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Estrogen Receptor-Related Receptor α Impinges on the Estrogen Axis in Bone: Potential Function in Osteoporosis

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The orphan nuclear estrogen receptor-related receptor α (ERRα) is expressed by osteoblastic cells and plays a functional role in osteoprogenitor proliferation and differentiation. To dissect further the role of ERRα in bone, we investigated the effects of estrogen (E2) on ERRα both *in vitro* and *in vivo*. Chronic treatment of fetal rat calvaria cells with E2-stimulated bone nodule formation and up-regulated ERRα mRNA expression at early (10 h and d 8) but not later times in culture, suggesting a link between ERRα and E2 during osteoprogenitor proliferation. ERRα mRNA levels were significantly lower in ovariectomized adult rat bones *vs.* those of sham-operated rats early (1 d and 1 wk) post surgery, but levels returned to control levels thereafter. ERRα is also expressed in osteoclasts (tartrate-resistant acid phosphatase + multinucleated cells) *in vivo* and *in vitro* (RAW 264.7 cells) and ovariectomization lowered the OPG/receptor activator of nuclear factor κB ligand expression ratio. Down-regulation of ERRα expression via antisense treatment of rat calvaria cells not only inhibited osteogenesis but also increased adipocyte colony formation and changed the OPG/receptor activator of nuclear factor κB ligand ratio. These data suggest that ERRα is regulated by estrogen in bone in which it may play a functional role at several levels (osteoblasts, adipocytes, and osteoclasts) in E2 deficiency diseases such as osteoporosis. (*Endocrinology* 143: 3658–3670, 2002)

NUCLEAR RECEPTORS ARE transcription factors involved in various physiological regulatory processes. The superfamily to which nuclear receptors belong comprises both ligand-dependent molecules such as the steroid hormone, thyroid hormone, retinoic acid, and vitamin D receptors and an increasing number of so-called orphan receptors for which no ligand has yet been determined (1–3). Similar to the classic ligand-dependent receptors, the orphan receptors display the same structural organization and contain the two more conserved domains: the DNA-binding domain (domain C) and the ligand-binding domain (domain E), but it is not yet known whether they have ligands that await identification or whether they act in a constitutive manner.

Two orphan receptors, estrogen receptor-related receptor (ERR) α and ERRβ (4), NR3B1 and NR3B2, respectively, according to the Nuclear Receptors Nomenclature Committee, 1999 (5), are closely related to the estrogen receptors (ERs) α and β (6, 7), NR3A1 and NR3A2, respectively. ERRα and ERRβ were identified by low-stringency screening of cDNA libraries with a probe encompassing the DNA-binding domain of the human ER. Recently, a third ERR, ERR3 or ERRγ, was identified by yeast two-hybrid screening with the glucocorticoid receptor-interacting protein 1 as bait (8). The DNA-binding domain region of ERRs and ERs is highly conserved; however, the other parts of the proteins share very little homology (4, 8). Sequence alignment of ERRα and the ERs, for example, reveals a high similarity (68%) in the 66 amino acids of the DNA-binding domain but only a moderate similarity (36%) in the ligand-binding E domain, which may explain the fact that ERRα does not bind estrogen. Although agonists of the ERRs have not yet been identified, the pesticides chlordane and toxaphene (9), the synthetic estrogen diethylstilbestrol (10), and the selective ER modulator 4-hydroxytamoxifen (11) have been suggested to be potential ligands for ERRα and ERRγ, respectively, and all act as antagonists. ERRα has been identified as a regulator of fat metabolism (12, 13) as well as a regulator of the human aromatase gene in breast, in which it is hypothesized to be critical for normal breast development (14, 15). Yang *et al.* (16) and Zhang and Teng (17) also showed that ERRα modulates the activating effect of estrogens on the lactoferrin promoter and suggested that ERRα may interact with ERs through protein-protein interaction.

Postmenopausal osteoporosis is a condition caused primarily by the severe decrease of serum estrogen levels after cessation of ovarian function. The absence of estrogen results in an increase in bone turnover (18) and a negative bone-remodeling balance, leading to bone loss and an increased fracture risk. The decrease in bone volume is accompanied by an increase in marrow adipose tissue (19, 20) and it has

Abbreviations: ALP, Alkaline phosphatase; aP2, fatty acid-binding protein; AS, antisense; BSP, bone sialoprotein; c/EBPα, CCAATT enhancer-binding protein α; E2, 17β estradiol; ER, estrogen receptors; ERRα, estrogen receptor-related receptor α; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPL, lipoprotein lipase; OCN, osteocalcin; OVX, ovariectomized; PPARγ, peroxisome proliferator-activated receptor γ; RANKL, receptor activator of nuclear factor κB ligand; RC, rat calvaria; S, sense; Sc, scrambled; TRAP, tartrate-resistant acid phosphatase.

been suggested that the increase in the number of adipocytes is due to a shift in production of adipocytes *vs.* osteoblasts from common bipotential precursors in the marrow cavity (21). A positive effect of estrogens on bone homeostasis has been documented in postmenopausal osteoporosis (18, 22) in which bone loss can be reversed by administration of natural or synthetic estrogens. Although the bone-preserving effect of estrogen replacement is indisputable, the molecular and cellular mechanism(s) mediating this effect remain unclear. ERs are expressed in osteoblasts (18, 23, 24), and estrogens have been found to elicit effects ranging from modulation of gene expression to regulation of proliferation in this cell type (18). In contrast, mice lacking a functional ERα or ERβ have only minor skeletal abnormalities (25, 26), suggesting that other isoforms of ERs (27) or mechanisms or receptors might be important during skeletal development. Given its homology to the ERs and the evidence that it may interact with ERα and modify estrogen effects on at least some genes, we hypothesized that ERRα may intervene in the signals induced by estrogen in bone. It has been shown, for example, that ERRα positively regulates the osteopontin gene (28, 29), an extracellular matrix molecule secreted by osteoblasts and thought to play a critical role in bone resorption (30, 31). More recently, we have found that ERRα plays a functional role in osteoprogenitor cell proliferation and differentiation at least *in vitro* and that these processes are exquisitely sensitive to changes in ERRα levels, with both up-regulation and down-regulation of the developmental sequence seen when ERRα is respectively up-regulated or down-regulated (32). In addition, ERRα is coexpressed with ERs in osteoblasts and therefore may modulate expression of common target genes in these cells (33).

Given these observations, it seemed important to determine whether ERRα impinges on the estrogen axis in bone. We report here that ERRα expression is increased by estrogen in a differentiation stage-specific manner in fetal rat calvaria (RC) cells *in vitro* and decreased after ovariectomy of rats, a well-established model of postmenopausal osteoporosis. We also found that down-regulation of ERRα expression via antisense (AS) treatment in RC cells, not only inhibited osteogenesis as reported previously (32) but also increased formation of adipocyte colonies and expression of adipocyte-associated markers, peroxisome proliferator-activated receptor γ (PPARγ), lipoprotein lipase (LPL), CCAATT enhancer-binding protein (c/EBPα), and the fatty acid-binding protein (aP2). Finally, we show that ERRα is expressed not only by osteoblasts but also by osteoclasts *in vivo* and by monocytic cells and through all developmental stages of osteoclasts differentiation *in vitro*. These data support the hypothesis that ERRα is regulated by estrogen in bone in which it may play a functional role at several levels (osteoblast, adipocyte, and osteoclast lineage cells) in estrogen-dependent diseases such as osteoporosis.

