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Retinoids Stimulate Periosteal Bone Resorption by Enhancing the Protein RANKL, a Response Inhibited by Monomeric Glucocorticoid Receptor*[S]

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H. Herschel Conaway[‡], Amir Pirhayati[§], Emma Persson[§1], Ulrika Pettersson[¶], Olle Svensson[‖], Catharina Lindholm[**], Petra Henning[**], Jan Tuckermann[‡‡], and Ulf H. Lerner[§2]**

*From the [‡]Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205, the Departments of [§]Molecular Periodontology, [¶]Clinical Pharmacology, and [‖]Orthopedic Surgery, Umeå University, SE-901 87 Umeå, Sweden, the **Center for Bone and Arthritis Research at the Institute for Medicine, Sahlgrenska Academy at the University of Gothenburg, SE-41345 Gothenburg, Sweden, and [‡‡]Tissue-specific Hormone Action, Leibniz Institute for Age Research, Fritz Lipmann Institute, D-07745 Jena, Germany*

Increased vitamin A (retinol) intake has been suggested to increase bone fragility. In the present study, we investigated effects of retinoids on bone resorption in cultured neonatal mouse calvarial bones and their interaction with glucocorticoids (GC). All-*trans*-retinoic acid (ATRA), retinol, retinalaldehyde, and 9-*cis*-retinoic acid stimulated release of ^{45}Ca from calvarial bones. The resorptive effect of ATRA was characterized by mRNA expression of genes associated with osteoclast differentiation, enhanced osteoclast number, and bone matrix degradation. In addition, the RANKL/OPG ratio was increased by ATRA, release of ^{45}Ca stimulated by ATRA was blocked by exogenous OPG, and mRNA expression of genes associated with bone formation was decreased by ATRA. All retinoid acid receptors (RARα/β/γ) were expressed in calvarial bones. Agonists with affinity to all receptor subtypes or specifically to RARα enhanced the release of ^{45}Ca and mRNA expression of *Rankl*, whereas agonists with affinity to RARβ/γ or RARγ had no effects. Stimulation of *Rankl* mRNA by ATRA was competitively inhibited by the RARα antagonist GR110. Exposure of calvarial bones to GC inhibited the stimulatory effects of ATRA on ^{45}Ca release and *Rankl* mRNA and protein expression. This inhibitory effect was reversed by the glucocorticoid receptor (GR) antagonist RU 486. Increased *Rankl* mRNA stimulated by ATRA was also blocked by GC in calvarial bones from mice with a GR mutation that blocks dimerization (GR^{dim} mice). The data suggest that ATRA enhances periosteal bone resorption by increasing the RANKL/OPG ratio via RARα receptors, a response that can be inhibited by monomeric GR.

Vitamin A (retinol) plays an essential role in numerous biological processes. These include such things as vision; the proliferation, differentiation, and apoptosis of cells; organ development and function; and the immune system (1–5). Numerous malformations and impaired vision, growth, organ function, and reproduction are noted with vitamin A deficiency (1–4).

Supplementation of the diet with vitamin A is a common occurrence in developed countries. In recent years, there has been debate over whether increased intake of vitamin A might promote skeletal fragility. Some studies have shown that high vitamin A intake and serum retinol levels are associated with an increased incidence of hip fracture or decreased bone mass (6–9). Other studies have shown no deleterious effects on bone mass and fracture risk and, in some instances, protection from bone loss when vitamin A is increased (10, 11).

Glucocorticoids (GCs)[3] are used extensively as anti-inflammatory agents, but unfortunately, the compounds have a number of undesirable side effects. A rapidly developing side effect of GC therapy is GC-induced osteoporosis, which represents the most common form of secondary osteoporosis (12–14). GC-induced osteoporosis is suggested to occur in two phases. An initial, rapid phase of bone loss is thought to be due to excessive bone resorption. This is followed by a second, slower phase believed to be due primarily to decreased bone formation (12).

GCs have been observed to oppose vitamin A in numerous biological systems (15–19), including bone (20). Previous studies have shown that both GCs and vitamin A derivatives (retinoids) are good stimulators of periosteal bone resorption, but when these compounds are added together, decreased resorption occurs (20). Neither the bone cells nor the mechanism(s) responsible for this negative co-operation have been defined.

Effects of vitamin A are mediated primarily by two families of nuclear hormone receptors, retinoic acid receptors (RARs) and

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[1] Present address: Dept. of Oncology, Umeå University, SE-901 87 Umeå, Sweden.

[2] To whom correspondence should be addressed: Dept. of Molecular Periodontology, University of Umeå, SE-901 87 Umeå, Sweden. Tel.: 46-90-785-62-90; Fax: 46-90-13-92-89; E-mail: Ulf.Lerner@odont.umu.se.

[3] The abbreviations used are: GC, glucocorticoid; ATRA, all-*trans*-retinoic acid; CT, calcitonin; DEX, dexamethasone; GR, glucocorticoid receptor(s); M-CSF, macrophage colony-stimulating factor; OPG, osteoprotegerin; RA, retinoic acid; RANKL, receptor activator of nuclear factor κB ligand; RAR, retinoic acid receptor; RXR, retinoic acid X receptor; D3, 1,25(OH)$_2$-vitamin D3; PTH, parathyroid hormone 1–34; AHPrBP, 3-amino-1-hydroxypropylidene-1,1-bisphosphonate; TTNPB, 4-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid.

the retinoid X receptors (RXRs) (21). Each receptor family is made up of three isoforms (α, β, and γ), produced by separate genes (22). All-*trans*-retinoic acid (ATRA), the major biologically active derivative of vitamin A, binds RAR partners of RAR/RXR heterodimers (22). Activated RAR/RXR heterodimers function as transcription factors, activating specific RA response elements for transcriptional regulation of target genes (21, 22).

GCs are believed to exert their actions primarily by genomic mechanisms utilizing the glucocorticoid receptor (GR) (23). The unligated GR is found in the cytoplasm of cells as a component of a protein complex composed of heat shock proteins, chaperons, and mitogen-activated protein kinases. Ligation of GR leads to dissociation from the complex and translocation into the nucleus. Within the nucleus, GR can alter gene expression by several mechanisms, including GR dimers activating gene transcription by binding to promoters of GR-regulated genes and interaction of GR monomers with transcription factors, such as AP-1, NF-κB, IRF-3, and STAT5 (23).

The effect of vitamin A on osteoclastogenesis and bone resorption has been studied the most thoroughly in rodent models (20, 24–26). Here, the action of ATRA is thought to be 2-fold. In neonatal mouse calvariae (20, 25), ATRA has been observed to be a potent stimulator of osteoclast formation and bone resorption. This is in contrast to studies with bone marrow cells and bone marrow macrophages, which show inhibition of osteoclastogenesis by ATRA and other retinoid derivatives (24). These studies suggest that ATRA may stimulate periosteal bone resorption while inhibiting cancellous bone resorption and are in good agreement with *in vivo* experiments in rodent models showing that retinoid administration leads to skeletal loss due to significant stimulation of periosteal resorption, which occurs at the same time that cancellous bone resorption is inhibited (26).

Osteoclasts are large multinucleated tartrate-resistant acid phosphatase-positive cells responsible for bone resorption. Macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) are products of stromal cells/osteoblasts that play key roles in regulating osteoclast formation (27–30). M-CSF binds to its receptor c-FMS and supports osteoclast survival, whereas RANKL enhances osteoclast differentiation and function by binding to RANK on osteoclast progenitor cells (27–30). Mice that are deficient in M-CSF, RANKL, or RANK do not have functional osteoclasts and develop osteopetrosis. Osteoprotegerin (OPG), a soluble protein released from stromal cells/osteoblasts, is another key factor regulating osteoclastogenesis (27–30). OPG functions as a decoy receptor for RANKL, blocking interaction between RANKL and RANK. Multiple fractures, numerous osteoclasts, and decreased trabecular bone volume are characteristic of mice with a targeted deletion of *Opg*.

GCs increase the RANKL/OPG ratio and increase bone resorption in neonatal mouse calvariae (31). ATRA has been reported to increase mRNA expression of RANKL in primary human osteoblast-like cultures and decrease mRNA expression and protein formation of OPG in MG-63 osteosarcoma cells (32). This suggests that osteoclastogenesis stimulated by retinoids may be due to an increased RANKL/OPG ratio; however, there are presently no data from bone or bone culture systems showing that vitamin A or derivatives can bring about changes in expression of RANKL and OPG that promote osteoclast formation and bone resorption.

In the current study, we have used neonatal mouse calvariae (a periosteal bone model) to investigate 1) how retinoids increase periosteal osteoclast formation and bone resorption and 2) how GCs inhibit this stimulation.

