Osteoblast differentiation influences androgen and estrogen receptor-α and -β expression

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Abstract

Significant levels of estrogen and androgens circulate in men and women, and both play an important role in bone metabolism. While it is well established that either estrogen or androgen replacement therapy is effective at ameliorating bone loss associated with hypogonadism, recent evidence nevertheless suggests that estrogen and androgens have distinct molecular actions on the skeleton. In this study, we have employed normal rat calvarial osteoblast cultures to characterize relative expression profiles of estrogen (ERα and ERβ) and androgen receptors (AR) during osteoblast differentiation. Normal osteoblast cultures can proceed through in vitro differentiation with distinct stages of proliferation, matrix maturation and mineralization in the appropriate differentiation medium containing ascorbic acid. Expression profiles of AR, ERα and ERβ in primary cultures during osteoblast differentiation were characterized both by semi-quantitative relative RT-PCR and by Western analysis. In cultures induced to differentiate by growth in the presence of ascorbic acid, the expression profile for each receptor was unique during the course of differentiation. ERα levels were elevated during matrix maturation and then declined during mineralization. ERβ expression was relatively constant throughout differentiation, exhibiting more constitutive expression. In contrast, AR levels were lowest during proliferation, and then increased throughout differentiation with highest levels in the most mature mineralizing cultures. Since steroid hormone action is generally mediated by specific cognate receptors, these results suggest that androgen actions may target cells during the mineralization stage of osteoblast differentiation, while estrogen action through either receptor isoform is more likely to affect osteoblasts earlier during matrix maturation. Interestingly, sex steroid receptor expression profiles did not exhibit the same patterns of regulation if osteoblast cultures were grown without ascorbic acid in medium that did not support extracellular matrix deposition. Thus, sex steroids may distinctly influence skeletal health by differential modulation of function during osteoblast differentiation.

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Introduction

It has long been recognized that the sex steroids estrogen and androgen play an important role in maintaining the adult skeleton. Hypogonadism in both sexes is associated with bone loss. Replacement therapy with either estrogen or non-aromatizable androgen (e.g. dihydrotestosterone) is effective at ameliorating this loss. Interestingly, recent evidence suggests that combination therapy with both estrogen and androgenic steroids is more effective than estrogen replacement in women (Watts et al. 1995, Raisz et al. 1996, Rosenberg et al. 1997, Barrett-Connor 1998). In ovariectomized animals, replacement with non-aromatizable androgens yields beneficial effects that are clearly distinct from those observed with estrogen replacement (Coxam et al. 1996). In addition, females with androgen-insensitivity syndrome show a decrease in bone mineral density, even with good estrogen compliance (Marcus et al. 2000). Blockade of the androgen receptor (AR) with the specific AR antagonist flutamide in intact females results in osteopenia (Goulding & Gold 1993). In addition, mutations in either estrogen receptor (ER) or in aromatase lead to the development of osteopenia in males (Smith et al. 1994, Morishima et al. 1995, Carani et al. 1997). Finally, in a recent study in which endogenous steroid production was blocked in normal elderly men, replacement with both estradiol and testosterone appears to have additive effects on markers of bone formation (Falahatini-Nini et al. 2000). Combined, these reports indicate that in both men and women it is probable that androgens and estrogens each have important, yet distinct, functions during bone development and in the subsequent maintenance of skeletal homeostasis in the adult (Vanderschueren et al. 1998, Bilezikian 2002). However, the contrasting effects of these steroids on bone cell metabolism are poorly characterized (see Wiren & Orwoll 1999 for review).
Bone is clearly a target tissue with respect to sex steroid action. Documentation of ER expression (Eriksen et al. 1988, Komm et al. 1988) and of AR mRNA and specific androgen-binding sites in normal human osteoblastic cells (Colvard et al. 1989) and in established cell lines (Benz et al. 1991, Orwoll et al. 1991, Zhuang et al. 1992) has been described. AR and ER protein relative abundance is described as similar in normal human osteoblastic cells (Colvard et al. 1989), suggesting that androgens and estrogens each play important roles in skeletal physiology.

Two isoforms of ER exist, ERα and ERβ (Kuiper et al. 1996). Reports describe distinct patterns of expression of ER isoforms in bone (Arts et al. 1997, Onoe et al. 1997, Bodine et al. 1998, Lim et al. 1999). AR expression has been characterized in bone slices (using immunocytochemical analysis), identifying AR in a variety of cells including bone-forming osteoblasts and osteocytes (Abu et al. 1997, van der Eerden et al. 2002). However, the levels of AR expression during osteoblast differentiation in vitro have not been described, nor have they been contrasted with ER expression profiles.