Materials and Methods

Cell culture

Osteoblasts. Cells were enzymatically isolated from the calvaria of 21-d Wistar rat fetuses that were killed by cervical dislocation; animal use and all procedures were approved by the University of Toronto Animal Care Committee. Cells obtained from the last four of five sequential digestion steps, populations II–V (34), were plated in T-75 flasks in αMEM containing 15% heat-inactivated fetal bovine serum (Flow Laboratories, McLean, VA) and antibiotics comprising 100 μg/ml penicillin G (Sigma, St. Louis, MO), 50 μg/ml gentamicin (Sigma), and 0.3 μg/ml Fungizone (Flow Laboratories). After a 24-h incubation, attached cells were washed with PBS to remove nonviable cells and other debris and then collected by trypsinization using 0.01% trypsin in citrate saline. Aliquots were counted with a Coulter counter (Coulter Electronics, Hialeah, FL), and the remaining cells were resuspended in the standard medium described above but lacking phenol red. The resuspended cells were plated into 100-mm tissue culture dishes at 10^5 cells/dish and in 24-well plates at 10^4 cells/well. After 24-h incubation, medium was changed and supplemented with 50 μg/ml ascorbic acid, 10 mM sodium β-glycerophosphate, and with or without vehicle (ethanol at 0.01%), 17β estradiol (E2) (10^{-8} to 10^{-11} M, Sigma), or ICI 182,780 (10^{-9} M, Tocris). For studies of E2 effects at early times after plating (10 h), 2% fetal calf serum (FCS) was used; 15% was used in all other experiments as indicated. Medium was changed every 2 d. All dishes were incubated at 37 C in a humidified atmosphere in a 95% air/5% CO_2 incubator.

Osteoclasts. RAW 264.7 (number TIB-71, American Type Culture Collection, Manassas, VA) were used to generate osteoclast-like cell using RANKL (receptor activator of nuclear factor κB ligand). Cells were plated overnight at a density of 2×10^4 cells/well in a 6-well plate in DMEM (Life Technologies, Inc., Rockville, MD) and 1% FCS. The next day the medium was changed to DMEM supplemented with 10% FCS and 30 ng/ml RANKL (Amgen, Inc., Thousand Oaks, CA). After 24 h, the medium was replaced by DMEM, pH 7.2 [13.53 g DMEM (Sigma), 0.78 g sodium bicarbonate (Sigma), 10% FCS, and 30 ng/ml RANKL (Amgen, Inc.)]. For each sample, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) or lysed for RNA extraction.

AS and sense (S) oligonucleotide treatment

RC cells were plated in 24-well plates at 10^4 cells/well. AS oligonucleotide inhibition of ERRα expression was accomplished with a 20-base phosphorothioate-modified oligonucleotide, localized to the A/B domain (32). Control dishes were treated with the complementary S oligonucleotide or no oligonucleotide. Briefly, oligonucleotide concentrations we found previously not to be toxic (0.5 μM to 2 μM) were added directly to cells during the differentiation phase (d 5, the end of proliferation, to d 11) in standard medium as above supplemented with 50 μg/ml ascorbic acid, 10 mM sodium β-glycerophosphate, and 10^{-8} M dexamethasone. Medium was changed every 2 d, and fresh oligonucleotides were added at each change. At d 15, cultures were terminated and mRNA was collected from some, and others were used for quantification of bone nodules and adipocyte colony formation.

Bone nodule and adipocyte colony quantification

For quantification of bone nodule and adipocyte colony formation, dishes or wells were fixed and stained by the Von Kossa technique (bone) or Sudan IV (adipocytes), and bone nodules and adipocyte colonies were counted on a grid (34, 35). Results are plotted as the mean number of nodules or adipocyte colonies ± SD of three wells for controls and each concentration of AS or S primers; results are representative of three independent experiments.

TRAP staining

Paraffin sections were deparaffinized in xylene, rehydrated (through 100%, 95%, and 70% ethanol and water), and washed twice with 1× PBS; cultured cells were fixed in 10% formalin for 10 min and then washed twice with 1× PBS. Fixed cells or sections were then incubated for 30 min and 1 h, respectively, at 37 C with freshly prepared TRAP staining solution [1 mg/ml naphthol AS MX phosphate (N-4875, Sigma)], 1% *N,N,*dimethylformamide (D-8654, Sigma), 0.6 mg/ml fast red TR (F-6760, Sigma), 2 mg/ml sodium tartrate (S-8640, Sigma) in 0.1 M acetate buffer, pH 5.9.

Ovariectomized rats

Four-month-old female Wistar rats, either ovariectomized (OVX) or sham-operated (Sham), were kept under standard laboratory conditions

for up to 4 wk. Animals were killed and the uteri weighed to ensure efficacy of the OVX surgery. Femurs were removed and samples for immunocytochemistry were processed and embedded in paraffin after fixing in 4% paraformaldehyde in PBS and decalcification for 2 wk. Total cellular RNA was prepared with Trizol reagent (Life Technologies, Inc.) from parallel femoral samples (collected at 1 d, 1 wk, 2 wk, and 4 wk after OVX) from which bone marrow had been rapidly flushed and bones had been snap-frozen in liquid nitrogen.

RT-PCR

Aliquots of total cellular RNA (1.5–5 μg) extracted with Trizol reagent (Life Technologies, Inc.) from RC and RAW 264.7 cells, and sham and OVX femurs were reverse transcribed using oligo dT and a first-strand synthesis kit (SuperScript II, Life Technologies, Inc.). PCR was performed with primers specific for ERRα, osteoblast-associated markers [osteocalcin (OCN), bone sialoprotein (BSP), and alkaline phosphatase (ALP); primers as in Ref. 32], adipocyte-associated markers (PPARγ, LPL, aP2, c/EBPα; primers below), osteoclast-associated markers (TRAP, cathepsin K; primers below), other molecules [osteoprotegerin (OPG), RANKL; primers below] markers, and the housekeeping gene ribosomal proteins L32 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The PCR mixture contained cDNA (1 μl), 1 μl deoxynucleotide triphosphate mix (10 mM), 10× PCR buffer, Q solution, 25 pmol primers, and 5 U *Taq* polymerase (QIAGEN, Valencia, CA). PCR was done for 25 cycles (94 C for 1 min, 55 C for 1 min, 72 C for 1 min, and a final elongation step of 7 min at 72 C) for OCN, BSP, ALP, and PPARγ; 22 cycles for L32 and ERRα; 30 cycles and 32 cycles for OPG and c/EBPα, respectively; 37 cycles (with annealing temperatures of 57 C) for RANKL; and 30 cycles (95 C for 30 sec, 60 C for 30 sec) for TRAP, cathepsin K, and GAPDH. Nested PCR or reamplification was required to visualize LPL (20 cycles and 13 cycles for first and second PCR, respectively, with annealing temperatures of 56 C) and aP2 (25 cycles for both PCR steps with annealing temperature of 55 C). The primers used were: PPARγ upstream, GCG GAG ATC TCC AGT GAT ATC; PPARγ downstream, TCA GCG ACT GGG ACT TTT CT; aP2 upstream, AAT TTG TAC TCT AAG; aP2 downstream, GTA ATC ATC GAA GTT TTC AC; LPL upstream, GTC TGA CCA ACA AGA AGG TC; LPL downstream, CAC TTA AGC TTC ATC ATC AG; LPL downstream (nested), GAG AAA TCT CGA AGG CCT GGT TG; c/EBPα upstream, CTT GCA GTT CCA GAT CGC AC; c/EBPα downstream, CAA CTC CAA CAC CTT CTG CTG; OPG upstream, TTG TGT GAC AAA TGT GCT CC; OPG downstream, GAC GTC TCA CCT GAG AAG; RANKL upstream, GTG GTC TGC AGC ATC GCT CTG; RANKL downstream, CGC TGG GCC ACA TCC AAC C; GAPDH upstream, TTCGACAGTCAGCCGCATCTTCTT; GAPDH downstream, CAGGCGCCCAATACGACCAAATC; TRAP upstream, AGCAGCCAAGGAGGACTACGTT; TRAP downstream, TCGTTGATGTCGCACAGAGG; cathepsin K upstream, TTAATTTGGGAGAAAAACCT; cathepsin K downstream, AGCCGCCTCCACAGCCATAAT.

The identity of all the PCR products was confirmed by sequencing the bands and comparison with published sequences (NCBI; BLAST search). Amplified bands were quantified by densitometric analysis and the results plotted represent the mean ± SD of triplicate determinations of one experiment, but similar results were seen in three independent experiments.

