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The Orphan Nuclear Estrogen Receptor–related Receptor α (ERR α) Is Expressed throughout Osteoblast Differentiation and Regulates Bone Formation In Vitro

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Abstract. The orphan nuclear estrogen receptor–related receptor α (ERR α), is expressed by many cell types, but is very highly expressed by osteoblastic cells in which it transactivates at least one osteoblast-associated gene, osteopontin. To study the putative involvement of ERR α in bone, we first assessed its expression in rat calvaria (RC) in vivo and in RC cells in vitro. ERR α mRNA and protein were expressed at all developmental stages from early osteoprogenitors to bone-forming osteoblasts, but protein was most abundant in mature cuboidal osteoblasts. To assess a functional role for ERR α in osteoblast differentiation and bone formation, we blocked its expression by antisense oligonucleotides in either proliferating or differentiating RC cell cultures and found inhibition of cell growth and a pro-

liferation-independent inhibition of differentiation. On the other hand, ERR α overexpression in RC cells increased differentiation and maturation of progenitors to mature bone-forming cells. Our findings show that ERR α is highly expressed throughout the osteoblast developmental sequence and plays a physiological role in differentiation and bone formation at both proliferation and differentiation stages. In addition, we found that manipulation of receptor levels in the absence of known ligand is a fruitful approach for functional analysis of this orphan receptor and identification of potential target genes.

Key words: osteoblasts • bone • nuclear receptor • estrogen related • differentiation

Introduction

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 (Gronemeyer and Laudet, 1995; Enmark and Gustafsson, 1996). The orphan receptors display the same structural organization as do the classic ligand-dependent receptors: the A/B domain located in the NH₂-terminal part of the protein harbors a ligand-independent transactivation function (AF-1); the C domain, which is the most conserved part of the molecule, is responsible for the specific DNA-binding activity; the E domain contains the ligand binding hydrophobic pocket and contributes to receptor dimerization and to the ligand-dependent transactivation function (AF-2).

Two orphan receptors, estrogen receptor–related receptor α (ERR α)¹ and ERR β ([Giguere et al., 1988]; NR3B1 and NR3B2, respectively, according to the Nuclear Receptors Nomenclature Committee, 1999 [Committee, 1999]) are closely related to the estrogen receptors ER α and ER β ([Green et al., 1986; Kuiper et al., 1996]; NR3A1 and NR3A2, respectively). ERR α was identified by low-stringency screening of cDNA libraries with a probe encompassing the DNA-binding domain of the human estrogen receptor (ER). Recently, a third ERR, ERR3 or ERR γ , was identified by yeast two-hybrid screening with the glucocorticoid receptor–interacting protein 1 (GRIP1) as bait (Hong et al., 1999). The DNA binding domain of ERRs and ERs is highly conserved; however, the other parts of the protein share very little homology (Giguere et al., 1988; Hong et al., 1999). Therefore, sequence alignment of ERR α and the ERs reveals a high similarity (68%) in the

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¹Abbreviations used in this paper: ALP, alkaline phosphatase; ANOVA, analysis of variance; COLLI, collagen type I α chain; ER, estrogen receptor; ERR α , estrogen receptor–related receptor α ; OCN, osteocalcin; OPN, osteopontin; RC, rat calvaria; RT, reverse transcription; UTR, untranslated region.

66 amino acids of the DNA-binding domain and a moderate similarity (36%) in the ligand-binding E domain, which may explain the fact that ERR α does not bind estrogen. Although ligands for the ERRs have not been clearly identified, the pesticides chlordane and toxaphene have been suggested to be potential ligands for ERR α (Yang and Chen, 1999). ERR α has been identified as a regulator of fat metabolism (Sladek et al., 1997; Vega and Kelly, 1997), as a regulator of the human aromatase gene in breast, a modulator of estrogen activity on the lactoferrin promoter (Yang et al., 1996; Zhang and Teng, 2000), and it has been hypothesized to be critical for normal breast development (Yang et al., 1998).

Postmenopausal osteoporosis is a condition caused primarily by the severe decrease of serum estrogen levels after cessation of ovarian function (Turner et al., 1994). ERs are expressed in osteoblasts (Eriksen et al., 1988; Komm et al., 1988; Turner et al., 1994) but mice lacking a functional ER α or ER β have relatively minor skeletal abnormalities (Korach, 1994; Windahl et al., 1999) suggesting that other mechanisms or receptors might be important during skeletal development. Due to their homology to the ERs, we hypothesized that ERR α may intervene in the signals induced by estrogen in bone. ERR α has a broad spectrum of expression, including fat, muscle, brain, testis, and skin (Bonnelye et al., 1997b). Strikingly, ERR α is also highly expressed in the ossification zones of the mouse embryo (in long bones, vertebrae, ribs, and skull), and is more widely distributed in osteoblast-like cells than is ER α (Bonnelye et al., 1997a). Moreover it has been shown that ERR α positively regulates the osteopontin (OPN) gene (Vanacker et al., 1998b), an extracellular matrix molecule secreted by osteoblasts and thought to play a role in bone remodeling (Denhardt and Noda, 1998).

Given these observations, we sought to assess more directly whether ERR α plays a functional role in osteoblast development and bone formation. Our findings show that ERR α is highly expressed and widely distributed in differentiating osteoblastic cells. They also indicate a critical role for ERR α in bone formation, with both up- and downregulation of bone nodule formation concomitant with up- and downregulation of ERR α expression *in vitro*. These data suggest that ERR α plays a widespread and physiologically relevant role in bone formation and predict specific ERR α target genes at different osteoblast developmental time windows.

Materials and Methods

Cell Culture

Cells were enzymatically isolated from the calvaria of 21-d Wistar rat fetuses by sequential digestion with collagenase as described previously (Bellows et al., 1986). Cells obtained from the last four of the five digestion steps (populations II–V) were plated in T-75 flasks in α -MEM containing 15% heat-inactivated FBS (Flow Laboratories) and antibiotics comprising 100 μ g/ml penicillin G (Sigma-Aldrich), 50 μ g/ml gentamycin (Sigma-Aldrich), and 0.3 μ g/ml fungizone (Flow Laboratories). After 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), and the remaining cells were resuspended in the standard medium described above. The resuspended cells were plated into 100-mm tissue culture dishes at 10^5 cells/dish, into 35-mm tissue cul-

ture dishes at 2×10^4 /dish, and in 24-well plates at 10^4 cells/well. After 24 h incubation, medium was changed and supplemented with 50 μ g/ml ascorbic acid, 10 mM sodium β -glycerophosphate, and with or without 10^{-8} M dexamethasone (Merck, Sharp, and Dohme, Canada, Ltd.). 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.

Northern Blots

Total RNA was extracted with guanidine from rat calvaria (RC) cells at different times of the culture corresponding to different stages of proliferation, differentiation, and bone nodule formation (Ausubel et al., 1996). Northern blots were prepared and hybridized with a 750-bp fragment corresponding to the rat 3' untranslated region (UTR) of ERR α (provided by J.M. Vanacker, CNRS, UMR 5665, Lyon, France) according to standard procedures (Chirgwin et al., 1979). Rat bone/liver/kidney alkaline phosphatase (ALP; Noda et al., 1987; gift of Dr. G.A. Rodan, Merck Research Laboratories, West Point, PA) was a 600-bp cDNA EcoRI fragment obtained by digesting pRAP54 with BssHII-XhoI to remove 1.8 kb of the 5' region and relegating the blunt ends. Rat OPN (provided by Dr. R. Mukherjee, McGill University, Montreal, Quebec, Canada) was a 700-bp cDNA BamHI-EcoRI fragment obtained by digesting the full-length cDNA with PvuI to remove 800 bp of 5' region and ligating the blunt ended fragment into SmaI cut pGEM-7Zf(+) vector (Promega). Rat osteocalcin (OCN) was a partial cDNA containing 350 bp of the 3'UTR isolated with OCN-specific primers from a λ gt11 library prepared from ROS 17/2.8 cells and rat L32 was generated from RC cell mRNA by PCR using specific primers (Liu et al., 1994).

Reverse Transcription PCR

Samples of total cellular RNA (1.5–5 μ g) were reverse transcribed using oligo(dT) and the first strand synthesis kit of SuperscriptTM II (GIBCO BRL). PCR was performed with specific primers specific for ERR α . Primers were as follows: ERR α upstream (3'UTR), CAG GAA AGT GAA TGC CCA GG; ERR α downstream (3'UTR), CTT TGC AGC AAA TAT ACA TT; L32 upstream, CAT GGC TGC CCT TCG GCC TC; and L32 downstream, CAT TCT CTT CGC TGC GTA GCC.

The PCR reaction mixture contained cDNA (1 μ l), 1 μ l dNTP mix (10 mM), 10 \times PCR buffer, Q solution, 25 pmol primers, and 5 U of Taq polymerase from Quiagen. 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 ERR α and L32. Amplimers were sequenced for verification.