In adults, there is continuous remodeling of bone that involves breakdown or resorption of the calcified extracellular matrix at localized sites followed by formation of new mineralized matrix. The osteoblast is the cell type responsible for formation of this new matrix. Mature, differentiated osteoblasts arise from precursor cells committed to the osteoblast lineage. In vivo osteoblasts undergo a developmental sequence progressing from committed proliferating precursors to mature, differentiated cells and, finally, to osteocytes embedded in the calcified matrix. We (Birnbaum & Wiren 1994, Birnbaum et al. 1995) and others (Aronow et al. 1990, Owen et al. 1990) have employed a well-characterized in vitro model, using normal rat osteoblastic cells isolated by collagenase digestion, that recapitulates these in vivo changes even compared with gene expression analysis performed in long bone (Weinreb et al. 1990, Zhou et al. 1994). In the presence of ascorbic acid and β-glycerophosphate, these cultures proceed through differentiation normally with the deposition of a collagenous extracellular matrix that mineralizes. However, this temporal sequence of differentiation does not proceed normally unless differentiation medium containing ascorbic acid is added, enabling appropriate extracellular matrix deposition (Aronow et al. 1990, Xiao et al. 2002). In this study, we have utilized normal rat osteoblastic cultures to characterize regulation of the AR, ERα and ERβ receptors at the level of both mRNA and protein expression during osteoblast differentiation.

This is the first report to characterize AR expression during specific stages of osteoblast differentiation, and to contrast the major sex steroid receptor expression profiles. We show that AR, ERα and ERβ mRNA and protein expression patterns are developmentally distinct, and that these changes are not evident in the absence of the development of a competent extracellular matrix. These results thus document divergent regulation of gene expression of the major sex steroid receptors, and suggest distinct actions of estrogen and androgens in the differentiating osteoblast population.

**Materials and Methods**

**Chemicals and reagents**

All the media, buffers, supplements and reagents for cell culture were obtained from Gibco BRL–Life Technologies (Grand Island, NY, USA) and Sigma Aldrich Co. (St Louis, MO, USA). Steroid hormones and other reagents were obtained from Sigma Aldrich Co.

**Cell culture**

Normal rat osteoblasts (rOB) were prepared by sequential collagenase digestion from neonatal rat calvaria as previously described (Birnbaum & Wiren 1994, Birnbaum et al. 1995), based on the protocol developed by Lian and Stein (Owen et al. 1990) and other laboratories (Binderman et al. 1974, Aubin et al. 1982). All animal procedures and animal care were reviewed and approved by the Portland, Oregon VA Medical Center Institutional Animal Care and Use Committee, and meet the NIH and the American Veterinary Medical Association guidelines for appropriate care and use of animals in research. Normal rat osteoblastic cells were used only as primary cultures without passage.

Cells were plated at 8000 cells/cm². Monolayer cultures were maintained for the first week in phenol-red free Ham’s F-12 with 5% fetal bovine serum (FBS). Beginning at day 7, cultures were switched to differentiation medium in phenol-red free BGJb (Fitzon-Jackson modification) supplemented with 5% FBS containing 50 µg/ml ascorbic acid (for appropriate collagen and extracellular matrix production). From day 14 on, 3 mM β-glycerophosphate (for appropriate mineralization) was added to the differentiation media. For some studies, cultures were grown entirely in phenol-red free Ham’s F-12 with 5% FBS without the addition of differentiation medium. Under these conditions without the elaboration of a complete collagenous extracellular matrix, osteoblastic cultures do not fully differentiate.

**Alkaline phosphatase activity**

Cells were plated in 6-well plates. Cell layers were washed twice with ice-cold phosphate-buffered saline (PBS), then harvested in 1 ml 50 mM Tris–HCl (pH 7·6), sonicated twice on ice and then centrifuged at 4 °C for 15 min at 1000 g. The supernatants were stored at −20 °C until analysis for alkaline phosphatase activity using p-nitrophenolphosphate as substrate. Absorbance was read
at 405 nm using a microplate reader. Alkaline phosphatase activity was expressed as nmol $p$-nitrophenol released/min per µg DNA. All analyses were done in six replicates. Each experiment was repeated two to three times.

**DNA content and calcium accumulation**

Cells were plated in 6-well plates. Cell layers were washed twice with ice-cold PBS, harvested in 50 mM Tris–HCl (pH 7·6), sonicated and then centrifuged for 15 min at 1000 $g$. The supernatants were analyzed for DNA content by dye-binding assay using bisbenzimid H 33258 from Hoechst reagents (Riedel-DeHagen AG, Seelze-Hannover, Germany). The DNA content was quantitated by measuring fluorescence at 458 nm spectrophotometrically, using purified calf thymus as a DNA standard (Labarca & Paigen 1980). All analyses were done in six replicates. For calcium accumulation experiments, wells were washed twice with ice-cold PBS and the cell matrix extracted by incubation twice with 0·5 ml 5% trichloroacetic acid for 2 h with rocking. Calcium concentrations were determined by the cresolphthalein complexone method using a kit from Sigma Aldrich Co. Each experiment was replicated two to three times.

