Receptor activator of nuclear factor-κB ligand (RANKL)/ RANK/osteoprotegerin system in bone and other tissues (Review)

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Abstract. The receptor activator of nuclear factor-κB ligand (RANKL)/RANK/osteoprotegerin (OPG) system was identified in the late 1990s, ending the search for the specific factors expressed by osteoblasts and stromal cells in order to regulate osteoclastogenesis. The identification of the RANKL/RANK/OPG system was a breakthrough in bone biology; however, the system not only works as a dominant mediator in osteoclast activation, formation and survival, but also functions in other tissues, including the mammary glands, brain and lymph nodes. Evidence has indicated that the existence of the RANKL/RANK/OPG system in these tissues suggests that it may have specific functions beyond those in bone. Disorders of the RANKL/RANK/OPG system are associated with certain human diseases, including postmenopausal osteoporosis, rheumatoid arthritis (RA), bone tumors and certain bone metastatic tumors. Genetic studies have indicated that the RANKL/RANK/OPG system may be a key regulator in the formation of lymph nodes and in the autoimmune disease RA, which further suggests that the immune system may interact with the RANKL/RANK/OPG system. The present review aimed to provide an overview of the role of the RANKL/RANK/OPG system in osteoclastogenesis, bone disease and tissues beyond bone.

Contents

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1. Introduction

In the adult skeletal system, bone is constantly renewed by the removal of old bone and the production of new bone tissue, a process mediated by osteoclasts and osteoblasts (1). This process requires the removal of trenches and tunnels of bone from the surfaces of trabeculae and cortical bone by osteoclasts (2). Osteoblasts subsequently fill these trenches by generating novel bone matrices within them (3). Regulation of the proliferation and activation of osteoclasts and osteoblasts results in modulation of the levels of bone resorption and formation (1). Various diseases, including osteoporosis, Paget's disease and rheumatoid arthritis (RA), may disturb the normal bone balance (4). Osteoclasts have a significant role in diseases that induce bone loss. The discovery of the receptor activator of nuclear factor-κB ligand (RANKL)/RANK/osteoprotegerin (OPG) signaling pathway in the late 1990s was a breakthrough in the elucidation of the regulatory mechanisms underlying osteoclastogenesis and bone resorption (5,6). The binding of RANKL to its receptor, RANK, results in the fusion, differentiation, activation and survival of osteoclasts (6).

OPG functions as a decoy receptor for RANKL, which prevents RANKL binding to RANK. OPG is therefore considered a protective factor against bone loss (7). During the last few decades, studies investigating the RANKL/RANK/OPG system have confirmed that the RANKL/RANK/OPG system is a master regulator of bone resorption (4). The RANKL/RANK/OPG system also has functions in other tissues, including as an immunomodulator and thermoregulator (8,9).

2. Identification of RANKL/RANK/OPG

Prior to the 1990s it was known that osteoclasts originated from mononuclear precursors in the myeloid lineage of hematopoietic cells, which also produce macrophages. Osteoclast progenitors (OCP) are able to differentiate into osteoclasts following macrophage-colony stimulating factor (M-CSF)

expression by osteoblasts/stromal cells. The phenomenon that mice developed osteopetrosis due to a lack of osteoclasts was identified in M-CSF knockout mice, which indicated that M-CSF was essential to osteoclastogenesis (10). However, M-CSF expressed by osteoblasts and stromal cells is unable to generate mature osteoclasts from osteoclast progenitor cells alone, indicating that additional factors are also essential for this process (6,11). These additional factors, expressed by osteoblasts, stromal cells or other cell types, remained elusive prior to the discovery of the RANKL/RANK/OPG system. The system was discovered independently by various groups using differing approaches (6,11,12).

OPG. The discovery of OPG was made independently by two groups. In 1997, Simonet *et al* (7) in the USA identified a novel molecule when they analyzed complementary DNA from the mouse intestine. The group subsequently revealed that transgenic mice overexpressing the OPG gene developed osteopetrosis due to a decrease in the number of osteoclasts, which confirmed that OPG had a significant role in osteclastogenesis.

