REVIEW

Targeting RANKL in metastasis

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Acting through its cognate receptor, receptor activator of nuclear factor-κB (RANK), RANK ligand (RANKL) is an essential mediator of osteoclast function and survival. Preclinical data have now firmly established that blockade of tumor-induced osteoclastogenesis by RANKL inhibition will not only protect against bone destruction but will also inhibit the progression of established bone metastases and delay the formation of de novo bone metastases in cancer models. In patients with bone metastases, skeletal complications are driven by increased osteoclastic activity and may result in pathological fractures, spinal cord compression and the need for radiotherapy to the bone or orthopedic surgery (collectively known as skeletal-related events (SREs)). Denosumab, a fully human monoclonal antibody against RANKL, has been demonstrated to prevent or delay SREs in patients with solid tumors that have metastasized to bone. In addition to its central role in tumor-induced osteolysis, bone destruction and skeletal tumor progression, there is emerging evidence for direct pro-metastatic effects of RANKL, independent of osteoclasts. For example, RANKL also stimulates metastasis via activity on RANK-expressing cancer cells, resulting in increased invasion and migration. Pharmacological inhibition of RANKL may also reduce bone and lung metastasis through blockade of the direct action of RANKL on metastatic cells. This review describes these distinct but potentially overlapping mechanisms by which RANKL may promote metastases.

Introduction

Receptor activator of nuclear factor (NF)-κB ligand (RANKL) and its cognate receptor RANK were initially characterized as essential factors for osteoclastogenesis (reviewed in Lacey *et al.*[1]). Mice in which either the *RANK* or *RANKL* genes are ablated exhibit severe osteopetrosis and an absence of osteoclasts.[2,3] RANKL and myeloid colony-stimulating factor are sufficient to generate osteoclasts from their myeloid precursors.[4] Osteoprotegerin (OPG), a decoy receptor for RANKL, inhibits osteoclasts and acts as a physiological regulator of bone resorption by counterbalancing RANKL activity.[5] The discovery of RANKL, RANK and OPG revealed the molecular mechanisms critical for osteoclastogenesis and the physiological control of bone remodeling.

Skeletal metastasis results from reciprocal engagement between tumor cells and normal host cells of the bone microenvironment (for example, osteoclasts, stromal cells, vascular cells and so on; **Figure 1**). The resultant bone destruction and skeletal complications are mediated by a pathologically increased rate of bone remodeling driven by marked increases in osteoclast activity. This cooperative interaction between the bone microenvironment and tumor is known as the vicious cycle and is critical for metastatic establishment and progression.[6] Thus, the increased bone turnover driven by osteoclastic bone resorption not only contributes to skeletal morbidity, but has also been hypothesized to contribute to both early tumor colonization and later progression in bone. Data supporting this hypothesis have been comprehensively described in a recent review by Weilbaecher *et al.*[7] Given that RANKL is essential for osteoclastogenesis, it is also an attractive therapeutic target for the prevention and treatment of metastatic bone disease.

The generation of the *RANK*- and *RANKL*-knockout mice revealed that, other than osteopetrosis and peripheral lymph node agenesis, the only tissue compartment that exhibited obvious functional defects was the development of the mammary gland during pregnancy.[8] In the absence of *RANK* or *RANKL*, lactation is impaired owing to a defective expansion of the lobulo-alveolar mammary epithelium at midgestation. This defect is associated with reduced mammary epithelial proliferation and survival, and it is now understood that RANKL is the major paracrine effector of progesterone's mitogenic action in mouse mammary and human breast epithelium.[9,10] RANKL protein is regulated by progesterone and is expressed within the