EXPERIMENTAL PROCEDURES

Materials—Mouse OPG fused to human IgG_1 Fc (OPG/Fc chimera), *Escherichia coli*-derived mouse RANKL Lys^{158}–Asp^{316} (catalog no. 462-TEC), the ELISA kits for mouse RANKL and mouse OPG, and recombinant human interleukin-1 receptor antagonistic protein were purchased from R&D Systems; ATRA, 9-*cis*-RA, TTNPB (Ro 13-7410) (4-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid), A7980 (AC-41848) (1-[2,4-dichlorophenyl)methyl-6,7,8,9-tetrahydro-3-phenyl-5H-imidazol[1,2-a]azepinium bromide hydrate), dexamethasone, hydrocortisone, RU 486 (17-hydroxy-11-(4-dimethylaminophenyl)17-(1-propynyl)-estra-4,9-dien-3-one), retinol, retinal-aldehyde, and essentially fatty acid-free bovine serum albumin were from Sigma; GR110 (Ro-41–5253) (4-[(*E*)-2-(7-heptoxy-4,4-dimethyl-1,1-dioxo-2,3-dihydrothiochromen-6-yl)prop-1-enyl]benzoic acid), GR103 (*N*-(4-hydroxyphenyl)retinamide), and GR104 (AM-580) (4-[(5,6,7,8-tetrahydro-5,6,8,8-tetramethyl-2-naphthalenyl)carboxamido]benzoic acid were from Biomol; bovine parathyroid hormone 1–34 (PTH) was from Bachem; α-modification of minimum essential medium and fetal calf serum (FCS) were from Invitrogen; CrossLabs® for Culture ELISA (CTX) was from Immunodiagnostics a/s; L-[5-^{3}H]proline was from PerkinElmer Life Sciences; [^{45}Ca]$CaCl_2$ was from Amersham Biosciences; oligonucleotide primers were from Invitrogen or Applied Biosystems; the HotStar Taq polymerase kit was from Qiagen Ltd.; the Kapa^{TM} Probe Fast quantitative PCR kit and Kapa2G^{TM} Robust HotStart Ready Mix were from Kapa; the PCR core kit was from Roche Applied Science; fluorescent labeled probes (reporter fluorescent dye VIC at the 5′-end and quencher fluorescent dye TAMRA at the 3′-end), high capacity cDNA reverse transcription kit, Power SYBR® Green PCR Master Mix, and the kits for quantitative real-time PCR were from Applied Biosystems; the RNAqueous®-4PCR kit was from Ambion Inc. (Austin, TX); culture dishes, multiwell plates, and glass chamber slides were from Nunc Inc.; and suspension culture dishes were from Corning Glass. Synthetic salmon calcitonin was a kind gift from Alejandra Martinez (Novartis Pharma AG); 1α,25$(OH)_2$-vitamin D3 (D3) was a kind gift from Hoffmann-La Roche (Basel, Switzerland); 3-amino-1-hydroypropylidene-1,1-bisphosphonate (AHPrBP) was a gift from Henkel KGaA (Düsseldorf, Germany); and indomethacin was a gift from Merck. D3, ATRA, 9-*cis*-RA, 13-*cis*-RA, TTNPB, A7980, GR103, GR104, GR110, and indomethacin were dissolved in ethanol. All other compounds were dissolved in either phosphate-buffered saline or culture medium.

Animals—CsA mice from our own inbred colony were used in most experiments if not otherwise stated. Mice with the GR

mutated and incapable of homodimerization (GR^{dim}) and corresponding wild type mice were also used. These mice have been described previously (33) and were backcrossed to the FVB/N background (34). Animal care and experiments were approved and conducted in accordance with accepted standards of humane animal care and used as deemed appropriate by the Animal Care and Use Committee of Umeå University.

Bone Organ Cultures—Mouse calvarial bone cultures were used for a bone resorption assay and for PCR analysis and ELISA measurements. Parietal bones from 5–7-day-old mice were microdissected and cut into either calvarial halves or four pieces (35). The bones were preincubated for 18–24 h in α-modification of minimum essential medium containing 0.1% albumin and 1 μmol/liter indomethacin (36). Following preincubation, the bones were extensively washed and subsequently cultured for up to 120 h in multiwell culture dishes containing 1.0 ml of indomethacin-free medium with or without test substances. The bones were incubated in the presence of 5% CO_2 in humidified air at 37 °C.

Measurements of Mineral Release—Mineral mobilization was assessed by analyzing the release of ^{45}Ca from bones prelabeled *in vivo* and dissected in quarters 4 days after injection of isotope. In most experiments, 2–3-day-old mice were injected with 1.5 μCi of ^{45}Ca, and the amounts of radioactivity in bone and culture medium were analyzed by liquid scintillation at the end of the culture period. For the time course experiments, the mice were injected with 12.5 μCi of ^{45}Ca, and radioactivity was analyzed at different time points by withdrawal of small amounts of culture medium. Isotope release was expressed as the percentage release of the initial amount of isotope (calculated as the sum of radioactivity in medium and bone after culture) (35). In some experiments, the data were recalculated, and the results were expressed as a percentage of the control, which was set at 100%. This allowed for accumulation of data from several experiments.

Measurements of Matrix Degradation—Bone matrix degradation was assessed by analyzing the release of ^{3}H from calvarial halves to culture medium. Mice were prelabeled 4 days before dissection with 10 μCi of [^{3}H]proline to label type 1 collagen, the main protein in bone matrix. The bones were dissected, preincubated, and cultured in α-modification of minimum essential medium with or without test substances as described above. The amount of ^{3}H in the medium at the end of the experiments was analyzed by liquid scintillation. At the end of the culture period, the bones were hydrolyzed, and the radioactivity in the hydrolysates and media was analyzed. The release of ^{3}H ([^{3}H]proline plus [^{3}H]hydroxyproline) parallels the release of [^{3}H]hydroxyproline and thus is a reliable indicator of collagen breakdown (37). In separate experiments, matrix degradation was assessed by analyzing the amount of type I collagen degradation fragments in culture media released from calvarial halves cultured as described above by using a commercially available ELISA.

Immunohistochemistry—At the end of culture, calvarial bones were fixed in 4% phosphate-buffered paraformaldehyde, decalcified in 10% EDTA in Tris buffer, pH 6.95, and embedded in paraffin. Sections were cut, deparaffinized, fixed in cold acetone, and subsequently treated with 3% H_2O_2 in PBS and an avidin/biotin blocking kit. After blocking with protein block, sections were incubated with unlabeled polyclonal rabbit anti-mouse cathepsin K (38) diluted 1:700 or normal rabbit serum as a negative control. After blocking with normal goat serum, biotin-labeled goat anti-rabbit serum was used as secondary antibody and was followed by incubation with the VECTASTAIN ABC kit and DAB substrate kit. All sections were counterstained with Mayer's hematoxylin and evaluated using a Leica Q500MC microscope (Leica, Cambridge, UK) by an observer blinded to the identity of the sections. The numbers of cathepsin K-positive multinucleated cells per section were determined.

Gene Expression—RNA was isolated from calvarial halves using the RNAqueous-4 PCR kit, according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 0.1–0.5 μg of total RNA using a high capacity cDNA reverse transcription kit. To ensure that there was no genomic DNA in the samples, negative controls that did not contain MultiScribe™ reverse transcriptase were included. Expression of mRNA was determined using semiquantitative RT-PCR or quantitative real-time PCR.

Semiquantitative RT-PCR analysis of the mRNA expression of *Calcr* (calcitonin receptor gene), *Acp5* (acid phosphatase gene), *Ctsk* (cathepsin K gene), *Rankl*, *Rank*, *Opg*, and *M-csf* was performed using either a HotStar Taq polymerase kit, a PCR core kit, or the Kapa2G™ Probe Fast quantitative PCR kit and compared at the logarithmic phase of the PCR. Sequences of the primers have been given previously (39–41).

Quantitative real-time PCR analysis of *Rankl*, *Rank*, *Opg*, *Calcr*, *Acp5*, *Ctsk*, *Akp1* (alkaline phosphatase gene), and *Ocn* (osteocalcin gene) was performed using the Kapa™ Probe Fast quantitative PCR kit with primers and probe as described in detail previously (40, 41). Quantitative real-time PCR analysis of *Rarα*, *Rarβ*, *Rarγ*, *M-csf*, c-*Fms*, and *Il-6* (interleukin-6 gene) was performed using a premade primer-probe mix from Applied Biosystems, whereas analysis of *Il-1β* (interleukin-1β gene) and *Tnf-α* (tumor necrosis factor-α gene) used SYBR Green. The amplifications were performed using a 384-well thermal cycler. Control assays included samples in which the reverse transcription reaction had been omitted and did not show any amplification (data not shown). To control for variability in amplification due to differences in starting mRNA concentrations, β-actin was used as an internal standard. The relative expression of target mRNA was computed from the target cycle threshold values and β-actin cycle threshold values using the standard curve method (62).

RANKL and OPG Protein Analyses—The protein synthesis of RANKL and OPG was assessed by measuring the levels of RANKL and OPG in calvarial bones and culture media using commercially available ELISA kits. Calvarial bones were dissected from 6–7-day-old mice (CsA) and divided into two halves along the sagittal suture. After preincubation, calvarial halves were individually incubated in 24-well plates in the absence or presence of test substances for 48 h. Calvarial cells were lysed with 0.2% Triton X-100, and the extracted bone samples and culture media were analyzed using the manufacturer's protocols for the ELISAs. The sensitivities of the immunoassays are 5 pg/ml.

