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## RANKL, a necessary chance for clinical application to osteoporosis and cancer-related bone diseases

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### Abstract

Osteoporosis is a common bone disease characterized by reduced bone and increased risk of fracture. In postmenopausal women, osteoporosis results from bone loss attributable to estrogen deficiency. Osteoclast differentiation and activation is mediated by receptor activator of nuclear factor- $\kappa$ B ligand (RANKL), its receptor receptor activator of nuclear factor- $\kappa$ B (RANK), and a decoy receptor for RANKL, osteoprotegerin (OPG). The OPG/RANKL/RANK system plays a pivotal role in osteoclast biology. Currently, a fully human anti-RANKL monoclonal antibody named denosumab is being clinically used for the treatment of osteoporosis and cancer-related bone disorders. This review describes recent advances in RANKL-related research, a story from bench to bedside. First, the discovery of the key factors, OPG/RANKL/RANK, revealed the molecular mechanism of osteoclastogenesis. Second, we established three animal models: (1) a novel and rapid bone loss model by administration of glutathione-S transferase-RANKL fusion protein to mice; (2) a novel mouse model of hypercalcemia with anorexia by overexpression of soluble RANKL using an adenovirus vector; and (3) a novel mouse model of osteopetrosis by administration of a denosumab-like anti-mouse RANKL neutralizing monoclonal antibody. Lastly, anti-human RANKL monoclonal antibody has been successfully applied to the treatment of osteoporosis and cancer-related bone

disorders in many countries. This is a real example of applying basic science to clinical practice.

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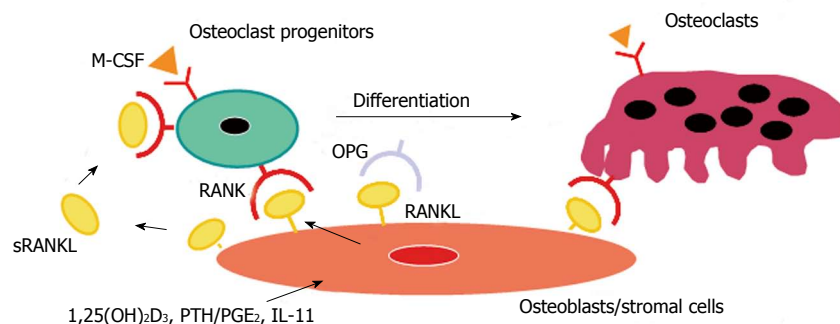
**Key words:** Osteoclast; Osteoblast; Receptor activator of nuclear factor- $\kappa$ B ligand; Denosumab; Receptor activator of nuclear factor- $\kappa$ B; Osteoprotegerin

**Core tip:** This review describes a success story from discovery of osteoprotegerin/receptor activator of nuclear factor- $\kappa$ B ligand (RANKL)/receptor activator of nuclear factor- $\kappa$ B (RANK) to clinical application of a fully human anti-RANKL monoclonal antibody to the treatment of osteoporosis and cancer-related bone disorders. RANKL is a key molecule for osteoclast differentiation and activation. Inhibition of RANKL activity with anti-RANKL antibody reduces osteoclastogenesis, resulting in inhibition of bone resorption. Three animal disease models of osteoporosis, hypercalcemia, and osteopetrosis by treating normal mice with soluble RANKL (sRANKL), adenovirus expressing sRANKL, and anti-mouse RANKL neutralizing antibody, respectively, can be established in 2-14 d and the establishment of these animal models could help accelerate research on bone metabolism.

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### INTRODUCTION

Morphogenesis and remodeling of bone depends on the integrated activity of osteoblasts which form bone and osteoclasts which resorb bone. There have been many



**Figure 1** A model illustrating a mechanism by which osteoblasts/stromal cells regulate osteoclast differentiation and activation. Three distinct signals stimulated by 1,25(OH)<sub>2</sub>D<sub>3</sub>, PTH/PGE<sub>2</sub>, and interleukin (IL)-11 induce receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) expression on osteoblasts/stromal cells. RANKL mediates a signal for osteoclastogenesis through RANK produced on osteoclast progenitors. Osteoprotegerin (OPG) inhibits osteoclastogenesis by interrupting the binding of RANKL and RANK. Macrophage colony-stimulating factor (M-CSF) produced by osteoblasts/stromal cells is also indispensable for proliferation and differentiation of osteoclast progenitors. Soluble RANKL (sRANKL) is produced by digestion of RANKL with metalloproteinases and may regulate osteoclastogenesis.

attempts to develop pharmaceuticals to treat osteoporosis and other metabolic bone disorders. The major difficulty in the development of such drugs is the lack of clarification of the mechanisms regulating differentiation of the bone cells including osteoblasts and osteoclasts. In the late-1990s, dramatic findings of the key factors of osteoclast differentiation opened a new era in research of osteoclast biology and the development of anti-resorptive pharmaceuticals for osteoporosis<sup>[1-11]</sup>.

