Androgen inhibition of MAP kinase pathway and Elk-1 activation in proliferating osteoblasts

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Abstract

Non-aromatizable androgens have significant beneficial effects on skeletal homeostasis independently of conversion to estradiol, but the effects of androgens on bone cell metabolism and cell proliferation are still poorly understood. Using an osteoblastic model with enhanced androgen responsiveness, MC3T3-E1 cells stably transfected with androgen receptor (AR) under the control of the type I collagen promoter (colAR-MC3T3), the effects of androgens on mitogenic signaling were characterized. Cultures were treated with the non-aromatizable androgen 5α-dihydrotestosterone (DHT) and the effects on osteoblast viability were determined as measured by an MTT assay. A complex response was observed in that continuous short-term DHT treatment enhanced osteoblast viability, but with longer-term DHT treatment inhibition was observed. The inhibition by DHT was prevented by the specific AR antagonist hydroxyflutamide, and was also observed in primary cultures of normal rat calvarial osteoblasts. In order to identify potential mediators of this effect, mitogenic pathway-specific cDNA microarrays were interrogated. Reduced hybridization of several genes important in MAP kinase-mediated signaling was observed, with the most dramatic effect on Elk-1 expression. Analysis of phosphorylation cascades demonstrated that DHT treatment inhibited phosphoERK1/2 levels, MAP kinase activation of Elk-1, Elk-1 protein and phosphoElk-1 levels, and downstream AP-1/luciferase reporter activity. Together, these data provide the first evidence that androgen inhibition of the MAP kinase signaling pathway is a potential mediator of osteoblast growth, and are consistent with the hypothesis that the MAP cascade may be a specific downstream target of DHT.


Introduction

The molecular pathways controlling bone formation either in normal individuals or in pathophysiological disease states are not well understood. This question is of critical importance since osteoporosis, a low bone mass disease associated with an increased risk of fracture, is the most prevalent degenerative disease in developed countries (Cooper & Melton 1996). It is well established that sex steroids are significant mediators of skeletal homeostasis and play an important role in maintaining bone mass. It is also well established that androgens have beneficial effects on skeletal homeostasis that do not involve their metabolism to estrogenic compounds. Nevertheless, the mechanisms that may underlie androgen actions in the skeleton remain, for the most part, unresolved (Wiren & Orwoll 2002).

There has been speculation that the positive effects of androgens on the skeleton may be mediated indirectly through increased muscle mass thought to enhance biomechanical responses, resulting in a beneficial effect on bone density. However, the effects of increased muscle mass on bone density may be complex and not directly correlated, as suggested by the phenotype of the myostatin null mouse. This model, the so-called ‘Mighty mouse’ with dramatic hypertrophy of muscle mass, nevertheless demonstrates no increase
in midshaft cortical bone (Hamrick et al. 2000). It has also been proposed that steroids can act non-sex-steroid specifically through non-genomic actions (Kousteni et al. 2001, 2002), although recent data suggest that classic genomic signaling may be the more significant regulator in bone (van der Eerden et al. 2002). Since osteoblastic (bone-forming) cells have been demonstrated to express the classic androgen receptor (AR) both in vivo and in vitro, it is likely that AR transactivation by androgen in the osteoblast to modulate gene expression has significant skeletal consequences.

While the effects of AR transactivation in the osteoblast remain unclear, one particularly controversial area with respect to androgen action is the effect on osteoblast proliferation, as both stimulation and inhibition have been reported. Benz et al. (1991) showed that prolonged androgen exposure in the presence of serum inhibited proliferation in a transformed human osteoblastic line (TE-85). Testosterone and 5α-dihydrotestosterone (DHT) were nearly equally effective regulators. Kasperk et al. (1997) reported that prolonged DHT treatment inhibited normal human osteoblastic cell proliferation in cultures pretreated with DHT. In contrast, the same group (Kasperk et al. 1989, 1990) demonstrated in osteoblast-like cells in culture (primary murine and passaged human) that a variety of androgens in serum-free medium increased proliferation. Finally, Hofbauer et al. (1998) examined the effect of DHT exposure on proliferation in hFOB/AR-6, an immortalized human fetal pre-osteoblastic (hFOB) cell line stably transfected with an AR cDNA under the control of the 3·6 kb fragment of the rat α1 (I)-collagen promoter. These cells represent an osteoblastic model with enhanced androgen responsiveness that employs a well-characterized clonal cell line that undergoes normal osteoblast differentiation (Franceschi et al. 1994).

The present study reports the first evidence for a distinct biphasic role for androgenic steroids in modulating viability of osteoblastic cells. In addition, we have found that androgens reduce the expression and/or inhibit the activity of several components of the MAP kinase pathway. Together these data provide evidence that androgen inhibition of the MAP kinase signaling pathway may be an important mediator of committed osteoblast growth and a specific downstream target of DHT in its growth-control pathway. Since the AP-1 transcription factor is also a significant regulator of osteoblast development, these findings may provide valuable insight into the molecular mechanisms of androgen action in bone.

Materials and methods

Materials

All the media, buffers, supplements and reagents for cell culture were obtained from GIBCO BRL-Life Technologies (Grand Island, NY, USA) and Sigma Chemical Co. (St Louis, MO, USA). Steroid hormones and other reagents were obtained from Sigma. [α-32P]UTP (800 Ci/mm) was purchased from DuPont NEN (Boston, MA, USA). The non-steroidal AR antagonist
hydroxyflutamide (O HF) was kindly provided by Schering-Plough (Klenisworth, NJ, USA).

