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Estrogen and Raloxifene Stimulate Transforming Growth Factor-β3 Gene Expression in Rat Bone: A Potential Mechanism for Estrogen- or Raloxifene-Mediated Bone Maintenance

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ABSTRACT

Estrogen or raloxifene (LY156758) prevent estrogen deficiency-induced bone loss in animals and humans. We demonstrated in the rat that a 22% reduction in bone mineral density generated by ovariectomy was associated with a 2-fold reduction of transforming growth factor-β3 (TGFβ3) messenger RNA expression in the femur. Administration of 17β-estradiol or raloxifene to ovariectomized rats restored both bone mineral density and TGFβ3 messenger RNA expression in the femur to levels measured in intact animals. In transient transfection assays, the promoter sequence from −38 to +110 of the human TGFβ3 gene, which contains no palindromic estrogen response element, was sufficient to mediate 17β-estradiol or raloxifene induced-reporter gene expression in presence of the estrogen receptor. Raloxifene activated TGFβ3 promoter as a full agonist at nanomolar concentrations. In the same cellular system, raloxifene inhibited the estrogen response element-containing vitellogenin promoter expression as a pure estrogen antagonist. In two well characterized osteoclast differentiation models, TGFβ3 significantly inhibited the differentiation and bone-resorptive activities of murine and avian osteoclasts. These findings suggest that regulation of TGFβ3 gene expression by raloxifene or estrogen in bone may be an important target to mediate bone maintenance. (*Endocrinology* **137:** 2075–2084, 1996)

MAINTENANCE OF bone mass is a dynamic process achieved by tightly coupled bone formation and resorption processes, so that no net gain or loss of bone mass is observed (1, 2). Multiple systemic and local factors such as cytokines, growth factors, steroids, and vitamin D play important roles to achieve normal bone balance (3–5). Recently, the gonadal steroid estrogen has attracted particular attention in bone research for its clinical effectiveness in preventing osteoporosis in women (6–8). Loss of estrogen at menopause is associated with a rapid reduction of bone mass, leading to porotic bones prone to fracture. Estrogen replacement can effectively prevent this rapid bone loss in postmenopausal women and in clinically relevant animal models such as ovariectomized (OVX) rats, establishing a protective effect of estrogen on the skeleton (7, 8). Although the beneficial effects of estrogen on bone are clear, the overall value of hormone replacement therapy (HRT) has been questioned due to estrogen-associated side-effects, including uterine stimulation and increased risk of endometrial cancer (9).

Recently, studies of raloxifene (LY156758), a nonsteroidal benzothiophene derivative, have provided intriguing observations (10). In the breast and uterus, raloxifene is a classical antiestrogen that inhibited the growth of mammary or endometrial carcinoma as well as estrogen-induced uterine tissue proliferation (11–13). However, in nonreproductive tissues, raloxifene prevented bone loss and lowered serum cholesterol with a pharmacological profile similar to that of estrogen in both OVX rats and postmenopausal women (14, 15). Such a tissue-selective estrogen agonist/antagonist profile of raloxifene has led to intensive preclinical and clinical investigations to explore the potential application of such chemical entities as ideal hormone replacement therapy (15).

Although compelling data in humans and animals demonstrating effectiveness of estrogen replacement for treating postmenopausal osteoporosis have accumulated rapidly in recent years, mechanistic understanding of how estrogen regulates bone homeostasis is still lagging behind. In reproductive tissues, estrogen-induced biological activities are mediated through the nuclear estrogen receptor (ER), which belongs to the steroid/thyroid hormone receptor superfamily (16, 17). Estrogen binding to ER induces a conformational change in the receptor that functions to activate gene transcription via a specific DNA element called the estrogen response element (ERE) (18). In bone, ER has been demonstrated to be expressed and function as a transcription factor (19, 20). Estrogen deficiency has also been associated with protein level changes of several growth factors, including transforming growth factor-β (TGFβ), insulin-like growth factor I (IGF-I), interleukin-1 (IL-1), IL-6, or tumor necrosis factor in bone tissue or osteoblast-like cells (21–24). However, whether transcriptional regulation of target genes is the key function for estrogen to regulate skeletal homeostasis and what target genes estrogen modulates in bone have not been fully elucidated.

To address these issues, we studied gene regulation by estrogen and raloxifene in femora of OVX rats. In this study, we examined gene expression of TGFβ, a key factor in bone

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formation, induction, and repair (21, 25), in response to ovariectomy and 17β-estradiol or raloxifene replacement. Three isoforms of TGFβ, TGFβ1, -2, and -3, have been identified in humans and animals (26) and shown to be expressed in bone tissues (4, 21, 27). We demonstrated that ovariectomy decreased the messenger RNA (mRNA) expression level of a specific TGFβ isoform, TGFβ3, in rat femur. Both 17β-estradiol and raloxifene elevated TGFβ3 gene expression to sham control levels within 2 h of injection. We also demonstrated that a non-ERE-containing, 150-bp nucleotide sequence in the 5′-untranslated region of the human TGFβ3 promoter could mediate 2- to a 6-fold up-regulation of chloramphenicol acetyltransferase (CAT) expression by 17β-estradiol or raloxifene in transient transfection assays, suggesting a transcriptional regulation of TGFβ3 by both agents. In osteoclast differentiation systems, TGFβ3 significantly inhibited osteoclast differentiation. Collectively, these data indicate that TGFβ3 may be a target gene for estrogen-mediated bone maintenance. Regulation of TGFβ3 by estrogen receptor may also use a non-ERE-mediated mechanism through which raloxifene functions as a *bona fide* agonist in bone.

Materials and Methods

Materials

Tamoxifen and 17β-estradiol were purchased from Sigma Chemical Co. (St. Louis, MO). Raloxifene (LY156758) was synthesized at the Lilly Research Laboratories (Indianapolis, IN) by a method originally reported by Jones *et al.* (10). The compound was greater than 98.5% pure, as determined by mass spectrometry and elemental analysis. ICI 164,384 was also synthesized at the Lilly Research Laboratories with greater than 98% purity. Recombinant human TGFβ3 was purchased from R&D Systems (Minneapolis, MN).

