

Roles of osteoclasts in pathological conditions

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Abstract

Bone is a unique organ crucial for locomotion, mineral metabolism, and hematopoiesis. It maintains homeostasis through a balance between bone formation by osteoblasts and bone resorption by osteoclasts, which is regulated by the basic multicellular unit (BMU). Abnormal bone metabolism arises from an imbalance in the BMU. Osteoclasts, derived from the monocyte-macrophage lineage, are regulated by the RANKL-RANK-OPG system, which is a key factor in osteoclast differentiation. RANKL activates osteoclasts through its receptor RANK, while OPG acts as a decoy receptor that inhibits RANKL. In trabecular bone, high turnover involves rapid bone formation and resorption, influenced by conditions such as malignancy and inflammatory cytokines that increase RANKL expression. Cortical bone remodeling, regulated by aged osteocytes expressing RANKL, is less understood, despite ongoing research into how Rett syndrome, characterized by MeCP2 abnormalities, affects RANKL expression. Balancing trabecular and cortical bone involves mechanisms that preserve cortical bone, despite overall bone mass reduction due to aging or oxidative stress. Research into genes like sFRP4, which modulates bone mass, highlights the complex regulation by BMUs. The roles of the RANKL-RANK-OPG system extend beyond bone, affecting processes such as aortic valve formation and temperature regulation, which highlight the interconnected nature of biological research.

KEY WORDS

BMU, bone resorption, DNA methylation, osteoclasts, RANKL, sFRP4

INTRODUCTION

Bone, an organ unique to vertebrates, supports locomotion, plays a crucial role in mineral metabolism, and serves as the site of hematopoiesis.^{1–4} Bone homeostasis is maintained through the balance between bone

formation by osteoblasts and bone resorption by osteoclasts, a process termed remodeling.^{5,6} Bone remodeling is a continuous regulatory process that progresses through seven sequential phases. (1) Quiescence, corresponding to 80%–95% of normal bone surfaces; (2) activation, which includes the recruitment of osteoclasts and

Abbreviations: AP-1, Activator protein 1; ATF4, Activating transcription factor 4; BAC, Bacterial Artificial Chromosome; BMPs, Bone morphogenetic proteins; BMU, Basic multicellular unit; Cbfa-1, Core-binding factor subunit alpha-1; CDX2, Caudal type homeobox 2; CpG, Cytosine and guanine dinucleotide; CRE, Canonical cAMP response element; CXCR4, C-X-C chemokine receptor type 4; GCTB, Giant cell tumor of bone; IL, Interleukin; JNK, c-Jun N-terminal kinase; M-CSF, Macrophage colony-stimulating factor; MeCP2, Methyl-CpG binding protein 2; MITF, Microphthalmia-associated transcription factor; NFATc1, Nuclear factor of activated T-cells, cytoplasmic 1; Ob, Osteoblasts; Oc, Osteoclasts; OCIF, Osteoclastogenesis inhibitory factor; OPG, Osteoprotegerin; PGE2, Prostaglandin E2; PKA, Protein kinase A; PKC, Protein kinase C; PTH, Parathyroid hormone; PTHrP, Parathyroid hormone-related protein; RANK, Receptor activator of nuclear factor κ B; RANKL, Receptor activator of NF- κ B ligand; Runx2, Runt-related transcription factor 2; sFRP4, Secreted frizzled-related protein 4; TBP, TATA box-binding protein; TNF, Tumor necrosis factor; TNFRSF, Tumor necrosis factor receptor superfamily; TNFSF, Tumor necrosis factor ligand superfamily; TRAcP, Tartrateresistant acid phosphatase; TRANCE, Tumor necrosis factor-related activation-induced cytokine; TSS, Transcription start site; VDRE, Vitamin D response element; WT, Wild-type.

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the retreat of bone-lining cells; (3) resorption by osteoclasts in Howship's lacunae in trabecular bone and 'cutting cones' in cortical bone; (4) reversal, marking the interval between the completion of resorption and the onset of bone matrix formation; (5) formation of bone matrix; (6) mineralization, with full calcification requiring approximately 3 to 6 months; and (7) termination, returning to the quiescent phase. Bone remodeling is organized by a local morphological unit, the bone remodeling unit (BRU), which is referred to as a 'packet' in trabecular bone⁷ and an 'osteon' in cortical bone⁸ (Figure 1). At the activation phase, the following processes take place: osteocytes, embedded within the bone matrix and possessing numerous processes to communicate with each other,

sense environmental changes, such as mechanical loading and hormonal states. These signals are then transmitted to the lining cells on the bone surface, which, in response to these stimuli, recruit osteoclasts and initiate bone resorption. It is also known that vascular endothelial cells, peripheral nerves, and T lymphocytes contribute to this regulation. Therefore, the BRU has now been extended to the functional conceptual unit, the basic multicellular unit (BMU), encompassing all bone-forming cells (osteoblasts, bone-lining cells, osteocytes), bone-resorbing cells (osteoclasts), their precursors, and associated cells (endothelial cells, neurons, etc.).⁹⁻¹¹ Osteolytic lesions associated with malignancy,¹² rheumatoid arthritis,^{13,14} postmenopausal osteoporosis,^{15,16} and senile