Osteoblast associated and other markers were also amplified by PCR using specific primers for rat OCN, OPN, ALP, bone sialoprotein (BSP), Cbfa1, collagen type I α chain (COLL1), C-fos, cyclin D1, Bax, and Bcl-2. 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, OPN, ALP, BSP, L32, Bax, and 30 cycles for Bcl-2, 32 cycles for c-Fos, 35 cycles for cyclin D1 and Cbfa1 (with annealing temperatures of 58°C and 62°C, respectively), and 23 cycles for COLLI with annealing temperature of 59°C.

Primers were OCN upstream: AGG ACC CTC TCT CTG CTC AC; OC downstream: AAC GGT GGT GCC ATA GAT GC; BSP upstream: CGC CTA CTT TTA TCC TCC TCT G; BSP downstream: CTG ACC CTC GTA GCC TTC ATA G; ALP upstream: CCC GCA TCC TTA AGG GCC AG; ALP downstream: TAG GCG ATG TCC TTG CAG C; OPN upstream: GCC ACT TGG CTG AAG CCT G; OPN downstream: GAA ACT CCT GGA CTT TGA CC; Cbfa1 upstream: CTT CAT TCG CCT CAC AAA C; Cbfa1 downstream: CAC GTC GCT CAT CTT GCC GG; cyclin D1 upstream: TCC CGC CAG CAG CAA GAC AC; cyclin D1 downstream: TGA GCT TGT TCA CCA GAA GC; c-Fos upstream: ATA GAG CCG GCG GAG CCG CG; c-Fos downstream: AAG CCC CGG TCG ACG GGG TG; Bax upstream: CCT TGG AGC AGC CGC CCC AG; Bax downstream: ATG TGG GCG TCC CGA AGT AGG; Bcl-2 upstream: GGG GAA ACA CCA GAA TCA AG; Bcl-2 downstream: AGA GAA GTC ATC CCC AGC CC; COLLI upstream: GGA GAG AGT GCC AAC TCC AG; COLLI downstream: CCA CCC CAG GGA TAA AAA CT.

Poly(A) PCR Library Selection

19 poly(A) PCR libraries representative of five transitional stages in osteoblast lineage progression were selected from >100 available amplified colonies (Liu et al., 1994; Candeliere et al., 1999; Aubin and Liu, 1996).

Stage A are replica-plated monolayer colonies committed to differentiate to the osteoblast lineage but not yet expressing type I α I collagen or ALP, both early markers of osteoprogenitor cells. Stage B and C colonies are progressively more mature, i.e., expressing type I α I collagen or both type I α I collagen and ALP, respectively. Stage D colonies represent multilayered cells and contain histologically identifiable cuboidal osteoblasts. Stage E colonies comprised terminal differentiation stages, with multilayered cells and mineralized bone matrix.

Total RNA was extracted using a mini-guanidine thiocyanate method as described previously (Liu et al., 1994). Globally amplified cDNA was synthesized by oligo(dT) priming, poly(A) tailed, and amplified by PCR with oligo(dT) primer (Liu et al., 1994). Total cDNA probe used as "housekeeping control" was prepared as described by Sambrook et al. (1989) from poly(A)⁺ mRNA isolated (Auffray and Rougeon, 1980) from mass populations of fetal RC cells grown in the presence of dexamethasone in which bone nodules were beginning to mineralize. Amplified cDNA (5 μ l) was run on 1.5% agarose gels, transferred onto 0.2- μ m pore size nylon membrane (ICN Biomedicals), and immobilized by baking at 80°C for 2 h. Prehybridization and hybridization were performed as described in Liu et al. (1994). After hybridization, the blots were washed at 65°C for 1 h each in 2 \times SSC/0.1% SDS and in 0.5 \times SSC/0.1% SDS. The blots were then exposed to phosphorimager screens (Molecular Dynamics), and digitized images obtained and quantified with the IPLab Gel program (Signal Analytics Corp.). After quantifying the data, the signal intensity for ERR α was standardized against total cDNA.

Western Blots

Total protein was extracted from confluent HeLa and RC cells according to standard methods (Ausubel et al., 1996). Western blot analyses were performed using a semidry system. Immunoblotting was performed with purified rabbit polyclonal antibody prepared against a mouse peptide (NH- $\text{KAEPASPDSPKGSSETETE-C}$) localized in the A/B domain (14–32 amino acids); blots were incubated overnight at room temperature with the polyclonal antibody diluted to 1/60, and binding was detected using HRP-conjugated goat anti-rabbit antibodies (1/3,000; Bio-Rad Laboratories) and chemiluminescence.

Immunocytochemistry

Immunolabeling of cultures was done essentially as described previously (Turksen and Aubin, 1991; Turksen et al., 1992). Cultures were rinsed with PBS, fixed with 3.7% formaldehyde in PBS, and permeabilized with methanol at –20°C. Frozen sections were fixed 10 min in cold acetone. Paraffine sections were treated deparaffined in xylene, then rehydrated in 100, 95, and 70% ethanol and water. After rinsing, cells in dishes or frozen paraffine sections were incubated for 1 h at room temperature with 10% normal serum in PBS for ERR α and in 3% BSA in PBS (denatured) for OCN, ALP, OPN, and BSP. After rinsing, cells or sections were incubated for 3 h with appropriate dilutions of purified rabbit polyclonal antibody described previously (1/50, anti-ERR α). The anti-rat OCN antiserum was provided by D. Modrowski (INSERM U349, Hospital Lariboisiere, Paris, France) and used at 1/100 dilution. The anti-OPN (MPIIB10) and anti-BSP (WWVIDI) antibodies were purchased from the Hybridoma Bank and used at a 1/300 and 1/100 dilution, respectively. The production and characterization of monoclonal antibody RBM 211.13 directed to rat bone/liver/kidney ALP have been described elsewhere (Turksen and Aubin, 1991; Turksen et al., 1992); it was used at a 1/100 dilution of purified ascites fluid. 10% normal serum in PBS or 3% BSA in PBS were used as negative controls. Nodules or calvaria sections were rinsed in PBS and incubated for 1 h at room temperature with secondary antibody CY-3-conjugated anti-rabbit (1/300 final dilution; Jackson ImmunoResearch Laboratories) for ERR α , OC, and ALP or secondary antibody anti-mouse (1/300 final dilution; Jackson ImmunoResearch Laboratories) for BSP and OPN. After rinsing, samples were mounted in Moviol (Hoechst Ltd.) and observed by epifluorescence microscopy on a ZEISS Photomicroscope III (ZEISS). For photography and printing, equal exposure times were used for specifically labeled and control cultures.

Nodule Quantification

For quantification of nodule formation, dishes or wells were fixed and stained by the Von Kossa technique and bone nodules were counted on a grid (Bellows et al., 1986; Bellows and Aubin, 1989). Results are plotted as the mean number of nodules \pm SD of three wells for controls and each

concentration of antisense or sense primers of three independent experiments and five dishes for pcDNA3 control and pcDNA3-ERR α of five independent experiments.

Cell Counting

For cell growth analysis, the cell layers were rinsed in PBS, released with trypsin and collagenase (1:1, vol/vol, of solutions described above), and the harvested cells were counted electronically. Results are plotted as the average of three counts for each of three dishes for control and pcDNA3-ERR α or three wells for each concentration of antisense or sense primers used.

ALP Histochemistry

The histochemical stain for ALP is a modification of Pearse's (1960). Cells were rinsed once with cold PBS and fixed in 10% cold neutral buffered formalin for 15 min, rinsed with distilled water, and left in distilled water for 15 min. Fresh substrate (10 mg Naphthol AS MX-PO4 [Sigma-Aldrich] dissolved in 400 μ l N,N-dimethylformamide, then added to 50 ml distilled water and 50 ml Tris-HCl [0.2 M, pH 8.3] and then 60 mg Red Violet LB salt [Sigma-Aldrich]), was filtered through Whatman's No. 1 filter directly onto the dishes, and incubated for 45 min at 20°C. The dishes were then rinsed in tap water, drained and stained with 2.5% silver nitrate for 30 min at room temperature (von Kossa stain), then rinsed three times with tap water. The dishes were finally air dried.

Transient Transfections

Primary RC cells were grown in 35-mm tissue culture dishes at 2 \times 10⁴/dish in α -MEM containing 10% heat-inactivated FBS (Flow Laboratories) and supplemented with 50 μ g/ml ascorbic acid, 10 mM sodium β -glycerophosphate, and 10^{–8} M dexamethasone. Cells were transfected at 50% of confluence according to the Effectene transfection protocol (QIAGEN) using a pcDNA3 empty plasmid as a control and pcDNA3-ERR α (mouse cDNA full-length from 1–2,211 bp; in the EcoRI cloning site) at 0.5 μ g of total DNA per transfection. As control of transfection efficiency, we used a CMV- β Gal vector. Nodules were counted at day 15. mRNA was extracted at 72 h, day 10 (beginning of nodules formation), and day 13 (formed nodules) after transfection.