Animals

Female, virus antibody-free, Sprague-Dawley rats (10–11 weeks old) were obtained from Charles Rivers Laboratories (Portage, MI) and group housed on a 12-h light, 12-h dark cycle with room temperature set at 22 C. The animals were allowed to acclimate for 1 week with *ad libitum* access to food and water. Bilateral oophorectomies were performed under ketamine (120 mg/kg)-xylazine (24 mg/kg) anesthesia. After recovery from the surgical anesthesia, animals were divided into treatment groups (OVX control and 17β-estradiol, raloxifene, or tamoxifen treatment), and compound administration was initiated. 17β-Estradiol (0.1–30 μg/kg·day), raloxifene, or tamoxifen (each at 0.01–1 mg/kg·day) were delivered sc in corn oil. All drugs were given in a volume of 0.1 mg/100 g BW. Experimental groups consisted of six animals per group (per dose). OVX and intact controls received vehicle injections.

After 5 weeks of treatment, rats were killed by carbon dioxide asphyxiation. The left femur was removed and frozen for subsequent single photon absorptiometry, which was performed on a Norland Digital Bone Densitometer (model 2780). Three transverse scans of 1 mm width were made, 6–7 mm proximal to the distal end of each femur. Measurements were made on randomized bones by an operator blinded as to the treatment group, who recorded the mean of three scan repetitions.

Ribonuclease (RNase) protection assay (RPA)

OVX and sham-operated rats were treated with a single injection of 17β-estradiol, raloxifene, or tamoxifen at respective doses of 0.1, 1, and 1 mg/kg. Control animals received a single injection of vehicle (corn oil). Animals were killed 2, 6, and 24 h after injection. Both femora of the rat were excised. Total RNA were extracted using the UltraSpec RNA Isolation System (Biotecx Laboratories, Houston, TX). Total RNA (20 μg) from each femur were analyzed for TGFβ mRNA expression using the RPA II Ribonuclease Protection Assay Kit (Ambion, Austin, TX). To generate isoform-specific riboprobes, TGFβ complementary DNA (cDNA) templates were prepared by PCR amplification of a rat liver cDNA pool using the following primers: TGFβ1: 5′-primer, TGCTCCAGCTCCACAGAG; 3′-primer, AATACGACTCACTATAGGGTGTTGGTTGTAGAGGGCA; TGFβ2: 5′-primer, CTTCGTGCCGTCTAATAATT; 3′-primer, AATACGACTCACTATA GGGCAACAACATTAGCAGGAGA; and TGFβ3: 5′-primer, GAATGGCTGTCTTTCGATGT; 3′-primer, AATACGACTCACTAT AGGGCCATGGTCATCTTCATTGT. cDNA templates generated by these primer sets correspond to regions from 665–832, 690–840, and 1196–1365 of rat TGFβ1, mouse TGFβ2, and mouse TGFβ3 cDNAs, respectively. ^{32}P-Labeled riboprobes were then generated by *in vitro* transcription of the cDNA templates using T7 polymerase. For internal gene expression control, a probe for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, expanding the region from 564–854 (GenBank, X02231), was also prepared and used as the control. After RNase digestion, protected probes were subjected to electrophoresis on 8 M urea-5% polyacrylamide gel to assure the proper sizes of the probes. The amount of radioactivity retained in the gel were quantitated using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA) and normalized by GAPDH expression from the same RNA sample.

Cell culture and transient transfection assay

Human MG63 osteosarcoma cells (American Type Culture Collection, Rockville, MD) were cultured in DMEM:F-12 (3:1) medium (with the addition of 0.1 μg/liter sodium selenite, 2.2 g/liter sodium bicarbonate, 4.9 mg/liter ethanolamine HCl, and 20 mM HEPES, pH 7.4) containing 10% FBS (Hyclone, Logan, UT) and 50 μg/ml tobromycin (Eli Lilly Co., Indianapolis, IN) at 37 C in 7% CO_2. For transfection, cells were seeded in DMEM:F-12 (3:1) medium containing 10% charcoal-dextran-treated FBS (Hyclone) 24 h before transfection. Reporter plasmid (TGFβCAT; 10 μg), ER expression plasmid (pCMVER; 5 μg), and reference plasmid (pSV-β-galactosidase vector, Promega Corp., Madison, WI; 5 μg) were cotransfected into cells by the calcium phosphate precipitation method (ProFection kit, Promega) and left on cells for 24 h. Cells were then switched to fresh medium containing 10% charcoal-dextran-treated FBS and treatment compounds for an additional 24 h. At the end of treatment, cellular proteins were extracted from cells in 0.25 M Tris buffer, pH 7.8, by three cycles of freeze/thawing (−80 C/37 C) in a dry ice-ethanol bath. Cell lysates containing 100 μg total proteins were used to assay for CAT and β-galactosidase activities. The acetylated and nonacetylated forms of labeled chloramphenicol were separated on TLC plates and analyzed using the β-scope 603 blot analyzer (Betagen Corp., Waltham, MA). CAT activity was defined by the percent conversion of acetylation normalized by β-galactosidase activity.

DNA plasmids used in transfection experiments

The ER expression plasmid, pCMVER, was obtained from Dr. Benita Katzenellenbogen and described previously (28). TGFβ promoter-CAT reporter plasmids used in this study were provided by Drs. Michael Sporn and Anita Roberts at the NIH. All of these plasmids were constructed using the same vector with an inserted TATA-less CAT gene. TGFβ1CAT was reported previously as phTG2 (29), which contains human TGFβ1 promoter sequence from −1132 to +11 in front of the CAT gene. TGFβ2CAT, previously reported as pB2–1728 (30), contains human TGFβ2 promoter sequence from −1728 to +3. TGFβ3CAT, reported as pB3–499 (31), contains the promoter sequence from −499 to +110 of the human TGFβ3 gene, whereas pB3–38 reporter plasmid contains a shorter sequence of TGFβ3 promoter (−38 to +110) in front of the CAT gene (31). The vitellogenin A2 promoter CAT plasmid, VIT-ERE-CAT (−331/−87VitCAT), was constructed according to the method of Klein-Hitpass *et al.* (18).