Immunohistochemistry

Immunolabeling of femurs was done essentially as described previously (36, 37). Paraffin sections were deparaffinized in xylene, rehydrated (through 100%, 95%, and 70% ethanol and water), rinsed in PBS, and then incubated for 1 h at room temperature with 10% normal serum in PBS. After rinsing in PBS, sections were incubated for 3 h at room temperature with a 1/50 dilution of anti-ERRα (32); 10% normal serum in PBS was used as negative control. Sections were then rinsed in PBS and incubated for 1 h at room temperature with secondary antibody CY-3-conjugated antirabbit (1/300 final dilution, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). After rinsing, samples were mounted in Moviol (Hoechst Ltd., Montréal, Québec, Canada) and observed by epifluorescence microscopy on a Zeiss photomicroscope III (Zeiss, Oberkochen, Germany). For photography and printing, equal exposure times were used for specifically labeled and control cultures.

Western blots

Total protein was extracted from RC cells at d 8 according to standard methods (38) and run on 7.5% SDS-PAGE gels, and Western blots were prepared in a semidry system. Immunoblotting was performed with a 1/60 dilution of anti-ERRα (32); blots were incubated overnight at room temperature and binding was detected using horseradish peroxidase-conjugated goat antirabbit antibodies (1/3000, Bio-Rad Laboratories, Inc., Hercules, CA) and chemiluminescence.

Statistical analysis

Results for PCR analysis and quantification of bone nodule and adipocyte colony number (AS/S experiments) were expressed as mean ± SD and analyzed statistically by ANOVA followed by *post hoc t* tests; statistical significance was taken as $P < 0.05$.

Results

ERRα expression and osteoblast differentiation are stimulated by estrogen in RC cell cultures

As well established, primary fetal RC cells undergo a proliferation-differentiation sequence when grown under appropriate conditions *in vitro* (39). For example, under the culture conditions used here (standard RC differentiation medium but lacking phenol red), expression of mRNA for early osteoblast markers (ALP, BSP) peaked by d 8–11, but late markers (OCN) were detectable only by d 11 and beyond (Fig. 1). To determine whether ERRα expression is regulated by estrogen over this developmental sequence, we treated these same cultures with different concentrations of E2. Treatment through proliferation and up to early differentiation phase significantly stimulated ERRα mRNA expression [50% at 10^{-10} M after 10 h (Fig. 2A); 40% at 10^{-8}, 10^{-10}, and 10^{-11} M, and 60% at 10^{-9} M at d 8 (Fig. 2B); and similarly at d 6 (not shown)], but treatment through later differentiation stages (d 11, 18) did not (Fig. 2C). Moreover, consistent with our previous observation that up-regulation of ERRα stimulates differentiation and bone nodule formation in RC cultures (32), the number of bone nodules formed was slightly (10%) but significantly increased by E2 (10^{-9} M) treatment, an effect blocked by ICI 182,780 (10^{-9} M) treatment (Fig. 3A). The 10^{-9} M ICI 182,780 also blocked the E2-stimulated increase in ERRα mRNA expression observed at 10 h (not shown) and d 8 (Fig. 3B) and the E2-stimulated increase in ERRα protein production at d 8 (Fig. 3C), suggesting that the increase in nodule formation and ERRα expression are mediated through ERs in RC cell cultures.

ERRα expression is decreased in bones of ovariectomized rats

The regulation of ERRα expression by E2 in RC cell cultures prompted us to ask whether similar regulation can be seen *in vivo*. To address this question, we used mRNA extracted from femurs of OVX rats, a model known to mimic the bone changes seen in postmenopausal osteoporosis. Visual inspection (Fig. 4A) and weights of the uteri dissected 4 wk after surgery showed the efficacy of surgery, with marked hypoplasia of OVX (0.16 g) *vs.* sham (0.8 g) uteri. Semiquantitative RT-PCR of RNA from femurs (from which bone marrow had been flushed) of animals at different times after surgery revealed that ERRα expression was significantly decreased 1 d and 1 wk after OVX but had rebounded

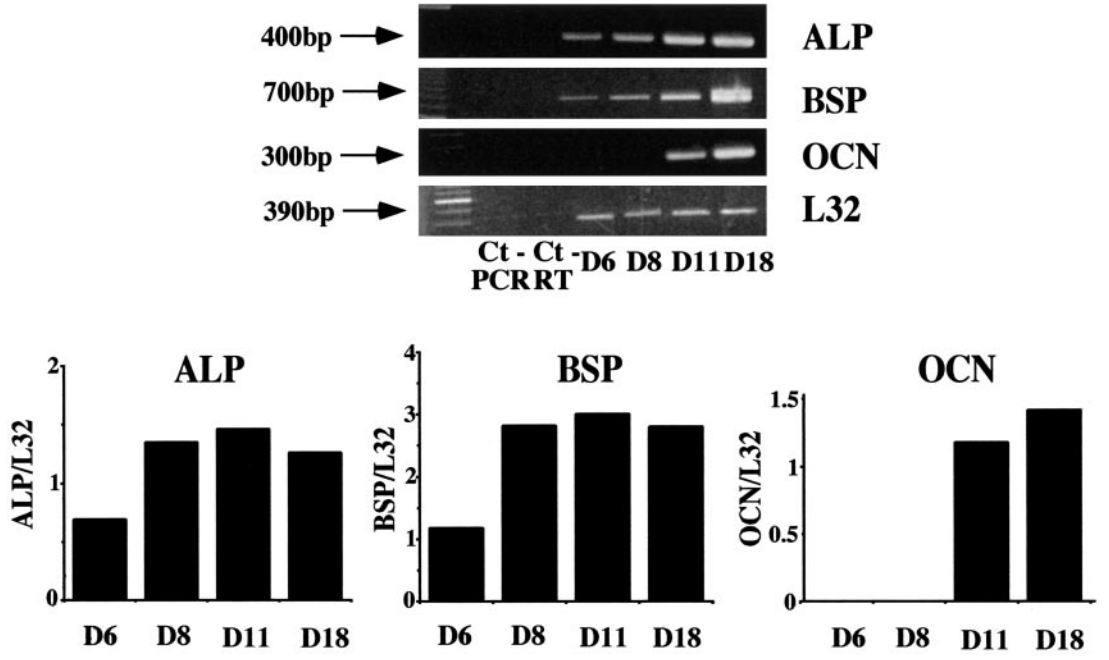

FIG. 1. Osteoblast developmental stages were confirmed in differentiating RC cell cultures by semiquantitative RT-PCR assessment of mRNA levels of three osteoblast markers (ALP and BSP, relatively early markers of osteoblast development, and OCN, a marker of osteoblast maturation). RC cells were cultured in phenol red free medium under standard differentiation conditions (*Materials and Methods*). ALP, BSP, and OCN band intensities were normalized against that of the housekeeping gene ribosomal protein L32.

to levels in sham-operated animals thereafter (Fig. 4B), consistent with the presence of abundant ERRα protein in osteocytes and active osteoblasts along bone surfaces in both OVX- and sham-operated animals at these later time points (Fig. 4C). Assessment of expression of mRNA for osteoblast-associated markers, as well as regulators of osteoclast formation OPG and RANKL (40–42), in the same samples showed that BSP, but not OCN, expression was markedly decreased early after OVX but also returned to control levels at late times (Fig. 4, D and E). Notably, RANKL and OPG expression were respectively increased and decreased at the earlier but not later time points after OVX (Fig. 4, D and E).

ERRα is expressed not only in osteoblasts but also in osteoclasts in vivo and in vitro

Immunohistochemistry of femoral sections from OVX rats revealed abundant expression of ERRα protein not only in osteoblasts and osteocytes but also in the numerous osteoclasts present at 4 wk after OVX (Fig. 5A, panel a). For comparison TRAP+ staining is shown on a serial section to confirm the osteoclastic nature of the cells expressing ERRα (Fig. 5A, panel b). To assess further ERR α mRNA expression by osteoclast lineage cells, RNA was extracted at various culture times from RAW cells, a monocyte-macrophage cell line that differentiates into osteoclasts after treatment by RANKL (43). ERRα mRNA is clearly expressed in RAW cell cultures at all times from the monocyte stage (d 1) to mature osteoclast stage (d 6) (Fig. 5B); expression levels tended to increase, although not significantly, as osteoclasts developed. For comparison, mRNA levels of two osteoclast markers, TRAP and cathepsin K, are shown in the same samples (Fig. 5C), and the TRAP staining of the RAW cells at d 3 (mononuclear cells) and at d 5 (polynucleated cells) (Fig. 5D) is also shown. Although it remains to be determined whether ERRα may play a cell autonomous role in osteoclast differentiation, given ERRα expression in osteoblasts (Fig. 4, C and E, and Ref. 32), we next asked whether ERRα may be involved in osteoblast-mediated osteoclast development. To address this, RANKL and OPG mRNA expression levels were assessed by RT-PCR in RNA samples extracted from RC cell cultures treated with ERRα-AS oligonucleotides from d 5 (after cells had reached confluence and proliferation was decreased) to d 11, a treatment regimen we have found previously to block effectively ERRα expression while concomitantly inhibiting osteoblast development and osteoblast-associated marker (*e.g.* BSP, OCN) expression (32). Both RANKL and OPG expression were significantly increased in ERRα AS-treated RC cells, compared with control or S-treated cultures (Fig. 5E), although in repeat experiments the increase in RANKL was usually higher than the increase in OPG, which would suggest that reduction in ERRα *in vitro* decreases the OPG/RANKL ratio.

