Journal of Cellular Biochemistry 103:335–345 (2008)

# Dexamethasone Promotes Osteoclastogenesis by Inhibiting Osteoprotegerin Through Multiple Levels

**Takeshi Kondo,[1] Riko Kitazawa,[1] Akira Yamaguchi,[2] and Sohei Kitazawa[1]***

[1]Division of Molecular Pathology, Department of Biomedical Informatics, Kobe University Graduate School of Medicine, Kobe, Japan
[2]Department of Oral Restitution, Oral Pathology, Graduate School of Tokyo Medical and Dental University, Tokyo, Japan

**Abstract** Increased bone fragility attributed to osteopenia is a serious side effect of glucocorticoid treatment. Glucocorticoid-induced bone loss is caused primarily by hypofunction and apoptosis of osteoblasts, and secondarily by accelerated bone resorption. To explore the mechanism whereby dexamethasone (Dex) stimulates osteoclastogenesis in the coculture system, we analyzed the effect of Dex on the expression of both mouse osteoprotegerin (OPG) and receptor activator of NF-κB ligand (RANKL). Dex reduced OPG transcripts and OPG protein secretion by the ST2 osteoblastic cells. Since mainly the c-Jun homodimer maintains the steady-state transcription of the OPG gene, we examined the effect of Dex on c-Jun signaling in ST2 cells. Western blotting disclosed that Dex decreased the amount of phospho-c-Jun protein (p-c-Jun) and, correspondingly, the amount of the phosphorylated p46 isoform of Jun N-terminal kinase (JNK). The amount of phospho-SEK1 also decreased after Dex treatment, while the amounts of phospho-ERK and p38 remained constant. Among mitogen-activated protein (MAP) kinase inhibitors, the JNK inhibitor mimicked the inhibitory effect of Dex on OPG promoter activity. On the other hand, Dex treatment per se showed a nominal increase of RANKL gene expression. A part of Dex-mediated OPG gene suppression was achieved by the suppression of β-catenin signaling. We speculate therefore that the bone resorptive action of Dex is mediated mainly by the inhibition of OPG by transrepressing the OPG gene through the AP-1 site, with a reduction (mediated mainly by the decrease in the p46 isoform of JNK) in the proportion of p-c-Jun in a JNK-dependent manner. J. Cell. Biochem. 103: 335–345, 2008. 

**Key words:** osteoprotegerin; dexamethasone; promoter; transrepression; c-Jun

Glucocorticoids (GCs) exert a multitude of effects on the immune response and are anti-inflammatory and immunosuppressive when administered therapeutically [Langenegger and Michel, 1999; Riccardi et al., 2000; Pitzalis et al., 2002]. Although GCs are used extensively to relieve inflammatory diseases, increased bone fragility attributed to osteopenia is a serious side effect of GC treatment. It is generally accepted that GC-induced bone loss is caused primarily by hypofunction and apoptosis of osteoblasts; GCs induce a 30% increase in osteoblast and osteocyte apoptosis [Weinstein et al., 1998]. At the same time, an increase in bone resorption markers (such as urinary deoxypyridinoline (DPD) excretion) is observed during GC therapy [Ardissone et al., 2002], indicating that accelerated bone resorption is also attributed to GC-induced bone loss.

Osteoclasts, derived from hematopoietic precursors of the monocyte/macrophage lineage, are multinucleated giant cells specialized in resorbing bone. Osteoclast precursors must interact with osteoblasts/stromal cells to differentiate into mature osteoclasts [Suda et al., 1992, 1999]. The protein involved in this interaction has been identified as receptor activator of NF-κB ligand (RANKL), also known as osteoclast differentiation factor (ODF) or tumor necrosis factor-related activation-induced cytokine (TRANCE) [Simonet et al., 1997; Wong

Grant sponsor: Grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan; Grant numbers: 18790243, 16590313, 16590278.

*Correspondence to: Sohei Kitazawa, Division of Molecular Pathology, Department of Biomedical Informatics, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan.
E-mail: kitazawa@med.kobe-u.ac.jp

Received 22 January 2007; Accepted 18 April 2007
DOI 10.1002/jcb.21414

et al., 1997b; Lacey et al., 1998; Nakagawa et al., 1998; Quinn et al., 1998; Yasuda et al., 1998]. A subset of osteoblasts/stromal cells, at the same time, secrete osteoprotegerin (OPG), a soluble member of the TNF receptor superfamily, that acts as a decoy receptor for RANKL and thereby prevents its interaction with the cognate receptor RANK [Anderson et al., 1997; Wong et al., 1997a; Kong et al., 1999]. OPG strongly inhibits osteoclast differentiation, survival, and function in vitro and bone resorption in vivo [Akatsu et al., 1998; Bucay et al., 1998; Hakeda et al., 1998]: mice lacking OPG show severe early onset of osteoporosis [Mizuno et al., 1998], whereas transgenic animals overexpressing OPG show osteopetrosis [Simonet et al., 1997]. RANKL and OPG are therefore the two final regulators of osteoclastogenesis, and many of the regulators of bone metabolism regulate bone volume by modulating osteoclastogenesis through this RANKL–OPG axis [Horwood et al., 1998; Murakami et al., 1998; Takai et al., 1998; Vidal et al., 1998; Brandstrom et al., 1998a,b; Nagai and Sato, 1999; Hofbauer et al., 2000]. Among these bone-seeking factors, 1α,25 dihydroxyvitamin D3 (1α,25(OH)2D3) alone [Hofbauer et al., 1998; Kondo et al., 2004] or in combination with dexamethasone (Dex) [Nagai and Sato, 1999] efficiently accelerate osteoclastogenesis by regulating the RANKL–OPG axis in a reciprocal manner. The effect of Dex per se on the RANKL–OPG axis varies, however, according to the nature or origin of osteoblastic/stromal cells. Dex upregulates the OPG gene in calvaria, a membranous bone that is resistant to GC-induced bone loss in the organ culture system [Swanson et al., 2006]. On the other hand, it downregulates OPG and upregulates RANKL reciprocally, thus increasing the RANKL/OPG ratio in the in vitro system using cultured human osteoblastic cell lines [Hofbauer et al., 1999; Harding et al., 2006].