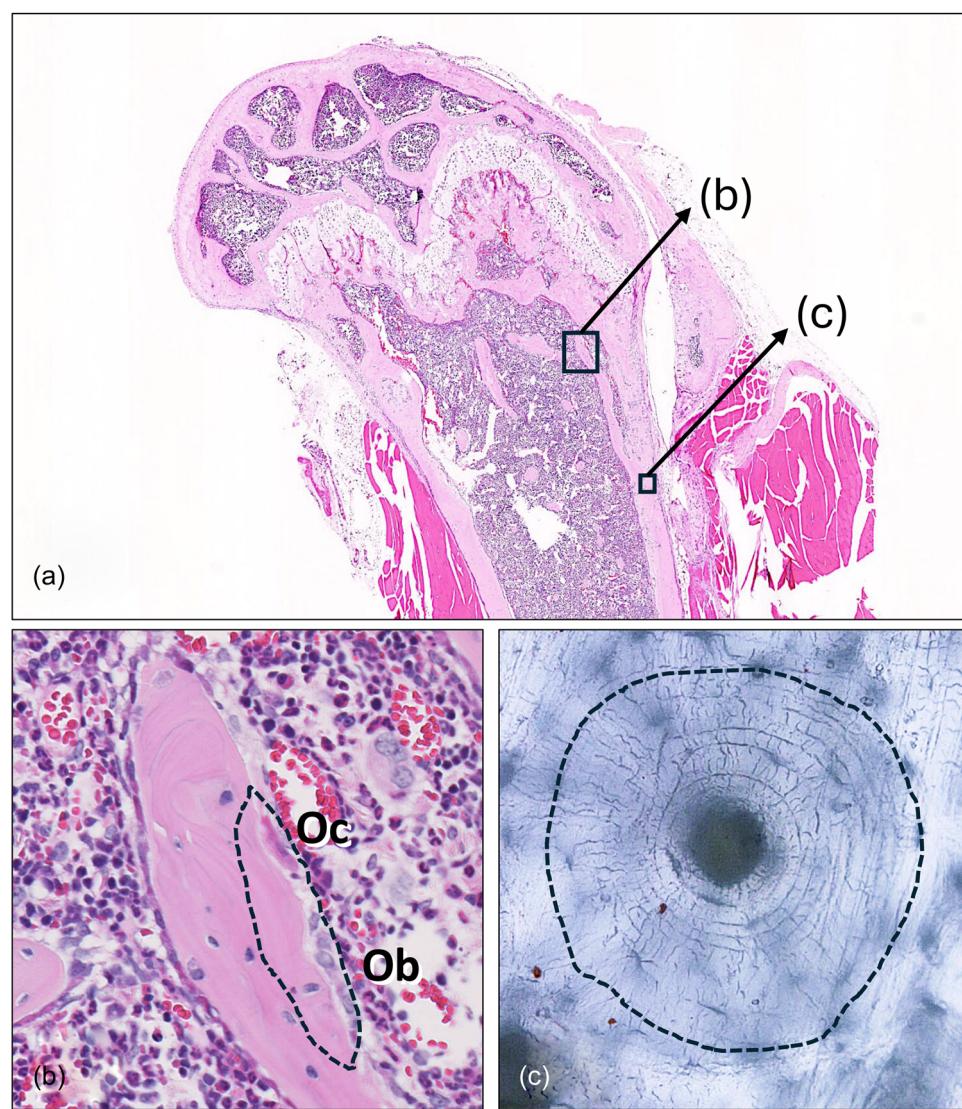


FIGURE 1 The difference in the basic multicellular unit (BMU) of mouse long bone (a) between cancellous (b) and cortical bone (c). In cancellous bone (b), the BMU corresponds to a dish-shaped region enclosed within the dotted line. Cuboidal osteoblasts (Ob) align along the surface of the trabeculae, with nearby osteoclasts (Oc) facilitating the coupling of bone formation and resorption. This unit is beneficial for short-term bone remodeling, as it rapidly stores and releases calcium. In contrast, in cortical bone (c), shown in cross-section, the BMU is composed of a structure called the osteon, enclosed within the dotted line. Centered around the Haversian canal, bone cells are arranged concentrically in lacunae, with each cell spreading out through a fine lacunar-canalicular network. This unit is specialized in maintaining the skeletal framework that supports the body, undergoing slow remodeling to prevent rapid loss of bone mass.

osteoporosis¹⁷ are caused by an imbalance within this BMU. Since the process of osteoclast formation and maintenance is dictated by the surrounding environment, particularly osteoblast lineage cells expressing the osteoclast differentiation factor, the receptor activator of NF- κ B ligand (RANKL),¹⁸⁻²¹ this review focuses on the mechanisms of gene expression related to osteoclast differentiation in pathological conditions.

ORIGIN OF OSTEOCLASTS AND MOLECULAR BASIS OF OSTEOCLASTOGENESIS

Osteoclasts, responsible for bone resorption, are highly specialized multinucleated giant cells that reside in the Howship's lacunae and construct clear zones and ruffled borders on the bone surface^{4,5} (Figure 2). That fact that

osteoclasts are derived from cells of the monocyte-macrophage lineage was clearly demonstrated by Dr. Nishikawa and his group at Kumamoto University: the genetic abnormality in op/op mice with osteopetrosis caused by loss of osteoclasts is caused by mutation in the M-CSF gene.²² Bone marrow macrophages cultured in the presence of M-CSF alone are, however, not sufficient for the formation of mature, functioning osteoclasts.²³ Several researchers, including our group, have attempted to isolate and identify the "osteoclast differentiation factor"; however, two major obstacles have impeded the identification: (1) the limited availability of osteoclasts that are almost exclusively located within hard bone tissue, and (2) the inability to culture and passage osteoclasts *in vitro*. Two studies from Japanese laboratories have helped overcome these impediments: (1) the development of a co-culture method that enables the formation of authentic osteoclasts *in vitro*,^{24,25} (2) the discovery of

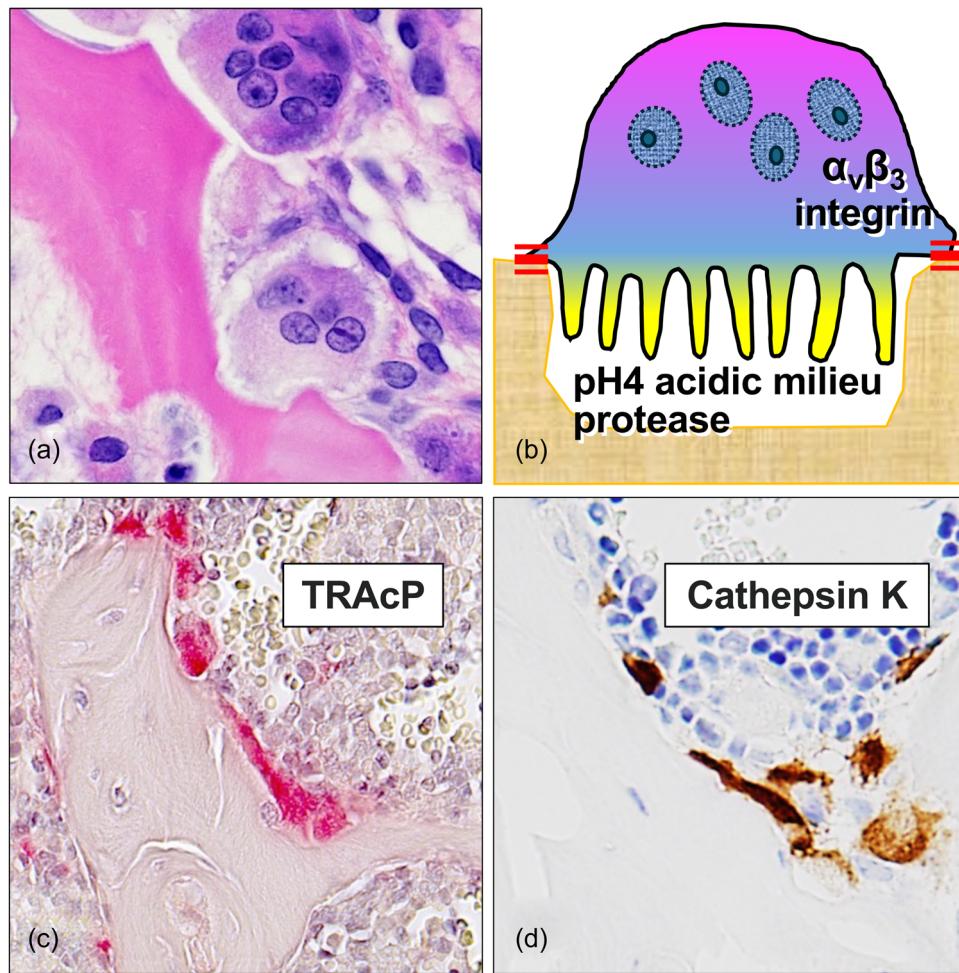


FIGURE 2 Characteristics of osteoclasts. Osteoclasts are polarized multinucleated giant cells located on the surface of bone, where they induce bone resorption, creating pits on the bone surface (a). A schematic representation of this process is shown in (b). Osteoclasts adhere firmly to the bone through $\alpha_v\beta_3$ integrins expressed on their surface, creating a space beneath the cell. In this space, osteoclasts secrete acid in an acidic environment with a pH of around 4, which dissolves the mineral components of the bone. Additionally, osteoclasts release proteolytic enzymes that degrade the protein components of the bone matrix, thereby eroding the bone surface. Common methods for identifying osteoclasts morphologically include histochemical techniques that use tartrate-resistant acid phosphatase (TRAcP) activity, which is abundant in osteoclasts (c), or immunohistochemistry using antibodies against the representative proteolytic enzyme cathepsin K (d).

