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Androgen Up-Regulates Epidermal Growth Factor Receptor Expression and Binding Affinity in PC3 Cell Lines Expressing the Human Androgen Receptor[1]

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ABSTRACT

Androgens are required for the optimal growth and development of both the normal prostate and steroid-sensitive prostate cancer. PC3 prostate cancer cell lines stably expressing the human androgen receptor (AR) and possessing an androgen-sensitive phenotype (PC3-hAR) were used to examine the role of the epidermal growth factor receptor (EGFR) in androgen-stimulated prostate cancer cell growth. Epidermal growth factor (EGF) and dihydrotestosterone (DHT) independently induced the growth of PC3-hAR cells. Moreover, EGF and DHT in combination exerted a synergistic effect on PC3-hAR cell growth. DHT-exposed PC3-hAR cells expressed a greater than 2-fold increase in EGFR mRNA and 50% more EGFR protein than controls. Time course radioligand-binding assays confirmed these findings by showing an elevation in EGF binding in the DHT-exposed PC3-hAR cells. In addition, radioligand competition-binding studies revealed a 2-fold increase in EGFR-EGF binding affinity in the PC3-hAR cells after DHT treatment. However, no enhancement of transforming growth factor α or EGF expression was detected because DHT did not affect the levels of these cytokines in the PC3-hAR cell lysate or conditioned media. Our observations suggest that DHT increases both EGFR number and receptor-ligand affinity in androgen-sensitive prostate cancer cells and that these effects correlate with increased EGF binding and an enhanced mitogenic response to EGF.

INTRODUCTION

The presence of androgen is essential for normal prostate differentiation, growth, and function, as well as the proliferation of steroid-sensitive prostatic adenocarcinomas (1, 2). However, the nature of the molecular mechanisms by which androgens induce prostate cell growth is obscure. Some insights have come from investigations of peptide mitogenic growth factors (for reviews, see refs. 3–5). Two such growth factors, epidermal growth factor EGF[3] and TGF-α, in conjunction with the EGFR, are important in the growth and development of normal and malignant tissues, including the breast and prostate (6–10). Moreover, EGFR expression is modulated by androgens in the steroid-responsive prostate cancer cell lines LNCaP and ALVA 101 (11, 12). Similarly, estrogens have been shown to increase both cell proliferation and TGF-α/EGF levels in estrogen-responsive breast cancer cells (13, 14). On the basis of these and other observations, it has been postulated that androgens may exert their mitogenic influence on prostate cancer cells by modulating the EGFR pathway (11, 12).

In an effort to investigate androgen-sensitive prostate cancer cell growth, we stably transfected the androgen-insensitive prostate cancer cell line PC3, which is thought to express little or no endogenous androgen receptor (15), with a normal human AR cDNA expression vector. These stably transfected PC3-hAR cell lines display an androgen-sensitive phenotype (16). Thus, the PC3-hAR cells provide a useful model for delineating the relationship between the EGFR pathway and androgens in steroid-sensitive prostate cancer cells. The results presented here demonstrate that the presence of an androgen, DHT, enhances the proliferative effect of EGF and increases both EGFR expression and binding affinity in the androgen-sensitive PC3-hAR cells. This analysis suggests a peptide growth factor pathway by which androgen stimulates prostate cancer cell growth.

MATERIALS AND METHODS

Hormones and Growth Factors. EGF from mouse submaxillary glands (receptor grade) was obtained from Collaborative Research (Bedford, MA). Mouse ^{125}I-labeled EGF (specific activity, 170 Ci/g) was from DuPont New England Nuclear (Boston, MA). DHT was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. The LNCaP (lymph node carcinoma of the prostate) and PC3 cell lines (bony metastatic site of prostatic adenocarcinoma) were obtained from American Type Culture Collection (Rockville, MD). The derivation of the PC3-hAR cell lines is described previously (16); briefly, the lipofectin technique (GIBCO-BRL, Gaithersburg, MD) was used to cotransfect the PC3 cell line with an expression vector pSG5, containing the human AR cDNA, and pSV2neo, containing the neomycin resistance gene (17). The PC3neo control line contains only the neomycin resistance vector. All cells were maintained at 37°C and 5% CO_2 in RPMI 1640 (GIBCO-BRL, Grand Island, NY) with added penicillin, streptomycin, L-glutamine, and 10% fetal bovine serum (all reagents from Sigma). All transfected cell lines were cultured in media containing 1 mg/ml gentamicin (Sigma). SFM consisted of RPMI 1640 without phenol red, with ITS+ premix [insulin-transferrin-selenium with BSA and linoleic acid (Collaborative Research)], penicillin, streptomycin, and L-glutamine.

