0163-769X/08/$20.00/0
*Printed in U.S.A.*

Endocrine Reviews 29(2):155–192

doi: 10.1210/er.2007-0014

# Receptor Activator of Nuclear Factor κB Ligand and Osteoprotegerin Regulation of Bone Remodeling in Health and Disease

Ann E. Kearns, Sundeep Khosla, and Paul J. Kostenuik

*Endocrine Research Unit (A.E.K., S.K.), Mayo Clinic College of Medicine, Rochester, Minnesota 55905; and Metabolic Disorders Research (P.J.K.), Amgen Inc., Thousand Oaks, California 91320*

**Osteoclasts and osteoblasts dictate skeletal mass, structure, and strength via their respective roles in resorbing and forming bone. Bone remodeling is a spatially coordinated lifelong process whereby old bone is removed by osteoclasts and replaced by bone-forming osteoblasts. The refilling of resorption cavities is incomplete in many pathological states, which leads to a net loss of bone mass with each remodeling cycle. Postmenopausal osteoporosis and other conditions are associated with an increased rate of bone remodeling, which leads to accelerated bone loss and increased risk of fracture. Bone resorption is dependent on a cytokine known as RANKL (receptor activator of nuclear factor κB ligand), a TNF family member that is essential for osteoclast formation, activity, and survival in normal and pathological states of bone remodeling. The catabolic effects of RANKL are prevented by osteoprotegerin (OPG), a TNF receptor family member that binds RANKL and thereby prevents activation of its single cognate receptor called RANK. Osteoclast activity is likely to depend, at least in part, on the relative balance of RANKL and OPG. Studies in numerous animal models of bone disease show that RANKL inhibition leads to marked suppression of bone resorption and increases in cortical and cancellous bone volume, density, and strength. RANKL inhibitors also prevent focal bone loss that occurs in animal models of rheumatoid arthritis and bone metastasis. Clinical trials are exploring the effects of denosumab, a fully human anti-RANKL antibody, on bone loss in patients with osteoporosis, bone metastasis, myeloma, and rheumatoid arthritis. (*Endocrine Reviews* 29: 155–192, 2008)**



**First Published Online December 5, 2007**

Abbreviations: BMC, Bone mineral content; BMD, bone mineral density; BMSC, bone marrow stromal cells; CaBr, breast cancer; CaP, prostate cancer; CTX, C-telopeptide; DDH, death domain homologous; DEX, dexamethasone; DXA, dual energy x-ray absorptiometry; IBA, ibandronate; IV BP, iv bisphosphonates; M-CSF, macrophage colony-stimulated factor; microCT, microcomputed tomography; MRI, magnetic resonance imaging; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; OB, osteoblast; OCIF, osteoclastogenesis inhibitory factor; ODF, osteoclast differentiation factor; OPG, osteoprotegerin; OPGL, OPG ligand; ORX, orchiectomy; OVX, ovariectomy; PD, pharmacodynamic; PDB, Paget's disease of bone; PK, pharmacokinetic; RA, rheumatoid arthritis; RANK, receptor activator of NF-κB; RANKL, RANK ligand; SRE, skeletal-related clinical event; TRAF, TNF receptor associated factor; TRAIL, TNF-related apoptosis-inducing ligand; TRANCE, TNF-related activation-induced cytokine; uNTX, urinary N-telopeptide; ZOL, zoledronic acid.

***Endocrine Reviews* is published by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

## I. Introduction

BONE REMODELING IS the lifelong process by which old bone is replaced by new bone. Bone remodeling also helps to maintain mineral homeostasis via the liberation of calcium and phosphorus into the circulation. The remodeling process occurs at discrete sites on cortical and cancellous bone surfaces and involves the integrated and sequential actions of osteoclasts and osteoblasts. The basic multicellular unit is the term given to this localized collaborative cellular team. The remodeling cycle leads to the formation of a new "osteon" via four steps: activation, resorption, reversal, and formation. A previously quiescent bone surface is "activated" by unknown signals that attract osteoclast precursors from the circulation to the skeleton where they fuse and form multinucleated cells. These multinucleated preosteoclasts attach to the bone surface, differentiate, and begin resorbing bone matrix (resorption phase). The completion of resorption is associated with apoptosis of osteoclasts, followed by a reversal phase during which additional cells including preosteoblasts move to the bone surface. Osteoblasts orchestrate the formation of new bone matrix and regulate its mineralization. The return of the bone surface to its quiescent state involves the apoptosis of osteoblasts, their incorporation into the bone as osteocytes, or their transformation into bone surface-lining cells.

In many pathological states, and perhaps in healthy adults, each bone remodeling cycle leads to a net loss of bone due to a modest deficiency in the amount of new bone formed relative to old bone removed (1, 2). This relative deficit in bone formation could be related at least in part to trabecular perforations that occur during the resorptive phase, leading to the loss of bone surface on which osteoblasts could deposit new bone (3). Acceleration of the bone remodeling rate, which is common in many metabolic bone diseases including postmenopausal osteoporosis, thereby leads to a greater rate of bone loss (1). Inhibition of bone remodeling has therefore become a major therapeutic strategy for preventing bone loss. This review will focus on the recent discovery of the OPG/RANKL/RANK signaling pathway (Fig. 1), its critical involvement in the cellular regulation of bone remodeling, and the effects of bone remodeling suppression via RANKL inhibition.

## II. Discovery and Validation of the OPG/RANKL/RANK Pathway

### *A. Description of proteins and methods of discovery*

The first component identified for a novel pathway regulating bone resorption and remodeling was osteoprotegerin (OPG), which was discovered in mice via a genomics-based approach. Expressed sequence tags were generated by sequencing randomly selected clones from a fetal rat intestine cDNA library. The relevant expressed sequence tag for OPG encoded an open reading frame with significant homology

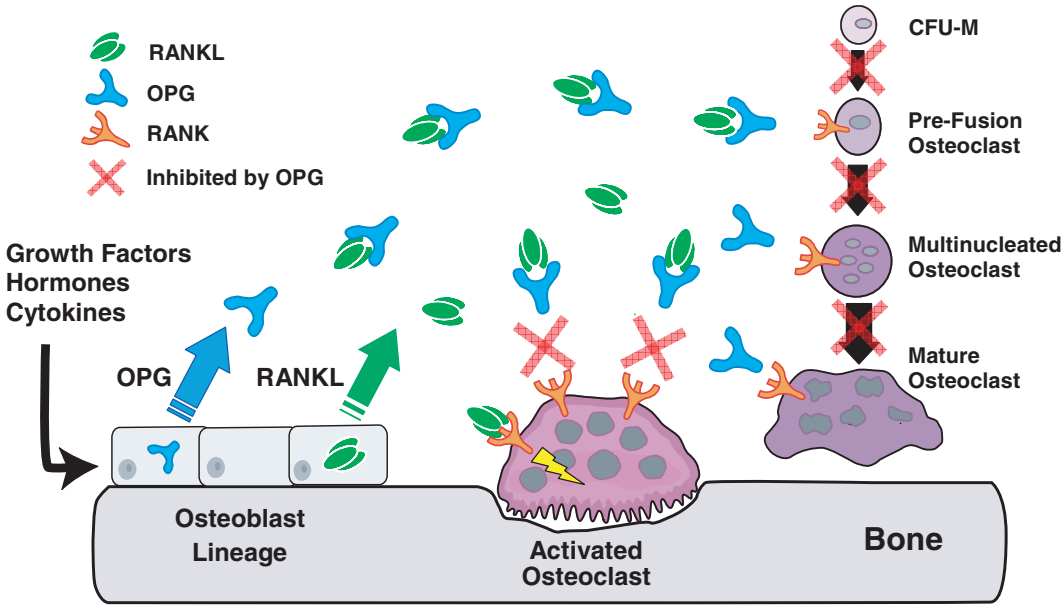


FIG. 1. Mechanisms of action for OPG, RANKL, and RANK. RANKL is produced by osteoblasts, bone marrow stromal cells, and other cells under the control of various proresorptive growth factors, hormones, and cytokines. Osteoblasts and stromal cells produce OPG, which binds to and thereby inactivates RANKL. The major binding complex is likely to be a single OPG homodimer interacting with high affinity with a single RANKL homotrimer (17). In the absence of OPG, RANKL activates its receptor RANK, found on osteoclasts and preosteoclast precursors. RANK-RANKL interactions lead to preosteoclast recruitment, fusion into multinucleated osteoclasts, osteoclast activation, and osteoclast survival. Each of these RANK-mediated responses can be fully inhibited by OPG. [Adapted from *Women's Health* 2:517–525, 2006 (266), with permission of Future Medicine Ltd.]

to TNF receptor 2. Subsequent cloning of the full-length gene revealed that it encoded a novel member of the TNF receptor family. The full-length rat OPG gene was overexpressed in its native soluble form in transgenic mice, under the control of a human apolipoprotein E promoter. These OPG transgenic mice were born with high bone mass, and this phenotype progressed as animals aged with no evidence of resolution. High bone mass in these animals was associated with marked reductions in osteoclast numbers and activity (4). The protein was therefore named "osteoprotegerin" based on its ability to protect bone. Independent of this effort, scientists in Japan isolated a protein secreted by cultured human fibroblasts that proved to be identical to OPG (5, 6). This molecule was referred to as osteoclastogenesis inhibitory factor (OCIF) based on its ability to suppress osteoclastogenesis *in vitro* (5). OPG/OCIF was shown soon thereafter to also suppress osteoclast activity (7–9), survival (10, 11), and adhesion to bone surfaces (12, 13). The name OPG was officially adopted by the nomenclature committee of the American Society of Bone and Mineral Research (ASBMR) (14). The preferred nomenclature and synonyms for the OPG/RANK/RANKL pathway are described in Table 1.

Structural domains of native OPG are described in Fig. 2 (adapted from Ref. 15). OPG is an atypical member of the TNF receptor family in that it is a secreted protein with no transmembrane domain and no direct signaling properties (4, 6). Secretion of OPG is mediated by a 21-residue signal peptide (4). OPG has three major structural motifs including four cysteine-rich TNF receptor domains, a heparin-binding domain, and two death domain homologous (DDH) regions. The DDH regions within native OPG have yet to be associated with any functional role (16), although recent evidence suggests that this domain contributes to the formation of homodimers (17). Unlike the intracellular DDH regions found in apoptosis-inducing proteins such as Fas, the DDH regions in OPG reside in an extracellular environment because OPG is secreted.

TABLE 1. Acronyms and synonyms for proteins from the OPG/RANKL/RANK pathway

| Acronym | Synonym |
|---|---|
| Ligand | |
| **RANKL** | **RANK ligand** |
| OPGL | Osteoprotegerin ligand |
| ODF | Osteoclast differentiation factor |
| TRANCE | TNF-related activation-induced cytokine |
| SOFA | Stromal osteoclast-forming activity |
| TNFSF-11 | TNF superfamily 11 |
| Receptor | |
| **RANK** | **Receptor activator of NF-κB** |
| ODAR | Osteoclast differentiation and activation receptor |
| TNFRSF-11A | TNF superfamily receptor 11A |
| Decoy receptor | |
| **OPG** | **Osteoprotegerin** |
| OCIF | Osteoclastogenesis inhibitory factor |
| TR-1 | TNF receptor-like molecule 1 |
| FDCR-1 | Follicular dendritic receptor 1 |
| TNFRSF-11B | TNF superfamily receptor 11B |

Acronyms and synonyms in *bold* are ASBMR-preferred terms for bone biology applications. References can be found within original JBMR manuscript. [Adapted from *J Bone Miner Res* 15:2293–2296, 2000 (14) with permission of the American Society for Bone and Mineral Research (ASBMR).]

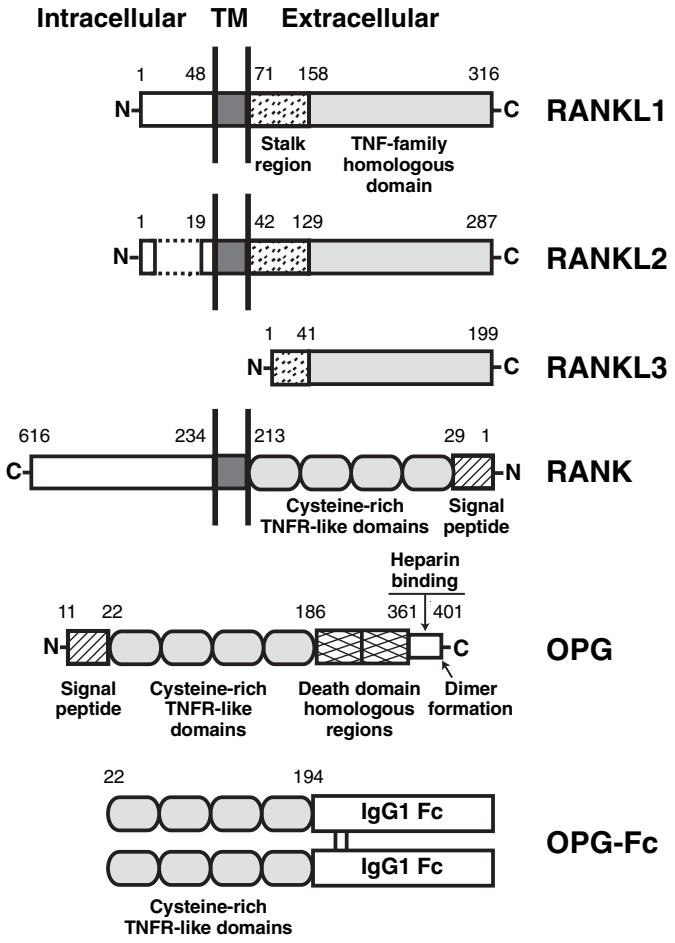


FIG. 2. Protein structures of RANKL, RANK, OPG, and OPG-Fc. Three isoforms of RANKL are described (44), each of which possesses similar C-terminal TNF-homology domains that are required for RANK activation (39). RANKL1 and RANKL2 isoforms possess a transmembrane (TM) domain and a stalk region that contains a proteolytic site that allows for cleavage of RANKL from cell surfaces (443). RANKL3 contains a truncated stalk region that possesses a proteolytic cleavage site, the function of which is unclear because this isoform lacks a transmembrane domain and is therefore secreted in the absence of proteolysis. RANK is a transmembrane protein with a large C-terminal cytoplasmic domain and an amino-terminal extracellular domain. Both RANK and OPG contain amino-terminal signal peptides as well as cysteine-rich TNF receptor-like domains that bind to RANKL. OPG lacks a transmembrane domain, consistent with its secretion as a soluble protein. OPG also includes two DDH regions, the roles of which are unknown. A heparin binding domain at the carboxy-terminus of OPG limits the half-life and distribution of the molecule, and this domain is also involved in dimer formation. Human OPG-Fc is a recombinant fusion protein that has been used in human clinical trials and in the majority of preclinical animal studies. This construct includes amino acids 22–194 of native OPG, comprising the minimal TNF receptor-like domains that mediate RANKL inhibition. This fragment lacks the signal peptide, DDH regions, and heparin binding domain of native OPG. The Fc fragment of human IgG1 was fused to the carboxy-terminus of this 22–194 fragment to maintain a dimeric molecule with a sustained circulating half-life. *Numbers in figure* represent amino acids. [Reproduced from S. Theoleyre *et al.*: *Cytokine Growth Factor Rev* 15:457–475, 2004 (15) with permission from Elsevier.]

OPG contains four cysteine-rich TNF receptor homologous domains that were necessary and sufficient for binding

to its target RANKL (17) and for osteoclast inhibition in cell culture studies (4, 16). Native OPG also possesses a heparin binding domain that might limit its circulating half-life in mice (18), perhaps via binding to cell surfaces (19). The heparin binding domain is also involved in homodimer formation (16, 17), but deletion of this region did not reduce the ability of OPG to inhibit osteoclastogenesis *in vitro* (16). OPG exists as monomeric and homodimeric forms, and in one study these two forms were shown to have similar potency for inhibiting osteoclastogenesis *in vitro* (18). However, more recent analyses using analytical ultracentrifugation to more accurately identify monomer *vs.* homodimer forms revealed that the OPG homodimer had 1000-fold greater affinity for RANKL than the monomeric form (17). This result suggests that the dimeric form of OPG is probably a more potent RANKL inhibitor than the monomeric form.

To enhance the pharmacological activity of native OPG, numerous constructs have been created wherein the signal peptide, heparin binding domain, and DDH domains were removed and the remaining peptide was fused to the Fc domain of human $IgG_1$ [*e.g.*, Fc-OPG (20–22) and OPG-Fc (23–26); Fig. 2]. The Fc fusion partner maintains the potent dimeric nature of OPG while significantly increasing its circulating half-life. The great majority of published studies describing the use of OPG in animals have relied on these types of recombinant OPG fusion proteins, whereas a few early studies demonstrated that native OPG was also capable of suppressing bone resorption in mice (7).

The major biological sources of OPG that modulate bone resorption are not well established. Osteoblasts or cells of that lineage have been shown to produce OPG (27–30). The physical proximity of osteoblasts and osteoclasts during bone remodeling creates opportunities for osteoblast-derived regulation of bone resorption. However, the production of OPG by cultured osteoblasts was shown to increase with cell differentiation, suggesting that mature osteoblasts are not well suited to support osteoclastogenesis (27). OPG is also produced by endothelial cells (31, 32), vascular smooth muscle cells (32), and other cells (33). The high concentrations of OPG extracted from healthy and diseased human blood vessels suggest that vascular cells might be major contributors to the circulating pool of OPG. Circulating OPG would still have significant potential to inhibit bone resorption by suppressing the differentiation of circulating osteoclast precursors (34).

The ability of OPG to suppress bone resorption and increase bone mass is related to its ability to bind via its TNF receptor domains to TNF domains within its natural ligand (17), now known as RANKL. RANKL has a storied history in bone biology that precedes its positive identification by more than a decade. As early as 1980 it was appreciated by Chambers (35) that cells of the osteoblastic lineage might be involved in promoting osteoclast recruitment and activity. A more formal hypothesis (36) and experimental confirmation in various model systems (37, 38) further supported this notion, but the identity of this "osteoclast activating factor" remained elusive. The eventual identification of this protein involved the use of OPG as a probe to recover an OPG binding protein that was initially referred to as osteoprotegerin ligand (OPGL) (39). An identical protein [osteoclast differentiation factor (ODF)] was independently identified from a cDNA library prepared from ST-2 stromal cells (40). This cell line had been previously shown to support osteoclast formation in a manner that could be completely inhibited by OPG (5). Although the essential role of OPGL/ODF in osteoclast biology was an important finding, this protein proved to be identical to one previously identified and referred to as receptor activator of nuclear factor κB ligand (RANKL) (41). RANKL, also referred to as TNF-related activation-induced cytokine (TRANCE) (42), was originally identified as a product of T cells. This TNF family member was able to promote dendritic cell survival and costimulation. The essential role of RANKL/TRANCE in bone resorption was confirmed soon thereafter (43). RANKL is the generally accepted acronym for this protein (14) (Table 1).

RANKL is a novel member of the TNF family of ligands. There are at least three forms of RANKL, two of which possess a transmembrane domain that positions the biologically active carboxy-terminus to the extracellular domain (*i.e.*, a type II transmembrane protein) (Fig. 2). One of these forms, RANKL2, is a shorter alternative splicing variant of RANKL1 (44). Both of these variants can remain on cell surfaces or can be proteolytically cleaved into soluble forms that possess osteoclast-stimulating activities within their TNF-homology domains (44, 45). RANKL is produced by numerous cell types including cells of the osteoblast lineage (46, 47) and activated T cells (42, 48, 49). T Cells express both soluble and membrane-bound forms of RANKL (49, 50), and both forms are implicated in focal bone erosions associated with inflammatory arthritis (26, 49). Cells of the osteoblast lineage can express RANKL on their surface in a manner that facilitates osteoclastogenesis *in vitro* via cell-to-cell contact with osteoclast precursors (29). Differentiation of cultured osteoblasts was associated with reduced RANKL expression and decreased ability to support osteoclastogenesis (27, 51), suggesting that the mature bone-forming osteoblast might not be capable of directing osteoclast activity via RANKL. Membrane RANKL has been suggested to be somewhat more potent than soluble RANKL in stimulating osteoclastogenesis *in vitro* (45, 52). However, soluble RANKL is measurable in the circulation, and serum RANKL has been shown in some studies to increase with stimulated bone resorption (26, 50, 53). Soluble recombinant RANKL is also capable of causing severe skeletal catabolism in mice (54, 55) and in rats (56). These results indicate that soluble RANKL has the potential to be an important physiological and pathological mediator of bone resorption.

RANKL is involved in numerous aspects of osteoclast differentiation and function. RANKL was implicated in the fusion of osteoclast precursors into multinucleated cells (39), their differentiation into mature osteoclasts (39), their attachment to bone surfaces (12), their activation to resorb bone (9, 39), and their continued survival by avoiding apoptosis (11). In most situations, RANKL probably relies on macrophage colony-stimulated factor (M-CSF, also known as CSF-1) as a cofactor for osteoclast differentiation (57, 58). However, it is interesting to note that unlike RANKL knockout mice, the osteoclast population recovers over time in mice lacking functional M-CSF (59). Vascular endothelial growth factor has been implicated as a compensatory factor that might maintain osteoclasts in the absence of functional M-CSF (60).

Recent preliminary evidence also suggests that RANKL can stimulate osteoclastogenesis and bone resorption in mice that lack functional M-CSF (47). These results suggest that RANKL plays a dominant role in the regulation of bone resorption, and no factor or combination of factors have been shown to be capable of restoring bone resorption when RANKL is absent.

The receptor that mediates all known activities for RANKL is called receptor activator of nuclear factor κB (RANK) (Figs. 1 and 2). RANK is a homotrimeric TNF receptor family member that was initially discovered from a bone marrow-derived dendritic cell cDNA library. RANK was identified as a receptor that mediated the ability of RANKL to promote the survival of cultured dendritic cells (41). An important role for RANK in osteoclastogenesis was reported by Nakagawa *et al.* (61), who referred to this TNF receptor family member as osteoclast differentiation factor receptor. The essential role for RANK in bone resorption was demonstrated soon thereafter by the high bone mass phenotype of RANK knockout mice, which were virtually devoid of osteoclasts (62, 63). RANK and RANKL knockout mice were virtual phenocopies of each other (62–64), which indicated that RANK and RANKL had few if any roles beyond their mutual interactions.

The binding and activation of RANK involve direct interactions between the extracellular receptor binding domain of trimeric RANKL and the extracellular cysteine-rich domains of trimeric RANK (17, 65). This interaction is thought to cause oligomerization of RANK and the subsequent activation of several signal transduction pathways. A simplified overview of major signaling pathways is described in Fig. 3. RANKL binding to RANK results in the recruitment of an adapter protein known as TRAF 6 (TNF receptor associated factor 6) to specific sites within the intracellular domain of RANK. TRAF 6 acts as a second messenger to activate various protein kinase pathways as well as transcription factors including nuclear factor-κB (NF-κB). Activated NF-κB translocates to the nucleus and up-regulates the expression of c-fos, which then interacts with nuclear factor of activated T cells (NFAT)-c1 to induce the transcription of osteoclastogenic genes. RANK signaling is far more complex than what is described above, and more details of these and other important pathways were recently described (66–69).

This brief historical summary recaps a remarkable 3-yr period in bone biology research (1997–1999), during which time the pieces to a long-sought picture of osteoclast biology were rapidly described in the scientific literature. The picture emerged from numerous independent laboratories around the world, using novel and distinct discovery platforms and model systems. Most satisfying scientifically was the consistency of observations and conclusions between research groups regarding the functions and essential roles of OPG, RANK, and RANKL. The consensus regarding this molecular triad is that OPG functions as a soluble decoy receptor by binding to RANKL, thereby preventing RANKL from binding and activating RANK (Fig. 1). OPG inhibition of RANKL leads to the rapid arrest of osteoclast formation, attachment to bone, activation, and survival.

### *B. Expression patterns and hormone/cytokine regulation of expression*

With some notable exceptions, the expression patterns of OPG, RANK, and RANKL genes would not obviously predict their biological roles. Expression of each gene product has been shown in numerous cells and tissues in which no known role for that gene product has been ascribed. OPG mRNA, for example, is highly expressed in the lung, heart, kidney, and placenta (4), and OPG has yet to demonstrate any physiological or pathological role in those organs. OPG mRNA is also expressed in liver, stomach, intestine, skin, bone, and most other human tissue (6) with the exceptions of brain, skeletal muscle, thymocytes, or peripheral blood lymphocytes (6, 70). At the cell culture level, OPG is expressed by bone marrow stromal cells (27), fibroblasts (6), endothelial cells (31, 71), lymphoid cells (70), smooth muscle cells (70), and other cell types.

RANK mRNA is expressed in bone, bone marrow, spleen, skeletal muscle, brain, heart, liver, lung, mammary tissue, and skin (61, 72, 73) and at the cellular level by osteoclasts,

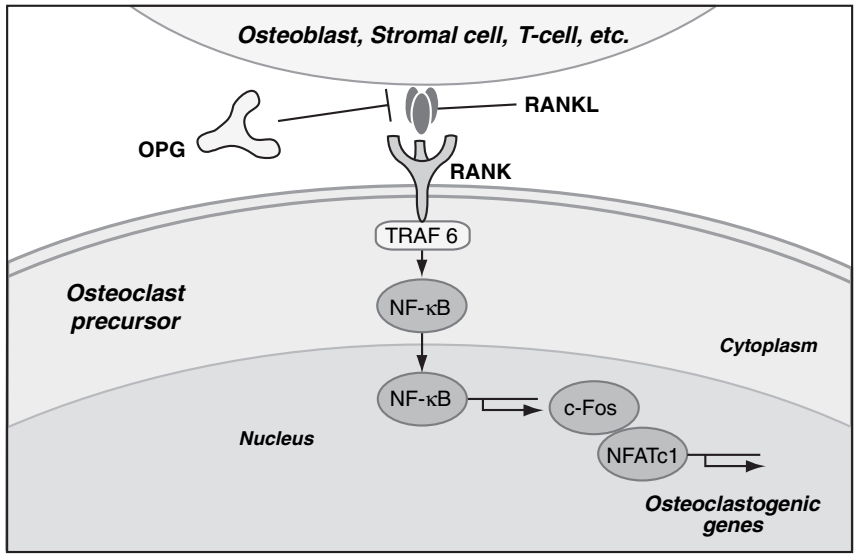


FIG. 3. Essential signaling pathways activated by RANKL interactions with RANK. RANKL, which is produced by osteoblasts, stromal cells, T cells, and other sources, activates RANK on the surface of osteoclasts and osteoclast precursors. RANK activation leads to the recruitment of the adaptor protein TRAF 6, leading to NF-κB activation and translocation of NF-κB to the nucleus. NF-κB increases c-Fos expression and c-Fos interacts with NFATc1 to trigger the transcription of osteoclastogenic genes. Activation of these pathways is prevented naturally by OPG, which prevents RANKL from activating RANK in the extracellular environment. RANKL-RANK interactions are also prevented by RANK-Fc (a truncated form of RANK that acts as a nonsignaling decoy receptor) and by denosumab, a fully human monoclonal antibody that binds and inhibits RANKL. [Adapted with permission from B. F. Boyce and L. Xing: *Arthritis Res Ther* 9:S1–S7, 2007 (66).]

osteoclast precursors, dendritic cells, chondrocytes, endothelial cells, fibroblasts and macrophages, and other cells (41, 61, 72, 74). RANKL is highly expressed in peripheral lymph nodes and bone, and also in spleen, thymus, Peyer's patches, intestine, brain, heart, skin, skeletal muscle, kidney, liver, lung, and in mammary tissue of pregnant mice (39, 40, 73, 75). Cells that express RANKL include osteoblasts, bone marrow stromal cells, activated T cells, B cells, fibroblasts, endothelial cells, chondrocytes, and mammary epithelial cells (31, 40, 42, 76, 77). Regulatory elements involved in the transcription of the OPG gene (78–81) and the RANKL gene (78, 79) have been recently described but are beyond the scope of this review.

Numerous growth factors, hormones, cytokines and drugs have been shown to influence the expression of OPG and RANKL, and an excellent summary of these regulatory responses was described recently (15) (Table 2). RANK tends to be minimally regulated, which is not unexpected because the modulation of OPG and/or RANKL levels is probably sufficient to control the signaling output of RANK. Numerous molecules that stimulate bone resorption have been shown to up-regulate RANKL expression and/or to down-regulate OPG expression. Reciprocal regulation of RANKL and OPG is often observed, a phenomenon that might serve to amplify proresorptive signals (82–84). Evidence in numerous disease models and settings suggests that the ratio of RANKL:OPG represents an important determinant of bone resorption (85–96), although exceptions to this concept have been noted (53, 97).

An illustration of reciprocal regulation of RANKL and OPG is found with PTH. PTH stimulates bone resorption in patients with hyperparathyroidism, and resorption is also stimulated when PTH is injected intermittently or infused continuously. In one study, PTH (1-34) was infused into parathyroidectomized rats for 6 h, after which time mRNA was extracted from bones and evaluated for expression of RANKL and OPG (88). PTH infusion led to a dose-dependent increase in RANKL mRNA and a dose-dependent decrease in OPG mRNA (Fig. 4). Intermittent PTH treatment in humans was also shown to increase serum RANKL and to reduce serum OPG (93), suggesting that OPG and RANKL might mediate the resorptive response to PTH independent of the mode of PTH administration.

TABLE 2. Regulators of OPG, RANKL, and RANK expression

| | OPG | RANKL | RANK | Refs. |
|---|---|---|---|---|
| 1,25-dihydroxyvitamin D | ↑ ↓ | ↑ | ↑ | 28, 103, 318 |
| Hormones | | | | |
| Estrogen | ↑ | ↓/— | | 46, 76, 84, 104–108 |
| Testosterone | ↑ ↓ | — | | 106, 219 |
| Glucocorticoid | ↓ | ↑ | | 254, 258, 259 |
| PTH | ↓ | ↑ | | 86, 88 |
| PTHrP | ↓ | ↑ | | 101 |
| Cytokines | | | | |
| IL-1 | ↑ ↓ | ↑ | ↑ | 28, 98, 99 |
| IL-4 | | | ↓/— | 109 |
| IL-7 | | ↑ | | 100 |
| IL-13 | ↑ | ↓ | — | 52 |
| IL-17 | ↓ | ↑ | — | 52 |
| TNF $\alpha$ | | ↑ | | 28, 52, 98 |
| Interferon $\gamma$ | ↑ | ↑ | ↑ | 52 |
| Prostaglandin E2 | ↓ | ↑ | | 28, 444 |
| Growth factors | | | | |
| TGF $\beta$ | ↑ | ↑ ↓ | — | 52 |
| Bone morphogenetic protein 2 | ↑ | | — | 28 |

↑, Increased expression; ↓, decreased expression; —, no change observed.

