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PERSPECTIVES

The Many Roles of RANKL-RANK Signaling in Bone, Breast and Cancer

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Abstract

Receptor activator of nuclear factor-κB ligand (RANKL), its signaling receptor RANK, and its natural decoy receptor OPG are members of the tumor necrosis factor (TNF) and TNF receptor superfamily and are best known for their essential role in controlling osteoclastogenesis. RANKL in bone has also been shown to serve as a chemoattractant for cancer cells, thus explaining the tropism of certain cancers such as breast and prostate cancer to preferentially metastasize to bone. Surprisingly, studies of genetically engineered mice demonstrated that RANKL-RANK signaling is also required for proper formation of a lactating mammary gland and, intriguingly, the development of mammary cancer. RANK-deficient mice show a markedly delayed development of hormone- and oncogen-driven tumorigenesis and RANKL-RANK signaling is required for the proliferation and survival of cancerous mammary epithelial cells. Here we review the physiological functions of RANKL-RANK and how this system might be a key to understanding breast cancer. *IBMS BoneKEy*. 2011 May;8(5):237-256.

The Classical Role of the RANKL-RANK-OPG Axis in Bone Remodeling

The discovery of three tumor necrosis factor (TNF) and TNF receptor family members revolutionized the understanding of bone biology since these molecules were shown to regulate osteoclastogenesis in normal bone homeostasis and also during the development of bone pathologies such as osteoporosis and arthritis. These molecules were the receptor activator of NF-κB ligand (RANKL) (1-4), its cognate signaling receptor RANK (1), and its natural decoy receptor osteoprotegerin (OPG) (5-7).

OPG (also called osteoclastogenesis inhibitory factor (OCIF), TNFRSF11B, TR1, or FDRC1) was the first molecule from this TNF receptor family to be discovered and was shown to inhibit osteoclastogenesis *in vitro* and *in vivo* (6;7). OPG lacks the typical transmembrane-spanning domain of TNF receptors, is secreted as a soluble glycoprotein (~55kDa) and forms disulfide-linked homodimers of ~110 kDa. OPG message is found in the brain, liver, lung, heart, kidney, skeletal muscle, skin, intestines, calvaria, stomach, testis, and placenta (5-9). Deletion of OPG from the mouse germline results in early-onset osteoporosis (10;11), demonstrating a critical requirement for OPG in the maintenance of postnatal bone mass. Furthermore, this finding suggested that OPG might neutralize a TNF-related factor that would by itself stimulate osteoclast development. Soon this factor was cloned and named OPG ligand (OPGL) or ODF (osteoclast differentiation factor) (3;4). Later it was realized that OPGL/ODF is identical to a molecule named RANKL and TRANCE (TNF-related activation-induced cytokine) which was first cloned due to its strong upregulation in T cells following antigen receptor stimulation (1;2;4). Throughout this review, we will use the now commonly accepted name for this protein, *i.e.*, RANKL.

RANKL is a type II transmembrane protein containing a membrane-anchoring domain, a connecting stalk, and a receptor-binding

ectodomain. Similar to OPG, RANKL forms stable, non-covalently associated trimers (12;13). Apart from high levels of expression in skeletal and primary as well as secondary lymphoid tissues, RANKL mRNA expression can also be detected in keratinocytes of the skin, mammary gland epithelial cells, the heart, skeletal muscle, the lung, stomach, placenta, thyroid gland, and brain (1;3;4;14-17). Human and mouse full-length RANKL are 317 amino acids and 316 amino acids long, respectively, and share 87% sequence homology (3). Three splice variants have been described in humans and mice, which harbor different N-termini (18;19). The longest isoform, RANKL1, harbors a 48 amino acid-long, N-terminal, intracellular domain, a transmembrane domain (49-71 amino acids) and a 245 amino acid-long extracellular domain. RANKL2 lacks parts of the intracellular domain (amino acids 14-44 of RANKL1) while RANKL3 lacks the whole intracellular domain as well as the transmembrane domain (lacking amino acids 1-118 of RANKL1) and is secreted as soluble protein. Cell membrane-bound RANKL (RANKL1 and RANKL2) can also be cleaved by specific metalloproteinases such as MMP-3, MMP-7, MMP-14, ADAM10, ADAM17 (also called TACE) and ADAM19, an alternative way to generate soluble RANKL of approximately 32-kDa (compared to the membrane-bound 40-45-kDa RANKL) (20-23).