Western Blot Analysis. Cells (7×10^6 for PC3neo and PC3-hAR and 3×10^6 for LNCaP) were lysed in RIPA buffer [20 mM HEPES-0.1% SDS-1% Triton X-100-0.5% sodium deoxycholate-5 mM EDTA-50 mM NaCl-25 mM sodium pyrophosphate-5 mM NaF-50 μM Na_3VO_4-1 mM phenylmethylsulfonyl flouride (pH 8.0; Sigma)] and centrifuged at 4°C. The supernatant was collected, brought to 1 ml total volume with TNT buffer [20 mM Tris-200 mM NaCl-1% Triton X-100 (Sigma)] and 20 μl of 20% protein A-Sepharose (Sigma), and rotated overnight at 4°C. Protein A-Sepharose had been preincubated in the presence of PG21 anti-human AR rabbit antiserum, which recognizes the first 20 amino acids of the human AR (kindly donated by Geoffrey Greene, University of Chicago). The samples were centrifuged, and the pellets were boiled in gel loading dye, resolved by 10% SDS-PAGE, and transferred to Hybond nitrocellulose membrane (Amersham, Buckinghamshire, England). The blots were incubated with a 2 μg/ml concentration of PG21 antiserum. Immunoreactive proteins were detected with the use of the enhanced chemiluminescence reagent system (Amersham).

Growth Assays. Cells were plated in 24-well plates (Costar, Cambridge, MA) at a density of 1×10^4 cells/well. These cells were grown in standard media for 24 h, at which point the media were changed to SFM containing 10 nM EGF, 5 nM DHT, or both. Media were replaced every 48 h. On day 6, cells were harvested by trypsinization (Sigma) and counted in a Coulter counter (Coulter Electronics Ltd., Luton, Beds., England). Cell viability was assessed by trypan blue (Sigma) exclusion with the use of a hemocytometer. Quadruplicate cultures were used for each assay.

RPAs. Total RNA from nonconfluent (70–80%) cell monolayers that had been grown in the presence or absence of 5 nM DHT in SFM for 48 h was isolated with RNAzol (TelTest, Friendswood, TX) according to the manufac-

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[3] The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; TGF, transforming growth factor; AR, androgen receptor; DHT, dihydrotestosterone; SFM, serum-free media; RPA, ribonuclease protection assay; IMEM, improved minimal essential media.

turer's instructions. RPAs were performed as described (18) and quantitated with the use of a Molecular Dynamics PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). For detection of EGFR mRNA, a 302-bp *SmaI-ClaI* cDNA fragment was inserted into pBlueScript SK+ (Statagene) for *in vitro* transcription of ^{32}P-labeled antisense RNA with T3 RNA polymerase (Stratagene). A 144-bp *PstI-HincII* fragment from the 5′ terminus of human β_2-microglobulin inserted into SP65 (19) was used for transcription of ^{32}P-labeled antisense RNA with SP6 polymerase (Stratagene). To normalize for the amount of RNA present in each sample, β_2-microglobulin antisense RNA served as an internal standard. The RPA presented here is representative of two independent experiments.

ELISA. Levels of EGFR and TGF-α were assessed with the use of sandwich-type ELISAs (Oncogene Science, Uniondale, NY). Cells (2.5×10^5) were plated in 75-cm^2 culture flasks (Costar) containing standard media. After 24 h the media were changed to SFM or SFM containing 5 nM DHT. The cells were incubated for 48 h, the culture media were removed and centrifuged, and the supernatant was saved. A solution of 2 mM EDTA in PBS (Sigma) was added to dislodge the cells. The cell suspension was centrifuged, and the pellet was resuspended in 10 volumes of receptor buffer [10 mM Tris-HCl (pH 7.4)-1.5 mM EDTA-10% glycerol-0.1% sodium azide-0.5 mg/ml leupeptin-1 μg/ml pepstatin-0.2 mM phenylmethylsulfonyl flouride (Sigma)]. Antigen extraction agent (Oncogene) was added, the sample was incubated at 4°C, centrifuged for 10 min, and the supernatant was retained for analysis. All determinations were made in triplicate. TGF-α was measured in cell lysate and media. Briefly, media were centrifuged and concentrated with the use of Amicon concentrators (Amicon, Beverly, MA). Protein concentration was measured with the use of the Pierce Micro BCA Protein Assay (Pierce Chemical Co., Rockford, IL) on a Bio-Rad Model 3550 Microplate Reader (Bio-Rad, Richmond, California). Samples were prepared in sample diluent (Oncogene), and the concentration of EGFR in fM/ml of sample was determined with the use of a standard absorbance curve. Numbers of EGFR molecules per cell were calculated from triplicate cell counts for each sample before antigen extraction.

RIA. EGF from cell lysates and media was prepared as above and measured with the use of a RIA (Amersham, Arlington Heights, IL). Murine EGF was used to construct a standard curve.