Almost simultaneously, Rodan and Martin (13) in Japan reported the same molecule independently. However, they had identified the molecule via an alternative approach. In the early 1980s, Rodan and Martin (13) hypothesized that osteoblasts regulated osteoclast formation, and that factors expressed by osteoblasts within bone are produced in response to known stimulators of bone resorption. Subsequent studies supported this hypothesis; however, the specific factors regulating osteoclastogenesis remained elusive. Rodan and Martin continued their search for these factors until they isolated a molecule that inhibited osteoclastogenesis, which was revealed to be identical to the molecule identified by Simonet *et al* (7). The discovery of OPG facilitated further studies into the mechanisms underlying osteoclastogenesis.

OPG is a member of tumor necrosis factor (TNF) receptor superfamily (TNFRS), also known as TNFRS member 11B (TNFRS11B) or osteoclastogenesis inhibitory factor (OCIF) and has a similar domain to CD40; therefore, it is able to bind to CD40 ligand. The original OPG comprises a 401 amino acid peptide with a 21-amino acid propeptide that is cleaved, resulting in a mature protein of 380 amino acids (7,14). In contrast to other members of the TNFRS, OPG lacks transmembrane and cytoplasmic domains and is highly expressed in soluble form in order to function as a decoy receptor for RANKL (7). OPG is expressed in numerous types of tissue, including the adult lung, heart, kidney, liver, thymus, lymphnodes, bone marrow, osteoblasts, vascular smooth muscle cells, B-lymphocytes and articular chondrocytes (15,16). However, the function of OPG in a number of these tissues has remained elusive.

Transgenic mice overexpressing OPG exhibited osteopetrosis, in contrast to OPG-deficient mice, which developed osteoporosis (16). The osteoprotective role of OPG was further confirmed by the identification of a 100-kb homozygous deletion of OPG in juvenile Paget's disease and an inactivating deletion in exon three of OPG in idiopathic hyperphosphatasia (17).

RANKL. OPG was immediately used as a probe to identify its ligand by the two groups who first reported the existence of OPG. They named the ligand OPG ligand (OPGL) and osteoclast differentiation factor (ODF), respectively (6,11). At present, OPGL/ODF is more commonly known as RANKL.

RANKL is a member of the TNF family and is highly conserved between species (7). Human RANKL is closely associated with TNF-related apoptosis-inducing ligand and Fas ligand, sharing ~34% and ~28% sequence homology, respectively (6).

The RANKL gene is located on human chromosome 13q14 and spans ~36 kb of genomic DNA, comprising six exons (8).

Human RANKL is a 317-amino acid peptide, which forms a 45 kDa membrane-associated protein. The soluble protein form of RANKL has a molecular weight of 31 kDa and is cleaved by matrix metalloproteinases (MMP)3 or 7 or a disintegrin and metallopeptidase, rendering it soluble but less active (18).

RANKL is expressed in numerous tissues, including bone and bone marrow, as well as lymphoid tissues (lymph nodes, thymus, spleen, fetal liver and Peyer's patches), mammary ligands and the brain, which suggested potential functions beyond those in bone (8,19). RANKL is a key regulator of osteoclastogenesis and mice lacking RANKL developed osteopetrosis due to osteoclast deficiency (20). The presence of M-CSF and RANKL was demonstrated to be necessary and sufficient for the complete differentiation of osteoclast precursor cells into mature osteoclasts (6,11). The expression of RANKL by synovial cells and activated T cells in RA contributes to bone loss and inflammation of the joints, characteristic of the disease (21). Therefore, RANKL presents a potential therapeutic target against bone destruction in RA.