Antisense and Sense Oligonucleotide Treatment

The resuspended RC cells were plated in 24-well plates at 10⁴ cells/well. Antisense oligonucleotide inhibition of ERR α expression was accomplished with a 20-base phosphorothioate-modified oligonucleotide, localized to the A/B domain, close to the start methionine. This domain is known not to be highly conserved among members of the nuclear receptor family. The ERR α antisense oligonucleotide sequence was: 5'-TCACCGGGGGTTTCAGTCTCA-3'. This sequence was rigorously analyzed by Blast search and no homologies were found; this included no homology with either ER α or ER β , ERR β or ERR γ , or any other currently known nuclear receptors. The corresponding sense and a scrambled oligonucleotide (5'-TCGGGCTCACACGGGGTTAGCT-3') were used as negative controls. Other control dishes were treated with no oligonucleotide. Preliminary experiments were done to determine effective oligonucleotide concentrations that were not toxic. 0.1 to 5 μ M oligonucleotides were added directly to cells either during the proliferation phase (days 1 to 6) and 0.5 to 2 μ M oligonucleotides were added during the differentiation phase (day 5 [end of proliferation] to 11) in standard medium as above supplemented with 50 μ g/ml ascorbic acid, 10 mM sodium β -glycerophosphate, and 10^{–8} M dexamethasone. Medium was changed every 2 d and fresh oligonucleotides were added. mRNA was collected at day 6 for the proliferation experiments and at day 15 for the differentiation experiments. Nodules were counted at 15 d.

Statistical Analysis

Results for PCR analysis, bone nodule number (antisense/sense experiments), and cell proliferation were expressed as mean \pm SD, and analyzed statistically by one-way analysis of variance (ANOVA) with treatment group as variance and Bonferroni and Tukey post tests with InStat software (v2.01; GraphPad Software). Results for bone nodule number (overexpression experiment) were expressed as mean \pm SD and analyzed statistically by the Mann-Whitney test and Welch test (InStat). Statistical significance was taken as $P < 0.05$.

Results

ERR α mRNA Is Expressed at All Developmental Stages of Osteoblast Differentiation and Maturation in RC Cells In Vitro

ERR α mRNA expression levels assessed over a proliferation-differentiation time course by Northern blotting of primary RC cell populations indicated that ERR α mRNA was expressed at all times analyzed, including proliferation (day 6), early nodule formation (day 10), and nodule mineralization (day 15) (not shown). However, because RC cell cultures comprise a heterogeneous mixture of cell types and osteoblasts at different differentiation stages, we sought to confirm that ERR α is expressed by osteoblast lineage cells and clarify its expression pattern over the proliferation-differentiation sequence of the osteoprogenitors. To do this, we used globally amplified (poly[A] PCR) cDNA pools prepared previously from single isolated osteoblast colonies at different stages of differentiation (Liu et al., 1994; Candelieri et al., 1999; Liu and Aubin, 1996). Colonies used were selected based on their molecular phenotypes (relative expression levels of COLLI, OPN, BSP, ALP, and OCN). ERR α was amplified in each cDNA pool with specific primers for sequences in the 3'UTR of ERR α and normalized to the relative amounts of total cDNA. ERR α mRNA was found to be expressed at all developmental times assessed including in colonies containing primitive progenitors, expressing only OPN (Fig. 1 A), in progressively more mature colonies expressing COLLI and OPN (Fig. 1 B) or COLLI, ALP, and OPN (Fig. 1 C), in multilayered colonies containing identifiable cuboidal osteoblasts also expressing

OCN (Fig. 1 D) and, finally, in mineralized bone nodules (Fig. 1 E).

ERR α Protein Is Expressed in Osteoblastic RC Cells In Vitro and in Fetal RC In Vivo

To determine whether ERR α protein is expressed in RC cell cultures, we performed immunocytochemistry. First, however, a Western blot of HeLa cell extracts was used to confirm the specificity of the ERR α antibody. As expected based on previously published data (Johnston et al., 1997; Shigeta et al., 1997), we detected a single immunoreactive band at 53 kD in HeLa (Fig. 2 A) and RC cell extracts (Fig. 3).

As predicted based on the relatively wide tissue distribution of ERR α mRNA in developing mouse embryos (Bonnelye et al., 1997b) and our results summarized above, ERR α protein was found to be widely distributed in most, if not all, cells in RC cultures at all times analyzed, including early proliferation stages, confluence (data not shown), when nascent bone nodules were forming (Fig. 2 B, a), and when nodules were mineralizing (Fig. 2 B, b). However, staining for ERR α was more intense in cuboidal osteoblasts associated with both early and late bone nodules than in the surrounding fibroblastic cells (Fig. 2 B, a and b). For comparison, protein expression of two well-established osteoblast markers, OCN (Fig. 2 B, d) and OPN (Fig. 2 B, e), both of which are coexpressed at high levels in the same cuboidal osteoblastic cells expressing ERR α , is also shown. As expected, ERR α was also highly expressed in cells expressing ALP and BSP (data not shown).

To extend the observations made in vitro to bones in vivo, we also performed immunocytochemistry on sections

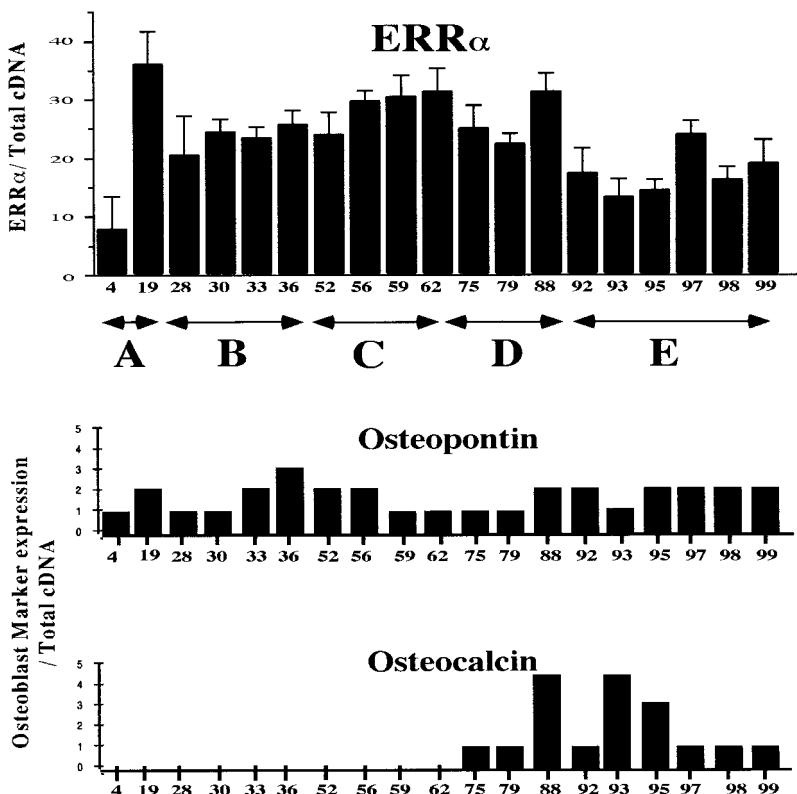


Figure 1. Detection of ERR α by RT-PCR in cDNA pools prepared from individual isolated colonies at different stages of osteoblast differentiation and characterized on the basis of molecular phenotype and expression of several known osteoblast lineage markers (OPN and OCN are shown; other osteoblast markers are not). The 19 cDNA pools shown represent several transitional stages: primitive progenitors (A), progressively more mature precursors (B, C, and D), and terminally differentiated, bone-forming osteoblasts (E). Although category order is progressive, the order of colonies within each category is random. ERR α mRNA was normalized with the relative amounts of total cDNA and detected at all developmental stages, with a trend to lower expression in the earliest precursors and most mature osteoblasts in mineralized nodules.