osteoclastogenesis inhibitory factor (OCIF)/osteoprotegerin (OPG), a potent inhibitor of osteoclastogenesis.²⁶ These studies led to the identification of an osteoclast differentiation factor/RANKL,^{19,21} and the elucidation of the molecular mechanisms required for osteoclast differentiation and maintenance. The discovery involved a fierce head-to-head competition²⁷ between the Japanese group¹⁹ and the Amgen group.²¹ Surprisingly, the gene ultimately identified as an osteoclast differentiation factor, universally referred to as RANKL in the field of bone metabolism,²⁸ was identical to TRANCE, previously identified as important molecule for dendritic cell differentiation derived from T lymphocytes.²⁹ RANKL transduces intracellular signals essential for osteoclast differentiation and activation by binding to its receptor RANK^{20,26,27,30,31} on osteoclast progenitor cells. On the other hand, OPG inhibits the action of RANKL as a soluble decoy receptor. The RANKL-RANK-OPG system is the key regulatory mechanism that controls the extent of bone resorption and is recognized as the primary regulator of the BMU²⁷ (Figure 3).

Currently, RANK, RANKL, and OPG are classified as members of the TNF superfamily under the designations TNFRSF11a, TNFSF11, and TNFRSF11b, respectively. In the present review, we use the terms RANK, RANKL, and OPG as conventions in the bone metabolism field.

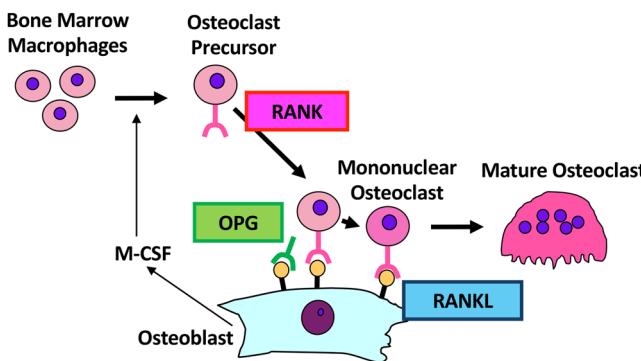


FIGURE 3 System regulating osteoclastogenesis. Receptor activator of NF- κ B ligand (RANKL) binds to its receptor, RANK, on osteoclast precursor cells, transmitting essential intracellular signals for osteoclast differentiation and activation. On the other hand, osteoprotegerin (OPG) acts as a soluble decoy receptor, inhibiting the action of RANKL. The RANKL-RANK-OPG system serves as a key regulatory mechanism that determines the balance between bone resorption and formation in both cancellous and cortical bone. RANKL is induced on the surface of osteocytes and osteoblasts, which support osteoclast differentiation by bone-resorption factors such as active vitamin D₃, PGE₂, and parathyroid hormone (PTH), and is inhibited by OPG. In inflammatory bone destruction, such as in rheumatoid arthritis, inflammatory cytokines induce RANKL expression on synovial fibroblasts. RANKL then transmits signals through its binding to RANK on osteoclast precursor cells, ultimately activating the nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), the master transcription factor responsible for osteoclast differentiation.

REGULATORY MECHANISM OF RANK, RANKL, AND OPG GENES

A gene promoter is a specific region of DNA where the transcription of a gene begins. It determines the transcription start site (TSS) and serves as the initiation point for gene expression. Identifying the TSS of mRNA is crucial for locating a promoter, because key transcriptional regulatory regions are typically situated nearby. By analyzing the nucleotide sequences surrounding the TSS, we can infer important characteristics of the promoter. Therefore, studying promoter structure in relation to gene expression can be seen as a form of molecular morphology, with nucleotide sequences acting as the structural blueprint. The core promoter is where general transcription factors bind, facilitating the recruitment of RNA polymerase. However, in addition to this essential function, various transcriptional regulatory factors also play significant roles in modulating gene expression. These factors are proteins that bind to specific sequences in the DNA, either promoting or inhibiting transcription by RNA polymerase. They contain domains that interact with other proteins, contributing to the regulation of transcription.

Furthermore, gene expression regulation is influenced by chromatin structure within the nucleus, which can toggle gene activity on or off. This process, known as epigenetics, creates specific patterns in specialized tissues and cells of multicellular organisms, playing a crucial role in tissue-specific transcriptional control. In this context, we will explore the mechanisms that regulate the transcription of genes involved in osteoclast formation, specifically focusing on RANK, RANKL, and OPG, based partly on our findings.

REGULATION OF RANK GENE TRANSCRIPTION

Basic Mechanisms of RANK Gene Transcription Regulation

The promoter region of the mouse RANK gene is notable for containing four TSSs, the absence of a TATA box, and the presence of GC-rich regions. Moreover, several candidate binding sites for transcriptional regulatory factors are located upstream of the core promoter. Among these factors, MITF and PU.1 are particularly important for monocyte differentiation and have been shown to bind to the promoter *in vitro*. Their expression can be induced by overexpression, while mutations in the binding sites lead to reduced activity, confirming the functional relevance of these predicted elements.³² RANK mRNA expression exhibits two key characteristics: first, it undergoes a modest increase following M-CSF administration; second, RANKL stimulates RANK, which leads to an elevation in RANK expression itself.³⁰ The response to M-CSF is

partially mediated through the AP-1 signaling pathway. Functional AP-1 binding sites within the RANK promoter have been identified as crucial components of the mechanism by which M-CSF induces RANK expression.³⁰ In the context of RANK activation through its interaction with the osteoclast differentiation factor RANKL, NFATc1 emerges as a critical mediator of downstream signaling from RANK. The presence of NFATc1 facilitates the activation of osteoclast-specific genes, such as cathepsin K and tartrate-resistant acid phosphatase (TRAcP). Three candidate NFATc1 binding sites have been identified within the RANK promoter, with one confirmed as a functional site. This indicates that RANK is a target gene of its own signaling pathway, establishing a positive feedback loop in which signals from RANK promote its own expression.³⁰ The presence of this positive feedback loop is vital for determining the definitive and irreversible path of cellular differentiation. In the presence of transcription factors like MITF and PU.1, M-CSF stimulates monocyte-macrophages to express RANK on their surface, thereby promoting their differentiation into a pre-osteoclast state. The interaction between RANK and RANKL suggests a pathway leading to irreversible differentiation into osteoclasts, as supported by promoter analyses.