**Construction of the AR expression plasmid**

The pBR327-based plasmid Col3·6-βgal-ClaPa containing the rat Col1a1 promoter sequence from −3518 to +115 and with the polyadenylation signal from bovine growth hormone served as the starting vector (Woitge et al. 2001). First, the BamHI site at −3145 in Col3·6-βgal-ClaPa was removed by introducing a point mutation to create the modified Col3·6Δ-βgal-ClaPa, using *in vitro* mutagenesis. PCR amplification was performed in 50 µl with 50 ng Col3·6-βgal-ClaPa plasmid, 0·4 µM each primer, 200 µM dNTPs and 2·5 U Pfu Turbo DNA high-fidelity polymerase. The primers used contain a point mutation (bold/underlined letters) that silences the BamHI site in the promoter sequence (forward: 5′-ACCCACACACCTAGGAGCCACCCACAGAATTTTG-3′ and reverse: 5′-GGAAATAGATCGGATCCCTAGGTGTGGGTG-3′). The reaction was 94 °C for 45 s, 55 °C for 45 s, and 68 °C for 20 min for 20 cycles. After the reaction, 10 U DpnI were added for 1 h at 37 °C. Bacteria were electroporated with the remaining DNA. Positive clones were identified by the BamHI restriction pattern.

To add BamHI sites to the rat AR cDNA ends, PCR primers were designed with BamHI ends as follows: forward GGATCCGATGAGGTTGAGTGTAGGTCT, reverse GGATCCGATGAGGTTGAGTGTAGGTCTCCTAGGTGTGTGGGTG (italics indicate BamHI recognition sequences). PCR conditions were 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 3 min for 30 cycles with 2·6 U Expand High Fidelity PCR System enzyme mix (Roche, Indianapolis, IN, USA) in a 50 µl reaction. The PCR product was T/A cloned in pCR 2·1-TOPO vector (Invitrogen, Carlsbad, CA, USA). Finally, the BamHI-rAR cDNA fragment was cloned into the BamHI site in the modified Col3·6Δ-βgal-ClaPa after removal of the βgal cDNA sequences, to give the expression constructs designated colAR. This construct has also been employed to generate transgenic mice with AR overexpression in the osteoblast lineage (MA Gentile, K Wiren, A Toombs, X-W Zhang, V Kasparcova, S Harada & K Jepsen, unpublished observations). The sequence and orientation of the AR insert was verified by sequencing.

**Cell culture and stable transfection**

The mouse clonal immortalized calvarial osteoblastic cell line MC3T3-E1 (passage 12–20) was obtained from Dr Peter Rotwein (Oregon Health and Science University). Monolayer cultures were maintained in minimal essential medium (MEM) with 2·38 g/l Hepes and 2·2 g/l NaHCO3 buffer, supplemented with 5% calf serum (CS). To select stable transfectants, clonal osteoblastic MC3T3-E1 cells were plated in 35 mm wells at 20 000 cells/cm² for 24 h before transfection in 5% CS media. Cells were stably cotransfected with 2 µg colAR or Col3·6Δ-βgal-ClaPa and 0·1 µg pRSV-neo plasmid DNA using GenePorter (Gene Therapy Systems, San Diego, CA, USA) in serum-free medium. Cells were grown for 48 h and were placed under selection with 500 µg/ml active G418 genitin sulfate. Transfection efficiency was approximately 80%, assessed in parallel cultures transfected with the control Col3·6Δ-βgal-ClaPa construct using a β-galactosidase (β-gal) staining kit (Invitrogen). Because of the high transfection efficiency, cultures were grown as stable pools (up to 100 independent colonies) for analysis, and were maintained throughout the study in the presence of G418 genitin sulfate at 500 µg/ml. For steroid treatments, cells were grown in 5% charcoal-dextran (cd)-stripped CS to remove endogenous steroids. Steroids were dissolved as stocks in ethanol and used at concentrations from 10⁻¹² M to 10⁻⁶ M. The final ethanol concentration in the media was no higher than 0·1%. O HF, a specific AR antagonist, was used at 10⁻⁵ M, a 1000-fold excess over the DHT concentration.

**Primary cultures and [³H]thymidine incorporation**

Normal rat osteoblast (rOB) cells were prepared by collagenase digestion from neonatal rat calvaria as previously described (Birnbaum & Wiren 1994, Birnbaum et al. 1995). Briefly, 21-day-old fetal rat calvaria were removed and digested with collagenase (Boehringer Mannheim, Indianapolis, IN, USA) in MEM for four 20 min intervals. The initial digestion products were discarded and the remaining three were pooled. Cells were plated at 8000 cells/cm² in MEM supplemented with 10% CS. Normal rOB cells were used only as primary cultures. All animal procedures and animal care
were reviewed and approved by the Portland, OR 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. [3H]thymidine incorporation was determined in cells plated in six-well tissue culture plates (35 mm) at a density of 4000 cells/cm² in MEM with 5% CS as previously described (Bliziotes et al. 2000). All analyses are done with triplicate samples, with experiments performed twice.

**Gel electrophoresis and Western blot analysis**

AR abundance was determined by immunoblotting as previously described (Wiren et al. 2002) with polyclonal rabbit AR antibodies (PA1-111A) purchased from Affinity Bioreagents, Inc. (Golden, CO, USA) and used at 4 µg/ml. The α-tubulin antibody (T9026) was a mouse monoclonal antibody purchased from Sigma and was used at 1:1000. PhosphoERK1/2 was analyzed by immunoblotting using a rabbit polyclonal antibody recognizing threonine and tyrosine phosphorylated pERK1/2 at 1:1000 (New England BioLabs, Inc., Beverly, MA, USA), or a monoclonal antibody recognizing total panERK1/2 at 1:5000 (panERK; Transduction Laboratories, Lexington, KY, USA). Similarly, a rabbit polyclonal antibody recognizing Ser-383 phosphorylated pElk-1, or a rabbit polyclonal antibody recognizing total panElk-1 used at 1:500 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Whole cell lysates were prepared with dephosphorylation inhibitors 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 phenylmethysulfonyl fluoride, 1 µg/ml leupeptin and 1 mM Na3VO4) containing 3% SDS. Equal amounts of cell extract (1·66 µg DNA/lane) were electrophoresed on a 7·5/10% SDS-polyacrylamide gel, and the separated proteins were transferred to an Immobilon-P polyvinylidene-difluoride transfer membrane (Millipore, Bedford, MA, USA). Conditions for washing were as previously described (Wiren et al. 2002). The membrane was incubated with either horseradish peroxidase-linked goat anti-rabbit IgG antibody (Bio-Rad Laboratories, Hercules, CA, USA) or goat anti-mouse IgG antibody (Bio-Rad) at 1:2000 for 1 h. Bound antibodies were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) on Kodak X-AR5 autoradiographic film.