Proresorptive agents that regulate OPG and/or RANKL include IL-1 (98, 99), IL-7 (100), IL-17 (52), TNF-$\alpha$ (52, 98), PTH (86, 88), PTHrP (101), and vitamin D (102, 103) (Table 2). Many molecules that suppress osteoclastogenesis and/or bone resorption have also been shown to regulate OPG and/or RANKL, including estrogen (46, 76, 84, 104–108), interferon-$\gamma$ (52), TGF-$\beta$ (52), IL-4 (109), and IL-13 (52) (Table 2). These studies have provided important molecular insights into the mechanisms by which some of these molecules might regulate bone resorption. OPG prevents the ability of proresorptive agents (IL-1, TNF-$\alpha$, PTH, PTHrP, vitamin D) to increase bone resorption in mice (21), which is consistent with the interpretation that those agents increase bone resorption by increasing the RANKL:OPG ratio. This conclusion must be tempered, however, by the fact that OPG and RANKL also play dominant roles in the regulation of normal bone remodeling. In the absence of RANK or RANKL, osteoclasts cannot form, function, or survive (62–64, 110, 111). The dominant role of RANKL in osteoclast biology creates practical limitations in using OPG as a research tool to evaluate the extent to which different proresorptive factors work through the RANK-RANKL pathway. For example, cell culture studies have shown that proresorptive agents including IL-1 and TNF-$\alpha$ can stimulate certain aspects of osteoclast activation or differentiation in a manner that cannot be entirely inhibited by OPG (112–114). Yet the direct injection of IL-1 and/or TNF-$\alpha$ into RANK knockout mice induced the appearance of few if any osteoclasts, with no evidence of

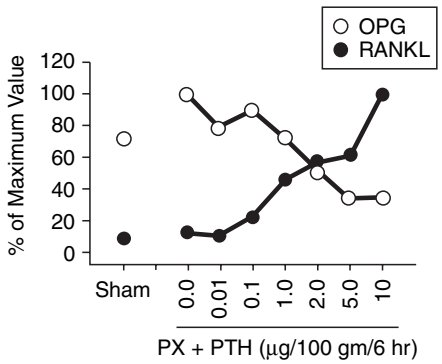


FIG. 4. PTH regulation of OPG and RANKL mRNA in parathyroidectomized (Px) rats. RNA was isolated from the distal femur of Px rats immediated after a 6-h infusion with vehicle or with different doses of human PTH 1-38. Bone mRNA was assessed by Northern blot analysis for expression of OPG and RANKL, the levels of which were standardized to the housekeeping gene GAPDH. This graphic presentation of Northern blot results depicts the relative values of OPG and RANKL mRNA at different dose levels of PTH. The highest levels of OPG mRNA were seen at the lowest levels of PTH infusion and decreased with increasing levels of PTH. In contrast, RANKL mRNA was lowest in the absence of PTH infusion and increased in a dose-dependent manner with increasing levels of PTH. These results illustrate the reciprocal regulation of OPG and RANKL in a manner that would amplify the proresorptive stimulus of PTH infusion. [Reproduced from Y. L. Ma *et al.*: *Endocrinology* 142:4047–4054, 2001 (88) with permission from The Endocrine Society.]

bone resorption (115) or hypercalcemia that was apparent in similarly challenged wild-type mice (63). A possible explanation for the apparent discordance between these *in vitro vs. in vivo* observations was provided when TNF-$\alpha$ was shown to require very low "permissive" levels of RANKL to stimulate osteoclasts (116). This low level of signaling might persist in cell culture studies despite apparent saturation by OPG, or alternatively culture conditions might include stimuli for osteoclast formation and survival that do not exist in animals. In support of the latter notion, OPG treatment of mice was able to fully prevent the proresorptive effects of IL-1 and TNF-$\alpha$ (21). Thus, whereas TNF-$\alpha$, IL-1, IL-8 (117), IL-6, IL-11 (118), and other molecules might have independent stimulatory effects on osteoclasts, the very existence of those osteoclasts remains dependent on RANK-RANKL signaling.

Unexpected sources of regulation for OPG and RANKL have been suggested by studies of signaling pathways for osteoblasts and osteocytes. An important signal transduction node for osteoblasts is $\beta$-catenin, a cytoplasmic protein that mediates signaling by members of the wnt (wingless) family of ligands. The genetic ablation of $\beta$-catenin in mice led to an osteopenic phenotype that appeared to be driven primarily by increased bone resorption (119). OPG mRNA expression was significantly reduced in osteoblasts isolated from these $\beta$-catenin-ablated mice. In contrast, mice with a gain-of-function mutation in $\beta$-catenin had high bone mass, and their cultured osteoblasts had increased expression of OPG mRNA. Other studies showed that the conditional knockout of $\beta$-catenin in osteoblasts resulted in increased expression of RANKL and decreased expression of OPG. Conversely, increased $\beta$-catenin activity in osteoblasts was shown to reduce RANKL and increase OPG expression (120). These observations raised the interesting possibility that wnt/$\beta$-catenin signaling in osteoblasts regulates bone mass by coordinating the activity of both osteoblasts and osteoclasts, with osteoclast regulation occurring via changes in OPG and RANKL.

The osteocyte is by far the most abundant cell type in bone. These cells are interconnected by numerous cellular processes that permeate bone matrix and are thought to play an important role in sensing mechanical strain (121). The mechanosensory function of osteocytes might ultimately be mediated via their connection to bone surfaces, where they have the potential to regulate osteoblasts and osteoclasts. Although osteocytes do not appear to express RANKL mRNA (122), a recent study suggests that these cells might regulate RANKL expression by other bone cells. The conditional ablation of osteoctyes in mice was associated with rapid bone loss in association with increased bone resorption, increased skeletal expression of RANKL mRNA, and an increased RANKL:OPG ratio (123).

### *C. Role of OPG, RANK, and RANKL in normal bone turnover*

The dominant physiological role of the RANK/RANKL/OPG pathway in the regulation of bone resorption was demonstrated in animal models wherein these molecules were genetically ablated or overexpressed or, in some cases, both. OPG overexpression was shown to result in increased cortical and cancellous bone mass in association with reduced osteoclast numbers (4). The essential physiological role of OPG in maintaining bone mass was shown by the severe osteopenic phenotype of OPG knockout mice (124–126). These mice exhibit increased bone turnover, cortical porosity, and reductions in cortical and cancellous bone volume and density. These deleterious changes are accompanied by spontaneous fragility fractures in the first weeks of life (124). Biomechanical testing revealed that cortical bone strength was reduced by 50% in OPG knockout mice compared with wild-type controls (125). The osteoporotic phenotype of OPG knockout mice tends to worsen with age, and the loss of even one OPG allele was associated with significant osteopenia (124). In addition to a deficit in bone quantity, OPG deficiency is associated with poor bone quality as well. Collagen within the cortical bone of OPG knockout mice has a woven rather than lamellar orientation, a phenomenon that is associated with pathologically elevated bone turnover (127). Mutations in the OPG gene in humans is associated with juvenile Paget's disease, and bone biopsies from such patients reveal a severe disruption in the normal architecture of cancellous bone (128).

These genetically based models of OPG deficiency are influenced by skeletal growth and development, which confound attempts to deduce the consequences of OPG deficiency on the mature skeleton. In the absence of conditional OPG knockout models, investigators have relied on injections of RANKL into adult rats and mice to create a model of relative OPG deficiency. RANKL injections led to rapid skeletal changes that closely resembled those associated with the lifelong absence of OPG: increased bone turnover, increased cortical porosity, weakened cancellous microarchitecture, and reductions in bone volume, density, and strength (54–56). Interestingly, the typical age-related bone loss seen in normal mice was correlated with increased skeletal expression of RANKL mRNA and reduced expression of OPG (89). These studies and others reveal that the adult mouse skeleton continues to rely on OPG and a positive OPG:RANKL ratio to maintain bone mass and strength.

Based on the established role of OPG in maintaining bone mass and the mechanistic basis of that role, the skeletal phenotypes of RANK and RANKL knockout mice were not unexpected. Genetic ablation of either RANK or RANKL led to severe osteopetrosis in association with a near total lack of osteoclasts (62–64, 110). A few notable skeletal differences were apparent between RANK and RANKL knockout mice compared with OPG transgenic mice. OPG transgenic mice exhibited an osteopetrotic phenotype, with high bone mass but without significant changes in the external shape of their long bones or defective tooth eruption (4). In contrast, RANK and RANKL knockout mice had more classical osteopetrotic features including high bone mass, shortened club-shaped long bones, and the failure of teeth to erupt. These differences suggest that a small residual level of RANK signaling, which is present in OPG transgenics (but not in RANK or RANKL knockout animals), was sufficient to establish normal bone shape and tooth eruption. This notion is supported by the skeletal phenotype associated with transgenic overexpression of RANK-Fc, a soluble extracellular portion of RANK that functions as a RANKL inhibitor (72). Similar to OPG

transgenic animals, RANK-Fc transgenic animals had high bone mass, normal bone shape, and normal tooth eruption. Unlike RANK and RANKL knockouts, RANK-Fc transgenics were able to maintain a greatly reduced but still present osteoclast population that may have permitted the establishment of normal bone shape and tooth eruption.

Two interesting hallmarks of recombinant OPG and RANKL are the rapidity of their effects on bone resorption and their ability to regulate osteoclast numbers. Soluble RANKL stimulates bone resorption within 1 h of injection in mice (9), and soluble OPG suppresses indices of bone resorption within 2 h of its injection (7). These kinetics are consistent with the demonstrated ability of RANKL and OPG to regulate the activity of mature active osteoclasts (9). OPG suppression of osteoclast numbers in mice and rats also occurs rapidly, with 50–60% reductions in osteoclasts noted within 12–24 h of treatment with an OPG-Fc fusion protein (11, 24). Maximal suppression of osteoclast numbers in these animal studies was greater than 90%. Reduced osteoclast numbers in mice was attributable to osteoclast apoptosis, which was apparent histologically within 6 h of OPG-Fc treatment (11). The prolonged duration of osteoclast suppression with OPG-Fc treatment was probably related to the long circulating half-life of the drug (24), as well as the time required to regenerate new osteoclasts from their precursor population once the drug is cleared (11).

The ability of OPG to cause consistent, rapid, and marked reductions in osteoclast numbers is relatively unique among antiresorptive agents. In contrast, bisphosphonates have a different mechanism of action that can be associated with normal or even increased osteoclast numbers despite clear antiresorptive effects (129–138). This phenomenon is consistent with previous observations that bisphosphonates suppress bone resorption via injury to osteoclasts when they solubilize bisphosphonate-contaminated bone (139, 140). Bisphosphonates do not appear to suppress osteoclastogenesis (129, 140, 141) and in some cases can paradoxically stimulate the formation of osteoclasts (133). Although bisphosphonates are clearly capable of inducing osteoclast apoptosis, this apoptotic response does not appear to be an essential component of their ability to suppress bone resorption (142). Whether the ability of OPG to suppress osteoclast formation, function, and survival has any clinical advantages over agents that act primarily or exclusively through the suppression of osteoclast function is not known. But the ability of bisphosphonates to suppress bone resorption without consistent reductions in osteoclast numbers suggests that bisphosphonates might act through a mechanism that is distinct from that of OPG.

### *D. Non-osseous roles for OPG, RANK, and RANKL*

*1. The vascular system.* Although the skeletal phenotypes associated with genetic knockout of OPG, RANK, and RANKL were somewhat predictable, a few unexpected phenotypes were also observed in these animals. OPG knockout animals developed medial calcification of the aorta and renal arteries, in association with elevated bone turnover (124). This vascular calcification could be prevented via transgenic overexpression of soluble OPG, indicating that systemic RANKL inhibition could prevent the vascular consequences of endogenous OPG deficiency (127). Bone turnover and RANKL levels are both systemically elevated in OPG knockout mice (143), and it is unclear whether their vascular calcification is related to high turnover, or to a relative increase in RANKL activity within blood vessels, or to other causes. Evidence at this point seems to favor high bone turnover as an etiological aspect of vascular calcification, at least in animal models. Recombinant OPG was shown to prevent vascular calcification induced by warfarin and by vitamin D in rats (144), and in each of these model systems the prevention of vascular calcification by OPG was associated with strong suppression of bone turnover. Bisphosphonate treatment of rats also suppressed vascular calcification in this warfarin model, in association with bone turnover suppression (145). The ability of OPG to prevent apoptosis of cultured endothelial cells (71, 146) and arterial smooth muscle cells (143) suggests that bone turnover suppression might not fully explain the ability of OPG to prevent vascular calcification. However, although bisphosphonates suppress vascular calcification (145), these agents have also been shown to suppress the proliferation, adhesion, migration, and survival of endothelial cells *in vitro* (147, 148) while inducing endothelial cell apoptosis *in vivo* (149). Thus, although OPG and bisphosphonates can have opposite effects on the survival of endothelial cells, both agents suppress bone turnover and prevent vascular calcification when tested in a similar animal model (144, 145). These phenomena are consistent with the interpretation that bone turnover is an important aspect of vascular calcification.

More evidence for a turnover-related mechanism of vascular calcification comes from recent studies using knockout mice that lack the ApoE gene, which predisposes them to atherosclerosis and vascular calcification. When these *ApoE* knockout mice were cross-bred with OPG knockout mice, bone turnover increased in association with greater calcification and advanced plaque progression. The absence of OPG had no apparent influence on circulating levels of total or HDL cholesterol (143). These interventional studies in preclinical models support the possibility that OPG serves a protective role in vascular disease. Based on this premise, osteoprotegerin has also been alternatively described as "vasculoprotegerin" (150, 151).

Evidence from human studies also suggests a relationship between bone turnover and vascular disease. Observational studies in postmenopausal women showed that aortic calcifications were inversely related to bone density and positively associated with fractures (152). It was therefore surprising that observational studies frequently show positive correlations between serum OPG levels and vascular disease, although the correlation between serum OPG levels and fractures or BMD is inconsistent as discussed in *Section IV.A* (151, 153–160). Recent review papers have summarized this literature more extensively (161–163). Possible interpretations of this positive relationship include OPG playing an active role in disease progression (157, 158), or OPG serving as a compensatory response to minimize disease progression (151, 154, 156, 164), or OPG representing a noncompensatory (neutral) response to disease (156, 165). Interventional studies with RANKL inhibitors will be needed to sort out these possibilities.

Although OPG therapy consistently suppresses vascular calcification in animal models (127, 144, 166), there are minimal data on the effects of OPG therapy on atherogenesis or other aspects of vascular disease. The clinical observational data described above highlight the need for further preclinical intervention studies on this subject. An ideal animal model would recapitulate the clinical picture of progressive atherosclerosis and vascular calcification in association with systemic increases in endogenous OPG levels. Those very conditions were recently described in an animal model involving LDL receptor knockout mice on a high-fat diet (167). Studies have been performed in similar mice to evaluate the consequences of therapeutic intervention with OPG in the presence of vascular disease and elevated endogenous OPG levels. LDL receptor knockout mice were put on a high-fat diet while being treated for 5 months with OPG. OPG treatment significantly reduced calcified lesion area in the aorta of these mice, without affecting atherosclerotic lesion size or number (166). OPG had no effect on concentrations of inflammatory cytokines in the aorta or on plasma cholesterol levels. These results support the hypothesis that OPG might reduce vascular calcification via suppression of bone turnover, with a neutral effect on atherosclerotic changes. Additional studies on RANKL inhibition in different atherogenesis models are warranted to corroborate these preliminary findings.

*2. Immune system development and function.* Another unexpected phenotype found in RANK and RANKL knockout mice was the total lack of lymph nodes (62, 64). Several other knockout mouse models also exhibit lymph node agenesis, but those models also exhibited a lack of Peyer's patches and follicular dendritic cells. Those findings led investigators to consider that these developmental processes were linked (168). However, RANK and RANKL knockout mice had normal Peyer's patches and intact splenic architecture (49, 62), indicating that the development of lymph nodes and secondary lymphoid organs can be differentially regulated. The RANK and RANKL knockout phenotypes prove that some level of RANK signaling is essential for the development of lymph nodes in mice. But beyond this observation, it has been difficult to ascribe an important role for RANK signaling in the function of immune systems in genetically intact mice or rats. It is noteworthy that in contrast to the effects of total RANKL ablation, prenatal inhibition of RANKL in OPG transgenic mice and rats did not lead to any observed changes in lymph node development or architecture (4, 182). Normal lymph node development occurred in OPG transgenic rats despite evidence that OPG was overexpressed in embryos by 100-fold during the gestational days that preceded the typical appearance of early lymphoid nodules. The low level of RANKL activity that persists in the presence of 100-fold overexpression of OPG was apparently sufficient to permit lymph node development, suggesting that total genetic ablation of RANK-RANKL signaling might be necessary to cause lymph node agenesis. Although RANKL knockout mice had no lymph nodes, there was no apparent intrinsic defect in immune cells from those animals, because T and B cells from RANKL knockout mice were able to populate the lymph nodes of normal wild-type mice (64). Examination of mice genetically deficient in RANKL, RANK, or OPG also revealed no overt dendritic cell defects (62, 64, 170). These observations suggest that the immune phenotype of RANK and RANKL knockout mice might be largely restricted to lymph node agenesis and the consequences thereof.

These findings notwithstanding, there is still significant interest in the potential immunomodulatory effects of RANKL and RANKL inhibition. Within the developed immune system, RANKL is expressed by T cells (42), RANK is expressed by dendritic cells (49), and OPG is expressed by B lymphocytes and dendritic cells (4, 70). *In vitro* studies show that binding of RANKL from T cells to RANK on dendritic cells regulates dendritic cell function and survival (42). While RANKL has the ability to stimulate dendritic cells, there is little evidence that RANKL inhibition leads to dendritic cell suppression. This is probably related to the central role of CD40L and CD40, found on T cells and dendritic cells, respectively, in mediating cross-talk between T cells and dendritic cells. This important pathway is apparently able to compensate for the total absence of RANK-RANKL signaling in knockout mouse studies (171, 172).

Several studies have shown that RANKL inhibition had no significant effects on the *in vivo* responses to immune challenges in genetically intact animals (171–174). Short-term (up to 2-wk) mouse studies have show that RANKL inhibition by OPG did not impair cell-mediated reactions such as contact hypersensitivity or granuloma formation, innate immunity, humoral responses to defined immune challenges (175), or influenza infection (173). Of greater interest, however, is the potential consequence of long-term RANKL inhibition on immune function. This question was addressed using OPG transgenic mice and rats, in which RANKL is inhibited continuously throughout life. Analysis of numerous cellular, innate, or adaptive immune responses in 4- to 6-month-old transgenic mice and rats showed no differences compared with normal wild-type controls (169, 174, 176). OPG transgenic rats in these studies had marked suppression of bone turnover and increased BMD, thereby demonstrating that RANKL inhibition can cause turnover suppression in the absence of measurable changes in the integrated response of the immune system (169). Similar findings have been noted in animal models of inflammatory bone loss, wherein RANKL inhibition effectively preserved bone while having no significant effects on the inflammatory component of disease (26, 110, 136, 177–181). These results suggest that RANKL likely plays a redundant role in the integrated function of the postnatal immune system, perhaps due to the overlapping and predominant role of the CD40-CD40L costimulatory pathway.

*3. Mammary gland development during pregnancy.* The other significant extraskeletal manifestation of total RANK/RANKL ablation in knockout mice was a defect in mammary gland development during pregnancy and lactation (73). Neither RANK nor RANKL was required for mammary gland development in mice before pregnancy, whereas pregnant RANK knockout mice failed to lactate and their litters did not survive. The mammary gland defect in RANK knockouts was attributed to the inability of sprouted alveolar buds

to differentiate and expand into mature lobuloalveolar structures. In contrast to the effects of total genetic ablation of RANK-RANKL signaling, the continuous inhibition of RANK-RANKL signaling does not appear to result in a failure of lactation. OPG transgenic rat and mouse pups are viable, show normal suckling behaviors, and survive to weaning without supplemental feeding (Ref. 4, and P. J. Kostenuik, unpublished observations). OPG is overexpressed in these animals by 100-fold during gestation and throughout life, and this level of OPG was associated with marked suppression of bone turnover (169, 182). These observations suggest that, as with lymph node development, a small permissive level of RANKL activity is sufficient to allow for mammary gland development during pregnancy in mice.

## III. Pharmacological Effects of RANKL Inhibition in Preclinical Models of Bone Disease

### *A. RANKL and RANKL inhibition in normal healthy animals*

Studies in normal healthy animals have revealed that OPG and RANKL play important ongoing roles in the maintenance of bone mass and in the regulation of normal bone remodeling. The purpose of bone remodeling in normal healthy animals is unclear, but candidate roles include calcium homeostasis (a spatially stochastic catalyst for remodeling) and repair of microdamage (a presumably targeted catalyst) (183). Normal healthy mice and rats do not typically undergo cortical remodeling, so it is difficult to claim that cortical remodeling in those systems is related to damage repair. The existence of targeted remodeling for microdamage repair in cancellous bone has not been established (183). Cancellous bone volume drops precipitously with aging in normal mice (89), so if cancellous remodeling was intended to replace microdamage, its effects on bone strength would be deleterious by virtue of substantial net bone loss. There is no evidence that suppression of bone remodeling in mice or rats results in reduced whole bone strength and abundant evidence (described in *Sections III.A*, *B*, and *F*) that suppression of bone remodeling increases bone strength in those species and in nonhuman primates.

The suppression of bone remodeling in normal rats by OPG leads to increased bone volume and density despite the significant coupling-related suppression of bone formation parameters (54–56). Furthermore, stimulation of bone remodeling in normal mice or rats by RANKL leads to reduced bone volume despite the significant coupling-related stimulation of bone formation (54–56). These observations suggest that remodeling-related changes in osteoblast output did not adequately counter the changes in osteoclast activity resulting from OPG or RANKL administration. This hypothesis must be tempered by the possibility that OPG and RANKL also influenced osteoclasts participating in bone modeling, which is more prevalent in younger growing animals and which is not countered by subsequent bone formation (184). Modeling-dependent bone formation and growth appear to be spared during RANKL inhibition because OPG had no influence on longitudinal bone growth in normal young rats (185), mice (186), piglets (187), or cynomolgus monkeys (25).

A brief summary of data from healthy gonad-intact rats and cynomolgus monkeys provides an illustration of the consequences of RANKL inhibition on normal bone remodeling, density, and strength. In rats, a single injection of OPG-Fc resulted in a rapid (within 12 h) and profound (up to 95%) reduction in the percentage of bone surfaces occupied by osteoclasts (24). Osteoclast numbers showed a gradual recovery from d 10 to 30 after injection as the drug was gradually cleared from the circulation. Osteoblast surface and biochemical markers of osteoblast activity were more modestly suppressed relative to osteoclast inhibition. These changes were associated with a progressive increase in cancellous bone volume and a significant increase in bone mineral density (BMD) at d 30 compared with vehicle-treated controls (24).

Assessments of bone strength and bone quality in normal healthy rodents treated with RANKL inhibitors have been limited to short treatment periods of less than 1 month. The marked turnover suppression associated with RANKL inhibition raises questions about the consequences of long-term therapy to bone strength and bone quality. These questions are particularly relevant if the repair of skeletal microdamage is a significant feature of normal bone remodeling. One approach to address this question involved the use of transgenic rats that overexpressed soluble OPG from a prenatal stage and throughout life (188). The transgene construct in these rats was the same one used to identify the critical role of OPG in regulating bone mass in transgenic mice (4). In transgenic rats, continuous overexpression of OPG for the first 6 months of life led to a 98% reduction in osteoclast surface in the lumbar vertebrae. This marked and sustained suppression was associated with 2-fold increases in extrinsic strength parameters of the vertebrae relative to age-matched wild-type (normal) control rats. These strength increases appeared to be primarily related to increases in bone mass. When strength parameters are standardized to bone mass parameters, one can indirectly examine the strength of the bone tissue itself (intrinsic bone strength). Intrinsic strength parameters in OPG transgenic rats were similar to values obtained in normal controls (188). These data suggest that long-term RANKL inhibition improves bone strength in normal animals primarily by increasing bone mass, while preserving the quality of bone matrix. Similar conclusions were supported by analyses of bone mass and bone strength in 12-month-old OPG-Tg rats (189).

Although bone mass is clearly a major attribute of bone strength, there are other components of bone strength that are not effectively captured by standard bone densitometry modalities such as dual energy x-ray absorptiometry (DXA) or peripheral quantitative computed tomography. These "bone quality" parameters include matrix mineralization (190), cortical porosity (191), cortical geometry (192), and trabecular microarchitecture (193, 194). The effects of recombinant RANKL on these parameters were recently described. In normal healthy mice, 10 d of recombinant RANKL injections led to reduced matrix mineralization, an 85% reduction in trabecular bone volume, deleterious changes in cortical geometry, and significantly reduced bone strength (54). In

normal healthy rats, 4 wk of RANKL infusion led to reduced matrix mineralization, decreased trabecular and cortical bone volume, reduced trabecular connectivity, and reduced bone strength (56).

Based on their mechanisms of action and their important roles in normal bone turnover, one might expect OPG and RANKL to have opposite effects on bone quality and strength parameters in normal animals. This seems to be the case because OPG treatment of normal rats was shown to increase matrix mineralization and improve bone strength (185). While RANKL caused deleterious effects on trabecular microarchitecture in normal rats (56) and reduced matrix mineralization in normal mice (56), OPG treatment was recently shown to have the opposite effects in normal mice. These data were presented in abstract form (195), and the lack of published data on the effects of OPG on trabecular microarchitecture in normal healthy animals warrants a brief description of those data. Briefly, 2- to 3-month-old Black Swiss/129 gonad-intact mice (n = 8 per group) were treated for 3 wk with either human OPG-Fc (5 mg/kg sc, twice a week) or with vehicle (saline). Distal right femurs and lumbar vertebrae were scanned *ex vivo* by microcomputed tomography (microCT) at 18-$\mu$m resolution for analyses of trabecular bone. OPG treatment was associated with significantly greater bone volume, volumetric BMD, bone matrix mineral density, trabecular thickness, number, and connectivity (Table 3). Representative microCT images of trabecular regions from the distal femur and lumbar vertebra are shown in Fig. 5. It is important to note that the magnitude of changes observed in this 3-wk study were greatly influenced by growth-related modeling that is typical of young mice. The magnitude and rate of such changes would not be achievable in adult humans, with or without bone disease. However, these data suggest that bone mass improvements with OPG are associated with evidence of improved trabecular architecture.

Nonhuman primates represent the most appropriate preclinical species for examining the skeletal response to therapeutic intervention. In young gonad-intact cynomolgus monkeys, 3 months of OPG treatment (25 mg/kg sc injections once monthly) led to significant reductions in biochemical markers of bone turnover, including serum alkaline phosphatase and urinary N-telopeptide (uNTX) (22). OPG treatment was associated with decreases in serum ionized calcium and phosphorus levels, which was an expected consequence of suppressed bone resorption and secondary increase in serum PTH levels. No clinical signs of hypocalcemia were noted in this toxicology study, perhaps due to an observed OPG-related increase in serum PTH that maintained serum calcium at an asymptomatic level. OPG treatment increased BMD and bone mineral content (BMC) in the proximal tibia and distal radius. There were no treatment-related clinical observations and no treatment-related histopathological findings in animals treated for 1 or 3 months with OPG (22). This study did not evaluate bone strength due to the relatively short period of treatment, but bone strength was assessed in another cynomolgus monkey study using a different RANKL inhibitor known generically as denosumab.

TABLE 3. MicroCT data from young gonad-intact female mice treated for 3 wk with either human OPG-Fc (5 mg/kg, twice weekly) or vehicle

| MicroCT parameter | Distal femur | Lumbar vertebra |
|---|---|---|
| Trabecular bone volume/total volume | +162[a] | +56[a] |
| Trabecular volumetric BMD (mg/cm$^3$) | +54[a] | +36[a] |
| Trabecular bone matrix mineral density (mg/cm$^3$) | +14[a] | +5[a] |
| Trabecular thickness | +13 | +22[a] |
| Trabecular no. | +70[a] | +28[a] |
| Trabecular spacing | −40[a] | −42 |
| Trabecular connectivity | +108[a] | +7 |

Data represent percentage change in OPG-treated animals relative to vehicle control after 3 wk of treatment.

[a] Significantly greater than vehicle controls, $P < 0.05$ by Student's *t* test (n = 8 per group).

Denosumab (previously known as AMG 162) is a fully human monoclonal antibody that inhibits RANKL. Twelve months of denosumab treatment in gonad-intact cynomolgus monkeys was associated with significant increases in bone strength at the femoral shaft (a purely cortical site) and at the lumbar spine (a predominantly cancellous site) (196). At both sites, bone strength was highly correlated with BMC ($R^2 = 0.75–0.85$), which suggests that the material properties of bone matrix (bone quality) were maintained during 12 months of denosumab therapy. Increments in bone strength in denosumab-treated animals were attributed primarily to increases in bone mass. It is important to confirm these findings in aged animals with high-turnover bone diseases, including animal models that are described in the next section.

### *B. RANKL and RANKL inhibition in ovariectomy models of bone loss*

Ovariectomy (OVX) represents a model of surgical menopause, wherein estrogen levels drop more precipitously compared with the slower decline seen in natural menopause. OVX is associated with increased bone turnover and reductions in BMD, volume, and strength in numerous species, including mice, rats, and cynomolgus monkeys. The therapeutic rationale for RANKL inhibition in OVX models was first suggested in 1997 when OPG was shown to fully prevent the increased osteoclasts and reduced bone volume associated with OVX of young rats (4). Those findings sparked intensive research into the question of whether estrogen might regulate OPG and RANKL in a manner that could contribute to the ability of estrogen to preserve bone mass. Abundant data from cell culture studies demonstrate that estrogen can increase OPG production (46, 84, 104, 108, 197), and in some cases estrogen also causes suppression of RANKL expression (84). The withdrawal of estrogen from cultures of a stromal cell line that supports osteoclastogenesis resulted in a dramatic reduction in their expression of OPG (197). Whether estrogen withdrawal also leads to decreased OPG levels in humans is currently unknown.

OVX has been shown to increase RANKL levels in various animal models (50, 198–201). OPG levels have been reported to increase (199) or decrease (198, 201) in association with OVX, and this difference in response could relate to variations in the animal model and/or the tissue source analyzed.

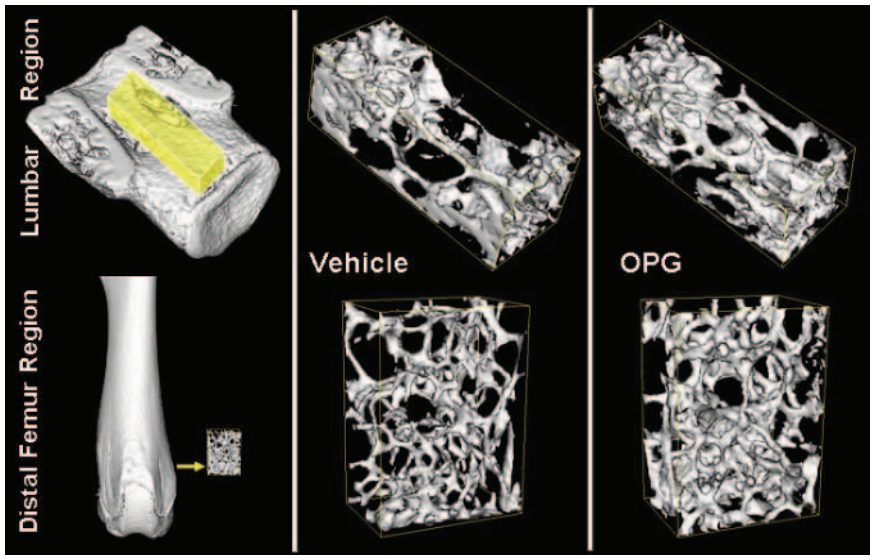


FIG. 5. Regions of analysis (*left panel*) and representative microCT images (*middle and right panels*) of trabecular regions from the lumbar vertebra (*bottom*) and distal femur (*top*) of mice treated for 3 wk with either human OPG-Fc (5 mg/kg, 2/wk) or vehicle. These samples represented the median value for trabecular bone volume for the lumbar vertebra (*top panels*) and the distal femur (*bottom panels*) from each group (n = 8 per group).

In humans, estrogen deficiency is associated with increased RANKL expression by bone marrow osteoblastic, T and B cells, whereas estrogen treatment reversed this increase (76). These associations are consistent with the interpretation that OPG and RANKL play important roles in mediating the effects of estrogen and estrogen withdrawal on bone. Definitive proof of these phenomena will be challenging, however, because the interventional use of OPG or other RANKL inhibitors results in suppression of osteoclasts regardless of the stimuli that drove their activity. It is possible that estrogen suppresses osteoclasts in a manner that does not rely entirely on the regulation of RANKL and/or OPG expression. In support of this idea, estrogen has been shown to directly suppress RANKL-induced osteoclast formation *in vitro* in the absence of osteoclast-supporting stromal cells (105).

Preclinical data from OVX animal studies highlight the therapeutic promise of RANKL inhibition for postmenopausal osteoporosis. One OVX study involved the use of very old rats, which were ovariectomized at 3 months of age and allowed to lose bone for 15 months before OPG treatment (202). Substantial osteopenia was evident at several skeletal sites before the initiation of OPG treatment, which was delivered for 24 wk. OPG treatment was associated with marked reductions in osteoclast surfaces and increased cancellous bone volume in the vertebrae and tibiae. OPG treatment also significantly increased lumbar and leg BMD, while increasing biomechanical strength parameters at the lumbar vertebrae and femoral shaft. In a separate study, OPG was shown to preserve bone density in OVX rats while fully preventing the deleterious OVX-related changes in cancellous microarchitecture (200).

A recent study in young adult OVX mice was the first to compare OPG with a potent bisphosphonate (alendronate) (203). Both agents prevented OVX-related decreases in spine and femur BMD, while increasing vertebral cancellous bone volume. Both agents also caused significant suppression of osteoclast and osteoblast numbers, as well as biochemical markers of bone turnover. In this study OPG, but not alendronate, increased the mechanical strength of the femoral shaft. OPG also showed significantly greater changes in femur BMD, vertebral bone volume, and osteoclast numbers when compared with alendronate (203).