Epigenetic Regulation of RANK Gene

As part of the epigenetic regulation, the presence of numerous CpG loci suggests that DNA methylation may play a significant role. Comprehensive epigenomic analyses have indeed demonstrated that ST2 bone marrow stromal cells exhibit a hypermethylated state, while RAW 264.7 pre-osteoclast macrophage lineage cells display a hypomethylated state, indicating the presence of cell type-specific DNA methylation patterns.³³ Additionally, this region demonstrates increased methylation with aging, which is correlated with the suppression of RANK gene expression and the consequent decrease in osteoclastogenesis in aged mice.¹⁸ Overall, epigenetic regulation through cytosine methylation in the vicinity of the RANK gene promoter is essential for the cell type-specific modulation of RANK expression, and the age-related increase in methylation may contribute to the diminished osteoclastogenic potential and functionality observed in senile osteoporosis.

REGULATION OF RANKL GENE TRANSCRIPTION

Most factors involved in bone resorption, which operate through osteoclast formation and activation, ultimately target RANKL expression. Indeed, pathways that promote osteoclastogenesis include vitamin D,³⁴

parathyroid hormone (PTH)/parathyroid hormone-related protein (PTHRP),¹² prostaglandin E₂ (PGE₂),³⁵ and cytokines such as interleukin (IL)-6 and IL-11, which engage the gp130 signaling pathway,³⁶ all of which are known to enhance RANKL gene expression. Therefore, elucidating the regulatory mechanisms that govern RANKL gene expression is essential for a comprehensive understanding of bone metabolism. In this context, we present the fundamental mechanisms of RANKL transcriptional regulation, as well as the control mechanisms exerted by these bone-resorbing factors.

Basic Mechanisms of RANKL Gene Transcription Regulation

Under non-stimulated conditions, RANKL mRNA expression is maintained at extremely low levels; however, upon exposure to bone-resorbing factors, it rapidly increases in osteoblast-like cells, akin to the activation of a switch. RANKL mRNA expression is also limited to specific cell types, such as osteoblasts and some T lymphocytes, under physiological conditions. The core transcriptional regulatory region of the mouse RANKL gene upstream of the 5' region contains a Runx2/Cbfa-1 binding site upstream of the inverted TATA and CCAAT boxes, with various transcription factor binding sites, including a vitamin D response element,³⁷ located approximately 1 kb upstream. The human RANKL gene promoter shares similar structural elements, indicating that the mechanism of osteoclast formation and bone resorption via RANKL is conserved as a calcium-mobilizing system across mammals. The structure of the inverted TATA box and CCAAT-box in the RANKL gene core promoter is very similar to the promoter structure of bone sialoprotein, which is also regulated by Runx2 in an osteoblast-specific manner. Due to the presence of three Runx2 binding sites within the RANKL promoter, we initially hypothesized that RANKL expression might also be regulated by Runx2 in an osteoblast-specific manner.³⁸ Indeed, *in vitro* studies confirmed that Runx2 binds to these three Runx2 binding sites in the RANKL gene core promoter.³⁸ Nevertheless, the transcriptional activity of transiently introduced promoters remained consistently high across different cell types, even when extended up to 10 kb in length. Furthermore, analyses using Runx2-deficient cell lines (C6 cells) demonstrated that Runx2 binding to the core transcriptional regulatory region is not critical for the basal transcriptional activity of RANKL or its cell-specific expression.³⁸ We therefore hypothesized that the mechanisms underlying the tissue-specific expression of RANKL, or the maintenance of its low-level expression under basal conditions, likely reside further upstream of 10 kb or within the intronic or 3' regions of the gene. Indeed, studies by

O'Brien et al.,³⁹ employing BAC clones, have identified a region situated 74 kb upstream of the RANKL transcription start site as critical for tissue-specific expression and activation by various bone-resorbing factors. Gene expression regulation at the chromatin level entails the binding of transcription factors at locations distant from the transcription start site, thereby influencing gene activation. Our investigations into histone acetylation as a marker of chromatin activation uncovered alterations in histone acetylation extending up to 40 kb upstream of the RANKL gene, reinforcing the notion of a chromatin-level regulatory mechanism governing RANKL expression.³⁸ Therefore, the regulation of RANKL gene expression likely occurs in at least two stages: (1) large-scale chromatin dissociation (activation) or aggregation (inactivation) through mechanisms that act tens of kilobases upstream, and (2) transcriptional regulation by factors that bind near the core transcriptional regulatory region. This two-stage regulatory model provides a framework for interpreting the mechanisms of action of various bone-resorbing factors. Understanding the intricate processes of transcriptional regulation necessitates a distinction between higher-order systems that modulate chromatin dynamics—such as condensation and decondensation—and lower-order systems where transcription factors directly influence gene expression by binding to accessible DNA regions following decondensation. This differentiation is crucial, as a single transcription factor can participate in both levels of regulation and may function as either an activator or a repressor, depending on the specific context and co-factors involved. Consequently, the regulatory mechanisms governing one gene may not be universally applicable to others, and each gene's regulation must be analyzed with care, taking into account the differentiation state of the cells.

Vitamin D-Mediated Regulation of RANKL Transcription

A putative VDRE is situated approximately 1 kb upstream of the mouse RANKL promoter, and the activity of exogenously introduced promoters is augmented following vitamin D administration.³⁴ Consequently, the initial hypothesis posited that vitamin D enhances RANKL expression through a straightforward mechanism, akin to other vitamin D target genes.³⁴ However, the vitamin D-induced increase in the activity of the exogenously introduced RANKL promoter was only about two-fold, which is significantly less than the switch-like on/off control observed in osteoblastic cells. Thus, it is suggested that chromatin-level changes involving extensive dissociation (activation) may also play a role in the vitamin D-mediated regulation of RANKL expression, and further investigations into the precise mechanisms are ongoing.

Regulation of RANKL Transcription via PTH and PTHrP

PTH and PTHrP, both known to promote bone resorption, activate signaling through the PKA and PKC pathways upon binding to receptors on osteoblasts. Transfection studies demonstrated that treatment with PTHrP in cells containing the mouse RANKL promoter resulted in an almost two-fold increase in transcriptional activity within 6 h, an effect that was inhibited by PKA inhibitors. Similarly, the PKA agonist forskolin produced comparable results, suggesting the involvement of the PKA pathway in PTHrP-induced RANKL transcription.¹² Although the mouse RANKL promoter does not contain a canonical cAMP response element (CRE), we identified a CRE-like sequence approximately 1 kb upstream, in proximity to the vitamin D response element (VDRE). Gel shift assays using oligonucleotides confirmed the binding of CREB and ATF2 to this region.¹² Dr. Karsenty's group has reported that this site is essential for the regulation of RANKL expression through ATF4, suggesting that signals from the sympathetic nervous system may ultimately modulate bone mass via the ATF4-CRE signaling pathway.⁴⁰ Given the high expression of ATF4 in bone, this ATF4-mediated regulatory system is likely to play a significant role in osteoblast-specific RANKL expression. Furthermore, it has been reported that PTH/PTHrP-mediated RANKL gene expression is regulated at the chromatin level, with PKA acting far upstream rather than downstream of the basal transcriptional regulatory region.⁴¹ RANKL expression mediated by PTH/PTHrP has also been shown to be regulated at two levels: chromatin dissociation and subsequent binding of transcription factors.