**RNA isolation and relative RT-PCR semi-quantitative analysis of mRNA expression**

RNA was isolated by the single-step acid guanidinium isothiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi 1987) using RNA Stat-60 (TelTest, Inc. Friendswood, TX, USA). Quantitation of RNA was performed by spectrophotometric determination at 260 nm. Contaminating DNA was removed by RQ1-DNase (Promega, Madison, WI, USA) digestion and phenol–chloroform extraction or by Zymo–spin column purification following the manufacturer’s recommendations (Zymo Research, Orange, CA, USA).

PCR primers were designed by using OLIGO Software from Molecular Biology Insights, Inc. (Cascade, CO, USA) for rat AR, ERα and ERβ and for the housekeeping gene glyceraldehyde–3–phosphate dehydrogenase (GAPDH) (see Table 1 for primer sequence). GAPDH expression was used as an internal control to correct for potential differences in RNA quantitation and variations in cDNA yield during RT. Primers specific for rat AR (using accession no. M23264), rat ERα (using accession no. Y00102), rat ERβ (using accession no. U57439) or rat GAPDH (using accession no. M17701) were selected based on their melting temperatures, and did not show significant homology to other sequences in the BLAST database.

Full-length cDNA was synthesized using RNaseH$^-$ reverse transcriptase (Superscript II RT; Life Technologies, Gaithersburg, MD, USA) and an oligo(dT)$^{12-18}$ primer (Life Technologies) with 4µg DNase I-treated total RNA in 20 µl. PCR amplification was performed with 2·5 units Taq DNA polymerase (Amplitaq) from Perkin-Elmer (Branchburg, NJ, USA) and 1/20 volume of cDNA with 5–10 pmol of sense and antisense primers in 50 µl. Each assay was tested to ensure that the annealing and dissociation temperatures resulted in optimal DNA amplification. To determine that the PCR reaction remained in the linear phase of amplification, aliquots from a 100 µl reaction were removed after the indicated number of PCR cycles (see Fig. 2). Each cycle consisted of denaturation at 94 °C for 30 s, annealing at 58 °C for ERβ, 62 °C for ERα and 58 °C for AR for 20 s, followed by extension at 72 °C for 30 s. A final extension step was carried out at 72 °C for 10 min. PCR for AR, ERα and ERβ products was carried out in a Perkin-Elmer 9600 thermocycler for 40 cycles (which remains in the linear range for steroid receptor mRNA, see Fig. 2B). Parallel reactions were performed using GAPDH primers from the same RT reactions for 25 cycles shown in Fig. 2A (see also Kassem et al. 1998).

<table>
<thead>
<tr>
<th>Receptor (accession no.)</th>
<th>RT-PCR primers (5’→3’)</th>
<th>Position</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat AR (M23264)</td>
<td>CCATGGGGTGTTGGTGGA</td>
<td>757–776</td>
<td>276</td>
</tr>
<tr>
<td></td>
<td>TCCCGAGCTAACCTGCCTCA</td>
<td>1013–1032</td>
<td></td>
</tr>
<tr>
<td>Rat ERα (Y00102)</td>
<td>CCGGTCTATGCGCAGCTGACATC</td>
<td>435–458</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>GTAGAAAGGGGAGGGGCGGTGTC</td>
<td>651–674</td>
<td></td>
</tr>
<tr>
<td>Rat ERβ (U57439)</td>
<td>TCCCCGGCAGCCACGTAAACC</td>
<td>454–474</td>
<td>262</td>
</tr>
<tr>
<td></td>
<td>TCCCTCTTGTGCTTTGGA</td>
<td>695–715</td>
<td></td>
</tr>
<tr>
<td>GAPDH (M17701)</td>
<td>CGGCAAGTCTAAAGCCAGT</td>
<td>183–203</td>
<td>609</td>
</tr>
<tr>
<td></td>
<td>TCATACTGCGAGTTTCTCC</td>
<td>771–791</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1 Primers used for RT-PCR analysis of sex steroid receptors**

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The RT-PCR products were then analyzed by electrophoresis on a 1.5% agarose gel and visualized by a UV transilluminator after staining with ethidium bromide. DNA size marker was the 123 bp DNA ladder from GIBCO BRL–Life Technologies. Positive control size markers were also generated from total RNA isolated from an appropriate tissue (data not shown), using lung for ERβ (Maruyama et al. 1998) and testis for both ERα (Pau et al. 1998) and AR (Wilson & McPhaul 1996), and/or from a cDNA plasmid source (human AR cDNA kindly provided by Dr Marco Marcelli (Baylor College of Medicine and VA Medical Center, Houston, TX, USA) and human ERα cDNA provided by Dr Pierre Chambron (CNRS, Illkirch Cedex, France). Negative controls routinely included a no-RT reaction (data not shown). The identity of all PCR products was confirmed using automated sequence analysis with the ABI Dye Terminator Cycle Sequencing System on an ABI 377 DNA sequencer (PE Applied Biosystems, Foster, CA, USA). A clean readable sequence analysis with the ABI Dye Terminator Cycle Sequencing System on an ABI 377 DNA sequencer (PE Applied Biosystems, Foster, CA, USA). A clean readable sequence was uniformly obtained.