Other OVX mouse studies have corroborated the benefits of RANKL inhibition in estrogen ablation settings, using less direct means of targeting RANKL. An adenovirus gene therapy vector was used to deliver OPG at therapeutic levels that were sufficient to fully prevent bone loss in an acute OVX model (204). An adenovirus-associated gene therapy vector was subsequently shown to deliver OPG at levels sufficient to reverse established OVX-induced bone loss in mice (205). A peptidomimetic approach has been applied to disrupt RANK-RANKL interactions. This small molecule mimic of OPG was delivered continuously by osmotic minipump for 28 d immediately after mice were ovariectomized, and this approach led to partial prevention of OVX-related bone loss (206). An anti-RANKL vaccine has been tested in another acute OVX mouse model, and this approach also led to partial prevention of OVX-related bone loss (207). The safety and efficacy of these experimental approaches to RANKL inhibition would require rigorous preclinical safety testing in animals before their consideration for human use.

The only data describing the effects of RANKL inhibition in OVX primates were recently reported in preliminary form (208, 209). Adult cynomolgus monkeys were subjected to OVX or sham surgeries and allowed to recover for 1 month. OVX cynos were then treated with vehicle or denosumab once per month for 16 months. Denosumab treatment was associated with rapid and sustained suppression of biochemical parameters of bone resorption and formation (208). These changes were accompanied by significant increases in cortical and trabecular BMC and BMD at multiple skeletal sites (208). Denosumab treatment was also associated with significant improvements in biomechanical parameters of bone strength at the lumbar spine, femur neck, and femur diaphysis (209). Strong linear correlations were observed between bone mass parameters and bone strength parameters at all skeletal sites (209). These results suggest that denosumab improved bone strength primarily by increasing bone mass, and that bone formed during denosumab therapy has normal material properties.

### *C. RANKL and RANKL inhibition in orchiectomy models of bone loss*

Preclinical orchiectomy (ORX) models have utility in examining the consequences of androgen ablation and androgen deprivation therapy on bone (210–212). An important distinction between standard ORX rat models and androgen deprivation therapy in humans is the existence of prostate cancer in the latter population and the lack thereof in ORX rats. Normal ORX rat models are appropriate to examine the isolated effects of androgen ablation on bone. The ORX rat can also be used as a model of late-onset male hypogonadism (andropause) (213). Within this context, one limitation of the ORX rat model is the immediacy of disease onset, which contrasts with the more gradual decline in androgen levels in aging men (214).

Androgens have been shown to directly suppress the formation (215) and the bone-resorbing activity of cultured osteoclasts (216). Androgens also suppress osteoclastogenesis in coculture model systems that are dependent on osteoblasts (217). Whether OPG and/or RANKL mediate the suppressive effects of androgens on osteoclasts in such coculture systems is not clear. Androgens have been shown to up-regulate (218) and down-regulate (219) OPG expression in cultured osteoblasts, whereas RANKL was unregulated by androgens in both studies. Skeletal mRNA levels of OPG and RANKL were recently shown to be increased by ORX in rats (220), but whether RANKL protein levels exceeded OPG levels after ORX is unknown.

Whether or not ORX causes bone loss via the OPG-RANKL axis, RANKL inhibition has clear therapeutic benefits in ORX rats. In a recent study, rats were sham-operated or orchiectomized and then treated with vehicle or with OPG for 6 wk. MicroCT assessment at wk 6 revealed significant deterioration of cancellous microarchitecture in vehicle-treated ORX rats, and OPG fully prevented these changes (221). DXA analysis at wk 6 revealed that vehicle-treated ORX rats had significantly decreased BMD, whereas histomorphometry showed significant ORX-related reductions in periosteal bone formation rate and cancellous bone volume. OPG treatment fully prevented each of these changes (222). Androgen replacement therapy has been shown to partially mitigate the ORX-related deficit in periosteal bone formation in rats (211). The ability of OPG also to prevent the ORX-related reduction in periosteal bone formation was an unexpected finding. OPG was recently shown to stimulate the proliferation and differentiation of cultured osteoblasts (203), which suggests a possible mechanism for this periosteal response. An alternative explanation is that OPG prevented ORX-related increases in endocortical and/or cancellous resorption, thereby preserving bone geometry in a manner that obviated the periosteal response that is typically associated with ORX. The ability of OPG to prevent bone loss in this study could be interpreted as evidence that ORX is associated with a functional increase in the RANKL:OPG ratio. This conclusion might be premature, however, because OPG suppressed osteoclast numbers in ORX rats to levels that were well below ORX or sham control values (222). This phenomenon once again emphasizes the essential role of RANKL for the existence of osteoclasts, independent of gonadal hormone status.

### *D. RANKL and RANKL inhibition in rheumatoid arthritis models*

Abundant research in rheumatoid arthritis (RA) models has produced perhaps the best evidence for a causal role of excessive RANKL activity in a bone loss setting. The intense interest in OPG-RANKL biology in RA is related in part to the multiple forms of osteoclast-mediated bone loss that are characteristic of the disease, including focal bone erosions at the bone-pannus junction of inflamed joints, subchondral (periarticular) bone loss adjacent to inflamed joints, and generalized systemic bone loss (223–227). The alignment between clinical observations and the attributes of preclinical RA models is close in terms of theoretical mechanism of bone loss and their manifestations (228).

Animal models of RA consistently show up-regulation of RANKL in inflamed joints (26, 49, 181, 225, 229, 230), and in some cases in serum as well (26). There are fewer data on OPG levels in preclinical RA models, but OPG protein levels were recently shown to be decreased in the inflamed joints of two well-characterized arthritic rat models (adjuvant and collagen-induced arthritis). RANKL protein levels were concomitantly increased in inflamed joints, leading to marked increases in the RANKL:OPG ratio. This ratio was positively correlated with focal bone erosions and with osteoclast activity (231). A detailed time course assessment of inflamed joints from adjuvant and collagen-induced rat arthritis models showed that RANKL protein levels were significantly increased before or simultaneous with the earliest appearance of bone erosions and osteoclast responses (26). These data suggest that RANKL is well positioned to play a causal role in the progression of erosive disease in these commonly used models of RA. Additional support for this notion comes from numerous animal studies that consistently show that RANKL inhibition is associated with marked suppression of bone erosions. Total genetic ablation of RANKL in knockout mice rendered the animals resistant to arthritis-related joint destruction, and osteoclasts were essentially absent in their joints (110). RANKL inhibition via recombinant OPG treatment also results in marked suppression of bone erosions and osteoclasts in several rodent models, including adjuvant arthritis (26, 49, 177, 232, 233), collagen-induced arthritis (26, 181, 233), and TNF-mediated arthritis (136, 234). OPG was also shown to reduce periarticular bone loss in arthritic rats (235), and OPG also prevented systemic bone loss in arthritic mice (180, 234, 236) and arthritic rats (26).

Although bone preservation is an important and expected outcome for OPG therapy, cartilage preservation is a primary goal of RA therapy. Joint space narrowing is an important clinical endpoint for evaluating the efficacy of RA treatment, and maintenance of articular cartilage is a significant component of this endpoint. Cartilage damage in RA is thought to be mediated at least in part by degradative enzymes such as matrix metalloproteinases, which are released by synovial fibroblasts. Inadequate synthesis of type II collagen, aggrecan, and proteoglycans might also contribute to the progression of cartilage loss (237). OPG, RANK, and RANKL are all expressed at the protein level in human articular cartilage, but their functional roles in cartilage have not been clearly demonstrated (238). Nonetheless, interest in possible roles

for this pathway in the maintenance of articular cartilage was fostered by early observations that OPG treatment provided substantial cartilage preservation in arthritic mice (49). Other arthritis models have confirmed the ability of OPG or RANKL ablation to preserve cartilage, as measured histologically by proteoglycan preservation (110, 177, 181). Subsequently, serum RANKL concentrations in RA patients were shown to correlate positively with urine levels of CTX-II, a type II collagen degradation product (239). This relationship is not necessarily a causal one, because serum RANKL levels are also correlated with overall disease progression in RA (53, 239). Evidence that RANKL plays a direct role in maintaining cartilage is currently minimal. Although human articular cartilage and primary articular chondrocytes express RANK protein, exogenous RANKL did not appear to activate signaling in cultured chondrocytes, suggesting that RANK might be functionally inactive in those cells (238). A more likely mechanism for chondroprotection is related to the well-established ability of OPG to preserve subchondral bone, which helps to preserve the geometry of the joint while preventing osteoclasts from undermining the epiphyses from within. This indirect mechanism of chondroprotection is consistent with observations that OPG has no significant impact on the inflammatory components of arthritis, such as joint swelling (49, 136, 177, 179–181, 235, 236, 240).

### *E. RANKL and RANKL inhibition in glucocorticoid-induced bone loss models*

Glucocorticoids have antiinflammatory and immunosuppressive effects and are used in numerous disorders including rheumatoid arthritis, Crohn's disease, and glomerulonephritis. Glucocorticoids also have remarkably complex effects on bone, virtually all of which are detrimental, and these influences have been summarized in recent reviews (241, 242). This section will focus on the potential roles of bone resorption and the OPG-RANKL axis in mediating the effects of glucocorticoids on bone. The most universally described skeletal consequence of glucocorticoid therapy is a reduction in BMD, which can lead to increased fracture risk (243–245). Mechanisms responsible for glucocorticoid-induced osteoporosis have been extensively studied, but consensus has yet to be achieved. It is widely believed that suppression of bone formation is a component of bone loss, whereas increased (or inappropriately "normal") bone resorption likely also contributes.

At the cellular level, glucocorticoids [mainly prednisolone, dexamethasone (DEX), and cortisol] have complex effects on osteoblasts and osteoclasts. Glucocorticoids have been shown to stimulate the proliferation of cultured osteoblasts and/or their precursors in some studies (246, 247), whereas other studies showed that osteoblast proliferation was inhibited by glucocorticoids (246, 248). These conflicting results may be related to differences in the stage of osteoblast differentiation, and the timing and duration of glucocorticoid exposure in different systems (246). Glucocorticoids can also inhibit osteoblast differentiation (249–251), osteoblast attachment to bone matrix (252), and osteoblast production of IGF-I (253). Bone marrow harvested from mice treated with prednisolone was shown to have markedly reduced osteoblastogenic capacity (251). In general, these studies point toward significant osteoblast suppression as a component of glucocorticoid-induced bone loss.

The reported effects of glucocorticoids on osteoclasts are only slightly less complex. In favor of a proosteoclast effect, DEX was able to directly promote osteoclastogenesis from bone marrow cells that were exposed in culture to the essential osteoclastogenic cofactors RANKL and M-CSF (254). Furthermore, conditioned medium from glucocorticoid-treated osteoblasts had increased ability to support osteoclastogenesis (254). However, cortisol has been shown to inhibit resorption in bone organ cultures (255), and bone marrow harvested from mice treated with prednisolone showed reduced osteoclastogenic potential (251). A bone xenograft model showed that glucocorticoids inhibited osteoclast recruitment and bone resorption, whereas delayed treatment with glucocorticoids resulted in stimulation of osteoclast activity and bone resorption (256). DEX was recently shown to prolong the lifespan of osteoclasts, while decreasing their individual capacity to degrade bone (257). Osteoclast responses to glucocorticoids appear to be greatly influenced by their stage of differentiation as well as the timing of glucocorticoid exposure.

To the extent that increased (or inappropriately "normal") bone resorption contributes to the skeletal effects of glucocorticoids, there is ample molecular evidence that OPG and RANKL might be involved in the process. Glucocorticoids have been shown to suppress OPG mRNA expression in primary human osteoblasts and in osteoblast cell lines (254, 258), while up-regulating RANKL expression (254). Glucocorticoids also suppressed OPG protein production by human fibroblasts (254). DEX and prednisolone were shown to inhibit OPG production and increase RANKL production by human osteoblasts, leading to increased RANKL:OPG ratio (259). Glucocorticoids also stimulate osteoblasts to produce M-CSF (260), which is an important cofactor for osteoclastogenesis (261).

The lack of concordance regarding the effects of glucocorticoids on bone formation and bone resorption in these model systems is multifactorial. One important component of glucocorticoid biology that is lacking in most cell culture models is the marked depletion of osteoblast numbers in animals treated with glucocorticoids (251). Osteoblasts are an important source of both OPG and RANKL, and the effects of glucocorticoid-mediated osteoblast depletion on the relative balance of OPG and RANKL have not been well established. Regardless of net effects of glucocortoids on the RANKL:OPG ratio *in vivo*, animal studies account for this variable and might therefore have greater relevance than cell culture models. A limitation with animal models of glucocorticoid-induced bone loss is that they typically fail to include underlying diseases for which glucocorticoids are typically prescribed. This can be an advantage as well because the lack of underlying disease provides an opportunity to examine the direct consequences of glucocorticoids on bone.

Glucocorticoids decrease BMD in rats (262, 263), mice (251), and minipigs (264). In mice, BMD loss with prednisolone was associated with decreased osteocalcin and decreased bone formation, with no histological or biochemical

evidence for increased bone resorption (251). In minipigs, prednisolone decreased biochemical parameters of bone resorption and bone formation while decreasing bone growth and bone strength (264). Prednisolone infusion in rats resulted in suppressed bone growth and decreases in cortical area and cortical strength (263). In another rat study, prednisolone infusion led to reductions in biochemical markers of bone resorption and bone formation and a paradoxical increase in cancellous bone volume (265). The prednisolone-related increase in bone volume in rats, confirmed in a study described immediately below (262), is not consistent with histological data from patients with glucocorticoid-induced osteoporosis (194) and is a recognized limitation of the model (251). That being said, the only data on the effects of OPG on glucocorticoid-induced bone loss was generated in rats, using sc implanted prednisolone pellets. Those data were presented in abstract form (262), and the main findings are described in Fig. 6 due to the lack of published data on RANKL inhibition in other glucocorticoid-induced bone loss models. In this study, 45 d of prednisolone were associated with significant bone loss and increased osteoclast surface, and OPG treatment prevented these changes. Despite the limitations of this model, the skeletal response to prednisolone in these rats shared several similarities to changes observed in humans, including significantly decreased BMD (243), histological evidence of increased bone resorption (194), and biochemical evidence of suppressed bone formation (244, 267, 268). The ability of OPG to markedly reduce osteoclasts and to prevent the prednisolone-related loss of BMD suggests that RANKL inhibition represents a potential new strategy for the treatment and prevention of glucocorticoid-induced bone loss.

### *F. RANKL and RANKL inhibition in disuse osteopenia models*

Disuse osteopenia and osteoporosis occurs under circumstances such as prolonged bed rest (269), spinal cord injury (270, 271), and spaceflight (272, 273). Spinal cord injury is associated with decreased BMD, increased fracture risk, and deterioration of cortical geometry (270, 271). Astronauts lose significant cortical and cancellous bone mass within the first few months of spaceflight (274) and exhibit cortical thinning in the femoral neck (273). These and other forms of osteoporosis (262, 263) represent a significant therapeutic challenge due to evidence of increased bone resorption and suppression of bone formation. Biochemical markers of bone resorption and osteoclast numbers are significantly elevated in spaceflight and bed rest (275, 276), consistent with an osteoclast-mediated contribution to bone loss. Animal models also suggest increased bone resorption in response to skeletal unloading (121, 277). Skeletal unloading also suppresses bone formation (277, 278) and increases osteocyte apoptosis (121). Although anabolic therapy with intermittent PTH is effective at restoring bone mass in some skeletal unloading studies (279), skeletal unloading has been shown to significantly diminish the anabolic potential of PTH (280, 281).

The therapeutic potential of RANKL inhibition in disuse osteopenia is of interest because bisphosphonate therapy does not appear to consistently preserve both cortical and cancellous bone mass in unloading models. Long-term disuse osteopenia in dogs was suggested to be less responsive to bisphosphonate therapy compared with the efficacy of bisphosphonates in osteoporosis models (282). There was no evidence of suppression of cortical bone resorption in unloaded dogs treated with bisphosphonates, and cortical bone strength in treated animals remained significantly lower than the strength of normally loaded control bones (282). Cortical bone seems to be particularly sensitive to unloading in rats (283), and bisphosphonates are frequently ineffective at improving cortical bone strength in rat unloading models (284–286), even when cancellous bone strength was significantly improved (286). Cortical bone resistance to bisphosphonates was also suggested in a human bed rest study, wherein bisphosphonate treatment suppressed osteoclast numbers in cancellous bone but not in cortical bone (276). The reasons for the apparent resistance to bisphosphonates in unloaded cortical bone are not clear. One possibility is that the lower surface area in cortical bone provides lower uptake of bisphosphonates.

RANKL inhibition for the prevention of disuse osteopenia is appealing also because there is evidence that RANKL could be involved in the skeletal response to loading and

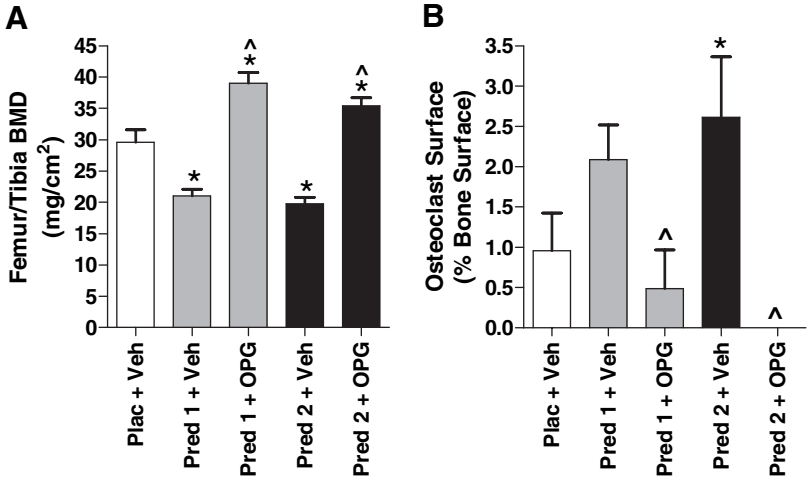


FIG. 6. Effects of RANKL inhibition via OPG on prednisolone-induced changes in bone. Slow-release prednisolone (Pred, 1 or 2 mg/kg·d) or placebo pellets (Plac) were implanted sc in normal young male rats for 45 d, during which time animals were treated with either vehicle (saline) or OPG-Fc (1 mg/kg, 3 times/wk by sc injection). A, BMD was measured by DXA on d 45 at the distal femur and proximal tibia. In vehicle-treated rats, both doses of prednisolone led to significant decreases in BMD compared with vehicle-treated rats with placebo pellets. OPG significantly increased BMD in animals treated with either dose of predisolone. B, Osteoclast surface was measured in histological sections of the proximal tibia and expressed as percentage of cancellous bone surface. High-dose prednisolone (2 mg/kg) led to a significant increase in osteoclast surface compared with Plac + vehicle (Veh) controls, and OPG treatment significantly reduced osteoclast surface in animals treated with either dose of prednisolone. Data represent means ± SD, n = 6–7 per group. *, Significantly different from Plac + Veh; ^, significantly different from respective Pred + Veh control; $P < 0.05$ by ANOVA.

unloading. Mechanical loading was shown to suppress osteoclast formation by decreasing RANKL expression in bone marrow stromal cells (287). In a cell culture model of unloading, simulated microgravity via vector averaging resulted in increased RANKL expression by a bone marrow-derived stromal cell line (288). Regardless of whether skeletal unloading promotes bone resorption and bone loss via increases in the RANKL:OPG ratio, animal studies in numerous unloading models show that RANKL inhibition consistently prevents cortical and cancellous bone loss.

Preclinical models of disuse osteopenia have been recently and extensively reviewed (289). Common rodent disuse models include tail suspension, sciatic neurectomy or crush, and spaceflight. OPG reduced cortical bone resorption and increased bone density in mouse nerve crush models (290, 291). OPG also prevented decreases in BMD and bone strength in a rat sciatic neurectomy model. This study showed strong correlations between femur BMD and femoral neck strength, consistent with the preservation of bone quality and improvements in bone mass (292). OPG preserved bone mass and increased cortical bone strength in tail-suspended mice (186). Another mouse tail suspension study showed that OPG preserved BMD in unloaded bones to a greater extent than was observed with two bisphosphonates (293). OPG was also able to prevent bone loss and improve cortical bone strength under the extreme conditions of microgravity. In that study, a single injection of OPG before a 12-d spaceflight caused significant increases in BMD and improved the strength of the femoral diaphysis of mice (294, 295). An elegant new mouse model of disuse osteopenia was recently described, in which botulism toxin was injected into the hindlimbs to induce temporary muscle paralysis (296). In this model, OPG treatment was shown to prevent deficits in cancellous bone volume as well as the unloading-related decreases in cortical thickness, volume, and strength (297). Skeletal unloading models in rats also show consistent bone preservation with OPG. OPG treatment of neurectomized rats improved BMD and bone strength at the femoral neck (292). OPG significantly increased the density and strength of cortical bone (298) in a rat unloading model that was nearly identical in design to one where bisphosphonate treatment had no such effects (284). More head-to-head studies, with a robust range of doses, are required before conclusions can be made regarding the differential effects of bisphosphonates *vs.* RANKL inhibitors in the prevention of cancellous or cortical bone loss in disuse osteopenia.

### *G. RANKL and RANKL inhibition in bone metastasis and multiple myeloma models*

Nearly 120 yr ago, British surgeon Stephen Paget observed that certain cancer cells had a predilection to metastasize to bone, and he proposed that the bone microenvironment provided a fertile "soil" for the localization and growth of particular types of cancer cells (299). Almost 100 yr later, it was reported that products released from resorbing bone provided a chemotactic stimulus for directed tumor cell migration (300). This provided clues as to possible mechanisms for tumor cell osteotropism, and shortly thereafter it was reported that bone resorption in bone organ cultures stimulated the proliferation of tumor cells that possessed bone metastasizing properties (301). Consistent with these culture studies, it was subsequently shown in rats that cancer cells immediately adjacent to bone surfaces had significantly greater proliferation rates compared with those that were distant from bone (302). Stimulation of bone resorption was shown to specifically increase the proliferation rate of metastatic cancer cells in bone, but not in other tissues (303). These observations, coupled with the ability of tumor cells to stimulate osteoclastic bone resorption (304), provided a powerful rationale for exploring bone turnover suppression as a therapeutic strategy for bone metastasis. This rationale is predicated on the interruption of a "vicious cycle" wherein bone resorption attracts tumor cells and stimulates their growth, whereas tumor cells promote osteoclast formation and activity, the result of which is more bone resorption that further attracts and stimulates cancer cells.

These seed-and-soil and vicious cycle theories have gained abundant experimental support, such that adjuvant therapies used today to control bone metastases target bone turnover rather than tumor cells. These lesions lead to skeletal-related clinical events (SREs) that include severe bone pain, pathological fracture, and spinal cord compression. Multiple myeloma also has deletrious effects on bone by virtue of increasing bone resorption while suppressing bone formation (305). Bisphosphonates are the only class of drugs currently approved for the reduction of SREs in patients with bone metastases or multiple myeloma (MM). These agents act by binding directly to mineralized bone matrix and directly inhibiting the activity of osteoclasts that attempt to degrade bisphosphonate-laden bone (140). In 1983 the bisphosphonate clodronate was shown to reduce hypercalcemia, bone pain, and osteolytic progression in breast cancer patients with bone metastases (306). In 1991, pamidronate was shown to reduce bone destruction in breast cancer patients with bone metastases and in myeloma patients (307) and prostate cancer patients with bone metastases (308). Ten years later, zoledronic acid (ZOL) was shown to reduce SREs in patients with bone metastases (309). These trials helped to establish the utility of bone turnover suppression as a therapeutic strategy for patients with bone metastases or myeloma.

Based on the dominant roles for OPG and RANKL in the regulation of bone resorption, numerous investigators have examined the potential role of this pathway in cancer cell regulation of osteoclasts. In the mid 1970s, the existence of "osteoclast stimulating factor" and "osteoclast activating factor" was theorized by Mundy *et al.* (310) and by Galasko (304), respectively. The existence of these factors was proposed to account for the local stimulation of osteoclasts that is associated with myeloma and other cancer cells in bone. The exact identity of these factors was not provided by these early studies, but the activities they described would later prove to have significant overlap with the biological attributes of RANKL. Some 30 yr later, the possible involvement of the RANK/RANKL/OPG pathway in bone metastasis has been described in numerous forms of cancer, including myeloma, and in dozens of preclinical bone metastasis models. In-depth review articles on these subjects have been recently published (311–313).

Cancer cells have been shown to regulate OPG and/or RANKL in a variety of ways, some of which are described in Fig. 7. With few exceptions, regulation of OPG and RANKL by cancer cells appears to increase the RANKL:OPG ratio in a manner that favors bone resorption. Moving clockwise from the cancer cell in Fig. 7, prostate cancer cells (CaP) (314–316) and MM cells (317–322) have been shown to express RANKL directly. Although breast cancer cells (CaBr) do not typically express RANKL directly (101, 323, 324), they can up-regulate RANKL expression by osteoblasts (OB) (101, 323) and bone marrow stromal cells (BMSC) (323–325). CaP cells can also up-regulate RANKL expression in osteoblasts (326). MM cells have been shown to up-regulate RANKL expression by BMSCs (327, 328), by T cells (48), and by endothelial cells (329). CaBr cells (101) and MM cells (327, 328, 330) can also down-regulate OPG production by osteoblasts and/or BMSCs. MM cells express syndecan on their surface, which sequesters and degrades heparin-binding proteins including OPG (331).

The functional consequences of these regulatory influences have not been exhaustively assessed. But as expected, OPG is capable of inhibiting the osteoclastogenic responses associated with direct RANKL production by cancer cells (317, 332) as well as in coculture systems where cancer cells induce other cells to express RANKL (316, 324, 329, 333). Furthermore, RANKL expression by human myeloma cells was shown to correlate with the propensity of those cells to cause bone destruction *in vivo* (317). These results are generally consistent with the ability of OPG and other RANKL inhibitors to suppress tumor-associated osteolysis in animal models of bone metastasis, as described in *Section III.H* and *I*.

### *H. RANKL and RANKL inhibition in animal models of breast cancer metastasis to bone*

Human MDA-231 breast cancer cells have been widely used in immunodeficient mice as an experimental bone metastasis model. These cells are capable of stimulating osteoclastogenesis and osteoclast activity *in vitro* (334), and their direct injection into the left ventricle of nude mice leads to the establishment of skeletal metastases that induce intense focal osteolysis (335). MDA-231 cells and other human breast cancer cells have been shown to express RANK (101, 336), which raises the possibility that RANKL and OPG might exert direct effects on RANK-expressing breast cancer cells. RANKL has been reported to stimulate signaling and invasiveness in human breast cancer cell lines (337), and RANKL was recently shown to stimulate the directed migration of human breast cancer cells in a manner that was inhibited by OPG (337). Although these direct responses to RANKL and OPG are intriguing, most animal models of breast cancer metastasis support the notion that bone turnover suppression is the primary mechanism by which RANKL inhibition prevents bone destruction and reduces skeletal tumor burden.

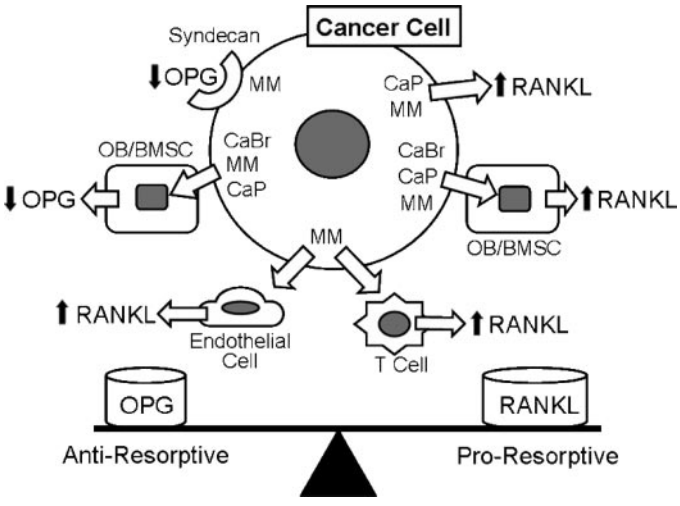


FIG. 7. Theoretical mechanisms by which cancer cells might promote bone resorption by regulation of OPG and/or RANKL. An annotated description is provided in *Section III.G*. Tumor-mediated increases in RANKL or decreases in OPG would tend to favor bone resorption.

The MDA-231 tumor model was used to examine the therapeutic potential of RANKL inhibition in the prevention of bone metastases and the control of skeletal tumor growth. OPG treatment at the time of tumor cell inoculation was able to completely prevent the radiographic appearance of lytic lesions (338). Histological assessment revealed that skeletal tumor burden was 75% lower in OPG-treated animals compared with vehicle controls. The mechanism for this reduction in tumor burden was probably related to OPG inhibition of bone destruction because tumor burden in numerous soft tissue organs was unaffected by treatment. OPG treatment virtually eradicated tumor-associated osteoclasts in this and other studies, which is a somewhat novel observation that relates to OPG's mechanism of action. OPG functions systemically as a soluble osteoclast inhibitor, and unlike bisphosphonates OPG does not require binding to bone surfaces for efficacy. This feature allows OPG and other soluble RANKL inhibitors such as RANK-Fc to eliminate osteoclasts within tumor foci (314). Consistent with this differential mechanism of action, two recent studies showed that OPG caused greater suppression of tumor-associated osteoclasts compared with bisphosphonate therapy in mice (339, 340).

Although prevention of bone metastases is a laudable therapeutic outcome, it is important that therapies are able to control the progression of established metastatic lesions in bone. OPG was tested in an MDA-231 treatment model, wherein treatment was delayed until osteolytic bone lesions were already apparent. These results were previously described in abstract form (341), and some key data are presented here. Experimental details are described in the legend for Fig. 8. Briefly, MDA-231 human breast cancer cells were injected into the left ventricle of immunodeficient mice, which were left untreated for 3 wk to allow for the development of lytic lesions in bone. One group was killed at wk 3 as a baseline control, and two other groups were treated with either vehicle or OPG-Fc for 7 d. During those final 7 d, radiographic lesion area and histological tumor burden increased by 3-fold in vehicle-treated animals, but there was no radiographic or histological progression in mice treated for 7 d with OPG. OPG treatment was also associated with a nearly complete elimination of tumor-associated osteoclasts (Fig. 8). This powerful antiosteoclast effect was a likely explanation for the reduction in skeletal tumor burden, based on the seed-and-soil hypothesis and the lack of OPG effects on extraskeletal tumor burden in this MDA-231 model (338).

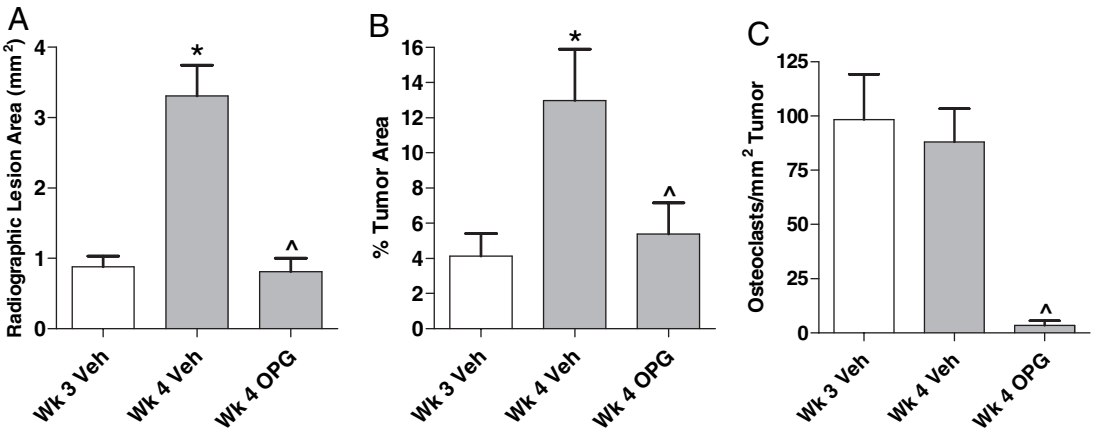


FIG. 8. Radiographic and histological assessment of bone metastases in mice treated with OPG. Human MDA-231 breast cancer cells were injected into the left ventricle of female Balb/c nu/nu mice. After 3 wk, mice were x-rayed to assess radiographic osteolysis and allocated to three groups with similar levels of bone destruction. One group was killed at wk 3 as a baseline control (Wk 3 Veh). Other groups were treated with either vehicle (Veh, saline) or with OPG-Fc (3 mg/kg) every second day for 7 d, then x-rayed and killed (Wk 4). Tibiae and femurs were analyzed by histomorphometry for skeletal tumor burden and tumor-associated osteoclasts per tumor area. A, Significant radiographic damage was apparent at wk 3 in vehicle controls, and by wk 4 the total radiographic lesions area increased by 3-fold in vehicle-treated mice. No radiographic progression was observed in mice treated during wk 4 with OPG. B, Histomorphometric assessment at wk 3 indicated the presence of metastases in femurs and tibiae, and by wk 4 there was a 3-fold increase in skeletal tumor burden (% tumor area) in vehicle-treated mice. OPG treatment during wk 4 was associated with no significant change in tumor burden relative to wk 3 controls. C, OPG treatment during wk 4 was associated with a 90% decrease in osteoclast numbers per square millimeter of tumor compared with either vehicle control group. Data represent means ± SD, n = 13–15 per group. *, Significantly different from Wk 3 Veh group; ∧, significantly different from Wk 4 Veh group; $P < 0.05$ by ANOVA.

