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Development and Characterization of a Conditionally Immortalized Human Osteoblastic Cell Line Stably Transfected With the Human Androgen Receptor Gene

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Abstract Androgens have significant beneficial effects on the skeleton. However, studies on the effects of androgens on osteoblasts are limited due to the absence of appropriate model systems that combine completness of the osteoblastic phenotype, rapid proliferation rate, and stable expression of the androgen receptor (AR). Thus, we stably transfected the conditionally immortalized human fetal osteoblastic cell line (hFOB) with the human wild-type AR (hAR) cDNA. Compared to nontransfected hFOB cells, constitutive hAR mRNA expression in three independent hAR-transfected hFOB clones (hFOB/AR) was 15-fold higher in hFOB/AR-16, 62-fold higher in hFOB/AR-2, and 72-fold higher in hFOB/AR-6 cells, respectively, as assessed by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Detectable constitutive levels of hAR mRNA by Northern blot analysis were present in hFOB/AR-2 and hFOB/AR-6 cells, but not in hFOB/AR-16 or hFOB cells, respectively. Treatment with 5α-dihydrotestosterone (5α-DHT) (10^{-8} M) for 24 h did not alter hAR mRNA steady state levels in the hFOB/AR cell lines. Nuclear binding studies demonstrated 152 ± 73 (mean ± SEM) functional hARs/nucleus in non-transfected hFOB cells, 3,940 ± 395 functional hARs/nucleus in hFOB/AR-2 cells, and 3,987 ± 823 hARs/nucleus in hFOB/AR-6 cells, respectively. Treatment with 5α-DHT increased the expression of a transiently transfected androgen response element-chloramphenicol acetyltransferase (ARE-CAT) reporter construct in hFOB/AR-6 cells in a dose- and time-dependent manner; no such effect was observed in transiently transfected hFOB cells lacking exogenously transfected hARs. Moreover, 5α-DHT-induced ARE-CAT expression was inhibited by the selective androgen receptor antagonist, hydroxyflutamide. In summary, we have developed and characterized androgen-responsive osteoblastic cell lines derived from normal human fetal bone that express physiological levels of functional hARs. These cell lines should provide a suitable model for further studies on the effects of androgens on osteoblast function, including the identification of potential androgen-regulated growth factors and cytokines. J. Cell. Biochem. 66: 542–551, 1997.

Key words: androgens; androgen receptor; bone cells; dehydroepiandrosterone; dihydrotestosterone; hydroxyflutamide; osteoblasts; stable transfection

INTRODUCTION

Androgens have major beneficial effects on the development and maintenance of the skeleton in both women and men [Bagatell and Bremner, 1996; Greenspan et al., 1989]. Clinically, androgen deficiency is associated with osteopenia, which is at least partially reversed by exogenous administration of androgens [Orwoll and Klein, 1995]. The mechanisms of androgen action on bone cells remain unclear, however. A major obstacle for such studies is the lack of appropriate model systems. Previous studies have used either human osteosarcoma cell lines or rodent systems [Benz et al., 1991; Takeuchi et al., 1994; Nakano et al., 1994; Gray et al., 1992; Masuyama et al., 1992], but it is unclear whether results obtained in these systems can be extrapolated to normal human

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bone cell biology. The use of primary human osteoblastic cultures [Kasperk et al., 1997], while physiologically relevant, is limited by their slow proliferation rate, early onset of senescence, and high interindividual variability.

Our group previously described the development of an immortalized human fetal osteoblastic cell line, hFOB [Harris et al., 1995a], which expresses the complete phenotype of the mature osteoblast, including production of alkaline phosphatase and osteocalcin, response to 1,25-dihydroxyvitamin D and parathyroid hormone, and the capacity to form mineralized nodules upon reaching confluence. Thus, the hFOB cells have phenotypic characteristics similar to those of human adult osteoblasts. These cells would be an ideal model system for studying androgen effects on bone, except for the fact that they express low levels of the androgen receptor (AR), which appears to be due to down-regulation as a result of long-term in vitro culture. Thus, while short-term primary cultures of human osteoblasts express up to 3,000 hARs per cell [Colvard et al., 1989] and up to 3,900 ERs per cell [Eriksen et al., 1988], hFOB cells have $<$200 hARs or hERs per cell [Harris et al., 1995a]. In order to use this system to study estrogen effects on bone, Harris et al. [1995b] developed subclones of the hFOB line that were stably transfected with an ER expression vector (hFOB/ER). These cells demonstrated the expected responses of estrogen-responsive target cells to estrogen treatment, including increases in endogenous progesterone receptor levels and up-regulation of the expression of an estrogen-response element-luciferase construct following estrogen treatment [Harris et al., 1995b]. Furthermore, the use of these cells has led to a better understanding of autocrine and paracrine mechanisms mediating the effects of estrogens, such as estrogen effects on proliferation [Robinson et al., 1997], on the insulin-like growth factor (IGF) system [Kassem et al., 1996], and on the transforming growth factor-β/bone morphogenetic protein (TGF-β/BMP) superfamily [Rickard et al., 1997] in osteoblastic cells.

The present study reports the development and characterization of subclones of hFOB cells that are stably transfected with an AR expression vector (hFOB/AR). These cells should facilitate direct study of androgen action on bone cells, including the identification of potential androgen-regulated growth factors and cytokines mediating androgen effects on bone.