Down-regulation of ERRα increases adipogenesis and adipocyte colony formation in RC cell cultures

As outlined earlier, in osteoporosis, decreased osteogenesis is accompanied by increased marrow adipogenesis, an observation that has led to the suggestion that adipocyte recruitment is favored over osteoblast recruitment from common precursors in this condition. We next asked whether adipocyte formation is altered concomitant with changes in osteogenesis and RANKL/OPG expression when ERRα levels are dysregulated. To address this question, we treated RC cells with ERRα AS, S, or scram-

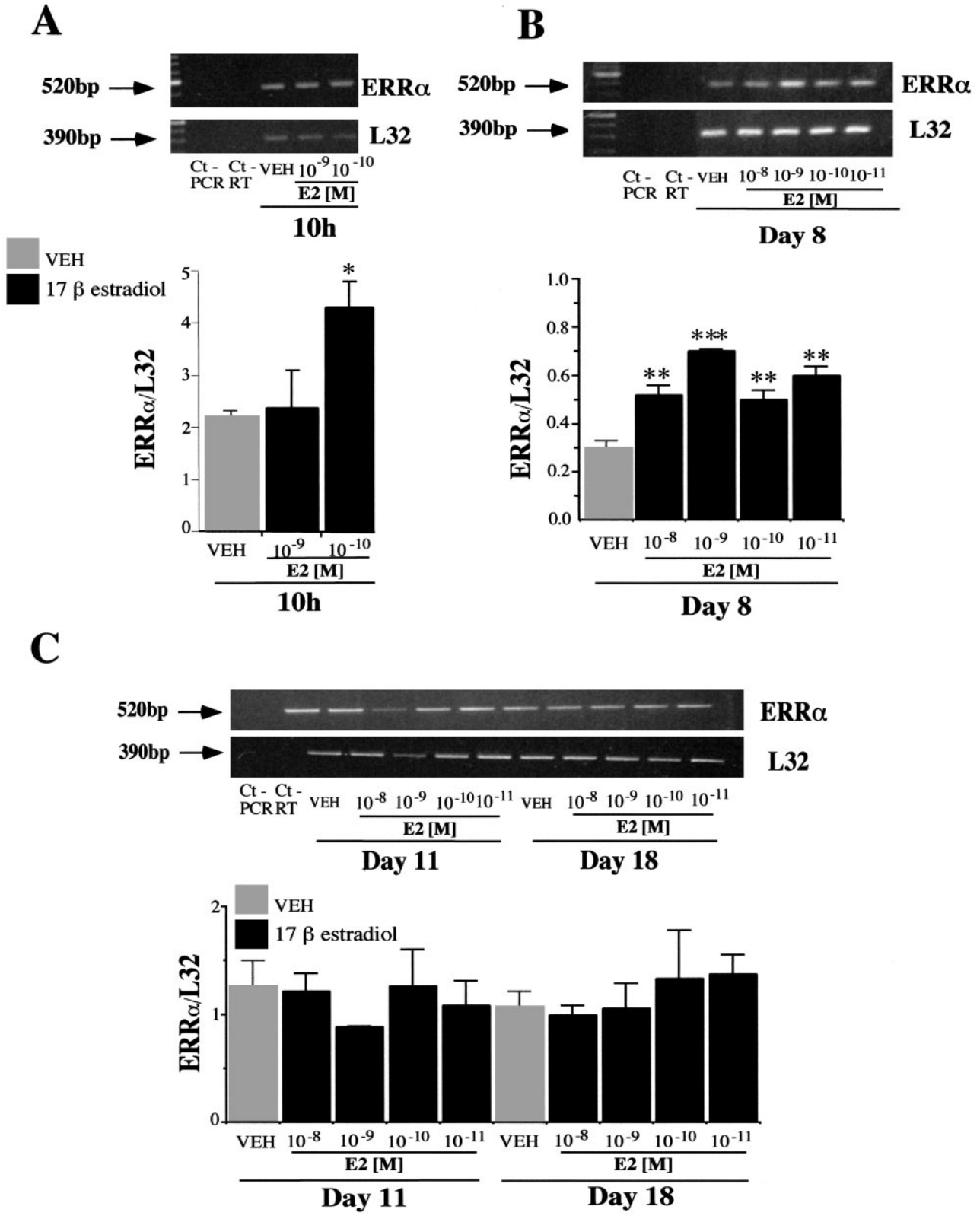

FIG. 2. Semiquantitative RT-PCR with ERRα-specific primers was used to assess expression of ERRα over the proliferation-differentiation time course in RC cells cultured as in Fig. 1 but in the presence (+E2) or absence (VEH) of E2 (10^{-8} to 10^{-11} M). E2 increased ERRα expression levels after 10 h (A) and at confluence (d 8) stages (B) but not during early nodule formation (d 11) and matrix mineralization (d 18) stages (C). ERRα band intensities were normalized against that of the housekeeping gene ribosomal protein L32. Results represent the mean ± SD for triplicate determinations for one experiment (*t* test, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, E2-treated *vs.* VEH). Similar results were seen in three independent experiments.