The important role of bone resorption in skeletal tumor growth was suggested by data from a recently reported study that directly compared OPG and bisphosphonate therapy in an MDA-231 nude mouse model of bone metastasis (340). Treatment of tumor-bearing mice with OPG or with ibandronate (IBA) resulted in similar and significant decrease in radiographic lesion area. Both drugs caused significant reductions in tumor-associated osteoclasts, histological tumor area, and tumor cell apopotosis. Although IBA caused a significant reduction in tumor-associated osteoclasts, OPG treatment caused their total elimination. The total absence of osteoclasts in OPG-treated mice provided an opportunity to explore the potential for direct bisphosphonate-mediated antitumor effects. As monotherapies, OPG and IBA were each associated with significant and similar suppression of tumor cell proliferation and induction of tumor cell apoptosis in bone. The combination of OPG + IBA showed no greater effects on tumor cell proliferation and apoptosis compared with OPG alone, which argues against a direct bisphosphonate-mediated antitumor effect. There are limited data on the concentrations of bisphosphonates within metastatic tumor foci, but one study in mice with bone metastases showed that the maximal concentration of one bisphosphonate (incadronate) within established bone metastases was about 50-fold lower than concentrations that were required to show *in vitro* suppression of the same cancer cells (342). Furthermore, the growth-inhibitory effects of incadronate on cultured tumor cells occurred at 100-fold higher concentrations than those needed to suppress osteoclasts. The existence of such high levels within bone seems unlikely, based on animal data showing that the uptake of radiolabeled bisphosphonate was no greater in bones with extensive metastatic disease compared with tumor-free bones (342). Although direct antitumor effects of bisphosphonates are scientifically difficult to rule out, the experimental use of OPG in numerous bone metastasis models has provided substantial evidence that turnover suppression is the principal means by which bisphosphonates and RANKL inhibitors reduce skeletal tumor burden (340, 343–345).

### *I. RANKL and RANKL inhibition in animal models of prostate cancer metastasis to bone*

While bone metastasis lesions in prostate cancer patients typically have an osteoblastic (osteosclerotic) appearance, there is evidence that bone resorption is also frequently increased in these lesions (346, 347). In animal models of prostate cancer metastasis, skeletal lesions frequently exhibit increased osteoclast activation and osteolysis (316, 348). OPG treatment was shown to markedly reduce osteoclast numbers in skeletal lesions that resulted from the direct intratibial injection of human C4–2B prostate cancer cells into immunodeficient mice (316). This therapeutic effect was accompanied by significant reductions in skeletal tumor burden and the prevention of radiographic bone destruction. OPG treatment had no significant effects on the sc growth of C4–2B tumors, which is consistent with the interpretation that OPG reduced skeletal tumor burden via suppression of bone turnover (316). A different human tumor cell line (LNCaP) that produces an osteoblastic response was used to examine the therapeutic effects of RANKL inhibition in mice. In this study, OPG significantly decreased the development of bone tumors and also reduced the progression of established tumors (348). These benefits were accompanied by significant reductions in osteoclast numbers, whereas OPG did not inhibit LNCaP cells grown sc or in culture. RANK-Fc was also used as a RANKL inhibitor in a mouse model wherein human LuCaP prostate cancer cells were injected

into bone. RANK-Fc treatment was associated with a significant reduction in serum prostate-specific antigen and decreased skeletal tumor burden, whereas RANK-Fc did not suppress the sc growth of LuCaP cells (314). Thus, in these two models, suppression of skeletal tumor burden was probably related to the antiresorptive effects associated with RANKL inhibition.

This notion is supported by recent studies that directly compared OPG and the bisphosphonate ZOL on the intratibial growth of human C4–2 prostate cancer cells. OPG and ZOL both suppressed tumor-associated osteolysis and decreased osteoclast surfaces in tumor-bearing bones (344). OPG and ZOL both significantly decreased skeletal tumor burden when delivered in a preventative manner, whereas only OPG caused significant suppression of skeletal tumor burden when treatment was delayed until lytic tumors were established (344). Although OPG had no significant effects on the proliferation of cultured C4–2 cells, high doses of ZOL (10–100 $\mu$M) were associated with significant C4–2 growth suppression. These data suggest that the ability of both OPG and ZOL to reduce skeletal tumor burden in this prostate cancer model was related to osteoclast suppression rather than by direct antitumor effects. Other human prostate cancer cell lines have been shown to express RANK and to respond directly to RANKL stimulation via activation of signal transduction and increased migration in culture (349). OPG inhibited RANKL-mediated migration of these RANK-expressing prostate cancer cells, so it is therefore possible that some human prostate tumors might respond directly to RANKL and therefore to RANKL inhibition.

### *J. RANKL and RANKL inhibition in animal models of multiple myeloma*

MM cells appear to have the broadest array of mechanisms to up-regulate bone resorption via the OPG-RANKL pathway. Unlike bone metastasis, MM cells originate in bone marrow where they clonally expand and spread to cause widespread osteolysis (350). Bone formation is typically suppressed in MM (351), which creates an additional therapeutic challenge that is not typically associated with bone metastases. Furthermore, widespread skeletal expansion of myeloma cells can lead to significant systemic bone loss (350). Systemic bone loss was a feature of a murine MM model, wherein RANKL-expressing 5T2MM myeloma cells were shown to cause extensive bone lesions in mice with increased osteoclasts, decreased BMD, and cortical thinning of long bones. OPG treatment prevented each of these changes (352). Another murine myeloma cell line (5T33MM), which also directly expresses RANKL, caused extensive skeletal tumor burden in mice such that 85% of bone marrow cells were of myeloma origin (353). OPG treatment of tumor-bearing mice led to significant reductions in tumor burden, including a reduction in serum paraprotein levels, and OPG-treated animals showed significantly longer survival than untreated mice. OPG had no significant effects on the survival or proliferation of cultured 5T33MM cells, and this survival benefit was therefore attributed to the eradication of tumor-associated osteoclasts (353). To the extent that myeloma cells might rely on the presence of osteoclasts for their continued growth (354), the ability of RANKL inhibitors to eradicate tumor-associated osteoclasts could provide an indirect antitumor effect that is independent of their ability to prevent bone destruction.

RANK-Fc, a RANKL inhibitor with a mechanism of action that is similar to that of OPG, also has therapeutic effects in myeloma models. Primary human myeloma cells were injected into immunodeficient mice bearing human bone xenografts, and these myeloma cells infiltrated the xenografts and induced osteoclast-mediated lytic lesions (328). RANK-Fc treatment, initiated after the establishment of skeletal lesions, prevented radiographic progression of disease and significantly reduced osteoclast numbers and tumor cell numbers in the xenografts. The antitumor effect of RANK-Fc was probably related to osteoclast suppression because RANK-Fc did not show direct cytotoxic effects on cultured myeloma cells (328). A human plasma cell leukemia cell line (ARH-77) also created extensive osteolytic lesions when injected into nude mice, leading to hind limb paralysis in 80% of animals. RANK-Fc prevented osteolysis in these mice and prevented hind limb paralysis, but without evidence of changes in skeletal tumor burden (328). ARH-77 cells are less dependent on the bone marrow microenvironment compared with MM cells, which may explain the lack of reduced tumor burden with RANK-Fc. The ability of RANK-Fc to prevent osteolysis in this model indicates that these plasma cell leukemia cells still rely on RANKL to cause bone destruction.

A comparison of RANK-Fc and bisphosphonates was recently described in a murine model, wherein primary human MM cells were injected into human bone xenografts. When these xenografts were implanted into immunocompromised mice, myeloma cells stimulated bone resorption and decreased BMD of the xenograft. Treatment of mice with RANK-Fc or with bisphosphonates resulted in decreased bone resorption and reduced skeletal tumor burden (345). RANK-Fc had no direct effects on cultured myeloma cells, which suggests that bone turnover suppression was the primary mechanism by which tumor burden was inhibited.

In contrast to these *in vivo* results, cell culture studies have suggested that OPG might serve as a paracrine survival factor for human MM cells by inhibiting the proapoptotic effects of TNF-related apoptosis-inducing ligand (TRAIL) (330). This is an intriguing observation, particularly in light of the ability of MM cells to down-regulate OPG production by osteoblasts or stromal cells (327, 328, 330) and to also sequester and degrade OPG via syndecan expression (331). Whether MM cells can promote their survival via this OPG-depleting mechanism is unclear because evidence suggests that OPG-TRAIL binding might have relatively low affinity compared with the affinity of TRAIL for its canonical receptors (355). Furthermore, the resistance of primary human myeloma cells and other cancer cells to TRAIL-induced apoptosis did not correlate with OPG expression by those cells (356, 357). Evidence for meaningful OPG-TRAIL interactions *in vivo* is minimal. If such interactions occurred, one might expect that TRAIL reduces OPG activity and vice versa. However, TRAIL-deficient knockout mice had no skeletal abnormalities, and TRAIL had no cytotoxic effects in OPG-deficient knockout mice (358).

Although there are few data from human studies on the

potential for OPG-TRAIL interactions, flow cytometry studies conducted with bone marrow aspirates from postmenopausal subjects showed that saturating levels of OPG did not reduce the ability of anti-TRAIL antibodies to recognize TRAIL-positive cells (76). It is also interesting to note that RANK-Fc, which has no TRAIL-binding activity, suppresses myeloma tumor burden in animal models (328, 345) similar to the effects of OPG (353). Direct lentiviral transfer of the human OPG gene into human myeloma cells led to their secretion of therapeutic levels of OPG, which was shown to prevent bone destruction in immunodeficient mice without altering their *in vitro* growth properties (359). Whether endogenous TRAIL was a component of this model or other myeloma models is not known. But recombinant OPG treatment has not been shown to increase tumor burden in bone or in other organs in any experimental models of myeloma or bone metastasis, including the MDA-231 studies described above. MDA-231 cells have been shown to be TRAIL-sensitive because the injection of recombinant TRAIL into MDA-231 tumor-bearing mice led to significant antitumor effects and increased mouse survival (360). Despite this antitumor effect, 14 d of daily high-dose TRAIL injections in these mice had no effects on bone parameters. Thus, an interactive role of OPG-TRAIL with regard to the tumor immunosurveillance, tumor cell growth, or bone turnover *in vivo* remains speculative. It is also worth noting that the only recombinant RANKL inhibitor that is currently being tested in clinical trials is denosumab, a fully human anti-RANKL antibody that has no TRAIL-binding properties (361).

### *K. RANKL and RANKL inhibition in fracture and fracture repair*

Fracture healing is an ordered process that gradually restores the structural integrity of bone, eventually returning it to approximately its original strength. Normal fracture repair after immobilization in casts is characterized by callus formation. The periosteal callus forms from the hematoma at the fracture site with the production of a collar of fibrous tissue, fibrocartilage, and hyaline cartilage around the fracture fragments. Subperiosteal new bone formation begins some distance from the fracture site and, through a process similar to endochondral ossification, advances toward the central and peripheral regions of the callus. A similar, less prolific response occurs at the endosteal surface. Over time, the callus is remodeled from randomly oriented woven bone to mature cortical bone.

The involvement of bone remodeling in fracture repair implicates the OPG/RANKL system in the repair process. OPG and RANKL mRNA expression was elevated in the fracture callus of mice during the repair of standard transverse long bone fractures (362). The biological implications of these changes are difficult to evaluate, but interventional studies with different RANKL inhibitory strategies suggest that RANKL plays a role in callus remodeling but not callus formation. The effects of recombinant OPG were studied in rats subjected to closed, transverse tibial fractures. In this study, OPG was delivered at a very high dose (10 mg/kg, three times per week) at the time of fracture and for 8 wk thereafter (363). This treatment regimen resulted in serum OPG levels (up to 35 $\mu$g/ml) that were at least 10,000-fold higher than levels found in normal rat serum. Histological assessment of fracture calluses at wk 3 and 8 showed greater than 90% reductions in osteoclast numbers in OPG-treated rats compared with time-matched vehicle controls. At wk 3, there were no reported differences in callus dimensions, BMC, or biomechanical strength of the callus between OPG- and vehicle-treated controls. These results indicate that continuous RANKL inhibition did not influence the formation of a normal facture callus. At wk 8, fracture calluses from OPG-treated rats were about 20% larger and had 30% greater BMC compared with vehicle controls. These findings are consistent with a delay in callus remodeling, a phenomenon that is typically associated with bisphosphonates and other antiresorptive therapies in similar models (364–366).

Biomechanical strength is perhaps the most relevant preclinical endpoint for fracture repair. Destructive mechanical tests, which are not feasible in humans, provide important information that integrates various aspects of the repair process. Although delayed callus remodeling in humans can indicate a risk for nonunion, it can also represent a benign and expected response to antiresorptive therapy. Data from fracture repair studies using bisphosphonates (364) or with the RANKL inhibitor RANK-Fc (367) have suggested that the delay in callus remodeling with antiresorptive therapy has a neutral or positive effect on biomechanical integrity by virtue of increasing callus dimensions. Consistent with this notion, the delayed callus remodeling associated with OPG treatment in rats resulted in larger calluses that were able to withstand the same amount of biomechanical force as did the calluses of untreated rats (363). Similar findings were obtained when OPG was delivered via a gene therapy vector in a similar rat model of fracture repair (368). OPG delivery was associated with a 3-fold reduction in osteoclast numbers in the fracture callus and a significant increase in callus BMC compared with vehicle controls. Despite these changes, the amount of force required to break the calluses was similar in OPG and vehicle controls (368).

Fracture repair studies were also performed in normal mice that were treated with the RANKL inhibitor RANK-Fc. In this study, RANK-Fc treatment was associated with the elimination of osteoclasts in long bone fracture calluses, but union occurred in all animals within 28 d (369). This study also provided the only available data on the effects of RANKL inhibitory treatment withdrawal on fracture calluses. Osteoclast numbers in fracture calluses were shown to return to normal levels within 15 d of stopping RANK-Fc treatment, and animals from this experimental group also achieved union. This finding highlights the potent yet reversible nature of osteoclast suppression with RANKL inhibitors. These preclinical fracture repair studies have provided experimental evidence to support the provocative yet supportable hypothesis that osteoclasts do not play an important role in the biomechanical aspects of fracture repair in mice or rats.

## IV. Role and Regulation of OPG/RANKL in the Human Skeleton

The pivotal role of the RANKL/RANK/OPG regulatory system in osteoclast formation and function has been defin-

itively demonstrated in animal studies and in cellular studies as discussed earlier. This evidence generates a great deal of interest in these cytokines as key regulators of skeletal metabolism in health and disease in humans. Investigations in humans under various conditions and disease states have largely examined serum levels of OPG and/or RANKL, but a few have examined tissue/cellular expression of these cytokines. From nonhuman studies, it is apparent that the relative expression of OPG and RANKL is critical to regulation of osteoclast activity and, in turn, bone turnover in health and disease. OPG is a secreted protein and is detectable in the peripheral circulation where it is found as a monomer, dimer, or bound to RANKL. The commercially available assays for OPG detect all these forms, but the importance of each form and the relative amounts of each present in the circulation in different conditions is uncertain. Heparin binding by native OPG is likely to limit its distribution and half-life (18) such that OPG measured in serum might have limited potential to influence osteoclasts within bone. Serum RANKL measurements also ignore the membrane-bound form, which could be an important source of RANKL in normal and/or disease states. To complicate matters further, OPG can bind to membrane RANKL in a manner that is displaceable by soluble RANKL (370), and OPG might also bind to cell membrane glycosaminoglycans in a manner that is displaceable by heparin (19). Additionally, human reference ranges for OPG and RANKL have not been clearly established. As outlined above, these proteins are expressed in a wide range of tissues, and therefore, the tissue source of circulating OPG and RANKL cannot be determined and may complicate the interpretation of levels in different situations. Many factors influencing the circulating levels of these cytokines have been identified (371). Age appears to be a major factor influencing OPG levels in nearly all studies (372–377).

### *A. Postmenopausal osteoporosis*

The central role of estrogen deficiency in the pathogenesis of osteoporosis in postmenopausal women has been clearly established. Estrogen deficiency results in increased bone resorption over bone formation and net bone loss. Estrogen has been demonstrated to up-regulate gene expression and protein synthesis of OPG in human osteoblastic cells (104, 378) *in vitro*. It is logical from animal and *in vitro* studies that the RANKL/OPG system is involved in the pathogenesis of postmenopausal osteoporosis. However, studies designed to assess the relation between serum OPG and RANKL levels and bone metabolism/osteoporosis in postmenopausal women have yielded conflicting results. There is general agreement that OPG levels increase after the menopause (373, 379–381) and with GnRH agonist therapy (382), both conditions of low estrogen levels. Residual estrogen levels after the menopause correlate with bone density and fracture risk, yet the relationship of estrogen levels with serum OPG levels in postmenopausal women has been inconsistent across studies, with some supporting a weak relationship (375) and others no relationship (373, 379) or a negative correlation (381). No relationship was found between serum OPG and serum estradiol during GnRH agonist therapy in premenopausal women in one small study (382). Serum OPG levels have been found to be positively related to BMD (375, 379), negatively related to BMD (374, 383), or unrelated to BMD (372, 373) in postmenopausal women. Similarily, the evidence is conflicting about an association between markers of bone turnover and circulating OPG because some studies report an association (154, 372, 375) or an association only among osteoporotic women (384), and others report no association (373, 379). Results of studies examining the relationship between serum OPG levels and fracture are also inconsistent. Low OPG levels were reported to be associated with prevalent vertebral fractures in two studies (379, 384), and high serum OPG levels were associated with fracture in another study (383). The risk of subsequent fractures in postmenopausal women was not associated with OPG levels (154). Collectively, the data regarding serum OPG levels in postmenopausal women have been conflicting, and their potential clinical applicability as a marker of bone disease is not yet proven.

Serum RANKL levels have also been investigated in postmenopausal women and reported not to be associated with menopausal status or BMD (379, 385) but to be associated with nontraumatic fracture (385). However, in one of the studies over half of the women (54.9%) had concentrations of RANKL that were undetectable (379), and in the other study 40% of samples were below the detection limit of the assay (385). This raises the concern that the current assay for RANKL may not be ideal for the study of serum levels of this cytokine in postmenopausal women. Levels of RANKL in the bone microenvironement may be more relevant, but only a few studies have attempted to investigate this. The relationship between menopause, estrogen use, and PTH on RANKL and OPG mRNA in human bone tissue samples was examined in pre- and postmenopausal women (386). No difference was found in expression of RANKL, OPG, or the ratio between premenopausal women, postmenopausal women, and postmenopausal women taking estrogen. In a separate study of cadaveric proximal femur bone biopsies, RANKL/OPG mRNA ratio was strongly associated with histomorphometric indices of bone turnover in trabecular bone (87). Similarly, the RANKL/OPG mRNA ratio in transiliac bone biopsies of women sustaining a hip fracture was increased compared with controls with osteoarthritis (387). In addition, RANKL/OPG mRNA ratio was significantly greater in bone from the proximal femur of hip fractures than controls with osteoarthritis (388). These studies suggest that a relative increase in the expression of molecular promoters of osteoclast formation and activity in the bone microenvironment may underlie the susceptibility to fracture, but more detailed prospective studies are needed to prove this conclusively. However, the data on tissue levels, unlike that regarding serum levels, are consistent with the notion that increased bone turnover is an important factor in fracture risk.

Few studies have examined the response to osteoporosis therapy and change in the OPG/RANKL/RANK system in postmenopausal women. Cell culture studies show a positive effect of estrogen (104, 378) and raloxifene (30) on OPG production by human osteoblastic cells, but correlation *in vivo* between estrogen levels and serum OPG levels has been inconsistent. In postmenopausal women receiving hormone replacement therapy, OPG levels declined by 3 months and

positively correlated with changes in bone turnover markers at 3 months (389). However, no association was seen between the change in OPG and BMD after 1 yr of estrogen therapy (389). Eghbali-Fatourechi *et al.* (76) examined the cell surface concentrations of RANKL in bone marrow cells (osteoblastic, T, and B cells) of premenopausal and postmenopausal women, with and without estrogen treatment. RANKL expression per cell correlated with bone resorption markers and inversely with serum estradiol levels (76). The findings suggested that up-regulation of RANKL on bone marrow cells is correlated with increased bone resorption induced by estrogen deficiency. In contrast, a study of oral bisphosphonates in postmenopausal women found a positive correlation between changes in serum OPG levels and BMD response (390). Serum RANKL levels in this study did not change during bisphosphonate treatment. The effects of bisphosphonates on serum OPG may reflect changes in osteoblast cell pool or OPG production. Similar effects of bisphosphonates on serum OPG levels have been reported in Paget's disease of bone (PDB) (see *Section IV.D*). Treatment with human PTH (1-34) in women receiving chronic glucocorticoid and hormone replacement therapy resulted in rapid increase in serum RANKL by 1 month and a decrease in serum OPG levels at 6 and 12 months of therapy (93). The interpretation of these results is complicated by the concomitant use of glucocorticoids and hormone replacement therapy.

### *B. Men*

There are fewer studies investigating the OPG/RANKL system in men, but they rely on similar methodology as those in women: measuring serum concentrations of these cytokines. The limitations to this approach have already been outlined. As with women, serum OPG levels increase with age in men (372, 373, 376, 380, 381, 391, 392). OPG levels in men have been correlated inversely with BMD (373, 392), borderline positively with BMD (372), or not at all (376). The relationship of bone turnover and OPG levels is also conflicting. An inverse correlation of OPG with osteocalcin has been reported (372, 380, 392). However, a positive correlation with bone resorption markers but no correlation with bone formation markers has been reported (373). Yet another study reported a negative correlation with bone resorption markers (376). The relationship of OPG levels and sex steroids in men has also been examined. A positive correlation with free testosterone index and free estradiol index has been reported by one group (376). A negative correlation with bioavailable testosterone and bioavailable estradiol (373) and also with free testosterone index (391) has been reported. Testosterone replacement appeared to lower OPG levels in men made acutely hypogonadal by GnRH agonist and who also received aromatase inhibitor blockade of estrogen production (106). Men with prostate cancer who receive androgen deprivation therapy experience bone loss. However, studies looking at serum OPG/RANKL have largely investigated these as a marker of bone metastasis progression and as such do not shed light on the independent contribution of androgen deprivation to serum levels. As in postmenopausal women, the data in men are conflicting regarding the utility of serum levels of OPG to reflect bone disease states.

### *C. Glucocorticoid treatment*

Glucocorticoids are used as treatment for a variety of conditions because of their modulation of inflammation and the immune system. The detrimental skeletal effects of glucocorticoid therapy include suppression of bone formation by increasing osteoblast apoptosis and, to a lesser extent, and perhaps indirectly, increase in bone resorption. However, glucocorticoids also reduce intestinal calcium absorption, increase renal calcium excretion, and disturb vitamin D metabolism. All of these actions have implications to adversely affect the skeleton. Investigations of glucocorticoid effects on bone in humans are complicated by the underlying disease state for which they are given, and drawing common conclusions about skeletal effects across disease states for which glucocorticoids are given can be problematic. Nonetheless, the effects of glucocorticoids on bone remodeling have been extensively studied in animal models, and a role of the OPG/RANKL/RANK system in their effects has been discussed earlier. As with most of the human studies of the OPG/RANKL/RANK system, those investigating these cytokines during glucocorticoid therapy have focused on evaluating serum levels. In a study of patients with Crohn's disease, serum OPG levels decreased, whereas RANKL tended to increase with glucocorticoid therapy compared with baseline (244). Similarily, in a group of patients with glomerulonephritis, serum OPG levels were decreased with glucocorticoids compared with baseline, whereas markers of bone formation were transiently decreased and a marker of bone resorption was increased (267). There also was a positive association between OPG and bone density in this group of patients. A cross-sectional study of cardiac transplant recipients found a positive correlation between serum OPG levels and BMD, and a lower average serum OPG level was seen in those with prevalent vertebral fractures (393). In the same study, serum OPG levels fell after transplant, and bone loss based on BMD measurement was positively associated with the decrease in OPG level (393). Transplant recipients received not only glucocorticoids but also other immunosuppressive agents that may have independent effects on bone remodeling (especially cyclosporine). Although it is difficult to draw firm conclusions from the few human studies to date, there does appear to be some consistency in the effect of glucocorticoids to lower serum OPG levels *in vivo* that may partially explain the adverse skeletal effects of these drugs.

### *D. Paget's disease of bone*

PDB is a focal disorder of accelerated and disordered bone remodeling. Osteoclast-mediated bone resorption is increased with a compensatory increase in new bone formation. However, the resulting bone at affected sites lacks normal organization and is a mosaic of woven and lamellar bone. Osteoclasts in pagetic bone are increased in number and size and have abnormal appearance with substantially more nuclei than normal and with nuclear and cytoplasmic inclusions. Osteoblasts in pagetic bone are also numerous

and active, rapidly synthesizing new bone. The increase in both bone formation and resorption at pagetic sites can be detected in the peripheral circulation where increased levels of both markers of formation and resorption are found. Although the exact etiology of Paget's disease remains to be determined, there is abundant evidence of the involvement of the OPG/RANKL/RANK system and the NF-κB signaling pathway in the pathogenesis of the disorder. RANKL expression is abundant in cultures of stromal cells or marrow cells derived from pagetic bone compared with nonaffected bone of the same individuals (394, 395). Inactivating mutations in the OPG gene have been demonstrated in juvenile PDB (396–398). Constitutive activation of RANK has been demonstrated in familial expansile osteolysis, another disorder with abnormal bone remodeling, and in familial early onset PDB (398). Most recently, mutations in the sequestosome 1 gene, a scaffold protein in the NF-κB signaling pathway, have been demonstrated in some cases of familial and sporadic PDB (399–404).

Complimenting the *in vitro* cell system and genetic evidence are several studies examining serum levels of OPG and RANKL in PDB and their response to treatment with bisphosphonates. OPG levels are consistently increased in patients with PDB compared with controls (405–407). This lends support to the concept of increased OPG circulating in conditions of elevated turnover potentially as a protective mechanism, although the relationship between OPG levels and bone turnover in unaffected individuals is less clear as discussed earlier. RANKL levels are elevated in PDB in some studies (406) but not in others (407). The response of OPG and RANKL levels in PDB to treatment with bisphosphonates has also been investigated. The primary mechanism of action of bisphosphonates is to inhibit bone resorption by impairing key metabolic pathways of osteoclasts, although some *in vitro* cell studies and animal studies suggest potential effects on osteoblasts (408–411). OPG levels in PDB have been reported to increase modestly after treatment with pamidronate (406) and risedronate (407) and to decrease after treatment with tiludronate (405). These differing results may be due to differences in the mechanism of action of amino bisphosphonates compared with non-amino bisphosphonates on osteoclasts and osteoblasts, or due to differences in study populations. The increase in OPG levels after bisphosphonate treatment that lowers bone turnover does not support the general concept that serum OPG levels correlate with bone turnover in all situations.

### *E. Rheumatoid arthritis*

RA is characterized by chronic inflammation of the synovium, the predominance of proerosive mediators, and subsequent destruction of bone and cartilage. Many investigations have implicated inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-11, TNF-α) as important mediators stimulating bone lysis in RA. However, the critical role of the OPG/RANKL/RANK system in bone remodeling and osteoclast development has led to the concept of "osteoimmunology" to more accurately encompass the complex interactions between the immune system and the skeletal system in RA and other inflammatory arthritis conditions. *In vitro* cell system models and animal models discussed earlier support this concept, and the human *in vivo* data will be reviewed here.

The production of inflammatory cytokines in the inflamed joint is likely to up-regulate RANKL and RANK (90, 412), which promotes osteoclast development and bone destruction. Synovial cells isolated from rheumatoid joints have been found to express RANKL, RANK, and OPG (90, 412–415). Activated T lymphocytes within arthritic joints have also been shown to express RANKL and therefore might contribute to the promotion of bone erosions (413, 416). Higher levels of RANKL expression have been observed in synovial tissue from active RA patients *vs.* those with quiescent disease (413). Also, in synovial tissue from active RA, RANKL expression was seen but OPG was not (33), and the ratio of OPG:RANKL in synovial fluid has been correlated positively with radiological scores (417). Both T cells and non-T cells have been observed to be the source of RANKL in the synovium (413, 418). These results suggest that the deficient production of OPG or the ratio of RANKL:OPG in the joint may underlie the development of erosions.

Peripheral measurements of serum or cellular levels of OPG and RANKL have also been investigated in RA. The ratio of circulating OPG/RANKL levels was shown to negatively correlate with progression of radiographic joint damage in newly diagnosed active RA patients (419). However, another group reported that whereas OPG and RANKL serum levels were higher in RA patients compared with controls, the ratio of OPG/RANKL was similar between the two groups, and no correlation was observed between disease score and serum levels in RA patients (53). RANKL and OPG mRNA levels have been found to be elevated in peripheral blood mononuclear cells in RA patients compared with controls, and the ratio of OPG/RANKL tended to be higher in the synovium than in the peripheral blood mononuclear cells in early RA patients (418). The use of serum levels of OPG or RANKL to understand disease progression requires additional study.

In addition to focal bone erosions and periarticular bone loss, systemic bone loss and increased fracture risk are important components of RA (224, 239, 420–422). The mechanisms responsible for bone loss at skeletal sites such as the lumbar spine and hip, in the absence of obvious inflammation, are not well understood. Certainly the high circulating levels of TNF-α and IL-1 that are common in RA have the potential to promote osteoclastogenesis throughout the skeleton (223, 237, 423–425). Systemic stimulation of bone resorption in RA patients is suggested by increases in circulating biochemical markers of bone resorption (426) and increased osteoclastogenic potential of their bone marrow (426, 427). Recently, serum RANKL levels were reported to be increased in RA patients, and serum RANKL was inversely correlated with BMD of the hip and positively correlated with circulating bone resorption markers (239). Furthermore, the RANKL:OPG ratio was significantly elevated in serum of RA patients, and this increase was predictive of joint destruction (419). These results are consistent with the notion that RANKL might play a role in systemic bone loss in RA patients, in addition to its putative role in focal bone loss.

Several studies have reported that treatment of RA is associated with changes in OPG and RANKL in the peripheral

circulation. Ziolkowska *et al.* (53) reported normalization of serum OPG and RANKL by 14–22 wk of therapy with infliximab, although the RANKL:OPG ratio did not change during therapy. Interestingly, higher baseline levels of OPG or RANKL were associated with larger decreases with therapy. Vis *et al.* (428) found that infliximab treatment in RA caused a decline in serum RANKL and the OPG/RANKL ratio but no change in serum OPG. Infliximab treatment was also associated with stabilization of BMD at the hip and spine but continued bone loss in the hand (428). This agrees with *in vitro* cell culture studies showing a decrease in RANK expression in peripheral blood mononuclear cells treated with infliximab and inhibition of RANKL expression and increase in OPG secretion in cultured synovial cells treated with infliximab, methotrexate, sulfasalazine, or hydroxychloroquine (429). Collectively, these investigations suggest that disease-modifying antirheumatic drugs exert some of their effects through the OPG/RANKL/RANK system.