EGF-binding Assays. Competition binding studies compared the effects of increasing concentrations of EGF on inhibition of ^{125}I-labeled EGF binding to EGFR in both control and DHT-exposed cells during a 5-h incubation at 4°C. Data were corrected for nonspecific binding, defined as cell-associated radioactivity remaining in the presence of 1×10^{-6} M EGF. Specific binding represented less than or equal to 12% of the total amount of ^{125}I-labeled EGF added to any sample (20). Binding assays were carried out on nonconfluent (70–80%) cell monolayers at a cell density of $1–1.5 \times 10^6$ cells/ml for PC3neo and PC3-hAR cell lines. At time zero, serum containing media were aspirated and SFM was added. After 24 h, the cells were incubated for an additional 48 h in SFM with or without 5 nM DHT. Cells were then washed with IMEM 108 (Biofluids, Rockville, MD) supplemented with 0.1% BSA (Sigma) and preincubated for 1 h with Buffer 1 [IMEM 108-40 mM HEPES (Sigma)-0.1% BSA (pH 7.4)]. The cells were next incubated in Buffer 1 with the addition of 20 pM ^{125}I-labeled EGF (30,000 cpm/ml) and increasing amounts of EGF (1×10^{-12} M through 1×10^{-6} M). The incubation was performed for 5 h at 4°C. The media were removed, and the radioactivity was recorded. The cells were washed twice with PBS containing 0.1% BSA. Cells were incubated with 1 N NaOH (Baker, Philipsburg, NJ), and the radioactivity was recorded. A hemocytometer was used to measure cell numbers from controls in which Buffer 1 was added, without EGF. cpm were normalized for 1.2×10^6 cells. All radioactivity was measured on a Micromedic 10/600 γ counter (Micromedic, Horsham, PA) in polystyrene tubes (Fisher, Pittsburgh, PA). DHT was initially dissolved in 100% ethanol, with less than 0.1% ethanol added to any cell incubation.

Time Course-binding Studies. Time course assays were carried out as above, but triplicate wells of cells were incubated for selected intervals with 20 pM ^{125}I-labeled EGF in Buffer 1 at 4°C. Specific binding was determined by subtracting nonspecific binding from the total radioactivity recorded. Nonspecific binding is defined as steady-state cell-associated radioactivity measured after the incubation of cells with 1×10^{-6} M unlabeled EGF and 20 pM ^{125}I-labeled EGF. cpm were normalized to 1×10^6 cells.

Statistical Analysis. Mathematical modeling of competition binding data depended on equations derived to analyze dissociation constants applicable to incubations at equilibrium (21). Barring the existence of scientific error, data from inhibition of ^{125}I-labeled EGF binding by EGF for a single population of high affinity binding sites can be theoretically modeled according to the equation

$$\text{cpm} = V\left(\frac{K_D}{(K_d + EGF\ \text{conc})}\right) + B \qquad (1)$$

where *cpm* = the γ emissions recorded per min; B = the nonspecific background, *i.e.*, the cpm when the concentration of unlabeled EGF is infinite; V = the difference between the cpm when the concentration of unlabeled EGF is 0 and the nonspecific background; *conc* = the concentration of unlabeled EGF; and K_d = the apparent dissociation constant.

Statistical analysis of competition binding assays involved the following nonlinear model, which was specified for the conditional median of cpm given concentration of EGF based on theoretical considerations presented in equation 1:

$$\text{median cpm} = V\left(\frac{K_D}{(K_d + \text{EGF conc})}\right). \qquad (2)$$

Diagnostic plots, however, indicated a lack of homogeneity of variance in cpm conditional on EGF concentration. To account for this heterogeneity, transform-both-sides with weighting models (22) were fit, allowing the conditional variance to depend on the concentration of EGF and on the conditional median of cpm. The following model for cpm conditional on EGF concentration was fit via maximum likelihood:

$$\text{cpm}(\lambda) = V\left(\frac{K_D}{(K_d + \text{EGF conc})}\right)^{(\lambda)} + (-\log_{10}\text{EGF conc})^{\theta}\sigma\epsilon \qquad (3)$$

where $\epsilon \sim N(0,1)$, and $y^{(\lambda)}$ is the Box-Cox transformation of y (23).

All inference, including confidence intervals and t tests, about the structural parameters (V, K_d, and B) was made conditional on the estimated values of the variance parameters θ and λ, *i.e.*, assuming the estimated values were the true values. Because the uncertainty from estimating the variance parameters was not accounted for in making inference, confidence intervals and P values may be too narrow and too small, respectively. However, this conditional method of inference is widely used and accepted.

Statistical analysis of ELISA, RIA, time course-binding assays, and proliferation studies involved a pooled two-tailed Student's t test, which was performed for all experimental conditions relative to control cells. The data were assumed to conform to a normal distribution in each experiment, and significant values were defined as $P < 0.05$.