The physiological significance of these different RANKL isoforms and RANKL shedding is still unclear. *In vitro* studies have shown that only RANKL1 is able to induce osteoclastogenesis when expressed on NIH3T3 cells while RANKL2 seems to be inactive (19). Soluble RANKL3, on the other hand, was reported to inhibit osteoclastogenesis when co-expressed with RANKL1 (19). In line with this finding it was shown that soluble RANKL shed by MMP14 inhibits osteoclastogenesis *in vitro* and *in vivo* (24). Mice deficient in MMP14 show decreased production of soluble RANKL by osteoblasts, increased numbers of osteoclasts and an osteoporosis phenotype (24). All these data indicate that soluble RANKL functions to counteract membrane-bound RANKL and to inhibit osteoclastogenesis. In contrast, using a rat model of osteolytic metastatic prostate cancer it has been reported that osteoclasts at the tumor-bone interface increase MMP7 expression, leading to an increase of soluble RANKL, which further promotes osteoclast activation/differentiation and therefore osteolysis (25). Similarly, another group has shown that mammary tumor cells can induce expression of the protease cathepsin G in osteoclasts, which again leads to increased soluble RANKL and increased osteoclastogenesis and osteolysis (26;27). Therefore, soluble RANKL can function as a positive or negative regulator of osteoclastogenesis, probably dependent on the physiological context.

The signaling receptor for RANKL, RANK, was cloned from a bone marrow-derived myeloid dendritic cell (DC) cDNA library enhancing DC survival (1) and, at around the same time, was identified as a signaling receptor involved in osteoclast differentiation *in vitro* (28;29). Human and mouse RANK (TNFRSF11A, OFE, ODFR, TRANCE-R, ODAR, CD265) are type I transmembrane glycoproteins of 616 and 625 amino acids, and comprise signal peptides of 29 and 30 amino acids, extracellular domains of 183 and 184 amino acids, transmembrane domains of 21 and 20 amino acids, and large cytoplasmic domains of 383 and 391 amino acids, respectively. Since TNF receptors, such as FAS, TNFR1, or TNFR2, generally assemble into trimeric complexes on the cell surface prior to ligand binding, it is inferred that RANK trimerization is a prerequisite for RANKL binding and signal transmission (30-33). RANK mRNA is most abundant in DCs, bone, skeletal muscle, the thymus, liver, colon, small intestine and adrenal glands (1;28;34). RANK protein can be detected on the surface of DCs (1;14;34), $CD4^+$ and $CD8^+$ T cells (35), Langerhans cells (17) and mammary epithelial cells in a dynamic fashion throughout pregnancy, with highest levels at day 15.5 of pregnancy in mice (16;36).

The essential physiological roles of RANKL-RANK were elucidated by knock-out studies

showing that both RANKL and RANK are indeed absolutely essential for the development of osteoclasts – genetic inactivation of RANKL or RANK results in a complete loss of mature osteoclasts leading to severely thickened bones, a disease called osteopetrosis (37-39). Corroborating these mouse studies, loss-of-function mutations in RANK have recently been reported in children suffering from severe osteopetrosis (40). On the other hand, over-activated RANKL-RANK signaling – either through increased expression of RANKL itself or the shortfall of inhibitory OPG – causes the development of too many bone-resorbing osteoclasts and osteoporosis. For example, mice lacking OPG suffer from osteopenia (10;11). Mouse experiments have also shown that injection of OPG leads to a rapid increase and injection of RANKL to a rapid decrease in osteoclast numbers within a few hours (41;42). In humans, activating mutations in RANK have been found in patients suffering from osteolysis (43) and loss-of-function mutations of OPG were found in patients with juvenile Paget's disease, which is characterized by increased bone remodeling leading to skeletal deformity, osteopenia and spontaneous fractures (44). Together these data show that this system is not only required but also sufficient to balance bone homeostasis.