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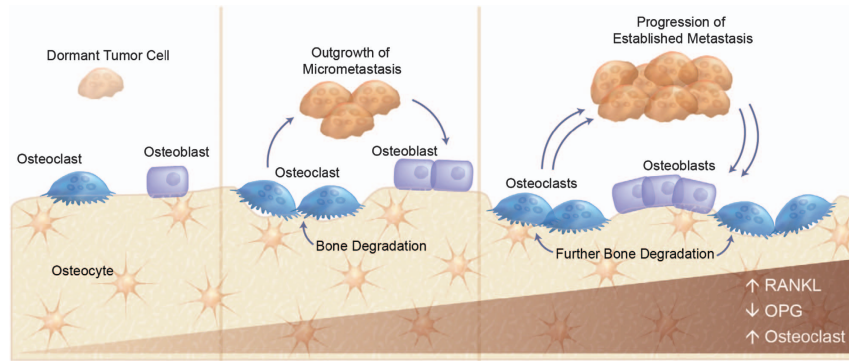
Figure 1 Interaction with the bone microenvironment supports tumor metastasis. OPG, osteoprotegerin; RANKL, RANK ligand.

estrogen receptor-positive/progesterone receptor-positive (ER + /PR +) luminal epithelium.[10,11] RANKL is positively correlated with breast proliferation and acts in a paracrine manner on RANK expressed in proliferating ER − /PR − breast cells.[9,11] In addition to mediating the mitogenic effect of progesterone, RANKL also controls the responsiveness of mammary stem cell and luminal progenitors[12] to progesterone.[13,14] These observations provided the initial rationale that RANKL might also directly stimulate tumor formation and metastatic progression through activation of epithelial cells.

Here we will review the preclinical *in vivo* and *in vitro* mechanistic evidence that RANKL blockade effectively inhibits both skeletal and nonskeletal metastasis. We will also review the clinical trials that have addressed the potential for RANKL inhibition to delay or prevent skeletal complications or bone metastases in cancer patients.

Role of RANKL in Pathologically-Induced Osteolysis and Bone Metastasis

In bone metastasis, RANKL-positive stromal cells have been observed at the tumor/bone interface adjacent to osteoclasts[15,16] and associated with tumor-induced osteolysis.[17] Early work using co-culture approaches demonstrated that, in contrast to the observations in progesterone-exposed mammary/breast tissue and primary breast cancer specimens (see below), breast tumor cells cultured *in vitro* did not express RANKL but could provoke increased stromal RANKL expression, leading to enhanced osteoclast formation.[18] This paradigm of stromal reaction to tumor involvement has been consistently observed across different model systems. The molecular mechanisms for upregulation of RANKL within bone stroma include transcriptional responses to tumor-associated cytokines and factors including interleukin (IL)-1β, IL-6, IL-8, IL-11, IL-17, macrophage inflammatory protein-1α, tumor necrosis factor-α, parathyroid hormone-related protein (PTHrP), prostaglandin E2 (PGE2) and CXCL13.[19,20] Interestingly, stimulation of the sympathetic nervous system has also been demonstrated to increase RANKL levels within osteoblast lineage cells.[12] Factors produced by tumors such as PTHrP, IL-1, PGE2, DKK-1 or epidermal growth factor also cause a decrease in local OPG expression by stromal and osteoblastic cells, thereby reducing local levels of the natural RANKL inhibitor.[18,21,22] Altogether, the net impact of tumor involvement on bone is to increase the RANKL:OPG ratio.