RESULTS

AR Expression in the PC3-hAR Cell Lines. In initial studies we characterized the expression of AR protein in the transfected clonal cell lines PC3-hAR-E, PC3-hAR-Q, and PC3-hAR-B and the control cell line PC3neo. For this purpose, whole cell lysates of PC3-hAR and PC3neo cells were immunoprecipitated with the use of an anti-AR antibody, PG21, and the immunoprecipitates were assessed with the

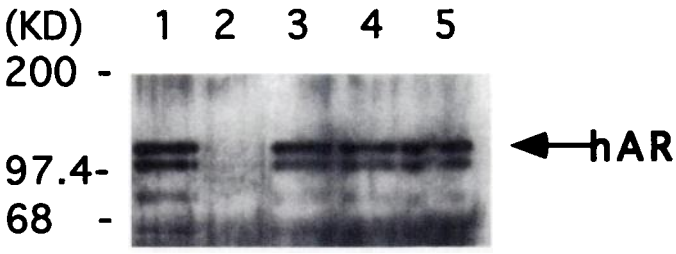

Fig. 1. Western analysis of human androgen receptor expression in stable PC3 transfectants. LNCaP (*Lane 1*), PC3neo (*Lane 2*), PC3-hAR-Q (*Lane 3*), PC3-hAR-B (*Lane 4*), and PC3-hAR-E (*Lane 5*) cell lysates were immunoprecipitated, resolved by SDS-PAGE, transferred to a nitrocellulose blot, and detected with the use of enhanced chemiluminescense. The *upper band*, representing the androgen receptor, is indicated by an *arrow* and was detected at M_r 110,000. *KD*, molecular weight in thousands. The lower bands presumably represent degradation products of the full length androgen receptor because they are variably present in similar assays.

Table 1 *Six-day EGF/DHT proliferation study*

Cell counts, expressed as mean values ± SD in thousands, of stably transfected PC3 cell lines, cultured for 6 days in the absence or presence of 10 nM EGF, 5 nM DHT, or both EGF and DHT.

Cell Line	SFM	EGF (10 nM)	DHT (5 nM)	EGF/DHT
PC3neo	127 ± 7	119 ± 8	121 ± 8	118 ± 10
PC3-hAR-B	21 ± 2	35 ± 2[a]	19 ± 4	34 ± 4[a]
PC3-hAR-E	21 ± 3	36 ± 5[a]	32 ± 3[a]	89 ± 9[a,b]
PC3-hAR-Q	20 ± 3	33 ± 5	36 ± 4[a]	96 ± 8[a,b]

[a] Statistically significant compared to SFM ($P < 0.05$).
[b] Statistically significant compared to EGF + DHT ($P < 0.05$).

use of Western analysis with the PG21 antibody (Fig. 1). This experiment revealed the expression of AR protein in the PC3-hAR-E, B, and Q cell lines, with the LNCaP cell line serving as a positive control for AR expression. No AR expression was detectable in the parental PC3neo line.

Proliferation Studies of PC3-hAR Cell Lines. Our previous studies have established that PC3-hAR cells are androgen sensitive, because they are still capable of some attenuated growth in SFM (16). In light of the modulation of the prostatic EGFR system by androgen (11, 12), we wished to evaluate both the separate and combined effects of EGF and DHT on PC3-hAR cell growth (Table 1). Administration of 10 nM EGF in SFM for a 6-day period resulted in increases in cell number of 71% for PC3-hAR-E cells, 67% for PC3-hAR-B cells, and 65% for PC3-hAR-Q cells, as compared to cells cultured in SFM alone. A similar growth-stimulatory effect was observed upon treatment with DHT; treatment with 5 nM DHT over 6 days produced a 52% increase in cell number in PC3-hAR-E cells and an 80% increase in PC3-hAR-Q cell number as compared to control cell number. However, no significant effects on growth by DHT were observed with the PC3-hAR-B cell line. When incubated in the presence of both EGF and DHT, a greater than 4-fold increase in cell