Clinically most relevant were the findings that hyperactivation of the RANKL-RANK axis is largely responsible for the osteoporosis seen in millions of postmenopausal women (45;46). For instance, the female sex hormone estrogen regulates the expression of the "protector of the bone" OPG (47) and loss of estrogen during menopause will also lead to decreased levels of OPG. This results in less RANKL inhibition and a shift towards osteoclastic bone resorption, explaining the gender bias of osteoporosis in postmenopausal women (7). In order to generate targeted therapies to treat osteoporosis, a RANKL-blocking antibody called denosumab has been generated. This antibody recently proved to be beneficial in clinical studies (48-50) and has therefore been approved for women with osteoporosis and for bone-related events in men undergoing chemotherapy to treat prostate cancer.

The RANKL-RANK-OPG Axis and the Interconnection Between Bone Loss, Mammary Gland Formation and Mammalian Biology

Surprisingly, RANKL and RANK were also found to be absolutely essential for the development of a lactating mammary gland and proliferation of mammary epithelial cells during pregnancy (16). Mammary gland morphogenesis proceeds in distinct steps, beginning with a fetal mammary anlage undergoing ductal elongation and branching during puberty. During pregnancy, ductal and alveolar epithelial cells expand and proliferate to increase ductal side branching and facilitate development of lobulo-alveolar structures, the so-called milk-producing units (51). Besides the block in osteoclastogenesis, RANKL- or RANK-deficient mice are unable to develop such lobulo-alveolar structures during pregnancy (16). Interestingly, RANKL expression in mammary epithelial cells is induced by the "pregnancy hormones" prolactin, progesterone and PTHrP, but not estradiol (16). RANKL and RANK protein expression gradually increase during mouse pregnancy, become prominently expressed at around P14.5 to P16.5, respectively, and decrease again thereafter (16;36;52). RANKL expression is localized in the luminal cells of the developing alveolar bud with RANK being predominantly detected in areas where lobular cells are branching (36). These data are consistent with the notion that RANKL-RANK regulate mammary epithelial cell proliferation during lobulo-alveolar morphogenesis in an autocrine/paracrine fashion (16). Thus, RANK and RANKL are expressed in a spatially- and time-restricted fashion and are essential for the development of a lactating mammary gland during pregnancy.

RANKL-RANK have also been implicated recently in mammary stem cell (MaSC) biology. It was shown that fluctuations of endogenous progesterone during the

estrous cycle and pregnancy lead to a pronounced increase of MaSC numbers (53;54). RANKL expression was shown to be induced in ductal luminal cells, while RANK was shown to be expressed on the MaSC-enriched basal cell compartment. Whole body anti-RANKL treatment of pregnant mice thereby reduces the capacity of the MaSC-enriched basal cell population to form colonies *in vitro* (54). Furthermore, experiments in our laboratory showed that expansion of progesterone receptor-negative MaSCs triggered by synthetic progesterone derivates is dependent on RANK expression (55). This notion is also supported by findings from Beleut *et al.* (56) showing that progesterone induces two waves of bulk mammary epithelial proliferation. The first one occurs within 24 hours, affecting progesterone receptor-positive cells, and is dependent on cyclinD1. The second and more prominent wave is triggered solely by RANKL, driving proliferation of progesterone receptor-negative mammary epithelial cells in a paracrine fashion, and lasts for up to 8 days (56). Together, these data suggest that RANKL-RANK could be involved in progesterone-induced expansion of MaSCs during the menstrual cycle and pregnancy. However, the exact functional and genetic relationships between RANKL-RANK and pregnancy hormones, stem cells and cell cycle regulatory molecules await further genetic elucidation *in vivo*.

Subsequent mouse studies showed that RANKL-RANK signaling is also sufficient to trigger mammary gland morphogenesis. For example, forced expression of RANK in mammary epithelial cells also induced mammary epithelial cell proliferation during pregnancy and defective lactation due to impaired differentiation of lobulo-alveolar structures (36). Similarly, overexpression of RANKL was shown to induce proliferation, ductal side-branching and formation of lobulo-alveolar structures even in mammary glands from mulliparous animals (57). Furthermore, when aged, these animals developed hyperplasias within the mammary epithelium due to the massive mammary epithelial proliferation triggered by RANKL (57). In addition, ectopic expression of RANKL was even shown to rescue the defects of progesterone receptor-deficient mammary epithelial cells to form lobulo-alveolar structures (56).