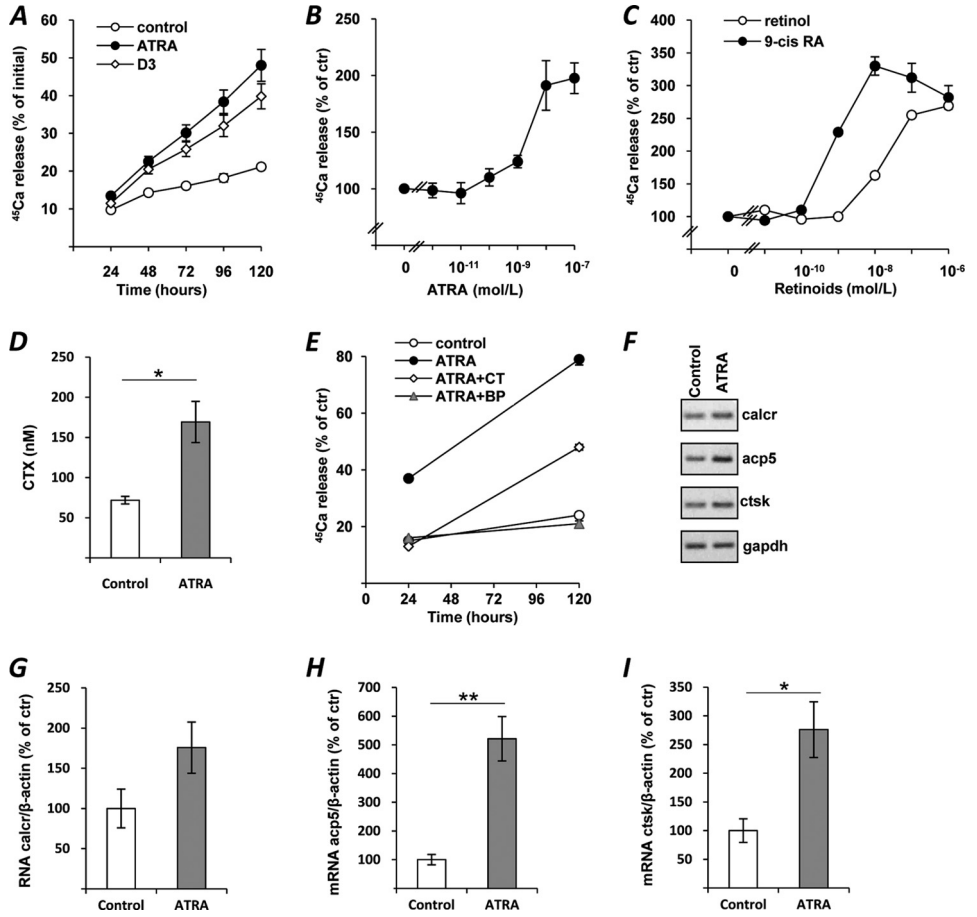

FIGURE 1. **Retinoids stimulate osteoclast differentiation and bone resorption in organ cultured mouse calvarial bones.** *A*, ATRA (10^{-7} M) and D3 (10^{-8} M) stimulated ^{45}Ca release from calvarial bones in a time-dependent manner. *B*, release of ^{45}Ca from calvarial bones cultured for 120 h with ATRA was concentration-dependent. *C*, releases of ^{45}Ca from calvarial bones cultured for 120 h with retinol and 9-*cis*-RA were concentration-dependent. *D*, release of the type I collagen degradation fragment (*CTX*) to medium by ATRA (10^{-7} M) for assessment of bone matrix degradation in 120-h culture. *E*, sustained and transient inhibitions of ^{45}Ca release by the bisphosphonate (*BP*) AHPrBp (10^{-4} M) and salmon calcitonin (10^{-9} M), respectively, in calvarial bones cultured for 120 h with ATRA (10^{-7} M). Shown are decreases of *Calcr*, *Acp5*, and *Ctsk* mRNA in calvarial bones cultured for 120 h with ATRA (10^{-7} M), assessed by both semiquantitative RT-PCR (*F*) and quantitative real-time PCR (*G–I*). Values are means of four or five observations (*A*, *D*, *E*, *G–I*) or 12–18 observations (*B* and *C*). *Error bars*, S.E. (when larger than the radius of the *symbol*).

Statistical Analyses—All statistical analyses were performed using one-way analysis of variance with Levene's homogeneity test and *post hoc* Bonferroni's test or, where appropriate, Dunnett's T3 test, or using the independent sample *t* test (SPSS for Windows; SPSS Inc., Chicago, IL). All experiments were performed at least twice with comparable results, and all data are presented as means ± S.E.

RESULTS

Stimulation of Osteoclastic Bone Resorption in Mouse Calvarial Bones by Retinoids—Enhanced resorption of periosteal bone by vitamin A is suggested to increase bone fragility. Initial experiments were performed to evaluate the effect of ATRA and related compounds using mouse calvariae, a periosteal bone model. ATRA (10^{-7} M) caused a time-dependent increased release of ^{45}Ca in cultured neonatal mouse calvarial bones that was significant ($p < 0.05$) at 24 h (Fig. 1*A*). The resorptive effect of ATRA was very similar to that obtained at an optimal concentration (10^{-8} M) of D3. D3 and PTH were employed as positive controls in the studies. Both are well characterized hormonal stimulators of calvarial bone resorption. When different concentrations of ATRA (10^{-12} to 10^{-7} M) were evaluated after incubation for 120 h, significant ^{45}Ca release was observed at 10^{-10} M and above, with half-maximal stimulation (EC_{50}) equaling $\sim 3 \times 10^{-9}$ M (Fig. 1*B*). Retinol and 9-*cis*-RA also dose-dependently stimulated ^{45}Ca release in calvarial bones, with calculated EC_{50} values of 3×10^{-8} M and 10^{-9} M, respectively (Fig. 1*C*). In separate experiments, retinaldehyde was also observed to be a good stimulator of ^{45}Ca release in calvarial bones (data not shown).

At 120 h, stimulation of mineral release by 10^{-7} M ATRA was associated with a 2.4-fold stimulation of matrix degradation, as assessed by the release of the type I collagen degradation fragment (*CTX*) from bones to media (Fig. 1*D*). This was in good agreement with results from experiments where matrix degradation was assessed by analyzing the release of ^{3}H from bones in which bone matrix collagen had been prelabeled *in vivo* with [^{3}H]proline. In these experiments, ATRA (10^{-7} M) caused a 2.3-fold enhancement of ^{3}H release, an observation similar to stimulations seen with D3 and PTH at optimally effective concentrations (10^{-8} M) (supplemental Fig. 1*A*).

The stimulation of ^{45}Ca release by ATRA (10^{-7} M) was inhibited by salmon CT (10^{-9} M) and the nitrogen-containing bis-

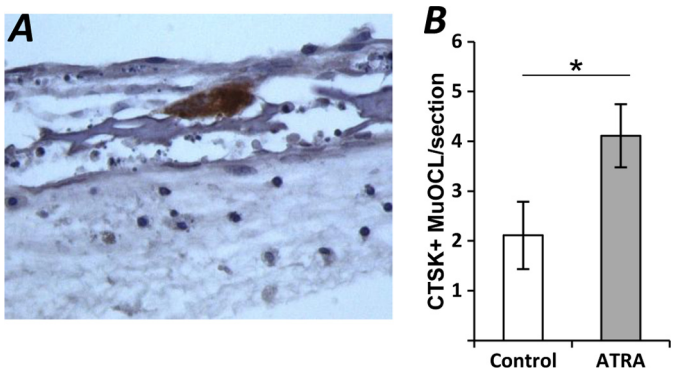

FIGURE 2. **ATRA (10^{-7} M) increased osteoclast formation in mouse calvarial bones cultured for 120 h.** Shown are cathepsin K-stained osteoclasts in ATRA (10^{-7} M)-stimulated calvarium (*A*) and the number of osteoclasts in unstimulated and ATRA (10^{-7} M)-stimulated calvarial bones (*B*).

phosphonate, AHPrBP (10^{-4} M) (Fig. 1*E*), two different but well documented inhibitors of osteoclast activity (42, 43). The hormone CT inhibits osteoclast function by stimulating cAMP formation and increasing cytosolic Ca^{2+} in osteoclasts (44), whereas nitrogen-containing bisphosphonates inhibit key enzymes in the mevalonate/cholesterol biosynthetic pathway in osteoclasts (45). Both compounds showed a strong inhibitory effect at 24 h, but the inhibition by CT was partially lost at 120 h, whereas that of AHPrBP was maintained for 120 h. Semiquantitative RT-PCR revealed that the enhancement of mineral and matrix degradation caused by ATRA was accompanied by enhanced mRNA expression at 48 h of the osteoclastic genes *Calcr*, *Acp5*, and *Ctsk* (Fig. 1*F*), an observation confirmed by quantitative real-time PCR (Fig. 1, *G–I*).

ATRA Stimulates Formation of Cathepsin K-positive Osteoclasts in Mouse Calvarial Bones—Direct confirmation of the osteoclastogenic action of ATRA was obtained by immunostaining of multinucleated bone surface ostoclasts for cathepsin K (Fig. 2*A*), the major collagenolytic enzyme in osteoclasts (46). Cathepsin K is highly expressed in multinucleated osteoclasts, and immunostaining revealed that treatment of calvarial bones with ATRA (10^{-7} M) for 120 h resulted in a 2-fold ($p < 0.05$) increase in osteoclast number (Fig. 2*B*).

Stimulation of Bone Resorption in Calvarial Bones by ATRA Is Due to Enhanced RANKL—M-CSF, RANKL, and OPG play essential roles in osteoclastogenesis, and experiments were performed to evaluate expression of these regulators and their receptors. In calvarial bones incubated with ATRA (10^{-7} M) for 10 h, analysis of gene expression by semiquantitative RT-PCR revealed that mRNA expression of *Rankl* was clearly enhanced, whereas the mRNA expression of *Opg* was decreased and that of *Rank* was unaffected (Fig. 3*A*). Additionally, the mRNA expression of c-*Fms* was enhanced, and that of *M-csf* was decreased by treatment with ATRA (Fig. 3*A*).

Quantitative real-time PCR showed that ATRA (10^{-7} M) caused a time-dependent increase of *Rankl* mRNA expression, which was delayed for 4 h, maximal at 12 h (8-fold stimulation), and then gradually leveled off at 48 h (2-fold stimulation) (Fig. 3*B*). ATRA caused a rapid decrease of *Opg* mRNA, which was maximal at 2 h (50% inhibition), remained inhibited for at least 12 h, and then leveled off with no significant effect observed at 48 h (Fig. 3*C*). A time-dependent enhancement of c-*Fms* mRNA was also noted with ATRA (Fig. 3*D*), whereas *M-csf* mRNA was time-dependently decreased over 48 h (Fig. 3*E*). No effect on *Rank* mRNA was seen at any time point (data not shown). The effect of ATRA on *Rankl* mRNA was concentration-dependent, with stimulation seen at concentrations similar to those causing enhanced ^{45}Ca release (Fig. 3*F*).

An increased RANKL/OPG ratio enhances osteoclastogenesis and bone resorption. When the protein level of RANKL was analyzed at 48 h, it was observed that ATRA (10^{-7} M) caused a 6.5-fold enhancement of RANKL protein in calvarial bones and a 20-fold increase in culture medium (Fig. 3*G*). In contrast, the protein level of OPG in bones and culture medium was unaffected by ATRA (Fig. 3*H*).

The importance of RANKL for the bone-resorptive effect of ATRA was assessed by adding OPG (300 ng/ml) to calvarial bones stimulated by ATRA (10^{-7} M). OPG substantially inhibited both the increased ^{45}Ca release (Fig. 3*I*) and the mRNA expression of *Ctsk* (Fig. 3*J*) stimulated by ATRA but did not affect *Rankl* mRNA (Fig. 3*K*).

ATRA Does Not Affect Bone Resorption Induced by RANKL—We have reported recently that ATRA inhibits osteoclast differentiation and formation in osteoclast progenitor cell cultures from mouse bone marrow stimulated by RANKL (24). In the present study, we tested to see if ATRA could affect ^{45}Ca release in mouse calvarial bones stimulated to resorb by recombinant RANKL. It was observed that ATRA did not antagonize RANKL in calvarial bones (Fig. 4). Thus, in contrast to bone marrow cells, ATRA does not impair osteoclastogenesis in mouse calvariae.