Quantitative analysis of the proteins was performed by volume densitometry using Gel Expert Software (Nucleotech, San Mateo, CA, USA) after scanning of the film (Hewlett Packard ScanJet 6100 C/T) in the linear range. Data are presented as the protein to α-tubulin ratio to correct for variations in protein loading and then normalized to control values for comparison between treatments. The data shown represent the means ± S.E.M.

**AR quantification**

For the steroid-binding study, both colAR-MC3T3 and col βgal-MC3T3 stable cultures were grown for 5 days. Cultures were maintained in MEM with 5% cd-CS then switched to serum-free medium for 24 h before harvesting cells at day 5. Whole cell homogenates were freshly prepared from osteoblastic cells after washing with PBS. Cells were detached with trypsin/EDTA then an equal volume of trypsin inhibitor at 1 mg/ml in PBS (Sigma) was added. Cells were then centrifuged at 1500 g for 10 min at 4 °C. The supernatant was aspirated and the cell pellet stored at −80 °C.

Frozen cell pellets consisting of approximately 5·0 × 10⁸ cells were thawed on ice in buffer (50 mM Tris–HCl, 1·5 mM EDTA, 0·5 mM dithiothreitol, 10% glycerol, Sigma protease inhibitor cocktail) and homogenized with a polytron (Brinkmann Instruments, Inc., Westbury, NY, USA). Following centrifugation at 40 000 g for 30 min, cytosols were removed from nuclear pellets. The residual pellets were then analyzed for DNA content by a dye-binding assay with bisbenzimide H 33258 (Hoechst Reagents, Riedel-DeHagen AG, Seelze-Hanover, Germany). The DNA content was quantified by measuring fluorescence at 356 nm excitation and 458 nm emission in a SF-330 spectrofluorometer (Varian, Palo Alto, CA, USA). The DNA content was then determined from a standard curve, using purified calf thymus DNA as a standard (Labarca & Paigen 1980).

ARs were quantified by titration analysis using [3H]methyltrienolone ([3H]R1881) (New England Nuclear, specific activity 70–87 Ci/mM) as previously described (Wiren et al. 1999).
Non-specific $[^3H]R1881$ binding was subtracted from total $[^3H]R1881$ binding to determine the specific $[^3H]R1881$ bound. Specific $[^3H]R1881$-binding capacity was estimated by one-site binding with non-linear regression and Scatchard plot analysis using Prism software (GraphPad Software, Inc., San Diego, CA, USA). The high-affinity ($K_d=1\times10^{-5} - 5\times10^{-10}$ M), specific binding of $[^3H]R1881$ was expressed in fmol/mg DNA and sites/cell. The sensitivity for the detection of specific androgen binding using these methods was 5 fmol/ml. The AR-binding assays were controlled by including LNCaP prostatic cells, containing known levels of AR.

**MTT assay for cell viability**

Cells were plated in 96-well dishes at 4000 cells/cm$^2$ in MEM with 5% CS. Cells were generally plated in control or hormone-containing media, and grown for 5 days before isolation. DHT treatment at $10^{-8}$ M was at the end of growth, either continuous (5 days) or for the last 48 or 72 h. The non-steroidal aromatic anti-androgen OHF has been shown to antagonize androgen regulation of gene expression, but has a relatively low affinity for the AR (Warriar et al. 1993). Thus, a several hundred-fold to 1000-fold molar excess of OHF over DHT is required for effective blockage of the DHT effect (Kemppainen et al. 1992). OHF was added 30 min before the addition of DHT, and was used at 1000-fold excess. After treatment, MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Chemicon International, Inc., Temecula, CA, USA) was added to each well for 5 h. The cells were removed from the incubator and acidic isopropanol was added to each well to solubilize the precipitated formazin substrate. After incubation and agitation for 1 h, the plates were analyzed at 570 nm using a BioRad model 570 microplate reader (Bio-Rad). Cells were plated with 12 wells/condition. Experiments were repeated at least three times. Results are presented as the means ± S.E.M.

**RNA isolation and cDNA microarray analysis of mRNA expression**

RNA was isolated by the single-step acid guanidinium isothiocyanate–phenol–chloroform extraction method using RNA Stat-60 (Tel-Test, Inc., Friendswood, TX, USA). Contaminating DNA was removed by Zymo-spin column purification following the manufacturer’s recommendations (Zymo Research, Orange, CA, USA). Commercially available application-specific gene expression cDNA arrays, designed to analyze genes important in mitogenesis, were purchased from GEArray (SuperArray, Bethesda, MD, USA). Each array consists of 56 spots containing specific cDNA fragments arrayed in duplicate (23 genes relating to mitogenic factor-mediated signal transduction), in addition to control sequences including PUC18 as a negative control and housekeeping genes glyceraldehyde 3-phosphate dehydrogenase and β-actin. Microarrays were employed following the manufacturer’s instructions. Briefly, cDNA was prepared from DNase-treated total RNA by reverse transcription with MMLV reverse transcriptase, radiolabeled using [$\alpha$-$^32$P]dCTP (3000 Ci/mM) with gene-specific primers. Hybridization was performed under precisely specified conditions to a positively charged nylon membrane containing the arrayed DNA. After washing, membranes were exposed to multi-sensitive storage phosphor screens and visualized using a Cyclone scanner (Packard Instrument Company, Inc., Meriden, CT, USA). Image quantification used OptiQuant software (Packard). All analyses were performed in the linear response range of the screen. Data analysis employed GEArray Analyzer v1.3 software, with background subtraction using the minimum value and normalization to the median value of the hybridization signals. Data are shown as the mean of two values.