In this study, we explored the mechanism whereby Dex stimulates osteoclastogenesis in the coculture system (using osteoblastic/stromal cells and bone marrow macrophages) by analyzing the effect of Dex on mouse OPG gene expression in osteoblasts. We used bone-marrow-derived osteoblastic/stromal cell line ST2 that corresponds to precursor osteoblastic cells in trabecular bone which is highly susceptible to GC-induced bone loss. We focused particularly on three major transcriptional mechanisms—AP-1 [Kondo et al., 2004], Runx2 [Thirunavukkarasu et al., 2000], and the Wnt/β-catenin signaling pathway [Glass et al., 2005]—that regulate OPG gene expression.

## MATERIALS AND METHODS

### Cell Culture and In Vitro Osteoclast-Like Cell Formation

Mouse bone marrow stromal cell line ST2 and C6 cells, established from the calvariae of *runx*-deficient mice, were cultured and maintained in αMEM (Sigma) supplemented with 10% fetal bovine serum (FBS, GIBCO-BRL). Both ST2 and C6 cells are categorized as immature mesenchymal cell lines capable of differentiating into osteoblastic cells. All cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. Mouse bone marrow mononucleated cells ($10^5/cm^2$), prepared as previously described [Kitazawa et al., 1995], were cocultured with ST2 cells ($10^4/cm^2$) in the presence of 1α,25$(OH)_2D_3$ ($10^{-8}$ M) and dexamethasone ($10^{-8}$ M) in multiwell plates (Becton Dickinson). At the end of the culture period, the cells were stained for tartrate resistant acid phosphatase (TRAP) with a commercial kit (Sigma), and the number of osteoclast-like TRAP positive multinucleated (more than 3) cells was counted.

### Northern Blot Analysis

Total RNA was extracted from ST2 cells by RNAzol (Tel-Test, Inc., Friendswood, TX). RNA samples (10 μg) were separated by denaturing electrophoresis in formaldehyde-agarose gels and stained with ethidium bromide. Total RNA was transferred onto Hybond N+ nylon membranes (Amersham Biosciences Corp., Piscataway, NJ) and immobilized by UV cross-linking. A 1,004 bp cDNA fragment from OPG and a 250 bp cDNA fragment from RANKL were generated by RT-PCR with the following pairs of primers.

- OPG sense: 5′-GAATGGACAACCCAGGAAACCC-3′;
- OPG antisense: 5′-GTGCTTGAGGGCATACATCAGG-3′;
- RANKL sense: 5′-TCCTAACAGAATATCAGAAGACAG-3′;
- RANKL antisense: 5′-AGGCTTGCCTCGCTGGGCCACATC-3′.

The sequence of OPG and RANKL cDNA was confirmed by direct sequencing. The cDNA fragments of OPG, RANKL, and GAPDH were labeled with [$\alpha$-$^{32}$P]dCTP by the random primer technique (Random Primer DNA labeling kit, TaKaRa, Japan). The membranes were hybridized with the labeled probe in 5× SSPE, 5× Denhardt's solution, 0.5% SDS, and 1× Background Quencher (Molecular Research Center, Cincinnati, OH) at 60°C for 16 h, washed in 2× SSPE containing 0.1% SDS, in 1× SSPE containing 0.1% SDS, and in 0.1× SSPE containing 0.1% SDS and then analyzed with image analyzer BAS2000 (FUJIX, Tokyo, Japan). The density of each band was analyzed with Image Gauge software (FUJIX, Tokyo, Japan).

### Quantitative Real-Time PCR

Total RNA (1 μg) isolated from each cell line was reverse transcribed to produce cDNA, which was then amplified and quantified by the ABI PRISM 7300 Real-Time PCR system (Applied Biosystems, Foster city, CA) using a set of primers and probes (assay ID: mouse RANKL (Mm00441908_m1), mouse OPG (Mm 00435452_m1), mouse Runx2 (Mm00501578_m1), and mouse β-catenin (Mm004803033_m1)) purchased from Applied Biosystems. The amount of mRNA was quantified relative to that of GAPDH in each reaction according to the manufacturer's protocol (Applied Biosystems).

### OPG ELISA Assay

The amount of OPG protein secreted into the culture medium by ST2 cells was analyzed by a sandwich ELISA procedure, with the use of ANALYZA (TECHNE Corporation, MN) according to the manufacturer's instructions.

### Transient Transfection Study

Nested deletion mutants generated by restriction enzyme sites (OPG: Luc-1487, Luc-1125, Luc-697, Luc-116; RANKL: Luc-1005, Luc-723, Luc-524, Luc-276) were ligated to a promoterless and enhancerless pGL3-Basic vector plasmid (Promega) as previously described [Kitazawa et al., 1999; Kitazawa and Kitazawa, 2002; Kondo et al., 2004]. For site-directed mutagenesis of the putative AP-1 binding site (−293/−287), the AP-1 core element (TGACTGA, −293/−287) in Luc-1487 was replaced with CTCCCTC by a recombinant PCR strategy as previously described [Kondo et al., 2004]. Plasmid DNA was purified with EndoFree Plasmid Maxi kit (QIAGEN). Each plasmid construct was cotransfected with the phRG-TK vector (Promega) into ST2 cells via liposome-mediated transfection, using Lipofectamine Reagent (Invitrogen), according to the manufacturer's instructions. pGL3-Control vector (Promega) was also transfected as a positive control. Transfected cells were cultured in αMEM (ST2) supplemented with 10% FBS. The cells were harvested 48 h after transfection, and luciferase activity from cell lysates was determined with a luminometer (Model ATP-3010, Advantec, Japan). The transcription efficiency of each construct was standardized by the activity of Renilla luciferase and of a basal luciferase construct.

### Immunoblotting

ST2 cells were treated with Dex ($10^{-8}$ M) for 5 min, 10 min, 30 min, 1, 3, 6, 12, 24 h, and the treated cells in 100 mm culture dishes were then lysed with 0.5 ml lysis buffer (50 mM Tris–HCl, pH 8.0, 100 nM NaCl, 0.2% Nonidet P-40, 0.2% sodium deoxycholate, 0.1% SDS, and 50 μm/ml aprotinin) and a protease inhibitor cocktail tablet (Boehringer Mannheim). The lysates were centrifuged at 15,000*g* for 10 min at 4°C and the supernatants were stored at −80°C. Equal amounts of protein were separated by SDS-PAGE, electrotransferred to nitrocellulose membranes (Trans-Blot) from Bio-Rad Laboratories (Hercules, CA), and immunoblotted with primary antibodies against c-Jun and c-Fos (from Santa Cruz Biotechnology) and phospho-c-Jun (Ser63), phosphorylated Jun N-terminal kinase (phospho-JNK (Thr183/Tyr185)), phosphorylated SAPK/Erk kinase (phospho-SEK1/MKK4 (Thr261)), phosphorylated p44/42 MAP Kinase (Erk1 and Erk2) (phospho-p44/42 MAP Kinase (Thr202/Tyr204)), phosphorylated p38 MAP Kinase (phospho-p38 MAP Kinase (Thr180/Tyr204)), β-catenin, and phosphorylated β-catenin (phospho-β-catenin (Ser33/37/Thr41)) (from Cell Signaling Technology). Detection of immunocomplexes was carried out with the enhanced chemiluminescence (ECL) kit and Hyperfilm ECL from Amersham.