ATRA Does Not Stimulate Bone Resorption via Prostaglandins or Osteotropic Cytokines—Calvarial bones produce prostaglandins and express several cytokines that can stimulate bone resorption, including IL-6, TNF-α, and IL-1. To assess if the resorptive effect of ATRA in the calvarial bones was secondary to stimulation of prostaglandins or of cytokines known to stimulate RANKL and bone resorption, we analyzed the mRNA expression of the inducible cyclooxygenase enzyme, *Cox-2* (cyclooxygenase-2 gene), together with *Il-6*, *Tnf-α*, and *Il-1β*, in bones stimulated by ATRA for 1–48 h. ATRA had no effect on the mRNA expression of any of these genes at any time point (data not shown), nor was ^{45}Ca release induced by ATRA affected by the cyclooxygenase enzyme inhibitors, indomethacin, flurbiprofen, and naproxen (supplemental Fig. 2*A*) or the interleukin-1 receptor antagonistic protein (supplemental Fig. 2*B*).

ATRA Inhibits Expression of Genes Associated with Bone Formation—In addition to stimulating periosteal bone resorption, retinoids have been suggested to decrease subperiosteal bone formation (26). When osteoblast genes associated with bone formation were evaluated, it was found that ATRA time-dependently inhibited the mRNA expression of *Ocn* (Fig. 5*A*). Furthermore, mRNA expressions of additional osteoblast genes associated with bone formation, *Akp1*, *Runx2*, *Osterix*, and *Procollagen α1(1)*, were substantially decreased by ATRA after 48 h of culture (Fig. 5, *B–E*).

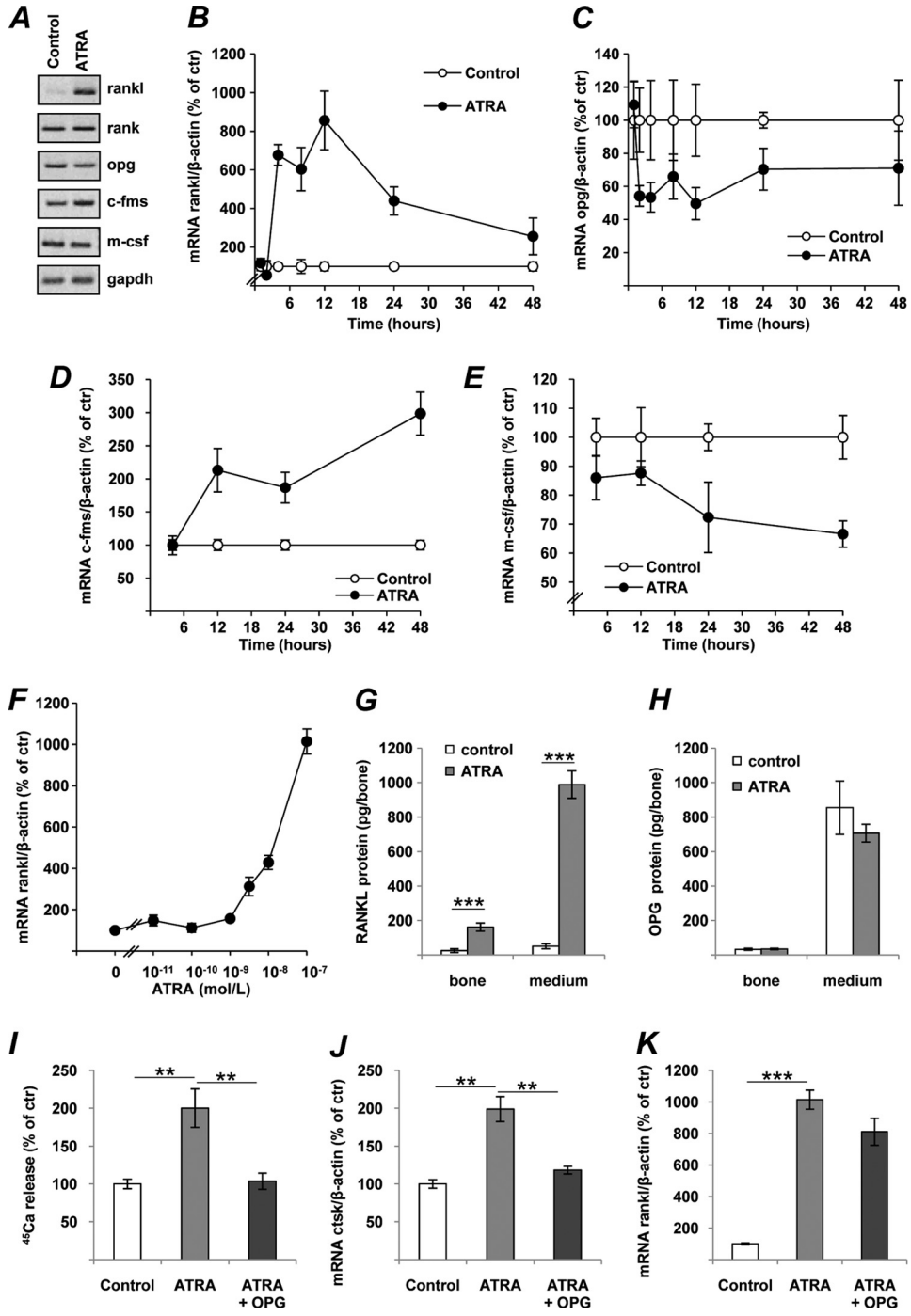

FIGURE 3. **The stimulation of bone resorption by ATRA in cultured mouse calvarial bones is due to increased RANKL.** ATRA (10^{-7} M) enhanced the mRNA expression of *Rankl* and c-*Fms* and decreased *Opg* and *M-csf* mRNA but did not affect *Rank* mRNA in calvarial bones cultured in the presence of the retinoid for 10 h (*A*). Increased *Rankl* mRNA (*B*) and decreased *Opg* mRNA (*C*) in calvarial bones cultured with ATRA (10^{-7} M) for 48 h were time-dependent and transient. Enhanced mRNA expression of c-*Fms* (*D*) and decreased expression of *M-csf* (*E*) elicited by ATRA (10^{-7} M) progressed with time. Up-regulation of *Rankl* mRNA in the calvarial bones by ATRA was concentration-dependent (*F*). Stimulation of calvarial bones with ATRA (10^{-7} M) for 48 h increased RANKL protein in bones and in the culture medium (*G*) without affecting OPG protein in bones or in medium (*H*). The stimulation of ^{45}Ca release from calvarial bones by ATRA (10^{-7} M) was abolished by co-treatment with recombinant OPG (300 ng/ml) (*I*). OPG decreased the increased mRNA expression of *Ctsk* (*J*) stimulated by ATRA (10^{-7} M) in calvarial bones without affecting *Rankl* mRNA (*K*). Values are means of four or five observations. *Error bars*, S.E. (when larger than the radius of the *symbol*).

Stimulation of Bone Resorption and RANKL in Mouse Calvarial Bones Is Mediated by RARα—We showed in an earlier study that RARα mediated the inhibition by ATRA of osteoclastogenesis in mouse bone marrow cells stimulated by RANKL (24). In the current experiments, a range of RAR agonists and an antagonist were used to determine which receptor was responsible for the stimulation of resorption by ATRA in neonatal mouse calvariae.

Quantitative real-time PCR analysis showed that calvarial bones expressed *Rarα*, *Rarβ*, and *Rarγ* mRNA and that

ATRA (10^{-7} M) caused a time-dependent, substantial (15-fold) stimulation of *Rarβ* mRNA, with no effects on *Rarα* or *Rarγ* mRNA expression (Fig. 6*A*). Enhancement of *Rarβ* mRNA by ATRA was dependent on the concentration of ATRA (Fig. 6*B*).

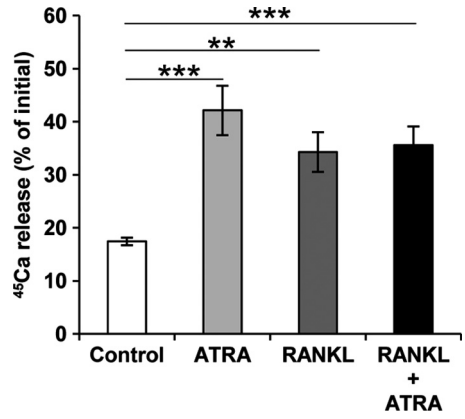

FIGURE 4. **ATRA does not affect RANKL-stimulated bone resorption.** Mouse calvarial bones were cultured for 120 h in the absence (control) or presence of either ATRA (10^{-7} M), RANKL (10 ng/ml), or their combination, and ^{45}Ca release was analyzed as a parameter of bone resorption. Values are means of five observations. *Error bars*, S.E.

The importance of RAR receptor subtypes was assessed by culturing calvariae in the presence of different agonists. ATRA (RARα/β/γ), TTNPB (pan-RAR agonist) (47), and GR104 (RARα) (48) concentration-dependently stimulated ^{45}Ca release from the calvarial bones with similar potencies (Fig. 5*C*). In contrast, A7980 (RARγ) (49) and GR103 (RARβ/γ) (50) did not affect ^{45}Ca release (Fig. 6*C*).

Assessment of gene expression showed that 10^{-7} M concentrations of ATRA, TTNPB, and GR104, but not A7980 or GR103, enhanced *Rankl* mRNA in calvarial bones at 24 h (Fig. 6*D*). None of the agonists affected the mRNA expression of *Opg* (data not shown).

The concentration-dependent stimulatory effect of ATRA (3×10^{-9} and 10^{-8} M) on *Rankl* mRNA was reversed by 10^{-7} M of the RARα antagonist GR110 (51) in a competitive manner (Fig. 6*E*). ATRA had no effect on *Opg* mRNA in either the presence or absence of GR110 (data not shown). Like ATRA, GR104 increased RANKL protein (Fig. 5*F*) but had no significant effect on OPG protein (Fig. 6*G*).