In conclusion, RANKL and RANK are essential and sufficient for the formation of the lactating mammary gland, an organ required for transmission of maternal calcium to neonates in mammals. In evolutionary terms, the formation of a lactating mammary gland is a relatively recent event, starting with the appearance of ancient mammals around 200 million years ago. In terms of evolution it appears that the RANKL-RANK-OPG axis – the master regulator of skeletal calcium release and osteoclastic bone resorption – has a second essential function, namely, the formation of a lactating mammary gland during pregnancy. Our results also provided an unexpected molecular and evolutionary explanation for gender bias and the high incidence of osteoporosis in females. The strong bias toward bone loss in postmenopausal women may be due to the fact that the RANKL-RANK-OPG axis is essential for reproduction and the survival of mammalian offspring.

The Role of the RANKL-RANK-OPG Axis in Cancer

After the RANKL-RANK-OPG triad was established as the crucial mediator of osteoclastogenesis and mammary gland development, it was proposed that this pathway might also play a role in primary and metastatic bone tumors and in breast tumors (58-60). Several studies have underscored this notion with clinical and preclinical evidence. Here, we summarize these studies and discuss new therapeutic interventions targeting RANK signaling in cancer.

Bone tumors

Primary bone tumors and metastatic bone lesions can be divided into osteoclastic and osteoblastic lesions (61). Osteoclastic lesions are characterized by increased bone

resorption dependent on osteoclasts, which leads to local destruction of bone, loss of skeletal integrity, fractures and spinal cord compression, and hypercalcemia as well as bone pain due to a more acidic environment generated by osteoclasts, which then affects pain receptors on neurons (61). Primary bone tumors or metastatic tumor cells stimulate bone resorption either by secreting osteoclastic factors themselves such as RANKL or by influencing the surrounding stroma to express factors like PTHrP, TGF-β, IL-8 or prostaglandin E2 (45;46). These factors have been shown to upregulate RANKL expression in osteoblasts, driving osteoclastogenesis and resulting in local bone destruction (62). Since the bone matrix is very rich in growth factors and cytokines, decomposition of the bone matrix subsequently releases more cytokines and growth factors, further fueling the cycle of tumor cell proliferation and RANKL secretion (61;63). This generates a perfect microenvironment for growth of local tumor cells or even generates a metastatic niche for seeding tumor cells from a breast or prostate tumor, thereby creating a vicious cycle of bone metastasis (58).

Osteosarcoma and metastatic bone lesions from prostate tumors, on the other hand, typically form osteoblastic lesions. These lesions have a sclerotic character with woven and/or osteoid bone formation and lead to an overall increase in bone remodeling and bone volume (64;65). Nevertheless, recent findings support the notion that there is substantial osteolytic activity within osteoblastic tumors. Bone resorption markers like N-telopeptide of type I collagen (NTX), a bone collagen breakdown product, are commonly upregulated in patients with an osteoblastic disease presentation (66). Increased bone resorption within osteoblastic lesions could also be confirmed histologically in humans (67). The importance of osteoclast function in sclerotic prostate bone metastasis could also be shown experimentally in mice (60;68-71) as well as therapeutically in clinical studies (70;72) (reviewed in (73-76)). When compared with healthy individuals, the serum RANKL/OPG ratio as measured by ELISA is significantly increased in patients with osteolytic pathologies like primary bone tumors or bone metastasis from breast, prostate, lung, multiple myeloma and renal cancers (63;77). Importantly, even osteoblastic lesions from some prostate cancers or from primary osteosarcomas have been shown to have lytic components and altered RANKL-RANK-OPG levels (63;78;79).

Osteosarcoma is the most frequent primary bone tumor in adolescents and children and originates from mesenchymal cells (64). RANK expression was reported for several mouse (POS-1 cells) (80) and human osteosarcoma cell lines (MNNG/HOS, Saos-2, MG-63) (81;82) and expression was also seen in about 50% of human osteosarcoma specimens (81). In co-culture experiments it has been reported that the human RANKL-expressing osteosarcoma cell line HOS can induce human blood monocytes to form multinucleated osteoclast-like cells (82). This could also explain the existence of multinucleated giant cells seen in about 3% of osteosarcomas (83) and the increased serum RANKL/OPG ratio in patients with high grade osteosarcomas (63). Further support for the importance of the RANKL-RANK-OPG triad in the pathology of osteosarcoma comes from a recent study in mice. In two osteosarcoma models, therapeutic OPG administration effectively reduced tumor incidence as well as tumor outgrowth and ultimately prolonged survival up to four-fold (84). Interestingly, the effect of OPG did not seem to be a direct effect on the osteosarcoma cells, but rather an indirect effect by inhibiting RANKL produced by the tumor microenvironment (84). Furthermore, using a novel, transgenic model of murine osteosarcoma it was shown that *Prkar1a* functions as a bone tumor suppressor, the loss of which induces RANKL overexpression. Interestingly, *Prkar1a* loss defines a molecular tumor subclass in mouse as well as in human osteosarcomas that display high RANKL expression (85). However, it remains to be shown if RANKL expression in those tumors is causally linked to tumorigenesis and if