Epigenetic Regulation of RANKL Gene

A study from Dr. Bird's group in the UK reported that the methylated structure of CpG sites near the T/A repeated sequence serves as a target sequence for the representative methylated cytosine-binding protein, MeCP2.⁴² Upon reviewing the reported sequence, we hypothesized that the TATA box and adjacent CpG sites in the promoter region of the RANKL gene may correspond to this MeCP2-target sequence. To investigate this, we conducted experiments using unmethylated sequences and sequences with a single methyl group *in vitro* to assess the binding affinity of TATA box-binding protein (TBP) and MeCP2.⁴³ In the absence of methylation, TBP binds to the TATA box of the RANKL gene promoter; however, introducing a single methyl group inhibits TBP binding, allowing MeCP2 to occupy the site. Furthermore, chromatin precipitation assays using ST2 bone marrow stromal cells demonstrated that TBP and MeCP2 bind to the RANKL gene promoter region in a mutually exclusive manner.⁴³ In

other words, when only one methylation occurs at the CpG site upstream of the TATA box, it acquires RANKL-binding properties and represses transcription by displacing TBP binding from the TATA box. We have proposed a novel model of epigenetics in the vicinity of the TATA box.⁴³ This unique epigenetic mechanism is also involved in the expression of the sFRP4⁴⁴ and CXCR4⁴⁵ genes as well as the RANKL gene.

REGULATION OF OPG GENE TRANSCRIPTION

The OPG mRNA in mice, as determined by primer extension analysis, exhibits at least three distinct TSSs, indicating the presence of multiple TSSs and a promoter characterized by complex regulatory mechanisms. The cloned upstream region of the mouse OPG gene (approximately 3.2 kb) lacks a TATA box, suggesting a regulatory profile similar to that of house-keeping genes. Consequently, OPG expression at both the mRNA and protein levels is likely regulated primarily through post-transcriptional mechanisms, particularly those affecting mRNA stability and protein synthesis, rather than relying solely on transcriptional regulation. Notably, the promoter region of the mouse OPG gene does not harbor binding sites for bone morphogenetic proteins (BMPs) or Runx2 (Cbfa1), which were predicted in the human counterpart.⁴⁶ However, an AP-1 binding-like site (TGACTGA) was identified approximately 290 base pairs upstream of the TSSs, and this site is presumed to play a critical role in regulating basal OPG mRNA levels.^{37,47} Vitamin D promotes osteoclastogenesis by positively regulating RANKL and negatively regulating OPG. The down-regulation of OPG mRNA in response to vitamin D is primarily due to reduced mRNA stability and a shorter half-life; additionally, vitamin D significantly inhibits OPG promoter activity. Importantly, no known negative VDREs were identified within the analyzed region of the mouse OPG promoter, and constructs with deletions or mutations in the AP-1 site did not show suppression by vitamin D. These findings suggest that the down-regulation of OPG transcription by vitamin D and other bone-resorbing factors occurs via AP-1-mediated transrepression, potentially through a mechanism involving decreased activation of c-Jun independent of JNK signaling.⁴⁷

TRABECULAR AND CORTICAL BONES AND THEIR BALANCE

We have outlined the transcriptional regulation of the RANK-RANKL-OPG system, which is central to the BMU. As previously mentioned, the structure and regulation of BMU differ between trabecular bone (packet)

and cortical bone (osteon), resulting in distinct mechanisms underlying disease development in each type of bone. This section will explain how abnormalities in the regulatory mechanisms of RANK, RANKL, and OPG gene expression are related to the pathologies of trabecular and cortical bone. Additionally, the balance between trabecular bone and cortical bone, and how it is regulated to protect cortical bone, will be discussed.

Trabecular bone and its pathological conditions

To regulate calcium for eggshell production, the medullary bone of chickens undergoes rapid turnover, with bone being formed and resorbed daily.⁴⁸ In mammals, high-turnover bone metabolism primarily occurs in the trabecular bone^{49,50} that is analogous to the medullary bone in birds. Therefore, the trabecular bone is a primary target in conditions such as hypercalcemia associated with malignancy,⁵¹ osteolytic bone metastases,¹² and bone destruction caused by rheumatoid arthritis.¹³ In these pathological osteolytic conditions, tumor-derived PTHrP⁵¹ and various inflammatory cytokines⁵² target osteoblasts lining the bone surface. This stimulation induces the expression of RANKL gene through responsive elements located on RANKL gene promoter, promoting active osteoclast formation (Figure 4a). Some breast cancer cells express the same adhesion molecule, $\alpha\beta\beta$ integrin,^{53,54} as osteoclasts, allowing them to adhere directly to the eroded bone surface,⁵⁵ creating the appearance of direct bone destruction by cancer cells (Figure 4b); however, the destruction is actually indirect through osteoclasts, however.

In the context of bone destruction in rheumatoid arthritis, it was initially hypothesized that osteoclasts might be triggered by the expression of RANKL by infiltrating T lymphocytes. However, upon creating a collagen-induced arthritis model and analyzing the expression of RANKL, RANK, and OPG, we observed that RANKL was primarily expressed by fibroblasts in the inflamed synovium. Within the synovium, macrophages were activated by RANKL and differentiated into mononuclear, RANK-positive, TRAcP-positive preosteoclasts. In essence, when articular cartilage is eroded and the synovial membrane comes into direct contact with bone tissue, mature multinucleated osteoclasts are recruited to the bone, resembling an assault launched from a landing craft, leading to rapid and extensive bone destruction.

Cortical bone and its pathological conditions

The cellular source of RANKL in cortical bone remodeling has long been unclear. However, it is now

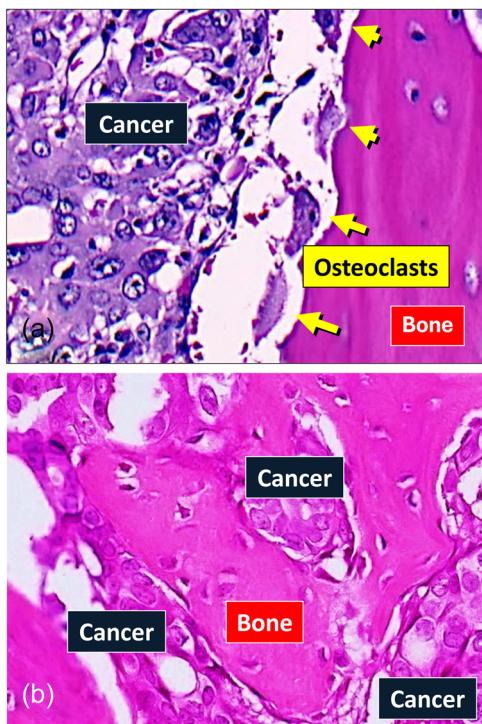


FIGURE 4 Osteoclasts in bone invasion and osteolytic bone metastasis of malignancy. In bone destruction caused by malignant tumor invasion and osteolytic bone metastasis, the tumor itself is incapable of directly resorbing bone. At the leading edge of bone invasion (a), numerous osteoclasts are induced between the cancer cells and the bone tissue (yellow arrows). Tumor-derived parathyroid hormone-related protein (PTHrP) promotes the expression of RANKL on the surface of osteoblasts, leading to the induction of osteoclasts. Similar to fruit encased in ice, which is given to polar bears at Japanese zoos, bone contains growth factors. Cancer cells crave the “fruit,” that is, the growth factors within the bone, but cannot break the “ice” themselves. The tumor secretes factors such as PTHrP, which stimulate osteoblast-lineage cells to induce RANKL and recruit osteoclasts. The “polar bear” (osteoclast) resorbs the bone, releasing the “fruit” (growth factors) contained within. As a result, the “fruit” (growth factors) released by the osteoclasts is then used by the cancer cells, creating a symbiotic relationship. Furthermore, as seen in (b), cancer cells in bone metastasis lesions sometimes appear in direct contact with bone tissue, giving the impression that the cancer cells themselves are eroding the bone. The same integrin, $\alpha_v\beta_3$, that functions as an adhesion molecule for osteoclasts is also expressed on the surface of breast cancer cells, allowing them to adhere to the resorption pits created by osteoclasts, much like a hermit crab occupying an empty shell.