#### Discovery of key factors to understand the molecular mechanism of osteoclastogenesis

We previously identified and cloned an osteoclastogenesis inhibitory factor named OCIF<sup>[2,3]</sup>. We used human fetal lung fibroblasts, IMR-90 cells, as the cell source and purified OCIF using the *in vitro* osteoclastogenesis assay established by Takahashi *et al*<sup>[12]</sup> and Udagawa *et al*<sup>[13]</sup>. Since fibroblasts are present ubiquitously in the body, it was surprising to find that these cells produce a novel osteoclastogenesis inhibitory factor. IMR-90 cells produce a number of cytokine and growth factors including hepatocyte growth factor (HGF)<sup>[14]</sup>. Suda *et al*<sup>[15]</sup> proposed a working hypothesis that osteoclasts are generated by cell-to-cell interaction with a hypothetical membrane-bound factor called osteoclast differentiation factor (ODF) on osteoblasts. To explore this hypothesis we attempted to find a novel osteoclastogenesis inhibitory factor that could be an inhibitor of the hypothetical factor, ODF<sup>[2,3]</sup>. Simonet *et al*<sup>[1]</sup> independently found the identical factor through the rat EST project and named it osteoprotegerin (OPG). They found a novel tumor necrosis factor (TNF) receptor family member in the comprehensive genomic sequencing project and identified it as an osteoclastogenesis inhibitor by overexpression of its cDNA in transgenic mice. Notably, two independent groups identified the same factor at almost the same time by different strategies.

Thereafter, Yasuda *et al*<sup>[4]</sup> and Lacey *et al*<sup>[5]</sup> independently identified a ligand of OCIF/OPG with expression cloning and named it ODF and OPG ligand (OPGL), respectively. ODF/OPGL was found to be identical to

TNF-related activation-induced cytokine (TRANCE)<sup>[16]</sup> and RANKL<sup>[17]</sup>, which were cloned as factors regulating T-cell and dendritic cell functions. We confirmed that ODF was the long-sought after ligand regulating osteoclast differentiation and activation<sup>[4]</sup>. As standard nomenclature of the same molecule, OPG and RANKL were proposed by the ASBMR President's Committee on Nomenclature, respectively<sup>[18]</sup>.

We further identified RANK as a receptor for RANKL on osteoclasts<sup>[6]</sup>. Although RANK was known to be a receptor for RANKL in the T-cell and dendritic cell interaction<sup>[17]</sup>, the receptor responsible for the RANKL-mediated osteoclastogenesis had not been identified. Some ligands of the TNF family bind to several receptors of the TNF receptor family. It was suspected that RANKL might bind to another member of the TNF receptor family, but not to RANK. We molecularly cloned the RANKL receptor from mouse osteoclast progenitors by panning and identified it as RANK<sup>[6]</sup>. A polyclonal antibody against soluble RANK (sRANK) mimicked the RANKL function by clustering of RANK. In contrast, sRANK and Fab fragment of anti-RANK polyclonal antibody completely inhibited RANKL-mediated osteoclastogenesis by binding to RANKL and RANK, respectively. Hsu *et al*<sup>[7]</sup> also led to the same conclusion using transgenic mice overexpressing soluble RANK. The importance of OPG/RANKL/RANK was demonstrated *in vivo* with gene-deficient mice<sup>[19-23]</sup>. The summary of these results is illustrated in the model of osteoclast differentiation (Figure 1). The details of OPG/RANKL/RANK are described elsewhere<sup>[8-11]</sup>.

To investigate the effects and functions of RANKL *in vivo*, transgenic mice overexpressing mouse soluble RANKL (sRANKL-TG mice)<sup>[24]</sup> and RANKL-deficient mice<sup>[21]</sup> were generated. They are useful animal models but it takes several months to interbreed them with other TG mice or gene-deficient mice. Alternatively, we attempted to establish three animal disease models by treating normal mice with either sRANKL, adenovirus vector harboring mouse sRANKL cDNA (Ad-sRANKL), or anti-mouse RANKL neutralizing mono-

clonal antibody. It takes two to 14 d to make these animal models using normal mice. Thus, the establishment of these quick animal models could help accelerate research on bone metabolism.

