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INVITED REVIEW

Discovery of the RANKL/RANK/OPG system

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Abstract
Almost a quarter century has passed since discovery of receptor activator of NF-κB ligand (RANKL). This discovery had a major impact on identification of mechanisms regulating osteoclast differentiation and function, establishment of a research field bridging bone and the immune system (osteoimmunology), and development of a fully human anti-RANKL neutralizing antibody (denosumab). Denosumab is now clinically available for treatment of osteoporosis and cancer-induced bone diseases in the US, Europe and many other countries, including Japan. Denosumab is a so-called blockbuster drug, with sales of 5.0 billion US dollars in 2019. This is a real success story from bench to bedside. In this review, the pivotal roles of the RANKL/RANK/OPG system in osteoclast differentiation and function are shown. RANKL is a ligand required for osteoclast generation, RANK is the receptor for RANKL, and osteoprotegerin (OPG) is a decoy receptor for RANKL. The review covers recent results showing the importance of RANKL on osteoblasts in regulation of osteogenesis and the role of RANKL-RANK dual signaling in coupling of bone resorption and formation, including demonstration of RANKL reverse signaling that we had previously hypothesized. Possible applications of anti-RANKL antibody in treatment of cancer are also discussed.

Keywords Receptor activator of NF-kB ligand (RANKL) · Osteoclasts · Osteoblasts · Reverse signaling · Denosumab · Immuno-oncology

Introduction

Bones are dynamic tissues in which bone resorption and formation are continuously repeated in a homeostatic mechanism of "coupling" between these processes. Osteoclasts are cells that resorb bone, while osteoblasts form bone. The bone volume resorbed by osteoclasts is strictly controlled to be equal to that formed by osteoblasts under normal conditions. If conditions such as menopause and aging cause bone resorption to increase relative to bone formation, metabolic bone diseases such as osteoporosis develop. Thus, there are precise mechanisms that control coupling between bone resorption and formation.

In their pioneering work, Rodan and Martin [1] hypothesized a role of osteoblasts in hormonal control of bone resorption. Osteoblasts are somehow involved in regulation of osteoclastogenesis. Suda et al. [2] advanced this hypothesis and proposed the presence of a hypothetical factor, osteoclast differentiation factor (ODF), that is produced on osteoblasts by bone resorbing factors such as 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$] and parathyroid hormone (PTH). There were many attempts to identify ODF, but all resulted in failure until the late 1990s, until discovery of the RANKL/RANK/OPG system. In this review, an overview of this system is given.

Discovery of three key factors in the molecular mechanism of osteoclastogenesis

Discovery of OCIF/OPG

Mouse coculture system

Until the late 1980s, it was difficult to prepare osteoclasts in in vitro culture, and as an alternative, these cells were directly prepared from bones. Takahashi et al. [3] established a mouse coculture system for osteoclasts, in which

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osteoblasts isolated from mouse calvaria were cocultured with spleen cells containing osteoclast progenitors. Osteoclasts formed only when osteoblasts and spleen cells were cocultured in the presence of $1,25(OH)_2D_3$. Suda et al. [2] hypothesized that direct contact between osteoblasts and osteoclast progenitors was essential for osteoclast differentiation (Fig. 1a). In this hypothesis, osteoclast progenitors are "seeds" and osteoblasts are "soil" that provide a suitable microenvironment for osteoclast formation in bone. Chambers [4] reported a similar hypothesis at almost the same time.

Hypothesis for osteoclast development

In the hypothesis proposed by Suda et al. [2], a membrane-bound factor (ODF) is induced on osteoblasts or

