSP-1* thrombospondin 1

proliferation of mammary epithelial cells. The authors concluded that a cell-autonomous homotypic mechanism underlies the contribution of RANK signaling to tumorigenesis [102].

The landmark study of Chen et al. provided strong evidence on the efficacy of RANKL inhibition for the management of osteosarcoma in animal models. The researchers demonstrated that loss of a single *Prkar1a* allele due to increase of RANKL levels is a key mechanism in osteosarcoma genesis regardless of either RB or protein p53 status in genetically engineered mouse model (GEMM). Moreover, increased RANKL levels proved to be associated with increased osteosarcoma aggressiveness. The importance of this RANKL effect was highlighted by the observation that whole-body RANKL deletion completely abrogated tumorigenesis, while osteoclastic RANK deletion in GEMM led to attenuated tumor initiation and prolonged life span; this impact was related to inactivation of osteoclastogenesis

and up-regulation of the tumor suppressor gene PTEN. To strengthen their observations, the authors treated GEMM with RANK-Fc, which resulted in a 50% prolongation of life span with 3 times decrease in lung metastases compared to non-RANK-Fc treated mice [103].

Recently, the seminal study of Navet et al. implicated intrinsic (tumoral) RANK signaling in the metastatic process of osteosarcoma. However, this prometastatic effect appeared not to be ascribed to direct promotion of tumor cell proliferation, but rather to chemoattraction of RANK expressing osteosarcoma cells by RANKL expressing cells in bone microenvironment (extrinsic) [98]. In this study, in vitro and in vivo experiments showed for the first time that RANK overexpressing osteosarcoma cells present increased migration capacity only in immune-deficient mice. Given that chemotherapy is known to induce immunosuppression, the authors postulated that in case of cancer resistant to treatment, RANK overexpressing cancer cells could contribute