RANKL inhibition could harbor a therapeutic effect.

Expression of RANKL-RANK has also been shown in giant cell tumors (GCTs), rare primary osteolytic neoplasms that show massive bone destruction of the epiphysis of long bones and are therefore also known as osteoclastomas (86;87). Mononucleated spindle-like stromal cells are thought to be the neoplastic, proliferating component of the tumor, which attract multinucleated osteoclast-like giant cells, hence promoting bone destruction (88;89). Interestingly, two studies have shown that RANKL is expressed solely by GCT stromal cells, whereas RANK is expressed exclusively by multinuclear osteoclast-like giant cells (88;89), suggesting that stromal RANKL might function as a chemotactic attractant as already reported for mammary epithelial as well as prostate cancer cells (58). Importantly, the first clinical phase II studies have shown that RANKL inhibition has therapeutic effects for patients suffering from GCTs (90). In addition, expression of RANKL has also been shown in neoplastic mononuclear cells of chondroblastoma specimens, another rare aggressive osteolytic bone tumor (91).

Multiple myeloma (MM) is a hematological malignancy affecting plasma cells (92). Plasma cells are terminally differentiated B cells that are generated in the bone marrow and can be found abundantly in the lymphatic system. Their task is to produce large amount of antibodies once they re-encounter the pathogen for which they were sensitized (92). Chromosomal abnormalities such as translocation of the immunoglobulin heavy-chain gene with several oncogenes are frequently seen in MM patients and might be important for tumor initiation and progression (92). After clonal expansion in the bone marrow, the production of pro-osteolytic cytokines like RANKL, IL-1, IL-6, TNF, MIP-1, HGF and PTHrP contribute to the local damage seen in bone (45;46).

Whether RANKL is expressed by neoplastic myeloma cells or the stroma is still a controversial question. While Pearse *et al.* and Giuliani *et al.* could not detect RANKL expression by immunohistochemistry, Western blotting and RT-PCR in myeloma cells (93;94) and argued that upregulation of RANKL in MM patients results from higher expression in the bone environment, Farrugia *et al.* and Sezer *et al.* showed strong cytoplasmic RANKL staining in myeloma cells by IHC and surface staining by flow cytometry, respectively (95;96). However, using microarray technology, Shaughnessy *et al*. could not detect RANKL expression in MM cells of 170 MM patients (97). These discrepancies might be due to the specific antibody used or differences in specimen preparation. Nevertheless, the soluble form of RANKL was also found to be upregulated in MM patients while OPG was downregulated and the RANKL/OPG ratio could be correlated with disease severity and survival (77;98;99). Giuliani *et al.*, for example, have reported that myeloma cells not only activate the bone microenvironment to produce RANKL but also stimulate T lymphocytes to express and secrete RANKL through the direct release of high levels of IL-6 and IL-7 (100). Furthermore, myeloma cells can also down-regulate OPG release from osteoblasts and bone marrow stromal cells as shown in co-culture experiments (93;101). Myeloma cells can bind, internalize and degrade OPG, thereby actively shifting the balance towards osteoclastogenesis and bone resorption (102). In addition, in two studies using a murine MM model, recombinant OPG-Fc, a fusion molecule between the stabilizing Fc part of immunoglobulin and OPG, was shown to inhibit osteolytic bone disease, to reduce tumor burden and to increase survival (103;104). Similar results were obtained in a xenograft MM model using RANK-Fc (93).