**Assays of transcriptional activity**

For the functional analysis of MAP kinase activity, we used a PathDetect Elk Trans-Reporting system (Stratagene, La Jolla, CA, USA) with a luciferase reporter. The system uses both a fusion construct containing the DNA-binding domain of the yeast transcription factor Gal4 linked to the carboxy-terminal transcriptional activation domain of Elk1 (Gal4-Elk1, pFA2-Elk1) cotransfected with a reporter gene containing five Gal4-binding sites upstream of a minimal promoter linked to luciferase (5 × Gal4-E1B-LUC, pFR-LUC). We also determined AP-1 transcriptional activation using the AP-1-driven luciferase reporter gene.
Statistical analysis

All data were analyzed using Prism software (GraphPad Software). Data for treatments were analyzed using single-factor ANOVA followed by Newman–Keuls post-hoc test for multiple comparisons. Treatment/time or treatment/cell line effects were analyzed by two-factor ANOVA, followed by a Newman–Keuls post-hoc test for multiple comparisons with the control. The individual contrast between treatment groups was made with an unpaired two-tailed Student’s t-test, with confirmation of DHT inhibition by a one-tailed Student’s t-test. Differences of $P < 0.05$ are considered statistically significant. Results are presented as the means ± S.E.M.

Results

Model of enhanced androgen responsiveness

AR levels are low as osteoblasts proliferate then increase during differentiation to reach maximal levels in mature osteocytic-like cells, as we have shown previously (Wiren et al. 2002). In order to enhance androgen responsiveness, particularly in early proliferating osteoblasts, cultures of the immortalized mouse calvarial osteoblastic cell line MC3T3-E1 were stably transfected with an AR expression construct under the control of 3·6 kb of the rat α1(I)-collagen promoter. The MC3T3-E1 osteoblastic cell line was chosen because it is extremely well characterized and exhibits normal osteoblast differentiation expression profiles (Franceschi et al. 1994). The col3·6 promoter was chosen since expression in osteoblastic cells is already well characterized, with both high levels of expression and significant expression throughout growth in culture (Kalajzic et al. 2002). Stably transfected colAR-MC3T3 cultures were isolated by selection with G418, and used as stable pools. Details of the construct are shown in Fig. 1, and described in Materials and methods.

To demonstrate AR expression in colAR-MC3T3 cultures, we performed Western analysis. As shown in Fig. 2A, Western analysis reveals an increase in receptor abundance from day 3 to day 5 as the colAR-MC3T3 cultures begin to reach confluence. Western analysis also demonstrated nearly twice the level of AR in colAR cells at day 8 vs control colβgal-MC3T3 cultures that were stably transfected with an irrelevant gene (β-gal driven by the same collagen promoter fragment; data not shown). Protein expression represents both endogenous AR and that from expression of the colAR construct. Real-time RT-PCR analysis of construct expression vs endogenous AR expression demonstrated that colAR expression was nearly double that of endogenous AR (data not shown). To further characterize AR protein levels, binding studies and Scatchard analyses were performed. AR levels were determined at day 5 by $[^{3}H]R1881$ binding in both colAR-MC3T3 and control colβgal-MC3T3 cultures. As shown in Table 1, analysis of AR binding in control colβgal-MC3T3 cultures (522 ± 16 fmol/mg DNA or 1885 ± 58 sites/cell) showed the level of AR slightly higher than other osteoblast-like cells such as U2-OS or SaOS-2 cells (Wiren et al. 1999). As expected from
the gene expression analysis shown in Fig. 2B, the levels of AR in colAR-MC3T3 cultures were significantly elevated nearly 3-fold (1569 ± 62 fmol/mg DNA or 5663 ± 221 sites/cell) compared with control cultures ($P < 0.01$). This level of receptor expression in the colAR-MC3T3 cells is in the high physiological range, below amounts seen in human prostatic carcinoma LNCaP cells (3971 ± 598 fmol/mg or 14 335 ± 2157 sites/cell), but comparable with kidney or normal ventral prostate (1351 ± 85 fmol/mg or 4877 ± 308 sites/cell).

We next examined transactivation of the AR in colAR-MC3T3 osteoblasts to confirm androgen responsiveness, determined with a reporter assay in transient transfections (Fig. 2B). We employed an androgen-responsive luciferase gene coupled to a promoter containing three tandem repeats of an ARE sequence, the ARE-LUC plasmid. ColAR-MC3T3 cultures were transiently transfected with ARE-LUC while proliferating, treated with DHT (1, 10 or 100 nM) for 54 h and luciferase activity was determined (Fig. 2B). Control col βgal-MC3T3 cultures were also analyzed. As can be seen, DHT stimulated AR transactivation nearly 3-fold at 10 nM DHT ($P < 0.05$) and 100 nM DHT ($P < 0.05$). Thus, the colAR-MC3T3 cultures represent an osteoblastic model of enhanced androgen responsiveness.