Reactive changes in the bone microenvironment to tumor involvement leads to locally increased RANKL:OPG levels, and experimental observations in bone metastasis indicate that this is the predominant RANKL signal leading to focally aggressive osteolytic bone destruction. The relative contributions of tumor cell-expressed RANKL and/or OPG to the net RANKL:OPG ratio in bone metastasis is less well defined. Tumor cells themselves can express OPG, and this can be modulated in the bone by estrogen signaling.[23,24] Although the preponderance of data indicates little (or variable) RANKL expression on tumor cells themselves, RANKL expression has been occasionally reported in primary tumors or tumor cell lines (reviewed in Dougall[19]), which would be predicted to cause osteolysis directly, leading to greater bone metastasis. Lack of specificity in reagents used to detect RANKL could explain the disparate observations. Expression analysis of RANKL in human primary tumors and potential relationships to metastatic outcomes are discussed later in this review but it is important to consider that the degree of RANKL expression observed in the primary tumor may not reflect the local influences on RANKL levels at the bone metastatic site. Bone microenvironment signals could induce RANKL or suppress OPG expression by metastatic tumor cells using mechanisms similar to those observed in stromal cells. Experimental methods using either real-time imaging of RANKL promoter/reporter constructs or careful analysis of host/tumor mRNA have shown that the bone environment can induce RANKL within prostate tumor,[25] head and neck squamous cell carcinoma (HNSCC)[26] or oral squamous carcinoma[27] cells (OSCC). In addition to cytokines/signals known to stimulate RANKL production or suppress OPG expression in stroma, other stimuli present in bone—such as αvβ3 integrin, CD44, TGF-β-dependent signaling[28] or the hypoxic environment of bone metastasis,[29]—may operate on metastatic tumor cells, leading to enhanced RANKL production. Interestingly, it has been hypothesized that the reactive skeletal environment may activate micrometastases to progress into overt bone metastasis by provoking epithelial-to-mesenchymal transition (EMT) of tumor cells.[30] The expression of RANKL on tumor cells undergoing EMT, as has been observed in prostate cancer[31] and HNSCC,[26] may be part of the EMT activation of metastatic outgrowth by further exacerbating bone resorption.

The *in vitro* observations that RANKL expressed on tumor cells can mediate osteoclastogensis directly without any requirement for osteoblasts or stromal cells (for example, Zhang *et al.*[32]) suggest that tumor-expressed RANKL, perhaps locally

induced within the skeleton, would contribute to bone metastasis beyond the stromal contribution. However, experimental evidence demonstrates that any tumor-expressed RANKL has relatively minimal (or variable) contributions (above and beyond stromal RANKL) to bone metastasis *in vivo*. For instance, by comparing a human-specific RANKL antibody with OPG-Fc, which inhibits both human and mouse RANKL, Morrissey *et al.*,[33] demonstrated that the stromal RANKL mediates bone destruction in a human prostate cancer xenograft model in mice. Using a similar approach in models of OSCC, Sato *et al.*[34] demonstrated that the stromal contribution of RANKL was consistently observed, whereas involvement of tumor cell RANKL in osteolysis was variable. These observations suggest the potential for tumor-expressed RANKL to further amplify the osteoclastic response in bone metastasis or to provoke bone resorption at early steps in bone colonization.

Pharmacological RANKL inhibitors would be active against RANKL, irrespective of the source (for example, bone stroma, infiltrating cells and tumor cells), and preclinical experimental bone metastasis studies using OPG-Fc or RANK-Fc support an essential role for RANKL in tumor-associated osteoclastogenesis and subsequent osteolytic bone destruction. Tumor-associated bone destruction in bone metastasis models of breast, lung, prostate, renal, OSCC, colon and epidermoid tumors is prevented by pharmacological inhibition of RANKL (reviewed in Roodman and Dougall[35]). Most studies have examined the role of RANKL in bone destruction and progression of established bone metastases; however, the reciprocal feedback of tumor cells and bone resorption might also actively contribute to tumor colonization and metastatic outgrowth, suggesting a role for RANKL at earlier stages of bone metastasis. Recent evidence from an *in vivo* model system has demonstrated that the localization of MDA-MB-231 breast cancer cells into bone induces significant contact-dependent changes of both osteoclast and osteoblast numbers before the development of bone lesions.[36] Defining the molecular mechanisms responsible for these early events is challenging, partly because of the limitations in technological identification of small numbers of tumor cells within bone. Only detailed histological analysis will reveal the cellular composition of small tumor foci, and subsequent identification of specific molecules associated with different cell types within these foci requires well-validated antibodies. Experimental models have been utilized to address whether skeletal metastases might be prevented or delayed through pharmacological blockade of RANKL and bone turnover in which OPG-Fc treatment was initiated either simultaneously with or before tumor cell-line inoculation. RANKL inhibition significantly delayed *de novo* formation of skeletal metastases.[37] In total, these data indicate that diverse cancer types employ multiple mechanisms involving RANKL as a common signaling pathway for tumor-induced osteolysis. Moreover, RANKL actively contributes to the vicious cycle of bone metastasis, mediating the processes of early tumor colonization and progression of bone metastasis.