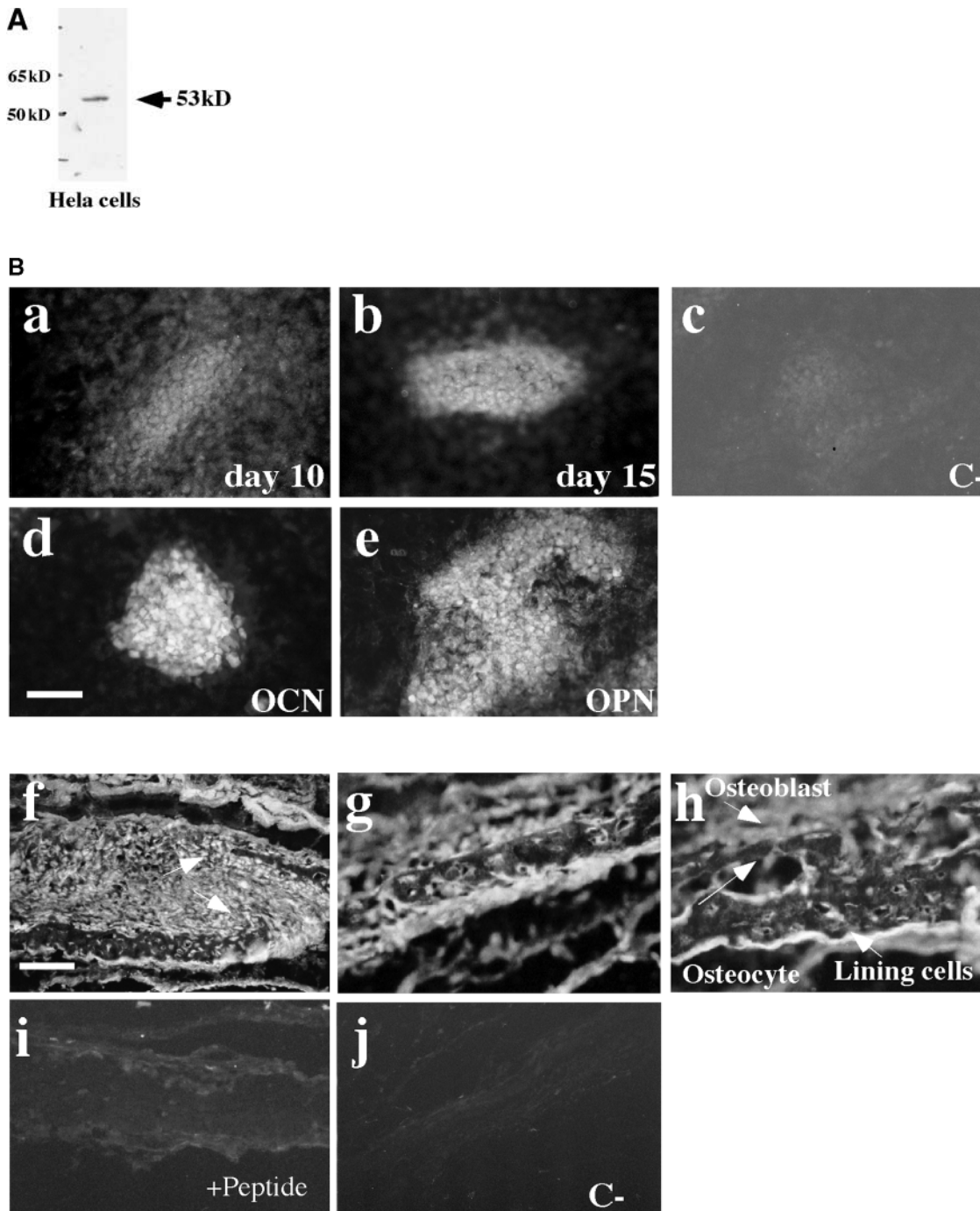


Figure 2. ERR α protein with apparent molecular mass of 53 kD is clearly detectable in whole-cell extracts obtained from HeLa cells (A). After SDS-PAGE (10% polyacrylamide), gels were blotted onto nitrocellulose, probed with purified rabbit polyclonal antibody directed against the A/B domain of ERR α , and detected with chemiluminescence. ERR α is detectable in vitro by immunolabeling of RC cells in early differentiation-nascent nodule formation stages (B, a) and later during matrix mineralization (B, b). Immunolabeling of cuboidal osteoblasts for OCN (B, d) and OPN (B, e) is shown for comparison. The negative secondary antibody control (incubation without rabbit anti-ERR α antibody) (C-) is shown in (B, c). Bar, 200 μ m (a-j). ERR α is also detectable in vivo in sections showing different regions of the 21-d fetal RC; at the osteogenic front (B, f), more mature growing bone trabecula (B, g), and remodeling bone (B, h). Preincubation of the ERR α antibody with the peptide used as immunogen abolishes ERR α labeling (B, i). The negative secondary antibody control (incubation without rabbit anti-ERR α antibody) is also shown (B, j).

of 21-d fetal RC, the same bones used for preparation of cell cultures. Consistent with the in vitro results, ERR α was found in all detectable cohorts of osteoblasts from those associated with nascent bone at the osteogenic front

(Fig. 2 B, f) to those in the more mature growing bone trabecula (Fig. 2 B, g) and remodeling bone (Fig. 2 B, h). Preincubation of the ERR α antibody with the peptide used as immunogen abolished all specific labeling (Fig. 2 B, i).

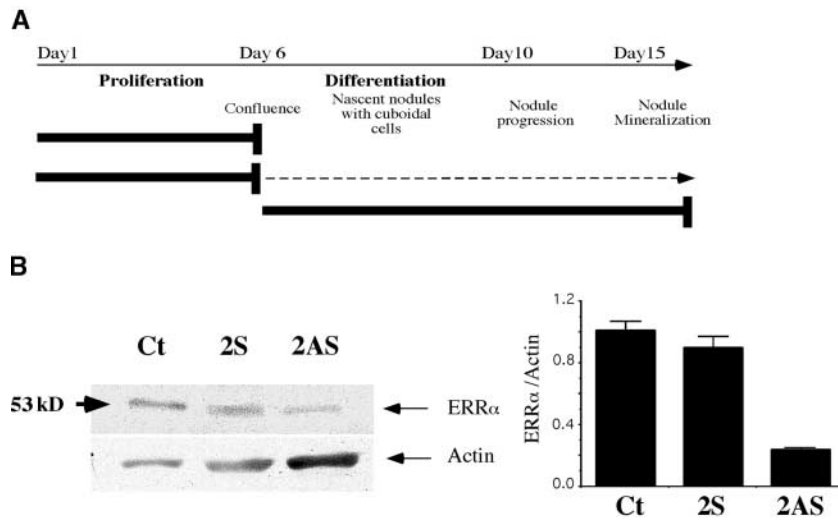


Figure 3. Inhibition of $ERR\alpha$ expression was accomplished with a 20-base phosphorothioate-modified antisense (AS) oligonucleotide, localized to the A/B domain of $ERR\alpha$ (see Materials and Methods for sequence). RC cells were treated at different developmental times from early proliferation stages through differentiation and mineralization as indicated in the summary (A). Control RC cells were treated either with the complementary sense (S) oligonucleotide or no oligonucleotide (Ct). The specificity and efficacy of the antisense treatment are evident from the Western blot, i.e., decreased intensity of label for $ERR\alpha$ protein after treatment with 2 μ M of antisense (AS, B), but not 2 μ M sense (S, B) or no oligonucleotide (Ct, B).

Consistent with our reverse transcription (RT)-PCR results on single bone nodules (see for example Fig. 1), $ERR\alpha$ was also detectable in sutural cells (arrows, Fig. 2 B, f), preosteoblasts, and osteocytes (Fig. 2 B, g and h). $ERR\alpha$ was also high in osteocytes present in postnatal (4 wk) RC (data not shown), suggesting that $ERR\alpha$ may be involved not only in the formation but also in the maintenance of bone.

Inhibition of $ERR\alpha$ Expression Blocks the Proliferation of RC Cells and Their Differentiation to Mature Bone-forming Osteoblasts

Given its expression in both proliferating osteoprogenitor cells and more mature osteoblasts and osteocytes, we next asked whether $ERR\alpha$ is a critical factor in osteoblast proliferation and/or differentiation. Antisense oligonucleotides form DNA-RNA duplexes with specific mRNA species, thereby blocking binding of the mRNA to the 40S ribosomal subunit and preventing translation (Reddy et al., 1994). As outlined in Materials and Methods, we designed antisense oligonucleotides to maximize specificity for $ERR\alpha$ and, by Blast searching, found no evidence for homology with either ERs or any other nuclear receptors; controls comprised both a scrambled and a sense oligonucleotide. Preliminary experiments were done to determine effective oligonucleotide concentrations that were not toxic (not shown) and the specificity of the antisense was confirmed by Western blot on RC cell extracts. After 24 h of treatment or not with sense or antisense oligonucleotides on RC cells at day 12, $ERR\alpha$ is detectable as expected in extracts of untreated cultures and those treated with 2 μ M sense oligonucleotides (Fig. 3 B) but is almost undetectable in cultures treated with 2 μ M antisense after quantification with actin used as control (Fig. 3 B). The specificity of the antisense was also confirmed by immunocytochemistry for $ERR\alpha$ on bone nodules (data not shown).

To dissect the possible involvement of $ERR\alpha$ in osteoblast differentiation and bone formation, we treated RC cells at different developmental times from early proliferation stages until mineralized nodule formation (summary, Fig. 3 A). Treatment of RC cells between days 1–6, the proliferation stage, caused a significant and specific dose-

dependent decrease, i.e., 13% at 0.5 μ M, 30% at 1 μ M, and 40% at 2 μ M, in cell number at day 6 in dishes treated with antisense compared with sense, scrambled, or untreated controls (Fig. 4 A). These results suggest that $ERR\alpha$ may play a role in the proliferation or very early differentiation phases of RC cells. To analyze the underlying mechanism of $ERR\alpha$ action during the proliferation phase, we assessed expression of early markers of osteoblast differentiation (ALP, BSP, OPN, Cbfa1, COLLI), proliferation (cyclin D1, c-Fos [data not shown]), and apoptosis (Bcl2, Bax) at day 6 (Fig. 4 B). BSP and Cbfa1 were reduced significantly by 35% (Fig. 4, B and C); cyclin D1 was also reduced. On the other hand, ALP, OPN, COLLI, c-Fos, Bax, and Bcl2 were not affected (Fig. 4, B and C, and data not shown). To determine whether treatment during the proliferation time window caused a sustained alteration in differentiation, we assessed terminal differentiation/bone nodule formation at day 15 in cultures treated between days 1–6 with antisense and then switched to normal differentiation medium. A significant decrease in mineralized bone nodule number, i.e., 29% at 1 μ M and 45% at 2 μ M antisense oligonucleotides compared with sense or untreated controls (Fig. 5 A), was seen. Concomitantly, we found that ALP and BSP expression were lower than levels seen in control or sense-treated cultures, whereas OPN, OCN, and COLLI were not significantly altered (Fig. 5, B and C).