## V. Clinical Therapeutics Targeting the RANK/RANKL/OPG System

Clinical studies were conducted to investigate the biological activity of two different recombinant OPG constructs, focusing on osteoporosis and cancer-induced bone disease settings. The first drug tested clinically was Fc-OPG, which consisted of the active TNF receptor domains of native OPG fused at its carboxy-terminus to the Fc fragment of human IgG1. In a phase 1 sequential dose escalation study in healthy postmenopausal women, a single sc dose of Fc-OPG led to dose-dependent reductions in markers of bone resorption, including uNTX. Suppression of uNTX occurred within 12 h after dose, reached a maximum of 80% below baseline at the highest dose tested (3.0 mg/kg), and was maintained for up to 45 d (20).

The second OPG construct to enter the clinic was OPG-Fc (also known as AMGN-0007; Fig. 2). With OPG-Fc, the Fc fragment of IgG1 was fused to the amino terminus of the active domain of OPG, which conferred a longer half-life compared with Fc-OPG. OPG-Fc was evaluated in a phase 1, active-controlled, dose escalation study in patients with MM or breast cancer-related bone metastases (23). A dose-dependent reduction in uNTX occurred within 1 d of dosing with OPG-Fc, and uNTX reached a nadir of approximately −60 and −84% in the MM and breast cancer strata, respectively, at the higher doses (1.0 and 3.0 mg/kg). Suppression of bone resorption was maintained throughout the duration of the study (56 d). The pharmacodynamic profile of OPG-Fc was generally similar to that observed with the open-label active control, pamidronate.

The results of these clinical trials suggested therapeutic potential for RANKL inhibition in the treatment of bone loss. The clinical development of these OPG constructs was stopped in favor of the development of denosumab, a fully human monoclonal antibody that inhibited RANKL. An important practical consideration was that denosumab had a significantly longer circulating half-life compared with OPG-Fc (430), which would allow for reduced dosing frequency.

Another potential advantage of a fully human anti-RANKL antibody is the low potential for immune responses (431), a theoretical attribute that has been borne out in clinical trials with denosumab. Denosumab and numerous other fully human therapeutic antibodies have been made in transgenic mice, wherein their murine Ig genes have been deleted and replaced with human orthologs (431, 432). Denosumab was obtained using Abgenix's transgenic XenoMouse system (Abgenix, Fremont, CA). These mice were immunized with human RANKL, leading to an immune response that resulted in antigen-specific fully human IgGs that bind and inhibit RANKL (433). One such IgG (AMG 162) was cloned and expressed in Chinese hamster ovary cells and purified for large-scale production of clinical-grade material.

### *A. Mechanism of action of denosumab*

Denosumab is a fully human monoclonal antibody with a high affinity ($K_d$ $3 \times 10^{-12}$ M) and specificity for RANKL and can thus bind and neutralize the activity of human RANKL in a similar fashion to the action of OPG. Denosumab does not cross-react with TNF-$\alpha$, TNF-$\beta$, TRAIL, or CD40 ligand (361).

Because the activity of denosumab is primate specific, it cannot be tested in mouse or rat models of bone disease. However, because OPG and denosumab have similar mechanisms of action, OPG has been used as a surrogate for denosumab to examine the effects of RANKL inhibition in numerous mouse and rat models of bone disease. Human OPG-Fc and denosumab were shown to have similar effects on suppressing bone resorption and increasing BMD in knockin mice that were genetically engineered to exclusively express a chimeric (human/murine) form of RANKL that is recognized by both OPG and denosumab (434). One potential advantage of denosumab is that it has a significantly longer half-life compared with human OPG-Fc (430), which promotes more sustained suppression of bone resorption and thereby permits infrequent dosing.

Ongoing phase 2 and 3 trials are evaluating denosumab in the treatment and prevention of postmenopausal osteoporosis, the treatment of bone loss associated with sex hormone ablative therapy in subjects with cancer, the treatment of bone erosions associated with RA, the treatment of bone metastases to delay or prevent skeletal-related events in subjects with advanced cancer, and the prolongation of bone metastasis-free survival in subjects with hormone-refractory prostate cancer.

In these studies, denosumab is administered sc, at intervals ranging from every 4 wk to every 6 months, depending on the therapeutic area being evaluated. This long dosing interval holds the potential to facilitate treatment compliance, which is increasingly being recognized as a major obstacle in therapy for these conditions.

### *B. Clinical studies of denosumab in postmenopausal women with osteoporosis*

Denosumab administration in postmenopausal women with low BMD resulted in gains in BMD at the total hip, femoral neck, and distal radius at 12 and 24 months (230).

Those gains in BMD were associated with decreases in bone resorption. This ongoing phase 2 study is evaluating the effects of denosumab (6, 14, or 30 mg every 3 months or 14, 60, 100, or 210 mg every 6 months), open-label oral alendronate 70 mg once weekly, and placebo on BMD of the lumbar spine, total hip, femoral neck, and distal radius at 1, 3, 6, and 12 months. Changes in bone turnover are also being regularly assessed by measurement of serum and urine telopeptides and bone-specific alkaline phosphatase.

Treatment with denosumab resulted in a mean increase in BMD at the lumbar spine of 3.0 to 6.7% (*vs.* an increase of 4.6% with alendronate and a loss of 0.8% with placebo), at the total hip of 1.9 to 3.6% (*vs.* an increase of 2% with alendronate and a loss of 0.6% with placebo), and at the distal third of the radius of 0.4 to 1.3% (*vs.* decreases of 0.5% with alendronate and 2.0% with placebo).

In a planned extension of this study, denosumab treatment for 2 yr resulted in persistent reductions in bone resorption and continued, significant increases in BMD from baseline in the 337 women (82%) who completed 2 yr of study (38 placebo, 259 denosumab, 40 alendronate) (435). Across dose groups, women treated with denosumab for 2 yr had significantly greater improvements in BMD compared with placebo ($P < 0.001$) at the lumbar spine, total hip, and distal radius; these improvements were similar to or greater than those observed in the open-label alendronate group. During yr 2, in response to denosumab, serum C-telopeptide (CTx) and uNTX/creatinine demonstrated continued suppression. The duration of the suppression of bone turnover appeared to be dose dependent. Optimal increases in BMD appeared to occur at denosumab doses of 30 and 60 mg, administered at 3 and 6 months, respectively.

### *C. Clinical studies of denosumab in rheumatoid arthritis*

Based on the implication of RANKL involvement in the bone and cartilage changes characteristic of RA and the positive impact of RANKL inhibition in rat models of RA (26), the effects of denosumab (60 or 180 mg every 6 months) are being investigated in subjects with RA (436–438). In addition to studying BMD and bone turnover marker changes, a study by Cohen *et al.* (436) is assessing bone erosions using magnetic resonance imaging (MRI). Significant reductions in the progression of bone erosion were reflected in MRI erosion scores from this population of 227 patients with mild to moderately active RA (for at least 6 months) who had received a single 180-mg dose of denosumab compared with placebo ($P < 0.019$).

Results from a 6-month interim analysis were published by Dore *et al.* (437) showing that treatment with denosumab resulted in significant increases in BMD at the lumbar spine, total hip, and femoral neck, as well as suppression of bone turnover markers serum CTx type I (CTx-I) and serum procollagen type I N-propeptide at 3 and 6 months compared with placebo. In addition, significant suppression of the cartilage turnover marker, CTx-II/creatinine, was observed at 3 months. Of particular note, approximately 40% of patients in each treatment group had baseline CTx-II levels below quantifiable limits. However, at 3 months, this percentage increased to 81 and 89% in the 60- and 180-mg denosumab groups, respectively, and then returned to approximately 40% in all groups at 6 months, before the next denosumab dose (438).

In addition, a larger proportion of patients in the placebo group had an increase in MRI erosion score from baseline to 6 months than either denosumab dose group [53, 42, and 30% in the placebo, 60-mg, and 180-mg groups, respectively ($P = 0.01$ for 180-mg group *vs.* placebo)]. Adjustment for baseline erosion score did not affect the results (436).

### *D. Clinical studies of denosumab in patients with advanced cancer with bone metastases or multiple myeloma*

In advanced cancer, bone is a common site of metastasis. Bone metastases lead to skeletal complications (such as vertebral or nonvertebral bone fractures, spinal cord compression, surgery, or radiation therapy to bone) that are associated with increased bone turnover. Studies in patients with metastatic bone disease (439–441) suggest that denosumab suppresses bone resorption and normalizes bone turnover, as measured by serum and urinary telopeptides. Although these studies provide intriguing results, the relationship between bone turnover suppression and clinical outcomes of skeletal metastasis with denosumab therapy remains to be better defined by data from larger prospective trials with sufficient statistical power. Large prospective trials comparing the effects of denosumab or bisphosphonates on skeletal-related events in metastatic cancer are currently under way.

A recent double-blind phase 1 study of denosumab published by Body *et al.* (442) in patients with bone metastases from breast cancer (n = 29 women) and patients with MM (n = 25; 14 men and 11 women) showed that denosumab was well tolerated, with an adverse event profile that was not significantly different from placebo patients. Denosumab therapy was associated with sustained decreases (≥84 d) in uNTX and serum NTX after a single sc dose (0.1, 0.3, 1.0, or 3.0 mg/kg). These decreases were similar in magnitude, but of numerically longer duration, *vs.* those observed in a comparator group receiving an iv dose of pamidronate 90 mg. At the 3.0 mg/kg dose, denosumab demonstrated mean half-lives of 46.3 and 33.3 d in patients with breast cancer and MM, respectively. Population pharmacokinetic (PK) and pharmacodynamic (PD) analyses reported by Peterson *et al.* (440) suggest that denosumab PK and PD in breast cancer patients with bone metastases are consistent with those observed in other patient populations, and treatment with denosumab caused rapid and substantial suppression (>60% from baseline) of uNTX.

Lipton *et al.* (439) reported interim (13-wk) data from a phase 2 study of 255 patients with breast cancer and bone metastases who had not been previously treated with iv bisphosphonates (IV BP). Subjects were stratified by chemotherapy or hormonal therapy and randomized to receive either denosumab or IV BP in one of six cohorts [five denosumab (double blind), one IV BP (open label)]. Based on PK and PD assessments demonstrating decreased uNTX in cancer patients in response to treatment with denosumab (442), the primary endpoint of this study was the percentage change from baseline to wk 13 in uNTX, corrected for creatinine. The denosumab 120-mg every 4 wk dose resulted in

the greatest percentage decrease from baseline in uNTX. Treatment with denosumab (n = 212) resulted in rapid and sustained suppression of bone turnover and was similar to IV BP at reducing the risk of skeletal-related events (439).

Suarez *et al.* (441) compared 49 (of a planned 135) cancer patients with bone metastases on established IV BP therapy who were randomized to receive denosumab (33 of 49) or continue with an established IV BP treatment (16 of 49; most of these patients received ZOL). Subjects had prostate (n = 24), breast (n = 20), or other (n = 5) cancers (including MM). The proportion of subjects with uNTX less than 50 nM bone collagen equivalent/mM creatinine at wk 13 was greater with denosumab (pooled arms) treatment than with continued IV BP treatment: 76% (95% confidence interval 60.3–91.2) *vs.* 38% (95% confidence interval 18.5–61.4; $P = 0.015$), respectively. Based on this interim assessment, denosumab appears to normalize uNTX more frequently than IV BP in patients with elevated uNTX, despite established IV BP therapy, across tumor types (441). Thus, it appears that these patients had become refractory to BP treatment, because they showed elevated uNTX despite receiving their established BP regimens. In contrast, patients who were switched to denosumab subsequently showed an overall normalization of uNTX levels. This study showed a safety profile similar to what might be typically observed in patients receiving standard chemotherapy regimens for the cancers studied.

### *E. Evaluation of adverse events profiles in clinical studies of denosumab*

The largest and most recent single-study sample in cancer patients receiving denosumab is that of the 255 breast cancer patients reported by Lipton *et al.* (439) (discussed in *Section IV.D*). These authors report a denosumab safety profile similar to that seen in advanced breast cancer patients receiving systemic cancer treatment.

The largest sample size published to date is the 24-month report by Lewiecki *et al.* (435) of data from osteopenic or osteoporotic women comparing denosumab (n = 314) with alendronate (n = 46) and placebo (n = 46). Few significant differences were observed between the profiles of adverse events in the denosumab groups and those in the placebo group and the alendronate group. Hypertension and urinary tract infections were reported more often in the denosumab group than the placebo group ($P = 0.01$), whereas dyspepsia and osteoarthritis appeared to occur with significantly greater frequency in the open-label alendronate group ($P = 0.01$).

Indeed, the percentages of patients experiencing adverse events (92.0, 93.5, and 93.5%, respectively) and serious adverse events (13.4, 13.0, and 8.7%, respectively) in the denosumab, alendronate, and placebo groups were similar (435). The incidence and nature of adverse events continue to be examined in ongoing studies of denosumab, including assessments for unusual immune responses, infections, and neoplasms.

## VI. Summary

The discovery of the OPG/RANKL/RANK pathway is a major advance in understanding the molecular orchestration of bone remodeling. The pivotal role of these cytokines in bone remodeling has been demonstrated in cell culture systems and healthy animals as well as animal models of disease. The data in humans are generally supportive of the importance of OPG and RANKL in normal bone physiology as well as in metabolic bone disease. Clinical trials that are evaluating the therapeutic potential of RANKL inhibition are under way.

## Acknowledgments

Scott Saunders (Meniscus Health Care Communications) and Amy Foreman-Wykert (Amgen Inc.) assisted in the preparation of this manuscript. We also thank David Ke for helpful comments regarding wnt regulation of OPG and RANKL.

Received May 25, 2007. Accepted November 15, 2007.

Address all correspondence and requests for reprints to: Paul J. Kostenuik, Amgen Inc., 1 Amgen Center Drive, M/S 15-2-B, Thousand Oaks, California 91320. E-mail: paulk@amgen.com

This work was supported by National Institutes of Health Grant AG004875 (to S.K.).

Conflicts of Interest: A.E.K. is involved in a clinical trial with Amgen and has received lecture fees from Amgen Inc. S.K. has nothing to declare. P.J.K. is an employee and shareholder in Amgen Inc.

## References

1. **Compston JE, Vedi S, Kaptoge S, Seeman E** 2007 Bone remodeling rate and remodeling balance are not co-regulated in adulthood: implications for the use of activation frequency as an index of remodeling rate. J Bone Miner Res 22:1031–1036
2. **Rodan GA, Martin TJ** 2000 Therapeutic approaches to bone disease. Science 289:1508–1514
3. **Eriksen EF** 1986 Normal and pathological remodeling of human trabecular bone: three dimensional reconstruction of the remodeling sequence in normals and in metabolic bone disease. Endocr Rev 7:379–408
4. **Simonet WS, Lacey DL, Dunstan C, Kelly M, Chang MS, Luthy R, Nhuyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliot R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarpley J, Derby P, Lee R, Boyle WJ** 1997 Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 89:309–319
5. **Tsuda E, Goto M, Mochizuki SI, Yano K, Kobayashi F, Morinaga T, Higashio K** 1997 Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. Biochem Biophys Res Comm 234:137–142
6. **Yasuda H, Shima N, Nakagawa N, Mochizuki SI, Yano K, Fujise N, Sato Y, Kanno T, Murakami A, Tsuda E, Morinaga T, Higashio K** 1998 Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis *in vitro.* Endocrinology 139:1329–1337
7. **Akatsu T, Murakami T, Ono K, Nishikawa M, Tsuda E, Mochizuki SI, Fujise N, Higashio K, Motoyoshi K, Yamamoto M, Nagata N** 1998 Osteoclastogenesis inhibitory factor exhibits hypocalcemic effects in normal mice and in hypercalcemic nude mice carrying tumors associated with humoral hypercalcemia of malignancy. Bone 23:495–498
8. **Hakeda Y, Kobayashi Y, Yamaguchi K, Yasuda H, Tsuda E, Higashio K, Miyata T, Kumegawa M** 1998 Osteoclastogenesis inhibitory factor (OCIF) directly inhibits bone-resorbing activity of isolated mature osteoclasts. Biochem Biophys Res Comm 251:796–801
9. **Burgess T, Qian YX, Kaufman S, Ring BD, Van G, Capparelli C, Kelley M, Hsu H, Boyle WJ, Dunstan CR, Hu S, Lacey DL** 1999 The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. J Cell Biol 145:527–538

10. **Akatsu T, Murakami T, Nishikawa M, Ono K, Shinomiya N, Tsuda E, Mochizuki SI, Yamaguchi K, Kinosaki M, Higashio K, Yamamoto M, Motoyoshi K, Nagata N** 1998 Osteoclastogenesis inhibitory factor suppresses osteoclast survival by interfering in the interaction of stromal cells with osteoclast. Biochem Biophys Res Comm 250:229–234
11. **Lacey DL, Tan HL, Lu J, Kaufman S, Van G, Qiu W, Rattan A, Scully S, Fletcher F, Juan T, Kelley M, Burgess TL, Boyle WJ, Polverino AJ** 2000 Osteoprotegerin ligand modulates murine osteoclast survival in vitro and in vivo. Am J Pathol 157:435–448
12. **O'Brien EA, Williams J, Marshall MJ** 2000 Osteoprotegerin ligand regulates osteoclast adherence to the bone surface in mouse calvaria. Biochem Biophys Res Comm 274:281–290
13. **O'Brien EA, Williams J, Marshall MJ** 2001 Osteoprotegerin is produced when prostaglandin synthesis is inhibited causing osteoclasts to detach from the surface of mouse parietal bone and attach to the endocranial membrane. Bone 28:208–214
14. **The American Society for Bone and Mineral Research President's Committee on Nomenclature** 2000 Proposed standard nomenclature for new tumor necrosis factor family members involved in the regulation of bone resorption. J Bone Miner Res 15:2293–2296
15. **Theoleyre S, Wittrant Y, Tat SK, Fortun Y, Redini F, Heymann D** 2004 The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. Cytokine Growth Factor Rev 15:457–475
16. **Yamaguchi K, Kinosaki M, Goto M, Kobayashi F, Tsuda E, Morinaga T, Higashino K** 1998 Characterization and structural domains of human osteoclastogenesis inhibitory factor. J Biol Chem 273: 5117–5123
17. **Schneeweis LA, Willard D, Milla ME** 2005 Functional dissociation of osteoprotegerin and its interaction with receptor activator of NF-κB ligand. J Biol Chem 280:41155–41164
18. **Tomoyasu A, Goto M, Fujise N, Mochizuki SI, Yasuda H, Morinaga T, Tsuda E, Higashio K** 1998 Characterization of monomeric and homodimeric forms of osteoclastogenesis inhibitory factor. Biochem Biophys Res Comm 245:382–387
19. **Irie A, Takami M, Kubo H, Sekino-Suzuki N, Kasahara K, Sanai Y** 2007 Heparin enhances osteoclastic bone resorption by inhibiting osteoprotegerin activity. Bone 41:165–174
20. **Bekker PJ, Holloway D, Nakanishi A, Arrighi M, Leese PT, Dunstan CR** 2001 The effect of a single dose of osteoprotegerin in postmenopausal women. J Bone Miner Res 16:348–360
21. **Morony S, Capparelli C, Lee R, Shimamoto G, Boone T, Lacey DL, Dunstan CR** 1999 A chimeric form of osteoprotegerin inhibits hypercalcemia and bone resorption induced by IL-1$\beta$, TNF-$\alpha$, PTH, PTHrP, and 1,25$(OH)_2D_3$. J Bone Miner Res 14:1478–1485
22. **Smith BB, Cosenza ME, Mancini A, Dunstan C, Gregson R, Martin SW, Smith SY, Davis H** 2003 A toxicity profile of osteoprotegerin in the cynomolgus monkey. Int J Toxicol 22:403–412
23. **Body JJ, Greipp P, Coleman RE, Facon T, Geurs F, Fermand JP, Harousseau JL, Lipton A, Mariette X, Williams CD, Nakanishi A, Holloway D, Martin SW, Dunstan CR, Bekker PJ** 2003 A phase I study of AMGN-0007, a recombinant osteoprotegerin construct, in patients with multiple myeloma or breast carcinoma related bone metastases. Cancer 97(3 Suppl):887–892
24. **Capparelli C, Morony S, Warmington K, Adamu S, Lacey D, Dunstan CR, Stouch B, Martin S, Kostenuik PJ** 2003 Sustained antiresorptive effects after a single treatment with human recombinant osteoprotegerin (OPG): a pharmacodynamic and pharmacokinetic analysis in rats. J Bone Miner Res 18:852–858
25. **Ominsky MS, Kostenuik PJ, Cranmer P, Smith SY, Atkinson JE** 2007 The RANKL inhibitor OPG-Fc increases cortical and trabecular bone mass in young gonad-intact intact cynomolgus monkeys. Osteoporos Int 18:1073–1082
26. **Stolina M, Adamu S, Ominsky MS, Dwyer D, Asuncion F, Geng Z, Middleton S, Brown H, Pretorius J, Schett G, Bolon B, Feige U, Zack D, Kostenuik PJ** 2005 RANKL is a marker and mediator of local and systemic bone loss in two rat models of inflammatory arthritis. J Bone Miner Res 20:1756–1765
27. **Gori F, Hofbauer LC, Dunstan CR, Spelsberg TC, Khosla S, Riggs BL** 2000 The expression of osteoprotegerin and RANK ligand and the support of osteoclast formation by stromal-osteoblast lineage cells is developmentally regulated. Endocrinology 141:4768–4776
28. **Hofbauer LC, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S** 1998 Osteoprotegerin production by human osteoblast lineage cells is stimulated by vitamin D, bone morphogenetic protein-2, and cytokines. Biochem Biophys Res Comm 250:776–781
29. **Udagawa N, Takahashi N, Yasuda H, Mizuno A, Itoh K, Ueno Y, Shinki T, Gillespie MT, Martin TJ, Higashio K, Suda T** 2000 Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function. Endocrinology 141:3478–3484
30. **Viereck V, Grundker C, Blaschke S, Niederkleine B, Siggelkow H, Frosch KH, Raddatz D, Emons G, Hofbauer LC** 2003 Raloxifene concurrently stimulates osteoprotegerin and inhibits interleukin-6 production by human trabecular osteoblasts. J Clin Endocrinol Metab 88:4206–4213
31. **Collin-Osdoby P, Rothe L, Anderson F, Nelson M, Maloney W, Osdoby P** 2001 Receptor activator of NF-κB and osteoprotegerin expression by human microvascular endothelial cells, regulation by inflammatory cytokines, and role in human osteoclastogenesis. J Biol Chem 276:20659–20672
32. **Olesen P, Ledet T, Rasmussen LM** 2005 Arterial osteoprotegerin: increased amounts in diabetes and modifiable synthesis from vascular smooth muscle cells by insulin and TNF-$\alpha$. Diabetologia 48:561–568
33. **Haynes DR, Barg E, Crotti TN, Holding C, Weedon H, Atkins GJ, Zannetino A, Ahern MJ, Coleman M, Roberts-Thomson PJ, Kraan M, Tak PP, Smith MD** 2003 Osteoprotegerin expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathies and osteoarthritis and normal controls. Rheumatology 42: 123–134
34. **Shalhoub V, Faust J, Boyle WJ, Dunstan C, Kelley MJ, Kaufman S, Scully S, van Auken M, Lacey DL** 1999 Osteoprotegerin and osteoprotegerin ligand effects on osteoclast formation from human peripheral blood mononuclear cell precursors. J Cell Biochem 72: 251–261
35. **Chambers TJ** 1980 The cellular basis of bone resorption. Clin Orthop Relat Res 151:283–293
36. **Rodan GA, Martin TJ** 1981 Role of osteoblasts in hormonal control of bone resorption: a hypothesis. Calcif Tissue Int 33:349–351
37. **Udagawa N, Takahashi N, Akatsu T, Sasaki T, Tamaguchi A, Kodama H, Martin TJ, Suda T** 1989 The bone marrow-derived stromal cell lines MC3T3–G2/PA6 and ST2 support osteoclast-like cell differentiation in cocultures with mouse spleen cells. Endocrinology 125:1805–1813
38. **Takahashi N, Akatsu T, Udagawa N, Sasaki T, Yamaguchi A, Moseley JM, Martin TJ, Suda T** 1988 Osteoblastic cells are involved in osteoclast formation. Endocrinology 123:2600–2602
39. **Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliot R, Colombero A, Elliot G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ** 1998 Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176
40. **Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki SI, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T** 1998 Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 95:3597–3602
41. **Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, DuBose RF, Cosman D, Galibert L** 1997 A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 390:175–179
42. **Wong B, Josien R, Lee SY, Sauter B, Li HL, Steinman RM, Choi Y** 1997 TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. J Exp Med 186:2075–2080
43. **Fuller K, Wong B, Fox S, Choi Y, Chambers TJ** 1998 TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. J Exp Med 188:997–1001
44. **Ikeda T, Kasai M, Utsuyama M, Hirokawa K** 2001 Determination of three isoforms of the receptor activator of nuclear factor-κB

ligand and their differential expression in bone and thymus. Endocrinology 142:1419–1426
45. **Hikita A, Yana I, Wakeyama H, Nakamura M, Kadono Y, Oshima Y, Nakamura K, Seiki M, Tanaka S** 2006 Negative regulation of osteoclastogenesis by ectodomain shedding of receptor activator of NF-κB ligand. J Biol Chem 281:36846–36855
46. **Bord S, Ireland DC, Beavan SR, Compston JE** 2003 The effects of estrogen on osteoprotegerin, RANKL, and estrogen receptor expression in human osteoblasts. Bone 32:136–141
47. **Nakamichi Y, Udagawa N, Yasuda H, Nakamura M, Kobayashi Y, Takahashi N** 2007 M-CSF independent mechanisms for osteoclastogenesis. J Bone Miner Res 22:S380
48. **Giuliani N, Colla S, Sala R, Moroni M, Lazzaretti M, La Monica S, Bonomini S, Hojden M, Sammarelli G, Barille S, Bataille R, Rizzoli V** 2002 Human myeloma cells stimulate the receptor activator of nuclear factor-κB ligand (RANKL) in T lymphocytes: a potential role in multiple myeloma bone disease. Blood 100:4615–4621
49. **Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, Capparelli C, Li J, Elliot R, McCabe S, Wong T, Campagnuolo G, Moran E, Bogoch ER, Van G, Nguyen LT, Ohashi PS, Lacey DL, Fish E, Boyle WJ, Penninger JM** 1999 Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature 402:304–309
50. **Nakamichi Y, Udagawa N, Kobayashi Y, Nakamura M, Yamamoto Y, Tamashita T, Mizoguchi T, Sato M, Mogi M, Penninger JM, Takahashi N** 2007 Osteoprotegerin reduces the serum level of receptor activator of NF-κB ligand derived from osteoblasts. J Immunol 178:192–200
51. **Deyama Y, Takeyama S, Koshikawa M, Shirai Y, Yoshimura Y, Nishikata M, Suzuki K, Matsumoto A** 2000 Osteoblast maturation suppressed osteoclastogenesis in coculture with bone marrow cells. Biochem Biophys Res Comm 274:249–254
52. **Nakashima T, Kobayashi Y, Yamasaki S, Kawakami A, Eguchi K, Sasaki H, Sakai H** 2000 Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-κB ligand: modulation of the expression by osteotropic factors and cytokines. Biochem Biophys Res Comm 275:768–775
53. **Ziolkowska M, Kurowska M, Radzikowska A, Luszczykiewicz G, Wiland P, Dziewczopolski W, Filipowicz-Sosnowska A, Pazdur J, Szechinski J, Kowalczewski J, Rell-Bakalarska M, Maslinski W** 2002 High levels of osteoprotegerin and soluble receptor activator of nuclear factor κB ligand in serum of rheumatoid arthritis patients and their normalization after anti-tumor necrosis factor α treatment. Arthritis Rheum 46:1744–1753
54. **Yuan YY, Lau AG, Kostenuik PJ, Morony S, Adamu S, Asuncion F, Bateman TA** 2005 Soluble RANKL has detrimental effects on cortical and trabecular bone volume, mineralization and bone strength in mice. J Bone Miner Res 20:S161–S162
55. **Yuan YY, Kostenuik PJ, Ominsky MS, Morony S, Adamu S, Simionescu DT, Basalyga DM, Asuncion F, Bateman TA** 24 November 2007 Skeletal deterioration induced by RANKL infusion: a model for high-turnover bone disease. Osteoporos Int 10.1007/s00198-007-0509-7
56. **Yuan YY, Kostenuik PJ, Morony S, Simionescu DT, Basalyga DM, Bateman TA** 2006 RANKL infusion as a disease model: implications for bone and vascular systems. J Bone Miner Res 21:S163
57. **Itoh K, Udagawa N, Matsuzaki K, Takami M, Amano H, Shinki T, Ueno Y, Takahashi N, Suda T** 2000 Importance of membrane- or matrix-associated forms of M-CSF and RANKL/ODF in osteoclastogenesis supported by SaOS-4/3 cells expressing recombinant PTH/PTHrP receptors. J Bone Miner Res 15:1766–1775
58. **Quinn J, Elliott J, Gillespie MT, Martin TJ** 1998 A combination of osteoclast differentiation factor and macrophage-colony stimulating factor is sufficient for both human and mouse osteoclast formation *in vitro*. Endocrinology 139:4424–4427
59. **Begg SK, Radley JM, Polland JW, Chisholm OT, Stanley ER, Bertoncello I** 1993 Delayed hematopoietic development in osteopetrotic (*op/op*) mice. J Exp Med 177:237–242
60. **Niida S, Kaku M, Amano H, Yoshida H, Kataoka H, Nishikawa S, Tanne K, Maeda N, Nishikawa SI, Kodama H** 1999 Vascular endothelial growth factor can substitute for macrophage colony-stimulating factor in the support of osteoclastic bone resorption. J Exp Med 190:293–298
61. **Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Yano K, Morinaga T, Higashio K** 1998 RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. Biochem Biophys Res Comm 253:395–400
62. **Dougall WC, Glaccum M, Charrier K, Rohrbach K, Brasel K, De Smedt T, Daro E, Smith J, Tometsko ME, Maliszewski CR, Armstrong A, Shen V, Bain S, Cosman D, Anderson D, Morrissey PJ, Peschon JJ, Schuh J** 1999 RANK is essential for osteoclast and lymph node development. Genes Dev 13:2412–2424
63. **Li J, Sarosi I, Yan XQ, Morony S, Capparelli C, Tan HL, McCabe S, Elliot R, Scully S, Van G, Kaufman S, Juan SC, Sun Y, Tarpley J, Martin L, Christensen K, McCabe J, Kostenuik PJ, Hsu H, Fletcher F, Dunstan CR, Lacey DL, Boyle WJ** 2000 RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis, and regulation of bone mass and calcium metabolism. Proc Natl Acad Sci USA 97:1566–1571
64. **Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan C, Lacey DL, Mak TW, Boyle WJ, Penninger JM** 1999 OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph node organogenesis. Nature 397:315–323
65. **Lam J, Nelson CA, Ross FP, Teitelbaum SL, Fremont DH** 2001 Crystal structure of the TRANCE/RANKL cytokine reveals determinants of receptor-ligand specificity. J Clin Invest 108:971–979
66. **Boyce BF, Xing L** 2007 Biology of RANK, RANKL and osteoprotegerin. Arthritis Res Ther 9:S1–S7
67. **Boyle WJ, Simonet WS, Lacey DL** 2003 Osteoclast differentiation and activation. Nature 423:337–342
68. **Lee ZH, Kim HH** 2003 Signal transduction by receptor activator of nuclear factor κB in osteoclasts. Biochem Biophys Res Comm 305:211–214
69. **Wada T, Nakashima T, Oliveira-dos-Santos AJ, Gasser J, Hara H, Schett G, Penninger JM** 2005 The molecular scaffold Gab2 is a crucial component of RANK signaling and osteoclastogenesis. Nat Med 11:394–399
70. **Yun TJ, Chaudhary PM, Shu GL, Frazer JK, Ewings MK, Schwartz SM, Pascual V, Hood LE, Clark EA** 1998 OPG/FDCR-1, a TNF receptor family member, is expressed in lymphoid cells and is up-regulated by ligating CD40. J Immunol 161:6113–6121
71. **Malyankar UM, Scatena M, Suchland KL, Yun TJ, Clark EA, Giachelli CM** 2000 Osteoprotegerin is an αVβ3-induced, NF-κB-dependent survival factor for endothelial cells. J Biol Chem 275:20959–20962
72. **Hsu H, Lacey DL, Dunstan C, Solovyev I, Colombero A, Timms E, Tan HL, Elliot G, Kelley MJ, Sarosi I, Wang L, Xia X, Elliot R, Chiu L, Black T, Scully S, Capparelli C, Morony S, Shimamoto G, Bass MB, Boyle WJ** 1999 Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proc Natl Acad Sci USA 96:3540–3545
73. **Fata JE, Kong YY, Li J, Sasaki T, Irie-Sasaki J, Moorehead R, Elliott R, Scully S, Voura EB, Lacey DL, Boyle WJ, Khoka R, Penninger JM** 2000 The osteoclast differentiation factor osteoprotegerin ligand is essential for mammary gland development. Cell 103:41–50
74. **Min JK, Kim YM, Kim YM, Kim EC, Go YS, Kan IL, Lee SY, Kong YY, Kwon YG** 2003 Vascular endothelial growth factor up-regulates expression of receptor activator of NF-κB (RANK) in endothelial cells. J Biol Chem 278:39548–39557
75. **Kartsogiannis V, Zhou H, Horwood NJ, Thomas RJ, Hards DK, Quinn J, Niforas P, Ng KW, Martin TJ, Gillespie MT** 1999 Localization of RANKL (receptor activator of NFκB ligand) mRNA and protein in skeletal and extraskeletal tissues. Bone 25:525–534
76. **Eghbali-Fatourechi G, Khosla S, Sanyal A, Boyle WJ, Lacey DL, Riggs BL** 2003 Role of RANK ligand in mediating increased bone resorption in early postmenopausal women. J Clin Invest 111:1221–1230
77. **Hofbauer LC, Shui C, Riggs BL, Dunstan CR, Spelsberg LC, O'Brien T, Khosla S** 2001 Effects of immunosuppressants on receptor activator of NF-κB ligand and osteoprotegerin production