RANK. Following the discovery of OPG and RANKL, the receptor of RANKL required identification. However, the receptor for RANKL had previously been identified as RANK (8). Human RANK is an amino acid peptide, comprising 616-amino acids, an N-terminal extracellular domain and a large C-terminal cytoplasmic domain, as well as a 28-amino acid signaling peptide and a 21 amino acid short transmembrane domain (8). RANK is primarily expressed by cells of the macrophage/monocyte lineage, including preosteoclastic cells, T and B cells, dendritic cells and fibroblasts (8,22). RANK is highly expressed on the surface of osteoclast progenitors and mature osteoclasts, which are able to translate osteoclastogenesis signals by binding to RANKL (6). A RANK knock-out mouse model developed osteopetrosis and exhibited an absence of osteoclasts (23). RANKL/RANK signaling has been extensively studied following the discovery of the ligand and its receptor (24,25). There are multiple pathways involved in the transduction of RANKL/RANK signaling in osteoclastogenesis, including nuclear factor-κB (NF-κB), c-Jun N-terminal kinase (JNK)/activator protein 1 (AP-1), c-Myc and calcinerin/nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) (26,27). Three pathways participate in the activation and survival of osteoclasts: Src, p38 and extracellular signal-regulated kinase (ERK) (28). Adaptor molecules, including growth factor receptor-bound protein 2 (Grb2) and TNF receptor-associated factor (TRAF)2/5/6 also mediate RANKL/RANK signaling (28).

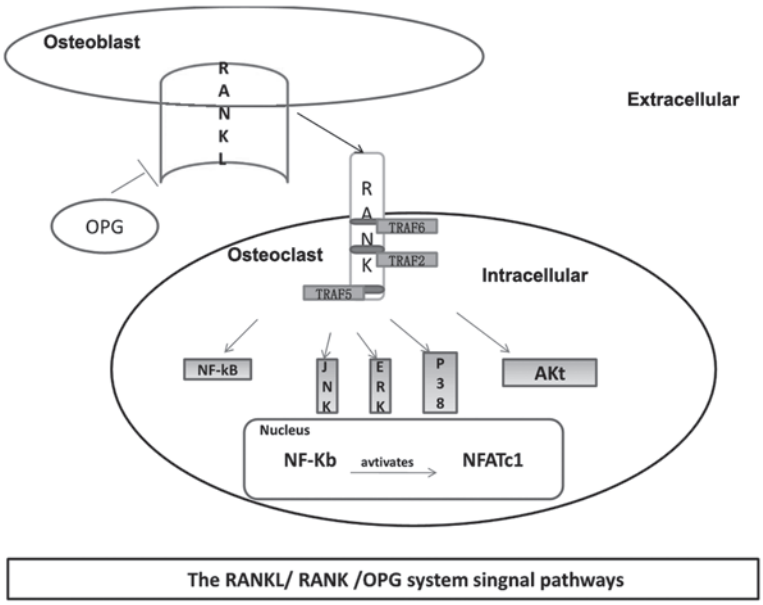

Figure 1. Major signaling pathways involved in osteoclastogenesis. Osteoblasts produce RANKL following the binding of RANKL to RANK on the surface of osteoclastic precursors, and TRAF2, 5 and 6 to the RANK cytoplasmic domain. Subsequently, multiple molecules are activated, including JNK, p38, ERK, Akt and NF-κB. Activated NF-κB translocates into the nucleus and interacts with NFATc1 to trigger osteoclastogenic gene transcription. TRAF, tumor necrosis factor receptor associated factor; RANK, receptor activator of nuclear factor-κB; RANKL, RANK ligand; JNK, c-Jun N-terminal kinase; p38, protein 38; ERK, extracellular signal-regulated kinase; NF-κB, nuclear factor κB; NFATc1, nuclear factor of activated T-cells, cytoplasmic 1; OPG, osteoprotegerin.

3. RANKL/RANK/OPG signaling in osteoclastogenesis

As described above, the binding of RANKL to RANK leads to osteoclastogenesis, which may be blocked by OPG acting as a decoy receptor for RANKL. The signaling pathways underlying RANKL/RANK have been extensively studied, and multiple pathways are involved in mediating RANKL/RANK signal transduction (Fig. 1).