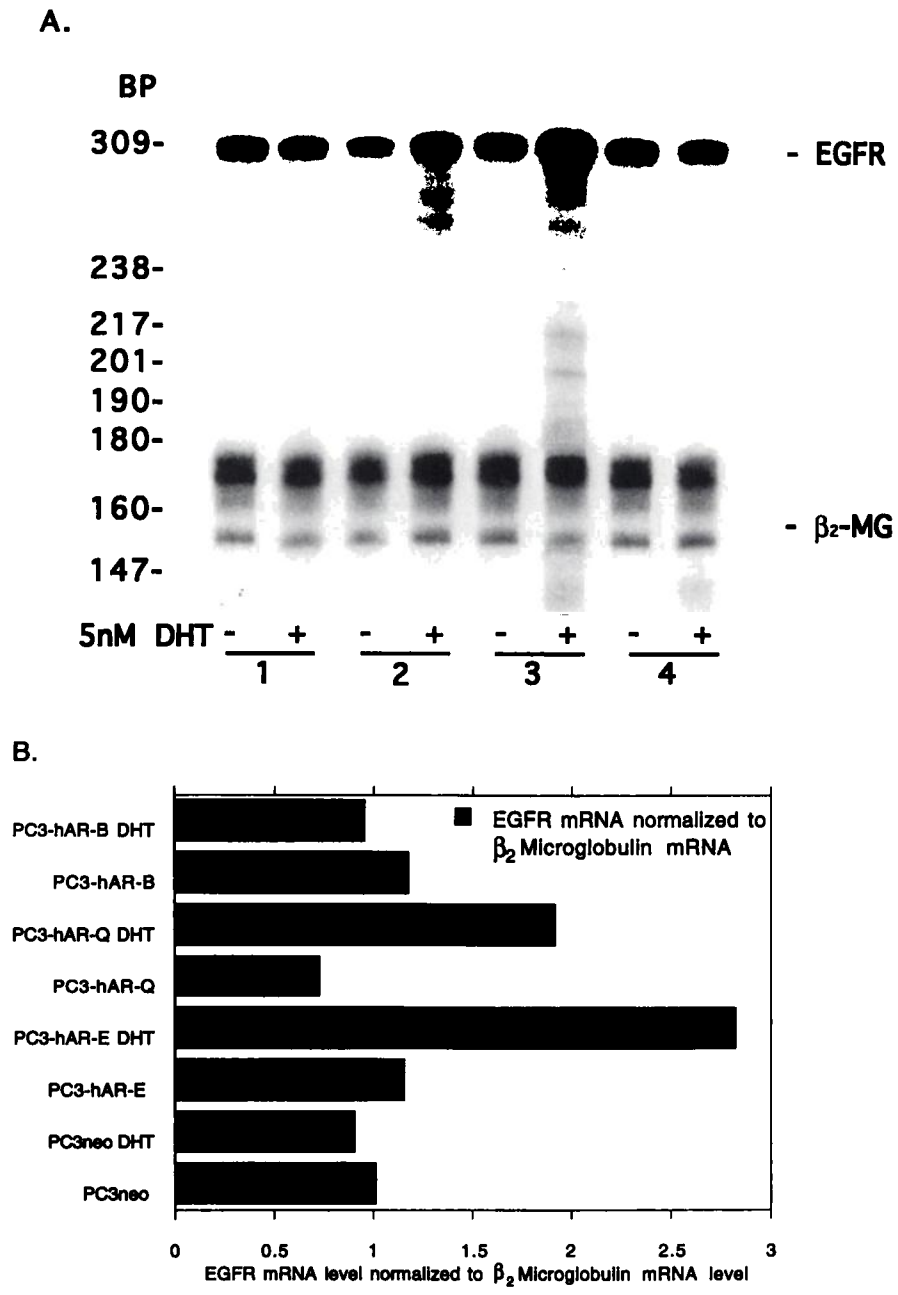

Fig. 2. *A*, RPA using ^{32}P-labeled antisense EGFR and β_2-microglobulin RNA riboprobes to assess the effect of 5 nM DHT on EGFR expression in stable PC3 transfectant cells. Total RNA was isolated from PC3neo (*1*), PC3-hAR-Q (*2*), PC3-hAR-E (*3*), and PC3-hAR-B (*4*) cells grown in the presence (+) or absence (−) of 5 nM DHT for 48 h. BP, nucleotide bp markers are ^{32}P-end-labeled DNA fragments of the PBR322 vector restricted with *MspI* (Promega Corporation, Madison, WI). *B*, PhosphorImager analysis of EGFR expression normalized to β_2-microglobulin expression.

number was recorded in both the PC3-hAR-E and PC3-hAR-Q cell lines *versus* control samples. This synergistic effect on proliferation by 10 nM EGF and 5 nM DHT was greater than the predicted additive response by 31% for PC3-hAR-E cells ($P < 0.05$) and 39% for PC3-hAR-Q cells ($P < 0.01$). The PC3-hAR-B cells demonstrated a growth response in EGF-DHT-containing media comparable to that observed in the presence of EGF alone.

Effect of Androgen on EGFR Expression in PC3-hAR Cell Lines. In view of previous reports of elevated EGFR expression in androgen-exposed prostate cancer cells (11, 12), we next examined the expression of *EGFR* gene transcripts in DHT-treated PC3-hAR cells. We theorized that androgen might serve to enhance the mitogenic effects of EGF by upregulating EGFR expression in the PC3-hAR cells. The effect of a 48-h treatment with 5 nM DHT on EGFR steady-state transcript levels in the PC3-hAR-E, B, Q, and PC3neo cell lines was assessed with the use of RPAs (Fig. 2). On the basis of the PhosphorImager analysis of these experiments, we determined that a greater than 2-fold increase in steady-state EGFR transcript level was induced in the PC3-hAR-E and PC3-hAR-Q cells after exposure to androgen. However, no fluctuation in mRNA level was seen in the PC3neo or PC3-hAR-B cells under comparable conditions.