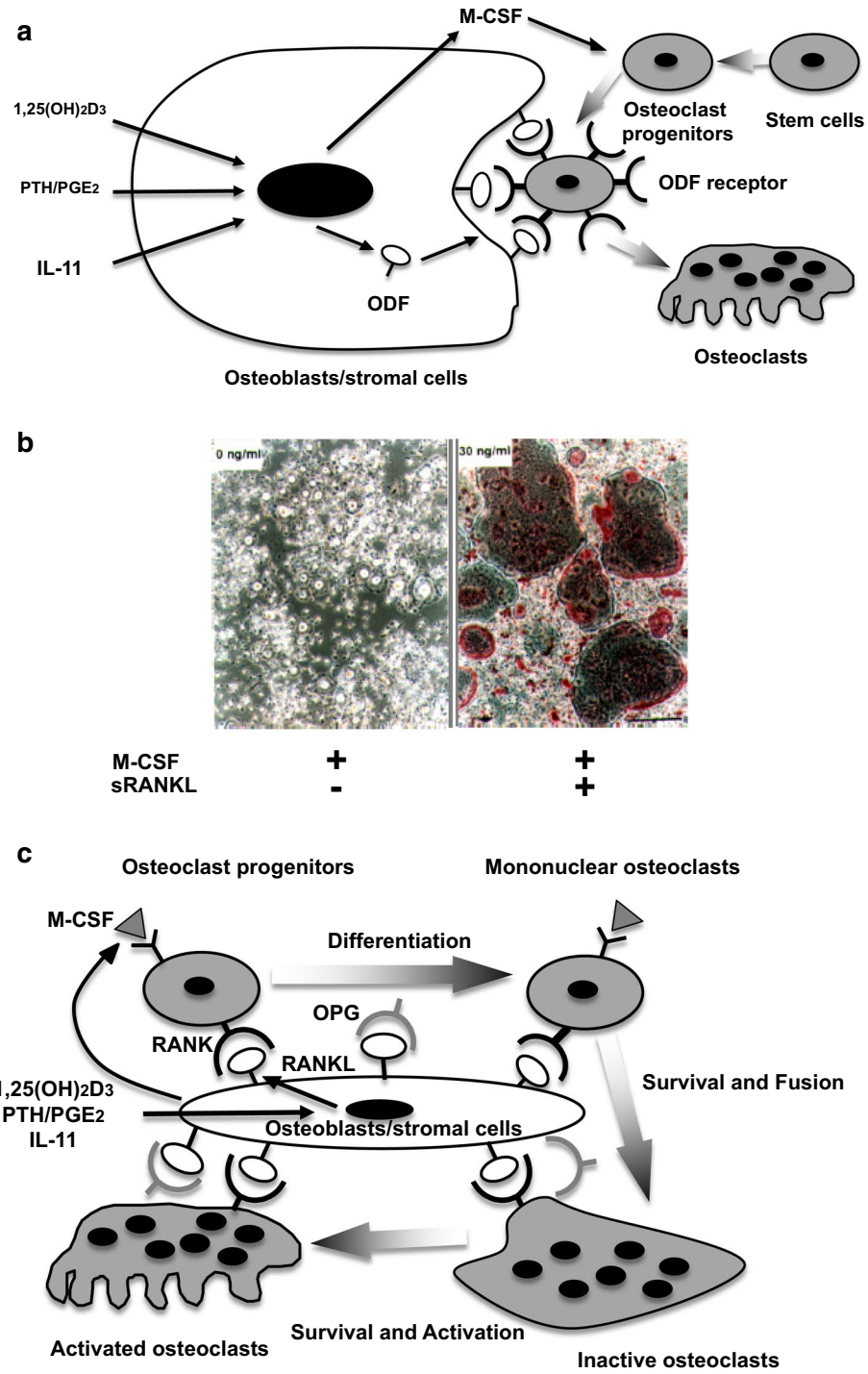

Fig. 1 Mechanisms of osteoclast development. **a** Hypothesis of osteoclast development (before RANKL identification). **b** Osteoclast formation from mouse spleen cells with sRANKL and M-CSF in the absence of osteoblasts (Yasuda et al. Proc Natl Acad Sci USA 1998;95:3597–3602 [14] Copyright (1998) National Academy of Sciences). **c** A model illustrating a mechanism through which osteoblasts/stromal cells regulate osteoclast differentiation and activation (after RANKL identification). *1,25(OH)$_2$D$_3$* 1,25-dihydroxyvitamin D_3, *PTH* parathyroid hormone, *PGE$_2$* prostaglandin E_2, *IL-11* interleukin-11, *ODF* osteoclast differentiation factor, *M-CSF* macrophage colony-stimulating factor, *RANK* receptor activator of NF-κB, *RANKL* receptor activator of NF-κB ligand, *sRANKL* soluble RANKL, *OPG* osteoprotegerin

stromal cells in response to bone-resorbing factors such as $1,25(OH)_2D_3$, PTH, prostaglandin E_2 (PGE_2), and interleukin (IL)-11 (Fig. 1a). Osteoclast progenitors with ODF receptor recognize ODF by cell-to-cell contact and differentiate to osteoclasts. Macrophage colony-stimulating factor (M-CSF) produced by osteoblasts/stromal cells is also required for osteoclast differentiation [5]. ODF is a hypothetical factor that is proposed to be commonly induced on membranes of osteoblasts/stromal cells by bone-resorbing factors such as $1,25(OH)_2D_3$, PTH and IL-11, and to differentiate osteoclast progenitors to osteoclasts with M-CSF through binding to ODF receptor on the progenitors [2].