MATERIALS AND METHODS

Materials

Cell culture medium and supplements were purchased from Sigma (St. Louis, MO). Culture flasks and dishes were obtained from Corning (Corning, NY). Restriction endonucleases, ligase, and *Taq* DNA polymerase were from Boehringer Mannheim (Indianapolis, IN) or Promega (Norwalk, CT). Unless otherwise stated, all other chemicals were of molecular biology grade and were purchased from Sigma. The random primer labeling kit (Decaprime II) was from Ambion (Austin, TX) and the radiolabeled nucleotides [α-^{32}P]-dATP, [α-^{32}P]-dCTP, [^{3}H]thymidine and [^{3}H]dihydrotestosterone were from DuPont-NEN (Boston, MA). A cDNA encoding the human androgen receptor (hAR) [Chang et al., 1988] and the plasmid p(PRE)$_2$PBL$_7$ containing glucocorticoid/androgen-hybrid responsive elements (5′: CTGTACAGGATGTTCTAGCTAC) located upstream of a promoter linked to the chloramphenicol acetyltransferase gene (ARE-CAT) [Denison et al., 1989] were kindly provided by Dr. Donald J. Tindall (Mayo Clinic, Rochester, MN). The pRSVβ-GAL plasmid containing the β-galactosidase gene (β-GAL) was a gift from Dr. Thomas C. Spelsberg (Mayo Clinic, Rochester, MN), and the pTRI-GAPDH plasmid encoding the human gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Ambion (Austin, TX). Hydroxyflutamide was kindly provided by Dr. Rudolph Neri (Schering-Plough, Kenilsworth, NJ).

Vector Construction

The cDNA sequence (3.2 kb) encoding the wild-type human AR [Lubahn et al., 1988] was ligated into the *Bam*HI site of the expression vector pHEGO-HYG (ATCC deposit No. 79994). This vector has been previously described in detail [Harris et al., 1995b]. It contains a CMV promotor inserted into the *Cla*I/*Hind*III site, the SV40 polyadenylation signal, and the hygromycin B resistance gene driven by the thymidine kinase promotor. The AR expression vector resulting from insertion of the AR cDNA into this vector was designated pAR-HYG (Fig. 1).

Stable Transfection

For stable transfection, hFOB cells (3×10^7) were resuspended in 400 μl of serum-free me-

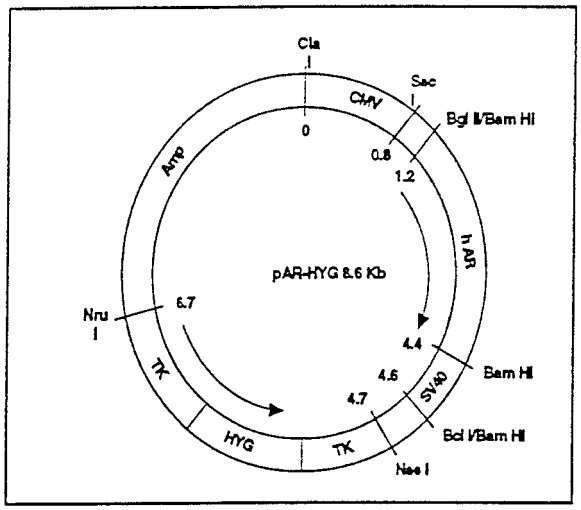

Fig. 1. Construction of the pAR-HYG vector. The human AR cDNA (hAR) was inserted downstream of the Cytomegalovirus (CMV) promotor and upstream of the SV40 polyadenylation signal of the pHEGO-HYG vector. *Arrows,* directions of transcription for the AR and hygromycin resistance (HYG) genes. Selected restriction enzyme sites are indicated, as well as the map location (clockwise in kb from the *Cla*I site).

dium containing 15 µg of the AR expression vector pAR-HYG vector (linearized with *Nae*I) and placed in an electroporation cuvette. The cells were then subjected to a pulse of 900 V/cm at 960 µFD in a BioRad electroporation device and incubated at 4°C for 10 more min. Transfected cells were then selected for hygromycin B resistance as previously described for the hFOB/ER line [Harris et al., 1995b]. Transfected cells were plated in growth medium (phenol red-free DMEM/Ham F12 medium, 1:1) supplemented with 10% (v/v) charcoal-stripped fetal bovine serum (csFBS) and geneticin (300 µg/ml) (base medium) and incubated for 72 h. Cells were then incubated in selective medium (base medium supplemented with 75 µg/ml hygromycin B) for 6 days until resistant single colonies were visible. Resistant colonies (100–500 cells) were trypsinized, passaged, and expanded in selective medium containing 100 µg/ml hygromycin B to $>10^7$ cells for cryopreservation. Sixteen hygromycin-resistant clones were obtained and subsequently screened. The hFOB/AR cells were maintained in base medium alternately supplemented with geneticin (300 µg/ml) or hygromycin B (100 µg/ml) to select for cells expressing the AR. The temperature-sensitive mutant SV40 large T antigen is most active at the permissive temperature (34°C)—the temperature used in all experiments with these cell lines.

Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For quantitative assessment of AR mRNA expression, hFOB/AR cell lines and nontransfected hFOB cells (5×10^6) were grown in serum-free medium supplemented with 0.1% (w/v) bovine serum albumin (BSA) in 75-cm^2 flasks. Cells were treated for 48 h with the specific nonsteroidal anti-androgen hydroxyflutamide at a concentration of 10^{-6} M to minimize any residual androgen activity. Cells were then treated with either vehicle or 5α-DHT (10^{-8}M) for 24 h at 34°C, and total cellular RNA was isolated using a commercially available kit (RNeasy) from Qiagen (Hilden, Germany). Synthesis of cDNA was performed in a 20-µl reaction using 2 µg of total RNA, 1× reverse transcriptase buffer, 1 mM each of dATP, dCTP, dGTP, and dTTP, 20 units of RNase inhibitor, 10 U of AMV reverse transcriptase, 200 pmol of random hexamers, and 800 pmol of poly-dT_{15} primers (all from Boehringer Mannheim) for 2 h at 42°C. Subsequent PCR reactions contained 1 µl of cDNA samples, 1 µM of each primer (Table I), 1 × PCR buffer, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.5 µl [α-^{32}P]dCTP (10 µCi/µl), 1.5 mM $MgCl_2$, and 0.5 units of *Taq* polymerase. Oligonucleotides (Table I) were synthesized at the Oligonucleotide Core Laboratory (Mayo Clinic, Rochester, MN). Thermal cycling was performed in a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT). PCR reaction profiles consisted of initial denaturation at 94°C for 2 min, cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 7 min. To ensure greater accuracy in quantitative assessment, the number of cycles necessary to reflect linear amplification kinetics was determined by terminating PCR reactions after 18 cycles and every three cycles thereafter, followed by a final post-extension step at 72°C for 7 min (data not shown). Linear amplification occurred at 24 cycles for GAPDH reactions and at 30 cycles for hAR reactions, respectively.

Amplified PCR products were analyzed by electrophoresis on 1% (w/v) agarose gel and visualized by ethidium bromide staining. For quantification, gel slices were counted in a liquid scintillation counter (Beckman), and values were normalized to GAPDH values to control for differences in RNA loading during cDNA

TABLE I. Oligodeoxynucleotide Primers Used for PCR

Target cDNA	Oligonucleotide sequence	cDNA size (bp)
hAR sense	5′ AAGCTCAAGGATG-GAAGTGCAG 3′	582
hAR antisense	5′ TGTTGCTGAAG-GAGTTGCATGG 3′	
GAPDH sense	5′ GGTATCGTG-GAAGGACTCAT 3′	473
GAPDH antisense	5′ TCCACCACCCTGT-TGCTGTA 3′	

synthesis. Coefficients of variation for triplicate reactions with hAR and GAPDH were between 4% and 10%, respectively.

Northern Blot Analysis

Ten µg of total RNA was separated on a 1% (w/v) agarose gel containing 2.2 M formaldehyde [Lehrach et al., 1977]. The RNA was then transferred to a positively charged nylon membrane (Hybond N^+, Amersham, Arlington Heights, IL) by capillary blotting [Thomas, 1980], using 20× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). Ethidium bromide staining of the gel and methylene blue staining (15 min in 5% acetic acid, followed by 5 min in 0.04 (w/v) methylene blue and 0.8 M sodium acetate, pH 4.0) [Herrin and Schmidt, 1988] of the membrane ensured equal loading, absence of degradation, and efficient transfer. Prehybridization (≥6 h) and hybridization (≥16 h) were carried out at 42°C (5× SSC, 10% (w/v) dextrane sulfate, 50% (v/v) formamide, 1% (w/v) lauroylsarcosine, and 0.25 mg/ml herring sperm DNA). The cDNA inserts were radiolabeled with 4 µl [α-^{32}P]dATP and 4 µl [α-^{32}P]-dCTP to a specific activity of approximately 10^9 cpm/µg DNA using a random primer DNA labeling kit [Feinberg and Vogelstein, 1983]. After stringent washing (3 × 5 min at room temperature in 2× SSC and 0.5% (w/v) N-lauroyl sarcosine, 3 × 20 min at 42°C in 0.1× SSC and 0.1% (w/v) N-lauroyl sarcosine) membranes were subjected to autoradiography on an intensifying screen at −80°C. Band intensity was quantified by densitometry (Pharmacia LKB Biotechnology, Piscataway, NJ). All experiments were carried out at least three times, and representative blots were shown. Control hybridization with GAPDH cDNA showed that approximately equal amounts of RNA were loaded.

Nuclear Binding Assay

Specific nuclear binding of tritiated androgens in hFOB/AR cell lines and nontransfected hFOB cells was measured using a nuclear binding assay as previously described [Colvard et al., 1988; Eriksen et al., 1988]. Briefly, cells at confluence were trypsinized and suspended in Eagle's Basal medium (Irvine Scientific, St. Anna, CA). Quadruplicate aliquots (5×10^5) were incubated with 10 nM of the synthetic androgen [17α-methyl-^{3}H]R1881(methyl-trienolone) from (Du Pont-NEN, Boston, MA) in the presence or absence of 1 µM of R1881, for 1 h at 22°C. The reaction was stopped by the addition of 1 ml cold (4°C) 5 mM HEPES, 0.2 mM EDTA, 0.1% (w/v) BSA, pH 7.4, and then centrifuged. Subsequently, the cells were resuspended in homogenization buffer (TTG buffer) containing 50 mM Tris, pH 7.4, 10% (v/v) glycerol, 10 mM KCl, 0.1% Triton X, and 0.1% (w/v) BSA. To harvest nuclear pellets, cells were homogenized with a glass homogenizer, placed onto 1.4 M sucrose in TTG buffer, and centrifuged for 20 min at 6,000*g* at 4°C. The supernatant was discarded, and the pellet was washed in cold TTG buffer and then centrifuged. One ml of 0.25 M sodium acetate in 100% ethanol was added to the pellet, incubated for 1 h at 22°C, and subsequently centrifuged at 1,000*g* for 5 min. The radioactivity from the supernatant was measured in a liquid scintillation counter (Beckman). DNA in replicates was measured using a modification [Colvard et al., 1988] of the diphenylamine method as previously described [Burton, 1956].