To evaluate whether this androgen-stimulated increase in steady-state EGFR mRNA in the PC3-hAR-E and PC3-hAR-Q cell lines is associated with an increase in EGFR protein expression, we performed ELISAs. Extracts from PC3neo and PC3-hAR cell lines incubated for 48 h in SFM with or without DHT were assayed, with resultant values of fM/ml of EGFR being normalized for protein and cell number (Table 2). In the absence of androgen, EGFR levels were shown to be similar in the PC3neo control line and PC3-hAR cells, and these receptor values reflected those in the literature (Ref. 10; Fig. 3). In contrast, the PC3-hAR-E and PC3-hAR-Q clones displayed a 55% ($P < 0.03$) and 53% ($P < 0.003$) increase in EGFR protein in cells incubated in DHT-supplemented SFM compared to cells grown in SFM alone. No such increase in EGFR number in PC3neo and PC3-hAR-B DHT-exposed cells was noted.

Table 2 *Expression of EGFR protein by ELISA*

EGFR protein expression in PC3neo and PC3-hAR cells cultured in the presence or absence of 5nM DHT, as measured by ELISA with fM/ml of EGFR normalized for μg of protein.

Cell line	Concentration of DHT	EGFR protein/cell extract (fm EGFR/μg protein ± SD)
PC3neo	0	1.67 ± 0.46
	5 nM	1.86 ± 0.67
PC3-hAR-E	0	1.89 ± 0.71
	5 nM	2.28 ± 0.61[a]
PC3-hAR-Q	0	1.69 ± 0.45
	5 nM	2.71 ± 0.74[a]
PC3-hAR-B	0	1.83 ± 0.34
	5 nM	1.77 ± 0.62

[a] Statistically significant compared to 0 DHT control ($P < 0.01$).

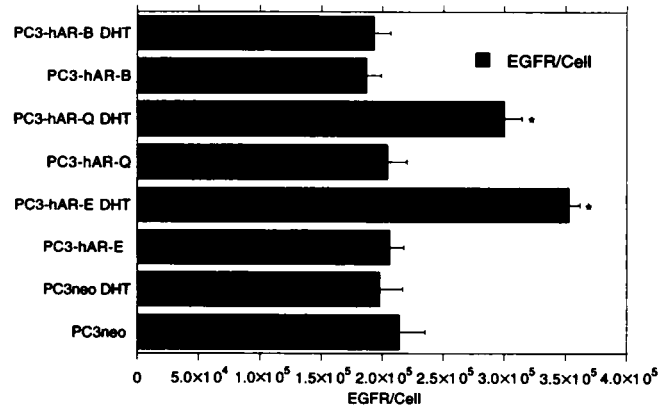

Fig. 3. The effect of androgen on EGFR number under serum-free conditions using ELISA protein quantification. Nonconfluent (70–80%) prostate cancer cell monolayers were incubated in the presence or absence of 5 nM DHT. After 48 h, the cells were harvested and counted prior to execution of EGFR ELISAs. *Columns*, mean; *bars*, SD. *, statistically significant ($P < 0.05$).

Table 3 *Expression of TGF-α and EGF protein levels by ELISA and RIA*

TGF-α and EGF expression in PC3neo and PC3-hAR cells grown in the presence or absence of 5 nM DHT for 48 h, as measured by ELISA and RIA, respectively.

Cell Line	Concentration of DHT	TGF-α protein[a] (fM TGF-α/μg cell protein ± SD)	EGF protein[b] (pg EGF/μg cell protein ± SD)
PC3neo	0	0.119 ± 0.029	2.40 ± 1.53
	5 nM	0.109 ± 0.035	2.85 ± 1.95
PC3-hAR-E	0	0.062 ± 0.055	2.46 ± 2.81
	5 nM	0.025 ± 0.024[c]	3.52 ± 3.52
PC3-hAR-Q	0	0.012 ± 0.009[c]	2.86 ± 1.66
	5 nM	0.021 ± 0.008[c]	3.09 ± 1.56
PC3-hAR-B	0	0.012 ± 0.001[c]	No data
	5 nM	0.005 ± 0.001[c]	No data

[a] ELISA.
[b] RIA.
[c] Statistically significant compared to PC3neo controls ($P < 0.05$).