Direct (Osteoclast-Independent) Pro-Metastatic Effects of RANKL

The observation that mouse mammary gland development during pregnancy is controlled by the intrinsic expression and functionality of RANK and RANKL within mammary tissue revealed a critical role for the RANKL pathway in epithelial biology. These findings provided the first experimental rationale that RANKL might directly control carcinogenesis and metastatic progression, distinct from its role in the promotion of osteoclastogenesis (**Figure 2**). In fact, the critical direct contribution of RANKL to breast tumor initiation and distant metastasis has now been demonstrated *in vivo*. During tumor formation, RANKL promotes the increased proliferation and survival of the normal and preneoplastic breast as well as the proliferative and nonproliferative (regenerative) expansion of the mammary stem/progenitor cell.[38–40] In combination, these RANKL-dependent mechanisms could expand the pool of breast epithelial cells undergoing transformation, thereby increasing mammary cancer risk. In support of this hypothesis, early RANKL inhibition profoundly blocks mammary tumor formation.[38,39] In addition, both *in vitro* and *in vivo* studies have indicated that RANKL can also promote distant metastasis via direct effects on RANK-expressing cells (**Figure 2**). In this section, we will review the *in vivo* data supportive of RANKL promotion of distant metastasis, followed by a review of mechanistic data suggesting that RANKL promotes metastasis
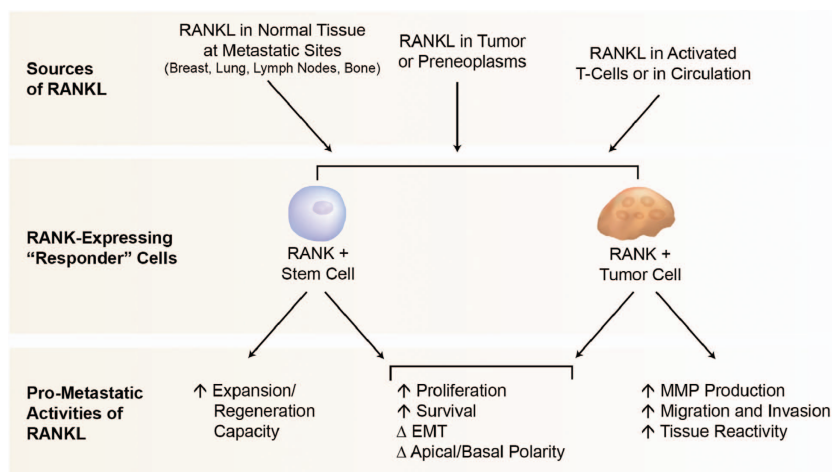
Figure 2 Mechanisms of RANKL-mediated promotion of metastasis. EMT, epithelial-to-mesenchymal transition; MMP, matrix metalloproteinase; RANKL, RANK ligand.

by acting directly on RANK-expressing cells, including tumor cells or a stem/progenitor cell compartment.

Working from the observation that RANKL directly stimulates B16F10 melanoma cell migration *in vitro* (see below), Jones *et al.*[41] tested whether a RANKL inhibitor would block B16F10 metastasis *in vivo* in an experimental bone metastasis model. Significant reductions in B16F10 skeletal metastasis were observed following OPG-Fc treatment, given simultaneously with tumor cell injection. Two lines of evidence from this study support the osteoclast-independent nature of this antimetastatic effect: first, the B16F10 subclone used did not stimulate a substantial osteoclast reaction to tumor cells in the bone; and second, in contrast to OPG-Fc, the bisphosphonate osteoclast inhibitor, zoledronic acid, did not reduce B16F10 bone metastasis. These observations are consistent with a direct role for RANKL in bone metastasis independent of its osteoclastogenic activity. This intracardiac experimental model predominately develops skeletal metastasis, which limits any inference on RANKL function toward metastasis to other sites. Moreover, it is difficult to experimentally dissect the distinct contributions of osteoclast-dependent and -independent effects of RANKL on metastasis within the bone compartment.[42]