To determine whether $ERR\alpha$ also plays a role in osteoblast differentiation independently of an effect on proliferation, we next treated RC cells with the antisense or control oligonucleotides from day 5 (after cells had reached confluence and proliferation was decreased) to day 11. Although cell number was decreased by day 15 (19% at 1 μ M and 35% at 2 μ M; data not shown) in antisense-treated cultures, a much larger dose-dependent decrease in mineralized bone nodule formation was seen, i.e., 30% at 0.5 μ M, 60% at 1 μ M, and 100% decrease at 2 μ M (Fig. 6, A–C); the sense and scrambled oligonucleotides had a nonspecific non-dose-dependent effect on nodule numbers (Fig. 6, A–C). A similar inhibition of bone nodule formation was also observed when we treated the osteoblastic cell line MC3T3-E1 with the antisense oligonucleotides

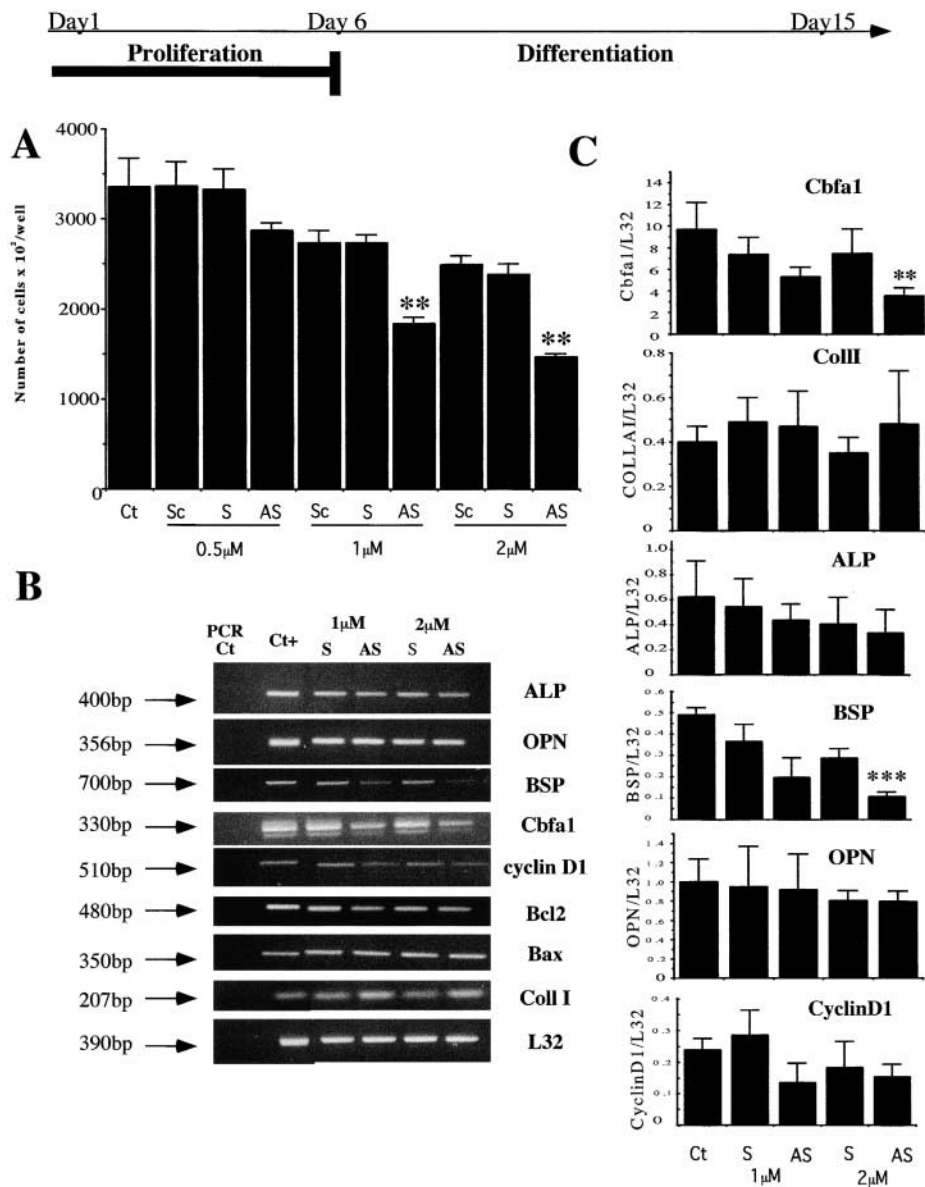


Figure 4. RC cells were treated with antisense (AS) or sense (S) oligonucleotides at 0.5, 1, and 2 μ M or no (Ct) oligonucleotide during the proliferation stage (days 1–6). Inhibition of $ERR\alpha$ protein synthesis decreased cell proliferation as evident from decreased cell number at day 6 (ANOVA, $P < 0.0001$; Bonferroni and Tukey post tests $**P < 0.01$, $***P < 0.001$ AS vs. S or Sc). Data are expressed as the cell number mean \pm SD of triplicate wells and are representative of three independent experiment (A). Total RNA was extracted from parallel wells and RT-PCR was performed on triplicate samples by using primers specific for early markers of osteoblast differentiation (ALP, BSP, OPN, cbfa1, COLLI), proliferation (cyclin D1), and apoptosis (Bcl2, Bax) at day 6 (B). The amount of PCR product for each marker was normalized to ribosomal protein L32 PCR product (C). Cbfa1 and BSP were significantly reduced in AS-treated cultures (ANOVA; $P < 0.05$ and $P < 0.001$, respectively; Bonferroni and Tukey post tests $**P < 0.01$, $***P < 0.001$ AS vs. S).

over a comparable time period (data not shown). In antisense-treated cultures, ALP-positive colonies were present and large in diameter but flat, suggesting that inhibition of $ERR\alpha$ blocked differentiation at an early stage such that progression to matrix deposition and maturation (Fig. 6 B) was reduced. Consistent with this interpretation, Cbfa1, BSP, and OCN were all decreased in antisense-treated cultures whereas OPN, COLLI, and ALP were not affected (Fig. 7, A and B). Immunocytochemistry confirmed the decrease in OCN- and BSP-expressing cells, but the maintenance of ALP expression in incipient bone nodules (Fig. 7 C).

Overexpression of $ERR\alpha$ Increases Differentiation and Bone Nodule Formation in RC Cells

To address further the putative functional role of $ERR\alpha$ in osteoblast differentiation suggested by the antisense oligonucleotide treatment, we next asked whether osteoblast differentiation and bone formation were altered when

$ERR\alpha$ is overexpressed in RC cells. $ERR\alpha$ overexpression was achieved by transient transfection of day 5 (50–60% confluent) cultures of RC cells with a CMV- $ERR\alpha$ construct. By using a CMV- β Gal control vector, we estimated that we reproducibly obtained a maximal efficiency of transfection of 10–15%, which resulted in a 30% increase in $ERR\alpha$ levels observed on Northern blots (Fig. 8 A). Consistent with the relatively low transfection efficiency, a small but statistically significant increase (15%) in the number of mineralized bone nodules formed by day 15 was observed (Fig. 8 B). In parallel, we assessed expression levels of osteoblast markers (ALP, OPN, OCN) 72 h after transfection, at day 10 when bone nodules were beginning to form and at day 13 when nodules were well developed. We observed an increase in ALP and OPN at 72 h (Fig. 8 C) and day 10 (data not shown) in cultures overexpressing $ERR\alpha$, consistent with previous data describing OPN as a target gene of $ERR\alpha$ in reporter assays (Bonnelye et al., 1997a; Vanacker et al., 1998b). OCN, a mature osteoblast marker expressed only at later differen-