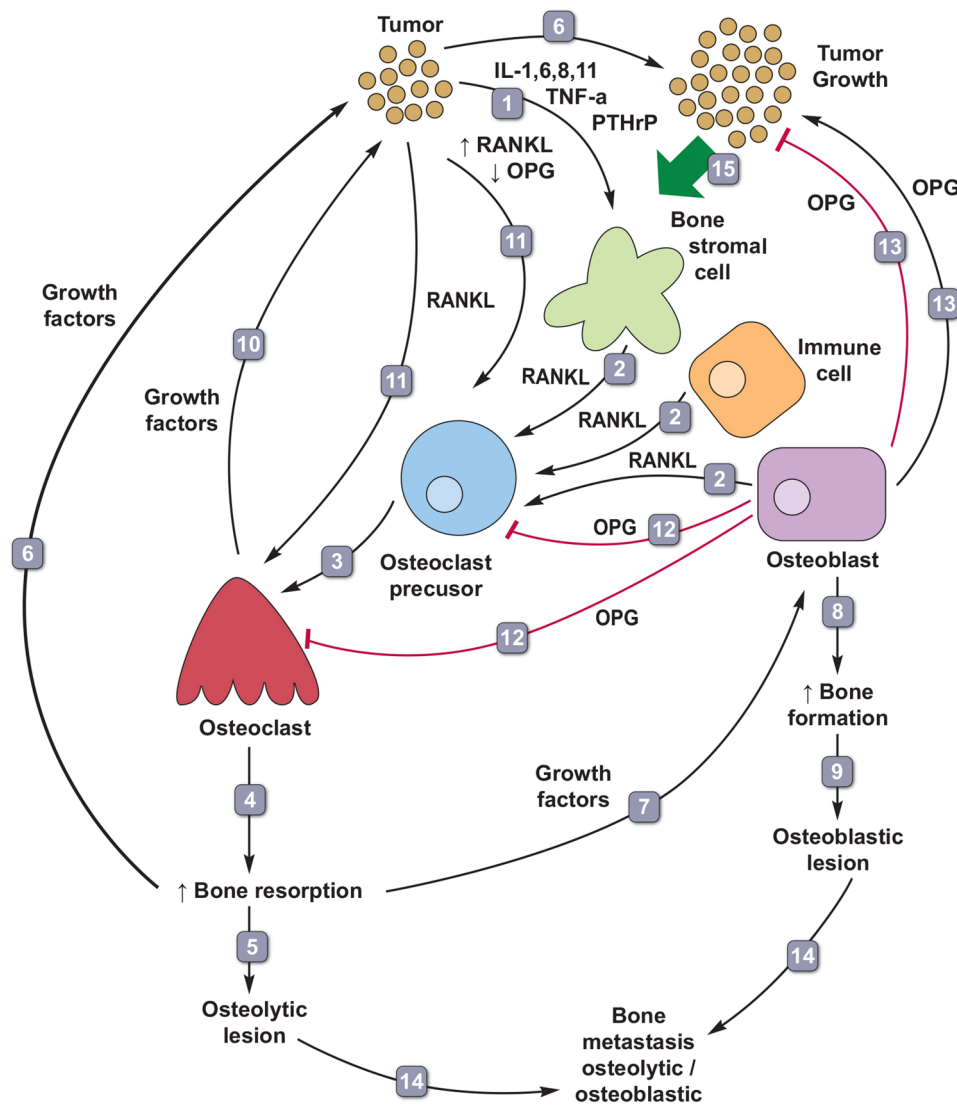


Fig. 1 The RANKL/OPG axis in the “vicious cycle” of the interplay among cancer cells, tumor microenvironment, and bone microenvironment (1) Cancer cells secrete cytokines and hormones/growth factors, including IL-1, IL-6, IL-8, IL-11, TNF- α , PTHrP, resulting in recruitment of bone stromal cells (e.g. osteoblasts, immune cells). (2) Bone stromal cells, mainly the osteoblasts and the immune cells, are induced by cancer cells to overexpress RANKL, which acts on osteoclast precursors. (3) RANKL promotes differentiation and activation of osteoclasts. (4) Increased osteoclastic activity leads to increased bone resorption, the hallmark of osteolytic lesions (5). Increased bone resorption, releases growth factors that result in tumor growth (6) and

potentially increased osteoblastic function (7), leading to increased bone formation (8), the hallmark of osteoblastic lesions (9). (10) Osteoclasts-derived growth factors nurture the tumor. (11) Cancer cells per se produce RANKL. (12) Osteoblasts produce OPG which abrogates the RANKL-induced differentiation and activation of osteoclasts and potentially either fosters or inhibits tumor growth (13). (14) Bone metastases represent a continuum of dysregulated bone remodeling extending from predominantly osteolytic lesion to predominantly osteoblastic lesions. (15) Tumor growth perpetuates the “vicious cycle” aggravating the skeletal tumor burden. ↑: increase; ↓: decrease

to a metastatic process mediated through RANKL/RANK signaling. Consequently, abrogation of this RANKL/RANK mediated metastatic effect via an antibody blocking RANKL was suggested by Navet et al. as a strategy to prevent metastasis in an immune-compromised context [98].

Furthermore, considering that RANKL proved to have no effect on proliferation but a positive effect on differentiation of RANK-expressing osteosarcoma cells, Navet et al.

hypothesized that RANK expressed by osteosarcoma cells could be a factor of good prognosis. However, RANKL/RANK signaling was observed to exert a prometastatic effect fostering the development of lung metastases. As the background of this aggressive behavior was suggested the RANKL-induced expression of MMP on RANK expressing osteosarcoma cells, which could promote the escape of cancer cells from the initial site. Additionally, RANKL

might also favor seeding of cancer cells to lungs through mechanisms still unknown, likely independently of RANK and OPG expression. Given that LGR4 is considered a third receptor of RANKL known to be involved in progression, migration and metastatic processes, the role of the RANKL/LGR4 interaction in the ontogenesis of osteosarcoma merits further exploration [98].

Navet et al. provided clinical data obtained through immunohistochemical detection of RANKL/RANK/OPG on biopsies of a cohort of osteosarcoma patients with or without metastases, showing no significant difference of intrinsic RANK expression between the two group of patients. In fact, RANKL availability in microenvironment proved to be the major determinant of metastatic dissemination. Decreased OPG/RANK ratio, observed in this study, was postulated to facilitate the interaction of RANKL available in the microenvironment with RANK expressed by osteosarcoma cells, thereby predisposing to metastatic dissemination [98].