Studies on RANKL function in lung metastasis models avoid the experimental challenges of discriminating between the indirect (for example, osteoclast-mediated) vs direct action (for example, acting on RANK-expressing cells) of RANKL function in the bone compartment. Data from these models provide strong evidence for direct promotion of metastasis, distinct from RANKL effects on osteoclasts. For instance, Gonzalez-Suarez *et al.*[38] recently evaluated mouse mammary tumor virus-neu (MMTV-neu) transgenic mice as a model to test the role of RANKL in spontaneous tumor development and lung metastases. In MMTV-neu mice, RANK-Fc treatment did not change median time to mammary tumor formation, but reduced the number of mammary tumors per mouse and significantly decreased spontaneous lung metastases. Tan *et al.*[43] confirmed these findings using complementary methods, observing significantly fewer (50%) spontaneous lung metastases in MMTV-neu mice crossed to the RANK-knockout mice vs MMTV-neu controls.[43] Using an orthotopic mammary tumor cell line (MT2) that spontaneously metastasizes to lungs, Tan *et al.*[43] also showed that treatment of mice with RANKL after MT2 tumor grafting to mammary gland increased the incidence and number of pulmonary metastases. Finally, knockdown of RANK within MT2 cells reduced lung metastases and inhibited the response to RANKL, suggesting that RANKL acted directly on RANK-expressing tumor cells.

'Gain-of-function' experiments *in vivo* have also provided evidence that the intrinsic expression of RANK on tumor cells enhances distant metastasis. For example, overexpression of RANK in MDA-MB-436 breast tumor cells increased the frequency and size of lung metastases after tail vein injection.[44] Similarly, a significantly greater metastatic growth rate in the bone was observed in high RANK-expressing MDA-MB-231 breast cells compared with RANK-negative MDA-MB-231 cells.[45] In both studies, the enhanced metastatic progression of RANK-expressing tumor cells at distant sites was much greater than tumor growth observed upon subcutaneous/orthotopic injection of tumor cells. In addition, knockdown of RANK expression in MDA-MB-231 cells prevented the increase in

bone metastasis caused by activation of the sympathetic nervous system.[12] These observations are consistent with the expression of RANKL at sites of lung and bone metastasis (and not within subcutaneous tumors) and suggest a direct pro-metastatic role for RANKL independent of tumor growth.

There is considerable *in vitro* and *in vivo* evidence for various pleiotropic mechanisms that would explain the direct (osteoclast-independent) stimulation of metastasis by RANKL. Multiple recent studies have demonstrated functional RANK protein expression on the surface of various cancer cell lines, and *in vitro* experiments suggest that RANKL stimulation will directly induce molecular pathways associated with tumor cell metastasis. Exposure of RANK-expressing human breast, lung, melanoma and prostate cancer cell lines to RANKL *in vitro* resulted in increased migration and invasion through collagen or fibronectin matrices.[41,44,46–50] The RANKL-dependent effect is correlated with upregulation of several factors involved in migration, angiogenesis and invasion. For instance, RANKL-dependent changes in MDA-231 breast cancer cells include matrix metalloproteinase-1(MMP1),[46] MMP9, the MMP inducer EMMPRIN/CD47 and vascular endothelial growth factor (VEGF).[51] The RANKL-stimulated migration and invasion of PC3 cells was correlated with increases in MMP9, IL-6, IL-8, VEGF and several members of the CXCL-family of chemokines,[48] whereas migration and invasion of A549 lung cancer cells induced by RANKL was correlated with increases in ICAM-1 expression.[50] Treatment of MT2 mammary tumor-bearing mice with RANKL resulted in decreased levels of maspin, a mammary serine protease inhibitor known to suppress metastases to diverse organs such as the lymph node, liver and lung.[52] In addition, EMT is known to be associated with greater motility, invasion and metastasis, and data suggest that RANKL-mediated EMT may also stimulate metastatic behavior. Constitutive activation of RANK induces EMT in the breast cell lines MCF10A and MDA-MB-436. The ability of RANK overexpression to increase pulmonary metastasis of MDA-MB-436 breast cancer cells was associated with EMT and increased cell migration and anchorage-independent growth.[44]