Isolation and molecular cloning of OCIF/OPG

Tsuda et al. [6] at Snow Brand Milk Products purified and identified a novel factor called osteoclastogenesis inhibitory factor (OCIF). OCIF was purified from conditioned medium of human primary fibroblasts (IMR-90 cells) with a combination of column chromatography and a bioassay using bone marrow cells treated with $1,25(OH)_2D_3$. The main points of the strategy were the suggestion of an inhibitor(s) of ODF in Suda's hypothesis [2]; and the expected presence of the inhibitor in conditioned medium of fibroblasts. Since ODF was a hypothetical factor, there was no inhibitor of the hypothetical factor at that time. Tsuda et al. proposed a new hypothesis, based on the Suda hypothesis. Thus, since fibroblasts are present in the whole body, it seemed unlikely that these cells would produce an inhibitor of osteoclast formation. We now know that IMR-90 cells produce a number of growth factors and cytokines [7].

We subsequently cloned human OCIF cDNA using the amino-acid sequences of the protein and identified it as a novel member of the tumor necrosis factor (TNF) receptor family [8]. OCIF is a secreted protein without a transmembrane domain that inhibits all in vitro osteoclast formation elicited through three distinct signaling pathways stimulated by $1,25(OH)_2D_3$, PTH and IL-11. Administration of OCIF to rats increased bone volume, accompanied by a decrease of active osteoclast number [8]. During preparation of the manuscript (Ref. [8]), Simonet et al. [9] at Amgen independently cloned the same molecule in the expressed sequence tag (EST) cDNA project. The Amgen group found a novel member of the TNF receptor family without functional information and produced transgenic (TG) mice overexpressing the protein to identify its function. The TG mice showed osteopetrosis with inhibition of osteoclast differentiation. The protein was named OPG.

A difference in the strategies at Snow Brand Milk Products and Amgen is that we aimed to identify a hypothetical inhibitor of osteoclastogenesis by purification and bioassay, while Amgen discovered a novel protein in EST cDNA sequencing by chance and identified it as an inhibitor of osteoclastogenesis. It is of note that the same molecule was independently identified at almost the same time using two different strategies. The American Society for Bone and Mineral Research (ASBMR) President Committee on Nomenclature proposed OPG as the name of choice [10]. The physiological role of OPG is apparent through the finding that OPG-knockout (KO) mice exhibit severe osteoporosis due to enhanced osteoclast formation [11, 12]. Thus, OPG is a potent osteoclastogenesis inhibitory factor.

Discovery of RANKL

Evidence for OPG-binding protein

During characterization of OPG, we found evidence for an OPG-binding protein. The mouse stromal ST2 cell line supports osteoclastogenesis from mouse spleen cells with $1,25(OH)_2D_3$ and dexamethasone (Dex) [13]. OPG bound to a single class of high affinity binding sites induced by $1,25(OH)_2D_3$ and Dex in ST2 cells [8]. If the binding sites on treated ST2 cells were occupied by OPG, the cells failed to support osteoclastogenesis from spleen cells. The time course of the increase in binding sites coincided with that of osteoclast formation. These results strongly suggested that the sites were involved in cell-to-cell signaling between stromal cells and osteoclast progenitors, and that OPG inhibits osteoclastogenesis by interrupting this signaling through its binding sites [8]. Cross-linking using radioactive OPG revealed that OPG bound to a 40-kDa protein induced on the treated cells. Taken together, these results raised the possibility that the 40-kDa OPG-binding protein is a ligand for OPG, and is identical to ODF [8]. Since members of the TNF receptor family bind to ligands of the TNF family, we assumed that ODF was a novel member of the TNF ligand family.

Molecular cloning of ODF

To identify the 40-kDa OPG-binding protein, we screened a cDNA expression library of ST2 cells treated with $1,25(OH)_2D_3$ and Dex using radioactive OPG. A cDNA clone encoding 316 amino acids (MW 36 kDa) was isolated [14]. The OPG-binding protein was a novel member of the TNF ligand family, as expected, with a type II transmembrane domain and extracellular C-terminal region. Expression of the gene was independently induced by $1,25(OH)_2D_3$, PTH and IL-11, and the protein was produced on osteoblasts. Coimmunoprecipitation of the protein and OPG complex with anti-OPG Ab showed that the size of the protein was 40 kDa. The soluble form of the protein together with M-CSF induced osteoclast formation from spleen cells in the absence of osteoblasts (Fig. 1b). This was the first time that osteoclasts had been generated from

their progenitors without coculture with osteoblastic cells, the common method for in vitro generation of osteoclasts. The osteoclasts formed numerous resorption pits on dentine slices. Fixed COS7 cells expressing the recombinant protein induced osteoclast formation from spleen cells, suggesting that the protein mediates cell-to-cell signaling essential for osteoclastogenesis. Taken together, the protein satisfied the major criteria for ODF in terms of its biological activity and regulation of its expression by bone-resorbing factors. Therefore, we concluded that the protein was ODF, a long-sought after ligand mediating an essential signal on osteoclast progenitors for their differentiation into osteoclasts [14] (Fig. 1c).