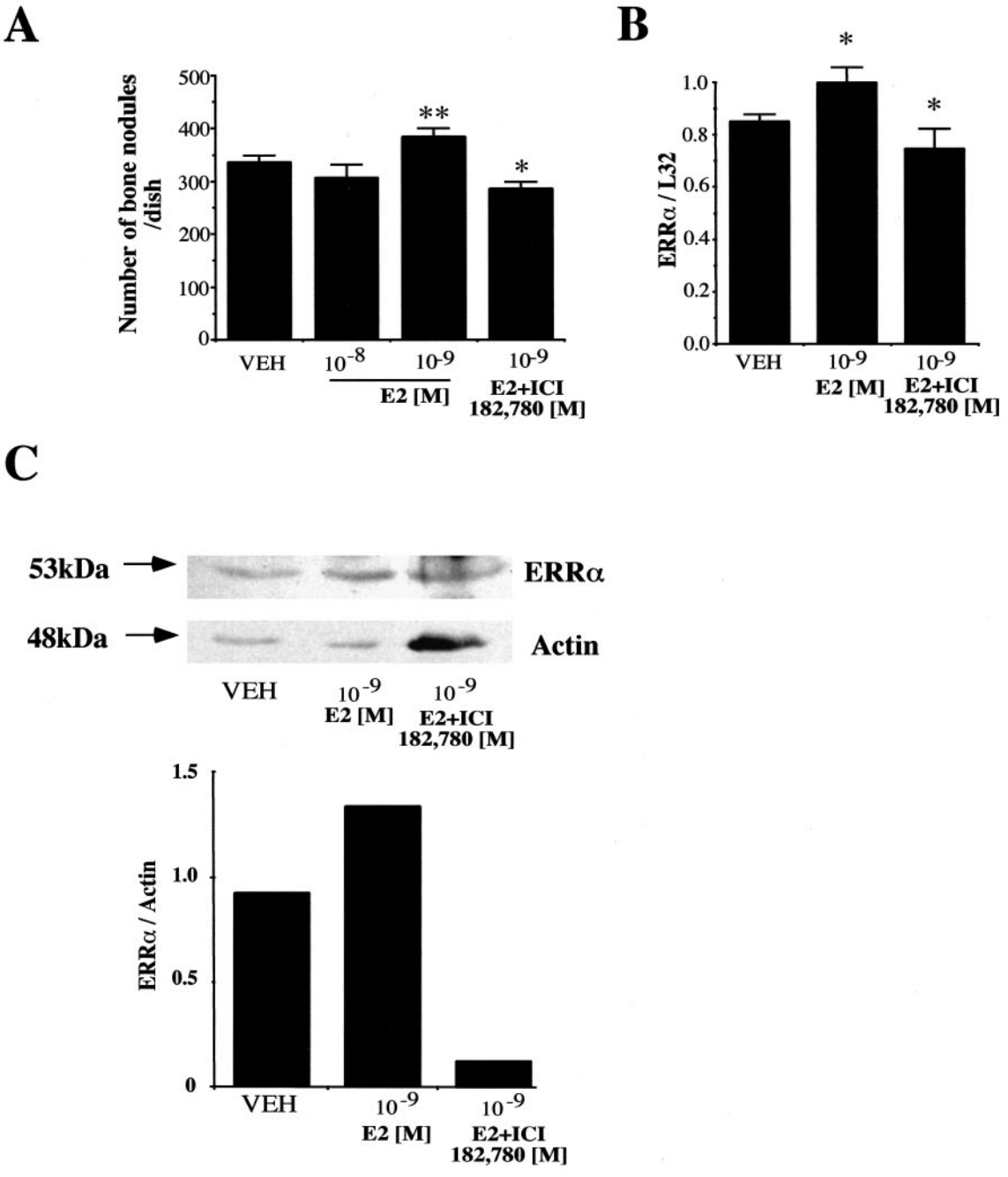

FIG. 3. Estrogen treatment increased the number of bone nodules formed in RC cultures grown as in Fig. 2, an effect blocked by 10^{-9} M ICI 182,780 (A). Semiquantitative RT-PCR and Western blotting showed that ERRα mRNA levels (B) and protein levels (C), respectively, were increased by E2 (10^{-9} M, d 8) treatment, an effect also blocked by 10^{-9} M ICI 182,780 (*t* test, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, E2-treated *vs.* VEH or E2-treated *vs.* E2+ ICI 182,780). Similar results were seen in three independent experiments.

bled (Sc) oligonucleotides and double-stained the wells at d 15 with von Kossa (black: mineralized bone nodules) and sudan IV (red lipid droplets: adipocyte colonies); double staining allowed simultaneous quantification of bone and adipocyte colonies in the same wells. Consistent with what we reported recently (44), ERRα AS (Fig. 6A, panels b and d) but not S (Fig. 6A, panels a and c) or Sc oligonucleotides dose-dependently inhibited bone nodule formation (von Kossa, black staining) when RC cells were treated from d 5 to d 11 (Fig. 6C). Interestingly, we also observed a concomitant increase in adipocyte colony formation (Sudan IV, red staining) in the same AS-treated but not control-treated cultures (Fig. 6, A and B). For example, in 2 μM AS-treated cultures, an almost complete inhibition of mineralized bone nodule formation was seen concomitant with a significant increase (53%) in adipocyte colony number (Fig. 6, A–C). Consistent with this observation, expression of several key markers of adipocyte differentiation (LPL, c/EBPα, PPARγ, and AP2) was increased (Fig. 6, D and E). S and Sc oligonucleotides had a nonspecific

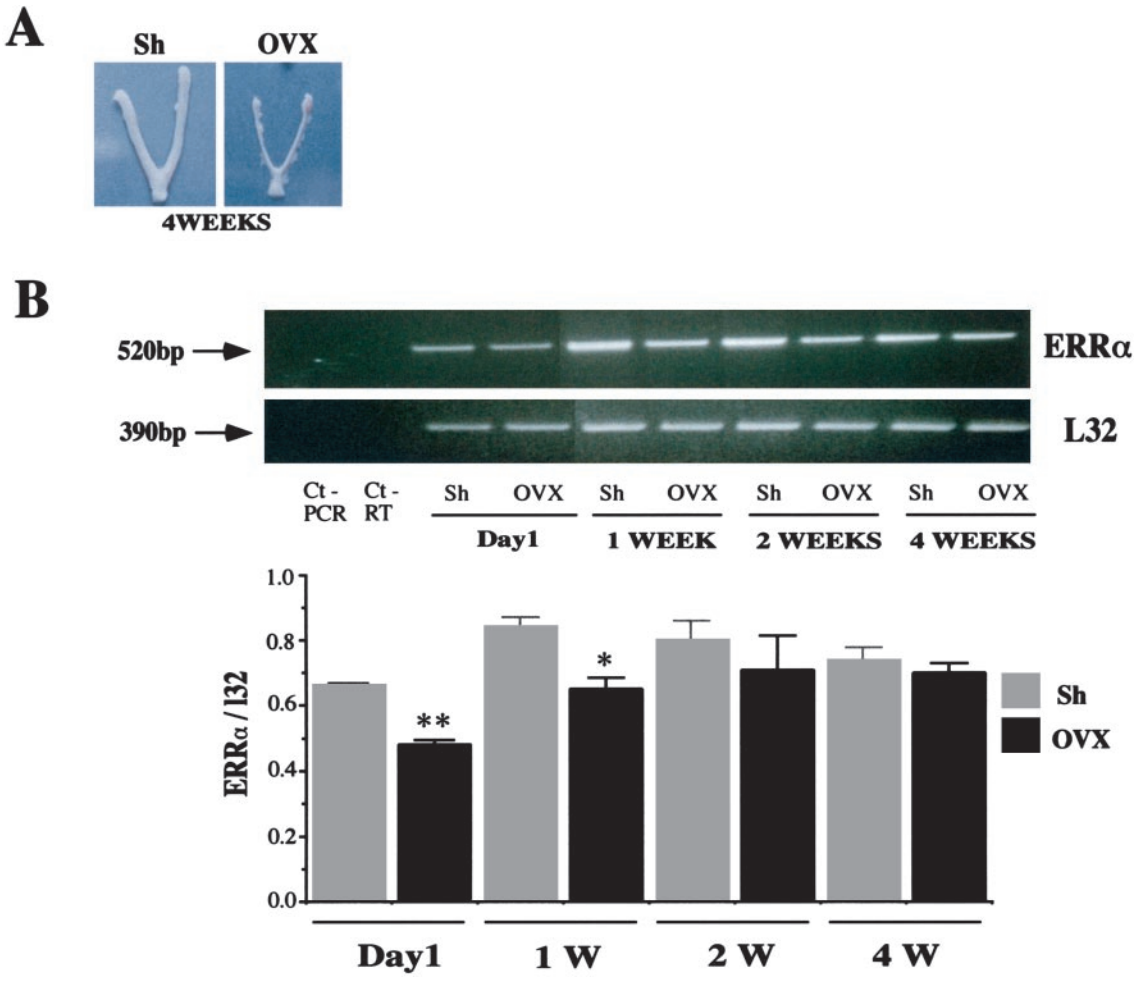

FIG. 4. ERRα mRNA expression levels were assessed by RT-PCR in RNA samples extracted from femurs (from which bone marrow had been flushed) of OVX or sham-operated (Sh) rats. The mRNA was extracted at d 1, 1 wk, 2 wk, and 4 wk after surgery. Marked uterine hypoplasia is obvious in OVX but not sham rats 4 wk after surgery (A). ERRα mRNA is down-regulated in OVX rats at d 1 and 1 wk but not later (B). In parallel, ERRα protein expression was assessed by immunohistochemistry in femoral sections of OVX and sham-operated rats 4 wk after surgery. ERRα protein is highly expressed in osteoblasts and osteocytes, shown in the secondary ossification zone (C, panels a and c) and in the cortical bone (C, panels b and d), in sham (panels a and b) and OVX (panels c anc d), respectively (C). The negative secondary antibody control (incubation without rabbit anti-ERRα antibody) is also shown (C, panel e). *Bar,* 200 μm. For comparison, mRNA levels for four bone markers, BSP, OCN, OPG, and RANKL, were assessed by PCR in the same samples (D and E). ERRα, BSP, OCN, OPG, and RANKL band intensities were normalized against that of the ribosomal probe L32. Results represent the mean ± SD for triplicate determination for one experiment (*t* test, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ OVX-treated *vs.* sham). Similar results were seen in three independent experiments.

nondose-dependent effect on adipocyte and bone colony formation (Fig. 6, B and C).