known that aged osteocytes express RANKL⁵⁶ that regulates remodeling at the osteon level. We are currently focusing our studies on Rett syndrome,⁵⁷ as it is characterized by cortical bone thinning.⁵⁸ Since Rett syndrome is caused by abnormalities in the MeCP2,⁵⁹ we investigated the relationship between MeCP2 expression and RANKL expression in osteocytes. As mentioned in the RANKL gene promoter structure, the RANKL gene promoter region contains a CpG sequence immediately upstream of the TATA box,^{34,60,61} and when this sequence undergoes

methylation, it becomes a typical MeCP2 binding site,^{42,43} suggesting a novel epigenetic regulatory mechanism, where a single CpG methylation event near the TATA box can control gene expression in an on/off manner (Figure 5). Initially, we hypothesized that this specific epigenetic mechanism plays a role in the tissue-specific expression of the RANKL gene. Our newly developed technique for visualizing single-base methylated cytosine in a sequence-specific manner³³ has revealed that single methylation near the TATA box occurs during the differentiation of osteoblasts into osteocytes (unpublished data). Since MeCP2 expression in osteocytes exhibits patchy negative staining (Figure 6), the progressive loss of MeCP2 protein in aged osteocytes suggests the presence of a remodeling mechanism whereby RANKL expression is reactivated in these MeCP2-negative osteocytes, leading to the induction of osteoclasts in aged cortical bone. This phenomenon of gene reactivation due to the loss of MeCP2 while methylation is present is known as methylation memory phenomenon, and is also involved in the reactivation of Cyclin D1⁶² and CDX2⁶³ expression in colorectal cancer.

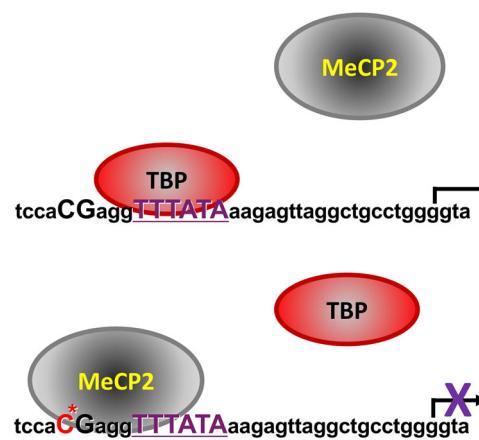


FIGURE 5 Schema of how single cytosine methylation regulates the expression of RANKL. A structure where the CpG site near the T/A repeat sequence is methylated has been described by Dr. Bird's group from the UK as a target sequence for MeCP2,⁴² a representative methyl-CpG binding protein. The CpG site near the TATA box in the promoter region of the RANKL gene is precisely this target sequence. As depicted in the upper panel, in the absence of methylation, TATA box-binding protein (TBP) binds to the TATA box of the RANKL gene promoter, leading to the transcription of mRNA. When methylation occurs at the CpG site upstream of the TATA box in the RANKL promoter region (highlighted in red text with an asterisk in the lower panel), it gains affinity for MeCP2, displacing TBP and thereby inhibiting transcription. This novel epigenetic model hypothesizes that it plays a crucial role in the on-off-on pattern of RANKL gene expression in cortical bone, as illustrated in Figure 6. The observation that Rett syndrome, characterized by abnormalities in MeCP2, presents not only with neurological symptoms such as autism, but also with cortical bone thinning, further supports the importance of DNA methylation and MeCP2 in cortical bone metabolism.

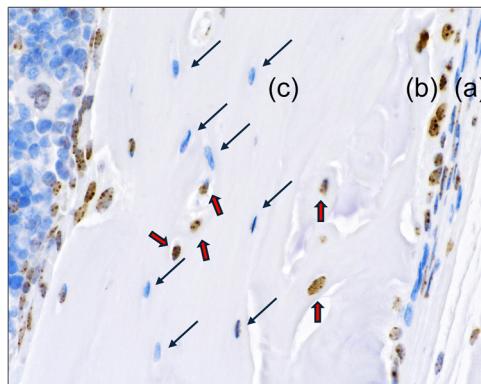


FIGURE 6 MeCP2 protein expression in cortical bone. Immunohistochemical analysis reveals that the expression of MeCP2 varies significantly among different cell types in cortical bone: (a) immature osteoblast progenitor cells and stromal cells, (b) osteoblasts on the bone surface, and (c) osteocytes within the bone matrix and lacunae. In region (a), most immature osteoblast progenitors and stromal cells show minimal expression of MeCP2. In contrast, the majority of osteoblasts on the bone surface (b) exhibit nuclear positivity for MeCP2. At the level of osteocytes (c), MeCP2 displays a characteristic heterogeneous staining pattern. While osteocytes (red arrows) are MeCP2-positive, adjacent osteocytes (black arrows) are MeCP2-negative. The loss of MeCP2 expression may lead to the reactivation of RANKL expression that was previously suppressed by MeCP2. This unique epigenetic mechanism suggests that older osteocytes in cortical bone signal osteoclasts with a “resorb me” message.

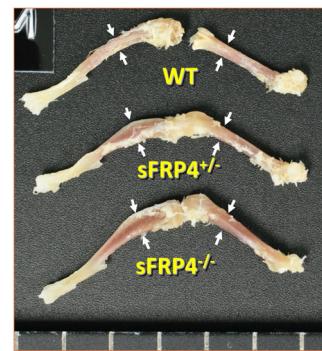


FIGURE 7 Phenotype of sFRP4 gene knockout mice, a factor balancing cancellous and cortical bone. During the longitudinal growth of mice, cancellous bone progressively decreases. Although cortical bone also diminishes, it is maintained at the expense of the resorption of cancellous bone. Our study on oxidative stress and bone tissue identified the molecule sFRP4 as a key regulator balancing cancellous and cortical bone. In collaboration with Dr. Aizawa from RIKEN, we generated sFRP4 knockout mice. Macroscopic analysis of the hindlimbs revealed that, compared with wild-type (WT) mice (top panel), heterozygous knockout ($sFRP4^{+/-}$) (middle panel) and homozygous knockout ($sFRP4^{-/-}$) (bottom panel) mice exhibited thicker bones, presenting an apparently robust phenotype.