by human osteoblastic and coronary artery smooth muscle cells. Biochem Biophys Res Comm 280:334–339
78. **Enomoto H, Shiojiri S, Hoshi K, Furuichi T, Fukuyama R, Yoshida CA, Kanatani N, Nakamura R, Mizuno A, Zanma A, Yano K, Yasuda H, Higashio K, Takada K, Komori T** 2003 Induction of osteoclast differentiation by Runx2 through receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin regulation and partial rescue of osteoclastogenesis in Runx2−/− mice by RANKL transgene. J Biol Chem 278:23971–23977
79. **Fu Q, Jilka RL, Manolagas SC, O'Brien CA** 2002 Parathyroid hormone stimulates receptor activator of NF-κB ligand and inhibits osteoprotegerin expression via protein kinase A activation of cAMP-response element-binding protein. J Biol Chem 277:48868–48875
80. **Thirunavukkarasu K, Halladay DL, Miles RR, Yang X, Galvin R, Chandrasekhar S, Martin TJ, Onyia JE** 2000 The expression of osteoblast-specific transcription factor Cbfa1 contributes to the expression of osteoprotegerin, a potent inhibitor of osteoclast differentiation and function. J Biol Chem 275:25163–25172
81. **Thirunavukkarasu K, Miles RR, Halladay DL, Yang X, Galvin R, Chandrasekhar S, Martin TJ, Onyia JE** 2001 Stimulation of osteoprotegerin (OPG) gene expression by transforming growth factor-β (TGF-β). J Biol Chem 276:36241–36250
82. **Nagai M, Sato N** 1999 Reciprocal gene expression of osteoclastogenesis inhibitory factor and osteoclast differentiation factor regulates osteoclast formation. Biochem Biophys Res Comm 257:719–723
83. **Nakagawa N, Yasuda H, Yano K, Mochizuki SI, Kobayashi N, Fujimoto H, Shima N, Morinaga T, Chikazu D, Kawaguchi H, Higashio K** 1999 Basic fibroblast growth factor induces osteoclast formation by reciprocally regulating the production of osteoclast differentiation factor and osteoclastogenesis inhibitory factor in mouse osteoblastic cells. Biochem Biophys Res Comm 265:158–163
84. **Bord S, Frith E, Irleand DC, Scott MA, Craig J, Compston JE** 2004 Synthesis of osteoprotegerin and RANKL by megakaryocytes is modulated by estrogen. Brit J Haematol 126:244–251
85. **Morabito N, Guadio A, Lasco A, Atteritano M, Pizzoleo MA, Cincotta M, La Rosa M, Guarino R, Meo A, Frisina N** 2004 Osteoprotegerin and RANKL in the pathogenesis of Thalassemia-induced osteoporosis: new pieces of the puzzle. J Bone Miner Res 19:722–727
86. **Lee SK, Lorenzo JA** 1999 Parathyroid hormone stimulates TRANCE and inhibits osteoprotegerin messenger ribonucleic acid expression in murine bone marrow cultures: correlation with osteoclast-like cell formation. Endocrinology 140:3552–3561
87. **Fazzalari NL, Kuliwaba JS, Atkins GJ, Forwood MR, Findlay DM** 2001 The ratio of messenger RNA levels of receptor activator of nuclear factor κB ligand to osteoprotegerin correlates with bone remodeling in normal human cancellous bone but not in osteoarthritis. J Bone Miner Res 16:1015–1027
88. **Ma YL, Cain RL, Halladay DL, Yang X, Zeng Q, Miles RR, Chandrasekhar S, Martin TJ, Onyia JE** 2001 Catabolic effects of continuous human PTH (1-38) *in vivo* is associated with sustained stimulation of RANKL and inhibition of osteoprotegerin and gene-associated bone formation. Endocrinology 142:4047–4054
89. **Cao J, Venton L, Sakata T, Halloran BP** 2003 Expression of RANKL and OPG correlates with age-related bone loss in male C57BL/6 mice. J Bone Miner Res 18:270–277
90. **Haynes DR, Crotti TN, Loric M, Bain GI, Atkins GJ, Findlay DM** 2001 Osteoprotegerin and receptor activator of nuclear factor κB ligand (RANKL) regulate osteoclast formation by cells in the human rheumatoid arthritic joint. Rheumatology 40:623–630
91. **Locklin RM, Khosla S, Turner RT, Riggs BL** 2003 Mediators of the biphasic responses of bone to intermittent and continuously administered parathyroid hormone. J Cell Biochem 89:180–190
92. **Grimaud E, Soubigou L, Couillaud S, Coipeau P, Moreau A, Passuti N, Gouin F, Redini F, Heymann D** 2003 Receptor activator of nuclear factor κB ligand (RANKL)/osteoprotegerin (OPG) ratio is increased in severe osteolysis. Am J Pathol 163:2021–2031
93. **Buxton EC, Yao W, Lane NE** 2004 Changes in serum receptor activator of nuclear factor-κB ligand, osteoprotegerin, and interleukin-6 levels in patients with glucocorticoid-induced osteoporosis treated with human parathyroid hormone (1-34). J Clin Endocrinol Metab 89:3332–3336
94. **Taranta A, Fortunati D, Longo M, Rucci N, Iacomino E, Aliberti F, Facciuto E, Migliaccio S, Bardella MT, Dubini A, Borghi MO, Saraifoger S, Teti A, Bianchi ML** 2004 Imbalance of osteoclastogenesis-regulating factors in patients with celiac disease. J Bone Miner Res 19:1112–1121
95. **Terpos E, Politou M, Szydlo R, Nadal E, Avery S, Olavarria E, Kanfer E, Goldman JM, Apperley JF, Rahemtulla A** 2004 Autologous stem cell transplantation normalizes abnormal bone remodeling and sRANKL/osteoprotegerin ratio in patients with multiple myeloma. Leukemia 18:1420–1426
96. **Terpos E, Szydlo R, Apperley JF, Hatjiharissi E, Politou M, Meletis J, Viniou N, Yataganas X, Goldman JM, Rahemtulla A** 2003 Soluble receptor activator of nuclear factor κB ligand-osteoprotegerin ratio predicts survival in multiple myeloma: proposal for a novel prognostic index. Blood 102:1064–1069
97. **Masi L, Simonini G, Piscitelli E, Del Monte F, Giani T, Cimaz R, Vierucci S, Brandi ML, Falcini F** 2004 Osteoprotegerin (OPG)/RANKL system in juvenile idiopathic arthritis: is there a potential modulating role for OPG/RANKL in bone injury? J Rheumatol 31:986–991
98. **Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S** 1999 Interleukin-1β and tumor necrosis factor-α, but not interleukin 6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. Bone 25:255–259
99. **Wei S, Kitaura H, Zhou P, Ross FP, Teitelbaum SL** 2005 IL-1 mediates TNF-induced osteoclastogenesis. J Clin Invest 115:282–290
100. **Toraldo G, Roggia C, Qian WP, Pacifici R, Weitzmann MN** 2003 IL-7 induces bone loss in vivo by induction of receptor activator of nuclear factor κB and tumor necrosis factor α from T cells. Proc Natl Acad Sci USA 100:125–130
101. **Thomas RJ, Guise TA, Yin JJ, Elliott J, Horwood NJ, Martin TJ, Gillespie MT** 1999 Breast cancer cells interact with osteoblasts to support osteoclast formation. Endocrinology 140:4451–4458
102. **Kondo T, Kitazawa R, Maeda S, Kitazawa S** 2004 1α,25 dihydroxyvitamin D3 rapidly regulates the mouse osteoprotegerin gene through dual pathways. J Bone Miner Res 19:1411–1419
103. **Quinn JM, Horwood NJ, Elliot J, Gillespie MT, Martin TJ** 2000 Fibroblastic stromal cells express receptor activator of NF-κB ligand and support osteoclast differentiation. J Bone Miner Res 15:1459–1466
104. **Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Spelsberg TC, Riggs BL** 1999 Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells. Endocrinology 140:4367–4370
105. **Shevde NK, Bendixen AC, Dienger KM, Pike JW** 2000 Estrogens suppress RANK ligand-induced osteoclast differentiation via a stromal cell independent mechanism involving c-jun repression. Proc Natl Acad Sci USA 97:7829–7834
106. **Khosla S, Atkinson EJ, Dunstan CR, O'Fallon WM** 2002 Effect of estrogen versus testosterone on circulating osteoprotegerin and other cytokine levels in normal elderly men. J Clin Endocrinol Metab 87:1550–1554
107. **Khosla S, Atkinson EJ, Dunstan CR, O'Fallon WM, Riggs BL** 2000 Estrogen and testosterone have opposite effects on circulating OPG levels following induction of hypogonadism and aromatase inhibition in normal elderly men: potential mechanism for differential effects of estrogen versus testosterone on bone resorption. J Bone Miner Res 16:S146
108. **Mitani M, Miura Y, Saura R, Kitagawa A, Fukuyama T, Hashiramoto A, Shiozawa S, Kurosaka M, Yoshiya S** 2005 Estrogen specifically stimulates expression and production of osteoprotegerin from rheumatoid synovial fibroblasts. Int J Mol Med 15:827–832
109. **Lubberts E, Joosten LA, Chabaud M, van den Bersselaar L, Oppers B, Coenen-de Roo C, Richards CD, Miossec P, van den Berg WB** 2000 IL-4 gene therapy for collagen arthritis suppresses synovial IL-17 and osteoprotegerin ligand and prevents bone erosion. J Clin Invest 105:1697–1710
110. **Pettit AR, Ji H, von Stechow D, Muller R, Goldring SR, Choi Y, Benoist C, Gravellese EM** 2001 TRANCE/RANKL knockout mice

are protected from bone erosion in a serum transfer model of arthritis. Am J Pathol 159:1689–1699
111. **Li P, Schwarz EM, O'Keefe RJ, Ma L, Boyce BF, Xing L** 2004 RANK signaling is not required for TNFα-mediated increase in CD11bhi osteoclast precursors but is essential for mature osteoclast formation in TNF α-mediated inflammatory arthritis. J Bone Miner Res 19:207–213
112. **Azuma Y, Kaji K, Katogi R, Takeshita S, Kudo A** 2000 Tumor necrosis factor-α induces differentiation of and bone resorption by osteoclasts. J Biol Chem 275:4858–4864
113. **Fuller K, Murphy C, Kirstein B, Fox SW, Chambers TJ** 2002 TNFα potently activates osteoclasts, through a direct action independent of and strongly synergistic with RANKL. Endocrinology 143:1108–1118
114. **Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Morinaga T, Higashio K, Martin TJ, Suda T** 2000 Tumor necrosis factor α stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. J Exp Med 191:275–285
115. **Li J, Morony S, Tan H, Qiu W, Warmington K, Porkess V, Dunstan CR, Kostenuik PJ, Lacey DL, Boyle W** 2001 Combination of TNF-α and IL-1β induced osteoclast formation and bone resorption is dependent on RANK signal transduction. J Bone Miner Res 16:S379
116. **Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL** 2000 TNF-α induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. J Clin Invest 106:1481–1488
117. **Bendre MS, Montague DC, Peery T, Akel NS, Gaddy D, Suva LJ** 2003 Interleukin-8 stimulation of osteoclastogenesis and bone resorption is a mechanism for the increased osteolysis of metastatic bone disease. Bone 33:28–37
118. **Kudo O, Sabokbar A, Pocock A, Itonaga I, Fujikawa Y, Athanasou NA** 2003 Interleukin-6 and interleukin-11 support human osteoclast formation by a RANKL-independent mechanism. Bone 32:1–7
119. **Glass DA, Bialek P, Ahn JD, Starbuck M, Patel MS, Clevers H, Taketo MM, Long F, McMahon AP, Lang RA, Karsenty G** 2005 Canonical wnt signaling in differentiated osteoblasts controls osteoclast differentiation. Dev Cell 8:751–764
120. **Holmen SL, Zylstra CR, Mukherjee A, Sigler RE, Faugere MC, Bouxsein ML, Deng L, Clemens TL, Williams BO** 2005 Essential role of β-catenin in postnatal bone acquisition. J Biol Chem 280:21162–21168
121. **Aguirre JI, Plotkin LI, Stewart SA, Weinstein RS, Parfitt AM, Manolagas SC, Bellido T** 2006 Osteocyte apoptosis is induced by weightlessness in mice and precedes osteoclast recruitment and bone loss. J Bone Miner Res 21:605–615
122. **Ikeda T, Utsuyama M, Hirokawa K** 2001 Expression profiles of receptor activator of nuclear factor κB ligand, receptor activator of nuclear factor κB, and osteoprotegerin messenger mRNA in aged ovariectomized rat bones. J Bone Miner Res 16:1416–1425
123. **Tatsumi S, Ishii K, Amizuka N, Li M, Kobayashi T, Kohno K, Ito M, Takeshita S, Ikeda K** 2007 Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. Cell Metab 5:464–475
124. **Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL, Boyle WJ, Simonet WS** 1998 Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 12:1260–1268
125. **Mizuno A, Amizuka N, Irie K, Murakami A, Fujise N, Kanno T, Sato Y, Nakagawa N, Yasuda H, Mochizuki SI, Gomibuchi T, Yano K, Shima N, Washida N, Tsuda E, Morinaga T, Higashio K, Ozawa H** 1998 Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. Biochem Biophys Res Comm 247:610–615
126. **Nakamura M, Udagawa N, Matsuura S, Mogi M, Nakamura H, Horiuchi H, Saito N, Hiraoka BY, Kobayashi Y, Takaoka K, Ozawa H, Miyazawa H, Takahashi N** 2003 Osteoprotegerin regulates bone formation through a coupling mechanism with bone resorption. Endocrinology 144:5441–5449
127. **Min H, Morony S, Sarosi I, Dunstan CR, Capparelli C, Scully S, Van G, Kaufman S, Kostenuik PJ, Lacey DL, Boyle WJ, Simonet WS** 2000 Osteoprotegerin reverses osteoporosis by inhibiting endosteal osteoclasts and prevents vascular calcification by blocking a process resembling osteoclastogenesis. J Exp Med 192:463–474
128. **Salmon P** 2004 Loss of chaotic trabecular structure in OPG-deficient juvenile Paget's disease patients indicates a chaogenic role for OPG in nonlinear pattern formation of trabecular bone. J Bone Miner Res 19:695–702
129. **Sato M, Grasser WA** 1990 Effects of visphosphonates on isolated rat osteoclasts as examined by reflected light microscopy. J Bone Miner Res 5:31–40
130. **Marshall MJ, Wilson AS, Davie MW** 1990 Effects of (3-amino-1-hydroxypropylidene)-1,1-bisphosphonate on mouse osteoclasts. J Bone Miner Res 5:955–962
131. **Evans RA, Howlett CR, Dunstan CR, Hills E** 1982 The effect of long-term low-dose diphosphonate treatment on rat bone. Clin Orthop 165:290–299
132. **Evans RA, Baylink DJ, Wergedal JE** 1979 The effects of two diphosphonates on bone metabolism in the rat. Metab Bone Dis Relat Res 2:39–48
133. **Marshall MJ, Holt I, Davie MW** 1993 Osteoclast recruitment in mice is stimulated by (3-amino-1-hydroxypropylidene)-1,1-bisphosphonate. Calcif Tissue Int 52:21–25
134. **Balena R, Toolan BC, Shea M, Markatos A, Myers ER, Lee SC, Opas EE, Seedor JG, Klein H, Frankenfield D, Quartuccio H, Fioravanti C, Clair J, Brown E, Hayes WC, Rodan GA** 1993 The effects of 2-year treatment with the aminobisphosphonate alendronate on bone metabolism, bone histomorphometry, and bone strength in ovariectomized nonhuman primates. J Clin Invest 92:2577–2586
135. **Herrak P, Gortz B, Hayer S, Redlich K, Reiter E, Gasser J, Bergmeister H, Kollias G, Smolen JS, Schett G** 2004 Zoledronic acid protects against local and systemic bone loss in tumor necrosis factor-mediated arthritis. Arthritis Rheum 50:2327–2337
136. **Redlich K, Hayer S, Maier A, Dunstan CR, Tohidast-Akrad M, Lang S, Turk B, Pietschmann P, Woloszczuk W, Haralambous S, Kollias G, Steiner G, Smolen JS, Schett G** 2002 Tumor necrosis factor α-mediated joint destruction is inhibited by targeting osteoclasts with osteoprotegerin. Arthritis Rheum 46:785–792
137. **Morony S, Warmington K, Adamu S, Asuncion F, Geng Z, Grisanti M, Tan HL, Capparelli C, Starnes C, Weimann B, Dunstan CR, Kostenuik PJ** 2005 The inhibition of RANKL causes greater suppression of bone resorption and hypercalcemia compared with bisphosphonates in two models of humoral hypercalcemia of malignancy. Endocrinology 146:3235–3243
138. **Yang Li C, Majeska RJ, Laudier DM, Mann R, Schaffler MB** 2005 High-dose risedronate treatment partially preserves cancellous bone mass and microarchitecture during long-term disuse. Bone 37:287–295
139. **Flanagan AM, Chambers TJ** 1991 Inhibition of bone resorption by bisphosphonates: interactions between bisphosphonates, osteoclasts, and bone. Calcif Tissue Int 49:407–415
140. **Sato M, Grasser W, Endo N, Akins R, Simmons H, Thompson DD, Golub E, Rodan GA** 1991 Bisphosphonate action: alendronate localization in rat bone and effects on osteoclast ultrastructure. J Clin Invest 88:2095–2105
141. **Muhlbauer RC, Bauss F, Schenk RK, Janner M, Bosies E, Strein K, Fleisch HA** 1991 BM 21.0955, a potent new bisphosphonate to inhibit bone resorption. J Bone Miner Res 6:1003–1011
142. **Halasy-Nagy JM, Rodan GA, Reszka AA** 2001 Inhibition of bone resorption by alendronate and risedronate does not require osteoclast apoptosis. Bone 29:553–559
143. **Bennett BJ, Scatena M, Kirk EA, Rattazzi M, Varon RM, Averill M, Schwartz SM, Giachelli CM, Rosenfeld ME** 2006 Osteoprotegerin inactivation accelerates advanced atherosclerotic lesion progression and calcification in older $ApoE^{-/-}$ mice. Arterioscler Thromb Vasc Biol 26:2117–2124
144. **Price PA, June HH, Buckley JR, Williamson MK** 2001 Osteoprotegerin inhibits artery calcification induced by warfarin and by vitamin D. Arterioscler Thromb Vasc Biol 21:1610–1616
145. **Price PA, Faus SA, Williamson MK** 2001 Bisphosphonates alendronate and ibandronate inhibit artery calcification at doses com-

parable to those that inhibit bone resorption. Arterioscler Thromb Vasc Biol 21:817–824
146. **Pritzker LB, Scatena M, Giachelli CM** 2004 The role of osteoprotegerin and tumor necrosis factor-related apoptosis-inducing ligand in human microvascular endothelial cell survival. Mol Biol Cell 15:2834–2841
147. **Wood J, Bonjean K, Ruetz S, Bellahcene A, Devy L, Foidart JM, Castronovo V, Green JR** 2002 Novel antiangiogenic effects of the bisphosphonate compound zoledronic acid. J Pharm Exp Ther 302: 1055–1061
148. **Bezzi M, Hasmim M, Bieler G, Dormond O, Ruegg C** 2003 Zoledronate sensitizes endothelial cells to tumor necrosis factor-induced programmed cell death: evidence for the suppression of sustained activation of focal adhesion kinase and protein kinase B/Akt. J Biol Chem 278:43603–43614
149. **Giraudo E, Inouse M, Hanahan D** 2004 An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. J Clin Invest 114:623–633
150. **Schoppet M, Preissner KT, Hofbauer LC** 2002 RANK ligand and osteoprotegerin: paracrine regulators of bone metabolism and vascular function. Arterioscler Thromb Vasc Biol 22:549–553
151. **Ziegler S, Kudlacek S, Luger A, Minar E** 2005 Osteoprotegerin plasma concentrations correlate with severity of peripheral artery disease. Atherosclerosis 182:175–180
152. **Schulz E, Arfai K, Liu X, Sayre J, Gilsanz V** 2004 Aortic calcification and the risk of osteoporosis and fractures. J Clin Endorinol Metab 89:4246–4253
153. **Schoppet M, Al-Fakhri N, Franke FE, Katz N, Barth PJ, Maisch B, Preissner KT, Hofbauer LC** 2004 Localization of osteoprotegerin, tumor necrosis factor-related apoptosis-inducing ligand, and receptor activator of nuclear factor-κB ligand in Monckeberg's sclerosis and atherosclerosis. J Clin Endocrinol Metab 89:4104–4112
154. **Browner WS, Lui LY, Cummings SR** 2001 Associations of serum osteoprotegerin levels with diabetes, stroke, bone density, fractures, and mortality in elderly women. J Clin Endocrinol Metab 86:631–637
155. **Jono S, Ikari Y, Shioi A, Mori K, Miki T, Hara K, Nishizawa Y** 2002 Serum osteoprotegerin levels are associated with the presence and severity of coronary artery disease. Circulation 106:1192–1194
156. **Schoppet M, Sattler AM, Shaefer JR, Herzum M, Maisch B, Hofbauer LC** 2003 Increased osteoprotegerin serum levels in men with coronary artery disease. J Clin Endocrinol Metab 88:1024–1028
157. **Kiechl S, Schett G, Wenning G, Redlich K, Oberhoolenser M, Mayr A, Santer P, Smolen J, Poewe W, Willeit J** 2004 Osteoprotegerin is a risk factor for progressive atherosclerosis and cardiovascular disease. Circulation 109:2175–2180
158. **Erdogan B, Aslan E, Bagis T, Gokcel A, Erkanli S, Bavbek M, Altinors N** 2004 Intima-media thickness of the carotid arteries is related to serum osteoprotegerin levels in healthy postmenopausal women. Neurol Res 2004:658–661
159. **Nitta K, Akiba T, Uchida K, Otsubo S, Takei T, Yumura W, Kabaya T, Nihei H** 2004 Serum osteoprotegerin levels and the extent of vascular calcification in haemodialysis patients. Nephrol Dial Transplant 19:1886–1889
160. **Ueland T, Yndestad A, Oie E, Florholmen G, Halvorsen B, Froland SS, Simonsen S, Christensen G, Gullestad L, Aukrust P** 2005 Dysregulated osteoprotegerin/RANK ligand/RANK axis in clinical and experimental heart failure. Circulation 111:2461–2468
161. **Hofbauer LC, Schoppet M** 2004 Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular disease. JAMA 292:490–495
162. **Sattler AM, Schoppet M, Schaefer JR, Hofbauer LC** 2004 Novel aspects on RANK ligand and osteoprotegerin in osteoporosis and vascular disease. Calcif Tissue Int 74:103–106
163. **Collin-Osdoby P** 2004 Regulation of vascular calcification by osteoclast regulatory factors RANKL and osteoprotegerin. Circ Res 95:1046–1057
164. **Crisafulli A, Altavilla D, Marini H, Bitto A, Cucinotta D, Frisina N, Corrado F, D'Anna R, Squadrito G, Adamo EB, Marini R, Romeo A, Cancellieri F, Buemi M, Squadrito F** 2005 Effects of the phytoestrogen genistein on cardiovascular risk factors in postmenopausal women. Menopause 12:186–192
165. **Anand DV, Lahiri A, Lim E, Hopkins D, Corder R** 2006 The relationship between plasma osteoprotegerin levels and coronary artery calcification in uncomplicated type 2 diabetic subjects. J Am Coll Cardiol 47:1850–1857
166. **Morony S, Tintut Y, Zhang Z, Cattley R, Dwyer D, Stolina M, Kostenuik PJ, Demer LL** 2008 Osteoprotegrin (OPG) inhibits vascular calcification without affecting atherosclerosis in ldlr$^{(-/-)}$ mice. Circulation 117:411–420
167. **Shao JS, Cheng SL, Charlton-Kachigian N, Loewy AP, Towler DA** 2004 Teriparatide (human parathyroid hormone (1-34)) inhibits osteogenic vascular calcification in diabetic low density lipoprotein receptor-deficient mice. J Biol Chem 278:50195–50202
168. **Theill LE, Boyle WJ, Penninger JM** 2002 RANK-L and RANK: T cells bone loss, and mammalian evolution. Ann Rev Immunol 20:795–823
169. **Stolina M, Dwyer D, Ominsky MS, Corbin T, Zack DJ, Van G, Bolon B, McCabe J, Kostenuik PJ** 2006 Rats overexpressing soluble OPG from a prenatal stage have high bone mass but no alterations in the development of lymphoid organs or innate immune response. J Bone Miner Res 21:S153
170. **Yun TJ, Tallquist MD, Aicher A, Rafferty KL, Marshall AJ, Moon JJ, Ewings MK, Mohaupt M, Herring SW, Clark EA** 2001 Osteoprotegerin, a crucial regulator of bone metabolism, also regulates B cell development and function. J Immunol 166:1482–1491
171. **Bachmann MF, Wong B, Josien R, Steinman RM, Oxenius A, Choi Y** 1999 TRANCE, a tumor necrosis factor family member critical for CD40 ligand-independent T helper cell activation. J Exp Med 189: 1025–1031
172. **Padigel UM, Kim N, Choi Y, Farrell JP** 2003 TRANCE-RANK costimulation is required for IL-12 production and the initiation of a Th1-type response to *Leishmania major* infection in CD40L-deficient mice. J Immunol 171:5437–5441
173. **Miller RE, Branstetter D, Jones J, Cowan L, Dougall WC** 2005 The RANKL inhibitor RANK-Fc has potent antiresorptive efficacy but does not affect host immune responses to influenza infection. J Bone Miner Res 20:S293
174. **Stolina M, Adamu S, Dwyer D, Middleton S, Asuncion F, Ominsky MS, Schett G, Feige U, Kostenuik PJ, Zack DJ** 2005 RANKL inhibition with osteoprotegerin (OPG) prevents bone loss but does not affect immune status in arthritic rats. J Bone Miner Res 20:S257
175. **Stolina M, Guo J, Faggioni R, Brown H, Senaldi G** 2003 Regulatory effects of osteoprotegerin on cellular and humoral immune responses. Clin Immunol 109:347–354
176. **Stolina M, Dwyer D, Morony S, Corbin TJ, McCabe J, Zack D, Kostenuik PJ** 2005 Rats and mice overexpressing soluble OPG have high bone mass but no alteration in immunological parameters or lymphocyte function. Arthritis Rheum 20:S708
177. **Campagnuolo G, Bolon B, Feige U** 2002 Kinetics of bone protection by recombinant osteoprotegerin therapy in Lewis rats with adjuvant arthritis. Arthritis Rheum 46:1926–1936
178. **Byrne FR, Morony S, Warmington K, Geng Z, Brown HL, Flores SA, Fiorino M, Yin SL, Hill D, Porkess V, Duryea D, Pretorius JK, Adamu S, Manuokian R, Danilenko D, Sarosi I, Lacey DL, Kostenuik PJ, Senaldi G** 2005 CD4+CD45RB$^{Hi}$ T cell transfer induced colitis in mice is accompanied by osteopenia which is treatable with recombinant human osteoprotegerin. Gut 54:78–86
179. **Zwerina J, Hayer S, Tohidast-Akrad M, Bermeister H, Redlich K, Feige U, Dunstan C, Kollias G, Steiner G, Smolen J, Schett G** 2004 Single and combined inhibition of tumor necrosis factor, interleukin-1, and RANKL pathways in tumor necrosis factor-induced arthritis. Arthritis Rheum 50:277–290
180. **Schett G, Redlich K, Hayer S, Zwerina J, Bolon B, Dunstan CR, Gortz B, Schulz A, Bergmeister H, Kollias G, Steiner G, Smolen JS** 2003 Osteoprotegerin protects against generalized bone loss in tumor necrosis factor-transgenic mice. Arthritis Rheum 48:2042–2051
181. **Romas E, Sims NA, Hards D, Lindsay M, Quinn J, Ryan P, Dunstan CR, Martin TJ, Gillespie MT** 2002 Osteoprotegerin reduces osteoclast numbers and prevents bone erosions in collagen-induced arthritis. Am J Pathol 161:1419–1427
182. **Stolina M, Dwyer D, Ominsky MS, Corbin T, Van G, Bolon B, Sarosi I, McCabe J, Zack D, Kostenuik PJ** 2007 Continuous RANKL inhibition in OPG transgenic mice and rats suppresses