Osteoclast differentiation assay

Chicken osteoclast differentiation model (32, 33). Monocytes were isolated from the medullary bone of femora and tibia from egg-laying hens maintained on a calcium-deficient diet for 2 weeks and aliquoted at a density of $3 \times 10^5/cm^2$. Cultures were incubated with 0.001–10 ng/ml

recombinant human TGFβ3 for 7 days in 5% CO_2-air at 39 C. Bone resorption was quantitated between days 5–7 by measuring ^3H release into the medium from bone particles (20–53 μm) prelabeled *in vivo* with [^3H]proline (34). Hiura *et al.* (33) showed previously that the mass of ^3H-labeled bone resorbed correlates with Howships lacunae resorbed in bone slices with r = 0.98.

Mouse osteoclast differentiation cultures. The coculture method of Takahashi *et al.* (35) was modified and used to study the effects of TGFβ3 on osteoclast differentiation. A stable cell line, BALC, which supported osteoclast differentiation, was derived from neonatal BALB/C calvariae. The BALC cells (75,000 cells/chamber) were cocultured in Lab-Tek chamber slides (four-chamber, Nunc Inc., Naperville, IL) with bone marrow cells (150,000 cells/chamber) obtained from murine femora (8-week-old female BALB/C mice; Jackson Laboratories, Bar Harbor, ME). The cocultures were maintained in RPMI plus 5% heat-inactivated FBS (Hyclone) and 1% antibiotic-antimycotic solution (Life Technologies, Grand Island, NY) and treated with 10 nM 1,25-dihydroxyvitamin D_3 (Biomol, Plymouth Meeting, PA) and 0.01–10 ng/ml recombinant human TGFβ3. Medium was changed every 48–72 h, and fresh 1,25-dihydroxyvitamin D_3 and TGFβ3 were added. On day 8, the cells were fixed with formalin and stained for tartrate-resistant acid phosphatase (TRAP). The number of TRAP-positive cells containing three or more nuclei was quantitated. These multinucleated cells were confirmed to be osteoclast-like, as demonstrated by their ability to form resorption lacunae when exposed to bovine cortical bone slices and the ability of calcitonin to inhibit TRAP-positive multinucleated giant cell formation and bone resorption (data not shown in this paper).

Results

Bone-sparing effects of estrogen and raloxifene

The effects of 17β-estradiol or raloxifene on bone in the OVX rat are shown in Fig. 1. As previously shown (14), the 5-week ovariectomy period produced a 22% reduction ($P \leq 0.05$) in the bone mineral density of the distal metaphyseal region of the femur. Administration of 17β-estradiol or raloxifene to OVX rats sc produced dose-related prevention of bone loss due to OVX, with respective ED_{50} values of 2 and 80 μg/kg. These results were comparable to those of previous studies with oral gavage administration of raloxifene (14). The minimal effective dose ($P \leq 0.05$) was 1 μg/kg for 17β-estradiol and 30 μg/kg for raloxifene. In this particular study, both 17β-estradiol and raloxifene resulted in bone density levels that reached the level measured in intact rats at 0.03 mg/kg for 17β-estradiol or 1 mg/kg for raloxifene. Tamoxifen, a partial estrogen agonist/antagonist, offered partial protection from ovariectomy-induced bone loss with an ED_{50} value of 0.7 mg/kg and a minimal effective dose ($P \leq 0.05$) of 0.3 mg/kg. A 50% protection of bone mineral density loss by tamoxifen was achieved at the dose of 1 mg/kg.

TGFβ mRNA expression in rat bone

Although the mechanism of estrogen deficiency-induced bone loss is largely unknown, it has been reported that ovariectomy reduced the concentration of TGFβ, a key coupling factor of bone remodeling, in rat bone tissue (4, 21, 25). To establish if and at what level ovariectomy/estrogen replacement influenced TGFβ expression, we measured TGFβ mRNA levels in the femora of these animals. As shown in Fig. 2A, femoral TGFβ1 mRNA expression in sham-operated control female rats (Sham + vehicle) varied slightly (1.5-fold) during the 24-h treatment period. The level of TGFβ1 mRNA expression in OVX rats was more consistent and at the highest level observed in sham control animals (OVX + vehicle). This level of TGFβ1 mRNA expression in OVX rats (OVX + vehicle) was not affected by treatment with either 0.1 mg/kg 17β-estradiol (OVX + E_2) or 1 mg/kg raloxifene (OVX + Ral). Expression of TGFβ2 showed a similar pattern as TGFβ1 in the femur, where neither estrogen (OVX + E_2) nor raloxifene (OVX + Ral) treatment affected the expression of this message in OVX rats (OVX + vehicle) significantly (Fig. 2B). In contrast, ovariectomy caused a consistent 2-fold reduction of TGFβ3 mRNA expression in the femur (OVX + vehicle *vs.* Sham + vehicle; Fig. 2C). This reduction of TGFβ3 mRNA expression was rapidly restored to the sham control level by