Transient Transfection and CAT Assay

For transient transfection, hFOB/AR cells and nontransfected hFOB cells were seeded into 6-well plates at a density of 5×10^5 cells/well in 2 ml of base medium without antibiotics and incubated for 24 h at 34°C. Cells were then washed with OptiMEM (Gibco-BRL, Grand Island, NY), and incubated for 5 h in 1 ml of OptiMEM containing 10 µg of ARE-CAT DNA, 10 µg of β-GAL and 8 µl of LipofectAMINE reagent (Gibco-BRL) according to the manufacturer's recommendations. One ml of base medium containing 20% (v/v) csFBS was added, and cells were incubated for 24 h. At 24 h after

treatment of the transfectants with either vehicle (ethanol), 5α-DHT, dehydroepiandrosterone (DHEA), or hydroxyflutamide, cells were harvested in 100 µl of 0.25 M Tris–HCl, pH 8.0 and lysed by three cycles of incubation on dry ice for 2 min and at 37°C for 5 min, followed by incubation at 70°C for 15 min. The CAT diffusion method was used to assess conversion of chloramphenicol to acetylated forms [Gorman et al., 1982]. Briefly, 80 µl of cell lysates was incubated with 0.25 M Tris–HCl (pH 8.0), 10 mM chloramphenicol, and 0.1 mM [^{3}H]-acetyl-coenzyme A at a final volume of 150 µl for 4 h at 37°C, followed by xylene extraction. To normalize ARE-CAT activities for differences in transfection efficiency, β-Gal activity was determined in 20 µl of cell lysates in a final reaction volume of 300 µl, using the method of Norton and Coffin [1985].

Statistical Analysis

Unless otherwise stated, all numerical values are expressed as mean ± SEM. Student's paired t-test was used to evaluate differences between the test sample of interest and the respective control sample. A P-value of <0.05 was considered significant.

RESULTS

Vector Construction and Stable Transfection of hFOB/AR Cells

Following stable transfection with the hAR, a total of 16 hygromycin B-resistant single colonies were identified. These hFOB/AR cells attached to the culture dishes within 8–12 h of plating and displayed a clonal growth pattern with dense nests of osteoblasts, which closely resembled the growth pattern observed in nontransfected hFOB cells (not shown). Furthermore, all hFOB/AR cell lines had a spindle-shaped morphology with small cytoplasmic processes and a centrally located nucleus, all of which were similar to the nontransfected hFOB cells (not shown). However, there were marked differences in proliferation rates. While subclone-16 had a doubling time similar to nontransfected hFOB cells (2 days), subclones 2 and 6 had doubling times of 4–5 days. No evidence of cell senescence has been observed after 30 passages for hFOB cells and after 20 passages for hFOB/AR cell lines, respectively, when cells were maintained at 34°C, the permissive temperature in these cell lines.

Constitutive hAR mRNA Expression in hFOB Cells and hFOB/AR Cell Lines

All sixteen of the hFOB/AR cell lines and the nontransfected hFOB cells were screened for the presence of hAR gene expression. Of these, three hFOB/AR cell lines (hFOB/AR-2, -6, and -16) displaying a range of hAR mRNA expression were further characterized and compared to nontransfected hFOB cells. As assessed by RT-PCR (Table I), no constitutive expression of hAR mRNA was detected in the hFOB cells based on agarose gel electrophoresis (Fig. 2A). By contrast, moderate levels of hAR mRNA were detected in hFOB/AR-16 at baseline. The highest levels of hAR mRNA were expressed by hFOB/AR-2 and -6 (Fig. 2A). Semiquantitative assessment of hAR mRNA/GAPDH ratios revealed 15-fold higher hAR mRNA levels in hFOB/AR-16 as compared to hFOB cells and 62- and 72-fold higher levels in hFOB/AR-2 and -6, respectively (Fig. 2B). For comparison, the human osteosarcoma cell line SaOS-2 showed a 46-fold higher hAR mRNA/GAPDH ratio, as compared to hFOB cells. Northern blot analysis revealed no detectable hAR mRNA levels in hFOB cells and hFOB/AR-16, but easily detectable hAR mRNA levels in hFOB/AR-2 and -6 (Fig. 3A). Laser scanning densitometry revealed approximately similar constitutive levels of hAR mRNA expression in hFOB/AR-2 and -6 (Fig. 3B).