Semi-quantitative analysis of the RT-PCR products was performed by volume densitometry using GelExpert Software (Nucleotech, San Mateo, CA, USA) from the negative image after scanning of the gel photograph (HP ScanJet 6100 C/T); all amplification product quantitation was performed by volume densitometry using GelExpert Software from the negative image after scanning of the photograph (HP ScanJet 6100 C/T); all amplification product quantitation was performed by volume densitometry using GelExpert Software from the negative image after scanning of the photograph (HP ScanJet 6100 C/T); all amplification product quantitation was performed by volume densitometry using GelExpert Software from the negative image after scanning of the photograph (HP ScanJet 6100 C/T); all amplification product quantitation was performed by volume densitometry using GelExpert Software from the negative image after scanning of the photograph (HP ScanJet 6100 C/T); all amplification product quantitation was performed by volume densitometry using GelExpert Software from the negative image after scanning of the photograph (HP ScanJet 6100 C/T); all amplification product quantitation was performed by volume densitometry using GelExpert Software from the negative image after scanning of the photograph (HP ScanJet 6100 C/T); all amplification product Quantitative analysis of the proteins was performed by volume densitometry using GelExpert Software from the positive image after scanning of the photograph (HP ScanJet 6100 C/T). Data are presented as the protein to GAPDH expression values at day 4, followed over the time-course of in vitro differentiation (means ± S.E.M.). Data from two to three separate RNA isolations from different experiments are presented, with RT-PCR performed twice on each sample.

Western blot analysis

Polyclonal rabbit antibodies recognizing ERβ (PA1-310B), AR (PA1-111A) and a mouse monoclonal antibody against ERα (MA1-310) were purchased from Affinity Bioreagents Inc. (Golden, CO, USA). The MA1-310 antibody maps to the N-terminus of the receptor and does not recognize ERα or other members of the steroid receptor family; affinity-purified PA1-310B ERβ antibody maps to the carboxyl-terminus of the receptor and does not recognize ERα or other members of the steroid receptor family; polyclonal PA1-111A AR, antibody maps to the N-terminus of the receptor and does not recognize other members of the steroid receptor family. The α-tubulin antibody (T9026) was a monoclonal antibody purchased from Sigma Aldrich Co. Normal ROB cultures were extracted in lysis buffer (1% NP40, 20 mM Tris HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin and 1 mM Na3VO4 containing 3% SDS. Extract normalized to DNA (166 µg DNA/ lane) was mixed with 2 × SDS loading buffer (125 mM Tris buffer, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 1 µg/ml bromophenol blue) and boiled for 3 min. Extracts were loaded onto a 7.5% SDS-polyacrylamide gel and electrophoresed. The separated proteins were transferred to an Immobilon-P polyvinylidene-difluoride transfer membrane (Millipore, Bedford, MA, USA). The membrane was incubated for 1 h in blocking PBS with 5% non-fat dry milk. The primary antibodies were applied in PBS with 0.05% Tween-20 (PBS-T) buffer for 1 h at room temperature. Dilutions of antibodies were as follows: AR antibody at 4 µg/ml; ERα at 2 µg/ml; ERβ at 1 µg/ml; α-tubulin at 1:1000. After washing five times with PBS-T buffer, the membrane was incubated with horseradish peroxidase-linked goat anti-rabbit IgG antibody (Bio-Rad Laboratories, Richmond, CA, USA) for ERβ and AR, or goat anti-mouse IgG antibody (Bio-Rad) for ERα and α-tubulin both at 1:2000 for 1 h. Bands representing ERα, ERβ and AR co-migrate with positive controls: ERα and ERβ recombinant protein (Affinity Bioreagents) and lysates from LNCaP cultures respectively (data not shown). Preliminary studies employed three different sources for ERα antibody (PA1-308 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:1000 dilution, MC-20 (Santa Cruz) at 1:500 dilution, and MA1-310 (Affinity Bioreagents) at 2 µg/ml), and demonstrated that MA1-310 provided the clearest results (data not shown). Bound antibodies were visualized by an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) on Kodak X-AR5 autoradiographic film.

Quantitative analysis of the proteins was performed by volume densitometry using GelExpert Software from the positive image after scanning of the photograph (HP ScanJet 6100 C/T). Data are presented as the protein to α-tubulin ratio normalized to expression values at day 3, followed over the time-course of in vitro differentiation (means ± S.E.M.).