Discussion

Our findings show that ERRα expression is regulated by estrogen in osteoblastic RC cells *in vitro* and bones *in vivo*, with both osteoblast and osteoclast lineage cells expressing this nuclear receptor. Moreover, inhibition of ERRα expression during the differentiation phase of RC cell cultures with phosphorothioate-modified AS oligonucleotides not only inhibits osteoblast development but also increases the expression of key regulators of osteoclastogenesis, RANKL and OPG and concomitantly increases adipocyte differentiation and the expression of adipocyte-associated markers. Taken together, the data suggest that ERRα impinges on the estrogen axis in bone in which it may play roles in osteoclastogenesis and the shift away from marrow osteogenesis and toward adipogenesis associated with osteoporosis and age-related osteopenia.

ERRα and the estrogen axis in osteoporosis

We have found that ERRα expression is regulated by estrogen in osteoblasts and bone. Specifically, ERRα expression is increased early during proliferative stages in RC cells *in vitro* after treatment with E2 at physiological concentrations and decreased early *in vivo* in femurs isolated from ovariectomized *vs.* sham-operated rats. It is important to note that these changes in ERRα expression *in vivo* reflect changes in expression by bone cells (see also below) because we flushed bone marrow from bones before RNA isolation. Our finding that E2 regulates ERRα in bone is consistent with an earlier report that estrogen regulates ERRα expression in another

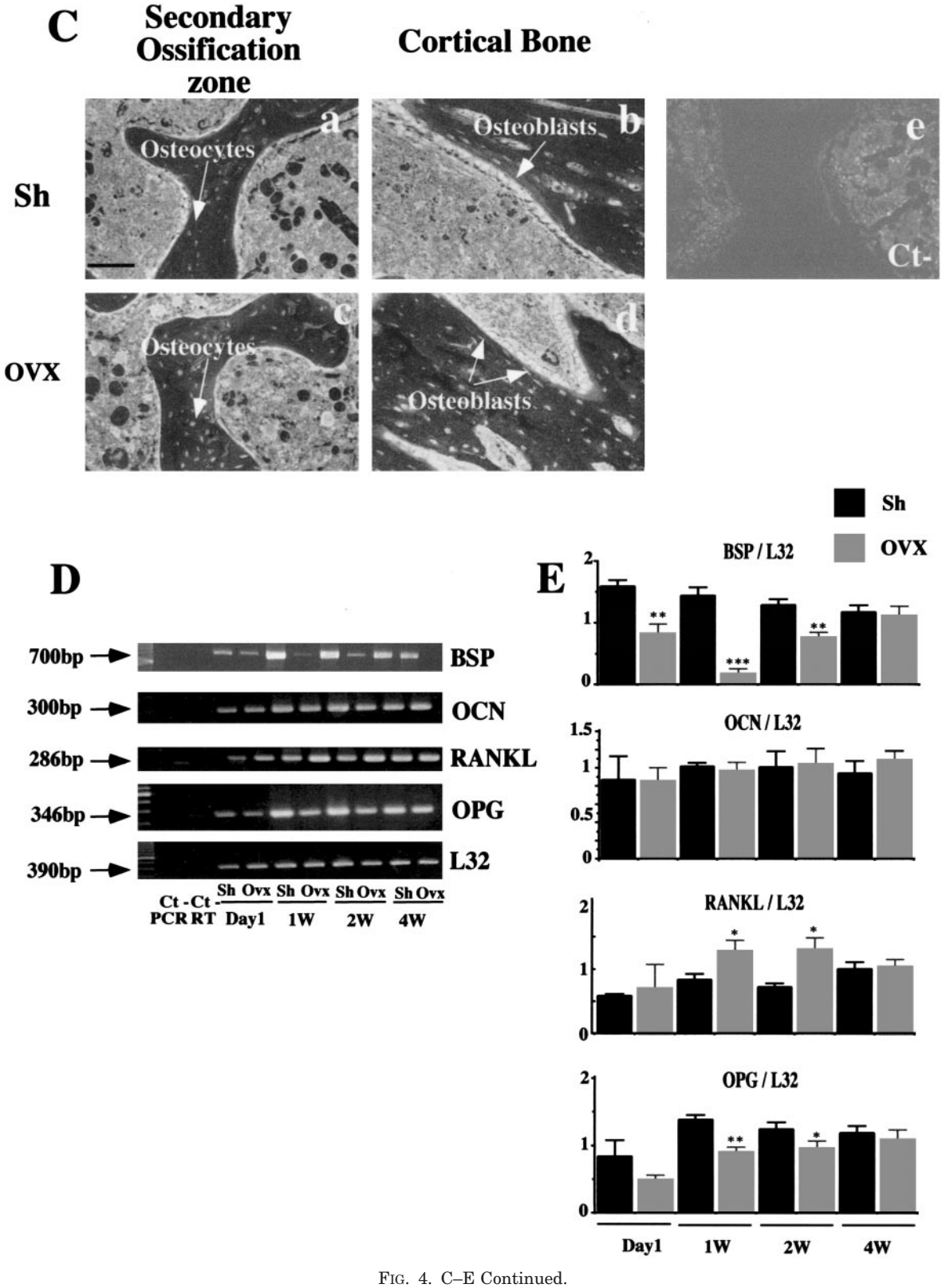

FIG. 4. C–E Continued.

tissue, *i.e.* the mouse uterus (45). Our observation that ERRα is regulated by estrogen in bone together with our recent finding that ERRα plays a functional role in osteoprogenitor cell proliferation and differentiation and that these processes are exquisitely sensitive to changes in ERRα levels (32), suggested to us that ERRα may be involved in the pathogenesis of postmenopausal osteoporosis. We found previously that inhibition of ERRα expression during the proliferation phase