Potential for Direct Pro-Metastatic Effect of RANKL via Action on Stem Cells

In breast cancer, the recently established role of RANKL in the proliferation and regenerative potential of normal mammary stem cells or early breast epithelial progenitor cells[13,14] suggests that RANKL-dependent activation of a cancer stem cell (CSC) or tumor-initiating cell (TIC) compartment may influence metastatic outcomes (**Figure 2**). The ability of RANKL to control canonical and noncanonical NF-κB pathways in mammary epithelia is also suggestive of a link to CSC,[53] as these latter pathways are functionally linked to breast CSC renewal.[54] Although unproven, it is hypothesized that CSC, given their distinct features from the bulk of proliferating tumor cells, are responsible for metastatic disease and resistance to therapies.[55] RANKL clearly regulates the activity of normal stem and descendant progenitor cells of the breast,[13,14,40] but what is the evidence that this pathway influences breast CSC? After genetic inactivation of RANK within the mammary epithelial lineage, the self-renewal capacity of the Lin-CD24 + CD49fhi stem cell-enriched population isolated from mammary tumors was substantially reduced, correlating with the overall reduction

in mammary tumor incidence observed in these mice after medroxyprogesterone acetate and 7,12-dimethylbenz[α] anthracene treatment.[39] As spontaneous metastases do not occur in this particular model, the association of this stem cell-enriched population with metastatic potential could not be determined. Using the experimental model of RANK over-expression in breast cells, Palafox *et al.*[44] observed increased expression of breast CSC and basal/stem cell markers and functional effects consistent with enhanced stem cell activities. Overexpression of RANK in immortalized but nontumorigenic MCF-10 cells increased the frequency of CD44 + CD24 − cells and the fraction of cells expressing either CD133 or CD10, phenotypic markers consistent with a stem cell-like fraction.[42] RANK overexpression in MCF10A cells confers the ability to grow in an anchorage-independent manner, a characteristic of tumor or stem cells. Stem cell functionality was also confirmed in RANK-overexpressing MCF-10 cells by demonstrating mammary outgrowth capacity after transplantation *in vivo*. In these experiments, RANK overexpression was also associated with increased tumorigenesis and metastasis suggesting that the observed increases in CSC numbers and activity may be relevant. HER-2 expression, a marker associated with breast CSC in humans[56] or TIC in mice[53] has now been shown to be modulated by the bone microenvironment. In a model mimicking the bone/tumor interactions, a co-culture of osteoblasts and MCF-7 breast cancer cells caused an increase in HER-2 that could be blocked by a RANKL inhibitor. RANKL treatment of MCF-7 cells increased the mammosphere-forming capacity, consistent with an effect on the stem cell activity.

Analysis of Human RANKL Pathway Expression and Association with Metastasis

An association of RANK/RANKL levels in tumors with patient outcome could support the pro-metastatic role of this pathway as described above. In primary breast cancer, RANK expression is more commonly observed in ER − /PR − tumors (50%) than ER + /PR + tumors (18%).[44] Higher RANK levels are positively associated with both higher proliferation index and pathological grade in primary breast cancer.[44] RANK/RANKL mRNA expression levels (determined by reverse transcription (RT)-PCR) were able to discriminate between node-negative and lymph node-positive/distant metastasis patients.[42] Higher RANK mRNA levels in primary breast tumors ($n = 295$) were significantly correlated with shorter overall survival.[57] Similarly, an analysis of primary breast tumors at the time of diagnosis (I-SPY 1 Trial; $n = 149$) demonstrated that higher RANK expression was not only significantly correlated with poorer recurrence-free survival but was also selectively associated with bone-dominant vs non-bone-dominant recurrence.[58] Using RT-PCR, analysis of primary renal cell carcinoma (RCC) samples ($n = 96$) indicated that high expression of RANKL or RANK mRNA was correlated with a significantly shorter disease-free survival and shorter bone metastasis survival.[59] In addition, lower OPG levels were significantly associated with poorer bone metastasis survival.