Lacey et al. [15] at Amgen cloned the same molecule, and called it OPG ligand (OPGL), after screening a cDNA expression library of the mouse myelomonocytic cell line, 32D, using fluorescence-activated cell sorting analysis with a fusion protein of OPG and an Fc-fragment (OPG-Fc). Soluble OPGL administered to mice induced rapid and severe hypercalcemia, which was inhibited by OPG treatment. We and others used a similar approach to identify ODF/OPGL as an OPG-binding protein [14, 15]. Contemporaneously, Anderson et al. [16] at Immunex and Wong et al. [17] in academia identified the same molecule regulating T-cell and dendritic cell functions, and called it RANKL and TNF-related activation-induced cytokine (TRANCE), respectively. Anderson et al. [16] reported that RANKL stimulated the activation of NF-κB in dendritic cells and it augmented the ability of the cells to stimulate T cells. In contrast, Wong et al. [17] reported that RANKL (TRANCE) stimulated c-Jun N-terminal kinase (JNK) specifically in cells of the T cell lineage and they suggested a role for RANKL in the regulation of the T cell-dependent immune response. As standard nomenclature for ODF/OPGL/RANKL/TRANCE, RANKL was proposed by the ASBMR President's Committee on Nomenclature [10].

RANKL-KO mice exhibit osteopetrosis with no osteoclasts, marrow spaces, or tooth eruption, indicating that RANKL is essential for osteoclast development [18]. These mice also have profound growth retardation, defects in early differentiation of T- and B-cells, and lack all lymph nodes. These results showed that RANKL is essential for osteoclast development in vivo.

Discovery of RANK

RANKL directly binds to osteoclast progenitors, suggesting that a membrane-bound receptor may be present on the cells [14, 15]. RANK, a novel member of the TNF receptor family, was known to be a receptor for RANKL in T-cell and dendritic cell interaction [16], but the receptor responsible for RANKL-mediated osteoclastogenesis had not been identified. Some ligands of the TNF family bind to several receptors of the TNF receptor family, and it was suspected that RANKL might bind to another member of the TNF receptor family, but not to RANK. We cloned the RANKL receptor from mouse osteoclast progenitors by panning and identified it as RANK [19]. A polyclonal Ab against soluble RANK (sRANK) mimicked RANKL function by clustering of RANK. In contrast, sRANK and a Fab fragment of anti-RANK polyclonal Ab completely inhibited RANKL-mediated osteoclastogenesis by binding to RANKL and RANK, respectively. While OPG inhibited RANKL-mediated osteoclastogenesis by interrupting the binding of RANKL to RANK, it had no effect on anti-RANK Ab-mediated osteoclastogenesis. Taken together, these results provided the first evidence that RANK is the sole signaling receptor essential for in vitro RANKL-mediated osteoclastogenesis and that OPG acts as a decoy receptor for RANKL to compete against RANK [19].

Hsu et al. [20] at Amgen made TG mice overexpressing sRANK and showed that the mice exhibited osteopetrosis, similar to OPG-TG mice, based on which RANK was predicted to be a receptor for RANKL in vivo. Dougall et al. [21] at Immunex found evidence that RANK was the receptor for RANKL in vivo by showing that RANK-KO mice had an almost identical phenotype to that of RANKL-KO mice. A summary of these results is illustrated in a model of osteoclast differentiation and activation (Fig. 1c). RANKL is important for differentiation, fusion, survival and activation of osteoclasts. We know that RANKL on osteocytes and osteoblasts regulates osteoclastogenesis in bone remodeling and modeling, respectively [22, 23].

Genetic and pharmacological models related to RANKL

Genetic models

To investigate the effects and functions of RANKL in vivo, we made TG mice overexpressing soluble RANKL (sRANKL) [24]. The sRANKL TG mice exhibited severe osteoporosis with an increase of osteoclasts. As mentioned above, RANKL-KO mice exhibit osteopetrosis with no osteoclasts [18]. X-ray images of the genetic models including wild-type (WT), sRANKL TG, and RANKL-KO showed a normal status, osteoporosis, and osteopetrosis, respectively (Fig. 2a). Recent studies using mice that specifically lack sRANKL showed that sRANKL is dispensable for physiological bone remodeling [25, 26].