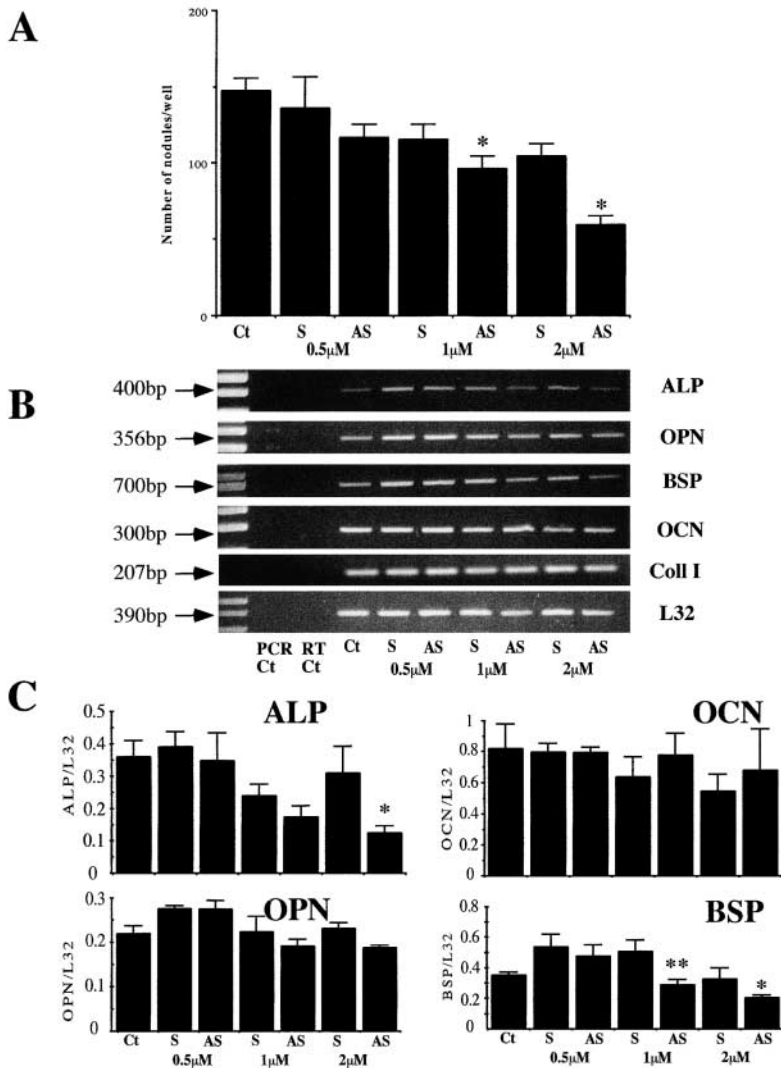
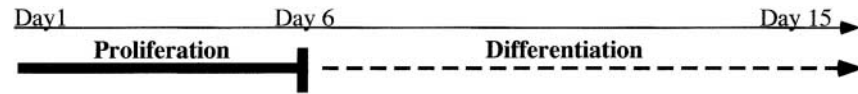


Figure 5. RC cells were treated with anti-sense (AS) or sense (S) oligonucleotides at 0.5, 1, and 2 μM or no (Ct) oligonucleotide during the proliferation stage (days 1–6) and then switched to normal differentiation medium, to determine if treatment during the proliferation time window caused a sustained alteration in differentiation; cultures were fixed at day 15. Inhibition of $ERR\alpha$ protein synthesis resulted in a decrease in the number of bone nodules (ANOVA; $P < 0.001$; Bonferroni and Tukey post tests, $*P < 0.05$ AS vs. S). Data are expressed as the mean number of nodules \pm SD of triplicate wells and are representative of two independent experiments; mineralized nodules were identified by von Kossa staining at day 15 (A). Total RNA was extracted from parallel wells at day 15 and RT-PCR performed on triplicate samples by using primers specific for osteoblast markers (ALP, BSP, OPN, OCN, COLLI) (B). Osteoblast marker PCR products were normalized to L32 PCR product (C). ALP and BSP were reduced significantly (ANOVA, $P < 0.001$) in both cases; Bonferroni and Tukey post tests $*P < 0.05$, $**P < 0.01$ AS vs. S.

tiation times (Fig. 1; see also Liu et al., 1994; Ducy et al., 1996), was undetectable at 72 h, but was increased at both day 10 (not shown) and 13 (Fig. 8 C).

Discussion

Our findings show that $ERR\alpha$ is expressed in fetal and adult RC in vivo and in RC cell cultures in vitro throughout all osteoblast differentiation stages, from early osteoprogenitors to mature osteoblasts. Inhibition of $ERR\alpha$ expression during the proliferation phase of RC cell cultures with phosphorothioate-modified antisense oligonucleotides decreased proliferation, as seen by a decrease in cell number and proliferation markers, and reduced differentiation, as seen by a decrease in both mineralized bone nodule formation and expression levels of osteoblast-associated markers. Inhibition of $ERR\alpha$ early during the differentiation phase also markedly decreased differentiation and bone nodule formation. Overexpression of $ERR\alpha$ by transient transfection in differentiating RC cell cultures

caused a small (15%) but significant increase in mineralized bone nodule formation and markers of osteoblast differentiation, consistent with the ~ 10 –15% transfection efficiency and increased $ERR\alpha$ mRNA expression seen in the primary cell cultures. Although the antisense experiments are not sufficient to formally link functionality of the receptor with biological activity during osteoblast proliferation and differentiation, taken together with the results of $ERR\alpha$ overexpression, the data suggest that $ERR\alpha$ may play a role in bone formation and turnover, with specific effects on proliferation, progression of differentiation, and mineralization activity of osteoprogenitors and more mature osteoblasts.

ERR α Is Expressed in Osteoblast Lineage Cells throughout Their Developmental Lifetime

We have found that $ERR\alpha$ is expressed at all detectable stages of osteoblast development, suggesting that $ERR\alpha$ may have a function in osteoblasts throughout their devel-

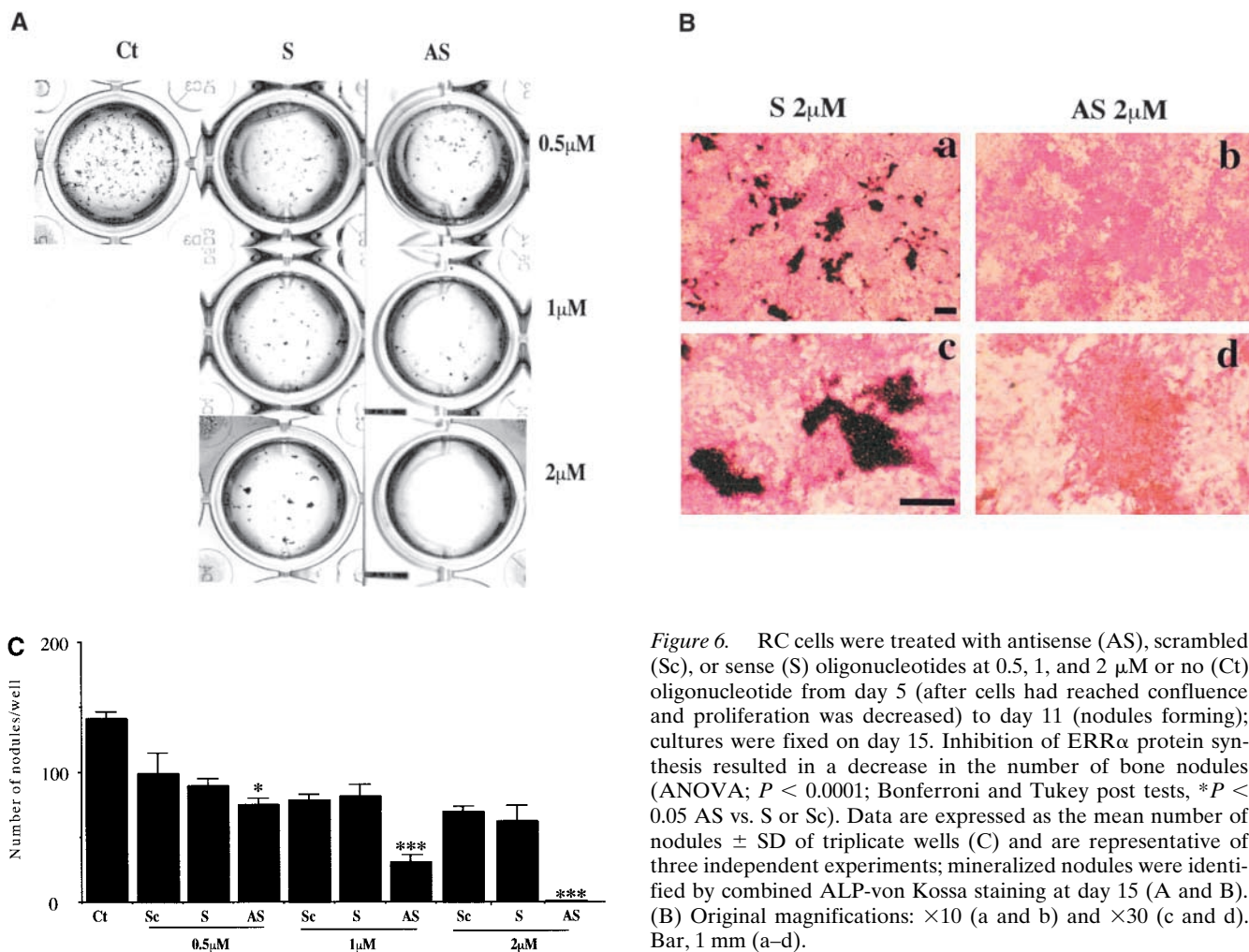
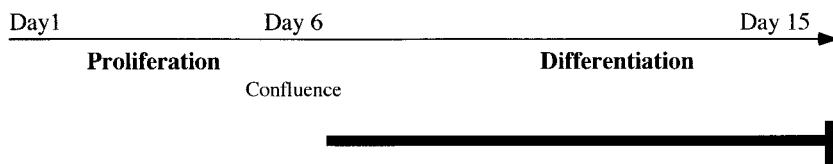