**Androgen inhibition during osteoblast proliferation**

As described above, the effects of androgen on osteoblast proliferation remain controversial. To examine the effect of androgens on osteoblast proliferation *in vitro*, we used colAR-MC3T3 cells grown in phenol red-free MEM with 5% cd-stripped serum, treated with the non-aromatizable androgen DHT. In these studies, the end-point of osteoblast growth/viability was assessed by an MTT colorimetric assay after treatment with $10^{-8}$ M DHT for either 2, 3 or 5 days. Interestingly, this time-course analysis revealed a complex pattern with stimulation of MTT activity with only 48 h of DHT treatment, but as the exposure time was extended to 3 or 5 days inhibition was observed (Fig. 3A). Analysis by two-way ANOVA revealed a significant interaction ($F=11.6, P<0.0001$), with individual *t*-tests showing significant differences after DHT treatment at all time points (enhanced at 2 days, $P<0.05$; and inhibited at 3 and 5 days, $P<0.01$). In order to determine whether this was an AR-mediated response, we used the specific AR antagonist OHF with and without DHT treatment. Significant inhibition with DHT treatment was observed after 3 days of treatment ($P<0.05$). However, coincubation of DHT with OHF abrogated the DHT inhibition (OHF+DHT vs DHT, $P<0.001$).
Although OHF alone slightly reduced MTT activity due to modest agonist effects of high concentration of OHF in the absence of androgen (Warriar et al. 1993), this inhibition did not reach significance compared with control. OHF alone was significantly different from DHT+OHF ($P<0.01$). Thus, the inhibition by DHT treatment was dependent on AR. Finally, using primary cultures of calvarial-derived rOB cells, we confirmed whether the inhibition of osteoblast growth with DHT treatment was observed in normal cells. The effect of DHT treatment on DNA synthesis was determined using $[^3H]$thymidine incorporation assays. As shown in Fig. 3C, DHT treatment (52 h; $10^{-10}$ M) significantly reduced DNA synthesis in cultures of normal rOB calvarial osteoblasts ($P<0.05$). Thus, DHT treatment can also reduce proliferation in normal osteoblastic cells.

Application-specific cDNA microarray analysis to identify mitogenic targets

While growth inhibition by DHT has been previously reported, the mechanism is not well characterized. To better understand signaling cascades important in DHT control of osteoblast growth, we interrogated mitogenic pathway-specific cDNA microarrays. We employed GEArray systems arrays, which are designed as application-specific arrays to characterize expression of genes involved in specific biological pathways. For these studies, we utilized the mitogenic pathway membranes to identify targets in the mitogenic cascade that may be influenced by androgen treatment. A representative image of the array membrane, using mRNA from control rOB cultures, shows the reliability of hybridization to the duplicate cDNA spots and the lack of background signal (Fig. 4A).

Following DHT treatment we observed reduced expression of several genes important in MAP kinase-mediated signaling, implicating this pathway as a target of androgen regulation. This was not surprising since MAP kinase cascades classically transmit and amplify signals involved in cell proliferation. In both colAR-MC3T3 and rOB osteoblast models, $10^{-8}$ M DHT treatment for 48 h in the presence of cd-stripped serum appeared to inhibit both ERK-1 and ERK-2, with the more robust effect on ERK-1 (Fig. 4C and D). However, the most dramatic effect of DHT treatment on hybridization intensity was observed with Elk-1 expression, with colAR-MC3T3 or rOB mRNA hybridization, demonstrating either a $\sim70\%$ or $\sim50\%$ reduction respectively (Fig. 4B). DHT

**Figure 2** Characterization of AR expression in stably transfected colAR-MC3T3 cultures. (A) AR expression by Western analysis. AR protein abundance in proliferating cultures of colAR-MC3T3 cells was determined in whole cell lysates by immunoblotting. AR abundance here represents both endogenous mouse AR, and rat AR expressed from the colAR construct. (B) ARE-LUC responsiveness in colAR-MC3T3 compared with control col $\beta$gal-MC3T3 cultures. Stably transfected colAR-MC3T3 and control col $\beta$gal-MC3T3 cultures were transiently transfected with an ARE luciferase promoter construct (ARE-LUC). AR transcriptional activation was assessed after $10^{-8}$ M DHT treatment for 54 h. Total cell lysates were analyzed for luciferase activity after normalization to $\beta$-gal as described in Materials and methods. Data are mean±S.E.M. of triplicate samples. Similar results were obtained in two experiments. *$P<0.05$; **$P<0.01$ (vs appropriate vehicle control).
inhibition of these genes was also enhanced in colAR-MC3T3 compared with parental MC3T3-E1 hybridizations (data not shown).

DHT inhibition of MAP kinase signaling

Mitogenic microarray analysis thus identified ERK1/2 and Elk-1 as potential targets for DHT regulation. In order to confirm results from the hybridization screen, Western analysis was performed. To determine the levels of ERK1/2 protein (Fig. 5A), colAR-MC3T3 cultures were treated with $10^{-8}$ M DHT for 48 h before isolation and analysis by immunoblotting with antibodies that recognize either total protein or only the dually phosphorylated activated phosphoERK1/2 (pERK1/2). Quantitative analysis of the Western results (Fig. 5B) showed modest 11% inhibition of total panERK expression in colAR-MC3T3 cultures that did not reach significance. However, the relative amount of active ERK1/2, assessed with phospho-specific antibody to phosphoERK1/2, was significantly reduced by 58% ($P<0.01$). These results suggest an additional inhibition of the kinase cascade, upstream of ERK1/2. As a downstream effector in the MAP kinase cascade, Elk-1 activity is directly controlled by phosphorylation by ERK1/2, thus regulating the ability of Elk-1 to activate or repress specific target genes (Yordy & Muise-Helmericks 2000). To further index the activity of ERK1/2, Elk-1 activation was measured with a Gal4/luciferase reporter construct in colAR-MC3T3 cultures, to assess Elk-1 transcriptional activity after ERK1/2 phosphorylation (Fig. 6). After treatment with $10^{-8}$ M DHT for 24 h, GAL4/luciferase reporter activity was significantly reduced nearly 41% ($P<0.05$). Thus, the functional consequence of the reduction in phosphorylated ERK1/2 assessed by Western analysis was confirmed with luciferase reporter analysis measuring the ability of ERK1/2 to stimulate Elk-1 transcription.