bone resorption without impairing lymphorganogenesis or functional immune responses. J Immunol 179:7497–7505
183. **Parfitt AM** 2002 Targeted and nontargeted bone remodeling: relationship to basic multicellular unit origination and progression. Bone 30:5–7
184. **Erben RG** 1996 Trabecular and endocortical bone surfaces in the rat: modeling or remodeling? Anat Rec 246:39–46
185. **Ross AB, Bateman TA, Kostenuik PJ, Ferguson VL, Lacey DL, Dunstan CR, Simske SJ** 2001 The effects of osteoprotegerin on the mechanical properties of rat bone. J Mater Sci Mater Med 12:583–588
186. **Bateman TA, Dunstan CR, Ferguson VL, Lacey DL, Ayers RA, Simske SJ** 2000 Osteoprotegerin mitigates tail suspension-induced osteopenia. Bone 26:443–449
187. **Kim HK, Morgan-Bagley S, Kostenuik PJ** 2006 RANKL inhibition: a novel strategy to decrease femoral head deformity after ischemic necrosis. J Bone Miner Res 21:1946–1954
188. **Ominsky MS, Corbin TJ, Li X, Morony S, Warmington KS, Geng Z, Grisanti MS, Tan HL, Shen V, Bolon B, McCabe J, Kostenuik PJ** 2005 RANKL inhibition increases extrinsic bone strength and maintains intrinsic bone strength in transgenic rats that continously overexpress a secreted osteoprotegerin transgene. J Bone Miner Res 20:S294
189. **Ominsky MS, Asuncion F, Barrero M, Niu Q, Corbin TJ, Dwyer D, Warmington KS, Grisanti M, Tan H, Li X, Stolina M, Kostenuik PJ** 2007 Transgenic overexpression of OPG results in increased bone mass and strength and decreased bone turnover in one-year-old female rats. J Bone Miner Res 22:S25
190. **Turner CH** 2002 Biomechanics of bone: determinants of skeletal fragility and bone quality. Osteoporos Int 13:97–104
191. **Nonaka K, Fukuda S, Aoki K, Yoshida T, Ohya K** 2006 Regional distinctions in cortical bone mineral density measured by pQCT can predict alterations in material property at the tibial diaphysis of the cynomolgus monkey. Bone 38:265–272
192. **Augat P, Reeb H, Claes LE** 1996 Prediction of fracture load at different skeletal sites by geometric properties of the cortical shell. J Bone Miner Res 11:1356–1363
193. **van der Linden JC, Homminga J, Verhaar JA, Weinans H** 2001 Mechanical consequences of bone loss in cancellous bone. J Bone Miner Res 16:457–465
194. **Dalle Carbonare L, Arlot ME, Chavassieux PM, Roux JP, Portrero NR, Meunier PJ** 2001 Comparison of trabecular bone microarchitecture and remodeling in glucocorticoid-induced and postmenopausal osteoporosis. J Bone Miner Res 16:97–103
195. **Ominsky MS, Li X, Grisanti M, Warmington KS, Tan HL, Kostenuik PJ** 2005 RANKL inhibition improves trabecular architecture in female mice. J Bone Miner Res 20:S294
196. **Atkinson J, Cranmer P, Saunders T, Niehaus M, Smith SY, Varela A, Ominsky MS, Cosenza ME, Kostenuik PJ** 2005 AMG 162, a fully human RANKL antibody, increases bone mass and bone strength in cynomolgus monkeys. J Bone Miner Res 20:S294
197. **Saika M, Inoue D, Kido S, Matsumoto T** 2001 17$\beta$-Estradiol stimulates expression of osteoprotegerin by a mouse stromal cell line, ST-2, via estrogen receptor-$\alpha$. Endocrinology 142:2205–2212
198. **Yoneda T, Ishimaru N, Arakaki R, Kobayashi M, Izawa T, Moriyama K, Hayashi Y** 2004 Estrogen deficiency accelerates murine autoimmune arthritis asociated with receptor activator of nuclear factor-$\kappa$B ligand-mediated osteoclastogenesis. Endocrinology 145:2384–2391
199. **Miyazaki T, Matsunaga T, Miyazaki S, Hokari S, Komada T** 2004 Changes in receptor activator of nuclear factor-$\kappa$B, and its ligand, osteoprotegerin, bone-type alkaline phosphatase, and tartrate-resistant acid phosphatase in ovariectomized rats. J Cell Biochem 93:503–512
200. **Li X, Asuncion F, Ominsky MS, Adamu S, Dwyer D, Stolina M, Tan HL, Grisanti M, Warmington KS, Kostenuik PJ** 2006 RANKL inhibition with OPG preserves bone density and trabecular architecture in ovariectomized rats. J Bone Miner Res 21:S304
201. **Wang FS, Ko JY, Lin CL, Wu HL, Ke HJ, Tai PJ** 2007 Knocking down dickkopf-1 alleviates estrogen deficiency induction of bone loss. A histomorphological study in ovariectomized rats. Bone 40:485–492
202. **Kostenuik PJ, Capparelli C, Morony S, Adamu S, Shimamoto G, Shen V, Lacey DL, Dunstan CR** 2001 OPG and PTH-(1-34) have additive effects on bone density and mechanical strength in osteopenic ovariectomized rats. Endocrinology 142:4295–4305
203. **Samadfam R, Xia Q, Goltzman D** 2007 Co-treatment of PTH with osteoprotegerin or alendronate increases its anabolic effect on the skeleton of oophorectomized mice. J Bone Miner Res 22:55–63
204. **Bolon B, Carter CR, Daris M, Morony S, Capparelli C, Hsieh A, Mao M, Kostenuik PJ, Dunstan CR, Lacey DL, Sheng JZ** 2001 Adenoviral delivery of recombinant osteoprotegerin (OPG) ameliorates bone resorption in a mouse ovariectomy model of osteoporosis. Mol Ther 3:179–205
205. **Kostenuik PJ, Bolon B, Morony S, Daris M, Geng Z, Carter C, Sheng J** 2004 Gene therapy with human recombinant osteoprotegerin reverses established osteopenia in ovariectomized mice. Bone 34:656–664
206. **Cheng X, Kinosaki M, Takami M, Choi Y, Zhang H, Murali R** 2004 Disabling of receptor activator of nuclear factor-$\kappa$B (RANK) receptor complex by novel osteoprotegerin-like peptidomimetics restores bone loss in vivo. J Biol Chem 279:8269–8277
207. **Spohn G, Schwarz K, Maurer P, Illges H, Rajasekaran N, Choi Y, Jennings GT, Bachmann MF** 2005 Protection against osteoporosis by active immunization with TRANCE/RANKL displayed on virus-like particles. J Immunol 175:6211–6218
208. **Ominsky MS, Schroeder J, Smith SY, Farrell JP, Kostenuik PJ, Atkinson JE** 2006 Denosumab (AMG 162, a fully human RANKL antibody) increases cortical and cancellous bone mass in aged ovariectomized cynomolgus monkeys. J Bone Miner Res 21:S72
209. **Ominsky MS, Schroeder J, Smith SY, Farrell DJ, Atkinson JE, Kostenuik PJ** 2007 Denosumab (AMG 162), a fully human RANKL antibody, improves cortical and cancellous bone mass and bone strength in ovariectomized cynomolgus monkeys. J Bone Miner Res 22:S23
210. **Gunness M, Orwoll E** 1995 Early induction of alterations in cancellous and cortical bone histology after orchiectomy in mature rats. J Bone Miner Res 10:1735–1744
211. **Turner RT, Wakley GK, Hannon KS** 1990 Differential effects of androgens on cortical bone histomorphometry in gonadectomized male and female rats. J Orthop Res 8:612–617
212. **Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC** 1997 The effects of androgen deficiency on murine bone remodeling and bone mineral density are mediated via cells of the osteoblastic lineage. Endocrinology 138:4013–4021
213. **Vandenput L, Boonen S, van Herck E, Swinnen JV, Bouillon R, Vanderschueren D** 2002 Evidence from the aged orchidectomized male rat model that 17$\beta$-estradiol is a more effective bone-sparing and anabolic agent than 5$\alpha$-dihydroxytestosterone. J Bone Miner Res 17:2080–2086
214. **Hijazi RA, Cunningham GR** 2005 Andropause: is androgen replacement therapy indicated for the aging male? Annu Rev Med 56:117–137
215. **Huber DM, Bendixen AC, Pathrose P, Srivastava S, Dienger KM, Shevde NK, Pike JW** 2001 Androgens suppress osteoclast formation induced by RANKL and macrophage colony-stimulating factor. Endocrinology 142:3800–3808
216. **Pederson L, Kremer M, Judd J, Pascoe D, Spelsberg TC, Riggs BL, Oursler MJ** 1999 Androgens regulate bone resorption activity of isolated osteoclasts in vitro. Proc Natl Acad Sci USA 96:505–510
217. **Chen Q, Kaji H, Sugimoto T, Chihara K** 2001 Testosterone inhibits osteoclast formation stimulated by parathyroid hormone through androgen receptor. FEBS Lett 491:91–93
218. **Chen Q, Kaji H, Kanatani M, Sugimoto T, Chihara K** 2004 Testosterone increases osteoprotegerin mRNA expression in mouse osteoblast cells. Horm Metab Res 36:674–678
219. **Hofbauer LC, Hicok KC, Chen D, Khosla S** 2002 Regulation of osteoprotegerin production by androgens and anti-androgens in human osteoblastic lineage cells. Eur J Endocrinol 147:269–273
220. **Franklin M, Bu SY, Lerner MR, Lancaster EA, Bellmer D, Marlow D, Lightfoot SA, Arjmandi BH, Brackett DJ, Lucas EA, Smith BJ** 2006 Dried plum prevents bone loss in a male osteoporosis model via IGF-I and the RANK pathway. Bone 39:1331–1342
221. **Li X, Ominsky MS, Asuncion F, Grisanti M, Tan HL, Warmington KS, Kostenuik PJ** 2006 RANKL inhibition by OPG prevents bone

loss and preserves trabecular architecture in orchiectomized rats. Calcif Tissue Int 78:S144–S145
222. **Li X, Geng Z, Kaufman S, Tan HL, Morony S, Grisanti M, Warmington KS, Ominsky MS, Kostenuik PJ** 2005 RANKL inhibition improves cortical bone geometry by stimulating periosteal bone formation in orchiectomized rats. J Bone Miner Res 20:S57
223. **Goldring SR, Gravellese EM** 2002 Pathogenesis of bone lesions in rheumatoid arthritis. Curr Rheumatol Rep 4:226–231
224. **Gough AK, Lilley J, Eyre S, Holder RL, Emery P** 1994 Generalised bone loss in patients with early rheumatoid arthritis. Lancet 344: 23–27
225. **Romas E, Bakharevski O, Hards DK, Karsogiannis V, Quinn J, Ryan P, Martin TJ, Gillespie MT** 2000 Expression of osteoclast differentiation factor at sites of bone erosion in collagen-induced arthritis. Arthritis Rheum 43:821–826
226. **Shimizu S, Shiozawa S, Shiozawa K, Imura S, Fujita T** 1985 Quantitative histologic studies on the pathogenesis of periarticular osteoporosis in rheumatoid arthritis. Arthritis Rheum 28:25–31
227. **Kapur RP, Yao Z, Iida M, Clarke CM, Doggett B, Xing L, Boyce BF** 2004 Malignant autosomal recessive osteopetrosis caused by spontaneous mutation of murine RANK. J Bone Miner Res 19: 1689–1697
228. **Bendele A, McComb J, Gould T, McAbee T, Sennello G, Chlipala E, Guy M** 1999 Animal models of arthritis: relevance to human disease. Toxicol Pathol 27:134–142
229. **Mori H, Kitazawa R, Mizuki S, Nose M, Maeda S, Kitazawa S** 2002 RANK ligand, RANK, and OPG expression in type II collagen-induced arthritis mouse. Histochem Cell Biol 117:283–292
230. **McClung MR, Lewiecki EM, Cohen SB, Bolognese MA, Woodson GC, Moffett AH, Peacock M, Miller PD, Lederman SN, Chesnut CH, Lain D, Kivitz AJ, Holloway DL, Zhang C, Peterson MC, Bekker PJ** 2006 Denosumab in postmenopausal women with low bone mineral density. N Engl J Med 354:821–831
231. **Stolina M, Dwyer D, Middleton S, Adamu S, Brown H, Asuncion F, Kostenuik PJ** 2006 Progression of inflammatory arthritis is associated with decreased OPG protein, increased RANKL protein and a greater RANKL:OPG ratio. Ann Rheum Dis 65:139
232. **Bolon B, Campagnuolo G, Feige U** 2002 Duration of bone protection by a single osteoprotegerin injection in rats with adjuvant-induced arthritis. Cell Mol Life Sci 59:1569–1576
233. **Schett G, Middleton S, Bolon B, Stolina M, Brown H, Zhu L, Pretorius J, Zack DJ, Kostenuik PJ, Feige U** 2005 Additive bone-protective effects of anabolic treatment when used in conjuction with RANKL and tumor necrosis factor inhibition in two rat arthritis models. Arthritis Rheum 52:1604–1611
234. **Redlich K, Gortz B, Hayer S, Zwerina J, Doerr N, Kostenuik PJ, Bergmeister H, Kollias G, Steiner G, Smolen JS, Schett G** 2004 Repair of local bone erosions and reversal of systemic bone loss upon therapy with anti-tumor necrosis factor in combination with osteoprotegerin or parathyroid hormone in tumor necrosis factor-mediated arthritis. Am J Pathol 164:543–555
235. **Neumann T, Oelzner P, Petrow PK, Thoss K, Hein G, Stein G, Brauer R** 2006 Osteoprotegerin reduces the loss of periarticular bone mass in primary and secondary spongiosa but does not influence inflammation in rat antigen-induced arthritis. Inflamm Res 55:32–39
236. **Saidneberg-Kermanac'h N, Corrado A, Lemeiter D, deVernejoul MC, Boissier MC, Cohen-Solal ME** 2004 TNF-$\alpha$ antibodies and osteoprotegerin decrease systemic bone loss associated with inflammation through distinct mechanisms in collagen-induced arthritis. Bone 35:1200–1207
237. **Abramson SB, Amin A** 2002 Blocking the effects of IL-1 in rheumatoid arthritis protects bone and cartilage. Rheumatology 41:972–980
238. **Komuro H, Olee T, Kuhn K, Quach J, Brinson DC, Shikhman A, Valbracht J, Creighton-Achermann L, Lotz M** 2001 The osteoprotegerin/receptor activator of nuclear factor κB/receptor activator of nuclear factor κB ligand system in cartilage. Arthritis Rheum 44:2768–2776
239. **Geusens P, Landewe R, Chen D, Dunstan C, Garnero P, van der Linden S, Boers M** 2002 Receptor activator of the nuclear factor κ-B ligand (RANKL) in serum is related to bone density, cartilage breakdown and inflammation in early active rheumatoid arthritis. J Bone Miner Res 17:S386
240. **Saidenberg-Kermanach'h N, Bessis N, Lemeiter D, De Vernejoul MC, Boissier MC, Cohen-Solal M** 2004 Interleukin-4 cellular gene therapy and osteoprotegerin decrease inflammation-associated bone resorption in collagen-induced arthritis. J Clin Immunol 24: 370–378
241. **Canalis E** 2003 Mechanisms of glucocorticoid-induced osteoporosis. Curr Opin Rheumatol 15:454–457
242. **Patschan D, Loddenkemper K, Buttgereit F** 2001 Molecular mechanisms of glucocorticoid-induced osteoporosis. Bone 29:498–505
243. **Israel E, Banerjee TR, Fitzmaurice GM, Kotlov TV, LaHive K, LeBoff MS** 2001 Effects of inhaled glucocorticoids on bone density in premenopausal women. N Engl J Med 345:941–947
244. **von Tirpitz C, Epp S, Klaus J, Mason R, Hawa G, Brinskelle-Schmal N, Hofbauer LC, Adler G, Kratzer W, Reinshagen M** 2003 Effect of systemic glucocorticoid therapy on bone metabolism and the osteoprotegerin system in patients with active Crohn's disease. Eur J Gastroenterol Hepatol 15:1165–1170
245. **Kanis JA, Johansson H, Oden A, Johnell O, de Laet C, Melton LJ, Tenenhouse A, Reeve J, Silman AJ, Pols H, Eisman JA, McCloskey EV, Mellstrom D** 2004 A meta-analysis of prior corticosteroid use and fracture risk. J Bone Miner Res 19:893–899
246. **McCulloch C, Tenenbaum HC** 1986 Dexamethasone induces proliferation and terminal differentiation of osteogenic cells in tissue culture. Anat Rec 215:397–402
247. **Bellows CG, Aubin JE, Heersche J** 1987 Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvarial cells *in vitro*. Endocrinology 121:1985–1992
248. **Walsh S, Jordan GR, Jefferiss C, Stewart K, Beresford JN** 2001 High concentrations of dexamethasone suppress the proliferation but not the differentiation or further maturation of human osteoblast precursors in vitro: relevance to glucocorticoid-induced osteoporosis. Rheumatology 40:74–83
249. **Ishida Y, Heersche JN** 1998 Glucocorticoid-induced osteoporosis: both *in vivo* and *in vitro* concentrations of glucocorticoids higher than physiological levels attenuate osteoblast differentiation. J Bone Miner Res 13:1822–1826
250. **Lian JB, Shalhoub V, Aslam F, Frenkel B, Green J, Hamrah M, Stein GS, Stein JL** 1997 Species-specific glucocorticoid and 1,25-dihydroxyvitamin D responsiveness in mouse MC3T3–E1 osteoblasts: dexamethasone inhibits osteoblast differentiation and vitamin D down-regulates osteocalcin gene expression. Endocrinology 138:2117–2127
251. **Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC** 1998 Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Invest 102:274–282
252. **Gronowicz GA, McCarthy MB** 1995 Glucocorticoids inhibit the attachment of osteoblasts to bone extracellular matrix proteins and decrease $\beta$1 integrin levels. Endocrinology 136:598–608
253. **McCarthy TL, Centrella M, Canalis E** 1990 Cortisol inhibits the synthesis of insulin-like growth factor-I in skeletal cells. Endocrinology 126:1569–1575
254. **Hofbauer LC, Gori F, Riggs BL, Lacey DL, Dunstan C, Spelsberg TC, Khosla S** 1999 Stimulation of osteoprotegerin ligand and inhibition of osteoprotegerin production by glucocorticoids in human osteoblastic lineage cells: potential paracrine mechanisms of glucocorticoid-induced osteoporosis. Endocrinology 140:4382–4389
255. **Liskova-Kiar M** 1979 Mode of action of cortisol on bone resorption in fetal rat fibulae cultured in vitro. Am J Anat 156:63–76
256. **Defranco DJ, Lian JB, Glowacki J** 1992 Differential effects of glucocorticoid on recruitment and activity of osteoclasts induced by normal and osteocalcin-deficient bone implanted in rats. Endocrinology 131:114–121
257. **Kim HJ, Zhao H, Kitaura H, Bhattacharyya S, Brewer JA, Muglia LJ, Ross FP, Teitelbaum SL** 2006 Glucocorticoids suppress bone formation via the osteoclast. J Clin Invest 116:2152–2160
258. **Vidal N, Brandstrom H, Jonsson KB, Ohlsson C** 1998 Osteoprotegerin mRNA is expressed in primary human osteoblast-like cells: down-regulation by glucocorticoids. J Endocrinol 159:191–195
259. **Humphrey EL, Williams J, Davie MJ, Marshall MJ** 2006 Effects of

dissociated glucocorticoids on OPG and RANKL in osteoblastic cells. Bone 38:652–661
260. **Rubin J, Biskobing DM, Jadhav L, Fan D, Nanes MS, Perkins S, Fan X** 1998 Dexamethasone promotes expression of membrane-bound macrophage colony-stimulating factor in murine osteoblast-like cells. Endocrinology 139:1006–1012
261. **Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K, Miyata T, Anderson DM, Suda T** 1999 Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor κB (RANK) receptors. J Exp Med 190:1741–1754
262. **Morony S, Lu J, Capparelli C, Dunstan CR, Lacey DL, Kostenuik PJ** 2001 Osteoprotegerin (OPG) prevents bone loss in a rat model of glucocorticoid-induced osteopenia. J Bone Miner Res 16:S148
263. **Ortoft G, Bruel A, Andreassen TT, Oxlund H** 1995 Growth hormone is not able to counteract osteopenia of rat cortical bone induced by glucocorticoid with protracted effect. Bone 17:543–548
264. **Ikeda S, Morishita Y, Tsutsumi H, Ito M, Shiraishi A, Arita S, Akahoshi S, Narusawa K, Nakamura T** 2003 Reductions in bone turnover, mineral, and structure associated with mechanical properties of lumbar vertebra and femur in glucocorticoid-treated growing minipigs. Bone 33:779–787
265. **King CS, Weir EC, Gundberg CW, Fox J, Insogna KL** 1996 Effects of continuous glucocorticoid infusion on bone metabolism in the rat. Calcif Tissue Int 59:184–191
266. **Lewiecki EM** 2006 Denosumab: a promising drug for the prevention and treatment of osteoporosis. Women's Health 2:517–525
267. **Sasaki N, Kusano E, Ando Y, Nemoto J, Iimura O, Ito C, Takeda S, Yano K, Tsuda E, Asano Y** 2002 Changes in osteoprotegerin and markers of bone metabolism during glucocorticoid treatment in patients with chronic glomerulonephritis. Bone 30:853–858
268. **Sharif M, Salisbury C, Taylor DJ, Kirwan JR** 1998 Changes in biochemical markers of joint tissue metabolism in a randomized controlled trial of glucocorticoid in early rheumatoid arthritis. Arthritis Rheum 41:1203–1209
269. **Leblanc AD, Schneider VS, Evans HJ, Engelbretson DA, Krebs JM** 1990 Bone mineral loss and recovery after 17 weeks of bed rest. J Bone Miner Res 5:843–850
270. **Modlesky CM, Slade JM, Bickel CS, Meyer RA, Dudley GA** 2005 Deteriorated geometric structure and strength of the midfemur in men with complete spinal cord injury. Bone 36:331–339
271. **Jiang SD, Dai LY, Jian LS** 2006 Osteoporosis after spinal cord injury. Osteoporos Int 17:180–192
272. **Vico L, Alexandre C** 1992 Microgravity and bone adaptation at the tissue level. J Bone Miner Res 7:S445–S447
273. **Lang T, LeBlanc A, Evans H, Lu Y, Genant H, Yu A** 2004 Cortical and trabecular bone mineral loss from the spine and hip in long-duration spaceflight. J Bone Miner Res 19:1006–1012
274. **Vico L, Collet P, Guignandon A, Lafage-Proust MH, Thomas T, Rehailia M, Alexandre C** 2000 Effects of long-term microgravity exposure on cancellous and cortical weight-bearing bones of cosmonauts. Lancet 355:1607–1611
275. **Smith SM, Nillen JL, LeBlanc A, Lipton A, Demers LM, Lane HW, Leach CS** 1998 Collagen cross-link excretion during space flight and bed rest. J Clin Endocrinol Metab 83:3584–3591
276. **Chappard D, Petitjean M, Alexandre C, Vico L, Minaire P, Riffat G** 1991 Cortical osteoclasts are less sensitive to etidronate than trabecular osteoclasts. J Bone Miner Res 6:673–680
277. **Grano M, Mori G, Minielli V, Barou O, Colucci S, Gianelli G, Alexandre C, Zallone A, Vico L** 2002 Rat hindlimb unloading by tail suspension reduces osteoblast differentiation, induces IL-6 secretion, and increases bone resorption in ex vivo cultures. Calcif Tissue Int 70:176–185
278. **Globus RK, Bikle DD, Morey-Holton ER** 1986 The temporal response of bone to unloading. Endocrinology 118:733–742
279. **Ma YF, Jee W, Ke HZ, Lin BY, Liang XG, Li M, Yamamoto N** 1995 Human parathyroid hormone-(1-38) restores cancellous bone to the immobilized, osteopenic proximal tibial metaphysis in rats. J Bone Miner Res 10:496–505
280. **Halloran BP, Bikle DD, Harris J, Tanner S, Curren T, Morey-Holton ER** 1997 Regional responsiveness of the tibia to intermittent administration of parathyroid hormone as affected by skeletal unloading. J Bone Miner Res 12:1068–1074
281. **Kostenuik PJ, Harris J, Halloran BP, Turner RT, Morey-Holton ER, Bikle DD** 1999 Skeletal unloading causes resistance of osteoprogenitor cells to parathyroid hormone and to insulin-like growth factor-I. J Bone Miner Res 14:21–31
282. **Li CY, Price C, Delisser K, Nasser P, Laudier D, Clement M, Jepsen KJ, Schaffler MB** 2005 Long-term disuse osteoporosis seems less sensitive to bisphosphonate treatment than other osteoporosis. J Bone Miner Res 20:117–124
283. **Allen MR, Bloomfield SA** 2003 Hindlimb unloading has a greater effect on cortical compared to cancellous bone in mature female rats. J Appl Physiol 94:642–650
284. **Kodama Y, Nakayama K, Fuse H, Fukumoto S, Kawahara H, Takahashi H, Kurokawa T, Sekiguchi C, Nakamura T, Matsumoto T** 1997 Inhibition of bone resorption by pamidronate cannot restore normal gain in cortical bone mass and strength in tail-suspended rapidly growing rats. J Bone Miner Res 12:1058–1067
285. **Apseloff G, Girten B, Weisbrode SE, Walker M, Stern LS, Krecic ME, Gerber N** 1993 Effects of aminohydroxybutane bisphosphonate on bone growth when administered after hind limb bone loss in tail-suspended rats. J Pharmacol Exp Ther 267:515–521
286. **Mosekilde L, Thomsen JS, Mackey MS, Phipps RJ** 2000 Treatment with risedronate or alendronate prevents hind-limb immobilization-induced loss of bone density and strength in adult female rats. Bone 27:639–645
287. **Rubin J, Murphy TC, Fan X, Goldschmidt M, Taylor WR** 2002 Activation of extracellular signal-regulated kinase is involved in mechanical strain inhibition of RANKL expression in bone stromal cells. J Bone Miner Res 17:1452–1460
288. **Kanematsu M, Yoshimura K, Takaoki M, Sato A** 2002 Vector-averaged gravity regulates gene expression of receptor activator of NF-κB (RANK) ligand and osteoprotegerin in bone marrow stromal cells via cyclic AMP/protein kinase A pathway. Bone 30:553–558
289. **Giangregorio L, Blimkie C** 2002 Skeletal adaptations to alterations in weight-bearing activity: a comparison of models for disuse osteoporosis. Sports Med 32:459–476
290. **Bateman TA, Dunstan CR, Lacey DL, Ferguson VL, Ayers RA, Simske SJ** 2001 Osteoprotegerin ameliorates sciatic nerve crush induced bone loss. J Orthop Res 19:518–523
291. **Simske SJ, Bateman TA, Ayers RA, Dunstan CR, Ferguson VL, Lacey DL** 1999 Effect of osteoprotegerin, pamidronate and ibandronate on the sciatic nerve crush model for disuse osteopenia. J Bone Miner Res 14:S523
292. **Mochizuki S, Fujise N, Higashio K, Tsuda E** 2002 Osteoclastogenesis inhibitory factor/osteoprotegerin ameliorates the decrease in both bone mineral density and bone strength in immobilized rats. J Bone Miner Metab 20:14–20
293. **Bateman TA, Lacey DL, Ferguson VL, Dunstan CR, Ayers RA, Simske SJ** 1999 Comparison of osteoprotegerin, pamidronate and ibandronate in the treatment of suspension disuse osteopenia. J Bone Miner Res 14:S528
294. **Bateman TA, Morony S, Ferguson VL, Simske SJ, Lacey DL, Warmington KS, Geng Z, Tan HL, Shalhoub V, Dunstan CR, Kostenuik PJ** 2002 Osteoprotegerin mitigates spaceflight-induced changes in mouse bone mass, density and mineral composition. J Bone Miner Res 17:S136
295. **Kostenuik PJ, Bateman TA, Morony S, Warmington K, Geng Z, Simske SJ, Ferguson VL, Dunstan CR, Lacey DL** 2002 OPG prevents relative osteopenia and deficits in skeletal strength in mice during a 12 day spaceflight. J Bone Miner Res 17:S209
296. **Warner SE, Sanford DA, Becker BA, Bain SD, Srinivasan S, Gross TS** 2006 Botox induced muscle paralysis rapidly degrades bone. Bone 38:257–264
297. **Warner SA, Srinivasan S, Kostenuik PJ, Gross TS** 2006 RANKL inhibition prevents the loss of bone volume and bone strength caused by Botox induced muscle paralysis. Trans Orthoped Res Soc 31:0189
298. **Ichinose Y, Tanaka H, Inoue M, Mochizuki S, Tsuda E, Seino Y** 2004 Osteoclastogenesis inhibitor factor/osteoprotegerin reduced bone loss induced by mechanical unloading. Calcif Tissue Int 75:338–343
299. **Paget S** 1889 The distribution of secondary growths in cancer of the breast. Lancet 1:571–573

300. **Orr FW, Varani J, Gondek MD, Ward PA, Mundy GR** 1979 Chemotactic responses of tumor cells to products of resorbing bone. Science 203:176–179
301. **Manishen WJ, Sivananthan K, Orr FW** 1986 Resorbing bone stimulates tumor cell growth. Am J Pathol 123:39–45
302. **Kostenuik PJ, Singh G, Suyama KL, Orr FW** 1992 A quantitative model for spontaneous bone metastasis: evidence for a mitogenic effect of bone on Walker 256 cancer cells. Clin Exp Metastasis 10:403–410
303. **Kostenuik PJ, Singh G, Suyama KL, Orr FW** 1992 Stimulation of bone resorption results in a selective increase in the growth rate of spontaneously metastatic Walker 256 cancer cells in bone. Clin Exp Metastasis 10:411–418
304. **Galasko C** 1976 Mechanisms of bone destruction in the development of skeletal metastases. Nature 263:507–509
305. **Berenson JR** 2002 Advances in the biology and treatment of myeloma bone disease. Semin Oncol 29:11–16
306. **Elomaa I, Blomqvist C, Grohn P, Porkka L, Kairento AL, Selander K, Lamberg-Allardt C, Holmstrom T** 1983 Long-term controlled trial with diphosphonate in patients with osteolytic bone metastases. Lancet 1:146–149
307. **Thiebaud D, Leyvraz S, von Fliedner V, Perey L, Cornu P, Thiebaud S, Burckhardt P** 1991 Treatment of bone metastases from breast cancer and myeloma with pamidronate. Eur J Cancer 27: 37–41
308. **Clarke NW, Holbrook IB, McClure J, George N** 1991 Osteoclast inhibition by pamidronate in metastatic prostate cancer: a preliminary study. Br J Cancer 63:420–423
309. **Berenson JR, Rosen LS, Howell A, Porter L, Coleman RE, Morley W, Dreicer R, Kuross SA, Lipton A, Seaman JJ** 2001 Zoledronic acid reduces skeletal-related events in patients with osteolytic metastases. Cancer 2001:1191–1200
310. **Mundy GR, Raisz LG, Cooper RA, Schechter GP, Salmon SE** 1974 Evidence for the secretion of an osteoclast stimulating factor in myeloma. N Engl J Med 291:1041–1046
311. **Blair JM, Zhou H, Seibel MJ, Dunstan CR** 2006 Mechanisms of disease: roles of OPG, RANKL and RANK in the pathophysiology of skeletal metastasis. Nat Clin Pract Oncol 3:41–49
312. **De Leenheer E, Mueller GS, Vanderkerken K, Croucher PI** 2004 Evidence of a role for RANKL in the development of myeloma bone disease. Curr Opin Pharmacol 4:340–346
313. **Dougall WC, Chaisson M** 2006 The RANK/RANKL/OPG triad in cancer-induced bone diseases. Cancer Metast Rev 25:541–549
314. **Zhang J, Dai J, Yao Z, Lu Y, Dougall W, Keller ET** 2003 Soluble receptor activator of nuclear factor κB Fc diminishes prostate cancer progression to bone. Cancer Res 63:7883–7890
315. **Brown JM, Corey E, Lee ZD, True LD, Yun TJ, Tondravi M, Vessella RL** 2001 Osteoprotegerin and RANK ligand expression in prostate cancer. Urology 57:611–616
316. **Zhang J, Dai J, Qi Y, Lin DL, Smith P, Strayhorn C, Mizokami A, Fu Z, Westman J, Keller ET** 2001 Osteoprotegerin inhibits prostate cancer-induced osteoclastogenesis and prevents prostate tumor growth in the bone. J Clin Invest 107:1235–1244
317. **Farrugia AN, Atkins GJ, To LB, Pan B, Horvath N, Kostakis P, Findlay DM, Bardy P, Zannettino A** 2003 Receptor activator of nuclear factor-κB ligand expression by human myeloma cells mediates osteoclast formation in vitro and correlates with bone destruction in vivo. Cancer Res 63:5438–5445
318. **Lai F, Cole-Sinclair M, Cheng WJ, Quinn J, Gillespie MT, Sentry JW, Schneider HG** 2004 Myeloma cells can directly contribute to the pool of RANKL in bone bypassing the classic stromal and osteoblast pathway of osteoclast stimulation. Brit J Haematol 126: 192–201
319. **Roux S, Meignin V, Quillard J, Guiochon-Mantel A, Fermand JP, Milgrom E, Mariette X** 2002 RANK (receptor activator of nuclear factor-κB) and RANKL expression in multiple myeloma. Brit J Haematol 117:86–92
320. **Sezer O, Heider U, Jakob C, Zavrski I, Eucker J, Possinger K, Sers C, Krenn V** 2002 Immunohistochemistry reveals RANKL expression of myeloma cells. Blood 99:4646–4647
321. **Sezer O, Heider U, Jakob C, Euker J, Possinger K** 2002 Human bone marrow myeloma cells express RANKL. J Clin Oncol 20:353–354
322. **Sezer O, Heider U, Zavrski I, Kuhne CA, Hofbauer LC** 2003 RANK ligand and osteoprotegerin in myeloma bone disease. Blood 101:2094–2098
323. **Kitazawa S, Kitazawa R** 2002 RANK ligand is a prerequisite for cancer-associated osteolytic lesions. J Pathol 198:228–236
324. **Chikatsu N, Takeuchi Y, Tamura Y, Fukumoto S, Yano K, Tsuda E, Ogata E, Fujita T** 2000 Interactions between cancer and bone marrow cells induce osteoclast differentiation factor expression and osteoclast-like cell formation in vitro. Biochem Biophys Res Comm 267:632–637
325. **Mancino AT, Klimberg VS, Yamamoto M, Manolagas SC, Abe E** 2001 Breast cancer increases osteoclastogenesis by secreting M-CSF and upregulating RANKL in stromal cells. J Surg Res 100:18–24
326. **Fizazi K, Yang J, Peleg S, Sikes CR, Kreimann EL, Daliani D, Olive M, Raymond KA, Janus TJ, Logothetis CJ, Karsenty G, Navone NM** 2003 Prostate cancer cells-osteoblast interaction shifts expression of growth/survival-related genes in prostate cancer and reduces expression of osteoprotegerin in osteoblasts. Clin Cancer Res 9:2587–2597
327. **Giuliani N, Bataille R, Mancini C, Lazzaretti M, Barille S** 2001 Myeloma cells induce imbalance in the osteoprotegerin/osteoprotegerin ligand system in the human bone marrow microenvironment. Blood 98:3527–3533
328. **Pearse RN, Sordillo EM, Yaccoby S, Wong BR, Liau DF, Colman N, Michaeli J, Epstein J, Choi Y** 2001 Multiple myeloma disrupts the TRANCE/osteoprotegerin cytokine axis to trigger bone destruction and promote tumor progression. Proc Natl Acad Sci USA 98:11581–11586
329. **Okada T, Akikusa S, Okuno H, Kodaka M** 2003 Bone marrow metastatic myeloma cells promote osteoclastogenesis through RANKL on endothelial cells. Clin Exp Metastasis 20:639–646
330. **Shipman CM, Croucher PI** 2003 Osteoprotegerin is a soluble decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand and can function as a pracrine survival factor for human myeloma cells. Cancer Res 63:912–916
331. **Standal T, Seidel C, Hjertner O, Plesner T, Sanderson RD, Waage A, Borset M, Sundan A** 2002 Osteoprotegerin is bound, internalized, and degraded by multiple myeloma cells. Blood 100:3002–3007
332. **Atkins GJ, Bouralexis S, Haynes DR, Graves SE, Geary SM, Evdokiou A, Zannettino A, Hay S, Findlay DM** 2001 Osteoprotegerin inhibits osteoclast formation and bone resorbing activity in giant cell tumors of bone. Bone 28:370–377
333. **Ono K, Akatsu T, Murakami T, Kitamura R, Yamamot M, Shinomiya N, Rokutanda M, Sasaki T, Amizuka N, Ozawa N, Hagata N, Kugai N** 2002 Involvement of cyclo-oxygenase-2 in osteoclast formation and bone destruction in bone metastasis of mammary carcinoma cell lines. J Bone Miner Res 17:774–781
334. **Grano M, Mori G, Minielli V, Cantatore FP, Colucci S, Zambonin Zallone A** 2000 Breast cancer cell line MDA-231 stimulates osteoclastogenesis and bone resorption in human osteoclasts. Biochem Biophys Res Comm 270:1097–1100
335. **Yoneda T, Michigami T, Yi B, Williams PJ, Niewolna M, Hiraga T** 1999 Use of bisphosphonates for the treatment of bone metastasis in experimental animal models. Cancer Treat Rev 25:293–299
336. **Holstead Jones D, Nakashima T, Sanchez OH, Kozieradzki I, Komarava SV, Sarosi I, Morony S, Rubin E, Sarao R, Hojilla CV, Komnenovic V, Kong YY, Schreiber M, Dixon JE, Sims SM, Khoka R, Wada T, Penninger JM** 2006 Regulation of cancer cell migration and bone metastasis by RANKL. Nature 440:692–696
337. **Hwang C, Chung H, Kang Y, Moon I, Yim C, Han K, Jang H, Park W, Yoon H, Han I** 2002 Effects of RANKL on breast cancer cell lines. J Bone Miner Res 17:S310
338. **Morony S, Capparelli C, Sarosi I, Lacey DL, Dunstan CR, Kostenuik PJ** 2001 Osteoprotegerin inhibits osteolysis and decreases skeletal tumor burden in syngeneic and nude mouse models of experimental bone metastasis. Cancer Res 61:4432–4436
339. **Tometsko ME, Roudier M, Canon J, Bryant B, Miller R, Jones J, Armstrong A, Dougall W, Chaisson ML** 2006 RANKL inhibition causes a greater suppression of tumor-induced osteoclastogenesis than zoledronate treatment *in vivo* and RANKL rescues osteoclasts from zoledronate killing *in vitro.* J Bone Miner Res 21:S346
340. **Zheng Y, Zhou H, Brennan K, Blair JM, Modzelewski J, Seibel**