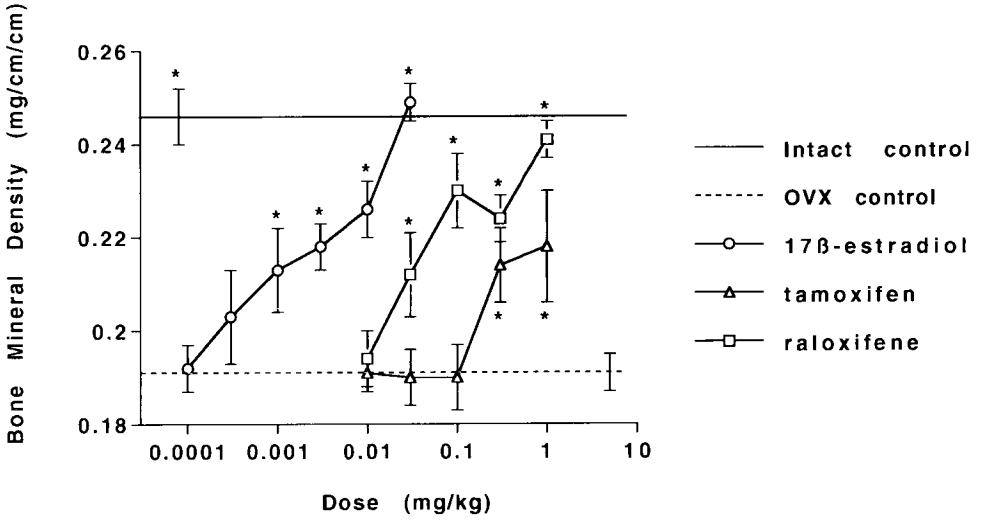

FIG. 1. Effects of 17β-estradiol, raloxifene, or tamoxifen on bone mineral density were determined in 75-day-old OVX rats. After ovariectomy, animals were given daily sc doses of the above agents at the indicated dosage levels for a period of 35 days, after which the animals were killed, and the left femur was removed. Bone mineral density was determined by single photon absorptiometry of the excised femur. The mean value of three transverse scans of the distal metaphyseal region (6–7 mm proximal to the distal end of the femur) was recorded. Statistical evaluation of the effects of 17β-estradiol, raloxifene, or tamoxifen on bone mineral density was made by one-way ANOVA with *post-hoc* Fisher's protected least significant difference analysis when indicated. Statistical significance was ascribed at $P \leq 0.05$. *, $P \leq 0.05$ *vs.* OVX control.

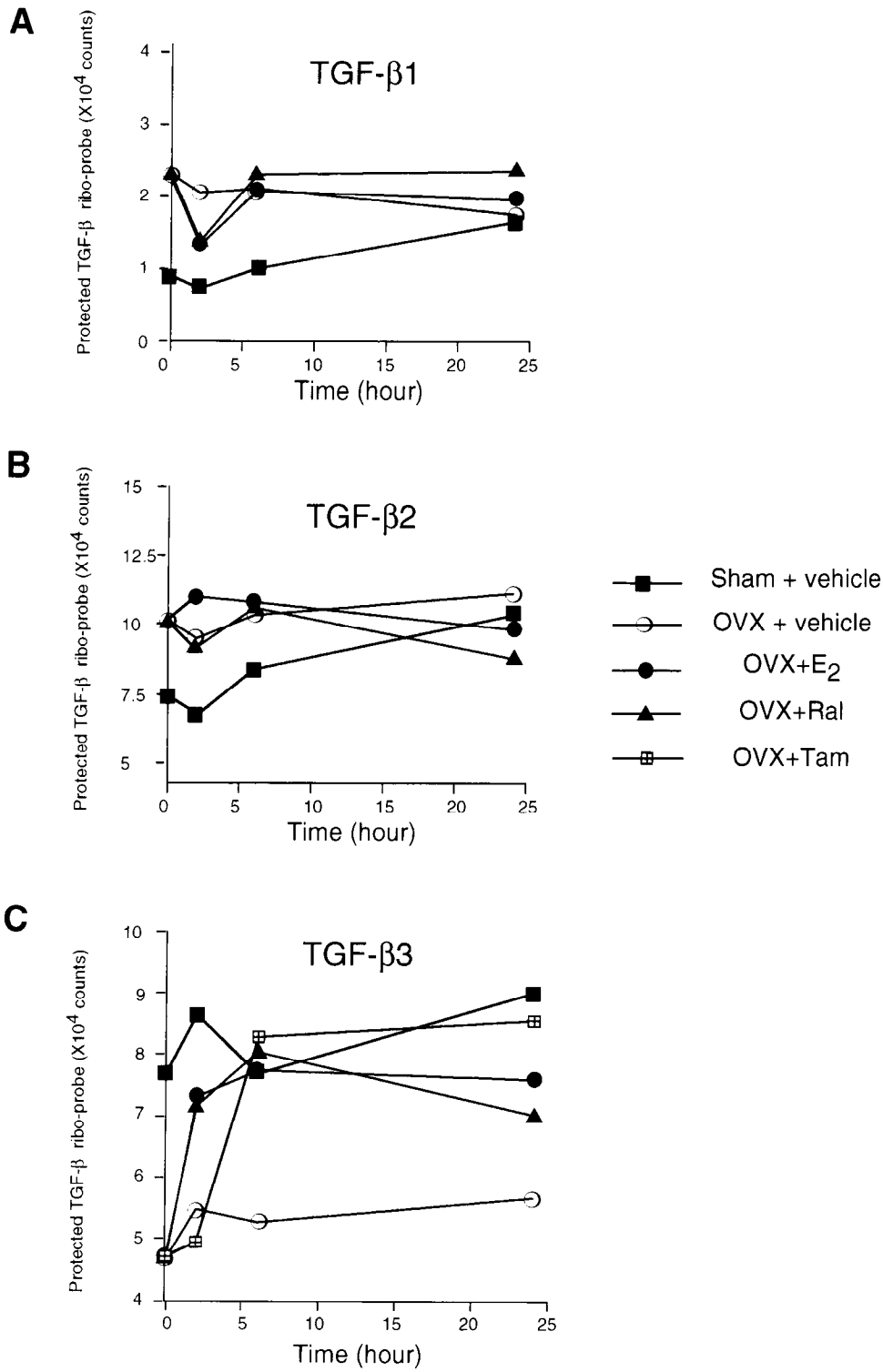

FIG. 2. Effects of ovariectomy and estrogen or raloxifene replacement on TGFβ mRNA expression in rat femora. Seventy-five-day-old Sprague-Dawley female rats were either sham operated or OVX 14 days before treatment. Treatment groups included 17β-estradiol (OVX + E_2) at 0.1 mg/kg, raloxifene (OVX + Ral) at 1 mg/kg or tamoxifen (OVX + Tam) at 1 mg/kg, each given sc. Sham control (Sham+vehicle) or OVX control (OVX+vehicle) rats received vehicle (corn oil) only. Animals were killed 2, 6, and 24 h after injection. Femora were excised for total RNA preparation. Levels of TGFβ mRNA expression in 20 μg total RNA were evaluated by RNase protection assays using isoform-specific TGFβ probes. Data were quantitated and normalized by GAPDH mRNA expression and plotted in the graph. Data are representative of two independently performed RPA assays.

either 17β-estradiol (0.1 mg/kg) or raloxifene (1 mg/kg) treatment within 2 h of injection. Tamoxifen injection (1 mg/kg) also restored TGFβ3 expression after 6 h of treatment.