Balancer of trabecular and cortical bone

Oxidative stress conditions such as diabetes and aging (senile osteoporosis or age-related osteoporosis) lead to a reduction in bone mass.⁶⁴ There is a mechanism whereby the reduction in bone mass primarily affects trabecular bone; however, efforts are being made to preserve cortical bone as much as possible.^{65,66} In other words, there is a higher-level regulatory function that maintains the balance between the BMUs present in trabecular and cortical bone. A comprehensive analysis of genes with increased expression in the osteoblast lineage under oxidative stress has been conducted and has identified the sFRP4 gene as one of the upregulated genes.^{44,67,68} We created genetically modified mice to analyze the function of sFRP4 at the individual level. The sFRP4 knockout mice appeared well-groomed, slender, and had large bones (Figure 7). Furthermore, as the mice aged, their phenotype remained largely unchanged. At first glance, it seemed that the loss of the sFRP4 gene rendered the mice resistant to the significant oxidative stress associated with aging. Surprisingly, however, micro-CT and histological examination of the aged mice revealed that, while the trabecular bone showed only minimal reduction, the cortical bone was significantly thinned⁶⁸ (Figure 8). In sFRP4 knockout mice, trabecular bone was protected from oxidative stress; however, the ability to maintain cortical bone,

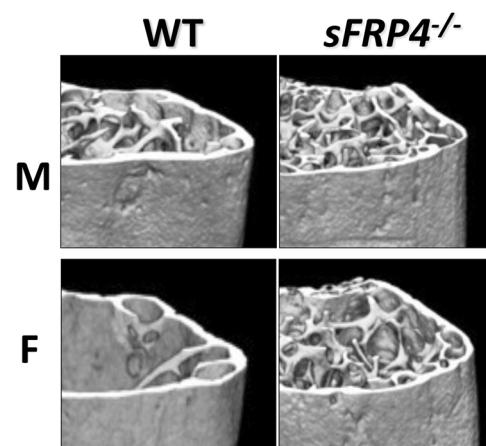


FIGURE 8 sFRP4 knockout mice are resistant to age-related cancellous bone loss, while cortical bone becomes compromised (as observed by micro-CT analysis). As wild-type (WT) mice age, both males (M) and females (F) experience a marked reduction in cancellous bone, a trend that is particularly pronounced in females. In contrast, sFRP4 knockout mice exhibit resistance to age-related cancellous bone loss, with well-maintained trabecular bone. However, their cortical bone becomes significantly thinner than that of WT mice, resulting in a disrupted balance between cancellous and cortical bone. Recently, sFRP4 deficiency has been described as the cause of Pyle disease,⁶⁹ a rare human bone disorder, where the bone structure closely resembles that of sFRP4 knockout mice, with an impaired balance between cancellous and cortical bone, making long bones prone to fracture. Analogous to financial management, where trabecular bone represents a checking account and cortical bone a savings account, a balancer allocates resources based on the state of income and expenditure. sFRP4 is considered one of the key regulators in this balancing process.

which primarily supports mechanical load, was compromised. This resulted in a disruption of the balance between trabecular and cortical bone. The sFRP4 gene was recently identified as the causative gene for a genetic condition known as Pyle's disease.⁶⁹ In human cases of Pyle's disease, the phenotype resembles that of sFRP4 knockout mice, which exhibit thicker bones that are nonetheless fragile and susceptible to fractures.⁷⁰ These findings strongly indicate that sFRP4 plays a critical role in regulating the balance between trabecular and cortical bone.

GIANT CELL TUMOR OF BONE AND RANKL REVERSE SIGNALING

Finally, we describe giant cell tumor of bone (GCTB), a bone tumor in which osteoclasts appear in extremely high numbers.^{71,72} GCTB accounts for approximately 5–10% of primary bone tumors and about 15–20% of benign bone tumors, making it a relatively common type of bone tumor. It typically develops in the epiphysis of young adults aged 20 to 40 years after the closure of the growth plate. It is most commonly found in the distal femur and proximal tibia, but it can also occur, though rarely, in vertebral bodies. As the disease progresses, it can cause bone destruction, pain, limited range of motion, pathological fractures, and, in rare cases, pulmonary metastasis.⁷² On plain X-rays, the characteristic finding is a localized cystic radiolucency in the epiphysis, with thinning or bulging of the cortical bone, often

referred to as a “soap bubble appearance.” Histopathologically, numerous osteoclast-like giant cells are observed, and initially, these giant cells were thought to be tumor cells themselves, which is why the tumor was named “giant cell tumor of bone.” Over time, two hypotheses regarding the tumor's origin have emerged: one suggesting tumorigenesis of monocyte-macrophages, the precursors of osteoclasts, and another proposing that the tumor consists of mesenchymal stromal cells, with the osteoclast-like giant cells appearing reactively. This debate continued for a long time. When tumor samples collected during surgery were cultured, the giant cells gradually disappeared, leaving only spindle-shaped stromal cells, which led to the dominant view that the tumor originates from mesenchymal stromal cells. However, the reason for the appearance of numerous giant cells, as well as the underlying genetic abnormalities, remained unclear for a long time. The identification of RANKL elucidated the molecular mechanisms of osteoclastogenesis, leading to the recognition that giant cell tumor of bone is a tumor of bone marrow-derived mesenchymal cells that highly express RANKL. However, it took an additional 15 years after the discovery of RANKL to identify the gene responsible for the tumor.⁷³ During this period, next-generation sequencing was developed, and genome analysis successfully identified gene mutations responsible for various tumors. Nevertheless, research into the causative gene for giant cell tumor of bone lagged behind, largely due to its histological peculiarity—where a small population of mesenchymal tumor cells coexists

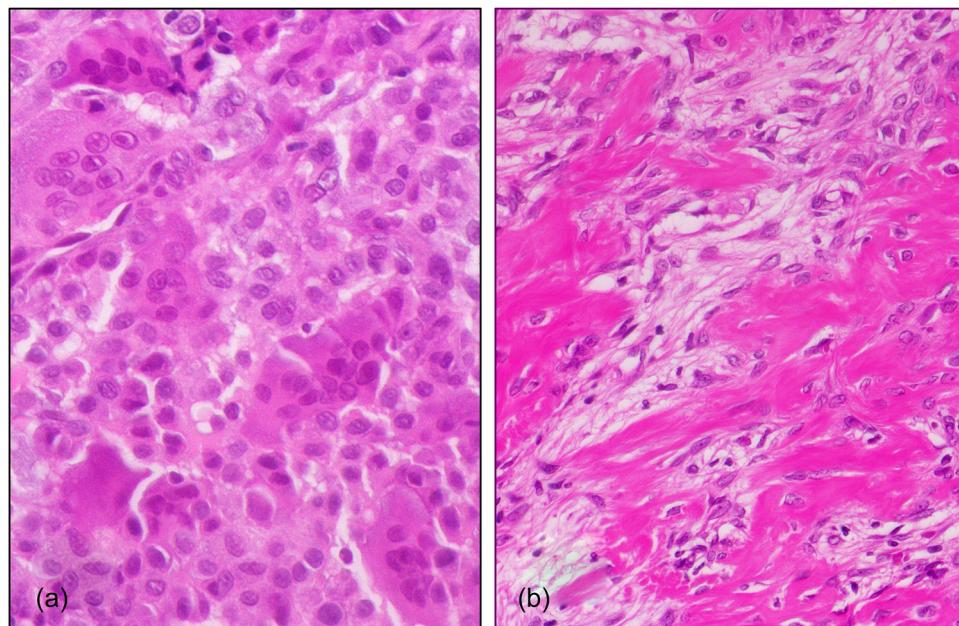


FIGURE 9 Giant cell tumor of bone (GCTB) before (a) and after (b) anti-RANKL antibody (Denosumab) treatment (H.E.). (a) illustrates the pre-treatment biopsy tissue, characterized by the presence of numerous multinucleated giant cells typical of GCTB. In contrast, (b) presents the post-treatment tissue, which shows a significant alteration in histological pattern. The hallmark features of GCTB have transitioned into a bone-forming tumor with osteoid formation.