## ESTABLISHMENT OF THREE ANIMAL MODELS OF METABOLIC BONE DISEASES

### ***A novel and rapid bone loss model by administration of GST-RANKL to mice***

Osteoporosis remains a major public health problem through its associated fragility fractures. Several animal models for the study of osteoporotic bone loss, such as ovariectomy (OVX) and denervation, require surgical skills and several weeks to establish<sup>[25-31]</sup>. We tried to establish a novel and rapid bone loss model by the administration of glutathione-S transferase (GST)-RANKL to mice<sup>[32,33]</sup> (Figure 2). GST-RANKL is a fusion protein of GST and the extracellular domain of human RANKL (aa 140-317). GST-RANKL showed stronger activity in osteoclastogenesis using mouse bone marrow macrophages (BMM) and the mouse macrophage cell line, RAW264 cells, respectively, compared with a commercially available soluble RANKL (sRANKL) (Figure 2A, B). Mice were injected intraperitoneally with GST-RANKL and used to evaluate existing anti-osteoporosis drugs. GST-RANKL decreased bone mineral density (BMD) within 50 h in a dose-dependent manner. The marked decrease in femoral trabecular BMD demonstrated by pQCT and the 3D images obtained by micro computer tomography (CT) were indistinguishable from those observed in the OVX model. Histomorphometry revealed significant increase in osteoclastic activity in the GST-RANKL-injected mice. In addition, serum biochemical markers of bone turnover such as calcium, C-terminal cross-linked telopeptides of collagen I (CTX), and tartrate-resistant acid phosphatase-5b (TRAP-5b) were also significantly increased in the GST-RANKL-injected mice in a dose-dependent manner. One of the gold standard models for osteoporosis is OVX which mimics osteoporosis in postmenopausal women. Moreover, the GST-RANKL-induced bone loss model was successfully applied to C57/B6 male and female mice, ICR mice, and Fisher rats. Very recently we successfully shortened the experimental period from 50 to 24 h and established a 1-d bone loss model with one injection of GST-RANKL in mice and rats (Tomimori *et al*, unpublished).

To apply this bone loss model in the evaluation of pharmaceuticals for osteoporosis, we tested bisphosphonates (BPs), PTH and a selective estrogen receptor modulator (SERM), which are commonly used for the treatment of osteoporosis. We successfully evaluated BPs and PTH within 4 d and 2 wk, respectively, and a combination of GST-RANKL injections and OVX allowed evaluation of a SERM in 18 d. As major pharma-

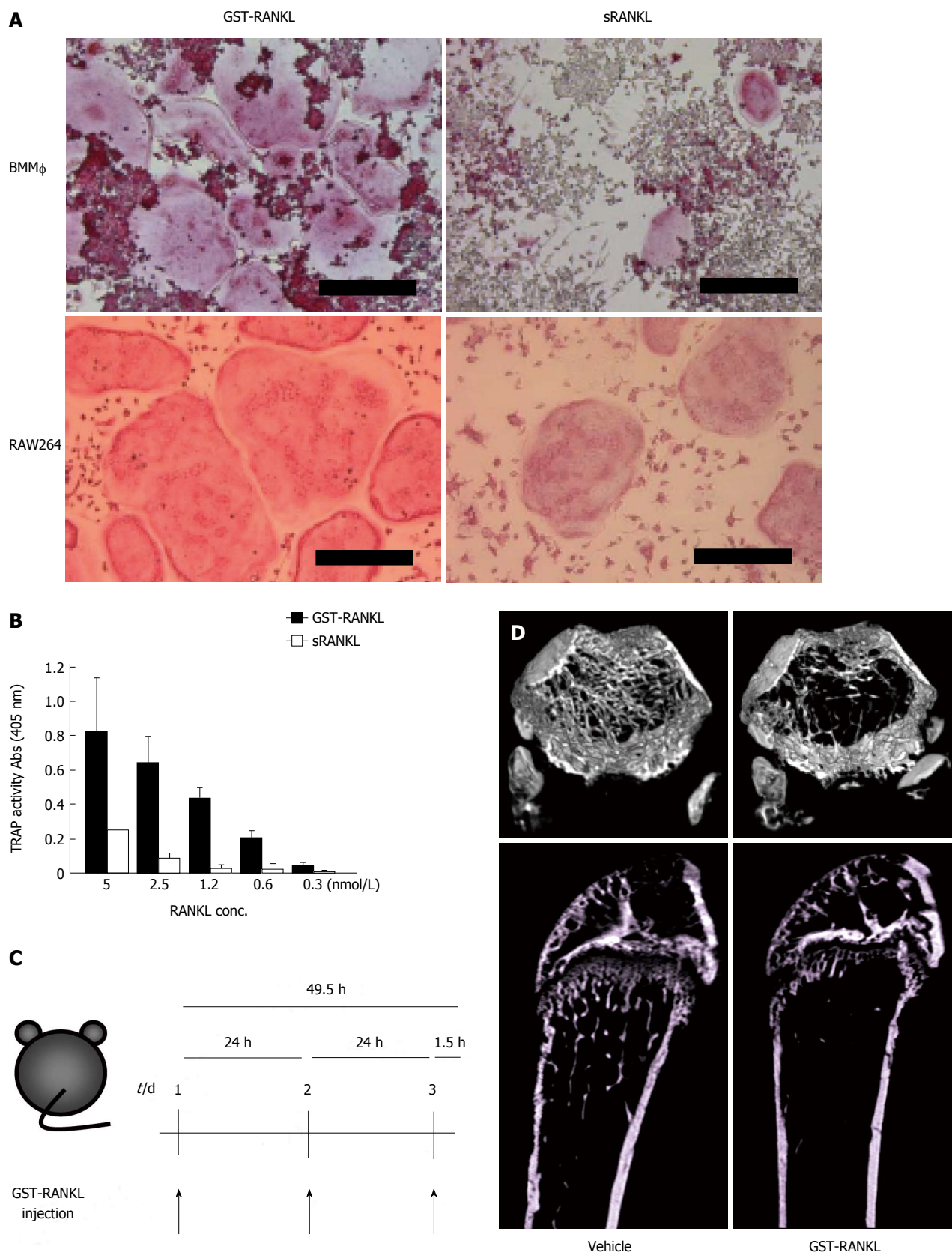
ceuticals for osteoporosis have been evaluated using the GST-RANKL-induced bone loss model, it could also be used to evaluate novel drug candidates. In fact, using the bone loss model we evaluated an inhibitor of Btk/Tec tyrosine kinases that are essential for signal transduction through RANK in osteoclast differentiation in approximately 50 h<sup>[32]</sup>.