Inducible hAR mRNA Expression in hFOB Cells and hFOB/AR Cell Lines

Inconsistent results have been reported with respect to the regulation of hAR mRNA expression by exogenous administration of androgens in a variety of tissues [Chang et al., 1995]. To determine whether hAR mRNA expression in fetal osteoblasts is regulated by 5α-DHT, hFOB cells and hFOB/AR cell lines 2, 6, and 16 were exposed to the specific nonsteroidal androgen hydroxyflutamide for 48 h (10^{-6} M) and then treated with 5α-DHT (10^{-8} M) for 24 h. As assessed by RT-PCR, there was a small increase in hAR/GAPDH ratios (1.31 ± 0.05-fold) after treatment with 5α-DHT as compared to vehicle-treated in hFOB cells ($P < 0.05$). However, no significant differences were detected by RT-PCR between treatment with vehicle or 5α-DHT in hFOB/AR-2, -6, and -16 (Fig. 2A, 2B). Northern blot analysis (Fig. 3A) with laser scanning densitometry (Fig. 3B) confirmed similar

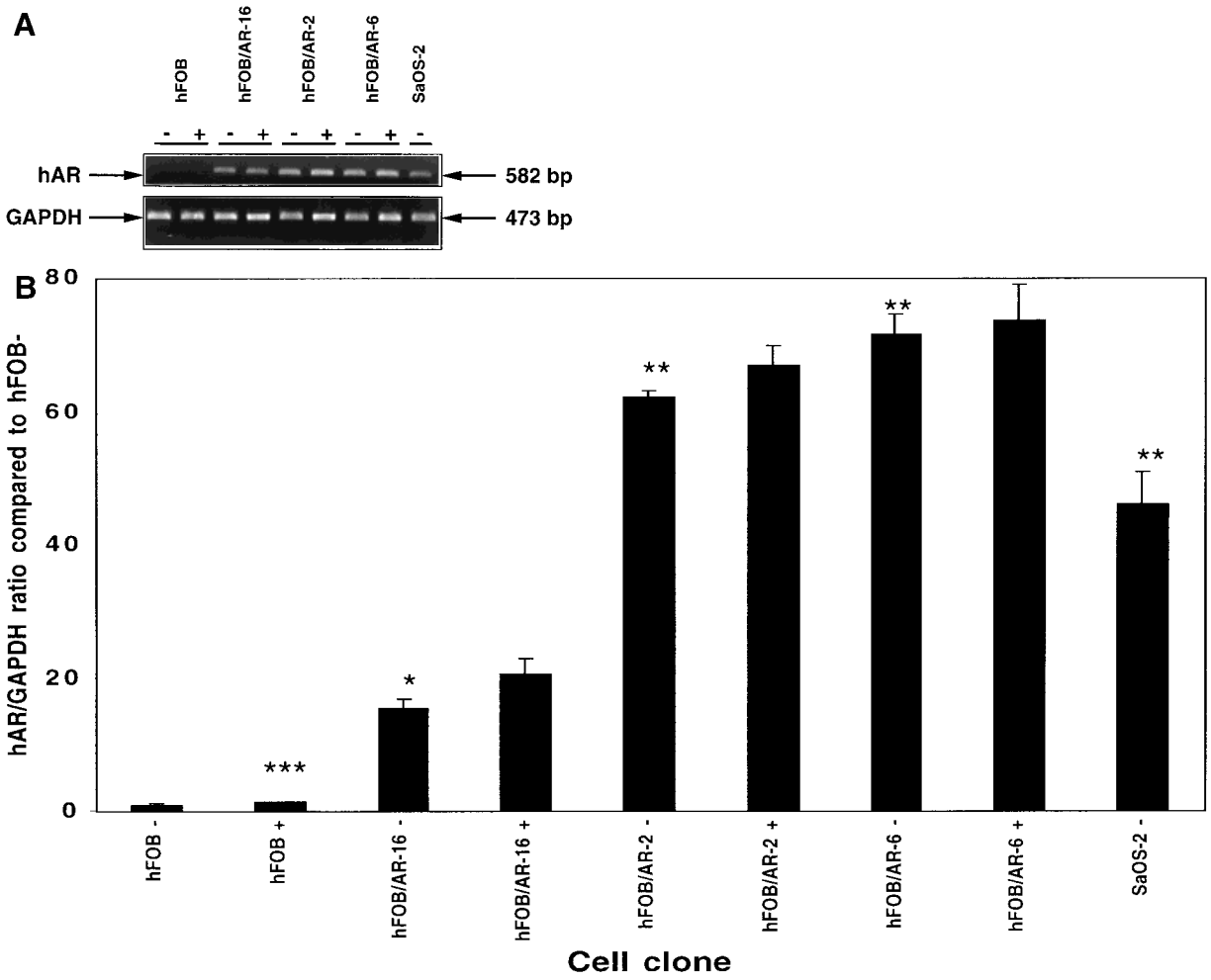

Fig. 2. RT-PCR analysis of constitutive and inducible expression of hAR mRNA in hFOB and hFOB/AR cell lines. **A:** RT-PCR of RNA derived from three hFOB/AR cell lines and nontransfected hFOB cells without (−) and with (+) treatment with 5α-DHT (10^{-8} M) for 24 h. Untreated SaOS-2 human osteosarcoma cells were included as a positive control. Sizes of PCR products with primers for hAR and GAPDH are given in bp. **B:** For quantification of the PCR products shown in **A,** radiolabeled nucleotide was incorporated into PCR products, and radioactivity was counted following gel electrophoresis and excision of gel slices. *P*-values between untreated samples (vs. hFOB cells): *$P < 0.001$; **$P < 0.0001$; *P*-values between treated and untreated samples: ***$P < 0.05$.

hAR mRNA steady-state levels in hFOB/AR-2 and -6 following treatment with either vehicle or 5α-DHT.