The involvement of OPG/RANKL/RANK axis in the colonization of the metastatic organ is not a prerogative for bone-homing tumors. In fact, the prometastatic effect of RANKL/RANK interaction in the setting of lung-homing osteosarcoma is not unexpected, given that RANKL/RANK is implicated in bone resorption, metastasis, and immune surveillance evasion [103]. The correlation of decreased OPG/RANK ratio with the potential of osteosarcoma to metastasize to lungs, observed by Navet et al., may indicate an anti-metastatic effect of OPG. Due to the dual role of OPG in carcinomas and the limited knowledge concerning OPG in sarcomas, the comparison of the role of OPG in the metastatic potential of these two types of cancer is daunting. These intriguing issues could represent an appealing field of future research.

The role of RANKL/RANK/OPG axis in multiple myeloma

Multiple myeloma (MM) is a rare clonal plasma cell neoplasm with an age-standardized incidence rate of 2.1 per 100,000 persons globally in 2016 [104]. The majority of patients ($\approx 90\%$) develop bone lesions, which result in serious SREs. Despite recent therapeutic advances, MM remains an incurable disease with substantial morbidity and mortality. Ongoing efforts for devising new therapeutic strategies capitalize on the cross-talk between MM cells and surrounding bone marrow components, which culminates in enhancement of growth of MM cells and bone destruction. Numerous studies have demonstrated that the BMSCs stimulated by adherent MM cells secrete plenty of cytokines that enhance the osteoclast-induced bone resorption, which in turn releases growth factors fostering the growth of MM cells [105]. The present article focuses on the key role of the

RANKL/OPG interplay in the pathogenesis of MM related bone disease.

The hallmark of the MM related bone destruction is the MM cells induced increase of RANKL/OPG ratio, ascribed to upregulation of RANKL expression and downregulation of OPG expression. MM cells directly secrete RANKL or stimulate its secretion by T lymphocytes and apoptotic osteocytes [106]. The syndecan-1 expressed on MM cells has been shown *in vitro* to bind the heparin-binding domain of OPG, resulting in internalization and eventually degradation of OPG [30]. Certain MM-derived factors inhibit osteoblasts formation, leading to decreased OPG production, namely the sclerostin, the dickkopf-1 (DKK1), and the secreted Frizzled-related protein (sFRP)2/3 (inhibitors of the canonical Wnt pathway), as well as the IL-7, which downregulates Runx2 [106]. The chemokines macrophage inflammatory protein (MIP)1- α and MIP1- β , secreted by MM cells, interact with the integrin ($\alpha 4\beta 1$) very late antigen-4 (VLA-4) and the vascular cell adhesion protein 1 (VCAM-1) on BMSCs to enhance both RANKL upregulation and OPG downregulation. Elevation of IL-3 in BM in MM patients acts in concert with RANKL and MIP1 to promote osteoclasts activation and bone destruction. Furthermore, activation of Notch signaling in MM cells and OCPs, evoked by BMSCs and MM cells, potentiates RANKL signaling [105].

Though beyond the scope of the present review, several additional mechanisms are implicated in the pathophysiology of MM, including: (i) overexpression of IL-6 by osteoclasts, BMSCs, and osteoblasts, resulting in MM cell expansion and bone destruction; (ii) production of tumor necrosis factor- α (TNF- α), B cell activating factor of TNF- α family (BAFF), and A proliferation inducing ligand (APRIL) by MM cells, boosting the survival thereof; (iii) production of numerous cytokines and growth factors by BMSC that foster MM cell proliferation, such as vascular endothelial growth factor (VEGF), SDF-1 α , and IGF-1 [107, 108].

Seum RANKL/OPG ratio was demonstrated as a prognostic factor for MM in the study of Terpos et al.: Patients with ratio value less than 1 showed a 5-year probability of survival rate of 89%, while all patients with a ratio greater than 3 survived for less than 4 years [108].