Activation of promoters of TGFβ genes by 17β-estradiol and raloxifene

The intriguing observation that raloxifene, an agent previously defined as an antiestrogen in reproductive functions, stimulated TGFβ3 mRNA production *in vivo* in a manner similar to 17β-estradiol led us to further examine the mechanism of this regulation. To investigate whether 17β-estradiol or raloxifene could modulate TGFβ3 expression directly through its promoter, we performed transient cotransfection experiments using a TGFβ promoter-CAT reporter construct and an ER expression plasmid in human MG63 osteosarcoma cells. As shown in Fig. 3A, TGFβ3CAT expression was sig-

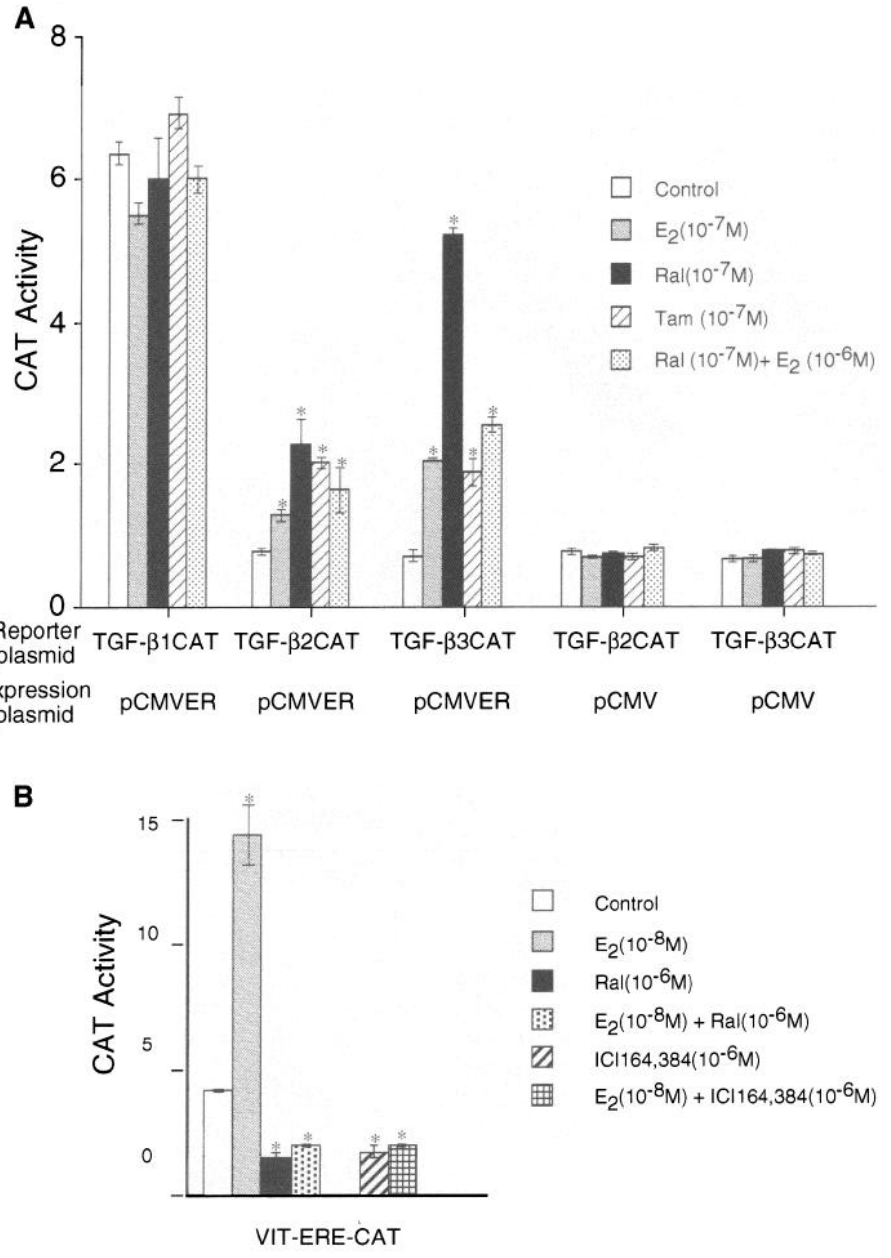

FIG. 3. ER-mediated TGFβ2 and TGFβ3 CAT activation by estrogen or raloxifene showed a novel ligand selectivity. A, Human osteosarcoma MG63 cells were cotransfected with each of the TGFβCAT plasmids, ER expression plasmid pCMVER or the arental plasmid pCMV and reference plasmid pSV-β-galactosidase vector. Cells were treated with 10^{-7} M each of raloxifene (*black bars*), 17β-estradiol (*gray bars*), tamoxifen (*hatched bars*), or a combination of raloxifene (10^{-7} M) and 17β-estradiol (10^{-6} M) (*dotted bars*) for 24 h after transfection. CAT activities were plotted as the mean and SD of three independently transfected samples. CAT activities were normalized by β-galactosidase activities for internal transfection control. B, Transfection experiments identical to those in A were performed using vitellogenin promoter containing CAT construct, VIT-ERE-CAT, in place of TGFβCAT constructs. Treatment included 10^{-8} M 17β-estradiol (*gray bar*), 10^{-6} M raloxifene (*black bar*), 10^{-6} M ICI 164,384 (*hatched bar*), and combinations of 10^{-8} M 17β-estradiol with 10^{-6} M raloxifene (*dotted bar*) or 10^{-6} M ICI 164,384 (*checkered box*). Data are representative of three independently performed transfection experiments. Each *data point* is presented as the mean and SD of triplicate samples obtained from a single experiment. In both A and B, data were analyzed using one-way ANOVA followed by a least significant difference multiple comparison test (Dunnett's). Statistical significance was ascribed at $P < 0.01$. *, $P < 0.01$ *vs.* vehicle control.

nificantly up-regulated by raloxifene, with a 7-fold increase, and by 17β-estradiol or tamoxifen, with 2-fold increases. TGFβ2CAT expression was also up-regulated by these agents, but only with a marginal 1.5-fold increase by 17β-estradiol and a 2-fold increase by raloxifene or tamoxifen. TGFβ1CAT expression, on the other hand, showed no response to either of these agents, although it was expressed at a higher basal level. Therefore, the promoter selectivity of 17β-estradiol or raloxifene activation *in vitro* paralleled the TGFβ isoform-specific regulation patterns by these two agents *in vivo* (Fig. 2). More interestingly, raloxifene up-regulated TGFβ2CAT and TGFβ3CAT more potently than