Statistical analysis

Linear regression analysis of PCR amplification was performed using r² as a measure of goodness-of-fit with Prism software (Graphpad, San Diego, CA, USA). This analysis was used to determine that PCR conditions were within the linear range for amplification.

Results

Characterization of in vitro differentiation in the normal rat calvarial osteoblast cell culture

In order to characterize the expression profiles of the major sex steroid receptors, we employed normal rat calvarial cultures derived by collagenase digestion as an in vitro
differentiation model. In Fig. 1 the temporal sequence of differentiation was followed in these cultures. DNA accumulation of the cultures increased up to day 11, and then remained constant or decreased slightly throughout the culture period up to day 26. Alkaline phosphatase activity was low until day 17, increased, then began to decline at day 26. Calcium deposition (mineralization of the matrix) was usually observed by day 20. After day 28, cells have been characterized as postmineralized cultures (Bodine et al. 1998), when osteocalcin levels begin to decline. These cultures can thus be characterized as progressing through distinct developmental stages in vitro, from proliferation to matrix maturation, mineralization and, finally, to postmineralized cultures, as previously described (Aronow et al. 1990, Owen et al. 1990, Birnbaum & Wiren 1994). This temporal sequence of differentiation can also be described by changes in gene expression as we have previously shown (Birnbaum & Wiren 1994), where collagen mRNA abundance was high during proliferation, then declined; osteopontin expression was elevated during both the proliferation and mineralization; alkaline phosphatase increased as proliferation slowed; and osteocalcin expression increased during the mineralization stage, then declined in postmineralization cultures. This temporal sequence of differentiation of osteoblastic cultures does not proceed normally unless differentiation medium containing ascorbic acid is added (Aronow et al. 1990), which can regulate transcription and post-transcriptional and post-translational processing of collagen (Nusgens et al. 2001).

Validation of mRNA expression assay using semi-quantitative RT-PCR

To better understand the action(s) of estrogen and androgen in the osteoblast population, we characterized the developmental profile of mRNA expression for the three major sex steroid receptors AR, ERα and ERβ in the normal rat osteoblast cultures. Semi-quantitative analysis of steroid receptor mRNA steady-state expression was determined by relative RT-PCR. The primer sequences for this analysis are shown in Table 1.

We first demonstrated that RT-PCR amplification products for the more abundant GAPDH mRNA species are linearly produced over a range of cycles from 21 to 29; linear regression analysis showed an $r^2=0.9891$ (Fig. 2A). A similar analysis was performed with ERα mRNA from samples with the highest level of expression for the sex steroid receptors (isolated from day-18 rat osteoblastic primary cultures; see Fig. 3). ERα amplification was shown to be in the linear range between 34 and 42 cycles with an $r^2=0.9938$ (Fig. 2B). All expression data are normalized to GAPDH levels to correct for differences in RNA quantitation and for potential variations in cDNA conversion during reverse transcription. GAPDH was
chosen as an internal control since its expression is not significantly affected by differentiation in this in vitro paradigm (data not shown).

Peak expression of ER isoform levels during osteoblast matrix maturation

RNA was isolated from rOB cultures at distinct stages of differentiation as described, and receptor expression was analyzed by semi-quantitative relative RT-PCR. AR, ERα and ERβ amplification was performed at 40 cycles; parallel reactions were performed using GAPDH primers for 25 cycles. Representative gels from the relative RT-PCR analysis from RNA isolated from normal rat osteoblastic primary cultures are shown in Fig. 3A. Data were first normalized to GAPDH expression. The normalized ratio for each specific receptor was then expressed relative to the level of expression in pre-confluent cultures (day 4), as shown in Fig. 3B. In order to fully characterize sex steroid receptor expression, Western analysis was also performed in a parallel fashion (see Fig. 4A). Quantitation of protein expression was accomplished after normalization to α-tubulin levels and the values expressed relative to the level at day 3 (Fig. 4B).

Modulation of ERα expression during osteoblastic differentiation was observed. Semi-quantitative analysis showed that ERα mRNA levels were low during proliferation, increased around twofold during matrix maturation and remained elevated, then declined in post-mineralizing cultures. The decline of ERα expression in post-mineralization cultures of normal rat calvarial cells has been described previously (Bodine et al. 1998). Interestingly, Western analysis of ERα protein levels showed changes generally parallel to the mRNA expression profiles in these cultures (Fig. 4B).