Of clinical relevance is the elevation of RANKL and RANKL/OPG ratio in MM in parallel with the increase of the disease stage and the severity of bone lesions. Furthermore, serum RANKL levels and RANKL/OPG ratios have been correlated significantly with angiogenic cytokines (Hepatocyte growth factor [HGF] and VEGF), and factors of disease activity, such as IL-6, $\beta 2$ -microglobulin, and lactate dehydrogenase (LDH) [109].

Novel insights into the origin of RANKL in MM patients are provided by the clinical study of Spanoudakis et al. The authors demonstrated the contribution of invariant NKT (iNKT) TCRV $\alpha 24$ J $\alpha 18$ /V $\beta 11$ cells—a subset of

CD1d-restricted, glycosphingolipid (GSL)-specific immunoregulatory T cells regulating a variety of immune responses—to the increase of RANKL in MM. iNKT cells from peripheral blood (PB) and BM of MM patients were shown to express higher levels of RANKL than PB iNKT cells from normal subjects, whereas the expression of RANKL in BM iNKT cells of MM patients is higher than that observed in BM T cells or autologous PB iNKT and T cells. Importantly, a strong association of the activity of bone resorption with RANKL overexpressed on iNKT cells in MM patients was demonstrated through assessment of β -C-terminal telopeptide levels. Interestingly, enrichment of BM with iNKT cells compared to PB was designated as a specific feature of MM. Finally, in this study, iNKT cells in normal individuals proved to be a richer source of surface and likely soluble RANKL than conventional T cells both *ex vivo* and *in vitro* [110].

Pitari et al. demonstrated that the miR-21, an OPG-targeting miRNAs acting as a negative regulator of OPG gene expression, was upregulated in BM adherent to MM cells, down-regulating OPG in HS-5 BM stromal cells. Consequently, the constitutive miR-21 inhibition significantly increased OPG production. Importantly, miR-21 was also shown to regulate RANKL production in BMSCs and osteoblasts via a feedback loop involving IL-6 and STAT3 signaling. Especially, miR-21 expression was stimulated by IL-6 in the presence of activated STAT3, while enhancing *per se* the phosphorylation of STAT3 via inhibition of PIAS3—a specific inhibitor of STAT3 phosphorylation. Phosphorylated STAT3 is known to stimulate the RANKL gene expression through IL-6 signaling; thus, the miR-21-induced STAT3 activation led to increased RANKL expression. Accordingly, the miR-21 inhibition is supposed to attenuate the osteolytic activity of BMSCs in a dual manner: increasing OPG, while decreasing RANKL production. This restoration of the aberrant RANKL/OPG ratio was shown to counteract the BM-related osteoclastic activity *in vitro* [111].

Additionally, silencing of miR-9718, which acts as a specific inhibitor of PIAS3, has been shown to attenuate RANKL-induced osteoclastogenesis *in vivo* [112].

In 2018, Food and Drug Administration (FDA) approved the administration of denosumab—a monoclonal antibody against RANKL—for prevention of skeletal-related events in MM patients, based on the landmark Phase III '482 study. Denosumab blocks pharmacologically the interface RANKL/RANK in a way reminiscent of the action of OPG [113]; yet, this issue is beyond the scope of the present review.

Exploration of the therapeutic potential of OPG

With the advent of current millennium, the development of the OPG construct OPG-Fc launched a new era in bone oncology, aiming at intercepting not only the cancer-related

osteolysis but also the tumor growth. This construct was generated via removal of the signal peptide, the heparin binding domain and the death domain of native OPG, followed by fusion of the remaining peptide to the Fc domain of human IgG1. OPG-Fc comprises aa 22-194 of native OPG, maintains the potent dimeric form, and has a prolonged half-life compared to native OPG. It has been demonstrated to inhibit hypercalcemia and bone resorption induced by IL-1b, TNF- α , PTH, PTHrP, and 1, 25(OH) $_2$ D3 [114].