17β-estradiol, which functioned as a partial agonist/antagonist of raloxifene in these promoter contexts. When 17β-estradiol was given alone, it partially up-regulated TGFβ2CAT and TGFβ3CAT expression (Fig. 3A, *gray bars*), whereas when a 10-fold molar excess of 17β-estradiol (K_d = 0.1 nM) was given in combination with 100 nM raloxifene (K_d = 0.2 nM), it inhibited the high level expression of TGFβ2CAT and TGFβ3CAT induced by raloxifene to its intrinsic partial activation level (Fig. 3A, *dotted bars*). This unusual ligand selectivity of TGFβ3 activation was more evident when compared to regulation of the classical ERE-containing vitellogenin promoter by these agents. When the vitellogenin promoter-CAT reporter plasmid (VIT-ERE-CAT) was cotransfected with pCMVER in MG63 cells, vitellogenin promoter responded to 17β-estradiol (E_2) with a 3-fold up-regulation (Fig. 3B, *gray bar*). Raloxifene, on the other hand, was not only incapable of up-regulating the vitellogenin promoter, but also suppressed the basal expression level of VIT-ERE-CAT by 2.5-fold (Fig. 3B, *black bar*). When given at a 100-fold molar excess, raloxifene completely blocked the up-regulation of VIT-ERE-CAT expression by 17β-estradiol (Fig. 3B, *dotted bar*), demonstrating a pure antiestrogen activity similar to that of the pure antiestrogen, ICI 164,384. As shown in the same graph, ICI 164,384 suppressed the basal activity of the vitellogenin promoter in the absence of 17β-estradiol and completely blocked the activity up-regulated by 17β-estradiol when given in combination (Fig. 3B). Such promoter-dependent regulation by raloxifene through ER is not a cell-specific event. In MCF-7, HeLa, and Chinese hamster ovary (CHO) cells, similar regulation patterns were observed on both TGFβ3 promoter and vitellogenin promoter in transfection assays (data not shown).

To demonstrate that up-regulation of TGFβ3 by 17β-estradiol or raloxifene and tamoxifen was mediated by ER, the parental expression plasmid pCMV was used to replace pCMVER in transfection assays. As shown in Fig. 3A, neither 17β-estradiol, tamoxifen, nor raloxifene was able to up-regulate TGFβ2CAT or TGFβ3CAT (pCMV lanes) expression above the control value in the absence of ER.

A 150-nucleotide region in TGFβ3 promoter that mediates 17β-estradiol- or raloxifene-induced TGFβ3 up-regulation

To further examine whether this unusual hormone selectivity of TGFβ3 promoter activation was dose dependent, pB3–38 CAT reporter plasmid (31), containing the promoter region of hTGFβ3 from −38 to +110, was cotransfected in MG63 cells with pCMVER. As shown in Fig. 4A, both raloxifene and 17β-estradiol up-regulated pB3–38 in a dose-dependent manner. Raloxifene was consistently a more potent activator of TGFβ3 than 17β-estradiol in the dose range tested in this study. The sequence composition from −38 to +110 of the hTGFβ3 gene is illustrated in Fig. 4B. Noticeably, no palindromic ERE sequence was present in this region.

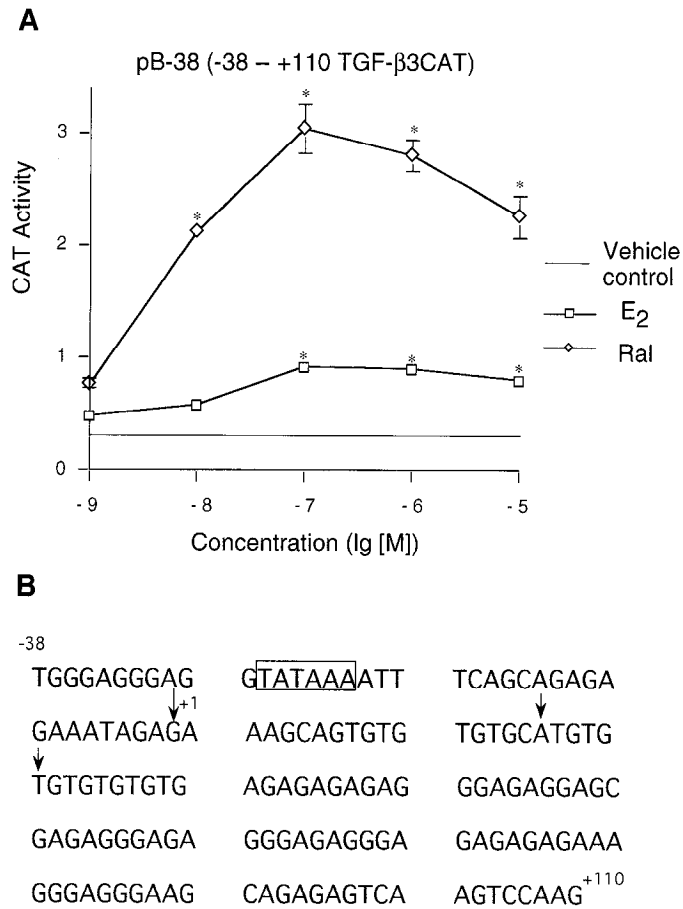

FIG. 4. Activation of TGFβ3 by 17β-estradiol and raloxifene was mediated through an 150-bp nucleotide sequence in the promoter region. A, pB3–38, a CAT reporter plasmid driven by the promoter sequence from position −38 to +110 of human TGFβ3, was cotransfected in MG63 cells with pCMVER. Cells were treated with 10^{-6}-10^{-9} M 17β-estradiol, raloxifene, or vehicle as control. CAT activity was normalized by β-galactosidase activity and plotted on the graph. Each *data point* is presented as the mean and SD of triplicate samples obtained from a single experiment. Data are representative of three independently performed transfection experiments. SDs for control, estrogen-treated, and 10 nM raloxifene-treated samples were smaller than the symbols. Statistical analysis was performed as described in Fig. 3A. Statistical significance was ascribed at $P < 0.01$. *, $P < 0.01$ *vs.* vehicle control. B, Nucleotide sequence of the region from −38 to +110 of the TGFβ3 promoter (31). *Arrowheads* indicate the major (+1) or minor transcription initiation sites. The TATA sequence is *boxed*. Numbers indicate nucleotide positions relative to the major transcription initiation site.