RANK, like other TNF family receptors, has no intrinsic protein kinase activating capacity to mediate signaling; therefore, adaptor molecules are required to bind to the intracytoplasmic domain of RANK (29). These adaptors include TNF receptor-associated factors (TRAFs), which bind to specific sites in the cytoplasmic domain of RANK and activate downstream molecules (25). TRAF2, 5 and 6 are the major TRAFS that bind to RANK; however, only TRAF6 appears to influence RANK signaling. This is based on the observation that in mice with deficiencies in TRAF2, 5 or 6, respectively, only TRAF6-deficient mice developed osteopetrosis (24). Of note, two separate groups identified osteopetrosis in TRAF6-deficient mice; one mouse model presented with a normal number of osteoclasts which were not active, whereas the other was found to have no osteoclasts (30,31). The reasons underlying the development of these two distinct phenotypes of TRAF6-deficient mice identified have remained elusive. Another adaptor is Grb2-associated binder (Gab) protein 2, a member of a protein family characterized by phosphorylation at tyrosine residues, which recruit signaling molecules containing Src homology-2 domains (32).

A preliminary step in the induction of downstream signaling following RANKL ligation to RANK is the binding of TRAFs to specific RANK motifs (Motif1, -2 or -3) on the cytoplasmic domain of RANK (33). Motif1 activates NF-κB and three mitogen-activated protein kinase pathways (JNK, ERK and p38) in response to RANKL stimulation. The existence of a protein complex containing TRAF6, TGF-β-activated kinase 1 and adaptor protein TAB2 is required for the activation of these pathways (25,33). RANKL/RANK additionally activates the Akt/PKB pathway via a complex comprising c-Src and TRAF6 at Motif1 (34). Therefore, following the binding of TRAFs to RANK multiple signaling pathways are activated. The majority of the signaling pathways associated with osteogenesis involve NFATc1. The binding of RANKL to RANK on the surface of osteoclast precursors recruits the adapter protein TRAF6, resulting in the activation of NF-κB via the phosphorylation and inactivation of inhibitory κ kinases (IKKs) and NF-κB inhibitory kinase (25). The binding of TRAF2 to TNFR triggers the activation of JNK; furthermore, interleukin (IL)-1 binds to IL-1 receptor and recruits MyD88, activating p38/ERK. These processes subsequently interact with the NFATc1 promoter to induce NFATc1 auto-amplification of NFATc1 and the subsequent transcription of specific genes, which mediate the completion of the differentiation process (27,35).

The binding of TRAF6 to RANK was found to increase intracellular calcium levels, which activated calcineurin and dephosphorylated and activated NFATc1, resulting in its translocation to nuclei in order to form a ternary complex with c-Fos and c-Jun at the NFATc1 gene promoter, inducing NFATc1 expression (27). Cyclosporine A, a calcineurin inhibitor and immunosuppressant, inhibits NFATc1 activation (36). Patients using immunosuppressants developed bone loss, which was mainly attributed to the inhibitory effects of immunosuppressants on osteoblasts (36). Osteoblasts regulate osteoclastogeneis via the expression of OPG and RANKL. The expression of OPG in osteoblasts is regulated by certain hormones, cytokines and the Wnt/β-catenin pathway (37). The Wnt/β-catenin pathway also regulates bone formation and differentiation in

osteoblasts. The jagged1/notch1 pathway in osteoblasts negatively mediates osteoclastogenesis by decreasing the number of osteoclast progenitors and decreasing RANKL expression in stromal cells (38). In conclusion, bone mass is regulated by osteoclasts and osteoblasts, and at least three pathways are involved in the regulation exerted by osteoblasts, including RANKL/RANK, Wnt/β-catenin and jagged1/notch1.