Compelling evidence derived from several mouse models sustain the antitumor effect of OPG [114]. Administration of OPG-Fc or RANK-Fc in mouse models of breast, prostate, colon, and non-small lung cell (NSCLC) cancer, as well as epidermal carcinoma and melanoma has resulted in significant amelioration of overall survival, prevention of bone metastases, and decrease of tumor burden in bone [115]. Moreover, OPG-Fc has been proved efficient in treatment of humoral hypercalcemia of malignancy [116]. Additionally, OPG-Fc has been depicted to reduce the skeletal tumor burden in a syngeneic model and a nude mouse model, wherein osteolytic lesions were generated by, respectively, colon adenocarcinoma (col 26) cells and MDA-MB-231-breast cancer cells. This effect was ascribed to elimination of osteoclasts, while no extraosseous metastatic site was affected [117]. Interestingly, an inhibitory effect of recombinant OPG on prostate cancer-related skeletal tumor osteoblastic burden has been observed, highlighting that the osteoclastic function is inherent to osteoblastic lesions [118]. Finally, administration of OPG in a mouse model of bone cancer pain has been shown to blunt both peripheral and central sensitization, reducing the prohyperalgesic peptide DYN in spinal cordal and the c-Fos in deep dorsal horn. These alterations likely emerge from inhibition of osteoclastic activity [119].

The above-mentioned work preceded the FDA approval of denosumab in bone oncology in 2010 [113, 120], an issue beyond the scope of the present review. However, the advent of denosumab has not overshadowed the evolving research on the therapeutic potential of OPG, which continues to be very informative.

In 2015, Ottewill et al. demonstrated *in vivo* that the administration of OPG-Fc can counteract the growth of dormant MDA-MB-231 cells disseminated in bone, interrupting the RANKL/RANK interface [121].

Miller et al., in 2014, reported the effects of OPG-Fc in combination with docetaxel as regards tumor-induced osteolysis, tumor burden, and survival in the H1299Luc mouse model of NSCLC metastasis in bone. In mice with established NSCLC bone metastases, administration of OPG-Fc as monotherapy led to a 84.1% skeletal tumor growth abrogation compared with vehicle control at the end of the study (day 21). Docetaxel (35 mg/kg) as monotherapy led to a 96.5% reduction in skeletal tumor burden, while the combination of OPG-Fc and docetaxel (35 mg/kg) resulted

in the most pronounced reduction in skeletal tumor burden at day 21 (99.7%) in comparison with either docetaxel alone ($p < 0.001$) or OPG-Fc alone ($p < 0.001$). The effect of docetaxel was attributed to reduced tumor burden, while the effect of OPG-Fc was ascribed to decreased osteoclastic activity. Moreover, RANKL inhibition via OPG-Fc completely averted the osteolytic bone lesions both as monotherapy and in combination with docetaxel [122]. Effective treatment of established bone metastases with an OPG-Fc armed conditionally replicating adenovirus (CRAd) in a murine model of osteolytic bone metastases of breast cancer has been reported by Cody et al., sustaining the antitumor potential of exogenous administration of OPG [123]. The combination of OPG-Fc with tamoxifen was reported to halt the skeletal metastatic bone disease in a mouse model of estrogen receptor-positive breast cancer, in 2012. In this model, OPG-Fc was shown to inhibit osteoclastic activity and prevent tumor-induced osteolysis, resulting in reduction of skeletal tumor burden. Tamoxifen as a single agent reduced MCF-7Luc tumor growth in the hind limbs. Combination of OPG-Fc with tamoxifen resulted in significantly enhanced inhibition of tumor growth compared with the inhibition observed with either single agent as monotherapy. OPG-Fc either as monotherapy or combined with tamoxifen, eliminated the osteolytic lesions [124]. Taken together, the above mentioned *in vitro* and *in vivo* data consolidate the indirect antitumor aspect of OPG ascribed to abrogation of RANKL.

Challenges in the therapeutic implementation of OPG

Despite the presumed antitumor effect of exogenous administration of OPG, the therapeutic implementation of OPG raises skepticism given the preclinical and clinical data sustaining the tumor-promoting effect of OPG. Indeed, the study of Zinonos et al. underscored the dual dynamics of OPG signaling, investigating the OPG overexpression in a xenogeneic murine model of osteolytic breast cancer: whereas OPG overexpression resulted in reduction of bone tumor burden and prevention of cancer-related osteolysis, it was correlated with increased propensity of cancer cells to colonize the lung [125].