Nuclear Binding Assay

To assess the number of functional hARs per nucleus, we measured specific nuclear binding of tritiated androgens in hFOB/AR cell lines and in the nontransfected hFOB cells using a nuclear binding assay. Nontransfected hFOB cells (n = 4) expressed 152 ± 73 hARs per nucleus, similar to the number of hERs previously reported in these cells [Harris et al., 1995b]. In contrast, significantly higher numbers of hARs/nucleus were detected in hFOB/AR-2 (3,940 ± 395 hARs/nucleus; n = 4; $P < 0.001$) and hFOB/AR-6 (3,987 ± 823 hARs/nucleus; n = 4; $P < 0.005$) in independent experiments.

CAT Assays

The cell line hFOB/AR-6 that displayed the highest levels of androgen receptors as assessed by RT-PCR, Northern blot analysis, and nuclear binding assay was used for direct assessment of androgen responsiveness. Following transient co-transfection of hFOB/AR-6 cells with the plasmids for ARE-CAT and β-Gal, the cells were treated for 48 h with 5α-DHT doses ranging from 10^{-11} M to 10^{-7} M or vehicle

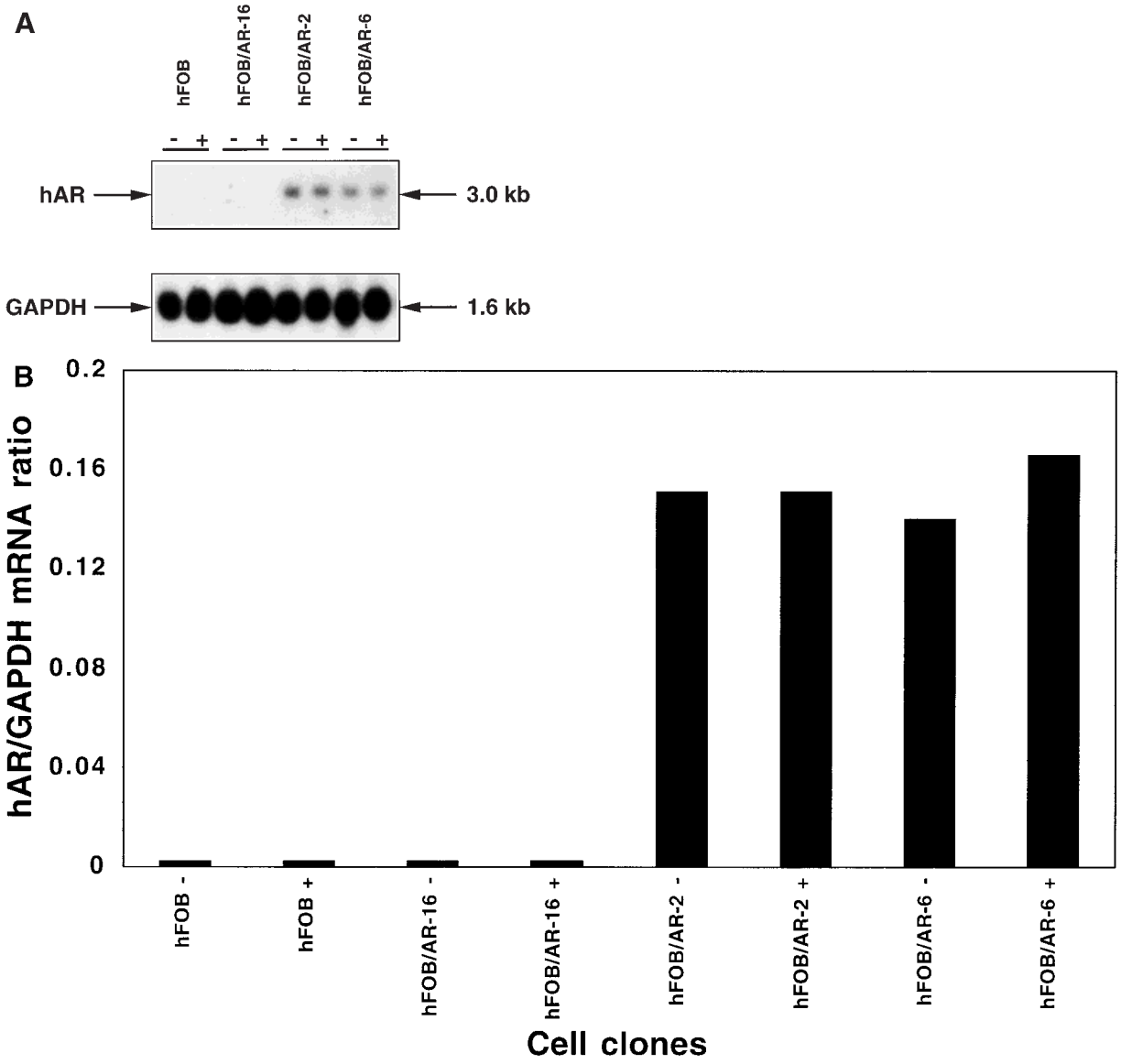

Fig. 3. Northern blot analysis of constitutive and inducible expression of hAR mRNA in hFOB and hFOB/AR cell lines. Untransfected hFOB cells and the cell lines hFOB/AR-2, -6, and -16 were treated with either vehicle or 5α-DHT (10^{-8} M) for 24 h. Ten μg of total RNA extracted from cell pellets was analyzed by electrophoresis, transferred to a nylon membrane, and hybridized to a [α-^{32}P]-labeled cDNA probe. **A:** Autoradiogram of hybridization with the probe for hAR (3.0 kb, top) and the housekeeping gene GAPDH (1.6 kb, bottom). **B:** Densitometric quantitation of the Northern blot shown in A. Values represent hAR/GAPDH ratios of a representative Northern blot.