We also evaluated a denosumab-like anti-human RANKL neutralizing monoclonal antibody as a new osteoporosis therapeutic drug candidate in 10 d<sup>[33]</sup>. We made anti-human RANKL monoclonal antibodies and selected a neutralizing antibody using the *in vitro* osteoclastogenesis assay with RAW264 cells. This antibody bound to and neutralized the activity of human but not mouse RANKL. The anti-human RANKL neutralizing antibody (100 g/mouse) or PBS was injected subcutaneously into mice 7 and 4 d before the GST-RANKL injection. Antibody treatment of the model mice completely inhibited the decrease in femoral trabecular BMD. In contrast, BMD in PBS-injected control mice was unaffected by the antibody treatment.

Notably, two or three injections of GST-RANKL induced a weak coupling, whereas longer treatments induced strong coupling<sup>[33]</sup>. Because OVX-induced bone loss is accompanied with a high turnover of bone remodeling, the GST-RANKL model is similar in mechanism to that of OVX-induced bone loss. Bahtiar *et al*<sup>[34]</sup> also used GST-RANKL to develop a high bone turnover model. Other models for high-turnover bone disease with sRANKL have been reported, using continuous infusion<sup>[35]</sup> and subcutaneous injections of sRANKL<sup>[36]</sup>. Some of the mice exhibited hypercalcemia. The infusion model requires a large amount of sRANKL and insertion of osmotic pumps subcutaneously into rats, while the injection model requires twice-daily subcutaneous injections into mice for 10 d. As a local bone loss model GST-RANKL was injected into mouse calvaria several times to induce osteoclastogenesis and bone loss near the injection sites within several days<sup>[37,38]</sup>.

A summary of the characteristics of the GST-RANKL-induced bone loss model in comparison to the OVX model is shown in Table 1. First of all, the GST-RANKL-induced bone loss model is rapid, being established within 24-50 h. Second, it is easy, as two or three intraperitoneal injections of sRANKL are sufficient to induce osteoporotic bone loss. Third, it is simple. The mechanism of bone loss in the model is simply due to a stimulation of osteoclast differentiation and activation with endogenous sRANKL. Lastly, it is useful for evaluation of major pharmaceuticals and/or candidates for osteoporosis. A Btk/Tec tyrosine kinase inhibitor, BPs, anti-human RANKL neutralizing monoclonal antibody, PTH and a SERM were evaluated within 50 h, 3 d, 10 d, 2 wk, and 18 d, respectively. Overall, the GST-RANKL model is the simplest, fastest, and easiest osteoporosis model and could be a gold standard for the evaluation of novel drug candidates of osteoporosis as well as OVX<sup>[32,33]</sup>.





**Figure 2 Establishment of GST-RANKL-injected bone loss model.** A: Osteoclasts were generated with 5 nmol/L glutathione-S transferase-receptor activator of nuclear factor- $\kappa$ B ligand (GST-RANKL) or sRANKL from bone marrow macrophages (BMM) and RAW264 cells in the presence and absence of M-CSF, respectively. The cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP). Bars = 250  $\mu$ m; B: Activities of GST-RANKL and sRANKL were compared using *in vitro* osteoclastogenesis assay with RAW264 cells by TRAP solution assay. The TRAP activity represents the number of osteoclasts. C: Experimental design. PBS (vehicle) or GST-RANKL was injected intraperitoneally at 24-h intervals for 3 d into 7-wk-old female mice. Mice were sacrificed 90 min after the last injection; D: Micro computer tomography 3D images of femurs of mice treated with vehicle or 2 mg/kg GST-RANKL. The upper and lower panels are 3D cross-sectional and longitudinal images, respectively.