### Mitogen-Activated Protein (MAP) Kinase Inhibitors

To assess their effect on OPG promoter activity (Luc-1487), the transfected cells were treated with three MAP kinase inhibitors: JNK

inhibitor (SP600125, BIOMOL Research Labs, 50 μM), ERK inhibitor (PD98059, Calbiochem, 10 μM), and p38 inhibitor (SB203580, Calbiochem, 30 μM).

### Transfection of Small Interfering (si)RNA

SiRNA against mouse Runx2 was synthesized by Ambion. Silencer Negative Control siRNA (Ambion) was used as a nonspecific control. For the transfection of siRNA, the cells were washed and resuspended in Opti-MEM (GIBCO). SiRNA was transfected into ST2 cells with the use of siPORT Amine Transfection Agent (Ambion) according to the manufacturer's instructions. The cells were then cultured for an additional 48-h before quantitative real-time PCR.

### Statistical Analysis

Data are expressed as means ± SD. Statistical analyses were carried out by Student's *t*-test. The level of significance was taken to be $P < 0.05$.

## RESULTS

### Coculture of ST2 Cells and Mouse Bone Marrow Macrophages

With Dex alone and with $1\alpha,25(OH)_2D_3$ and Dex, ST2 cells supported the formation of TRAP-positive multinucleated cells (Fig. 1A,B). The expression of OPG transcripts was suppressed by treatment with Dex alone and with Dex and $1\alpha,25(OH)_2D_3$ (Fig. 1C).

### Dose Response and Time Course of OPG Protein Production After Dex Treatment Assessed by ELISA

ELISA showed that the secretion of OPG protein into the culture medium decreased significantly after the treatment of ST2 cells with as little as $10^{-9}$ M of Dex (Fig. 2A); moreover, this strong inhibitory effect of Dex was observed as early as 6-h after the treatment and lasted for at least 48-h (Fig. 2B) during which no cytotoxic effect was observed.

### OPG and RANKL mRNA Expression in ST2 Cells

Northern blot analysis showed that a 24-h treatment with Dex ($10^{-9}$ M) suppressed the OPG mRNA expression in ST2 cells (Fig. 3, upper panel), whereas it slightly increased the expression of RANKL mRNA (Fig. 3, lower panel) in a reciprocal manner.

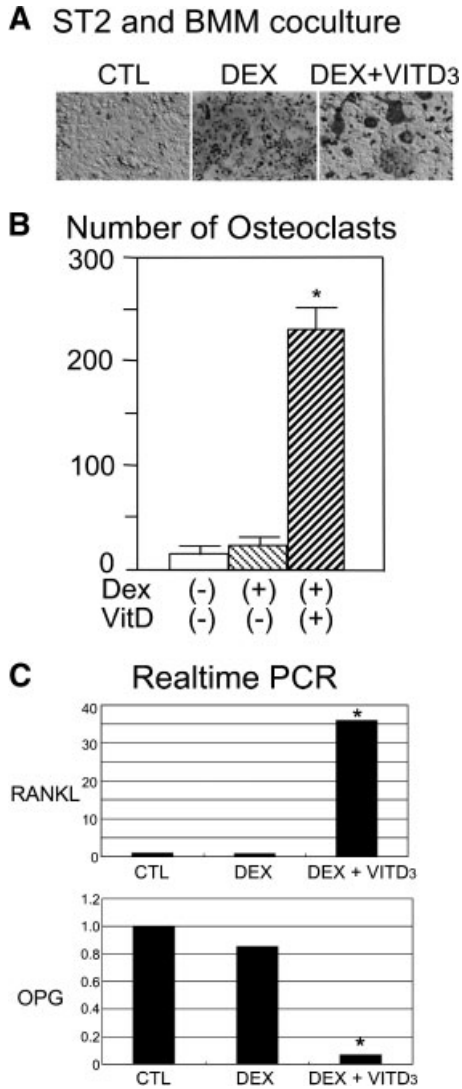


**Fig. 1.** Coculture of ST2 cells and mouse bone marrow macrophages (BMM). **A**,**B**: Mouse bone marrow macrophages ($10^5$/cm$^2$) and ST2 cells ($10^4$/cm$^2$) were cocultured with and without 10 nM $1\alpha,25(OH)_2D_3$ and dexamethasone (Dex) for 7 days and then stained for TRAP. With Dex alone (middle panel) and with $1\alpha,25(OH)_2D_3$ and Dex (right panel), ST2 cells supported the formation of TRAP-positive multinucleated cells. The number of osteoclast-like TRAP-positive multinucleated (more than 3 nuclei) cells was counted (B). **C**: By quantitative RT-PCR, in the presence of 10 nM Dex and $1\alpha,25(OH)_2D_3$, RANKL mRNA increased significantly. On the other hand, the expression of OPG transcripts was suppressed by treatment with Dex alone and with Dex and $1\alpha,25(OH)_2D_3$. Results are expressed as the relative mRNA amount standardized by GAPDH.