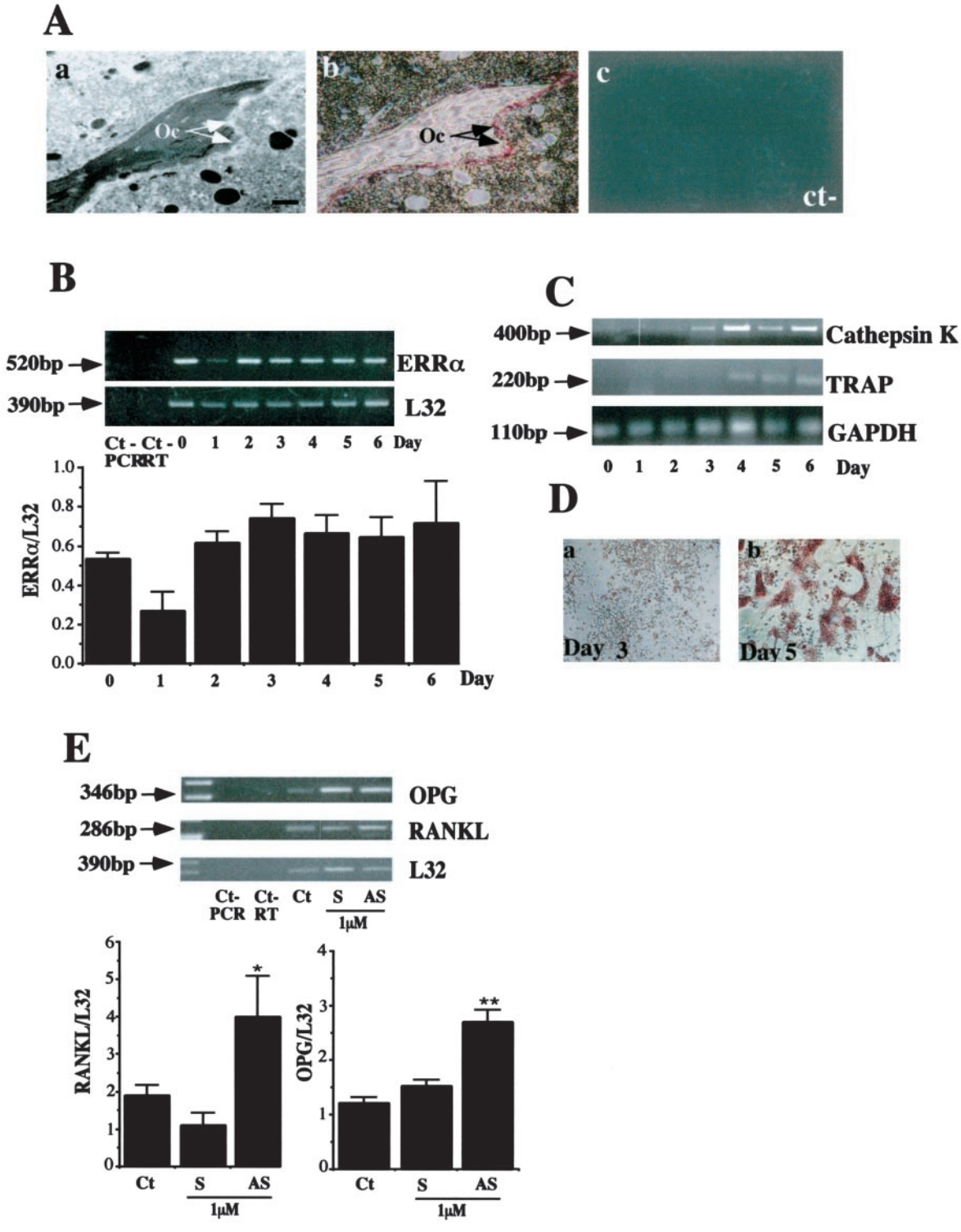

FIG. 5. Immunolabeling for ERRα in sections of femoral bone from OVX rats 4 wk after surgery. ERRα intensely labels large multinucleated cells (A, panel a) that are stained for TRAP in a serial section (A, panel b) identifying these cells as osteoclasts; the negative control (incubation with secondary antibody without rabbit anti-ERRα primary antibody) is also shown (A, panel c). Semiquantitative RT-PCR with ERRα-specific primers was used to assess expression of ERRα in the mouse osteoclast precursor cell line RAW 264.7 at various time points of differentiation (d 0: uninduced; d 1 to d 6: RAW264.7 cells after 1–6 d of treatment with RANKL) (B). For comparison, mRNA levels for two osteoclast markers, cathepsin K and TRAP, were assessed by PCR in the same samples (C) and TRAP+ staining of RAW cells at d 3 (mononuclear cells) and d 5 (polynuclear cells) is also shown (D). RC cells were untreated (Ct) or treated with ERRα AS or S oligonucleotides at 1 μM from d 5 (after cells had reached confluence and proliferation was decreased) to d 11 (nodules forming); total RNA was extracted from wells at d 15 (bone mineralized nodules) and RT-PCR was performed on triplicate samples by using primers specific for OPG and RANKL (E). Results represent the mean ± SD for triplicate determinations of one experiment (*t* test, *, $P < 0.05$, **, $P < 0.01$ AS *vs.* S); similar results were seen in three independent experiments. *Bar,* 200 μm (A).

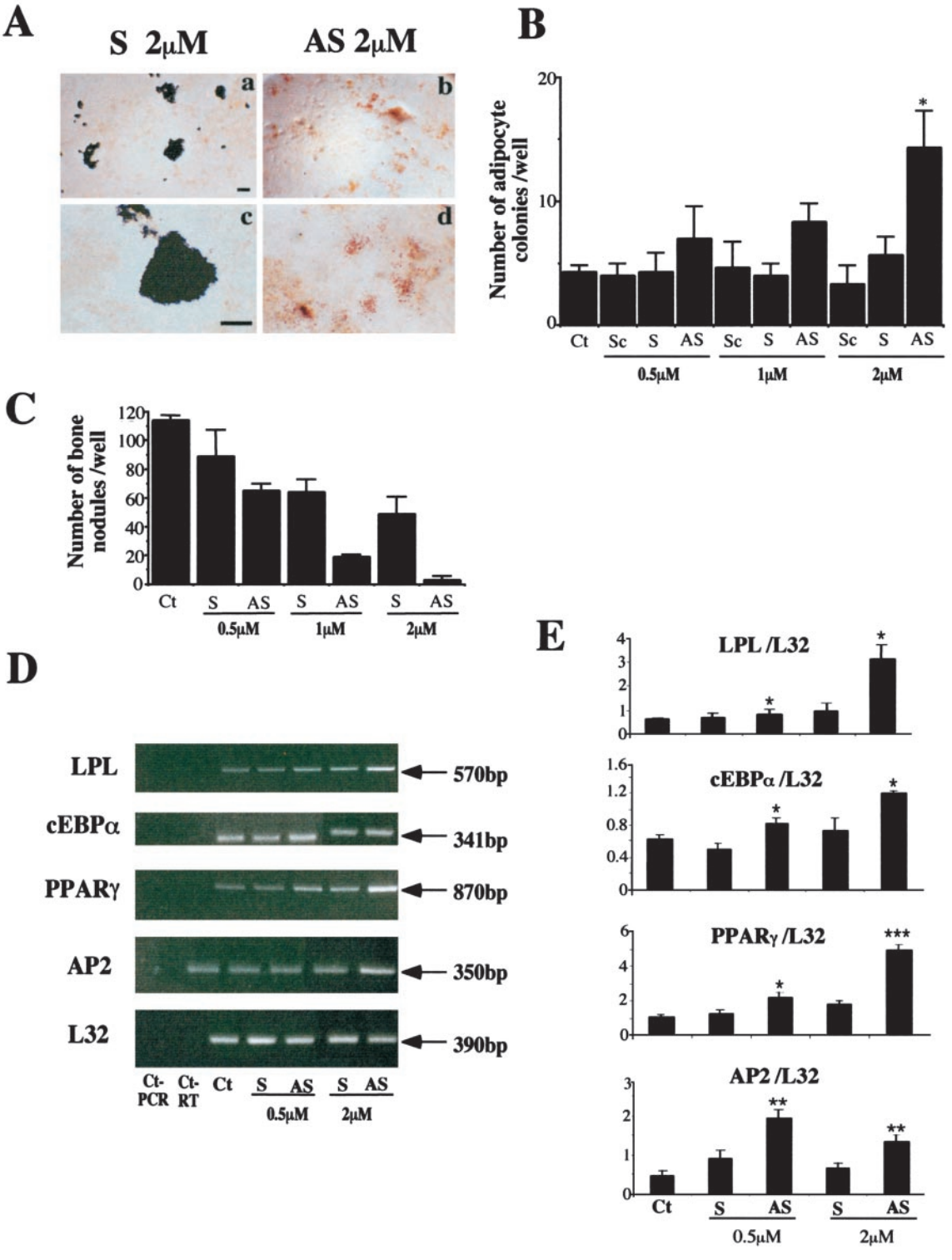

FIG. 6. RC cells were untreated (Ct) or treated with ERRα AS, Sc, or S oligonucleotides at 0.5, 1, and 2 μM from d 5 (cells had reached confluence and proliferation was decreased) to d 11 (nodules were forming); cultures were fixed on d 15 and double stained for lipid droplets (Sudan IV; *red staining*) and for mineralized bone nodules (von Kossa staining; *black staining*) (A). Inhibition of ERRα protein synthesis resulted in an increase in adipocyte colonies (A, panel a *vs.* b, panel c *vs.* d) and (B) and a concomitant decrease in the number of bone nodules (A, panel a *vs.* b, and c *vs.* d) and (C). Data are expressed as the mean number of nodules/well or adipocyte colonies/well ± SD of triplicate wells (*t* test, *, $P < 0.05$, **, $P < 0.01$ AS *vs.* S or Sc) and are representative of three independent experiments (B and C). Total RNA was extracted from parallel wells at d 15 and RT-PCR performed on triplicate samples by using primers specific for adipocyte markers (LPL, c/EBPα, PPARγ, and AP2) (D). Adipocyte marker PCR products were normalized to L32 PCR product. Results represent the mean ± SD for triplicate determinations of one experiment (*t* test, *, $P < 0.05$, **, $P < 0.01$ AS *vs.* S) (E); similar results were seen in three independent experiments. Original magnifications, ×10 (panels a and b) and ×30 (panels c and d). *Bar,* 1 mm (A, panels a–d).

of RC cell cultures decreased proliferation, as seen by a decrease in cell number, and reduced differentiation, as seen by a decrease in mineralized bone nodule formation. Inhibition of ERRα early during differentiation phase also markedly decreased differentiation and bone nodule formation. Taken together, these data suggest that ERRα plays a role in bone formation and turnover with specific effects on the progression of osteoprogenitors to more mature bone-forming osteoblasts. The decrease of ERRα expression that we have now found at early times after ovariectomy (d 1 and 1 wk) of rats suggests that ERRα may be among the first players involved in the pathogenesis of the osteopenia characterized later by a decrease in osteoblast proliferation and biosynthetic activity. Interestingly, BSP, a major constituent of bone matrix, is also acutely and markedly down-regulated in bones from ovariectomized rats, consistent with its marked down-regulation in RC cell cultures in which ERRα is inhibited (32).