Effect of Androgen on EGF and TGF-α Expression in the PC3-hAR Cell Lines. A number of previous studies have reported the existence of an EGF/TGF-α autocrine growth loop in PC3 cells (10). An increase in EGF/TGF-α production by androgen might result in an enhancement of such an autocrine growth loop in the PC3-hAR cells, and thus account for the observed synergistic effect of DHT and EGF on PC3-hAR cell growth. To test this theory we undertook TGF-α ELISAs and EGF RIAs, designed to detect any variation in TGF-α or EGF levels in the DHT-exposed cells or their culture media as compared to controls. Basal levels of TGF-α were detected in both the conditioned media (data not shown) and cell lysates of the PC3neo and PC3-hAR cell lines, with no differences observed between DHT and control groups (Table 3). A decrease in overall cell-associated TGF-α was observed in the PC3-hAR cells as compared to the PC3neo controls. All of the cell lines tested with RIAs exhibited expression of EGF in both lysate and media. Exposure to 5 nM DHT for a period of 48 h did not alter the steady-state level of EGF present in the cell lysates (Table 3) or media (data not shown) of any cell line studied in comparison with controls.

Modulation of EGF-EGFR Binding Affinity in PC3-hAR Cell Lines Exposed to Androgen. In addition to elevating EGFR expression in the PC3-hAR cells, androgen might also alter the EGFR-EGF-binding affinity and thereby potentiate the mitogenic signaling activity of the EGFR system. To address this possibility we carried out competition binding studies involving EGFR-EGF interactions in androgen-free or androgen-supplemented media (Fig. 4). The inhibition of ^{125}I-labeled EGF by EGF in all cell lines studied can be described by EGF interaction with a single population of high affinity binding sites. No significant differences in apparent K_d were observed between the PC3-hAR-B and PC3neo cells treated with DHT *versus* control cells. However, a statistically significant increase in receptor-ligand affinity was observed in the PC3-hAR-E ($P < 0.001$) and PC3-hAR-Q ($P < 0.005$) cells cultured in media containing androgen. As predicted by our ELISA data, an increase in maximal EGF binding was also observed in the PC3-hAR-E and PC3-hAR-Q cells exposed to DHT. It is important to note that in the absence of androgen no significant variation in derived EGFR-EGF dissociation constants was noted between the transfected lines and the PC3neo control line.