with a majority of non-tumorigenic osteoclast-like giant cells. It was not until 2013, through exome analysis using a large number of clinical samples, that the causative gene for giant cell tumor of bone was reported to be in the histone protein H3.3 (H3F3A, G34W).⁷³ Interestingly, mutations in the H3.3 gene are frequently observed in pediatric high-grade gliomas and other malignancies.⁷⁴ In contrast, similar bone-related diseases show different genetic patterns: chondroblastoma has mutations in a different region of the H3.3 (H3F3B, K36M) gene,⁷³ while giant cell reparative granuloma, previously thought to be a reactive condition, does not exhibit these mutations.⁷³ This suggests that, in tumors or tumor-like conditions of the bone that form giant cells, where morphological differentiation is challenging, analyzing the presence and pattern of H3.3 mutations can help confirm the diagnosis. In fact, H3.3 mutations are highly disease specific. Now, mutation-specific antibodies are commercially available for routine diagnostic use, providing valuable support in immunohistochemical differential diagnosis.^{75,76} In the treatment of GCTB, in addition to the traditional approach of surgical curettage and artificial bone grafting, the use of the anti-RANKL antibody (denosumab) to inhibit osteoclastogenesis has been introduced, yielding certain therapeutic benefits.⁷⁷ Anti-RANKL antibody targets not the tumor cells themselves but the secondary osteoclast-like giant cells induced by the tumor. In GCTB, suppressing osteoclastogenesis and reducing bone resorption are intended to limit tumor expansion, reduce the risk of pathological fractures, and decrease the proliferative effects of bone-derived growth factors on tumor cells.

We had the opportunity to compare pre-treatment biopsy tissue with post-treatment surgical specimens in a case of GCTB treated with denosumab. Figure 9a shows the pre-treatment biopsy tissue, while Figure 9b shows the post-treatment tissue, revealing a marked change in the histological pattern. Pre-treatment, the tissue displayed typical GCTB features with numerous osteoclast-like giant cells, but post-treatment, it transformed into a bone-forming tumor containing osteoid. Upon genetic analysis of this bone-forming tumor tissue, we confirmed the presence of the GCTB-specific H3F3A p.G34W mutation, verifying that the tumor had undergone histological modification due to the treatment (data not shown). These observations suggest that while anti-RANKL antibody therapy effectively suppresses the formation of osteoclast-like giant cells, it does not inhibit the proliferation of mesenchymal tumor cells (likely corresponding to pre-osteoblasts). Over time, only the spindle-shaped cells may proliferate again, and in this regrowth phase, the ability to form osteoclasts may be lost. Consequently, following RANKL-targeted therapy, the tumor may undergo a phenotypic change into a bone-forming tumor. The observation that bone-forming tumors being induced by RANKL-targeted therapy has

led to the hypothesis that RANKL itself may transmit a reverse signal into cells, promoting bone differentiation. In fact, modified antibodies that have only neutralizing activity against RANKL reduce osteoblast-mediated bone formation while inhibiting osteoclast maturation and bone resorption. However, when modified antibodies capable of activating RANKL reverse signaling

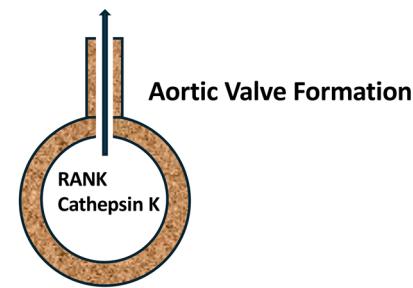
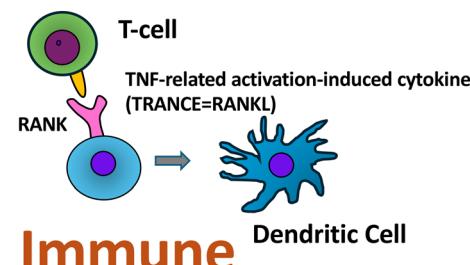
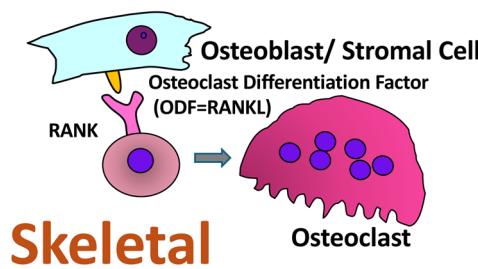


FIGURE 10 Diverse Functions of the RANKL-RANK-OPG System: The RANKL-RANK-OPG system is involved not only in osteoclast formation but also in dendritic cell differentiation, aortic valve development, and even thermoregulation. The RANKL-RANK-OPG system, which regulates osteoclast differentiation, is critically important in both the physiological and pathological states of bone tissue. When this osteoclast differentiation factor was finally identified, many researchers in bone metabolism were surprised to discover that it was identical to TRANCE, a molecule previously recognized as crucial for dendritic cell differentiation from T lymphocytes. Moreover, overexpression of OPG or variant RANK, as identified by our research, leads to cardiac abnormalities in mice (unpublished data). RANK signaling induces Cathepsin K expression in tissue macrophages during heart development, which is essential for aortic valve formation. Additionally, this system plays a role in the temperature fluctuations associated with the menstrual cycle. Thus, systems initially thought to function in a specific context can play completely different roles in various biological processes.

are administered, they not only suppress bone resorption but also prevent the inhibition of bone formation.⁷⁸ This suggests that RANKL reverse signaling in osteoblasts functions as a promising pharmacological target that promotes bone formation.²⁷

CONCLUSIONS

Discussed here is the RANKL-RANK-OPG system in the context of bone tissue. However, it has functions beyond bone tissue.⁷⁹ For example, it is involved in dendritic cell differentiation,²⁹ aortic valve formation,⁸⁰ temperature regulation,⁸¹ lymph node development,⁸² and mammary gland development.⁸³ These findings typically demonstrate that signaling pathways traditionally linked to specific events can also be involved in completely different processes. (Figure 10). This underscores how advancements in research in one area can contribute to progress in other fields and highlights the importance of comprehensive research in disciplines like pathology.

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CONFLICT OF INTEREST STATEMENT

Sohei Kitazawa is an Editorial Board member of Pathology International and a co-author of this article. To minimize bias, they were excluded from all editorial decision-making related to the acceptance of this article for publication.

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