ERβ levels show constitutive expression

In contrast to ERα, ERβ mRNA levels remained fairly constant once proliferation slows (Fig. 3B). Again, Western analysis revealed a similar pattern of ERβ protein expression, with high relative levels and little change during differentiation (Fig. 4B). This result is consistent with previous findings (Onoe et al. 1997). The most notable difference between ERα and ERβ expression

Figure 2 Analyses of linearity of amplification products in RT-PCR reaction for GAPDH and ERα mRNA expression. (A) Linearity of GAPDH amplification. Reverse transcription was conducted with 4 μg DNase I-treated total RNA. Amplification using receptor primers was performed with Taq DNA polymerase and 1/10 volume of cDNA in 100 μl PCR was carried out for 21, 23, 25, 27 or 29 cycles with aliquots removed at the number of PCR cycles indicated. The RT-PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized on a u.v. transilluminator. Semi-quantitative analysis of GAPDH mRNA steady-state expression by RT-PCR was performed using GelExpert Software after scanning the negative image of the photographed gels. Data are expressed in arbitrary units. The Pearson’s r² value was determined by linear regression using Prism software. As shown, amplification products between 21 and 29 cycles remain within the linear range (with a Pearson’s r²=0.9891). This experiment was performed twice with similar results. (B) Linearity of steroid receptor ERα amplification. Reverse transcription and amplification were as described for GAPDH reactions. Aliquots were removed at 34, 36, 38, 40 or 42 cycles for the less abundant ERα mRNA. The RT-PCR products were analyzed as described above. As shown, amplification products between 34 and 42 cycles remain within the linear range (with a Pearson’s r²=0.9938). This experiment was performed twice with similar results.
patterns was that ERβ expression remained relatively elevated even in postmineralizing cultures while ERα levels were declining.

Figure 3  Expression analyses of ERα, ERβ, and AR during in vitro differentiation in normal rOB cultures. (A) Normal rOB cells were cultured for the indicated number of days during proliferation, matrix maturation, mineralization, and postmineralization stages. Total RNA was isolated and subjected to relative RT-PCR analysis using primers specific for rat ERα, ERβ, and AR or rat GAPDH (see Table 1). Reverse transcription was conducted as described in Fig. 2 with PCR carried out for 40 cycles for the steroid receptors, with parallel reactions performed using GAPDH primers for 25 cycles (all in the linear range as described in Fig. 2). Bands for rat ERα at the predicted 240 bp, rat ERβ at 262 bp, rat AR at 276 bp and GAPDH at 609 bp are shown. (B) Analyses of ERα, ERβ, and AR mRNA relative abundance. Semi-quantitative analysis of mRNA steady-state expression by relative RT-PCR was performed using GelExpert Software after scanning the negative image of the photographed gels. Data are expressed in arbitrary units as the ratio of receptor abundance to GAPDH expression, then normalized to expression values at day 4 in pre-confluent cultures. Data represent means ± S.E.M.

Figure 4  Western blot analysis of ERα, ERβ, and AR protein levels during differentiation in normal rOB cultures. (A) Proteins extracted from normal rOB primary cultures were separated by a 7-5% SDS-polyacrylamide gel. The separated proteins were transferred to an Immobilon-P polyvinylidine-difluoride transfer membrane. PA1-310B (ERβ), PA1-111A (AR), MA1-310 (ERα) and T9026 (α-tubulin) antibodies were used for detection. Bound antibodies were visualized by an enhanced chemiluminescence ECL detection system. (B) Quantitative analysis of the proteins was performed by volume densitometry. Data are presented as protein/α-tubulin ratio normalized to expression values at day 3, followed over the time-course of in vitro differentiation (means ± S.E.M.).

AR, ERα and ERβ levels vary uniquely during osteoblast differentiation

AR mRNA levels showed complex regulation during in vitro differentiation. In contrast to the patterns of ER expression, AR mRNA abundance appeared highest in the later stages of osteoblast development during mineralization (Fig. 3B). AR mRNA levels were robustly
up-regulated over threefold through differentiation to an osteocyte-like cell. AR protein expression by Western analysis showed a similar profile, with AR levels highest in very mature cultures (Fig. 4B). This pattern of AR expression suggests the possibility of androgen regulation of osteoblast proliferation, but with an important role during mineralization/postmineralization.

**Influence of normal osteoblast differentiation on sex steroid receptor expression profiles**

The *in vitro* differentiation of normal primary osteoblastic cultures described here is an established model of bone development that is dependent on appropriate expression of the collagenous extracellular matrix, requiring the addition of differentiation medium containing ascorbic acid (Aronow *et al.* 1990). To examine whether the changes in sex steroid receptor expression we observed were influenced by normal osteoblast differentiation, cultures were grown without differentiation medium in the absence of ascorbic acid or β-glycerophosphate. Under conditions where elaboration of a competent extracellular matrix is lacking, sex steroid receptor mRNA expression levels were relatively constant after cultures reached confluence (Fig. 5), suggesting the co-ordinate regulation of sex steroid receptors with other markers of osteoblast differentiation and the influence of extracellular matrix-mediated events.