DHT inhibition of Elk-1

We next examined the effect of DHT treatment on expression of Elk-1, since the inhibition of Elk-1 was the most dramatic regulation observed with the mitogenic array analysis. Elk-1 (also known as p62 ternary complex factor, LEF/TCF) is a downstream target of ERK1/2 kinase activity as just described. As with ERK1/2, Western analysis was performed (Fig. 7A) to confirm the inhibition of Elk-1 expression observed with the hybridization screen. Cultures of colAR-MC3T3 were grown for 5 days and treated with $10^{-8}$ M DHT for 48 h before isolation. Quantification of the Western analysis is shown in Fig. 7B. Consistent with the down-regulation observed in the array analysis, Elk-1 protein levels detected with panElk-1 antibody, normalized to $\beta$-tubulin loading controls, were reduced nearly 40% ($P<0.05$). The relative amount of active phosphoElk-1 (pElk-1), again assessed with pElk-1 antibody normalized to $\alpha$-tubulin loading controls, was significantly reduced by 57% ($P<0.01$). Thus, the inhibition of

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Table 1 Analysis of AR levels in colAR-MC3T3 cultures. Specific [3H]R1881 binding capacity was estimated by non-linear regression, one-site binding and Scatchard plot analysis as described in Materials and methods. AR abundance in the colAR-MC3T3 cultures is ∼3-fold higher than control col $\beta$gal-MC3T3 cultures, and higher than U2-OS osteosarcoma cells (another androgen-responsive osteoblastic cell line). AR concentrations were similar to those observed in normal (non-castrate) rat prostate, but below those in human prostatic carcinomas.
pElk-1 levels was even more robust than the inhibition of total Elk-1 protein synthesis, consistent with the upstream inhibition of ERK1/2 signaling. These results suggest that DHT treatment inhibits several steps in the MAP kinase pathway that may play a role in the reduction of osteoblast growth observed.

**DHT inhibition of AP-1 activity**

Since Elk-1 mediates MAP kinase/growth factor stimulation of the c-fos promoter and is a c-fos protooncogene regulator (Davis 1995), we determined whether DHT treatment also influenced c-fos activity. We chose to index this particular

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**Figure 3** Complex effect of androgen on DNA accumulation in colAR-MC3T3 and normal rOB calvarial osteoblast cultures. (A) Kinetics of DHT response in proliferating colAR-MC3T3 cultures measured with colorimetric MTT. Cultures of colAR-MC3T3 continuously with 10^{-8} M DHT for 2 days led to increased MTT accumulation, but longer treatment for 3 or 5 days resulted in inhibition. Data are means±S.E.M. of six to eight dishes with six wells/dish. *P<0·05; **P<0·01 (vs control). (B) Specificity of AR mediation of DHT inhibition analyzed with the AR antagonist OHF. Cultures of colAR-MC3T3 were treated continuously with DHT at 10^{-8} M or in combination with OHF at 1000-fold excess (10^{-5} M) added 30 min before DHT. DHT inhibition was abrogated in the presence of OHF. Data are means±S.E.M. of eight dishes with six wells/dish. *P<0·05 (vs control); aP<0·001 (vs DHT); bP<0·01 (vs OHF alone). (C) Effect of DHT on DNA synthesis in normal rOB cultures. Primary cultures were grown as described in Materials and methods for [3H]thymidine assays. Cultures were treated with 10^{-10} M DHT for the last 52 h of treatment in serum-free medium. DHT treatment inhibited proliferation in normal osteoblastic cells. Data are means±S.E.M. of three samples per condition, repeated three times. Similar results were obtained with DNA accumulation measured with Hoechst staining. *P<0·05.
end-point in MAP kinase/growth factor signaling by measuring activation of a promoter containing the AP-1 consensus sequence using reporter assay. AP-1 is a homodimer or heterodimer composed of c-Jun/c-Jun or c-Jun/c-Fos subunits, which acts as a transcription factor at the AP-1 site to regulate gene expression. The luciferase reporter assay used to evaluate AP-1 activity employs the AP-1/LUC construct, containing four tandem repeats of the AP-1 consensus sequence with the β-globin promoter linked to the luciferase gene. DHT regulation of AP-1 activity was determined by luciferase assay using AP-1/LUC as shown in Fig. 8. ColAR-MC3T3 cultures were transiently transfected as described in Fig. 6. Proliferating cultures were treated with either vehicle control or $10^{-8}$ M DHT for 6, 24 or 54 h before the extracts were harvested for luciferase determinations.
Interestingly, complex kinetics of regulation after DHT treatment were again observed with AP-1 (functional Fos/Jun) activity. Analysis by two-way ANOVA showed a significant interaction between time and treatment ($F=13.83$, $P<0.0001$). Short-term DHT treatment for 6 h stimulated AP-1 transcriptional activation ($P$, $0.05$), analogous to the stimulation of MTT activity with shorter treatment times (shown in Fig. 3A). However, with longer incubation times out to 54 h, AP-1 activity in osteoblastic cells was significantly reduced ($P$, $0.001$), again similar to the complex kinetics observed with DHT regulation of MTT activity.