**Table 1 Comparison of features between ovariectomy and GST-RANKL bone loss models**

Technique	OVX model	GST-RANKL bone loss model
Technique	OVX	Intraperitoneal injections
Term for establishment	> 4 wk	24-50 h
Term for evaluation of BP	> 4 wk	3 d
Term for evaluation of PTH	> 4 wk	14 d
Term for evaluation of SERM	> 4 wk	18 d
Term for evaluation of anti-human RANKL	No	9 d
Term for evaluation of Tec tyrosine kinase inhibitor	NA	50 h
Evaluation of male animals	No	Yes
Term for pharmacological experiments	Several mon	Several wk/d
Advantages	Human disease model	Rapid, easy, simple, and inducible model

GST-RANKL: Glutathione-S transferase-receptor activator of nuclear factor- $\kappa$ B ligand; OVX: Ovariectomy; NA: Not available; BP: Bisphosphonates; SERM: Selective estrogen receptor modulator; PTH: Parathyroid hormone.

**Table 2 Comparison of serum soluble RANKL concentrations among various mouse models**

Mouse model	sRANKL (ng/mL)	Phenotype
Ad-sRANKL injection (High)	1500	Severe osteoporosis /hypercalcemia
Ad-sRANKL injection (Low)	233	Severe osteoporosis /hypercalcemia
sRANKL-Tg mice	30	Severe osteoporosis
GST-RANKL injection	3.5 <sup>1</sup>	Osteoporosis
Wild mice	0.1	Normal

<sup>1</sup>Serum glutathione-S transferase-receptor activator of nuclear factor- $\kappa$ B ligand (GST-RANKL) concentration 4 h after injection of 1 mg/kg GST-RANKL. sRANKL: Soluble RANKL; Ad-sRANKL: Harboring mouse sRANKL cDNA.

### **A novel mouse model of hypercalcemia with anorexia by overexpression of sRANKL using an adenovirus vector**

Hypercalcemia is a significant complication in human malignancies, including squamous cell, renal cell, and breast carcinomas. Humoral hypercalcemia of malignancy (HHM) is caused by the overproduction of parathyroid hormone related protein (PTHrP) by tumors<sup>[39,40]</sup>. PTHrP mobilizes calcium from bone by inducing the expression of RANKL<sup>[41]</sup>. RANKL is overexpressed in the marrow microenvironment in myeloma patients; the RANKL to OPG ratio is markedly increased compared to that in healthy controls<sup>[42]</sup>.

Symptoms of hypercalcemia manifest as a reflection of the extent and rate of increase of serum ionized calcium. Mild to moderate hypercalcemia is usually asymptomatic<sup>[43]</sup>, whereas moderate to severe hypercalcemia is usually symptomatic<sup>[44]</sup> and includes anorexia, constipation, vomiting, nausea, weakness and mental confusion.

In a previous study, we generated sRANKL-TG mice<sup>[24]</sup>.

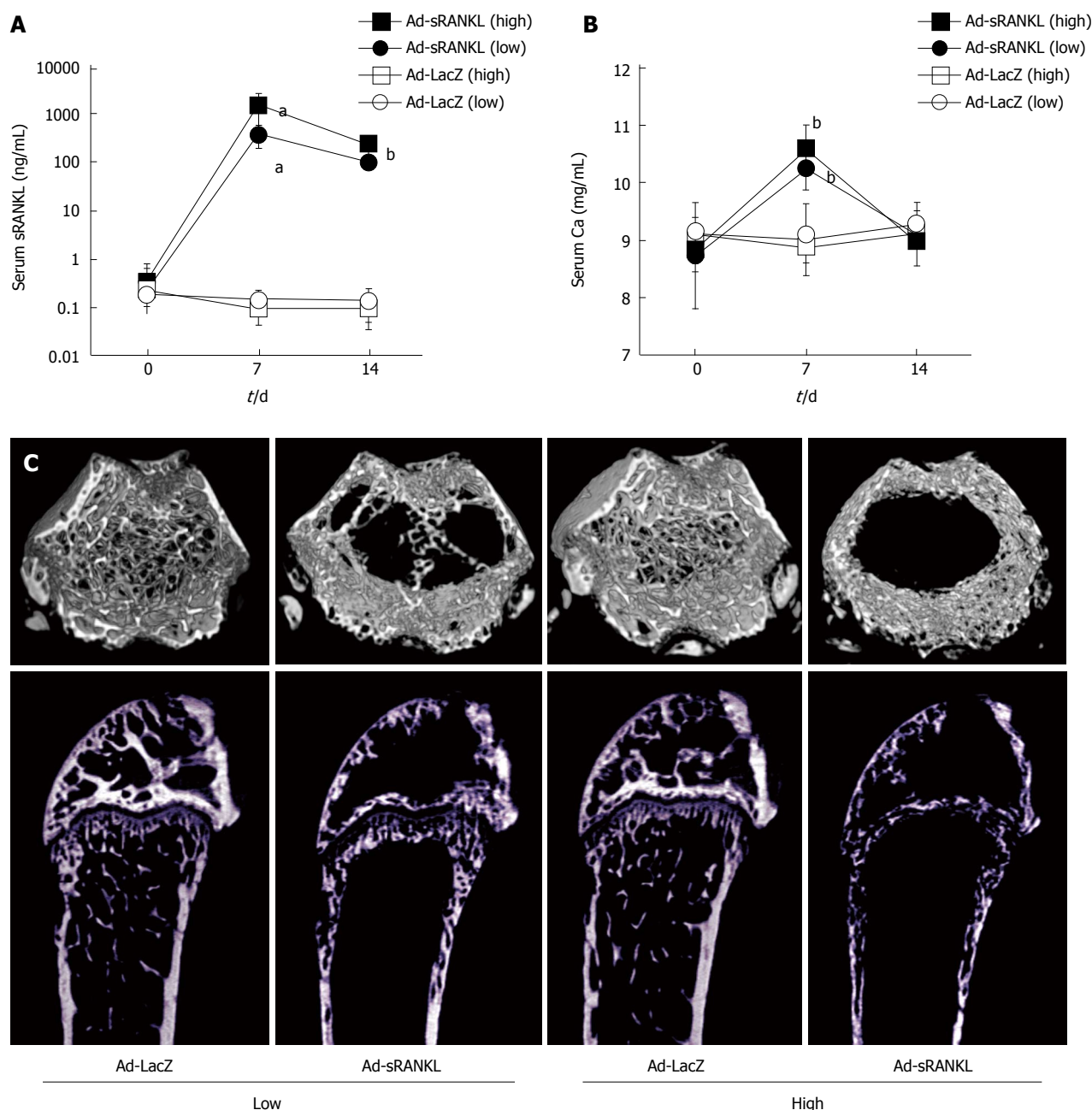
The sRANKL-TG mice exhibited severe osteoporosis accompanied with enhanced osteoclastogenesis, but no hypercalcemia. To analyze the relationship between the serum concentration of sRANKL and hypercalcemia and generate a simple and quick hypercalcemia model, Ad-sRANKL was injected intraperitoneally into male C57BL/6 mice<sup>[45]</sup>. Table 2 summarizes the results of serum sRANKL and calcium levels. Serum sRANKL increased markedly on day 7, while serum calcium increased with a peak on day 7 and returned to the baseline level on day 14. Food intake and body weight significantly declined on day 7. Taken together, the mice appeared to have anorexia as a symptom of hypercalcemia.