Pharmacological models

Genetic animal models are useful, but several months are needed to interbreed the mice with other TG mice or

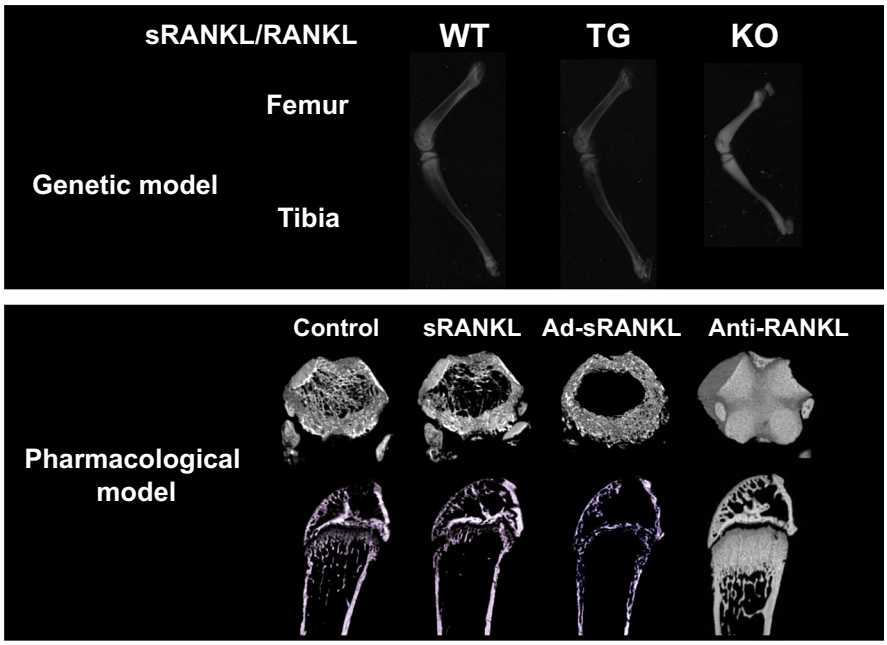

Fig. 2 Bone images in RANKL-related models. **a** X-ray images of genetic models including WT, TG, and KO mice. **b** Micro-CT images of pharmacological models including control, sRANKL-, Ad-sRANKL-, and Anti-RANKL-Ab administration to normal mice. *RANKL* receptor activator of NF-κB ligand, *sRANKL* soluble RANKL, *WT* wild type mice, *TG* sRANKL transgenic mice, *KO* RANKL-knockout mice, *Ad-sRANKL* adenovirus harboring sRANKL

KO-mice. As an alternative, we established three pharmacological animal models: (1) a novel rapid bone loss model by administration of glutathione-S transferase-RANKL fusion protein to mice (sRANKL) [27]; (2) a novel mouse model of hypercalcemia with anorexia by overexpression of sRANKL using an adenovirus vector (Ad-sRANKL) [28]; and (3) a novel mouse model of osteopetrosis by administration of a denosumab-like anti-mouse RANKL neutralizing monoclonal Ab (clone OYC1, Anti-RANKL) [29].

The sRANKL-mediated bone loss model was rapidly established within 24–50 h. Two or three intraperitoneal injections of sRANKL are sufficient to induce osteoporotic bone loss with an increase of osteoclasts. This model is useful for evaluation of pharmaceuticals and/or candidates for treatment of osteoporosis [27]. The Ad-sRANKL injection model showed very severe osteoporosis and hypercalcemia with anorexia. The serum sRANKL level in this model was about 1.5 μg/ml, while that in WT mice was 0.1 ng/ml [28]. In the anti-RANKL Ab-treated osteopetrosis model, a single subcutaneous injection of anti-RANKL Ab increased bone mass with marked decreases in osteoclast surface and number, as well as decreases in osteoblast surface, mineral apposition rate (MAR), and bone formation rate (BFR) after 2 weeks [29]. Micro-CT images of the pharmacological models including control, sRANKL-, Ad-sRANKL-, and anti-RANKL-Ab administration to normal mice showed a normal status, osteoporosis, severe osteoporosis, and osteopetrosis, respectively (Fig. 2b). Two to 14 days are required to make these animal models. These inducible models of osteoporosis and osteopetrosis in normal mice exhibit exact mirror images in terms of the change in bone mass and are useful for research on osteoclast biology and bone metabolism in vivo.