**Discussion**

Androgens have significant beneficial effects on skeletal homeostasis independently of conversion to estradiol, but the mechanisms are not well understood (Wiren & Orwoll 2002). The aim of this study was to elucidate the effects of the non-aromatizable androgen DHT on committed osteoblast growth by characterizing the mitogenic signaling cascade. Androgen-responsive colAR-MC3T3 cells stably transfected with full-length rat AR under the control of the 3·6 kb fragment of the rat $\alpha_1$ (I)-collagen promoter were employed. Treatment with DHT for several days in early non-confluent cultures inhibited osteoblastic viability. Inhibition of DNA synthesis was also observed in primary cultures of normal rat calvarial osteoblastic cells. Screening with application-specific cDNA arrays, targeted for genes important in mitogenesis, identified several genes important in the MAP kinase cascade as down-regulated after DHT treatment in both colAR-MC3T3 and rOB cultures. The most robust effect was inhibition of gene expression of Elk-1, confirmed by immunoblotting analysis of Elk-1 protein. In addition, inhibition of Elk-1 phosphorylation/activation levels was greater than the reduction of protein abundance alone, consistent with the reduced activation of ERK1/2 determined with both ERK1/2 phospho-specific Western analysis and MAP kinase/Elk-1 activation reporter analysis. Activation of AP-1, a downstream target of Elk-1, was also inhibited.

The effect of androgen exposure on osteoblast proliferation has been controversial; both stimulation and inhibition of osteoblast proliferation have been reported (Kasperk et al. 1989, 1990, 1991).
hormone treatment, in populations of proliferating androgen-responsive osteoblasts. Thus, some of the disparate reports in the literature regarding androgen effects on osteoblast proliferation may be the result of differences in DHT exposure times.

Dichotomous stimulatory or inhibitory responses to androgen exposure, depending on the time of treatment, were observed for both MTT activity and AP-1 transcriptional activity. The stimulatory response seen with the shorter treatment times may reflect enhancement of MAP kinase activation by androgens reported in osteoblastic cells and other transfected cell models (Kousteni et al. 2001) since treatment time in those studies was limited. Non-classic actions of both androgen and estrogen may be important in the control of cell growth or MAP kinase activation in some (Peterziel et al. 1999, Falcone et al. 2002, Marino et al. 2002) but not all settings (Singleton et al. 2003), and perhaps may not be the major contributor in bone (van der Eerden et al. 2002). The predominant effect of DHT treatment we observed was dramatic down-regulation of MAP kinase signaling, particularly the downstream target Elk-1. We have also shown androgen inhibition of osteoblast growth/viability to be dependent on functional AR, since the anti-androgen OHF completely abrogates androgen inhibition. Thus, long-term DHT exposure inhibits DNA synthesis and MTT activity and inhibits potential effectors, including ERK1/2 phosphorylation, Elk-1 protein levels and phosphorylation, and AP-1 transcriptional activity in osteoblastic cells.

Since little is known about signaling cascades important in DHT control of osteoblast growth, we interrogated mitogenic pathway-specific cDNA microarrays to identify candidate genes. Results showed reduced hybridization of genes important in MAP kinase-mediated signaling, implicating this pathway and predominantly Elk-1 as a target of androgen regulation. In fact, both Elk-1 protein abundance and phosphorylation showed dramatic down-regulation with DHT treatment. Thus, DHT reduces the pool of Elk-1 and in addition attenuates the level of phosphorylation of the Elk-1 that remains. This latter effect may arise in part from the reduced phosphorylation/activity of upstream ERK1/2 we observed. Although the reduction in phosphoERK1/2 could be responsible for the reduction in Elk-1 activation, there are other possibilities since both Jun N-terminal kinase (JNK)
and p38 can phosphorylate Elk-1. JNK1, JNK2 and p38 MAP kinase were also spotted on the mitogenic pathway arrays we employed. For JNK1 and JNK2, the hybridization intensities were also apparently reduced in colAR-MC3T3 analyses, but were near background in the normal rOB analysis and were thus not further evaluated in these studies. It has also been demonstrated that protein kinase B (PKB) can regulate Elk-1 activity through inhibition of the Ras-Raf-MEK-ERK pathway. In glioblastoma cells with constitutively active PKB, both Elk-1 and the SRE-containing c-fos were inhibited after a reduction in ERK (Galetic et al. 2003). It is also possible that the reduction in phosphorylation could be mediated by specific phosphatases. Interestingly, a recent report identified the dual-specificity MAP kinase phosphatase, MKP-1, as one likely mediator of glucocorticoid inhibition of osteoblast growth (Engelbrecht et al. 2003). Thus, it will now be of interest to determine the mechanism through which DHT transactivation of AR results in reduced ERK1/2 activity, as well as the reduced expression of Elk-1 protein and phosphorylation.

Since Elk-1 belongs to the ETS-domain family of transcription factors and is a c-fos proto-oncogene regulator, we determined whether DHT treatment influenced AP-1 transactivation of a luciferase reporter construct. AP-1 activity is a reflection of both the levels and/or the phosphorylation of Fos, increased by MAP kinase activation through all three MAP kinase cascades and by protein kinase C activation, and of Jun, which is also sensitive to MAP kinase activation predominantly through the JNK pathway (Whitmarsh & Davis 1996). Thus, AP-1 regulation is complex and can be influenced by multiple mechanisms. Our results suggest that one mechanism by which DHT modulates osteoblast activity may be through inhibition of the ability of AP-1 to activate transcription, consistent with the observation that androgens modestly inhibit c-fos expression in bone cells (Bodine et al. 1995). This is of interest, since it has been reported that some members of AP-1, for example c-fos, may in part underlie the phenomenon termed phenotype suppression in proliferating osteoblasts. Thus, high levels of AP-1 may lead to a reduction in differentiation during proliferation (Owen et al.