Inhibition of osteoclast differentiation by TGFβ3

Next, we addressed the potential biological consequences of elevated TGFβ3 production in bone by 17β-estradiol or raloxifene. Although TGFβ1 and -2 have been demonstrated to be potent inhibitors of osteoclast differentiation in different *in vitro* and *in vivo* models, such as rat long bone and human osteoclast cell cultures (36), TGFβ isoforms have been known to have different or even opposite effects on the same biological processes (37). To evaluate the effects of TGFβ3 on osteoclast differentiation, we used two well characterized osteoclast differentiation models. In a chicken osteoclast differentiation system (32, 33), attachment-selected monocytes from the medullary bone of egg-laying hens were cultured in the presence of TGFβ3 for 1 week. The osteoclast-specific differentiation markers of bone resorption were evaluated between days 5–7. As shown in Fig. 5A, TGFβ3 significantly inhibited the resorption activity of these cells in a dose-

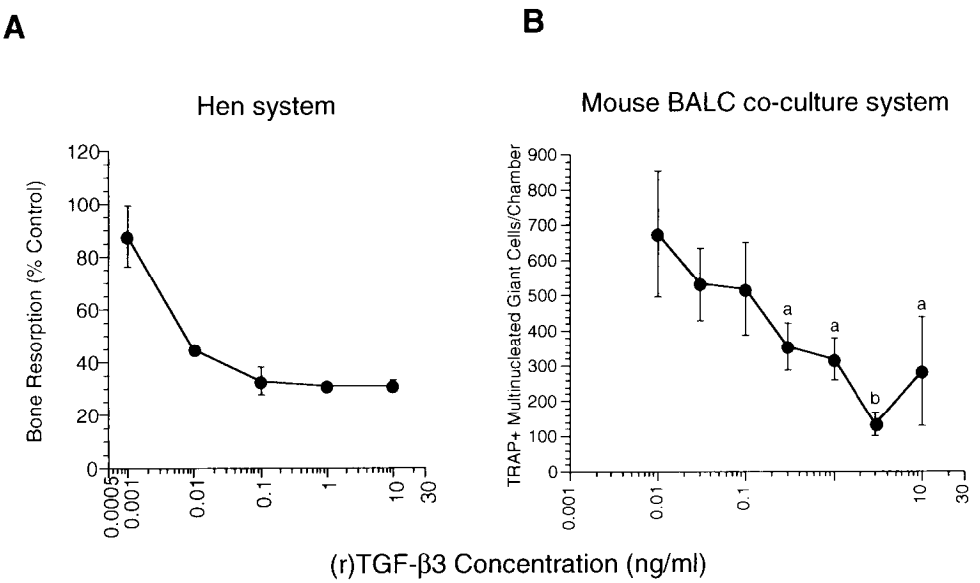

FIG. 5. Inhibition of osteoclast differentiation and bone resorption by TGFβ3 *in vitro*. A, The effects of TGFβ3 on differentiation and bone-resorbing activity of chicken osteoclast were evaluated in the chicken osteoclast differentiation model. Monocyte cultures from the medullary bone of egg-laying hens were incubated with 0.001–10 ng/ml recombinant human TGFβ3 for 7 days in 5% CO_2-air at 39 C. On day 7, monocyte cultures were incubated with *in vivo* [^{3}H]proline-prelabeled bone particles (20–50 μm) (21) to measure ^{3}H release into the medium. Data are presented as resorption (micrograms) compared to that in untreated controls (mean ± SD; n = 3–4). B, Effects of TGFβ3 on murine osteoclasts. Murine calvarial-derived cells, BALC, were cocultured with bone marrow cells in the continuous presence of 10 nM 1,25-dihydroxyvitamin D_3, and 0.01–10 ng/ml recombinant human TGFβ3 for 8 days. On day 8, the cultures were fixed with formalin and stained for TRAP. The number of TRAP-positive cells containing three or more nuclei were quantitated and are plotted in the figure. Each *data point* represents the mean and SD of four chambers. Data were analyzed using a one-way ANOVA followed by a least significant difference multiple comparison analysis (Tukey-Kramer) to determine the differences between individual groups. The level of significance was set at $P < 0.05$ compared to the control. a, $P < 0.05$ compared to the 0.01 ng/ml group. b, $P < 0.05$ compared to the 0.01, 0.03, and 0.1 ng/ml groups. Data are representative of three experiments using different cell populations.

responsive manner. Similar effects on osteoclast differentiation were observed with the mouse model of Takahashi *et al.* (35). *In vitro* osteoclast differentiation was observed when calvarial-derived BALC cells were cocultured with bone marrow-derived osteoclast progenitor cells in the presence of 1,25-dihydroxyvitamin D_3. As shown in Fig. 5B, the addition of TGFβ3 inhibited the formation of TRAP-positive osteoclast-like cells in a dose-dependent manner. These multinucleated cells were demonstrated to have the ability to form resorption lacunae when exposed to bovine cortical bone slices. Furthermore, calcitonin was able to inhibit TRAP-positive multinucleated giant cell formation and bone resorption.

TGFβ has been reported to be produced by both osteoblasts and osteoclasts (38, 39). In addition, studies have demonstrated that osteoclasts can activate latent TGFβ. Thus, the levels of TGFβ added to the cultures could have been slightly higher than indicated by the amount of exogenous TGFβ added. To evaluate whether endogenously produced or activated TGFβ was a major source of the observed osteoclast differentiation, we incubated the coculture system with TGFβ-neutralizing antibodies. No statistically significant difference in TRAP-positive osteoclast-like cell numbers was observed between cultures with and without antibodies, indicating that the effects observed in Fig. 5 were largely due to TGFβ3 added exogenously to the cultures (data not shown here). Additionally, inhibitory effects of TGFβ3 were observed in a porcine osteoclast differentiation model. Thus, we conclude that TGFβ3 inhibits osteoclast differentiation and bone resorption, which may be part of the mechanism by which 17β-estradiol and raloxifene prevent the loss of bone due to estrogen deficiency.