Our data are also in keeping with the observation that BSP is a useful biochemical marker of altered bone turnover in postmenopausal women (46, 47) and support the hypothesis that BSP is an important target gene of ERRα, a possibility that we are currently investigating (48). Consistent with this latter view, BSP has recently been described as an early response gene after estrogen treatment, increasing as early as 4 h after initiation of treatment (49). Interestingly, we found that key regulators of osteoclast formation and activity, RANKL and OPG (40–43), are also acutely regulated after ovariectomy in rats. Consistent with the view that reductions in the OPG/RANKL ratio increase osteoclast formation and bone resorption (50, 51) and recent evidence that a decreased OPG/RANKL ratio is seen after estrogen treatment of OVX female mice (52), we found that RANKL, essential for osteoclastogenesis, was up-regulated, whereas OPG, the decoy receptor, was down-regulated after OVX. This latter observation is also consistent with two studies describing stimulation of the expression of OPG by E2 *in vitro* (53, 54). The timing of the increase in the number of osteoclasts in our OVX rat model (between d 4 and 7 after ovariectomy) (55) is consistent with the regulation of expression of RANKL and OPG we observed. Indeed, inhibition of osteoclastogenesis is one of the main mechanisms by which estrogen is thought to prevent estrogen-deficient bone loss, and growing evidence supports the hypothesis that estrogens down-regulate osteoclast formation by blunting the production of IL-1, IL-6, and TNF (22), cytokines that enhance stromal cell production of RANKL (53, 56).

Together with our observation that down-regulation of ERRα expression via AS strategies in RC cells *in vitro* leads to increased levels of RANKL, the data suggest that ERRα may be a key new molecular mediator of estrogen action in bone. Clearly, further work must be done to elucidate the mechanism (direct or indirect) by which ERRα expression is regulated by estrogen. ERα may be one candidate because its own expression is regulated by estrogen in osteocytes (57). We have shown recently that ERα and ERRα are coexpressed in certain cohorts of osteoblasts *in vivo* (calvaria, femurs) and *in vitro* (RC and bone marrow stromal cell cultures) (33). Together with our observation that the estrogen-induced increase in ERRα expression after estrogen treatment is abolished when RC cells are treated by the ER antagonist ICI 182,780, it seems likely that ERα and/or ERβ may have a direct effect on ERRα expression in osteoblasts.

We also found that ERRα is expressed in osteoclasts *in vivo* (femurs of OVX rats) and *in vitro* (RAW cells before and after differentiation), which may explain the fact that, at 2 and 4 wk after surgery (in which the number of osteoclasts is known to increase and ERRα expression levels appear relatively normal in osteoblasts/osteocytes in these bones), we did not observe any difference between ERRα mRNA levels in femurs from OVX- *vs.* sham-operated animals. ERα has been reported to be expressed in osteoclasts (18), and recently estrogen was shown to reduce the ability of osteoclasts to degrade bone matrix by regulating matrix metalloproteinases and cysteine proteinases such as cathepsin K (58). Yang *et al.* (16) and Zhang and Teng (17) also showed that ERRα modulates the activating effect of estrogen on the lactoferrin promoter and suggested that ERRα may interact with ERs through protein-protein interactions, which makes ERRα a potential new partner of ERα in osteoclasts as well as in osteoblasts.

ERRα and adipocyte formation

Inhibition of ERRα levels by AS oligonucleotide treatment of RC cell cultures during differentiation phase not only blocks osteoblast differentiation but also increases adipogenesis, as evidenced by both an increase in adipocyte colony number and expression of several genes known to be involved in triglyceride synthesis, such as the early marker of adipocyte conversion, LPL; the fatty acid-binding protein aP2 (21); and PPAR γ (59), which acts synergistically with c/EBPα to coordinate the adipocyte differentiation cascade (60). These observations are particularly interesting in view of the fact that the decrease in bone volume associated with osteoporosis is accompanied by an increase in marrow adipose tissue and fat content in general (20, 61). There is other evidence that ERRα plays a role in adipogenesis and the metabolic activity of fat tissue. It is known to be expressed in brown fat during mouse development and is more highly expressed in brown *vs.* white fat (12, 62).

ERRα has also been shown to be up-regulated during adipocyte differentiation in the HIB cell model and to bind to the promoter of the medium chain acyl-coenzyme A dehydrogenase gene, a pivotal enzyme in the mitochondrial fatty acid b-oxidation cycle (12, 13). Furthermore, ERRα has been shown to be a regulator in breast of the human aromatase gene (14), which is an enzyme involved in the conversion of testosterone to estrogen and known to have a function in fat metabolism because aromatase deficiency results in obesity (63). Although some of these observations appear discrepant with our observations, it seems likely that differences in ERRα regulatory activities may exist between established lines *vs.* primary cell models, as has been seen with other regulators of adipogenesis in other studies. In addition, it seems crucial to assess whether ERRα plays a direct or indirect role in regulation of adipogenesis *in vivo*. Consistent with our data, it was recently demonstrated that endogenous estrogen decreases fat content in female mice, an effect that appears to be mediated by ERα but not ERβ (64–

66). As already mentioned, although the mechanism by which ERRα is up-regulated by estrogen *in vitro* and *in vivo* remains to be determined, nevertheless, our data are consistent with ERRα playing a role in adipogenesis and the effect of estrogen on adipogenesis.

As already outlined, the decrease in bone volume associated with osteoporosis is accompanied by an increase in marrow adipose tissue. One mechanism that has been postulated to account for this apparent reciprocity is an imbalance in the commitment or differentiation of common progenitors of the osteoblast and adipocyte lineages toward adipogenesis. Support for this comes from a variety of experimental manipulations *in vitro* (21, 67). Interestingly, however, the number of adipocyte colonies formed in ERRα AS-treated RC cell cultures is significantly lower than the number of bone colonies lost (*e.g.* 15 adipocyte colonies *vs.* 60 bone colonies). Although a possible lack of optimization of adipogenic conditions cannot be excluded, the fact that those colonies that do form contain cells with abundant patent lipid droplets suggests that the conditions do support terminal adipocyte differentiation. Thus, our data suggest that ERRα is not involved in changes in fate choice of bipotential progenitors toward the adipocyte lineage but rather is involved in regulation of a preadipocyte pathway parallel to that of committed osteoprogenitors. This suggests that ERRα may have two independent functions: one on osteoprogenitors-osteoblasts as an activator of osteoblast differentiation and bone formation and one on preadipocytes-adipocytes as an inhibitor of adipogenesis.

Summary

Although the molecular basis of the function of ERRα in these different cell types remains to be determined, our results suggest that ERRα is important not only in osteoblast but also in osteoclast and adipocyte development and that it may have a function in estrogen deficiency/postmenopausal osteoporosis via actions on all three cell types. Based on our data, the decrease of ERRα expression in the OVX rat model of postmenopausal osteoporosis may participate in regulation of the decrease in bone formation and increase in bone resorption and adipocyte number, phenomena also observed in postmenopausal osteoporosis in humans. Therefore, blocking the decrease of ERRα seen after ovariectomy or estrogen deficiency or appropriate use of agonists and antagonists of ERRα may provide a novel therapeutic approach for osteopenic disorders such as osteoporosis.

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