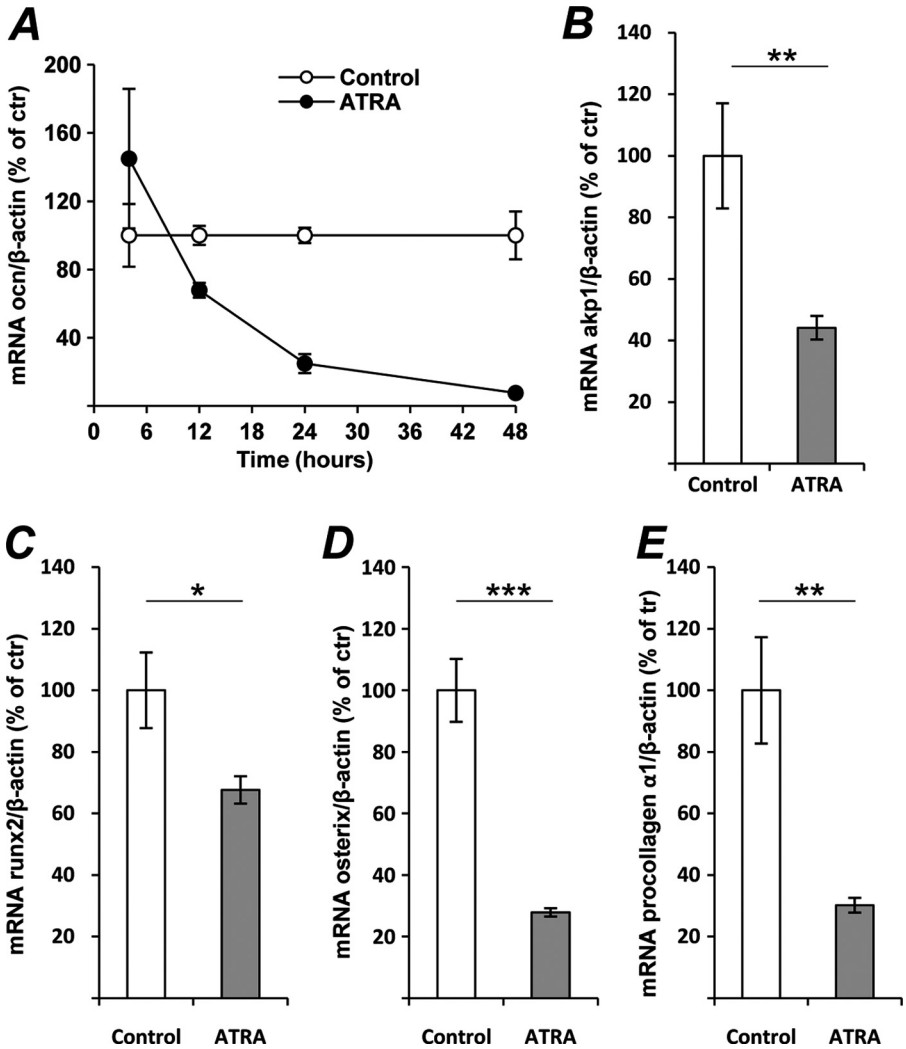

FIGURE 5. **ATRA decreased mRNA expression in cultured mouse calvarial bones of genes associated with extracellular matrix formation and mineralization.** ATRA (10^{-7} M) time-dependently decreased the mRNA expression of *Ocn* (*A*). ATRA also decreased the mRNA expression of *Akp1* (*B*), *Runx2* (*C*), *Osterix* (*D*), and *Procollagen α1* (*E*) in bones treated with the retinoid for 48 h. Values are means of four observations. *Error bars*, S.E. (when larger than the radius of the *symbol*).

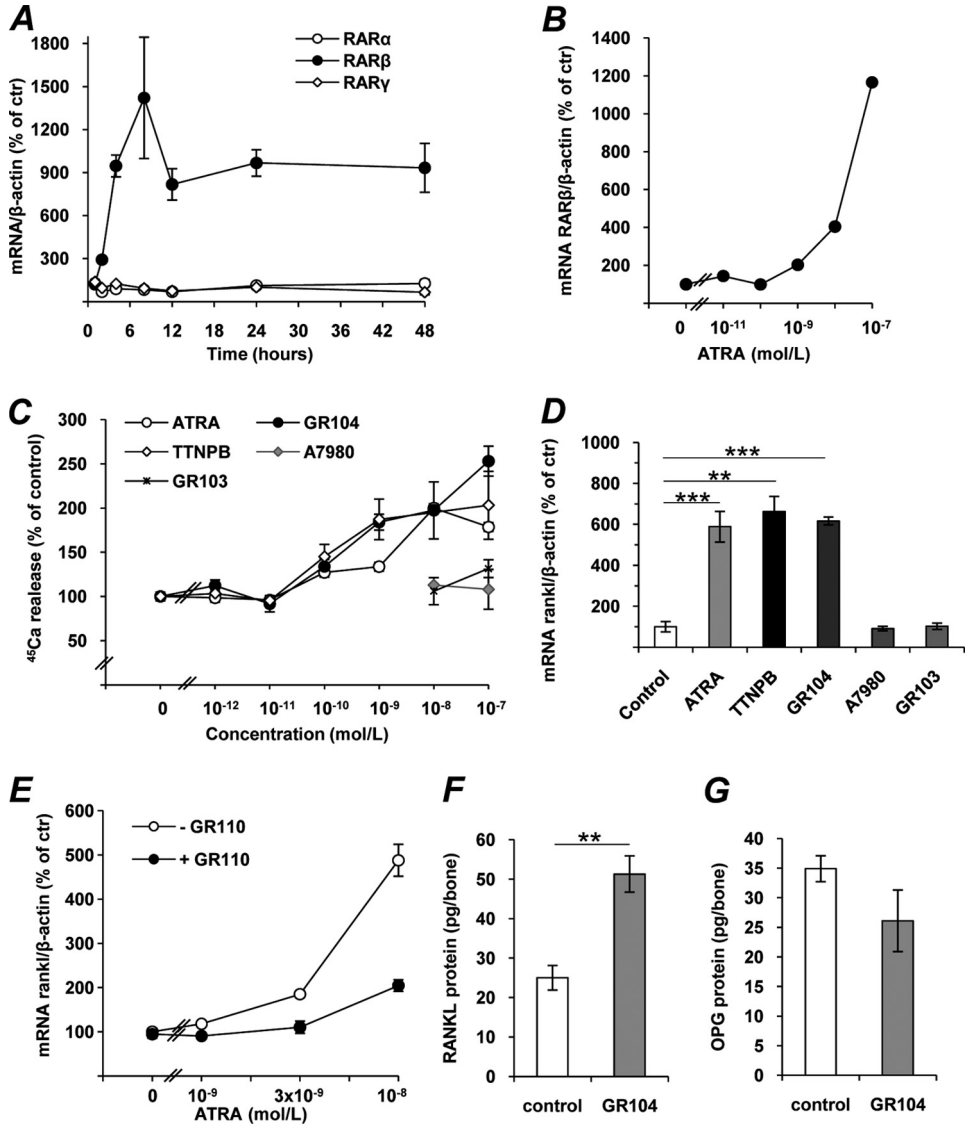

FIGURE 6. **Stimulation of bone resorption and RANKL by retinoids is caused by activation of RARα.** RARα, RARβ, and RARγ were expressed in mouse calvarial bones, and RARβ was time-dependently increased by ATRA (10^{-7} M) (*A*), an effect dependent on the concentration of ATRA (*B*). ATRA and agonists with affinity to either all RARs (TTNPB) or specifically to RARα (GR104) stimulated ^{45}Ca release in mouse calvarial bones with similar potencies, whereas agonists with affinity to RARβ/γ (GR103) or RARγ (A7980) had no effect (*C*). Similarly, 10^{-7} M concentrations of ATRA, TTNPB, and GR104, but not A7980 or GR103, enhanced *Rankl* mRNA after 24 of treatment (*D*). The concentration-dependent stimulatory effect of ATRA on *Rankl* mRNA in calvarial bones was inhibited by the RARα antagonist GR110 (10^{-7} M) (*E*). The RARα-specific agonist GR104 (10^{-7} M) enhanced RANKL protein (*F*) but did not affect OPG protein (*G*). Values are means of four or five observations. *Error bars*, S.E. (when larger than the radius of the *symbol*).

Stimulation of ^{45}Ca Release and RANKL in Mouse Calvarial Bones by ATRA Is Antagonized by Glucocorticoids—Numerous studies have shown that glucocorticoids oppose effects of retinoids, but the mechanisms responsible for this are unclear (15–20). We have reported previously that glucocorticoids increase RANKL and stimulate resorption in neonatal mouse calvarial bones (31). In the current study, we found that DEX reduces, in a concentration-dependent manner, ^{45}Ca release stimulated by ATRA (10^{-7} M) and 9-*cis*-RA (10^{-7} M) in neonatal mouse calvariae. The concentration of DEX causing this inhibition (IC_{50} of ~30 nM) is the same as the concentration of DEX that marginally stimulated (IC_{50} of ~30 nM) ^{45}Ca release (Fig. 7*A*). In addition, ^{45}Ca release induced by ATRA (10^{-7} M) was decreased by hydrocortisone (IC_{50} of ~100 nM) (supplemental Fig. 2*A*). In contrast to the inhibition noted with glucocorticoids, no inhibition of ^{45}Ca release was observed when either ATRA (10^{-7} M) or 9-*cis*-RA (10^{-7} M) was added to calvarial bones treated with varying concentrations of D3 or PTH (supplemental Fig. 2, *B* and *C*). When DEX (10^{-6} M) was added to bones stimulated by different ATRA and 9-*cis*-RA concentrations, there was a shift of the dose-response curves to the *right* (Fig. 7, *B* and *C*).