Figure 7 Androgen inhibition of MAP kinase downstream effector signaling: Western analysis of Elk-1 expression. (A) Elk-1 abundance and phosphorylation after DHT treatment. Western analysis was performed as indicated in Fig. 6 with equal amounts of proteins from cellular lysates from colAR-MC3T3 vehicle-treated or cultures treated with $10^{-8}$ M DHT for 2 days. Membranes were incubated with Elk-1 antibodies either to the protein (pan) or phospho-specific that recognize only phosphorylated Elk-1, to determine Elk-1 abundance and phosphorylation after DHT treatment. Blots were stripped and reprobed with antibody to α-tubulin to assess protein loading. (B) Quantitative analysis of Westerns determining the levels of Elk-1 abundance and phosphorylation status. Quantitative analysis was performed as described in Materials and methods, and showed DHT treatment reduced total panElk-1 by 40% and pElk-1 over 57%. Since the reduction in pElk-1 is greater than that observed for the protein itself, these results suggest that upstream signaling is also reduced, consistent with inhibition of pERK1/2 and MAP kinase luciferase. Data are means±S.E.M. of six samples per condition. *$P<0.05$; **$P<0.01$.

and conversely reductions in AP-1 may enhance differentiation.

Two major issues are raised based on the findings reported here: what is the consequence of inhibition of immature osteoblast proliferation on skeletal maturation/homeostasis by continuous androgen exposure, and is there control of androgen access to the osteoblast that may regulate the dichotomous response to androgen? Intuitively, inhibition of osteoblast growth by DHT treatment would appear inconsistent with the beneficial effects of androgens on skeletal health. However, this inhibition may have some positive consequences since inhibition of early osteoblast proliferation may lead to enhanced differentiation as the cells mature (Owen et al. 1990a). This is supported by the finding that continuous inhibition of MAP kinase signaling promotes early osteoblastic differentiation and mineralization (Higuchi et al. 2002), and by reports that DHT treatment can enhance osteoblast differentiation. Positive effects of androgen treatment on osteoblast differentiation and matrix production include increases in alkaline phosphatase activity and mRNA expression, and type Iα-1 collagen protein and mRNA levels (Benz et al. 1991, Gray et al. 1992), and effects on mineral accumulation (Kapur & Reddi 1989, Takeuchi et al. 1994). Combined, these results suggest that androgens may play a role in the regulation of bone matrix production and/or organization. This is particularly intriguing, since we have previously demonstrated that the highest levels of AR are seen in the most differentiated mineralizing cultures (Wiren et al. 2002). Whether or not control of androgen access occurs to modulate biphasic responses, through binding protein changes or changes in the cellular locations of phosphorylated intermediates for example, has not been explored.

**Figure 8** Androgen inhibition of MAP kinase downstream signaling: analysis of AP-1 reporter activation. AP-1 transcriptional activation after DHT administration in colAR-MC3T3 cultures was determined by luciferase assay using AP-1 luciferase containing four tandem repeats of the AP1 consensus sequence linked to luciferase. Cultures were treated with $10^{-8}$ M DHT for 6, 24 and 54 h. The data are expressed as percent of control vs DHT. This result shows DHT treatment for short times stimulates AP-1 activation, but longer treatment for 24 or 54 h inhibits AP1 heterodimer transactivation/function in colAR-MC3T3 cells. Data are means±S.E.M. of six samples per condition obtained in two experiments. *$P<0.05$; ***$P<0.001$ (vs control).
In this report, we have determined the effect of DHT treatment during osteoblast proliferation using a model of enhanced androgen responsiveness, the colAR-MC3T3 cells. The colAR-MC3T3 cultures are stably transfected with full-length rat AR under the control of the 3·6 kb fragment of the rat \( \alpha_1 \) (I)-collagen promoter. ColAR-MC3T3 cultures show increased AR levels demonstrated by analysis of functional binding with \( [\text{H}]1881 \), reflecting both endogenous AR and colAR construct production, and enhanced ARE transactivation that is androgen dose-dependent. The colAR-MC3T3 cultures exhibit a level of AR (\( \sim 5700 \) sites/cell) similar to that found in ventral prostate and other classic androgen target tissues, and is slightly elevated compared with another stably transfected osteoblastic model, the conditionally immortalized hFOB cell line with \( \sim 4000 \) sites/cell (Hoffbauer et al. 1997). Even though bone is clearly a target tissue with respect to androgen action, the mechanisms by which androgens exert their effects on bone biology are not well understood. Evidence does suggest that androgens act directly on the osteoblasts through the AR. With enhanced responsiveness, the colAR-MC3T3 stably transfected cell culture model may be particularly useful in examining the effects of androgen on the osteoblast differentiated phenotype. Studies determining the consequence of DHT treatment on osteoblast differentiation in colAR-MC3T3 cultures are ongoing.

In summary, DHT inhibits several steps in MAP kinase signaling including ERK1/2 phosphorylation and activity, Elk-1 expression and phosphorylation and AP-1 transcriptional activity. This decrease in MAP kinase signaling may underlie the reduction in osteoblast viability observed with longer DHT treatments characterized in this report. These results suggest one of the mechanisms by which DHT modulates osteoblast viability is through inhibition of MAP kinase signaling and Elk-1 downstream targets like AP-1. Together these data provide the first evidence that androgen inhibition of the MAP kinase signaling pathway, particularly at the level of Elk-1, may be an important mediator of committed osteoblast growth and thus a specific downstream target of DHT in its growth-control pathway. While androgen exposure will most likely have other effects on osteoblast function and gene expression, it is interesting to speculate that DHT exposure, through inhibition of MAP kinase signaling pathway(s) and immature osteoblast proliferation, may result in an enhanced differentiated state in osteoblasts. This effect may be skeletal site- or target cell-specific and may be influenced by AR number. Since the AP-1 transcription factor is also a significant regulator of osteoblast development, these findings also provide valuable insight into the molecular mechanisms of androgen action in bone and suggest an underlying complexity for androgen regulation of the osteoblast. Further studies will be required to establish how, where and which cell type in the skeleton is influenced by androgen exposure, and how the seemingly disparate effects of DHT on bone cell function translate into the effects reported in association with androgen action in vivo.

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