One study has reported that 89% of bone metastasis samples (total $n = 74$, representing at least 13 different tumor types) expressed RANK protein using immunohistochemistry.[60] Santini *et al.*[60] also indicated that the incidence of RANK protein expression was very high (68%) in primary tumor samples and

the degree/incidence of RANK expression was not significantly different when compared between primary and bone metastatic samples. Although this study reports RANK protein expression in a variety of primary tumors and bone metastases, the extent of expression observed (for example, 65% for primary breast tumors) is considerably higher than that reported by our own groups (<20% for ER + /PR + and <50% for ER − /PR − primary breast tumors).[38,44] It will be important to resolve whether these discrepancies are due to differences in the specificity of immunohistochemistry achieved with different reagents/methodologies, independent scoring systems or differences in tumor histotype distributions analyzed in the different studies.

Interestingly, with the exception of the RCC study,[59] none of the aforementioned studies showed any association of primary tumor RANKL levels, as single variables, with patient outcome. This may reflect relatively low levels and/or infrequent incidence of RANKL expression in primary tumors or limitations in mRNA detection for RANKL using different mRNA detection platforms. The progesterone-dependent regulation of RANKL in breast also indicates that RANKL levels will be dynamically regulated over the menstrual cycle. Alternatively, RANKL levels in the primary tumor may not be an important driver of metastasis, as high baseline RANKL levels are observed in specific tissue sites of distant metastases, such as peripheral lymph nodes, lung and bone.[61] Activation of the β2 adrenergic receptor, common in chronic stress and depression, increases RANKL levels in osteoblasts/bone stroma as well as in the lung and liver tissue.[12] Altogether, these sources could plausibly provide a local pro-metastatic signal to RANK-expressing cells. The higher functional expression of RANK in the primary tumor may therefore be a more critical determinant of metastatic functionality conferred by the RANKL/RANK pathway. Although there are reports of soluble RANKL in serum of cancer patients, it is still not clear whether this provides a relevant pathophysiological signal for metastasis. Comparative analysis of circulating vs tissue RANKL has indicated that the systemic and local regulation of RANKL production are discrete processes,[62,63] suggesting that the local source of RANKL may be more relevant for metastatic disease.

Clinical Bone Metastasis Studies with Denosumab

Denosumab is a fully human IgG2 monoclonal antibody that binds human RANKL with high affinity.[1] In an integrated analysis of three randomized double-blind phase 3 trials, denosumab was shown to be superior to the prior standard of care, zoledronic acid, for the prevention or delay of skeletal complications in patients with advanced cancer and bone metastases.[64] In an additional study in men with nonmetastatic castration-resistant prostate cancer, denosumab prolonged bone metastasis-free survival (hazard ratio (HR) = 0.85; 95% confidence interval (CI): 0.73, 0.98; $P = 0.028$) and delayed the time to first bone metastasis (HR = 0.84; 95% CI: 0.71, 0.98; $P = 0.032$) compared with placebo.[65] This is the first clinical study successfully demonstrating that targeting the bone compartment makes this microenvironment less hospitable for metastatic establishment and progression. Taken together, these clinical data demonstrate that RANKL inhibition can block osteoclast-mediated bone destruction and

provides a potential new approach for the treatment of bone metastases.