4. RANKL/RANK/OPG system interacts with the immune system

Bone was previously thought to be a stable inactive organ which provided support for muscles, and protected vital organs and hematopoietic marrow (39). However when bone modeling and remodeling was identified, the key regulators underlying these processes were identified to be bone resorption and formation by osteoclasts and osteoblasts, respectively (40). Bone marrow has long been known for its hematopoietic function in the generation of blood and immune progenitor cells. However, further information regarding the association between bone and the immune system remained elusive prior to the discovery of the RANKL/RANK/OPG system. Osteoblasts, stromal cells and T cells express RANKL and secrete M-CSF and TNF simultaneously. The binding of RANKL and M-CSF to RANK and colony stimulating factor 1 receptor, respectively, induces osteoclastogenesis (41). TNF positively influences the process by binding to its receptor. In the immune system, T cells participate in a variety of processes and also secrete cytokines, including TNF, IL-4 and IL-1, which are also involved in osteoclastogenesis. Following the identification of such associations between the immune system and bone, the field of 'osteoimmunology' was created (42).

Over the past decade, rapid progress was made in the field of osteoimmunology, elucidating the interactions and shared mechanisms between the skeletal and immune systems. One example of such an interaction is that between bone and the immune system in patients with RA. RA is an autoimmune disease resulting in a chronic, systematic inflammatory disorder, which may involve numerous tissues and organs, but principally attacks flexible joints causing bone loss (43). It has been demonstrated that activated T cells, osteoblasts and stromal cells in RA joints overexpress RANKL, leading to the activation of osteoclasts, which enhances bone destruction (44). Furthermore, activated T cells may secrete cytokines, including TNF, IL1, IL17 and IL6, which also contribute to bone loss. However, T cells also produce anti-osteoclastogenic cytokines, including interferon (IFN)-γ and IFN-β, which counteract the action of RANKL (26). Of note, IFN-γ and IFN-β inhibit osteoclastogenesis via differential mechanisms. IFN-γ suppresses osteoclastogenesis by inducing rapid degradation of TRAF6 (26). The binding of IFN-γ to IFN γ receptor 1 recruits the signal transducer and activator of transcription (Stat)1, resulting in the activation of Stat1. Activated Stat1 activates the proteasome, mediating the poly-ubiquitination of TRAF6 and therefore inducing TRAF6 degradation (26,45). By contrast, a series of *in vitro* and *in vivo* experiments demonstrated that IFN-β regulates osteoclastogenesis via a negative feedback mechanism involving c-Fos, a critical factor involved in osteoclastogenesis (46). RANKL or RANK knock-out mice not only develop osteopetrosis, but additionally lack lymph nodes (47), which suggests that RANKL/RANK may influence the formation of the lymph nodes, potentially via alterations to the function of lymph node inducer cells. Therefore, the RANKL/RANK system is a critical regulator of lymph node formation (48).

5. RANKL/RANK/OPG system in bone-associated diseases

Osteoporosis. Osteoporosis has a high prevalence amongst the elderly, in particular postmenopausal females and patients receiving glucocorticoid treatment. The major reason underlying the susceptibility of postmenopausal females to osteoporosis is their characteristic lack of estrogen, which has been demonstrated to be a significant regulator of bone density (49). Osteoporosis is characterized by decreased bone mass and density, which may result in an increased risk of fracture (50). Studies have revealed a significant role of RANKL and RANK in osteoporosis, since the number and activation of osteoclasts is responsible for the severity of bone loss in osteoporosis (51). It has been revealed that human bone marrow cells from untreated early postmenopausal females demonstrated higher expression levels of RANKL in comparison to those in an estrogen-treated group (52). Glucocorticoid-induced osteoporosis is also mediated by the RANK/RANKL/OPG system: Glucocorticoids stimulate RANKL expression by osteoclasts and inhibit OPG synthesis, enhancing osteoclast differentiation and proliferation (53).