RANKL reverse signaling

WP9QY (W9) is a peptide designed to be structurally similar to one of the cysteine-rich domains in TNF receptor type I, and is known to bind to TNFα and block its activity [30]. W9 also binds to RANKL and inhibits RANKL-induced osteoclastogenesis in vitro and in vivo [31]. We provided the first evidence that W9 enhances osteoblastic differentiation/mineralization in in vitro and increases bone mass in vivo [32]. Histomorphometrical analysis of mice treated with W9 showed that the peptide had a weak inhibitory effect on osteoclast number and surface in distal femoral metaphysis, but markedly increased MAR and BFR in femoral diaphysis. As a RANKL antagonist, it is surprising that W9 has a bone anabolic effect in vivo. However, knockdown of RANKL expression in treated osteoblastic cells reduces the effect of W9, and W9 has a weak effect on RANKL-KO osteoblasts in vitro. These results show that RANKL is involved in W9-mediated osteoblastogenesis [32]. We have hypothesized that W9 exerts its bone anabolic activity through RANKL reverse signaling [32–34] (Fig. 3a).

It is well known that RANKL transmits the osteoclast differentiation signal through RANK. Since W9 is an artificial synthetic peptide that can bind to RANKL, we further hypothesized that the endogenous ligand for RANKL is RANK. Binding of RANKL and RANK may transmit a bidirectional signal to activate osteoclasts and osteoblasts through the RANKL forward and reverse signals,

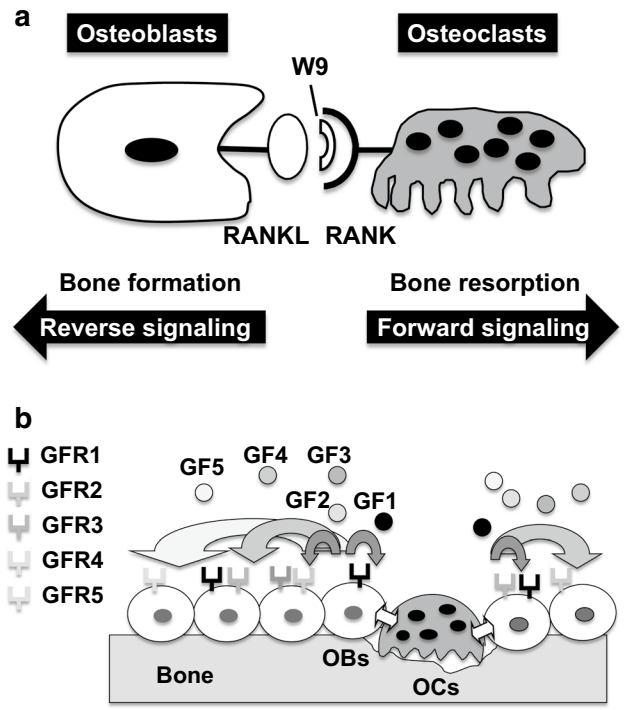

Fig. 3 Hypotheses of regulation of coupling between bone resorption and formation. **a** Bidirectional signaling through RANKL/RANK may control bone remodeling. **b** Activated osteoclasts stimulate osteoblasts to produce GFs and GFRs through RANKL reverse signaling. GFs and GFRs produced by osteoblasts may work in an autocrine/paracrine manner. *W9* WP9QY, *RANKL* receptor activator of NF-κB ligand, *RANK* receptor activator of NF-κB, *GF* growth factor, *GFR* growth factor receptor, *OB* osteoblast, *OC* osteoclast

respectively (Fig. 3a). Reverse signaling occurs among members of the TNF family, including TNFα, CD40L, and FasL, and a membrane-bound ligand transmitting its signal as a receptor [35]. OPG and RANK both bind to RANKL, and the greater osteoclastogenesis and osteoblastogenesis found in OPG-KO mice [11, 12] suggest that bidirectional signaling is enhanced without OPG, because OPG is a decoy receptor for RANKL. The observation of similar phenotypes of RANKL- and RANK-KO mice also supports this hypothesis [18, 21].

Once signaling through RANKL is activated in osteoblasts, the cells produce many growth factors (GFs) and their receptors (GFRs) to activate differentiation in an autocrine manner. These GFs and GFRs produced by RANKL reverse signaling may subsequently and sequentially affect neighboring cells to further activate differentiation in a paracrine manner (Fig. 3b). The reverse signal from RANK on osteoclasts to RANKL on osteoblasts, and the forward signal from RANKL to RANK may play important roles in coupling of bone formation and resorption.