In an effort to better understand the inhibitory interaction of glucocorticoids and retinoids, calvarial bones were either treated with ATRA and DEX separately or co-cultured with the two agents for periods of 0–24 and 24–72 h. Release of ^{45}Ca in bones co-treated for 72 h with DEX and ATRA was significantly decreased at 24–72 h in comparison with bones only exposed to ATRA (Table 1). In contrast, when bones were cultured in the presence of ATRA for 24 h and then co-cultured for an addi-

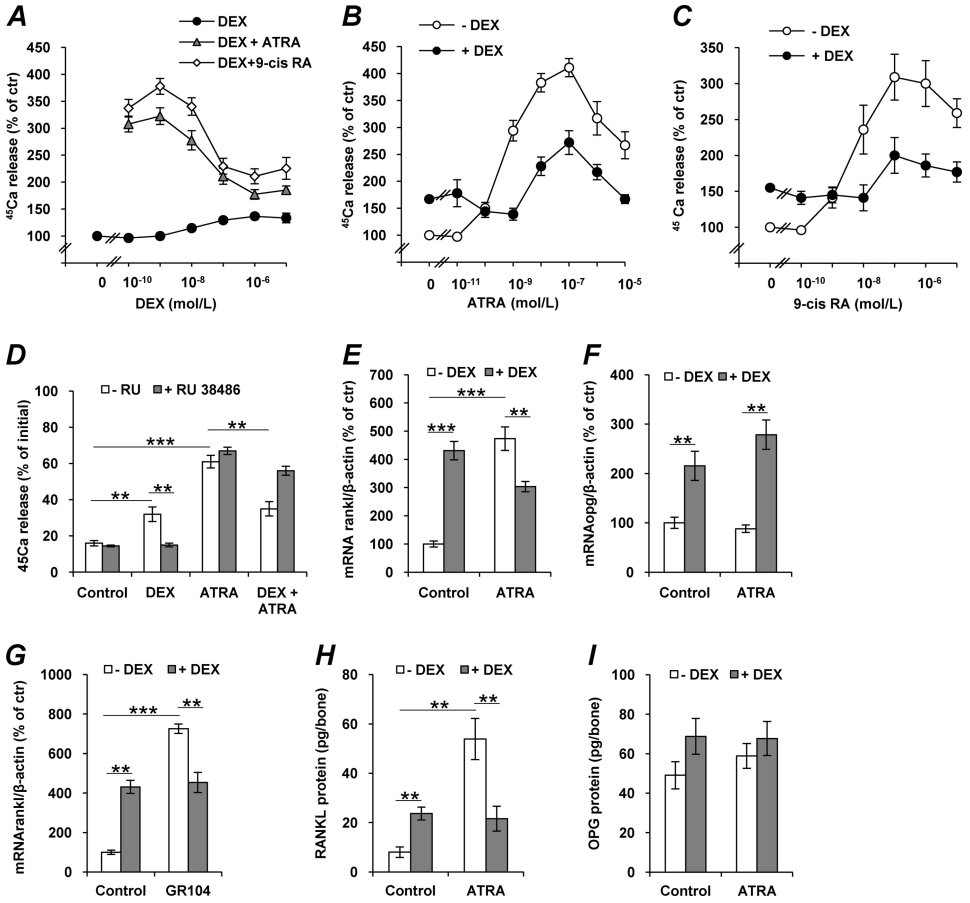

FIGURE 7. **DEX antagonizes the stimulatory effect of retinoids on bone resorption and RANKL in mouse calvarial bones, effects dependent on activation of the GR.** DEX concentration-dependently decreased the stimulatory effects of 10^{-7} M ATRA and 9-*cis*-RA) on ^{45}Ca release, at the same concentrations causing a modest stimulation of ^{45}Ca release in mouse calvarial bones (*A*). The inhibitory effect of DEX (10^{-6} M) on ^{45}Ca release induced by retinoids was seen at a wide range of concentrations of ATRA (*B*) and 9-*cis*-RA (*C*). The inhibitory effect of DEX (10^{-6} M) on ATRA (10^{-7} M)-induced ^{45}Ca release was reversed by the glucocorticoid receptor antagonist RU 486 (10^{-6} M) (*D*). DEX (10^{-6} M) enhanced *Rankl* mRNA by itself while reducing ATRA (10^{-7} M)-induced *Rankl* mRNA (*E*). DEX (10^{-6} M) enhanced *Opg* mRNA, but no interaction with ATRA (10^{-7} M) was observed (*F*). The stimulatory effect of the RARα-specific agonist GR104 (10^{-7} M) on *Rankl* mRNA was reduced by DEX (10^{-7} M) (*G*). DEX (10^{-7} M) enhanced RANKL protein in mouse calvarial bones and decreased the stimulatory effect of ATRA (10^{-7} M) on bone RANKL protein (*H*), whereas no interaction was observed on bone OPG protein (*I*). Values are means of four or five observations (*D–I*) or 10–12 observations (*A–C*). *Error bars*, S.E. (when larger than the radius of the *symbol*).

TABLE 1

Comparison of interactions between ATRA and dexamethasone upon ^{45}Ca release from mouse calvarial bones

Values are means ± S.E. for 10 bones.

Additions[a]		^{45}Ca release	
0–24 h	24–72 h	0–24 h	24–72 h
		%	%
		7.9 ± 0,2	13.8 ± 0.4
DEX	DEX	8.8 ± 0.4[b]	18.7 ± 1.3[b]
ATRA	ATRA	12.4 ± 0.8[b]	35.5 ± 2.2[b]
DEX + ATRA	DEX + ATRA	9.9 ± 0.1[b]	25.9 ± 1.0[b,c]
ATRA	ATRA + DEX	14.6 ± 0.6[b]	36.6 ± 2.1[b]
ATRA	ATRA + sCT[d]	12.9 ± 1.0[b]	22.8 ± 1.7[b,c]

[a] DEX, ATRA, and synthetic salmon calcitonin were added to final concentrations of 1 μmol/liter, 0.1 μmol/liter, and 1 nmol/liter, respectively.
[b] Significantly different from control ($p < 0.05$).
[c] Significantly different from ATRA alone ($p < 0.01$).
[d] Synthetic salmon calcitonin.

tional 48 h (24–72 h) in the presence of both ATRA and DEX, the ^{45}Ca release that occurred during the 0–24 h and 24–72 h incubations was not different from the ^{45}Ca release that occurred in the bones exposed only to ATRA (Table 1). However, when bones were cultured with ATRA for the first 24 h and then co-cultured with ATRA and the osteoclastic inhibitor calcitonin (43, 44) for the subsequent 48 h, significant inhibition by calcitonin was noted (Table 1). These results indicate that resorption can be suppressed at later time points but that the critical time window for interference by DEX occurs in the first phase of ATRA action.

The glucocorticoid receptor antagonist RU 486 (10^{-6} M) (52) significantly decreased ^{45}Ca release stimulated by DEX (10^{-6} M) in calvarial bones but did not affect the resorptive response to ATRA (10^{-7} M; Fig. 7*D*). Importantly, RU 486 reversed the inhibition of ATRA-stimulated ^{45}Ca release caused by DEX (Fig. 7*D*). This indicates that the inhibitory effect of DEX on resorption stimulated by ATRA is mediated by the GR.

Both ATRA (10^{-7} M) and DEX (10^{-6} M) enhanced *Rankl* mRNA in the calvarial bones (Fig. 7*E*). When calvarial bones were co-treated with ATRA (10^{-7} M) and DEX (10^{-6} M), the expression of *Rankl* mRNA was decreased relative to the expressions observed with the individual compounds (Fig. 7*E*). In contrast, ATRA did not affect *Opg* mRNA. DEX caused a 2-fold stimulation, and co-treatment with ATRA and DEX was not different from DEX treatment alone (Fig. 7*F*). When the

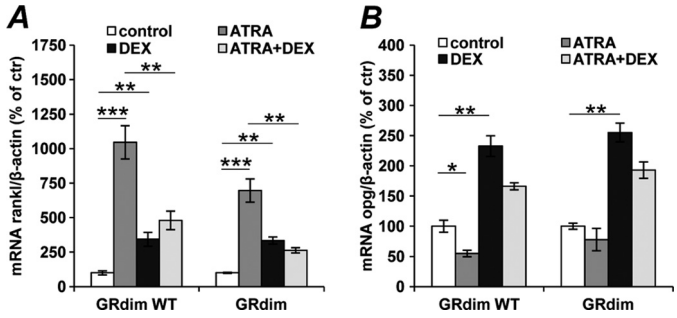

FIGURE 8. **Inhibitory effect of DEX on ATRA-induced *Rankl* mRNA is dependent on the monomeric GC.** Stimulatory effects of ATRA and DEX on *Rankl* mRNA as well as the inhibitory effect of DEX on ATRA induced *Rankl* mRNA were observed in calvarial bones from mice having a glucocorticoid receptor mutation rendering the receptor unable to dimerize (GR^{dim}) as well as in bones from corresponding wild type mice (GR^{dim} WT) (*A*). DEX stimulated *Opg* mRNA in calvarial bones from GR^{dim} and GR^{dim} wild type mice without interaction with ATRA being observed (*B*).

RARα agonist GR104 (10^{-7} M) was evaluated, it was found that *Rankl* mRNA was increased by GR104 alone but that co-treatment with DEX (10^{-6} M) resulted in a diminished response (Fig. 7*G*), similar to the findings with ATRA.

In agreement with the mRNA expression data, expression of RANKL protein in calvarial bones was increased by ATRA (10^{-7} M) but decreased by co-treatment with DEX (10^{-6} M) (Fig. 7*H*). RANKL protein in culture medium was also increased by ATRA but decreased by DEX co-treatment (data not shown). Neither ATRA, DEX, nor ATRA plus DEX significantly affected OPG protein in the calvarial bones (Fig. 7*I*). Stimulation of RANKL protein by GR104 (10^{-7} M) was also suppressed by DEX (10^{-6} M) (data not shown).

Dimerization of Glucocorticoid Hormone Receptor Is Not Necessary for Inhibition by DEX of ATRA-induced RANKL—Inhibition of ATRA by DEX is most likely mediated by either homodimerized GR or monomeric GR (23). ATRA (10^{-7} M) and DEX (10^{-6} M) caused 7–10-fold and 3–4-fold increases, respectively, of *Rankl* mRNA in wild type calvarial bones and in bones from mice where the GR is mutated (GR^{dim}) and incapable of homodimerization (Fig. 8*A*). Stimulation of *Rankl* mRNA by ATRA was substantially reduced by co-treatment with DEX in bones from both wild type and GR^{dim} mice (35) (Fig. 8*A*). ATRA caused a slight decrease of *Opg* mRNA expression in bones from wild type mice, and DEX increased *Opg* mRNA ~2.5-fold in wild type and GR^{dim} mice (Fig. 7*B*), whereas values of *Opg* mRNA for co-treatment of ATRA and DEX were no different from DEX alone (Fig. 8*B*). These results suggest that the monomeric GR was responsible for the inhibition of ATRA.