In addition, increases in markers for bone resorption (TRAP-5b) and formation (ALP, alkaline phosphatase) with a marked decrease in BMD measured by dual-energy X-ray absorptiometry were observed on day 14. The severe bone loss was confirmed by microCT (Figure 3). These results reflect accelerated bone formation following activation of osteoclasts, indicating coupling between bone formation and resorption.

Serum sRANKL level in the Ad-sRANKL group on day 7 was 15000 times higher than those in wild type mice. In sRANKL-TG mice, serum sRANKL was within normal range around 30 ng/mL<sup>[24]</sup>. Serum sRANKL level in the Ad-sRANKL group was about 50 times higher than that in the transgenic mice, which do not exhibit hypercalcemia even though they have severe osteoporosis and enhanced osteoclastogenesis. These observations suggest that 30 ng/mL sRANKL in serum is insufficient to induce hypercalcemia and that severe osteoporosis with enhanced osteoclastogenesis does not always accompany hypercalcemia. There may be a threshold sRANKL concentration for induction of hypercalcemia with anorexia.

Several experimental animal models of hypercalcemia have been described: a model with vitamin D treatment, a tumor transplant model and an infusion model using PTHrP<sup>[46-49]</sup>. These models have not shown a clear relationship between body weight loss and anorexia. Sato *et al*<sup>[49]</sup> showed that the recovery from hypercalcemia is accompanied by an improvement in body weight using a model of HHM, but the association of body weight loss with a decrease in food intake was not clearly shown in this model.

In summary, we established a novel model of hypercalcemia in normal mice injected intraperitoneally with Ad-sRANKL<sup>[45]</sup>. Overexpression of sRANKL activated osteoclasts to resorb bone, resulting in an increase in serum calcium. Hypercalcemic mice exhibited typical symptoms such as anorexia and weakness. The Ad-sRANKL-injected hypercalcemia model is the first one in which overexpressed sRANKL directly activates osteoclasts to increase serum calcium level. This simple and rapid model mimics HHM in terms of exhibiting anorexia and weakness and could be useful for investigating coupling between bone formation and resorption in high-turnover bone diseases, as well as for ex-



**Figure 3 Establishment of Ad-sRANKL-injected osteoporosis/hypercalcemia model.** Time-dependent changes in serum soluble receptor activator of nuclear factor- $\kappa$ B ligand (sRANKL) (A) and Ca (B) levels. Adenovirus vector harboring mouse sRANKL cDNA (Ad-sRANKL) or Ad-LacZ was injected intraperitoneally into 6-wk-old male C57BL/6 mice at  $1.0 \times 10^9$  pfu/mouse in high-dose groups and  $3.0 \times 10^8$  pfu/mouse in low-dose groups. Serum levels of sRANKL and Ca were measured on day 0, 7 and 14 after injection. Data are presented as the mean  $\pm$  SD ( $n = 5$  or  $6$ ). Ad-sRANKL high dose (filled squares), Ad-sRANKL low dose (filled circles), Ad-LacZ high dose (open squares), Ad-LacZ low dose (open circles). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs Ad-LacZ control; C: Micro computer tomography images of femurs in each group of mice. All mice were sacrificed on day 14 after injection and femurs were collected. The upper and lower panels are 3D cross-sectional and longitudinal images, respectively.