Figure 6. RC cells were treated with antisense (AS), scrambled (Sc), or sense (S) oligonucleotides at 0.5, 1, and 2 μM or no (Ct) oligonucleotide from day 5 (after cells had reached confluence and proliferation was decreased) to day 11 (nodules forming); cultures were fixed on day 15. Inhibition of $\text{ERR}\alpha$ protein synthesis resulted in a decrease in the number of bone nodules (ANOVA; $P < 0.0001$; Bonferroni and Tukey post tests, $*P < 0.05$ AS vs. S or Sc). Data are expressed as the mean number of nodules \pm SD of triplicate wells (C) and are representative of three independent experiments; mineralized nodules were identified by combined ALP-von Kossa staining at day 15 (A and B). (B) Original magnifications: $\times 10$ (a and b) and $\times 30$ (c and d). Bar, 1 mm (a–d).

opmental lifetime (see also below). In addition to its expression in fetal calvaria, $\text{ERR}\alpha$ is also highly expressed in adult calvaria (and other fetal and adult bones, data not shown) and is expressed throughout osteogenesis in adult rat bone marrow stromal cell cultures (Bonnelye and Aubin, in preparation), suggesting that it may function throughout the lifetime of the organism and may be required not only for bone formation but also for maintenance. In adult quiescent bone, labeling appears highest in osteocytes, which are thought to be mechanosensors that send strain-related signals to lining cells located at the bone surface through the canicular syncytium (Huiskes et al., 2000), leading to recruitment of osteoblasts among other effects. The ability of $\text{ERR}\alpha$ to transactivate the OPN promoter in osteoblasts suggests a possible mechanism by which $\text{ERR}\alpha$ may contribute to mechanical stress responses. OPN is expressed in osteocytes and has been postulated previously to play a bone role in response to mechanical stress; for example, the enhancement of osteo-

clastic bone resorption and suppression of osteoblastic bone formation in response to reduced mechanical stress do not occur in the absence of OPN (Ishijima et al., 2001). The high levels of expression of $\text{ERR}\alpha$ in osteocytes suggest that it may regulate OPN in this cell type and that dysregulation of $\text{ERR}\alpha$ expression may alter OPN expression and concomitantly modify the response to mechanical stress.

ERR α and Proliferation

Consistent with its expression in proliferating osteoblastic populations, we found that antisense oligonucleotide-induced downregulation of $\text{ERR}\alpha$ inhibits proliferation of RC cell populations, an inhibition that appears to have consequences on bone nodule formation at later times (see below). The decrease in proliferation of RC cells was somewhat unexpected, given our previous observation that $\text{ERR}\alpha$ expression appears to correlate with exit from proliferation and the onset of the differentiation process in at

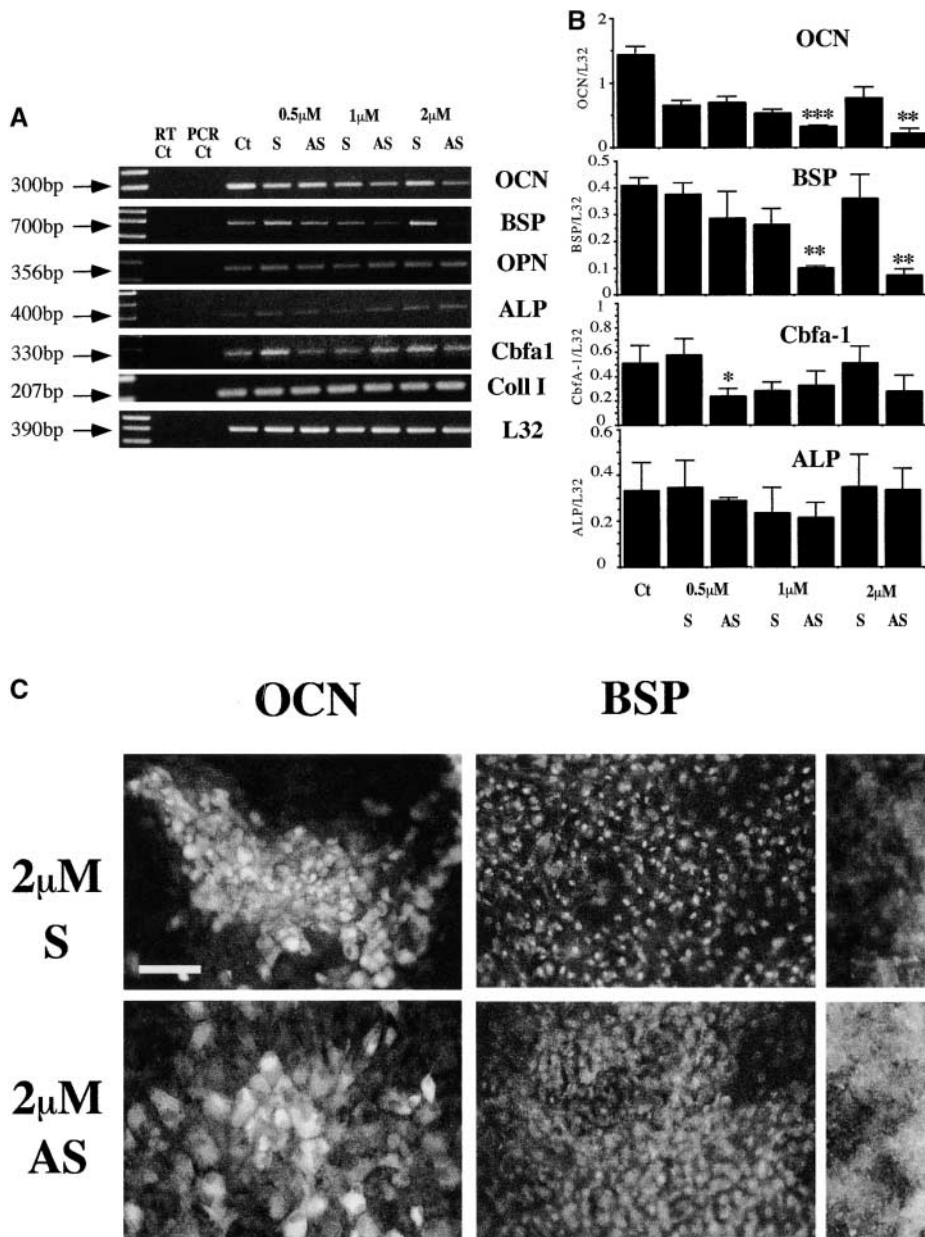


Figure 7. Total RNA was extracted from parallel wells at day 15 and RT-PCR performed on triplicate samples by using primers specific for osteoblast markers (ALP, BSP, OPN, OCN, COLLI, Cbfa1) (A). Osteoblast marker PCR products were normalized to L32 PCR product. Cbfa1, OCN, and BSP were reduced by antisense (AS) treatment (ANOVA; $P < 0.05$, $P < 0.001$, $P < 0.001$, respectively; Bonferroni and Tukey post tests $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ AS vs. S) (B). Immunocytochemistry confirmed the decrease in OCN- and BSP-expressing cells but the maintenance of ALP expression in developing bone nodules after treatment with 2 μM sense/antisense (C). Bar, 200 μm.

least certain other cell types, including the nervous system, the epidermis, and muscles in the developing mouse (Bonnellye et al., 1997b). This suggests that $ERR\alpha$ may play cell type-specific functions and is in keeping with its detection from the onset of osteogenesis in vivo (Bonnellye et al., 1997a) and its presence in all osteoblastic cells including the earliest detectable osteoprogenitors (current data).

The molecular basis for the $ERR\alpha$ effect on proliferation is of interest. As OPN has been described as a target gene of $ERR\alpha$ in vitro promoter-reporter assays (Bonnellye et al., 1997a; Vanacker et al., 1998a), and as OPN is highly expressed in many proliferative populations including osteoprogenitors (Fig. 1) and in many tumor cell lines (for a review, see Denhardt and Noda, 1998), it is one candidate target in the proliferation time window for antisense-induced downregulation. However, we found no detectable downregulation of this molecule by antisense oligonucleotide treatment during this developmental time

window, although it is clearly sensitive to regulation by $ERR\alpha$ later during the differentiation phase (see below). The mechanisms underlying this apparently differentiation stage-specific regulation of OPN and perhaps other target genes of $ERR\alpha$ is not yet clear; however, differentiation stage-specific expression of other coactivators or repressors including $ERR\alpha$ binding partners is one interesting hypothesis. We also found no significant changes in the antisense-treated RC populations in expression levels of a variety of proliferation and apoptosis/survival-associated genes expressed in osteoblasts including c-fos, Bcl-2, and Bax, although we cannot exclude possible changes in activity. However, we did observe a significant decrease in cyclin D1, a regulator of G1 phase progression. Interestingly, estrogen induces cell proliferation by stimulating progression through the G1 phase of the cell cycle (Wakeling et al., 1991; Clarke et al., 1992), and induction of cyclin D1 expression is a critical feature of the mitogenic response to