### Promoter Activity of Each Deletion Construct (OPG)

A series of deletion constructs of the mouse OPG promoter (Luc-116, Luc-697, Luc-1125, Luc-1487) was transfected into ST2 cells and

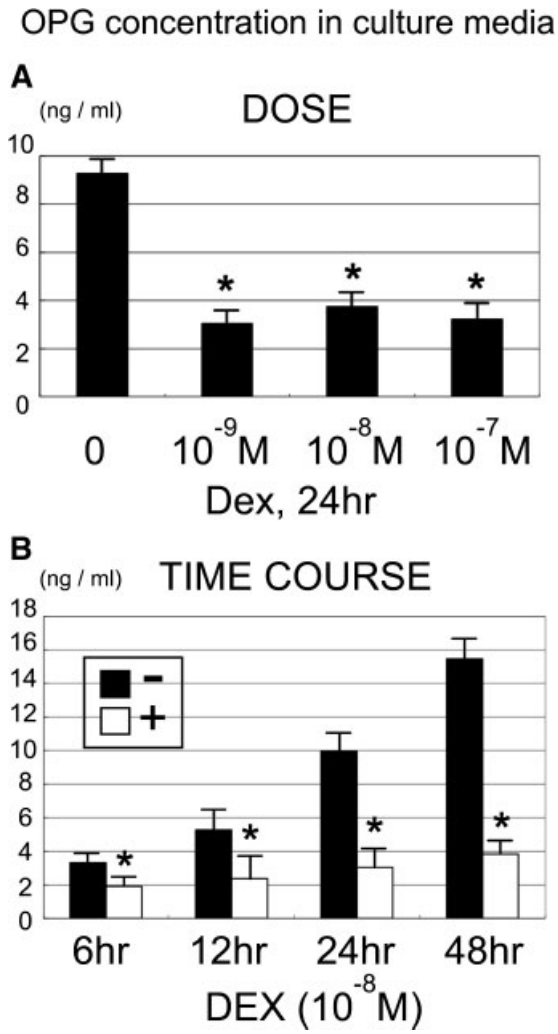


**Fig. 2.** Dose response and time course of OPG protein production after Dex treatment assessed by ELISA. To assess the effect of Dex on OPG at the protein level, the amount of OPG protein secreted by ST2 cells was measured. The cells were treated with either the vehicle (ethanol) or Dex. The secretion of OPG protein decreased after treatment with as little as $10^{-9}$ M of Dex (**A**). The strong inhibitory effect of Dex on OPG secretion was observed as early as 6 h after treatment and lasted for at least 48 h (**B**). $*P < 0.05$ versus non-treated cells.

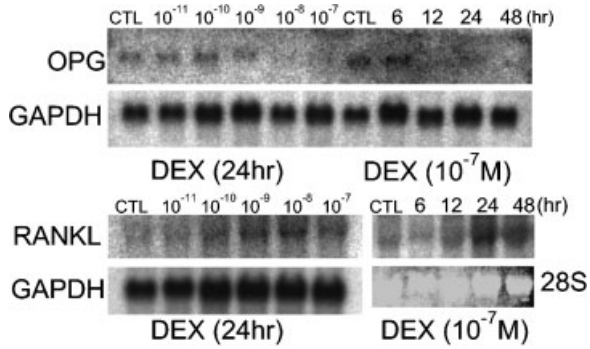


**Fig. 3.** OPG and RANKL mRNA expression in ST2 cells. Northern blot analysis showed that a 24-h treatment with Dex ($10^{-7}$ M) suppressed the expression of the OPG gene in ST2 cells (**upper panel**). On the other hand, Dex ($10^{-7}$ M) slightly increased the expression of RANKL mRNA in a reciprocal manner (**lower panel**).

subjected to luciferase assay. Mirroring Northern blot analysis, Dex reduced the promoter activity of Luc-1487 to 50% (Fig. 4A). Furthermore, introduction of mutation to the AP-1 site (−293/−287) showed a reduction in luciferase activity and completely nullified the inhibitory effect of Dex on the promoter (Fig. 4B).

### Promoter Activity of Each Deletion Construct (RANKL)

A series of deletion constructs of the mouse RANKL promoter (Luc-1005, Luc-723, Luc-524 Luc-185) was transfected into ST2 cells and subjected to luciferase assay (Fig. 5). Dex slightly increased RANKL promoter activity. Deletion mutant studies suggested that Dex upregulated RANKL through the putative GRE half sites (−642/−628) or the AP-1 sites in the promoter.

### Phosphorylation of MAP Kinase and Effects of MAP Kinase Inhibitors On Promoter Activity (OPG)

Since the mutation of the AP-1 site negated Dex-driven OPG suppression and since AP-1 is a major downstream effector for MAPK, we focused on the mitogen-activated protein (MAP) kinase signaling cascade. In agreement with the transient transfection studies, Western blot analysis (Fig. 6A) showed that the amount of phosphorylated c-Jun protein (p-c-Jun) decreased 1hr after treatment of ST2 cells with Dex, whereas the total amount of c-Jun protein decreased after 12 h. Corresponding to the decrease of p-c-Jun, the amount of phosphorylated p46 isoform of JNK decreased significantly 30 min after Dex treatment, whereas that of phosphorylated p54 isoform decreased after 12 h. The amount of phosphorylated SEK1 also decreased significantly 30 min after Dex treatment. In contrast, the amounts of p-ERK and p-p38 remained constant. The transfected cells were treated with three MAP kinase inhibitors: JNK inhibitor (SP600125), ERK inhibitor (PD98059) and p38 inhibitor (SB203580) to assess their effect on OPG promoter activity (Fig. 6B). Of these, only the