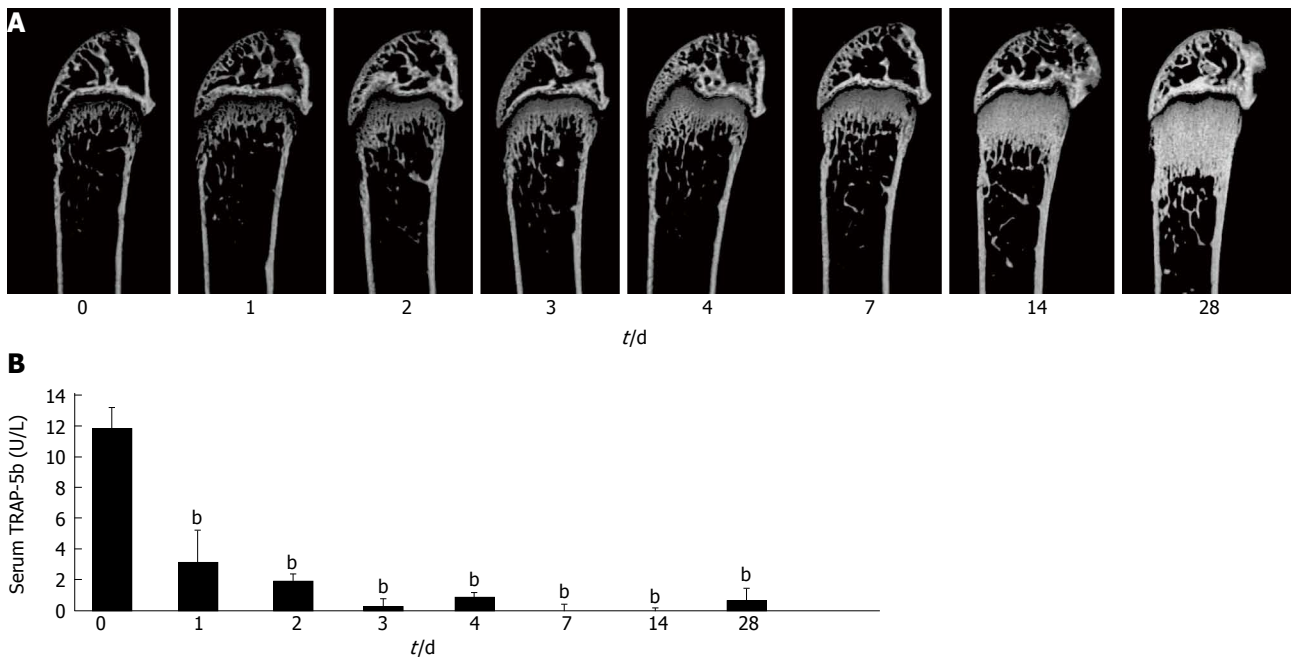
aming hypercalcemia with anorexia<sup>[45]</sup>.

#### **A novel mouse model of osteopetrosis by administration of a denosumab-like anti-mouse RANKL neutralizing monoclonal antibody**

A fully human anti-RANKL monoclonal antibody (denosumab) is clinically used for the treatment of osteoporosis and cancer-related bone disorders<sup>[50-54]</sup>. It is a strong inhibitor of RANKL and is very stable in the

blood stream for several months after single subcutaneous injection. Since denosumab does not cross react with rodent RANKL, its evaluation *in vivo* can be done only with non-human primates<sup>[55,56]</sup> or human RANKL-knock-in mice (HuRANKL mice)<sup>[57]</sup>. After replacing the exon 5 in mouse RANKL with that in human RANKL, denosumab can bind to and neutralize the chimeric mouse e/human RANKL in the HuRANKL mice. Cynomolgus monkeys have been used for the preclinical





**Figure 4 Establishment of anti-RANKL-injected osteopetrosis model.** A: Time course of the effect of anti-mouse receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) neutralizing antibody (clone, OYC1) on bone mass (day 0-28). OYC1 (5 mg/kg) was administered subcutaneously to 6-wk-old female mice ( $n = 5$  or 6) on day 0. Mice were sacrificed on day 0, 1, 2, 3, 4, 7, 14, and 28. Bone structure in the trabecular area was analyzed by micro computer tomography; B: Serum TRAP-5b levels were measured by ELISA. Data are shown as the mean  $\pm$  SD. <sup>b</sup> $P < 0.01$  (ANOVA) vs value on day 0.

animal experiments of denosumab<sup>[55,56]</sup>. To investigate the effect of RANKL inhibition in normal mice, we prepared anti-mouse RANKL neutralizing monoclonal antibody (OYC1) and established a novel mouse model of osteopetrosis by administration of the anti-mouse RANKL antibody to normal mice<sup>[58]</sup>.