**MJ, Dunstan CR** 2007 Inhibition of bone resorption, rather than direct cytotoxicity, mediates the anti-tumor actions of ibandronate and osteoprotegerin in a murine model of breast cancer bone metastasis. Bone 40:471–478
341. **Morony S, Warmington K, Tan H, Shalhoub V, Chow J, Dunstan CR, Lacey DL, Kostenuik PJ** 2002 OPG inhibits the progression of bone destruction and skeletal tumor burden in mice with established osteolytic MDA-231 breast cancer metastases. J Bone Miner Res 17:S147
342. **Usui T, Tanaka S, Sonoda T, Ozawa Y, Teramura K, Nakamura E, Watanabe T, Hibuchi S** 1997 Drug disposition of incadronate, a new bisphosphonate, in rats with bone metastases. Xenobiotica 27:479–487
343. **Kostenuik PJ** 2004 Revisiting the seed and soil theory of bone metastasis: new tools, same answer. J Musculoskel Neuron Interact 4:375–376
344. **Quinn JE, Brown LG, Zhang J, Keller ET, Vessella RL, Corey E** 2005 Comparison of Fc-osteoprotegerin and zoledronic acid activities suggest that zoledronic acid inhibits prostate cancer in bone by indirect mechanisms. Prostate Cancer Prostatic Dis 8:253–259
345. **Yaccoby S, Pearse RN, Johnson CL, Barlogie B, Choi Y, Epstein J** 2002 Myeloma interacts with the bone marrow microenvironment to induce osteoclastogenesis and is depenendent on osteoclast activity. Brit J Haematol 116:278–290
346. **Clarke NW, McClure J, George N** 1992 Disodium pamidronate identifies differential osteoclastic bone resorption in metastatic prostate cancer. Br J Urol 69:64–70
347. **Keller ET** 2002 The role of osteoclastic activity in prostate cancer skeletal metastases. Drugs Today 38:91–102
348. **Yonou H, Kanomata N, Goya M, Kamijo T, Yokose T, Hasebe T, Nagai K, Hatano T, Ogawa Y, Ochiai A** 2003 Osteoprotegerin/osteoclastogenesis inhibitory factor decreases human prostate cancer burden in human adult bone implanted into nonobese diabetic/severe immunodeficient mice. Cancer Res 63:2096–2102
349. **Mori K, Le GB, Charrier C, Battaglia S, Heymann D, Redini F** 2007 DU145 human prostate cancer cells express functional receptor activator of NFκB: new insights in the prostate cancer bone metastasis process. Bone 40:981–990
350. **Kyle RA, Gertz MA, Witzig TE, Lust JA, Lacy MQ, Dispenzieri A, Fonseca R, Rajkumar SV, Offord JR, Larson DR, Plevak ME, Therneau TM, Greipp PR** 2003 Review of 1027 patients with newly diagnosed multiple myeloma. Mayo Clin Proc 78:21–33
351. **Hjorth-Hansen H, Seifert MF, Borset M, Aarset H, Ostlie A, Sundan A, Waage A** 1999 Marked osteoblastopenia and reduced bone formation in a model of multiple myeloma bone disease in severe combined immunodeficiency mice. J Bone Miner Res 14:256–263
352. **Croucher PI, Shipman CM, Lippitt J, Perry M, Hijzen A, Brabbs AC, van Beek E, Holen I, Skerry TM, Dunstan CR, Russell GR, van Camp B, Vanderkerken K** 2001 Osteoprotegerin inhibits the development of osteolytic bone disease in multiple myeloma. Blood 98:3534–3540
353. **Vanderkerken K, De Leenheer E, Shipman C, Asosingh K, Willems A, van Camp B, Croucher P** 2003 Recombinant osteoprotegerin decreases tumor burden and increases survival in a murine model of multiple myeloma. Cancer Res 63:287–289
354. **Garrett IR, Dallas SL, Bonewald LF, Boyce BF, Dallas M, Radl J, Mundy GR** 1995 Evidence for bidirectional interactions between osteoclasts and myeloma cells. J Bone Miner Res 10:S195
355. **Truneh A, Sharma S, Silverman C, Khandekar S, Reddy MP, Deen KC, McLaughlin MM, Srinivasula SM, Livi GP, Marshall LA, Alnemri ES, Williams WV, Doyle ML** 2000 Temperature-sensitive differential affinity of TRAIL for its receptors: DR5 is the highest affinity receptor. J Biol Chem 275:23319–23325
356. **Lincz LF, Yeh TX, Spencer A** 2001 TRAIL-induced eradication of primary tumour cells from multiple myeloma patient bone marrow is not related to TRAIL receptor expression or prior chemotherapy. Leukemia 15:1650–1657
357. **Naumann U, Wick W, Beschorner R, Meyermann R, Weller M** 2004 Expression and functional activity of osteoprotegerin in human malignant gliomas. Acta Neurpath 107:17–22
358. **Sedger LM, Glaccum MB, Schuh J, Kanaly ST, Williamson E, Kayagaki N, Yun T, Smolak P, Le T, Goodwin R, Gliniak B** 2002 Characterization of the in vivo function of TNF-α-related apoptosis-inducing ligand, TRAIL/Apo2L, using TRAIL/Apo2L gene-deficient mice. Eur J Immunol 32:2246–2254
359. **Doran PM, Turner RT, Chen D, Facteau SM, Ludvigson LM, Khosla S, Riggs BL, Russell SJ** 2004 Native osteoprotegerin gene transfer inhibits the development of murine osteolytic bone disease induced by tumor xenografts. Exp Hematol 32:351–359
360. **Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh J, Lynch DH** 1999 Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med 5:157–163
361. **Elliott R, Kostenuik PJ, Chen C, Kelley M, Hawkins N, Housman J, McCabe J, Mukku V, Sullivan JK, Dougall W** 2006 Denosumab is a selective inhibitor of human receptor activator of NF-κB ligand that blocks osteoclast formation in vitro and in vivo. Eur J Cancer Suppl 4:62
362. **Kon T, Cho TJ, Aizawa T, Yamazaki M, Nooh N, Graves D, Gerstenfeld LC, Einhorn TA** 2001 Expression of osteoprotegerin, receptor activator of NF-κB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. J Bone Miner Res 16:1004–1014
363. **Ulrich-Vinther M, Andreassen TT** 2005 Osteoprotegerin treatment impairs remodeling and apparent material properties of callus tissue without influencing structural fracture strength. Calcif Tissue Int 76:280–286
364. **Cao Y, Mori S, Mashiba T, Westmore MS, Ma L, Sato M, Akiyama T, Shi L, Komatsubara S, Miyamoto K, Norimatsu H** 2002 Raloxifene, estrogen, and alendronate affect the processes of fracture repair differently in ovariectomized rats. J Bone Miner Res 17:2237–2246
365. **Li J, Mori S, Kaji Y, Mashiba T, Kawanishi J, Norimatsu H** 1999 Effect of bisphosphonate (Incadronate) on fracture healing of long bones in rats. J Bone Miner Res 14:969–979
366. **Madsen JE, Berg-Larsen T, Kirkeby OJ, Falch JA, Nordsletten L** 1998 No adverse effects of clodronate on fracture healing in rats. Acta Orthop Scand 69:532–536
367. **Delos D, Yang X, Ricciardi BF, Myers ER, Bostrom M, Camacho NP** 2007 The effects of RANKL inhibition on fracture healing and bone strength in a mouse model of osteogenesis imperfecta. J Orthop Res 25:1–12
368. **Ulrich-Vinther M, Schwarz EM, Pederson FS, Soballe K, Andreassen TT** 2005 Gene therapy with human osteoprotegerin decreases callus remodeling with limited effects on biomechanical properties. Bone 37:751–758
369. **Flick LM, Weaver JM, Ulrich-Vinther M, Abuzzahab F, Zhang X, Dougall WC, Anderson D, O'Keefe RJ, Schwarz EM** 2003 Effects of receptor activator of NFκB (RANK) signaling blockade on fracture healing. J Orthop Res 21:676–684
370. **Tat SK, Pardrines M, Theoleyre S, Couillad-Battaglia S, Heymann D, Redini F, Fortun Y** 2006 OPG/membranous-RANKL complex is internalized via the clathrin pathway before a lysosomal and a proteosomal degradation. Bone 39:706–715
371. **Rogers A, Eastell R** 2005 Circulating osteoprotegerin ligand and receptor activator for nuclear factor κB ligand: clinical utility in metabolic bone disease assessment. J Clin Endorinol Metab 90:6323–6331
372. **Indridason OS, Franzson L, Sigurdsson G** 2005 Serum osteoprotegerin and its relationship with bone mineral density and markers of bone turnover. Osteoporos Int 16:417–423
373. **Khosla S, Arrighi HM, Melton L, Atkinson EJ, O'Fallon WM, Dunstan C, Riggs BL** 2002 Correlates of osteoprotegerin levels in women and men. Osteoporos Int 13:394–399
374. **Kudlacek S, Schneider B, Woloszczuk W, Pietschmann P, Willvonseder R, Austrian Study Group on Normative Values of Bone Metabolism** 2003 Serum levels of osteoprotegerin increase with age in a healthy adult population. Bone 32:681–686
375. **Rogers A, Saleh G, Hannon RA, Greenfield D, Eastell R** 2002 Circulating estradiol and osteoprotegerin as determinants of bone turnover and bone density in postmenopausal women. J Clin Endocrinol Metab 87:4470–4475
376. **Szulc P, Hofbauer LC, Heufelder AE, Roth S, Delmas PD** 2001

Osteoprotegerin serum levels in men: correlation with age, estrogen, and testosterone status. J Clin Endocrinol Metab 86:3162–3165
377. **Yano K, Tsuda E, Washida N, Kobayashi F, Goto M, Harada A, Ikeda K, Higashio K, Yamada Y** 1999 Immunological characterization of circulating osteoprotegerin/osteoclastogenesis inhibitory factor: increased serum concentrations in postmenopausal women with osteoporosis. J Bone Miner Res 14:518–527
378. **Michael H, Harkonen PL, Vaananen HK, Hentunen TA** 2005 Estrogen and testosterone use different cellular pathways to inhibit osteoclastogenesis and bone resorption. J Bone Miner Res 20:2224–2232
379. **Mezquita-Raya P, De la Higuera M, Fernandez Garcia D, Alonso G, Ruiz-Requena ME, de Dios Luna J, Escobar-Jimenez F, Munoz-Torres M** 2005 The contribution of serum osteoprotegerin to bone mass and vertebral fractures in postmenopausal women. Osteoporos Int 16:1368–1374
380. **Oh ES, Rhee EJ, Oh KW, Lee WY, Baek KY, Yoon KH, Kang MI, Yun EJ, Park CY, Choi MG, Yoo HJ, Park SW** 2005 Circulating osteoprotegerin levels are associated with age, waist-to-hip ratio, serum total cholesterol, and low-density lipoprotein cholesterol levels in healthy Korean women. Metabolism 54:49–54
381. **Trofimov S, Pantsulaia I, Kobyliansky E, Livshits G** 2004 Circulating levels of receptor activator of nuclear factor-κB ligand/osteoprotegerin/macrophage-colony stimulating factor in a presumably healthy human population. Eur J Endocrinol 150:305–311
382. **Uemura H, Yasui T, Umino Y, Niki H, Takikawa M, Saito S, Furumoto H, Irahara M** 2003 Circulating osteoprotegerin in women during GnRH-agonist treatment and their relationship with mineral components and biomarkers of bone turnover. Bone 33:860–866
383. **Jorgensen HL, Kusk P, Madsen B, Fenger M, Lauritzen JB** 2004 Serum osteoprotegerin (OPG) and the A163G polymorphism in the OPG promoter region are related to peripheral measures of bone mass and fracture odds ratios. J Bone Miner Metab 22:132–138
384. **Fahrleitner-Pammer A, Dobnig H, Piswanger-Solkner C, Bonelli C, Dimai HP, Leb G, Obermayer-Pietsch B** 2003 Osteoprotegerin serum levels in women: correlation with age, bone mass, bone turnover and fracture status. Wien Klin Wochenschr 11:291–297
385. **Schett G, Kiechl S, Redlich K, Oberhollenzer F, Weger S, Egger G, Mayr A, Jocher J, Xu Q, Pietschmann P, Teitelbaum S, Smolen J, Willeit J** 2004 Soluble RANKL and risk of nontraumatic fracture. JAMA 291:1108–1113
386. **Seck T, Diel I, Bismar H, Ziegler R, Pfeilschifter J** 2001 Serum parathyroid hormone, but not menopause status, is associated with the expression of osteoprotegerin and RANKL mRNA in human bone samples. Eur J Endocrinol 145:199–205
387. **Abdallah BM, Stilgren LS, Nissen N, Kassem M, Jorgensen H, Abrahamsen B** 2005 Increased RANKL/OPG mRNA ratio in iliac crest bone biopsies from women with hip fractures. Calcif Tissue Int 76:90–97
388. **Tsangar H, Findlay DM** 2004 Increased expression of IL-6 and RANK mRNA in human trabecular bone from fragility fracture of the femoral neck. Bone 35:334–342
389. **Han KO, Choi JT, Choi HA, Moon IG, Yim CH, Park WK, Yoon HK, Han K** 2005 The changes in circulating osteoprotegerin after hormone therapy in postmenopausal women and their relationship with estrogen responsiveness on bone. Clin Endocrinol (Oxf) 62:349–353
390. **Dobnig H, Hofbauer LC, Viereck V, Obermayer-Pietsch B, Fahrleitner-Pammer A** 2006 Changes in the RANK ligand/osteoprotegerin system are correlated to changes in bone mineral density in bisphosphonate-treated osteoporotic patients. Osteoporos Int 17:693–703
391. **Gannage-Yared MH, Fares F, Semaan M, Khalife S, Jambart S** 2006 Circulating osteoprotegerin is correlated with lipid profile, insulin sensitivity, adiponectin and sex steroids in an ageing male population. Clin Endocrinol (Oxf) 64:652–658
392. **Oh KW, Rhee EJ, Lee WY, Kim SW, Baek KH, Kang MI, Yun EJ, Park CY, Ihm SH, Choi MG, Yoo HJ, Park SW** 2005 Circulating osteoprotegerin and receptor activator of NF-κB ligand system are associated with bone metabolism in middle-aged males. Clin Endocrinol (Oxf) 62:92–98
393. **Fahrleitner A, Prenner G, Leb G, Tscheliessnigg KH, Piswanger-Solkner C, Obermayer-Pietsch B, Portugaller HR, Berghold A, Dobnig H** 2003 Serum osteoprotegerin is a major determinant of bone density development and prevalent vertebral fracture status following cardiac transplantation. Bone 32:96–106
394. **Menaa C, Reddy SV, Kurihara N, Maeda H, Anderson D, Cundy T, Cornish J, Singer FR, Bruder JM, Roodman GD** 2000 Enhanced RANK ligand expression and responsivity of bone marrow cells in Paget's disease of bone. J Clin Invest 105:1833–1838
395. **Roodman GD, Windle JJ** 2005 Paget disease of bone. J Clin Invest 115:200–208
396. **Janssens K, De Vernejoul MC, de Freitas F, Vanhoenacker F, van Hul W** 2005 An intermediate form of juvenile Paget's disease caused by a truncating TNFRSF11B mutation. Bone 36:542–548
397. **Middleton-Hardie C, Zhu Q, Cundy H, Lin JM, Callon K, Tong PC, Xu J, Grey A, Cornish J, Naot D** 2006 Deletion of aspartate 182 in OPG causes juvenile Paget's disease by impairing both protein secretion and binding to RANKL. J Bone Miner Res 21:438–445
398. **Whyte MP** 2006 Paget's disease of bone and genetic disorders of RANKL/OPG/RANK/NF-κB signaling. Ann NY Acad Sci 1068:143–164
399. **Falchetti A, Di Stefano M, Marini F, Del Monte F, Mavilia C, Strigoli D, De Feo ML, Isaia G, Masi L, Amedei A, Cioppi F, Ghinoi V, Bongi SM, Di Fede G, Sferrazza C, Rini GB, Melchiorre D, Matucci-Cerenic M, Brandi ML** 2004 Two novel mutations at exon 8 of the Sequestosome 1 (SQSTM1) gene in an Italian series of patients affected by Paget's disease of bone (PDB). J Bone Miner Res 19:1013–1017
400. **Hocking LJ, Lucas GJ, Daroszewska A, Mangion J, Olavesen M, Cundy T, Nicholson GC, Ward L, Bennett ST, Wuyts W, van Hul W, Ralston S** 2002 Domain-specific mutations in sequestosome 1 (SQSTM1) cause familial and sporadic Paget's disease. Hum Mol Genet 11:2735–2739
401. **Hocking LJ, Lucas GJ, Daroszewska A, Cundy T, Nicholson GC, Donath J, Walsh JP, Finlayson C, Cavey JR, Ciani B, Sheppard PW, Searle MS, Layfield R** 2004 Novel UBA domain mutations of SQSTM1 in Paget's disease of bone: genotype phenotype correlation, functional analysis, and structural consequences. J Bone Miner Res 19:1122–1127
402. **Johnson-Pais TL, Wisdom JH, Weldon KS, Cody JD, Hansen MF, Singer FR** 2003 Three novel mutations in SQSTM1 identified in familial Paget's disease of bone. J Bone Miner Res 18:1748–1753
403. **Laurin N, Brown JP, Morisette J, Raymond V** 2002 Recurrent mutation of the gene encoding sequestosome 1 (SQSTM1/p62) in Paget disease of bone. Am J Hum Genet 70:1582–1588
404. **Layfield R, Hocking LJ** 2004 SQSTM and Paget's disease of bone. Calcif Tissue Int 75:347–357
405. **Alvarez L, Peris P, Guanabens N, Vidal S, Ros I, Pons F, Filella X, Monegal A, Munoz-Gomez J, Ballesta AM** 2003 Serum osteoprotegerin and its ligand in Paget's disease of bone: relationship to disease activity and effect of treatment with bisphosphonates. Arthritis Rheum 48:824–828
406. **Martini G, Gennari L, Merlotti D, Salvadori S, Franci MB, Campagna S, Avanzati A, De Paola V, Valleggi F, Nuti R** 2007 Serum OPG and RANKL levels before and after intravenous bisphosphonate treatment in Paget's disease of bone. Bone 40:457–463
407. **Mossetti G, Rendina D, De Filippo G, Viceconti R, Di Domenico G, Cioffi M, Postiglione L, Nunziata V** 2005 Interleukin-6 and osteoprotegerin systems in Paget's disease of bone: relationship to risedronate treatment. Bone 36:549–554
408. **Pan B, Farrugia AN, To LB, Findlay DM, Green J, Lynch K, Zannettino A** 2004 The nitrogen-containing bisphosphonate, zoledronic acid, influences RANKL expression in human osteoblast-like cells by activating TNF-α converting enzyme (TACE). J Bone Miner Res 19:147–154
409. **Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC, Bellido T** 1999 Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. J Clin Invest 104:1363–1374
410. **Reinholz GG, Getz B, Pederson L, Sanders ES, Subramaniam M, Ingle JN, Spelsberg TC** 2000 Bisphosphonates directly regulate cell proliferation, differentiation, and gene expression in human osteoblasts. Cancer Res 60:6001–6007
411. **Viereck V, Emons G, Lauck V, Frosch KH, Blaschke S, Grundker**

**C, Hofbauer LC** 2002 Bisphosphonates pamidronate and zolendronic acid stimulate osteoprotegerin production by primary human osteoblasts. Biochem Biophys Res Comm 291:680–686
412. **Gravellese EM, Manning C, Tsay A, Naito A, Pan C, Amento E, Goldring SR** 2000 Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. Arthritis Rheum 43:250–258
413. **Crotti T, Smith MD, Ahern MJ, Findlay D, Kraan M, Tak PP, Haynes D** 2002 Receptor activator NF-κB ligand (RANKL) expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathy, osteoarthritis, and from normal patients: semiquantitative and quantitative analysis. Ann Rheum Dis 61:1047–1054
414. **Shigeyama Y, Pap T, Kunzler P, Simmen BR, Gay RE, Gay S** 2000 Expression of osteoclast differentiation factor in rheumatoid arthritis. Arthritis Rheum 43:2523–2530
415. **Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, Takaoka A, Yokochi T, Oda H, Tanaka K, Nakamura K, Taniguchi T** 2000 T-Cell mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-γ. Nature 408:600–605
416. **Pettit AR, Walsh NC** 2006 RANKL is expressed at the pannus-bone interface at sites of articular bone erosion in rheumatoid arthritis. Rheumatology 45:1068–1076
417. **Skoumal M, Kolarz G, Haberhauer G, Woloszczuk W, Hawa G, Klingler A** 2005 Osteoprotegerin and the receptor activator of NF-κB ligand in the serum and synovial fluid: a comparison of patients with longstanding rheumatoid arthritis and osteoarthritis. Rheumatol Int 26:63–69
418. **Vanderborght A, Linsen L, Thewissen M, Geusens P, Raus J, Stinissen P** 2004 Osteoprotegerin and receptor activator of nuclear factor-κB ligand mRNA expression in patients with rheumatoid arthritis and healthy controls. J Rheumatol 31:1483–1490
419. **Geusens PP, Landewe R, Garnero P, Chen D, Dunstan CR, Lems WF, Stinissen P, van der Heijde D, van der Linden S, Boers M** 2006 The ratio of circulating osteoprotegerin to RANKL in early rheumatoid arthritis predicts later joint destruction. Arthritis Rheum 54:1772–1777
420. **Haugeberg G, Orstavik RE, Uhlig T, Falch JA, Halse JI, Kvien TK** 2002 Bone loss in patients with rheumatoid arthritis: results from a population-based cohort of 366 patients followed for two years. Arthritis Rheum 46:1720–1728
421. **Lodder MC, de Jong Z, Kostense PJ, Molenaar E, Staal K, Voskuyl AE, Hazes J, Kijkmans B, Lems WF** 2004 Bone mineral density in patients with rheumatoid arthritis: relation between disease and low bone mineral density. Ann Rheum Dis 63:1576–1580
422. **Van Staa TP, Geusens P, Bijlsma J, Leufkens H, Cooper C** 2006 Clinical assessment of the long-term risk of fracture in patients with rheumatoid arthritis. Arthritis Rheum 54:3104–3112
423. **Kumar S, Votta BJ, Rieman DJ, Badger AM, Gowen M, Lee JC** 2001 IL-1 and TNF-induced bone resorption is mediated by p38 mitogen activated protein kinase. J Cell Physiol 187:294–303
424. **O'Gradaigh D, Ireland D, Bord S, Compston JE** 2004 Joint erosion in rheumatoid arthritis: interactions between tumour necrosis factor α, interleukin 1, and receptor activator of nuclear factor κB ligand (RANKL) regulate osteoclasts. Ann Rheum Dis 63:354–359
425. **Redlich K, Hayer S, Ricci R, David JP, Tohidast-Akrad M, Kollias G, Steiner G, Smolen JS, Wagner EF, Schett G** 2002 Osteoclasts are essential for TNF-α-mediated joint destruction. J Clin Invest 110:1419–1427
426. **Garnero P, Jouvenne P, Buchs N, Delmas PD, Miossec P** 1999 Uncoupling of bone metabolism in rheumatoid arthritis patients with or without joint destruction: assessment with serum type I collagen breakdown products. Bone 24:381–385
427. **Toritsuka Y, Nakamura N, Lee SB, Hashimoto J, Yasui N, Shino K, Ochi T** 1997 Osteoclastogenesis in iliac bone marrow of patients with rheumatoid arthritis. J Rheumatol 24:1690–1696
428. **Vis M, Havaardsholm EA, Haugeberg G, Uhlig T, Voskuyl AE, van de Stadt RJ, Dykmans BA, Woolf AD, Kiren TK, Lems WF** 2006 Evaluation of bone mineral density, bone metabolism, osteoprotegerin and receptor activator of the NFκB ligand serum levels during treatment with infliximab in patients with rheumatoid arthritis. Ann Rheum Dis 65:1595–1599
429. **Lee CK, Lee EY, Chung SM, Mun SH, Yoo B, Moon HB** 2004 Effects of disease-modifying antirheumatic drugs and antiinflammatory cytokines on human osteoclastogenesis through interaction with receptor activator of nuclear factor κB, osteoprotegerin, and receptor activator of nuclear factor κB ligand. Arthritis Rheum 50:3831–3843
430. **Kostenuik PJ** 2005 Osteoprotegerin and RANKL regulate bone resorption, density, geometry and strength. Curr Opin Pharmacol 5:618–625
431. **Weiner LM** 2006 Fully human therapeutic monoclonal antibodies. J Immunother 29:1–9
432. **Lonberg N** 2005 Human antibodies from transgenic animals. Nat Biotechnol 23:1117–1125
433. **Green LL** 1999 Antibody engineering via genetic engineering of the mouse: XenoMouse strains are a vehicle for the faclie generation of therapeutic human monoclonal antibodies. J Imm Meth 231:11–23
434. **Kostenuik PJ, Warmington K, Grisanti M, Morony S, Geng Z, Tan HL, Christensen K, Sullivan J, Nguyen H, Adamu S, Asuncion F, Ominsky MS, Li X, McCabe J** 2005 RANKL inhibition with AMG 162, a fully human MAb, causes sustained suppression of bone resorption and increased BMD in knockin mice expressing humanized RANKL. J Bone Miner Res 20:S259
435. **Lewiecki EM, Miller PD, McClung MR, Cohen SB, Bolognese MA, Liu Y, Wang A, Siddhanti S, Fitzpatrick LA, for the AMG 162 Bone Loss Study Group** 2007 Two-year treatment with denosumab (AMG 162) in a randomized phase 2 study of postmenopausal women with low bone mineral density. J Bone Miner Res 22:1832–1841
436. **Cohen SB, Valen P, Ritchlin C, Schechtman J, Peterfy C, van der Heijde D, Zhou L, Newmark R, Tsuji W** 2006 RANKL inhibition with denosumab reduces progression of bone erosions in patients with rheumatoid arthritis: month 6 MRI results. Arthritis Rheum 54:S831
437. **Dore R, Hurd E, Palmer W, Shergy W, Zhou L, Newmark R, Tsuji W** 2006 Denosumab increases bone mineral density in patients with rheumatoid arthritis. Arthritis Rheum 54:S240
438. **Lane NE, Iannini M, Atkins C, Haraoui B, Zhou L, Tsuji W, Newmark R** 2006 RANKL inhibition with denosumab decreases markers of bone and cartilage turnover in patients with rheumatoid arthritis. Arthritis Rheum 54:S225
439. **Lipton A, Steger GG, Figueroa J, Alvarado C, Solal-Celigny P, Body JJ, de BR, Berardi R, Gascon P, Tonkin KS, Coleman R, Paterson AH, Peterson MC, Fan M, Kinsey A, Jun S** 2007 Randomized active-controlled phase II study of denosumab efficacy and safety in patients with breast cancer-related bone metastases. J Clin Oncol 25:4431–4437
440. **Peterson MC, Jang G, Kim W, Gurrola E, Kinsey A, Dansey R** 2006 Selection of a phase 3 dose regimen for denosumab based on pharmacokinetic (PK), pharmacodynamic (PD), and safety data from multiple subcutaneous (SC) dosing regimens in breast cancer patients (pts) with bone metastases (BM). J Clin Oncol 24:142s
441. **Suarez T, Fizazi K, Rahim Y, Wilson J, Fan M, Jun S, Lipton A** 2006 A randomized trial of denosumab (AMG 162) versus intravenous (IV) bisphosphonates (BP) in cancer patients (pts) with bone metastases (BM) on estabilised IV BP and evidence of elevated bone resorption. J Clin Oncol 24:483s
442. **Body JJ, Facon T, Coleman RE, Lipton A, Geurs F, Fan M, Holloway D, Peterson MC, Bekker PJ** 2006 A study of the biological receptor activator of nuclear factor-κB ligant inhibitor, Denosumab, in patients with multiple myeloma or bone metastases from breast cancer. Clin Cancer Res 12:1221–1228
443. **Lum L, Wong BR, Josien R, Becherer JD, Erdjument-Bromage H, Schlondorff J, Tempst P, Choi Y, Blobel CP** 1999 Evidence for a role of a tumor necrosis factor-α (TNF-α)-converting enzyme-like protease in shedding of TRANCE, a TNF family member involved in osteoclastogenesis and dendritic cell survival. J Biol Chem 274:13613–13618
444. **Brandstrom H, Jonsson KB, Ohlsson C, Vidal O, Ljunghall S, Ljunggren O** 1998 Regulation of osteoprotegerin mRNA levels by prostaglandin $E_2$ in human bone marrow stromal cells. Biochem Biophys Res Comm 247:338–341