Clinical studies with denosumab, a fully human monoclonal antibody directed against RANKL, showed that a single subcutaneous dose of denosumab leads to a rapid and sustained reduction of osteoclastic bone resorption measured by urinary NTX (105) and inhibited skeletal events in phase II and phase III trials without affecting primary tumor burden in MM patients (106-109). Interestingly, MM is not

the only hematopoietic malignancy where RANKL-RANK-OPG may play a pathogenic role. In a recent study of adult T-cell leukemia (ATL), it has been shown that RANKL is highly expressed in ATL cells derived from patients with hypercalcemia, whereas cells from ATL patients with normal calcium levels showed normal RANKL expression (110;111). Thus, RANKL-RANK appear to be involved in skeletal-related events such as bone loss observed in multiple hematological malignancies and further studies will be required to evaluate potential benefits of anti-RANKL therapy.

Breast Cancer

RANKL-RANK interactions have been shown to be essential for the proliferation and differentiation of mammary epithelial cells during pregnancy (16). Moreover, RANKL expression is under the control of female sex hormones (such as progesterone) (16), and the majority of breast cancers (~70%) express steroid hormone receptors (estrogen receptor, progesterone receptor) at the time of diagnosis, which is associated with a pro-proliferative role of these hormones (112). Therefore, we initially speculated that this system might pay a role in hormone-driven breast cancer (16;58-60).

Interestingly, several reports have also shown that RANK is expressed in both primary and metastatic tumors of different origin (113). In particular, RANK overexpression is frequently found in breast cancer patients and breast cancer-derived cell lines (58). While RANKL treatment of various RANK-expressing breast and prostate cancer cell lines did not increase their proliferation, it triggered cytoskeletal changes and induced cell migration towards the source (58). Importantly, inhibition of RANKL with OPG selectively abrogated metastasis and tumor burden in an *in vivo* melanoma model of bone metastases (58). Thus, RANKL may also act as a chemotactic factor for RANK-expressing tumor cells, which might help to shed some light as to why certain cancers show a strong prevalence for bone metastases. In such a model, RANKL would serve as one of the long sought-after “soil” factors that facilitate metastasis to bone (114).

Recently, our group and Gonzalez-Suarez *et al*. have shown that the RANKL-RANK system is also a key regulator of hormone (progestin)- and oncogene (Neu)-driven breast cancer development (55;115). Mechanistically, RANKL promotes the proliferation of mammary gland epithelial cells, rescues these cells from apoptotic cell death after DNA damage, and controls tumor stem cell renewal (55;115). In addition, Gonzalez-Suarez *et al*. and Tan *et al*. could show that RANKL-RANK strongly influence metastases in the Neu-driven tumor model (115;116). In particular, Tan *et al*. showed that infiltrating, regulatory T cells produce RANKL within mammary carcinomas, which, in turn, activates RANK-expressing neoplastic mammary epithelial cells, stimulating metastasis (116).

While OPG expression is confined to ducts undergoing columnar changes in normal breast tissue, OPG has been reported to be expressed in about 55% of breast tumors and in breast cancer cell lines (117-119). In breast tumors, OPG expression as measured by RT-PCR was positively correlated with a low tumor grade and estrogen receptor status (118;119). Interestingly, in addition to its function as a decoy receptor for RANKL, OPG has also been shown to bind to the TNF-related apoptosis-inducing ligand (TRAIL), thereby protecting breast cancer cell lines from TRAIL-induced apoptosis (117;120). This may explain how breast tumor cells evade TRAIL-induced elimination by the host immune system and how they survive in the bone, protected by BMSC-secreted OPG. Furthermore, it has been shown that MCF-7 cells overexpressing OPG form bigger tumors when transplanted orthotopically into the mammary fat pad or into the tibia of a mouse (121). However, this increased tumor growth was not due to decreased TRAIL-dependent apoptosis but due to increased proliferation (121). This is in contrast with reports of therapeutically administered recombinant OPG-Fc, where OPG leads to

reduced breast tumor growth and limited osteolysis in mice (59;121). These data suggest that OPG might serve tumor-promoting as well as tumor-suppressive functions within breast tumors.