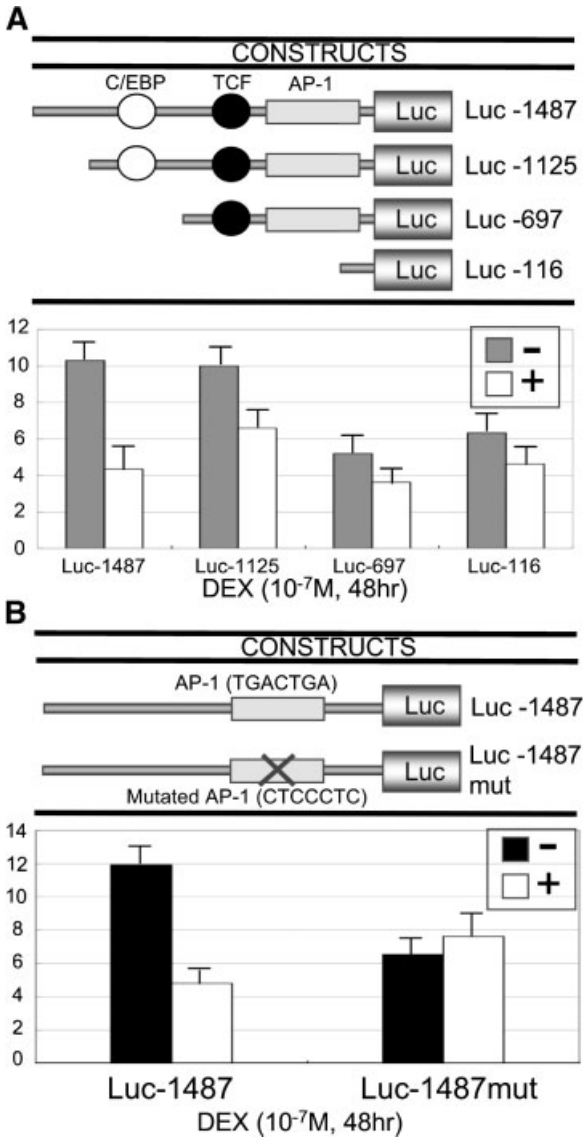


**Fig. 4.** Promoter activity of each deletion construct (OPG). A series of deletion constructs of the mouse OPG promoter (Luc-116, Luc-697, Luc-1125, Luc-1487) was transfected into ST2 cells and subjected to luciferase assay. Transfected cells were treated with Dex to assess its effect on OPG promoter activity. Mirroring Northern blot analysis, Dex reduced the promoter activity of Luc-1487 to 50% (**A**). Furthermore, introduction of mutation to the AP-1 site showed a reduction in luciferase activity and completely nullified the inhibitory effect of Dex on the promoter (**B**).

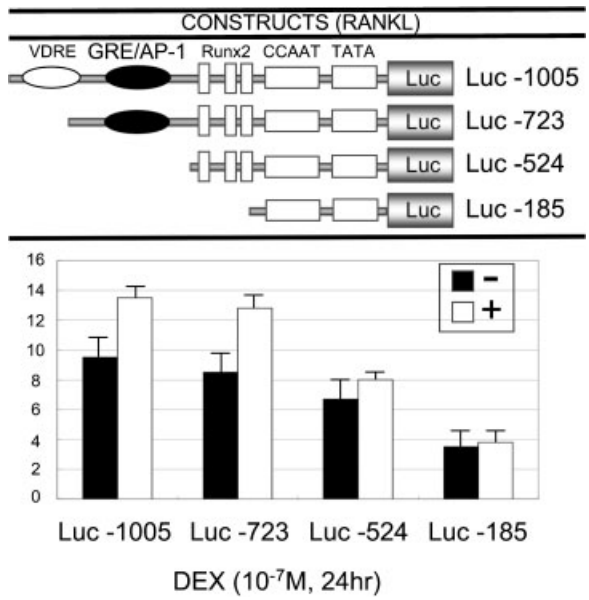


**Fig. 5.** Promoter activity of each deletion construct (RANKL). A series of deletion constructs of the mouse RANKL promoter (Luc-1005, Luc-723, Luc-524, Luc-185) was transfected into ST2 cells and subjected to luciferase assay. Transfected cells were treated with Dex to assess its effect on RANKL promoter activity. Dex slightly increased RANKL promoter activity. Deletion mutant studies suggested that Dex upregulated RANKL through the putative GRE half sites (−642/−628) or the AP-1 sites in the promoter.

JNK inhibitor (SP600125) mimicked the inhibitory effect of Dex.

### Involvement of Runx2 and β-Catenin Signaling in Dex-Mediated OPG Suppression

Because Runx2 and β-catenin signaling pathways are reportedly involved in OPG expression [Thirunavukkarasu et al., 2000; Glass et al., 2005], we tested the effect of Dex on these two factors. Dex treatment downregulated the Runx2 transcript and the β-catenin protein (Fig. 7A). Real-time RT-PCR analysis of Runx2-deficient mouse-derived stromal cell line C6 revealed that while steady-state transcription of the OPG gene in C6 cells was 2 times that in ST2 cells (Fig. 7B), Dex reduced OPG expression to the same extent (84% and 81%, respectively) in both cells (Fig. 7B). Although Dex reduced Runx2 expression in ST2 (Fig. 7A), Runx2 knockdown, established by the transfection of Runx2 siRNA, showed a nominal reduction in OPG mRNA expression in ST2 cells (Fig. 7B), indicating that the inhibitory effect of Dex on OPG expression is independent of Runx2. On the other hand, OPG promoter activity of Luc-1487 was reduced to 70% in ST2 cells treated with LiCl (Fig. 7C), an agonist of the β-catenin signaling pathway, indicating that the Wnt-β-catenin signaling pathway is partially involved in the Dex-mediated downregulation of OPG.