Evidence for our hypothesis was recently found by Ikebuchi et al. [36] in vivo, with the further suggestion of the importance of extracellular vesicles with RANK secreted from osteoclasts regulating osteoblastogenesis. Modulators of RANKL reverse signaling such as W9 and RANKL agonist Abs may be promising drug candidates for treatment of metabolic bone diseases. W9 accelerated BMP-2-induced calvarial bone regeneration and stimulated osteoblast differentiation in mice [37], and recovered alveolar bone loss by suppressing osteoclastogenesis and enhancing osteoblastogenesis in OPG-KO mice [38]. Local administration of W9 also promoted bone formation in a rat femur delayed-union model [39]. An artificial RANKL bifunctional Ab inhibiting osteoclastogenesis and activating RANKL reverse signaling prevented decreased bone formation in ovariectomized mice [36].

Therapeutic potential of anti-RANKL Ab in immuno-oncology

Denosumab was developed by Amgen and is now widely used in treatment of osteoporosis and cancer-induced bone diseases. Clinical trials of denosumab in patients with prostate and lung cancers have shown significantly improved overall survival [40, 41]. A retrospective analysis of denosumab treatment of patients with non-small-cell lung cancer (NSCLC) and bone metastases also showed good overall survival [42]. RANKL antagonists have anticancer effects through (1) direct action; (2) osteoclast-dependent indirect action; and (3) T-cell dependent indirect action (Table 1).

Table 1 Mechanisms through which RANKL antagonists have anticancer effects

Target	Tissue	Site of action	Mechanism
Cancer cells	Tumor tissues	RANK-positive cancer cells	Inhibition of RANKL-dependent proliferation of cancer cells
Osteoclasts	Bone tissues	Cancer cells in bone tissues	Interruption of a vicious cycle by inhibiting bone resorption
mTECs	Thymus	Various cancer cells	Generation of tumor-reactive T-cells by inhibiting development of mTECs

RANKL receptor activator of NF-κB ligand, *RANK* receptor activator of NF-κB, *mTECs* medullary thymic epithelial cells

Direct action of RANKL on RANK-positive cancer cells

RANKL is involved in metastasis of melanoma cells to bones [43]. Recent study has reported that sRANKL triggers bone metastasis by exerting chemotactic activity in tumor cells such as melanoma and breast cancer [26]. Involvement of RANKL in metastasis of prostate cancer cells is also known [44]. It is reported that RANK drives lung cancer even though the source of RANKL is not identified [45]. Systemic sRANKL and/or local RANKL production by cells in the tumor microenvironment would be the source. RANKL/RANK signaling plays a pivotal role in proliferation of mammary cancer cells [46, 47]. OPG-Fc or RANK-Fc inhibits metastasis and proliferation by binding to RANK-positive cancer cells [43, 46, 47]. Tumor-infiltrating regulatory T (Treg) cells produce RANKL and stimulate metastasis of RANK-positive mammary cancer cells [48]. RANKL on Treg cells acts directly on RANK on the mammary cancer cells.

Osteoclast-dependent indirect action of RANKL on cancer cells

Osteoclasts generated by stimulation with RANKL on osteoblasts and osteocytes resorb bones. GFs such as transforming growth factor (TGF)-β and insulin-like growth factor (IGF)-1 are released from bones, and stimulate cancer cells to proliferate and release bone-resorbing factors such as PTH-related protein (PTHrP) and IL-6. These factors stimulate osteoblasts and osteocytes to produce RANKL. Repetition of these processes forms a so-called vicious cycle, through which cancer cells in bones proliferate. This cycle can be interrupted by anti-RANKL Ab [49].

T-cell dependent indirect action of RANKL on cancer cells

Akiyama et al. [50] showed that RANKL-RANK signaling plays crucial roles in development of medullary thymic epithelial cells (mTECs) that express tissue-specific antigens (TSAs) during embryogenesis. Development of mTECs is stimulated in OPG-KO mice and abrogated in RANKL-KO mice. Thymic central tolerance is a critical process that not only prevents autoimmunity, but also presents a challenge to generation of antitumor immune responses. The mTECs eliminate self-reactive T cells by displaying a diverse repertoire of TSAs that are also shared by tumors. Therefore, while protecting against autoimmunity, mTECs simultaneously limit generation of tumor-specific effector T cells by expressing tumor self-antigens [50]. We and others have shown that anti-RANKL Ab inhibits development of mTECs, which suppresses growth of tumors transplanted in nude mice and prolongs survival of mice transplanted with cancer cells by producing tumor-reactive T cells with tumor-specific antigens [51, 52]. Inhibition of RANKL rescues these T cells from thymic deletion. Transplantation of splenic lymphocytes of mice treated with anti-RANKL Ab reduces the growth of tumors transplanted in nude mice, which suggests that anti-RANKL Ab enhances cancer immunity via T-cells.