In clinical phase I and II trials, denosumab also led to a rapid and sustained decrease in bone resorption as measured by urinary NTX/creatinine levels in breast cancer patients (105;108;122-124). In the first published phase III trial of patients with hormone receptor-positive, non-metastatic breast cancer and low bone mass, administration of denosumab resulted in consistent significant increases in bone mineral density (BMD) versus placebo. Twice-yearly administration was enough to observe those effects regardless of patient sub-group or skeletal site (124;125). Results of phase III trials with advanced breast cancer patients with bone metastasis show that denosumab is superior to the common treatment with zoledronic acid in delaying or preventing skeletal-related events and is generally well-tolerated (126;127). Thus, the RANKL-RANK-OPG axis might not only be involved in bone remodeling and mammary gland morphogenesis during pregnancy, but also seems to be functionally linked to breast cancer and breast cancer metastasis.

Prostate Cancer

Similar to breast tumor patients, about 80% of prostate tumor patients will develop bone metastases, which represent a serious complication of the disease and lead to high morbidity and mortality (128;129). However, unlike breast tumors and most other solid tumors, prostate tumors form osteoclastic and even more frequently osteoblastic bone lesions. Accumulating data show that bone resorption by osteoclasts plays an important role even in osteosclerotic lesions and in prostate bone metastasis formation (63;78;79;130).

RANK and RANKL expression have been detected in prostate tumor cells (58;68;69;120;130-134) and in primary and secondary prostate tumors (130). Functionally, RANKL has been shown to directly affect proliferation, migration and transcription of tumor suppressor genes in prostate tumor cells (130;131). Expression of RANKL, RANK and OPG is positively correlated with prostate tumor grade and metastatic spread (130;135). Serum OPG levels were also reported to be increased in patients with advanced prostate cancer and it has been proposed that serum OPG levels could serve as a marker of bone metastatic spread in prostate tumor patients (72;135;136). Indeed, Jung *et al.* have shown that serum OPG levels are a strong independent predictor of prostate cancer-related death (72).

In animal models, inhibition of RANKL-RANK by either recombinant or endogenously expressed OPG or by RANKL-Fc inhibited formation of bone tumors after intratibial inoculation of prostate tumor cells and diminished established, tumor-induced osteoblastic lesions (68-70;137). In a rat model of metastatic prostate cancer, Lynch *et al.* (25) showed that osteoclasts at the bone-tumor interface are stimulated to produce and secrete MMP-7, a metalloprotease capable of shedding RANKL from the cell surface of osteoblasts. This soluble RANKL will in turn promote further bone destruction by inducing osteoclastogenesis. Thus, MMP-7-deficient mice have reduced soluble RANKL levels and show decreased tumor-induced osteolysis (25).

Importantly, similar to breast cancer patients with bone metastasis, denosumab was better than zoledronic acid for prevention of skeletal-related events in men with bone metastases from castration-resistant prostate cancer (138).

All these data indicate that the RANKL-RANK-OPG triad has a critical impact on the development of metastasis. One question is whether this is mediated solely through the regulation of osteoclastogenesis and/or whether RANKL exerts additional, cell-autonomous functions in breast, prostate and melanoma cells. Indeed, RANKL stimulation has been shown to enhance chemotactic migration, to increase

proliferation of prostate tumor cells, and to upregulate expression of chemokines, which are known to enhance metastasis, such as GM-CSF, VEGF-A, IL-1, IL-6, IL-8, TNF-α or metalloproteinases such as MMP-9 and ADAM8 (16;58;131). Transformed prostate epithelial cells also show reduced expression of the metastasis suppressor gene Maspin after RANKL stimulation (139), further paving the way for metastasis. However, proper genetic models are needed to assess the function of RANKL-RANK in primary and metastatic prostate cancer.

Conclusion

RANKL-RANK are absolutely essential for osteoclastogenesis as well as the formation of a lactating mammary gland. Furthermore, RANKL-RANK signaling not only plays a central role in various bone pathologies but is also functionally linked to primary breast as well as prostate tumors and the formation of bone metastases. Clinical studies have already proven the therapeutic benefit of RANKL inhibition. Whether RANKL inhibition can also be used for prevention of breast cancer, as predicted from mouse studies, now needs to be determined in clinical trials.

Conflict of Interest: Dr. Penninger reports that IMBA, his host institute, is planning to submit a patent on blocking RANKL/RANK for future treatment/prevention of breast cancer, and that he owns shares in Amgen, a company that developed RANKL-blocking antibodies. Dr. Schramek: none reported.

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