Rheumatoid arthritis. RA is an autoimmune disease, which induces joint inflammation and bone destruction. Within the inflamed joints, activated T cells overexpress RANKL, thereby contributing to bone loss (54). At the site of bone resorption in patients with RA, synovial T cells express RANKL and an overexpression of RANKL messenger RNA is present, which contributes to osteoclast differentiation and activity (55). Modifying anti-rheumatic drugs which are used in RA treatment reduce the RANKL/OPG ratio and suppress osteoclast formation, cofirming the significance of RANKL and RANK signaling in RA (56).

RANKL/RANK/OPG and bone heredopathia. Disorders of the RANKL/RANK/OPG system may result in osteoporosis or osteopetrosis. Therefore, studies have been performed in order to elucidate the mechanisms underlying bone heredopathia, in particular heredopathia that induces osteoporosis or osteopetrosis.

It was previously reported that two patients with juvenile Paget's disease, an autosomal recessive disorder characterized by enhanced bone remodeling, osteopenia and risk of fractures, had 100 kb homozygous deletions within the OPG gene (57). Furthermore, idiopathic hyperphosphatasia, an autosomal recessive bone disease characterized by an enhanced rate of bone turnover and associated with deformities of long bones, kyphosis and acetabular protrusions in affected children, was found to contain a deletion in exon three of the OPG gene (58).

Certain patients with familial Paget's disease presented with a mutation in exon one of RANK, which resulted in an increase in the activation of RANK-mediated NF-κB

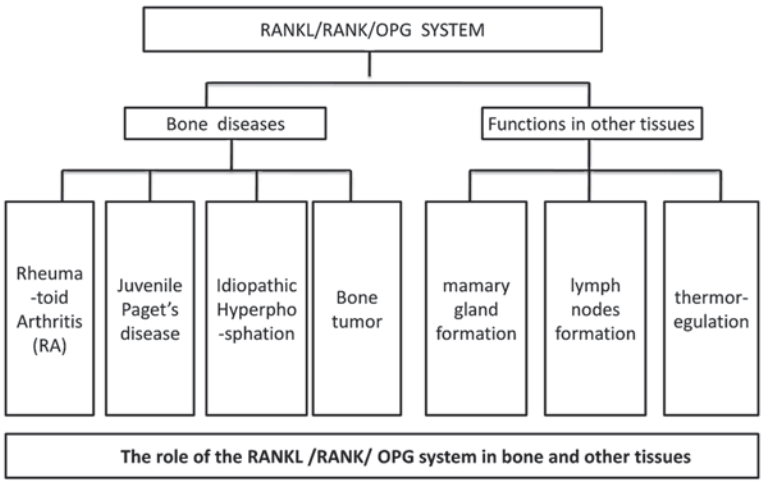

Figure 2. The RANKL/RANK/OPG system is associated with bone diseases and other physiological and pathological conditions. Disorders of the RANKL/RANK/OPG system result in rheumatoid arthritis, juvenile Paget's disease, idiopathic hyperphosphatasia and bone tumors. Furthermore, the RANKL/RANK/OPG system is also required for the formation of certain tissues, including the mammary glands and lymph nodes, and is involved in thermoregulation. RANK, receptor activator of nuclear factor-κB; RANKL, RANK ligand; OPG, osteoprotegerin.

signaling and enhanced the number of osteoclasts and osteolysis (59). Similarly, familial expansile osteolysis, a rare autosomal dominant disorder characterized by focal areas of enhanced bone resorption, contains a mutation in the signal peptide region of the RANK protein (59). In 2007, autosomal recessive osteopetrosis, which is frequently associated with normal or elevated numbers of non-functional osteoclasts, was demonstrated to contain mutations in the gene coding for RANKL (60).

Bone tumors. Certain bone tumors, including multiple myeloma and osteosarcoma, produce not only RANKL but also cytokines, which stimulate osteolysis and bone metastasis (61,62). Breast and prostate cancer are the most common malignant tumors associated with bone metastasis.