The abovementioned clinical studies were designed to address skeletal effects and were not designed to test an anticancer effect of denosumab. Interestingly, a *post hoc* analysis indicated that treatment with denosumab was associated with improved overall survival compared with zoledronic acid in a subgroup of patients with lung cancer from the phase 3 trial of denosumab versus zoledronic acid for the prevention of skeletal-related events (SREs) in patients with bone metastases from advanced solid tumors or multiple myeloma.[66,67] The significantly improved survival observed among patients with advanced lung cancer who received denosumab could be explained by a greater decrease in bone turnover achieved with denosumab compared with zoledronic acid. In addition, the mechanisms described in this review, such as interruption of tumor cell interactions in the bone microenvironment or direct inhibition of RANKL action on RANK-expressing lung tumor cells, may also have contributed to this finding.[67]

Conclusions and Future Directions

In summary, preclinical studies have demonstrated the central involvement of RANKL in the establishment and progression of the bone tumor in addition to tumor-induced bone destruction. Clinical studies have extended these observations and proven the causal role of RANKL-mediated osteoclastogenesis in skeletal complications in patients with existing bone metastases as well as the establishment of new bone metastases. In addition to expression within the bone stroma and certain tumor types, RANKL is now known to be expressed within osteocytes or activated T cells, although the specific contribution of RANKL within these cell types to bone metastasis is yet to be established. Beyond the well-characterized ability to reduce skeletal metastasis, a review of the recent literature provides evidence, with both orthotopic and transgenic mouse spontaneous metastasis models, that RANKL inhibition reduces nonskeletal metastasis.

Future preclinical studies will be important to determine whether any of the individual pro-metastatic mechanisms directly induced by RANKL on RANK-expressing cells *in vitro* would explain the profound ability of RANKL inhibition to block distant metastasis to nonskeletal sites *in vivo*. Alternatively, the direct pro-metastatic effect of RANKL may be due to multiple, pleiotropic RANKL-dependent responses acting in concert on RANK-expressing cells. The various effects, including increased migration/invasion, increased EMT induction, increased MMP production, increased levels of angiogenic factors, decreased levels of protease inhibitors, increased survival of a metastatic stem cell and so on would be predicted to enhance metastatic behavior independent of tissue site. Although it is clear that RANK expression on tumor or stem/progenitor cells is necessary for the pro-metastatic stimuli, the relevant source(s) of RANKL is still under investigation. Acting in an autocrine manner, RANKL may stimulate tumor cell migration and invasion of RANK-expressing cancer cells, as reported for prostate cancer C42b cells *in vitro* for instance.[68] This autocrine effect may be most relevant in tumor types that frequently express RANK and RANKL, such as lung adenocarcinoma.[69] Further analysis of RANK and RANKL expression

in primary or metastatic sites, using well-validated reagents, may provide additional insights into the relationship of this pathway and metastatic outcomes in patients and molecular mechanisms that influence RANK and RANKL expression.

In breast cancer, preclinical observations suggest that RANKL promotes metastasis via two distinct but overlapping mechanisms: stimulation of the tumor-induced osteoclastogenesis-associated bone metastasis and stimulation of RANK-expressing tumor cells. In addition, the newly defined role for RANKL in normal stem and progenitors cells of the breast, along with the known ability of RANK signaling to regulate canonical and noncanonical NF-κB pathways associated with self-renewal and activity of CSCs, has prompted the hypothesis that the profound antimetastatic effect of RANKL inhibition observed in the MMTV-neu mammary cancer models *in vivo* may also include mechanisms acting via decreased activity or survival of the TIC or metastasis-initiating cells.

The efficacy of RANKL inhibition has been clearly demonstrated in the prevention of SREs in patients with bone metastasis caused by a variety of tumors, but given the breast-specific effects of RANKL, breast cancer may provide a unique opportunity to investigate both osteoclast-independent and -dependent pre-metastatic activities of RANKL. Currently, denosumab is being tested to determine whether RANKL inhibition prolongs bone metastasis-free survival and disease-free survival in the adjuvant breast cancer setting.[70]

Conflict of Interest

WD is an employee of Amgen Inc. The remaining authors declare no conflict of interest.

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