(ethanol). The maximal response was observed at a dose of 10^{-8} M, with a subsequent decrease at 10^{-7} M (Fig. 4). By contrast, treatment with the adrenal androgen dehydroepiandrosterone (DHEA) (10^{-8} M) did not increase CAT activity. The hFOB cells that were not transfected with the hAR did not demonstrate a significant increase in CAT activity (1.34 ± 0.16 vs. 1.0 ± 0.58; $P = 0.69$) following transient transfection with ARE-CAT/β-GAL and treatment with 10^{-8} M 5α-DHT. To assess the kinetics of transcriptional activation, transiently co-transfected hFOB/AR-6 cells were treated with 5α-DHT at a dose of 10^{-8} M. CAT activity increased after 36 hrs and was 4.8-fold higher after 48 h, compared to baseline (Fig. 5).

The specificity of ARE-CAT activation by androgens in hFOB/AR-6 was assessed using the specific anti-androgen hydroxyflutamide. As shown in Figure 6, hydroxyflutamide significantly attenuated the increase in ARE-CAT activation due to 5α-DHT, and had no effect

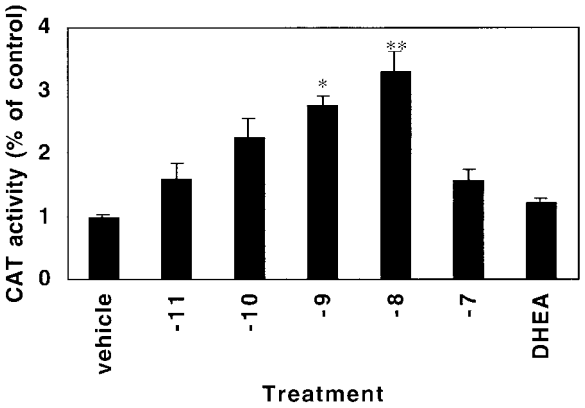

Fig. 4. Dose response of ARE-CAT activation by 5α-DHT in hFOB/AR-6 cells. Following transient transfection of cells with the plasmids for ARE-CAT and β-Gal, cells were treated for 48 h with different doses of 5α-DHT (10^{-11} M–10^{-7} M) or DHEA (10^{-8} M) and assayed for CAT activity. Values represent the mean of CAT/β-Gal ratios (normalized to vehicle) ±SE of two separate experiments (*$P < 0.01$, **$P < 0.05$).

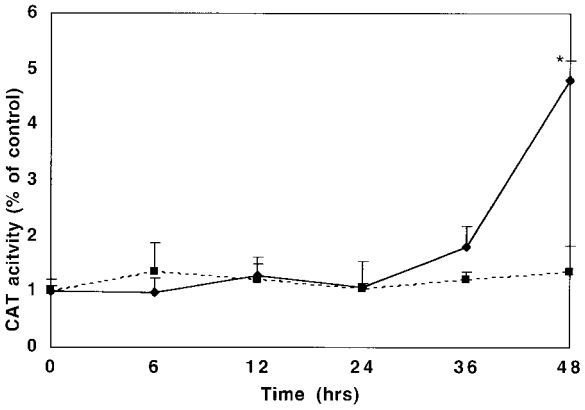

Fig. 5. Time course of ARE-CAT activation by 5α-DHT in hFOB/AR-6 cells. Following transient transfection with ARE-CAT and β-GAL plasmids, hFOB (dotted line, rectangles) and hFOB/AR-6 cells (plain line, diamonds) were exposed to 5α-DHT (10^{-8} M) for 0, 6, 12, 24, 36, and 48 h and cell lysates were assessed for CAT activities and normalized for β-Gal activity. Values represent the mean of CAT/β-Gal ratios ±SE of two separate experiments (*$P < 0.05$).

on ARE-CAT activation when administered alone.