The majority of patients with breast or prostate cancer (>75%) experience skeletal complications due to bone metastases (63). Breast cancer cells frequently express parathyroid hormone-related protein (PTHrP), which enhances osteoclastogenesis by increasing RANKL expression on the surface of osteoblasts. This process may result in osteolysis, increasing the release of growth factors, which in turn stimulate the proliferation of tumor cells and increase the production of PTHrP by cancer cells (64). Prostate cancer cells may express RANK and stimulate IKKa activation, which inhibits the expression of maspin, a known metastasis suppressor of prostate epithelial cells (63,65). Therefore, RANKL/RANK is associated with specific bone tumors and metastases.

The functions of the RANKL/RANK/OPG system in bone-associated diseases are summarized in Fig. 2.

6. RANKL/RANK/OPG system beyond bone

The discovery of the RANKL/RANK/OPG system led to experiments involving knock-out mice deficient in RANKL, RANK or OPG. Of note, neither RANK nor RANKL knock-out mice are able to form alveolar mammary gland structures during pregnancy, resulting in an absence of lactating mammary glands and leading to the death of newborn pups (66). Further studies indicated that RANKL expression in the mammary epithelial cells was induced by pregnancy hormones, including prolactin, progesterone and PTHrP. Certain RANKL/RANK downstream pathways regulate the formation of mammary glands, including the IKKa, NF-κB and cyclin D1 signaling pathways (67,68).

RANKL or RANK knock-out mice are not only deficient of mammary glands but additionally lack lymph nodes (20,47). Further studies revealed that the transfer of normal bone marrow to RANKL or RANK knock-out mice did not rescue lymph-node formation, which suggested that the RANKL mutant lymphocytes were not the main cause underlying the defective lymph nodes (48). However, the precise mechanism underlying this phenomenon has remained elusive.

RANKL and RANK are also expressed in tissues other than bone, including lung, kidney, spleen, thymus and mammary glands (69,70). The function of RANKL/RANK in the brain was not elucidated until 2009 (9). RANKL/RANK is localized to the lateral septal nucleus of the hypothalamus in the brain, an area associated with thermoregulation, suggesting a potential role for RANKL/RANK in mediating thermoregulation (9). A series of experiments were performed, which revealed that injection of recombinant RANKL into mice resulted in marked hyperthermia and these RANKL-induced phenomena were attenuated following pre-treatment with OPG (9). By contrast, peripheral intraperitoneal injection of RANKL did not alter body temperature nor activity (9). Therefore, central but not peripheral RANKL/RANK signaling is required to induce hyperthermia. The functions of the RANKL/RANK/OPG system beyond bones are summarized in Fig. 2.

7. Conclusion

The discovery of RANKL/RANK/OPG in the 1990s was a breakthrough in the elucidation of the biology of bone resorption and formation. Disorders of the RANKL/RANK/OPG system result in a variety of diseases, including osteoporosis and RA. RANKL/RANK signaling not only has a significant

role in bone but also functions in other tissues. The system regulates lymph-node formation, mammary-gland development, fever control and certain metastatic tumors.

Since the RANKL/RANK/OPG system functions in numerous tissues and its disorder is associated with multiple diseases, it presents a significant potential therapeutic target. A series of pharmacological experiments have been performed, the results of which indicated that OPG- and RANK-Fc inhibited bone loss in models of sex-steroid deficiency, glucocorticoid-induced osteoporosis, RA, multiple myeloma and metastatic bone disease (71,72). However, OPG- and RANK-Fc have not been taken to further clinical trial stages due to concerns regarding potential side effects on the immune system (43). A promising drug targeting the RANKL/RANK/OPG system is denosumab (73), which is a monoclonal antibody against RANKL. Phase II and III clinical trials of denosumab have not identified any significant side effects (74,75). Furthermore, curative effects have been observed in patients with various bone disorders, including postmenopausal osteoporosis, RA, multiple myeloma and metastatic bone disease (72). However, whether the long-term inhibition of RANKL has any adverse effects remains to be elucidated.

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