Mechanisms through which RANKL inhibition can exert therapeutic effects

As mentioned above, there are at least three mechanisms that can explain the anticancer effects of RANKL antagonists (Table 1). (1) Inhibition of the direct action of RANKL on RANK-positive cancer cells, such as mammary and prostate cancer cells; thus, the targets are limited to RANK-positive cancer cells. (2) Inhibition of the osteoclast-dependent indirect action of RANKL on cancer cells; this approach for interrupting a vicious cycle is only applicable to primary or secondary (metastatic) cancer cells in bones. (3) Inhibition of T-cell dependent indirect action of RANKL on cancer cells, using RANKL antagonists that enhance cancer immunity by generating tumor-reactive T cells with tumor-specific antigens through inhibiting development of mTECs. This approach is applicable to various cancer cells, but may be limited to younger patients due to thymic involution with age. The approach may also be a double-edged sword that cuts two ways: generation of tumor-reactive T cells, but also of self-reactive T cells that show autoimmune side effects. Denosumab has been widely used for treatment of osteoporosis and cancer-induced bone diseases, and thus, the risk may be low in elderly patients. However, it will be important to ensure patient safety in clinical trials, especially for younger patients.

Application of anti-RANKL Ab to immuno-oncology

Immune checkpoint inhibitors (ICIs) such as anti-programmed cell death protein 1 (PD-1) Ab and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) Ab are used clinically for treatment of many cancers. Binding of programmed cell death 1 ligand 1 (PD-L1) on cancer cells to PD-1 on T cells prevents the T cells from killing the cancer cells. Inhibition of the interaction of PD-L1 and PD-1 with an ICI (anti-PD-L1 Ab or anti-PD-1 Ab) allows the T cells to kill the cancer cells. In contrast, binding of B7 on dendritic cells to CTLA-4 on T cells prevents T cells from killing the cancer cells (Table 2).

Smyth et al. [53] reported a case of rapidly advancing metastatic melanoma with aggressive and symptomatic bone metastases treated with a combination of ipilimumab (anti-CTLA-4 Ab) and denosumab for palliation. This was the first

Table 2 Mechanisms through which anti-RANKL Ab and ICIs exert their activities in cancer immunity

Ab	Tissue/cell	Target	Mechanism
Anti-PD-1 Anti-PD-L1	Tumor tissues	PD-L1/PD-1	Activation of existing tumor-reactive T-cells through blocking inactivation by cancer cells. (Effector phase)
Anti-CTLA-4	T-cells	B7/CTLA-4	Activation of existing tumor-reactive T-cells through blocking inactivation by dendritic cells. (Priming phase)
Anti-RANKL	Thymus	RANKL/RANK	Generation of tumor-reactive T-cells by inhibiting development of mTECs. (Central tolerance)

RANKL receptor activator of NF-κB ligand, *ICI* immune checkpoint inhibitor, *PD-1* programmed cell death protein 1, *PD-L1* programmed cell death 1 ligand 1, *CTLA-4* cytotoxic T lymphocyte protein 4, *RANK* receptor activator of NF-κB, *mTECs* medullary thymic epithelial cells

report showing a synergistic effect of an ICI with denosumab in a patient. Similar observations have been reported in mice, showing the remarkable synergistic effects of anti-PD-1, anti-PD-L1, anti-CTLA-4 and anti-RANKL Abs on cancer immunity [53, 54]. Since restriction of cancer immunity takes place in the thymus and periphery, a combination of immunotherapies targeting central (i.e. thymic) and peripheral tolerance should work synergistically. This mechanism strongly supports the above findings. Several clinical trials are currently in progress to study the efficacy of denosumab in combination with ICIs for melanoma (CHARLI), renal cell carcinoma (KEYPAD), and NSCLC (POPCORN). It is likely that we will witness a historic moment in immune-oncology in the near future.

Conclusion

The discovery of the RANKL/RANK/OPG system at the bench has resulted in the bedside translation of denosumab within 12 years. This is a tremendous success in both basic and translational research in bone biology in the last decade. Translation of basic research to clinical applications is achieved by addressing the biological significance of basic findings, and this approach to science can save the lives of patients. Establishment of the mechanisms of osteoclastogenesis has created the new field of osteoimmunology, while denosumab has improved the quality of life for patients with osteoporosis and cancer-induced bone diseases. Further successes are likely in determining the mechanisms of RANKL/RANK dual signaling in regulation of the bone and immune system, and in improving cancer immunity using combinations of denosumab and ICIs.

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Compliance with ethical standards

Conflict of interest Dr. Hisataka Yasuda is an employee of Oriental Yeast Co., Ltd.

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