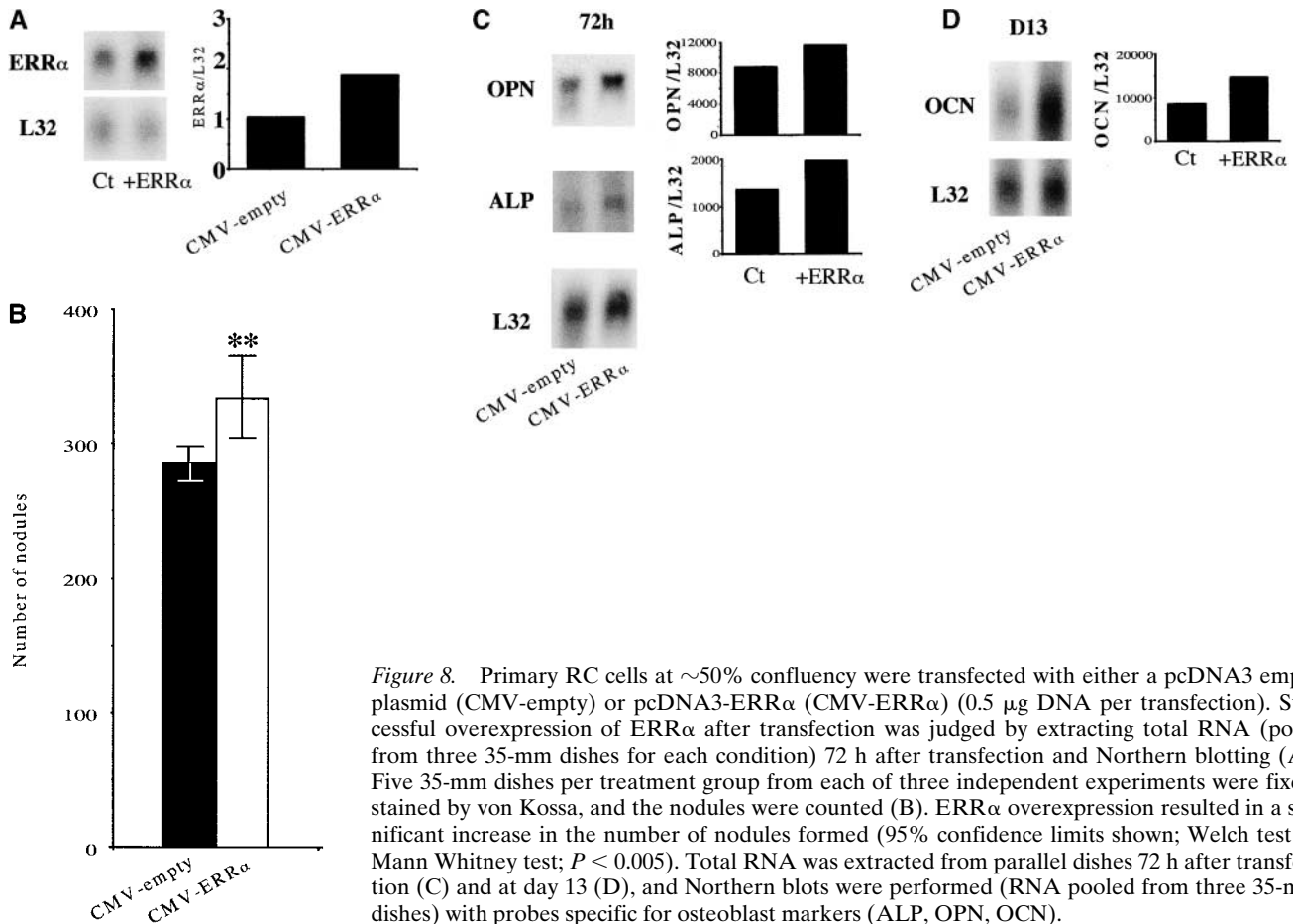


Figure 8. Primary RC cells at ~50% confluency were transfected with either a pcDNA3 empty plasmid (CMV-empty) or pcDNA3-ERR α (CMV-ERR α) (0.5 μ g DNA per transfection). Successful overexpression of ERR α after transfection was judged by extracting total RNA (pools from three 35-mm dishes for each condition) 72 h after transfection and Northern blotting (A). Five 35-mm dishes per treatment group from each of three independent experiments were fixed, stained by von Kossa, and the nodules were counted (B). ERR α overexpression resulted in a significant increase in the number of nodules formed (95% confidence limits shown; Welch test or Mann Whitney test; $P < 0.005$). Total RNA was extracted from parallel dishes 72 h after transfection (C) and at day 13 (D), and Northern blots were performed (RNA pooled from three 35-mm dishes) with probes specific for osteoblast markers (ALP, OPN, OCN).

estrogen. Recently, Sabbah et al. have described a region in the cyclin D1 promoter that confers regulation by estrogens in the human mammary carcinoma cells MCF7 (Sabbah et al., 1999). ERR α has been described as a modulator of the ER-mediated response of the human lactoferrin gene promoter (Yang et al., 1996), a mechanism that may also underlie its ability to regulate cyclin D1.

ERR α , Osteoblast Differentiation, and Matrix Mineralization

Our findings suggest a critical role for ERR α in bone formation, with both up- and downregulation of bone nodule formation concomitant with up- and downregulation of ERR α expression in vitro. Even in a background of high endogenous ERR α expression, further upregulation of ERR α by transfection of RC cells with a full-length ERR α expression vector late in the proliferation time window increased bone nodule formation by an amount approximately equivalent to the transfection efficiency of the population. Concomitantly, bone markers were also upregulated. Whether the increase in osteoprogenitor differentiation and bone nodule formation is a consequence of upregulation of any of these bone markers, or results from regulation of another currently unknown ERR α target gene, remains to be explicitly tested. Further insight into mechanisms may also be gained from experiments in which forced expression of ERR α is carried out with systematic time course and dose response studies.

Downregulation of ERR α either during proliferation phase or earlier or later in the differentiation sequence also had marked inhibitory effects on differentiation and bone nodule formation. Although one can speculate that the decrease seen when cells are treated only transiently during the proliferation phase reflects the downregulation of cyclin D1 and decreased proliferation of osteoprogenitors among other cells, the decrease could also reflect the concomitant downregulation of the bone "master" gene Cbfa1 (Ducy et al., 1997; Komori et al., 1997) and/or BSP, both of which are upregulated early during osteoprogenitor cell differentiation (Aubin and Liu, 1996; Malaval et al., 1999); we also found that BSP and ERR α are coexpressed in very early osteoprogenitors (Liu et al., 1994). The latter possibility is consistent with the finding that downregulation of ERR α after proliferation has largely ceased (antisense treatment from day 5–11) results in complete inhibition of mineralized bone nodule formation and concomitant downregulation of Cbfa1, BSP, and OCN. These observations, together with the data on increased bone formation when ERR α is upregulated early, suggest that at least part of the effect of ERR α on osteoblast differentiation and bone formation occurs early during the differentiation sequence, such that differentiation may not progress beyond a certain point when ERR α levels are low. In keeping with this hypothesis, large flat but ALP-positive colonies are present in antisense-treated cultures, and a

few cells express diminished levels of other osteoblast markers (Fig. 6).

Manipulation of ERR α levels in this study has served as a powerful approach to elucidating ERR α function in osteoblast development and bone formation in the absence of a known ligand. ERR α and the ERs share only moderate similarity (36%) in the ligand-binding E domain, which may explain the fact that ERR α does not bind estrogen. Although the pesticides chlordane and toxaphene, two organochlorine compounds with estrogen-like activity, have been reported to be antagonists of ERR α (Yang and Chen, 1999), suggesting that ERR α may act as a ligand-dependent repressor, no other ligands/agonists have yet been reported. On the other hand, the possibility of constitutive transactivation remains of interest. Constitutive activation of transcription of the lactoferrin gene by ERR α has been reported (Zhang and Teng, 2000). The constitutive transactivation was seen in cells cultured in phenol red-free medium supplemented with dextran-charcoal-treated serum, suggesting that ERR α may indeed function as a constitutive transactivator of at least some genes, although the experiments do not exclude the possibility of an endogenous ligand in particular cell types. Whether osteoblastic cells may be capable of producing an endogenous ligand or whether ERR α is functioning as a constitutive transactivator of at least some genes (e.g., BSP, Cbfa1, OCN) in this cell lineage remains to be determined. In the meantime, modulation of levels of this orphan receptor offers an alternative strategy for functional analysis and identification of potential target genes. Such modulation of receptor itself may also contribute to understanding of bone diseases characterized by decreased osteoblast numbers and reduced bone mass such as osteoporosis and arthritic conditions and to therapeutic strategies to deal with them.

In conclusion, ERR α is expressed in osteoblastic cells in vitro and in vivo and appears to have a function in proliferation, differentiation, bone nodule formation, and mineralization in RC cells. ERR α is expressed in fetal and adult calvaria and long bone, suggesting that ERR α may be involved not only in the formation but also in the maintenance and turnover of bone throughout the lifetime of the organism. These results indicate that ERR α may have an important function in the formation and turnover of the skeleton but the mechanism by which it does so, i.e., through convergence on the ER pathways or by other mechanisms, remains to be established. They also suggest that agonists and antagonists of ERR α may be useful as therapeutic agents in a wide variety of bone metabolic and other diseases affecting bone.

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Note added in proof: After this manuscript was submitted, a paper appeared reporting that diethylstilbestrol is a ligand for ERR α (Tremblay, G.B., T. Kunath, D. Bergeron, L. Lapointe, C. Champigny, J.-A. Bader, J. Rossant, and V. Giguere. 2001. *Genes Dev.* 15:833–838).

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