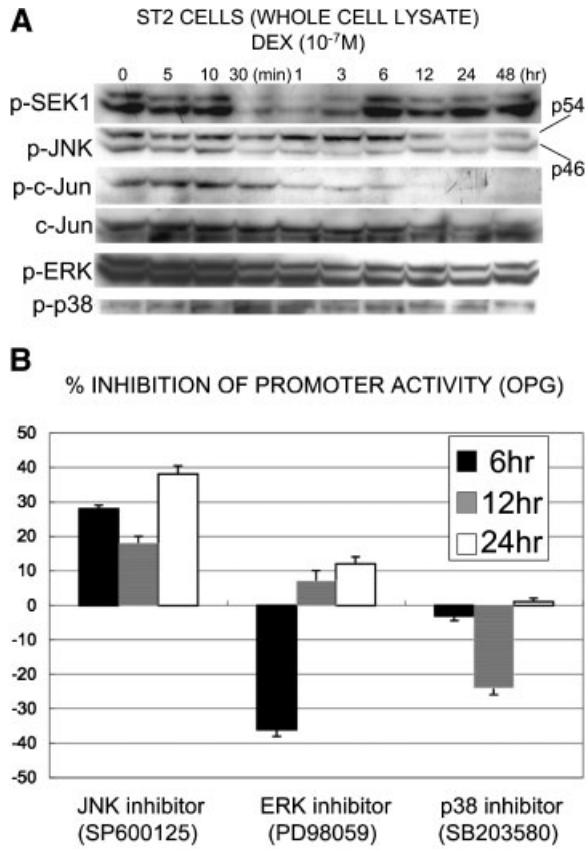


**Fig. 6.** Phosphorylation of MAP kinase and effects of MAP kinase inhibitors on promoter activity (OPG). **A**: ST2 cells were treated with Dex ($10^{-7}$ M) for 5 min, 10 min, 30 min, 1, 3, 6, 12, 24, 48 h, and whole cell lysates prepared from the treated cells were subjected to Western blot analysis. The antibodies used were against c-Jun, phospho-c-Jun (serine 63), phosphorylated Jun N-terminal kinase (JNK, threonine 183/tyrosine 185), phosphorylated SAPK/Erk kinase (SEK1, threonine 261), phospho-p44/42 MAP Kinase (p-ERK, threonine 202/tyrosine 204) and phosphorylated p38 MAP Kinase (p-p38, threonine 180/tyrosine 182). In agreement with the transient transfection studies, the amount of phosphorylated c-Jun protein (p-c-Jun) decreased 1hr after treatment of ST2 cells with Dex, whereas the total amount of c-Jun protein decreased after 12 h. Corresponding to the decrease of p-c-Jun, the amount of phosphorylated p46 isoform of JNK decreased significantly 30 min after Dex treatment, whereas that of phosphorylated p54 isoform decreased after 12 h. The amount of phosphorylated SEK1 also decreased significantly 30 min after Dex treatment. In contrast, the amounts of p-ERK and p-p38 were constant. **B**: The transfected cells were treated with three MAP kinase inhibitors: JNK inhibitor (SP600125), ERK inhibitor (PD98059), and p38 inhibitor (SB203580) to assess their effect on OPG promoter activity (Luc-1487). Only the JNK inhibitor (SP600125) mimicked the inhibitory effect of Dex. The inhibitory effect of each MAP kinase inhibitor is expressed as % inhibition of promoter activity. Results are expressed as means ± SD of three assays.

## DISCUSSION

GC-induced bone loss is now interpreted primarily as hypofunction and apoptosis of osteoblasts during the progressive phase, and secondarily as accelerated bone resorption especially during the rapid or early phase. Although the latter process is a major therapeutic target of GC-induced bone loss, its precise mechanism is not fully understood. In this study, we analyzed the effect of Dex on mouse OPG and RANKL expression and found that Dex accelerated osteoclastogenesis mainly by suppressing OPG expression at both transcriptional and protein secretion levels of ST2 osteoblasts.

Dex treatment per se, and in combination with $1\alpha,25(OH)_2D_3$, reciprocally regulated RANKL and OPG mRNA expression, although its exclusive effect on RANKL and OPG was small (Fig. 1). Additionally, OPG secretion into the culture media was modulated by as little as $10^{-9}$ M of Dex and as early as 6 h after treatment (Fig. 2), indicating, as is often the case with GC function, that Dex modulates OPG expression at multiple levels—from mRNA transcription to protein secretion. Since three major transcriptional mechanisms, namely AP-1 [Kondo et al., 2004], Runx2 [Thirunavukkarasu et al., 2000], and the canonical Wnt/β-catenin signaling pathway [Glass et al., 2005], regulate OPG gene expression, we extended our study to explore Dex function on the OPG gene at the transcriptional level, with special focus on the three mechanisms above.

First, as we have reported [Kondo et al., 2004], mainly the c-Jun homodimer binds to the AP-1 site in the mouse OPG promoter and maintains steady-state transcription of the OPG gene. Several mechanisms by which GCs inhibit AP-1 activity have been proposed [Hirasawa et al., 2003]: (1) suppression of the activation of JNK; (2) competitive reduction of the interaction between AP-1 and the transcriptional coactivator CREB binding protein (CBP) or p300 via the interaction of glucocorticoid receptor (GR) with CBP/p300; (3) inhibition of the binding of AP-1 to DNA by direct binding of GR to AP-1; and (4) induction of the inhibitory protein, glucocorticoid-induced leucine zipper (GILZ). In the first mechanism, it is hypothesized that GR disrupts the binding of JNK to c-Jun resulting in the inhibition of the phosphorylation of c-Jun by JNK [Hirasawa et al.,