DISCUSSION

Increased vitamin A intake has been suggested to result in thinner, more fragile bones (6–9). One mechanism proposed to play a role in this increased fragility is enhanced periosteal osteoclastogenesis (26). In the present study, neonatal mouse calvarial bone (a periosteal bone model) was employed to evaluate the effects of vitamin A and derivatives on osteoclast formation and function.

Vitamin A, retinaldehyde, ATRA, and 9-*cis*-RA were all found to be potent stimulators of ^{45}Ca release in cultured neonatal mouse calvarial bones. Retinaldehyde, which serves as an intermediate in the irreversible production of ATRA from retinol, had not been observed previously to be a stimulator of bone resorption. In addition to ^{45}Ca release, increased bone matrix breakdown and mRNA expression of genes associated with osteoclastogenesis (*Calcr*, *Acp5*, and *Ctsk*) were observed with ATRA treatment. The ^{45}Ca release elicited by ATRA in calvarial bones was also decreased by two well known inhibitors of osteoclast-mediated bone resorption, the amino bisphosphonate, AHPrBP (42), and the polypeptide hormone, salmon CT (43).

Unlike the sustained inhibition noted with AHPrBP, inhibition by CT was transient, which is characteristic of the "escape" phenomenon known to occur with CT (43). The inhibition by AHPrBP and salmon CT, combined with the stimulatory effects of ATRA, suggests that the resorption observed with vitamin A and the retinoids was due to stimulation of osteoclast differentiation and function.

Stimulation of osteoclastic bone resorption by most agents is thought to be indirect and due to stimulation of an increased RANKL/OPG ratio in osteoblasts/stromal cells (27–30). Stimulation of an increased RANKL/OPG ratio by ATRA was clearly observed in the present study. Although *Opg* mRNA was transiently decreased by ATRA, there was no change in OPG protein. In contrast, both *Rankl* mRNA and RANKL protein were found to be significantly elevated in calvariae by ATRA. The importance of RANKL for the bone-resorptive effect of ATRA was further confirmed by showing that an excess of exogenous OPG inhibited both mineral mobilization and osteoclast gene expression stimulated by ATRA without affecting *Rankl* mRNA. Earlier experiments showing increased *Rankl* mRNA in primary human osteoblast-like cultures and decreased mRNA expression and protein formation of OPG in MG-63 osteosarcoma cells had suggested that an increased RANKL/OPG might be important for the action of ATRA (32), but the present study is the first to show a clear linkage between an increased RANKL/OPG ratio and osteoclast formation and bone resorption by ATRA.

At the time of dissection, low numbers of osteoclasts are present in the 6–7-day-old calvariae used for study. These are lost during the preculture period, when the calvarial bones are exposed to basic medium and indomethacin. During subsequent treatment with osteoclastic stimuli, enhanced resorption is due to increased multinucleated osteoclast formation from mononucleated osteoclast progenitor cells (53). In addition to increasing the RANKL/OPG ratio, enhancing expression of osteoclastic genes (*Calcr*, *Acp5*, and *Ctsk*), and stimulating ^{45}Ca release and bone matrix breakdown, immunohistochemical analysis revealed that ATRA treatment of calvarial bones resulted in a 2-fold increase in multinucleated cathepsin K-staining bone surface osteoclasts.

Inflammatory cytokines, such as IL-6, TNFα, and Il-1β, as well as prostanoids, especially PGE_2, are known to be good stimulators of bone resorption, including calvarial bone resorption (54). In the present study, we evaluated if resorptive actions of retinoids might be indirect and mediated by such things as prostanoids or osteotropic cytokines. There was no indication that the stimulation of osteoclastogenesis and bone resorption by ATRA was mediated by these agents. Increased mRNA

expressions of the cytokines and COX-2 were not noted with ATRA treatment. Furthermore, no effect of prostaglandin inhibitors or the interleukin-1 receptor antagonist protein was observed in experiments with ATRA.

RARs can be activated by ATRA and 9-*cis* RA, whereas RXRs are activated by 9-*cis* RA. RARs form heterodimers with RXRs, and these heterodimers and RXR homodimers function as transcription factors, activating RA response elements in the promoter regions of target genes. Most retinol signaling in cells is thought to be mediated by ATRA binding to RAR in RAR/RXR heterodimers. Equally potent stimulations of calvarial ^{45}Ca release had been reported previously for ATRA and 9-*cis*-RA (25). In the present study, ^{45}Ca releases stimulated by ATRA and 9-*cis*-RA were similar, with EC_{50} values equaling 3×10^{-9} M and 10^{-9} M, respectively. It is still not clear if 9-*cis*-RA is formed physiologically in cells and what role this isomer may play as a specific ligand for RXR (22). Vitamin A and retinaldehyde were observed to be less potent stimulators of ^{45}Ca release than either ATRA or 9-*cis*-RA, perhaps reflecting a requirement for metabolism of these compounds to ATRA.

Constitutive mRNA expression of the ATRA receptor subtypes (RARα, RARβ, and RARγ) was observed in calvarial bones. ATRA increased expression of RARβ, but the significance of this is unclear at present. An earlier study employing a pan-RAR-antagonist and a RARβ/γ agonist suggested that RARβ/γ was responsible for stimulation of *Rankl* mRNA by ATRA in human primary osteoblast cultures (32); however, no evaluation using a range of RAR agonists to study RANKL formation and bone resorption had been accomplished. The present study employed a pan-RAR-agonist; RARα, RARγ, and RARβ/γ agonists; and a RARα antagonist to confirm that ^{45}Ca release and RANKL mRNA and protein stimulated by ATRA in neonatal mouse calvariae were RARα-mediated.

There are many examples of GCs opposing retinoid actions in biological systems, but the mechanisms involved have not been defined (15–20). The current study indicates that the negative interaction of GCs with retinoids is dependent on monomeric GR decreasing RANKL formation in neonatal mouse calvariae.

DEX was observed to decrease *Rankl* mRNA, RANKL protein, and ^{45}Ca release in calvarial bones when co-cultured with ATRA. Inhibition of ^{45}Ca release by DEX was noted in the first 24 of 72-h cultures, suggesting that a relatively early event in osteoclast differentiation was being blocked by the GC. The action of DEX to oppose ATRA-induced bone resorption was inhibited by RU 486.

The GC antagonist, RU 486, (mifepristone), has both anti-progesterone and anti-GC activity (52) and has been used as a GC antagonist in the treatment of Cushing syndrome (56). The relative binding activity of RU 486 at the GR is estimated to be approximately 3 times that of DEX and 10 times that of hydrocortisone. Inhibition by RU 486 suggests that the suppression of ATRA activity in calvarial bones by GC was mediated by the GR.

Inhibition of ATRA by GC was also observed in calvariae from GR^{dim} mice. GR^{dim} mice are mice that have the point mutation A4586T introduced into the GR D-loop (33). GR in GR^{dim} mice cannot homodimerize and activate GC response elements in regulatory sequences of GC target genes but can function by protein-protein interaction and modulate the function of other transcription factors, such as AP-1, NFκB, IRF-3, and STAT5 (57). Suppression of the activity of these transcription factors by monomeric GC is thought to play an important role in the anti-inflammatory actions of GC, whereas many side effects of GC have been hypothesized to be due to GR homodimerization (33, 58, 59). A recent study has suggested that monomeric GR can interact with AP-1 to cause inhibition of IL-11 in osteoblasts (34), and it is possible that interaction of monomeric GR with transcription factors may be responsible for GC decreasing the stimulation of RANKL and osteoclastogenesis by ATRA.

In vivo experiments with increased ATRA intake in rodents have found significant bone loss attributed to periosteal resorption occurring at the same time that cancellous bone resorption is suppressed (26). In an earlier *in vitro* study, we found an RARα-mediated inhibition of osteoclastogenesis in RANKL-stimulated bone marrow cells treated with ATRA (24); however, when ATRA was added to calvariae treated with RANKL in the current experiments, no inhibition of ^{45}Ca release was noted. This observation, coupled with the potent RARα-mediated stimulation of osteoclastogenesis and bone resorption observed in calvarial bones, suggests that differences in periosteal and bone marrow microenvironments may be responsible for the different responses to ATRA or, alternatively, that different periosteal and bone marrow osteoclast precursors may be present and respond differently to ATRA.

Retinoids appear to have different actions at various stages of osteoblastogenesis, and there is not good agreement presently on the effects of retinoids in osteoblasts. A recent study has suggested that vitamin A regulates *Bmp2* (bone morphogenetic protein 2 gene) mRNA expression and plays an important role in osteoblastogenesis and bone formation (60), but retinoids have also been reported to decrease bone formation, including subperiosteal bone formation (26). In the present study, decreases in mRNA expression of osteoblast genes (*Ocn*, *Akp1*, *Runx2*, *Osterix*, and *Procollagen* $\alpha 1$) in calvarial bone following ATRA treatment suggested that retinoids may not only increase bone resorption in calvariae but probably decrease bone formation as well.

If cortical bone thinning and suppression of osteoclastogenesis in cancellous bone play prominent roles in fracture incidence in humans following increased intake of vitamin A, this would be substantially different from conditions, such as postmenopausal osteoporosis and glucocorticoid excess, where the increased incidence of fracture is due primarily to cancellous bone loss. These different paradigms for bone fragility may help explain some of the differing outcomes in studies evaluating vitamin A intake and fracture risk. In support of this view, a recent hypervitaminosis A study in rodents has suggested that increased bone fragility associated with thinning of cortical bone may occur with little decrease in bone mass (61).

In conclusion, the data from the current study confirm the resorptive effects of vitamin A and derivatives such as ATRA in periosteal bone and suggest that this stimulation of osteoclast formation and bone mineral and matrix breakdown is due to enhanced RANKL formation and an increased RANKL/OPG

ratio. Experiments employing synthetic compounds targeted to RAR receptors suggested that calvarial responses stimulated by ATRA were mediated by RARα. Moreover, it was determined that the monomeric GR was responsible for glucocorticoids opposing the stimulation of RANKL and periosteal osteoclast formation and bone resorption by ATRA.