Single subcutaneous injection of the antibody markedly increased bone mass in a time-dependent manner for 4 wk (Figure 4A). Histomorphometry showed remarkable decreases in osteoclast surface and number, as well as decreases in osteoblast surface, mineral apposition rate, and bone formation rate after 2 wk. These results are consistent with the previous report on HuRANKL mice treated with denosumab<sup>[57]</sup>, which showed a negative coupling between bone resorption and formation. Decreases in bone resorption marker (TRAP-5b) and formation marker (ALP) were also observed in anti-RANKL-antibody-treated mice. There was almost no serum TRAP-5b activity for 4 wk, and anti-RANKL antibody was detected in serum of the treated mice even 4 wk after the injection (Figure 4B).

Histological and microCT analyses showed that the anti-RANKL antibody-treated mice exhibit an osteopetrotic phenotype, similar to the observation in OPG-treated mice<sup>[1,3]</sup>. The osteopetrotic phenotype was evident 2 d after a single injection in normal mice. The effect of a single injection (5 mg/kg body weight) of anti-RANKL antibody on bone mass is roughly equivalent to that of three daily injections (24 mg/kg body weight) of OPG, indicating that the efficacy and stability of anti-RANKL antibody *in vivo* was much higher than those of OPG<sup>[3,58]</sup>.

Osteopetrosis is generally caused by failure of osteoclast-mediated resorption of skeleton. There are numerous mouse models of osteopetrosis without osteoclasts, including c-fos-deficient mice<sup>[59]</sup>, op/op mice<sup>[60]</sup>, RANKL-deficient mice<sup>[21]</sup> and RANK-deficient mice<sup>[22,23]</sup>. The anti-RANKL antibody-treated mouse is an inducible osteopetrosis model. It is possible to investigate the difference between BPs and anti-RANKL antibody in normal mice. It is also possible to test the effects of switching pharmaceuticals, *e.g.*, BP to denosumab and PTH to denosumab, and to test the effects of combinations of pharmaceuticals, *e.g.*, PTH and denosumab. We have demonstrated that the combination of a denosumab-like anti-mouse RANKL monoclonal antibody and PTH synergistically increases bone mass in normal mice. We also showed that PTH increases bone formation in osteoclast-deficient mice treated with the anti-RANKL antibody, suggesting that PTH requires no osteoclasts for its bone anabolic activity. The anti-mouse RANKL neutralizing antibody (OYC1) is a surrogate antibody for denosumab and is useful for investigating unidentified functions of RANKL in mice<sup>[58]</sup>. In fact, several important functions of RANKL were identified in other tissues in addition to bones. They include fever control in the brain<sup>[61]</sup>, proliferation of mammary gland epithelial cells<sup>[62]</sup> and stem cells<sup>[63,64]</sup>, proliferation of mammary cancer cells<sup>[65,66]</sup>, hematopoiesis in bone marrow<sup>[67]</sup>, development of epithelial cells in the thymic medulla<sup>[68]</sup>, lymphogenesis<sup>[69]</sup>, proliferation of regulatory T cells *via* activation of dendritic cells<sup>[70]</sup>, and development of Microfold cells in intestinal epithelium<sup>[71]</sup>.

These inducible models of osteoporosis and osteo-

petrosis using normal mice exhibit exactly mirror images in terms of the change in bone mass and are useful to advance research on osteoclast biology as well as bone metabolism *in vivo*.

## IMPLICATIONS OF DISCOVERING THE OPG/RANKL/RANK SYSTEM

The discovery of the OPG/RANKL/RANK system guided us to the mechanisms of osteoclast differentiation and activation<sup>[8-11]</sup>. Inhibition of the RANKL/RANK signal in bone can increase bone mass and is useful for treatment of osteoporosis. OPG and soluble RANK have been developed as pharmaceutical candidates, and anti-human RANKL neutralizing antibody (denosumab) has been clinically used for osteoporosis and cancer-related bone disorders<sup>[50-54]</sup>. The past decade has witnessed significant progress in the development of the anti-human RANKL neutralizing antibody as a pharmaceutical agent. This is an outstanding story starting from the discovery of RANKL and advancing to the clinical application of anti-RANKL antibody<sup>[72]</sup>.

At present denosumab is clinically used for the treatment of osteoporosis and cancer-related bone diseases in Japan, Europe, United States and many other countries. A phase II clinical trial for rheumatoid arthritis is ongoing in Japan. The future treatment option of rheumatoid arthritis thus looks promising.

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