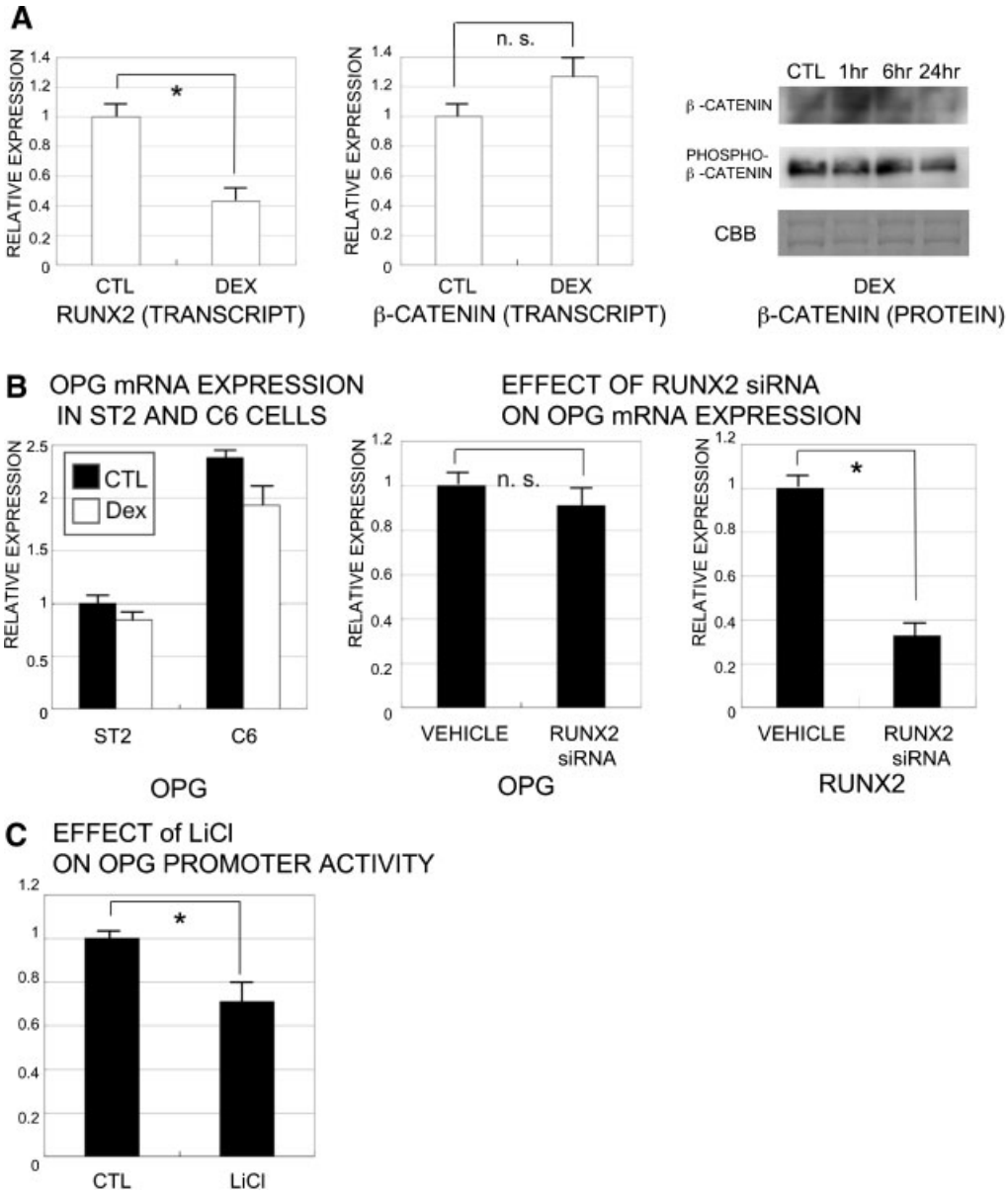


**Fig. 7.** Involvement of Runx2 and β-catenin signaling in Dex-mediated OPG suppression. **A**: Real-time RT-PCR analysis revealed that Dex decreased Runx2 mRNA expression in ST2 cells (**left panel**). Dex decreased β-catenin protein by a 24-h treatment (**right panel**) although it slightly increased β-catenin mRNA expression. Dex slightly increased phospho-β-catenin protein by a 6-h treatment. **B**: The steady-state transcription of the OPG gene in C6 cells, established from calvariae of *runx2*-deficient mice, was 2 times that in ST2 cells. Dex reduced OPG expression to the same extent in both cells. The transfection of Runx2 siRNA slightly decreased OPG mRNA expression in ST2 cells. **C**: LiCl (30 μM), an agonist of the β-catenin signaling pathway, reduced the promoter activity of Luc-1487 to 70%.

2003]. In addition, since GR is phosphorylated by JNK [Rogatsky et al., 1998], Hirasawa et al. [2003] have speculated that the interaction between GR and JNK is involved in the inhibition of the phosphorylation of c-Jun. In the present study, Western blot analysis showed that the amount of phospho-c-Jun protein (p-c-Jun) decreased after the treatment of ST2 with Dex; correspondingly, the amount of the phosphorylated p46 isoform of JNK decreased as early as 30 min after the treatment, whereas that of p54 decreased after 12 h (Fig. 6A), suggesting that Dex inhibits the c-Jun signaling pathway by suppressing both p46 and p54 isoforms of JNK simultaneously but separately. In contrast, the amounts of p-ERK and

p-p38 remained constant after Dex treatment, suggesting that Dex selectively modulates the JNK pathway in ST2 cells. This was further confirmed by the assessment with MAP kinase inhibitors showing that selective JNK inhibitor SP600125 mimicked the action of Dex on OPG gene promoter activity (Fig. 6B). Our data suggest that Dex negatively regulates OPG by transrepressing the OPG gene through the AP-1 site via a reduction in the proportion of p-c-Jun in a JNK-dependent manner; also the reduction is mediated mainly by the decrease in the p46 isoform. Reportedly, $1\alpha,25(OH)_2D_3$ also suppresses OPG gene expression via the AP-1 site but in a JNK-independent manner [Kondo et al., 2004]. Taken together, these results may explain the synergistic suppressive action of Dex and $1\alpha,25(OH)_2D_3$ on OPG expression through both JNK-dependent and JNK-independent pathways.

Second, since the cloning of the human OPG gene promoter region has revealed that Runx2, osteoblast-specific transcription factor, transactivates the gene [Thirunavukkarasu et al., 2000], we tested the effects of Runx2 on mouse OPG gene expression and on the inhibition of OPG by Dex. While the steady-state transcription of the OPG gene in C6 cells was two times that in ST2 cells, Dex reduced OPG expression to the same extent in both cells, indicating that the inhibitory effect of Dex on OPG expression is independent of Runx2. Moreover, Runx2 knockdown by the transfection of Runx2 siRNA showed a minimal effect on OPG mRNA in ST2 cells. Together with the fact that canonical binding elements for Runx2 are not present within the mouse OPG promoter region (1-3261) we have previously tested [Kondo et al., 2004], Runx2 may not be essential for either mouse OPG gene expression or Dex-mediated mouse OPG gene suppression.

As for the third mechanism, OPG expression is regulated by the Wnt-β-catenin signaling pathway through three potential TCF binding sites within the mouse OPG promoter [Glass et al., 2005]. In the present study, because Dex treatment downregulated the β-catenin protein expression (Fig. 7A, right panel), and LiCl, an agonist of the β-catenin signaling, also downregulated OPG promoter activity (Fig. 7C), Wnt-β-catenin signaling may be partially involved in Dex-mediated OPG gene suppression. Indeed, in cultured human osteoblasts, β-catenin protein expression is downregulated by glucocorticoid treatment through the enhancement of the production of dickkopf-1 [Ohnaka et al., 2005]. A similar mechanism may be involved in Dex-driven OPG suppression in mouse ST2 cells.

On the other hand, Dex slightly increased the expression of RANKL mRNA. Deletion mutant studies suggested that Dex upregulated RANKL through either the putative GRE half sites (−642/−628) or the AP-1 sites in the promoter. We speculate therefore that GCs per se promote osteoclastogenesis mainly by inhibiting OPG and partly by concurrently stimulating RANKL in a reciprocal manner, thereby enhancing bone resorption. Recently, the paper by the Teitelbaum group has shown that glucocorticoids modulate osteoclastogenesis by affecting M-CSF activation of RhoA, Rac, and Vav3 systems in osteoclasts. Also, $Gr^{oc-/-}$ mice are spared the impact of Dex on osteoclasts and their precursors [Kim et al., 2006]. Because M-CSF and the RANKL–OPG axis are two major regulators of osteoclasts, these data show that glucocorticoids play a prominent role in osteoclastogenesis by influencing multiple levels of multiple systems both directly and indirectly.

## ACKNOWLEDGMENTS

This work was supported in part by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (18790243 to TK, 16590313 to RK, and 16590278 to SK). We thank Shuichi Matsuda and Noriko Sakamoto for excellent technical assistance.

## REFERENCES

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.

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.

Ardissone P, Rota E, Durelli L, Limone P, Isaia GC. 2002. Effects of high doses of corticosteroids on bone metabolism. J Endocrinol Invest 25:129–133.