Discussion

The cellular and molecular targets of estrogen action in bone have been the focus of investigation for understanding of estrogen-mediated bone maintenance. Previous data indicated that estrogen may exert its biological functions in bone by influencing the expression of local factors that play important roles in skeletal homeostasis. To identify target genes of estrogen regulation in bone, we examined *in vivo* and *in vitro* regulation of the TGFβ by 17β-estradiol or a selective ER modulator (SERM), raloxifene. Our data provide insights in understanding the mechanisms by which estrogen and raloxifene act on bone.

First, using the OVX rat model, we demonstrated TGFβ3 as a potential target gene under 17β-estradiol regulation in intact rat bone. The data presented in this study suggest that withdrawal of estrogen in OVX rats or postmenopausal women causes decreased expression of TGFβ3 in bone. 17β-Estradiol or raloxifene can transcriptionally up-regulate TGFβ3 gene in bone. This increased level of TGFβ, therefore, will inhibit osteoclast differation and bone loss. A closer examination of the data presented in this study suggests that TGFβ3 may not be the sole causative agent in estrogen-mediated bone metabolism as one compares the *in vivo* bone

protective activities of raloxifene and 17β-estradiol to the *in vitro* activities of these agents to transactivate TGFβ3 promoter. In OVX rats, 17β-estradiol is 30- to 40-fold more potent than raloxifene to protect against ovariectomy-induced bone density loss. However, in transient transfection assays, raloxifene was 4-fold more potent than 17β-estradiol (Fig. 4). This may be explained by the observations that estrogen affects multiple factors in bone, including IGF-I, IL-1, and IL-6, which all play crucial roles in bone metabolism (23, 24). Future studies concerning whether TGFβ, IGF-I, IL-6, IL-1, and other factors were regulated by estrogen sequentially or simultaneously in bone in a spatially and temporally specific manner will further establish the mechanism by which estrogen regulates skeletal homeostasis.

Second, estrogen and raloxifene regulation of TGFβ is isoform specific. Our data showed that TGFβ3 was activated by 17β-estradiol or raloxifene both *in vivo* and *in vitro*. TGFβ2 was activated by raloxifene weakly as determined by *in vitro* promoter analysis. However, such a weak promoter response was not translated into a message change in intact animals, possibly due to the lower sensitivity of the promoter. TGFβ1 demonstrated no response to either raloxifene or estrogen in intact bone tissue or *in vitro* promoter analysis. Such differential promoter sensitivity of TGFβ genes in response to hormonal regulation suggests a divergence of their biological activities and regulation. TGFβs are multifunctional growth modulators that play a central role in numerous physiological and pathological processes by mediating cell growth and differentiation (40, 41). Although all three isoforms share the same receptor systems and are interchangeable in most *in vitro* assay systems, each isoform is expressed in a developmental and tissue-selective fashion (42). Distinct properties of each of the isoforms in several biological systems have been noted. For instance, in an adult rodent cutaneous wound-healing model, reduction of scarring required the addition of TGFβ3 and the neutralization of TGFβ1 and -2 simultaneously (37). TGFβ2 and TGFβ3, but not TGFβ1, inhibit the survival of cultured chick ciliary ganglionic neurons (43). TGFβ1 and TGFβ3 are more potent than TGFβ2 in inhibiting the migration of aortic endothelial cells (44). Furthermore, although TGFβ2 and TGFβ3 share homology in their promoter regions, the promoter of TGFβ1 is quite divergent, leading to speculation that each gene is distinctively regulated in a spatially and temporally specific manner. The data presented in this study provide additional experimental evidence to support such a hypothesis.

Third, our data established a direct promoter regulation of TGFβ gene by the ER. Cross-talk between steroid hormones and TGFβ has been observed at many different levels to coordinate specific events of cell proliferation and differentiation (42). The demonstration of direct gene regulation of the multifunctional growth factor, TGFβ, by estrogen may provide a molecular understanding of many estrogen-related activities observed in man and animals. For instance, TGFβ is a factor known to play important roles in regulating cardiovascular functions, including smooth muscle cell proliferation, low density lipoprotein receptor up-regulation, and nitric oxide synthase modulation (45–47). TGFβ has also been known to suppress cancer cell proliferation (48), stimulate apoptosis in different tissue or cell types (49, 50), and provide neuronal protection by regulating BCL-2 protein expression and calcium homeostasis (51). Expression of TGFβ is highly responsive to external stimuli in the brain (52). Hence, through TGFβ regulation, we may gain further mechanistical understanding of estrogen-regulated biological processes in the central nervous system, cardiovascular system, and cancer progression, as well as reveal potential new biological functions previously unknown to estrogen.

Lastly, data presented in this study suggest that TGFβ3 might represent a novel regulatory pathway of gene regulation by ER distinct from ERE-containing genes. The difference of TGFβ3 activation from ERE activation was suggested by the lack of ERE in its promoter and the reversed ligand preference for TGFβ3 activation by ER shown in Figs. 3 and 4. Previously, raloxifene was shown to exhibit a classical antiestrogenic profile in regulating the progesterone receptor gene, a conventional ERE-containing estrogen-inducible gene, in breast cancer cells and rat uterus on which gene 17β-estradiol functioned as the full agonist (53, 54). We have confirmed in this study a pure antiestrogenic activity of raloxifene on another ERE-containing gene, the vitellogenin gene. In the TGFβ3 promoter system, however, the preferred ligand was raloxifene, whereas 17β-estradiol functioned as a partial agonist/antagonist that activated the TGFβ3 promoter partially and only at pharmacological concentrations. Based on such observations, we speculate that an endogenous ligand different from 17β-estradiol may be the functional "hormone" of TGFβ3 activation *in vivo*. The SERM molecule, raloxifene, mimics the endogenous ligand as a *bona fide* agonist. Further elucidation of the response element, ligand requirement, as well as ER functions in TGFβ3 activation by ER followed by identification of other potential genes under the new regulatory pathway may further address the molecular mechanism through which SERM molecules, such as raloxifene, function as a tissue-selective estrogen agonist/antagonist. In conclusion, our study provided a molecular target for estrogen regulation of skeletal homeostasis through which raloxifene functions as a *bona fide* agonist in bone.

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