**Discussion**

The sex steroids estrogen and androgen have poorly characterized, but distinct, effects on skeletal homeostasis. In this report, we characterized the distinct expression profiles of AR, ERα and ERβ during *in vitro* osteoblast differentiation using normal rat calvarial-derived primary osteoblastic cultures as a differentiating osteoblastic model system. Using semi-quantitative analysis of mRNA steady-state expression as determined by relative RT-PCR with confirmation by Western analysis, we document distinct expression profiles for each receptor type that are dependent on the stage of osteoblast differentiation. AR levels were highest in mature cultures, while ERα expression peaked during matrix deposition and ERβ expression was relatively constant. In contrast, receptor expression profiles did not exhibit this pattern of regulation in primary cultures grown in medium that does not support either the elaboration of a normal collagenous extracellular matrix or full osteoblast differentiation.

Much of the long-term biologic response to a steroid is mediated by binding to its cognate receptor protein to regulate gene expression (Katzenellenbogen *et al.* 1996). The level of response may reflect the level of expression of the receptor itself (Webb *et al.* 1992, Dean & Sanders 1996, Katzenellenbogen *et al.* 1996). Consistent with this observation, we have described enhanced androgen action in osteoblasts with elevated AR expression (Wiren *et al.* 1999). In this report, we have shown distinct expression profiles for the major sex steroid receptors during osteoblast differentiation. Interestingly, studies that employed osteoblast cultures that do not fully differentiate, cultured without the addition of ascorbic acid or β-glycerophosphate, demonstrate that much of the change in sex steroid receptor expression is abrogated if osteoblasts do not undergo normal differentiation. This interdependence of sex steroid receptor expression and osteoblast differentiation suggests a fundamental and biologically relevant role for sex steroids in normal osteoblast differentiation. Thus, differential responses to both androgen and estrogen could occur during discrete stages of osteoblast differentiation.

This is the first report to show modulation of AR expression during osteoblast *in vitro* differentiation; distinct from ER isoform expression, AR levels are highest during later stages of osteoblast differentiation after the cells reach confluence in mineralizing osteocyte cultures. This result was observed in normal primary osteoblast cultures, and is
consistent with the localization of AR described in intact human bone undergoing endochondral ossification identified using immunocytochemical analysis (Abu et al. 1997). In developing bone from young adults, ARs were predominantly expressed in active osteoblasts at sites of bone formation and in osteocytes embedded in the bone matrix. This expression profile may also be consistent with certain effects of androgen during osteoblast proliferation but with distinct actions later in osteocyte-like cells. Further, a recent report, published while this manuscript was under review, demonstrated a significant level of AR expression generally in cells of the osteoblast lineage, including osteoblasts, osteocytes and osteoclasts, by in situ and immunocytochemical analysis in the trabecular compartment of the tibial metaphysis (van der Eerden et al. 2002). Interestingly, data indicated preferential nuclear staining of AR in males at sexual maturity, suggesting activation and translocation of the receptor in bone when steroid levels are elevated.

Although aromatization of testosterone to estrogen metabolites can also play a significant role in mediating the effects of androgens in bone (Vanderschueren et al. 1996, 1997, Carani et al. 1997), the importance of AR, independent of ER, in mediating androgen effects has also been demonstrated in a variety of settings. For example, in addition to data implicating a role for AR in development and maintenance of the adult skeleton already described (see Goulding & Gold 1993, Coxam et al. 1996, Marcus et al. 2000), intact ERα or ERβ null (single or double knockout) male mice do not show reduced trabecular bone mineral density compared with controls (Lindberg et al. 2002). This suggests that neither ERα nor ERβ is required for maintenance of normal trabecular bone mineral density in adult male mice. Consistent with this, Vanderschueren and Bouillon’s group has shown that testosterone prevents orchidectomy-induced bone loss in ERα knockout mice (Vandenput et al. 2001). They have also demonstrated that the ER ligand ICI 182,780, which binds both ERα and ERβ and antagonizes most estrogen-mediated actions, does not impair the bone-sparing effects of testosterone in orchidectomized rats (Vandenput et al. 2002). These accumulated data suggest an important role for androgens both in influencing bone size and in maintaining adult bone mass independent of ER.

The expression profiles for the estrogen receptors, ERα and ERβ, are distinct from AR. ERα mRNA levels were low during proliferation, increased during matrix maturation, then declined in postmineralizing cultures. This result is consistent with a previously published report where ERα mRNA expression was low during proliferation and increased with confluence and mineralization, but declined precipitously in more osteocytic-type cells (Bodine et al. 1998). When examined qualitatively in bone slices, ERα levels are similar in osteoblasts and osteocytes in some (Bord et al. 2001) but not all reports (for example see Kusec et al. 1998). This potential in vivo discrepancy with these results may be partially explained by the fact that mechanical forces are not present in normal culture. Since ERα has been shown to be important in the mechanism(s) by which mechanical force or strain modulates bone (for example see Cheng et al. 2002), strain may influence ERα levels. ERα mRNA expression has also been characterized in a variety of dexamethasone-treated osteoblastic or marrow progenitor cultures, which have generally shown increased expression with differentiation (Arts et al. 1997, Onoe et al. 1997, Oreffo et al. 1999). A role for ERα and ERβ in mediating androgen effects has also been suggested by data in a recent report showing induction of osteoblast differentiation with overexpression of ERα and to a lesser extent ERβ (Lambertini et al. 2002).