Brandstrom H, Jonsson KB, Ohlsson C, Vidal O, Ljunghall S, Ljunggren O. 1998a. Regulation of osteoprotegerin

mRNA levels by prostaglandin E2 in human bone marrow stroma cells. Biochem Biophys Res Commun 247:338–341.

Brandstrom H, Jonsson KB, Vidal O, Ljunghall S, Ohlsson C, Ljunggren O. 1998b. Tumor necrosis factor-alpha and -beta upregulate the levels of osteoprotegerin mRNA in human osteosarcoma MG-63 cells. Biochem Biophys Res Commun 248:454–457.

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.

Glass DA II, 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.

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 Commun 251:796–801.

Harding G, Mak YT, Evans B, Cheung J, MacDonald D, Hampson G. 2006. The effects of dexamethasone and dehydroepiandrosterone (DHEA) on cytokines and receptor expression in a human osteoblastic cell line: Potential steroid-sparing role for DHEA. Cytokine 36: 57–68.

Hirasawa N, Izumi S, Linwong W, Ohuchi K. 2003. Inhibition by dexamethasone of interleukin 13 production via glucocorticoid receptor-mediated inhibition of c-Jun phosphorylation. FEBS Lett 554:489–493.

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 Commun 250:776–781.

Hofbauer LC, Gori F, Riggs B, Lacey DL, Dunstan CR, 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.

Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL. 2000. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. J Bone Miner Res 15:2–12.

Horwood NJ, Elliott J, Martin TJ, Gillespie MT. 1998. Osteotropic agents regulate the expression of osteoclast differentiation factor and osteoprotegerin in osteoblastic stromal cells. Endocrinology 139:4743–4746.

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.

Kitazawa R, Kitazawa S. 2002. Vitamin $D_3$ augments osteoclastogenesis via Vitamin D-responsive element of mouse RANKL gene promoter. Biochem Biophys Res Commun 290:650–655.

Kitazawa S, Ross FP, McHugh K, Teitelbaum SL. 1995. Interleukin-4 induces expression of the integrin alpha v beta 3 via transactivation of the beta 3 gene. J Biol Chem 270:4115–4120.

Kitazawa R, Kitazawa S, Maeda S. 1999. Promoter structure of mouse RANKL/TRANCE/OPG/ODF gene. Biochem Biophys Acta 1445:134–141.

Kondo T, Kitazawa R, Maeda S, Kitazawa S. 2004. 1 alpha,25 dihydroxyvitamin $D_3$ rapidly regulates the mouse osteoprotegerin gene through dual pathways. J Bone Miner Res 19:1411–1419.

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 CR, 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.

Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capperelli 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.

Langenegger T, Michel BA. 1999. Drug treatment for rheumatoid arthritis. Clin Orthop Relat Res 366:22–30.

Mizuno A, Amizuka N, Irie K, Murakami A, Fujise N, Kanno T, Sato Y, Nakagawa N, Yasuda H, Mochizuki S, 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 Commun 247:610–615.

Murakami T, Yamamoto M, Ono K, Nishikawa M, Nagata N, Motoyoshi K, Akatsu T. 1998. Transforming growth factor-beta1 increases mRNA levels of osteoclastogenesis inhibitory factor in osteoblastic/stromal cells and inhibits the survival of murine osteoclast-like cells. Biochem Biophys Res Commun 252:747–752.

Nagai M, Sato N. 1999. Reciprocal gene expression of osteoclastogenesis inhibitory factor and osteoclast differentiation factor regulates osteoclast formation. Biochem Biophys Res Commun 257:719–723.

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 Commun 253:395–400.

Ohnaka K, Tanabe M, Kawate H, Nawata H, Takayanagi R. 2005. Glucocorticoid suppresses the canonical Wnt signal in cultured human osteoblasts. Biochem Biophys Res Commun 329:177–181.

Pitzalis C, Pipitone N, Perretti M. 2002. Regulation of leukocyte-endothelial interactions by glucocorticoids. Ann NY Acad Sci 966:108–118.

Quinn JMW, 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.

Riccardi C, Zollo O, Nocentini G, Bruscoli S, Bartoli A, D'Adamio F, Cannarile L, Delfino D, Ayroldi E, Migliorati G. 2000. Glucocorticoid hormones in the regulation of cell death. Therapie 55:165–169.

Rogatsky I, Logan SK, Garabedian MJ. 1998. Antagonism of glucocorticoid receptor transcriptional activation by the c-Jun N-terminal kinase. Proc Natl Acad Sci USA 95:2050–2055.

Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone L, Shimamoto G, DeRose M, Elliott 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, Amgen EST program, 1997. Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. Cell 89:309–319.

Suda T, Takahashi N, Martin TJ. 1992. Modulation of osteoclast differentiation. Endocr Rev 13:66–80.

Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. 1999. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 20:345–357.

Swanson C, Lorentzon M, Conaway H, Lerner UH. 2006. Glucocorticoid regulation of osteoclast differentiation and expression of receptor activator of nuclear factor κB (NF-κB) ligand, osteoprotegerin, and receptor activator of NF-κB in mouse calvarial bones. Endocrinology 147: 3613–3622.

Takai H, Kanematsu M, Yano K, Tsusa E, Higashio K, Ikeda K, Watanabe K, Yamada Y. 1998. Transforming growth factor-beta stimulates the production of osteoprotegerin/osteoclastogenesis inhibitory factor by bone marrow stromal cells. J Biol Chem 273:27091–27096.

Thirunavukkarasu K, Halladay DL, Miles RR, Yang X, Galvin RJ, Chandrasekhar S, Martin TJ, Onyia JE. 2000. The 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.

Vidal ON, Sjogren K, Eriksson BI, Ljunggren O, Ohlsson C. 1998. Osteoprotegerin mRNA is increased by interleukin-1 alpha in the human osteosarcoma cell line MG-63 and in human osteoblast-like cells. Biochem Biophys Res Commun 248:696–700.

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.

Wong BR, Josien R, Lee SY, Sauter B, Li HL, Steinman RM, Choi Y. 1997a. 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.

Wong BR, Rho J, Arron J, Robinson E, Orlinick J, Chao M, Kalachikov S, Cayani E, Barlett FS III, Frankel WN, Lee SY, Choi Y. 1997b. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. J Biol Chem 272:25190–25194.

Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. 1998. A novel molecular mechanism modulating osteoclast differentiation and function. Proc Natl Acad Sci USA 95:3597–3602.