However, it is highlighted that OPG-Fc is featured by absence of the death domain and the heparin binding domain of full length OPG. This discriminating feature might endow OPG-Fc with an antitumor potential, given that glycosaminoglycans binding to aa 195–401 domain of full length OPG (the region absent in OPG-Fc) have been implicated in the modulation of its bioactivity and its function [126]. Moreover, the proteins assigned to transduce tumor-promoting signals have been shown to bind the heparin binding domain

of OPG; thus, the truncated form OPG-Fc (aa 1–194) is expected to be deprived of the tumor-promoting effects of full length OPG (aa 1–401).

The administration of the recombinant osteoprotegerin construct AMGN-0007 initially led to suppression of bone resorption in MM and breast carcinoma patients in a phase I study [127]; however, this construct was abandoned early due to fear of stimulating an immune response against endogenous OPG.

Interestingly, the scientific attention has been drawn to emerging innovative experimental strategies exploiting the antitumor potential of OPG, which worth further exploration. Firstly, Lamoureux et al. in a landmark study showed that OPG transgene expression in a rat osteosarcoma model can disrupt the osteosarcoma progression via inhibiting RANKL-induced osteolysis [98]. Secondly, as discussed earlier, inhibition of miR-21 in MM could be proven a therapeutic option for MM-related bone disease [111]. Thirdly, further research is warranted to investigate whether mesenchymal stromal cells (MSCs) transfected with adenovirus carrying the OPG gene could be endorsed as a novel treatment of osteosarcoma [128]. Finally, Higgs et al. attempted to harness the RANKL binding property of OPG averting the binding of TRAIL. The authors introduced a therapeutic approach based on genetically engineered MSCs expressing OPG mut Y49R and F107A that retain RANKL binding while abolishing TRAIL binding. This strategy proved to be effective *in vivo*, diminishing osteolytic tumor burden in a mouse model of tumor-induced osteolysis [129].

Conclusions and future perspectives

OPG has been revolutionizing the perception of bone biology for over 2 decades. A growing body of evidence points to the key role of OPG in bone oncology concerning both bone metastatic disease and primary bone tumors. The tumor-promoting aspect of OPG is synthesized by a direct—protumorigenic, proangiogenic, prometastatic—effect and an indirect effect exerted through abrogation of the antitumor potential of RANKL and TRAIL. The antitumor aspect of OPG signaling is exerted through inhibiting tumor-promoting potential of RANKL and TRAIL signaling.

More light should be shed into the role of OPG in giant cell tumor of bone (GCTB). GCTB is a primary bone tumor with a unique profile, arising from GCTB neoplastic cells—dysfunctional mesenchymal stromal-like cells—that express RANKL, which interacts with RANK expressed by multinucleated osteoclastic giant cells, promoting the osteolytic activity of the latter. This interaction is already targeted pharmaceutically by denosumab; however, OPG merits further exploration in the setting of this tumor [130, 131].

An overarching aim is to identify and modify the factors that determine whether the clinical outcome of the balance among OPG, RANKL, and TRAIL results in elimination or, on the contrary, progression of cancer. Translating biological insights concerning the multifaceted role of OPG in bone oncology into clinical settings raises some outstanding questions: (1) How does the simultaneous presence of biologically relevant concentrations of TRAIL and RANKL influence the outcome of OPG signaling, given that RANKL and TRAIL bind to OPG with equal affinity? (2) Do the ratios RANKL/OPG, RANKL/TRAIL, OPG/TRAIL, RANK/OPG determine the interplay OPG/RANKL/TRAIL? (3) Could OPG, RANKL, RANK serve as predictive and/or prognostic biomarkers of primary bone tumors and/or skeletal metastatic disease? (4) Is there any difference as regards the clinical significance between the serum concentrations of OPG, RANKL, RANK, TRAIL and the tissue expression thereof? (5) Does the diversity of methods used for evaluation of OPG expression in pertinent literature affect the interpretation of the results? (6) How is interpreted the discrepancy between in vitro and in vivo data concerning the OPG-induced abrogation of TRAIL apoptotic signal [132]?

Further research aiming to decipher the expanding repertoire of OPG ligands and downstream signaling cascades could unravel new druggable molecular pathways, empowering the individualization of metastatic and primary bone cancer therapeutics.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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