Previous reports of ERβ expression show some inconsistencies between various differentiating osteoblast models. In dexamethasone-treated primary rat osteoblastic cultures, ERβ gene expression is consistently and highly expressed over a 28-day culture period (Onoe et al. 1997). In contrast, in dexamethasone-treated immortalized human osteoblastic cultures, ERβ expression increased dramatically over the culture period up to day 21 (Arts et al. 1997). These and other discrepancies seen between reports may be explained by the use of distinct models or species, osteoblast donor age, hormone treatments used to induce differentiation or the levels of steroid hormones or serum in the medium, and/or different quantitation methodologies that could influence differentiation and gene expression. Furthermore, contributions by in vivo circulating factors or biomechanical forces would not be present. Here we report ERβ mRNA and protein expression as relatively constant even in late cultures once proliferation slows, again consistent with expression osteocytes and in late cultures as previously shown (Onoe et al. 1997). Interestingly, ERβ expression characterized in adult bone slices by indirect immunoperoxidase staining indicates ERβ-positive osteoblastic and osteocytic cells are present during both intramembranous and endochondral ossification (Braidman et al. 2001).

It has been shown that ERα and ERβ are functionally distinct, even in bone. Animals with null mutations in genes for either ERα or ERβ show distinct skeletal abnormalities (Korach et al. 1996, Lindberg et al. 2001, Windahl et al. 2001). Furthermore, distinct signal pathways can be activated by ERα and ERβ (Jones et al. 1999, Shapiro et al. 2000, Fournier et al. 2001). In terms of responses in differentiating osteoblastic cells, a recent report documents distinct regulation by ERα or ERβ in stably transfected immortalized fetal osteoblastic cells resulting in differing regulation of select genes (Waters et al. 2001). Interestingly, it has also been suggested that heterodimers may form between ERα and ERβ (Pace et al. 1997). Thus, the ratio between the two isoforms may be important in mediating osteoblastic responses to estrogen, which may have significance given that ERβ
overexpression can inhibit activation of ERα (Ishibashi et al. 2001). Differences in estrogen regulation of gene expression between day 14 and day 30 cultures previously described (Bodine et al. 1998) may thus reflect the higher level of ERβ isoform expression relative to ERα in the mature late cultures that we have observed here.

Clinical data demonstrate that estrogen replacement therapy tends to suppress bone resorption with only modest effects on bone formation, but the cell type responsible for this response has not been established. Androgen replacement data have shown that a predominant effect of this steroid in vivo is to enhance bone formation markers (Falahati-Nini et al. 2000). Again, the cell type(s) responsible for these responses are not established. We have employed a normal primary differentiating culture system in these studies, derived from calvaria. Calvaria develop by way of intramembranous ossification with the direct formation of bone in the absence of a cartilage anlage. In a preliminary report using immunocytochemical analysis in bone sections, it has been shown that ERα and ERβ are expressed in distinct compartments in bone sections: ERβ is more ubiquitously expressed with higher levels in cancellous areas while ERα is more predominant in cortical bone (Bord et al. 2001). A similar relative reduction in ERβ gene expression in cortical bone was also reported (Onoe et al. 1997). However, contrasting results have been reported (Lim et al. 1999), where expression of either isoform was undetectable in cortical bone by RT-PCR analysis. While our data are consistent with both distinct actions and distinct osteoblastic populations as targets for these hormones, we have not examined expression in cell types from other bone areas. Nevertheless, we have observed distinct and stage-specific expression profiles for the sex steroid receptors, so that, at least at the level of regulation of expression, differences between the receptors exists. Thus, complex regulation in osteoblasts at distinct stages of differentiation may partially underlie the improved outcome of patients receiving both estrogen and androgen hormones (Watts et al. 1995, Raisz et al. 1996, Barrett-Connor 1998). Increased understanding of sex steroid receptor regulation may also aid in the understanding of the pathogenesis of age-related bone loss in both males and females.

In summary, sex steroid receptor expression pattern is distinctly modulated during and is influenced by normal osteoblast differentiation. Thus estrogen and androgen actions may be target-cell specific, and fundamentally inter-related with osteoblast differentiation. These results suggest an underlying complexity and subtlety for estrogen and androgen regulation of osteoblast expression in bone, and imply differences in the way each steroid may modulate function in different populations of osteoblasts through distinct signal transduction pathways. Thus, one way sex steroids may distinctly influence skeletal health is by differential modulation of osteoblast function during osteoblast proliferation and differentiation. Further studies will be required to establish how, where and which cell type in bone is specifically influenced by estrogen and androgen exposure.

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