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REFERENCES

1. Mark, M., Ghyselinck, N. B., and Chambon, P. (2006) *Annu. Rev. Pharmacol. Toxicol.* **46,** 451–480
2. Mark, M., Ghyselinck, N. B., and Chambon, P. (2009) *Nucl. Recept. Signal.* **7,** e002
3. Wald, G. (1968) *Science* **162,** 230–239
4. Moise, A. R., Noy, N., Palczewski, K., and Blaner, W. S. (2007) *Biochemistry* **46,** 4449–4458
5. Pino-Lagos, K., Benson, M. J., and Noelle, R. J. (2008) *Ann. N.Y. Acad. Sci.* **1143,** 170–187
6. Melhus, H., Michaëlsson, K., Kindmark, A., Bergström, R., Holmberg, L., Mallmin, H., Wolk, A., and Ljunghall, S. (1998) *Ann. Intern. Med.* **129,** 770–778
7. Feskanich, D., Singh, V., Willett, W. C., and Colditz, G. A. (2002) *JAMA* **287,** 47–54
8. Promislow, J. H., Goodman-Gruen, D., Slymen, D. J., and Barrett-Connor, E. (2002) *J. Bone Miner. Res.* **17,** 1349–1358
9. Michaëlsson, K., Lithell, H., Vessby, B., and Melhus, H. (2003) *N. Engl. J. Med.* **348,** 287–294
10. Ribaya-Mercado, J. D., and Blumberg, J. B. (2007) *Nutr. Rev.* **65,** 425–438
11. Caire-Juvera, G., Ritenbaugh, C., Wactawski-Wende, J., Snetselaar, L. G., and Chen, Z. (2009) *Am. J. Clin. Nutr.* **89,** 323–330
12. Canalis, E., Mazziotti, G., Giustina, A., and Bilezikian, J. P. (2007) *Osteoporos. Int.* **18,** 1319–1328
13. Hofbauer, L., and Rauner, M. (2009) *Mol. Endocrinol.* **23,** 1525–1531
14. Compston, J. (2010) *Nat. Rev. Rheumatol.* **6,** 82–88
15. Goldman, R. (1985) *Cancer Res.* **45,** 3118–3124
16. Ng, K. W., Manji, S. S., Young, M. F., and Findlay, D. M. (1989) *Mol. Endocrinol.* **3,** 2079–2085
17. Oberg, K. C., and Carpenter, G. (1989) *Mol. Endocrinol.* **3,** 915–922
18. Roberts, A. B., Anzano, M. A., Lamb, L. C., Smith, J. M., and Sporn, M. B. (1984) *Cancer Res.* **44,** 1635–1641
19. Violette, S. M., King, I., and Sartorelli, A. C. (1989) *J. Invest. Dermatol.* **93,** 165–168
20. Conaway, H. H., Grigorie, D., and Lerner, U. H. (1997) *J. Endocrinol.* **155,** 513–521
21. Bastien, J., and Rochette-Egly, C. (2004) *Gene* **328,** 1–16
22. Mic, F. A., Molotkov, A., Benbrook, D. M., and Duester, G. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 7135–7140
23. Kassel, O., and Herrlich, P. (2007) *Mol. Cell. Endocrinol.* **275,** 13–29
24. Conaway, H. H., Persson, E., Halén, M., Granholm, S., Svensson, O., Pettersson, U., Lie, A., and Lerner, U. H. (2009) *FASEB J.* **23,** 3526–3538
25. Kindmark, A., Melhus, H., Ljunghall, S., and Ljunggren, O. (1995) *Calcif. Tissue Int.* **57,** 242–244
26. Kneissel, M., Studer, A., Cortesi, R., and Susa, M. (2005) *Bone* **36,** 202–214
27. Teitelbaum, S. L., and Ross, F. P. (2003) *Nat. Rev. Genet.* **4,** 638–649
28. Lerner, U. H. (2004) *Crit. Rev. Oral. Biol. Med.* **15,** 64–81
29. Kearns, A. E., Khosla, S., and Kostenuik, P. J. (2008) *Endocr. Rev.* **29,** 155–192
30. Wada, T., Nakashima, T., Hiroshi, N., and Penninger, J. M. (2006) *Trends Mol. Med.* **12,** 17–25
31. Swanson, C., Lorentzon, M., Conaway, H. H., and Lerner, U. H. (2006) *Endocrinology* **147,** 3613–3622
32. Jacobson, A., Johansson, S., Branting, M., and Melhus, H. (2004) *Biochem. Biophys. Res. Commun.* **322,** 162–167
33. Reichardt, H. M., Kaestner, K. H., Tuckermann, J., Kretz, O., Wessely, O., Bock, R., Gass, P., Schmid, W., Herrlich, P., Angel, P., and Schütz, G. (1998) *Cell* **93,** 531–541
34. Rauch, A., Seitz, S., Baschant, U., Schilling, A. F., Illing, A., Stride, B., Kirilov, M., Mandic, V., Takacz, A., Schmidt-Ullrich, R., Ostermay, S., Schinke, T., Spanbroek, R., Zaiss, M. M., Angel, P. E., Lerner, U. H., David, J. P., Reichardt, H. M., Amling, M., Schütz, G., and Tuckermann, J. P. (2010) *Cell Metab.* **11,** 517–531
35. Ljunggren, O., Ransjö, M., and Lerner, U. H. (1991) *J. Bone Miner. Res.* **6,** 543–550
36. Lerner, U. H. (1987) *J. Bone Miner. Res.* **2,** 375–383
37. Brand, J. S., and Raisz, L. G. (1972) *Endocrinology* **90,** 479–487
38. Zenger, S., Hollberg, K., Ljusberg, J., Norgård, M., Ek-Rylander, B., Kiviranta, R., and Andersson, G. (2007) *Bone* **41,** 820–832
39. Ahlen, J., Andersson, S., Mukohyama, H., Roth, C., Bäckman, A., Conaway, H. H., and Lerner, U. H. (2002) *Bone* **31,** 242–251
40. Schwab, A. M., Granholm, S., Persson, E., Wilkes, B., Lerner, U. H., and Conaway, H. H. (2005) *Endocrinology* **146,** 4349–4361
41. Palmqvist, P., Lundberg, P., Persson, E., Johansson, A., Lundgren, I., Lie, A., Conaway, H. H., and Lerner, U. H. (2006) *J. Biol. Chem.* **281,** 2414–2429
42. Carano, A., Teitelbaum, S. L., Konsek, J. D., Schlesinger, P. H., and Blair, H. C. (1990) *J. Clin. Invest.* **85,** 456–461
43. Wener, J. A., Gorton, S. J., and Raisz, L. G. (1972) *Endocrinology* **90,** 752–759
44. Del Fattore, A., Teti, A., and Rucci, N. (2008) *Arch. Biochem. Biophys.* **473,** 147–160
45. Russell, R. G. (2011) *Bone* **49,** 2–19
46. Garnero, P., Borel, O., Byrjalsen, I., Ferreras, M., Drake, F. H., McQueney, M. S., Foged, N. T., Delmas, P. D., and Delaissé, J. M. (1998) *J. Biol. Chem.* **273,** 32347–32352
47. Pignatello, M. A., Kauffman, F. C., and Levin, A. A. (1997) *Toxicol. Appl. Pharmacol.* **142,** 319–327
48. Teng, M., Duong, T. T., Klein, E. S., Pino, M. E., and Chandraratna, R. A. (1996) *J. Med. Chem.* **39,** 3035–3038
49. Piu, F., Gauthier, N. K., Olsson, R., Currier, E. A., Lund, B. W., Croston, G. E., Hacksell, U., and Brann, M. R. (2005) *Biochem. Pharmacol.* **71,** 156–162
50. Fanjul, A. N., Delia, D., Pierotti, M. A., Rideout, D., Yu, J. Q., Pfahl, M., and Qiu, J. (1996) *J. Biol. Chem.* **271,** 22441–22446
51. Toma, S., Isnardi, L., Raffo, P., Riccardi, L., Dastoli, G., Apfel, C., LeMotte, P., and Bollag, W. (1998) *Int. J. Cancer* **78,** 86–94
52. Philbert, D., Costerousse, G., Gaillard-Moguilewsky, M., Nedelae, L., Nique, F., Tournemine, C., and Teutsch, G. (1991) in *Antihormones in Health and Disease* (Agarwal, M. K., ed) pp. 1–17, Karger, Basel, Switzerland
53. Lerner, U. H., Johansson, L., Ranjsö, M., Rosenquist, J. B., Reinholt, F. P., and Grubb, A. (1997) *Acta Physiol. Scand.* **161,** 81–92
54. Liu, Y. C., Lerner, U. H., and Teng, Y. T. (2010) *Periodontology 2000* **52,** 163–206
55. Deleted in proof
56. Nieman, L. K., Chrousos, G. P., Kellner, C., Spitz, I. M., Nisula, B. C., Cutler, G. B., Merriam, G. R., Bardin, C. W., and Loriaux, D. L. (1985) *J. Clin. Endocrinol. Metab.* **61,** 536–540
57. Herrlich, P. (2001) *Oncogene* **20,** 2465–2475
58. Reichardt, H. M., Tuckermann, J. P., Göttlicher, M., Vujic, M., Weih, F., Angel, P., Herrlich, P., and Schütz, G. (2001) *EMBO J.* **20,** 7168–7173
59. Tuckermann, J. P., Reichardt, H. M., Arribas, R., Richter, K. H., Schütz, G., and Angel, P. (1999) *J. Cell Biol.* **147,** 1365–1370
60. Tanaka, K., Tanaka, S., Sakai, A., Ninomiya, T., Arai, Y., and Nakamura, T. (2010) *Bone* **47,** 1006–1012
61. Lind, T., Lind, P. M., Jacobson, A., Hu, L., Sundqvist, A., Risteli, J., Yebra-Rodriguez, A., Rodriguez-Navarro, A., Andersson, G., and Melhus, H. (2011) *Bone* **48,** 496–506
62. Applied Biosystems (1997) *User Bulletin #2, ABI Prism 7700 Sequence Detection System*, Applied Biosystems, Carlsbad, CA