DISCUSSION

Studies on the effects of androgens on normal, human osteoblasts are limited by the lack of appropriate model systems that combine a normal and complete osteoblastic phenotype, a high degree of homogeneity, and a fast proliferation rate with the stable expression of physiologically relevant levels of hARs. We describe the development and characterization of human osteoblastic cell lines stably expressing high levels of hARs as assessed by semiquantitative RT-PCR and Northern blot analysis. Moreover, the functional activity of the hARs in these cells was confirmed by a nuclear binding assay that detects only ligand-binding hARs bound to nuclear acceptor sites [Colvard et al., 1989]. These human osteoblastic cell lines displayed a broad range of different hAR levels that may reflect differences in transfection efficiency, including cellular uptake and nuclear integration of the pAR-HYG. The use of different hFOB/AR cell lines will facilitate studies that assess the effect of different levels of hAR on normal human osteoblasts. Of these, the number of hARs in the hFOB/AR-2 and -6 cell lines is similar to that present in the epithelium of the prostate, which contains 2,000–12,000 hARs/nucleus [Chang et al., 1995]. The number of hARs/nucleus in these two cell lines is also similar to the number of hARs present in normal human osteoblasts in primary culture (≤3,000 hARs per cell) [Colvard et al., 1989]. Thus, these clones express "physiological" levels of hARs, both as present in reproductive tissues and in bone cells. Moreover, using transient transfection of a reporter gene construct, 5α-DHT stimulated CAT activity in the cell line hFOB/AR-6 in a time- and dose-dependent fashion, indicating functional transcriptional activation conferred by the hAR. By contrast, the adrenal androgen DHEA did not stimulate CAT activity. Androgen response was blocked by co-treatment with the pure anti-androgen, hy-

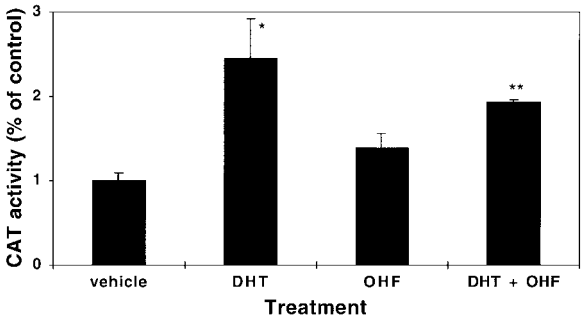

Fig. 6. Specificity of ARE-CAT activation by androgens. Inhibition by the specific anti-androgen, hydroxyflutamide. Transiently transfected hFOB/AR-6 cells were treated with vehicle, 5α-DHT (10^{-8} M), the specific anti-androgen hydroxyflutamide (10^{-6} M), or both 5α-DHT (10^{-8} M) and hydroxyflutamide (OHF) (10^{-6} M) for 48 h. Cell lysates were assessed for CAT and β-GAL activities. Values represent the mean (normalized to vehicle) ±SE of three independent experiments (*$P < 0.05$, **$P < 0.001$).

droxyflutamide, indicating that the observed effects are specific and mediated directly by the hAR.

The hFOB/AR cell lines have several advantages over previously described systems [Marie, 1994], such as a high degree of homogeneity due to their clonal origin, stable expression of physiologically relevant levels of functionally active hARs, and a relatively rapid growth rate. Recently, local mediators of estrogen effects have been identified using the analogous hFOB/ER cells. In this system, increased production of IGF binding protein-4, which is inhibitory to IGF action, may be responsible for estrogen-induced growth inhibition of hFOB/ER cells [Kassem et al., 1996; Robinson et al., 1997]. In addition, selective up-regulation of BMP-6 at the RNA and protein level by estrogen has recently been reported in hFOB/ER cells [Rickard et al., 1997]. In an analogous manner, the hFOB/AR cells should provide a useful model system to define androgen effects and its local mediators in human osteoblasts. Previous studies using murine osteoblasts have suggested that TGF-β may mediate some of the effects of androgens on bone cells [Kasperk et al., 1990]. The hFOB/AR cells should greatly facilitate such studies, and potentially lead to the identification of novel androgen-regulated genes in human osteoblasts.

Despite these advantages, we recognize several potential limitations of this system. First, since the parent cell line, the hFOB cells, is of fetal origin, the observed effects of androgens in the hFOB/AR cells may be limited to fetal osteoblasts and may not necessarily be the same as in adult human osteoblasts. Second, while hFOB/AR cells express physiological levels of the AR, the regulation of the AR in these cells is likely different from the regulation of the endogenous AR in osteoblasts, since the hAR construct in hFOB/AR cells is driven constitutively by the CMV promotor and lacks 5′ regions that are crucial for transcriptional regulation of the AR [Mizokami and Chang, 1994; Wiren et al., 1997]. Thus, while previous studies have found both up- and down-regulation of endogenous AR gene expression following activation of the AR by androgens [Chang et al., 1995], and the parent hFOB cells appeared to have a small increase in endogenous AR mRNA levels following DHT treatment, we did not detect any regulation of the stably transfected AR by DHT in hFOB/AR cells. Our data also indicate that, unlike DHT, the adrenal androgen DHEA does not directly activate the hAR in equimolar doses. These findings are consistent with previous observation that DHEA does not bind to the AR and is an androgen only by conversion to testosterone or DHT [Grover and Odell, 1975]. Alternatively, the recent description of specific binding sites for DHEA [Meikle et al., 1992] raises the possibility that the observed biological effects of DHEA may be mediated by a different receptor.

In summary, we have developed and characterized androgen-responsive human fetal osteoblast cell lines derived from normal human bone that constitutively express high levels of functionally active hARs and respond to treatment with androgens in a specific dose- and time-dependent fashion. These cells should provide a useful model system to better define androgen effects on bone, including the regulation of growth factors and cytokines. In addition, such studies may also provide insights into the role of androgens in the pathogenesis of disorders such as osteoporosis associated with testosterone deficiency, and potentially with aging.

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