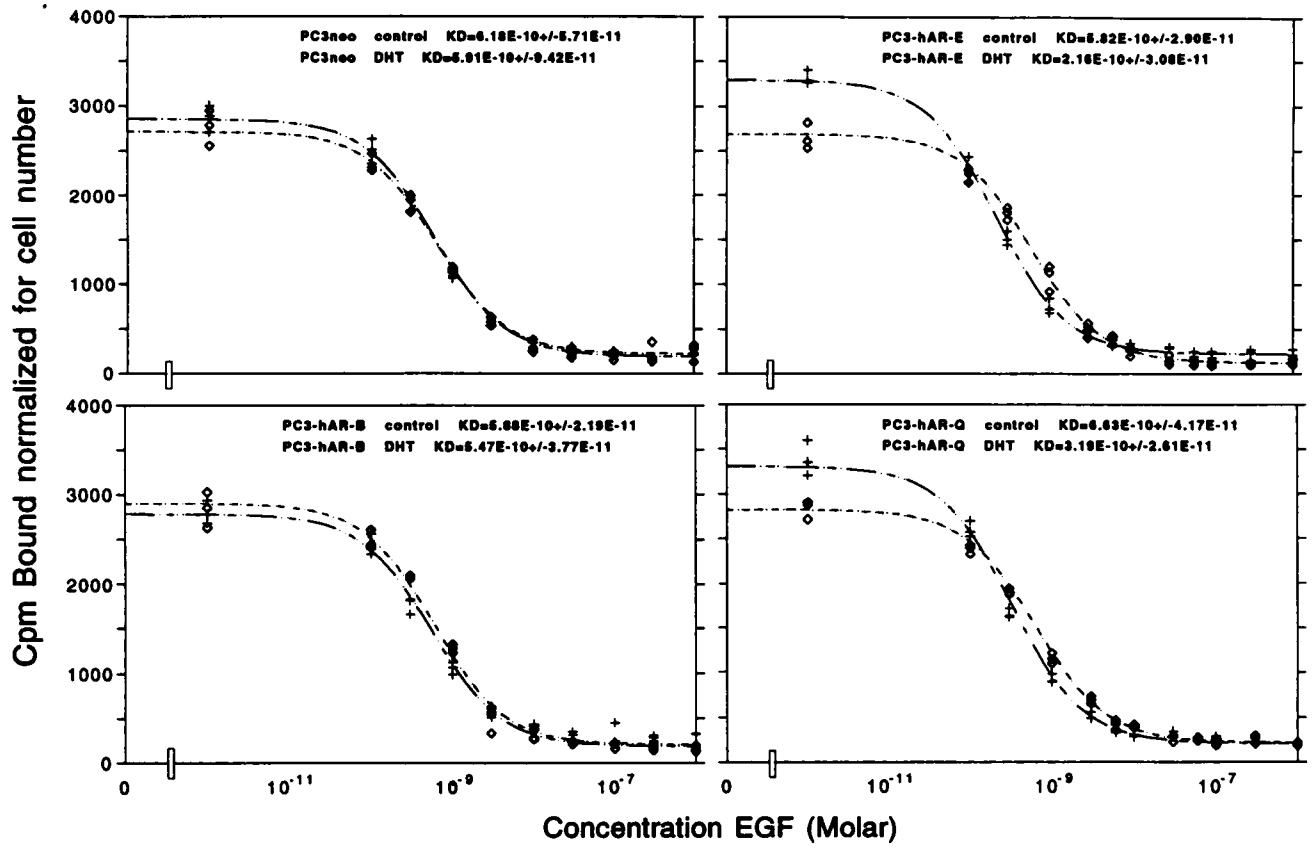

Fig. 4. EGFR-EGF-binding affinity as determined by the displacement of ^{125}I-labeled EGF by nonradioactive EGF. Prostate cancer cell line monolayers were cultured in the presence (+, · — ·) or absence (◇, - · -) of 5 nM DHT for 48 h, at which point they were incubated with 20 pM ^{125}I-labeled EGF and increasing amounts of nonradioactive EGF at 4°C for 5 h. Binding assays were carried out on nonconfluent (70–80%) cell monolayers at a cell density of 1–1.5 × 10^6 cells/ml for PC3neo and PC3-hAR cell lines. cpm were normalized for cell number. All curves were mathematically modeled to a single high affinity EGF binding site. All determinations were made in triplicate. *KD*, calculated dissociation constant ± SD.

We next utilized time course radioligand-binding experiments in an attempt to further investigate the ability of DHT to up-regulate EGF binding in the PC3-hAR cells, as reflected by the ELISA and competition binding study results. Nonconfluent cells were cultured for 48 h with or without 5 nM DHT in SFM, then incubated for selected time intervals with ^{125}I-labeled EGF. The cells were washed and then lysed, with the amount of receptor-bound ^{125}I-labeled EGF in the cell lysate reported in cpm (Fig. 5). In the absence of androgen, no difference was observed in the overall EGF binding capacity of the PC3neo *versus* the PC3-hAR cells. Importantly, the PC3-hAR-E ($P < 0.05$) and PC3-hAR-Q ($P < 0.02$) cell lines showed a statistically significant increase in overall EGF binding when cultured with androgen. Both the PC3neo and PC3-hAR-B cell lines showed no fluctuation in ligand binding in the presence of DHT in SFM, as compared to controls cultured in SFM alone. Thus, the DHT-induced increase in EGFR protein observed in the ELISAs correlated with an increase in overall EGF binding in the PC3-hAR-E and PC3-hAR-Q cell lines exposed to androgen.

DISCUSSION

In this paper we have utilized AR-negative PC3 cells expressing ectopic hAR to study the interactions of the AR with the EGFR system. The PC3-hAR cell lines display an androgen-sensitive phenotype and thus serve as a useful model for delineating the functions of the AR in steroid-sensitive prostate cancer cells. However, it is important to note that the PC3-hAR cells can grow slowly in steroid-free conditions and, therefore, do not entirely recapitulate an androgen-dependent prostate cancer cell phenotype.

The results presented here demonstrate that a physiological concentration of androgen induces proliferation, increases EGFR expression, and elevates EGFR-EGF binding affinity in the androgen-sensitive PC3-hAR cell lines *in vitro*. It has been established that both EGF and TGF-α can initiate proliferation of androgen-sensitive prostate cancer cells (11, 12). Our studies suggest that EGF can produce a similar mitogenic response in PC3 cell lines with a genetically reconstituted androgen-sensitive phenotype. More significantly, our research indicates that although EGF and DHT independently induce proliferation in the PC3-hAR cell lines, their combined effect on growth was of a synergistic nature.

In an attempt to elucidate a possible molecular mechanism to explain these observations, we undertook RPAs, ELISAs, and radioligand binding studies to reveal whether any changes in EGFR expression or binding characteristics occurred in PC3-hAR cells cultured in the presence of androgen. Expression of AR protein in the PC3-hAR-E and PC3-hAR-Q cells serves to up-regulate EGFR mRNA expression in the presence of androgen. Although exposure to androgens was also noted to elevate EGFR mRNA in the ALVA 101 cell line (11), there is no reported androgen response element in the region of the *EGFR* gene (24, 25). The majority of tumors overexpressing EGFR do so via transcriptional mechanisms, with EGFR mRNA levels closely paralleling EGFR protein expression (26). An EGFR-specific transcription factor has been reported (27), and an EGFR transcriptional repressor has been identified in HeLa and A431

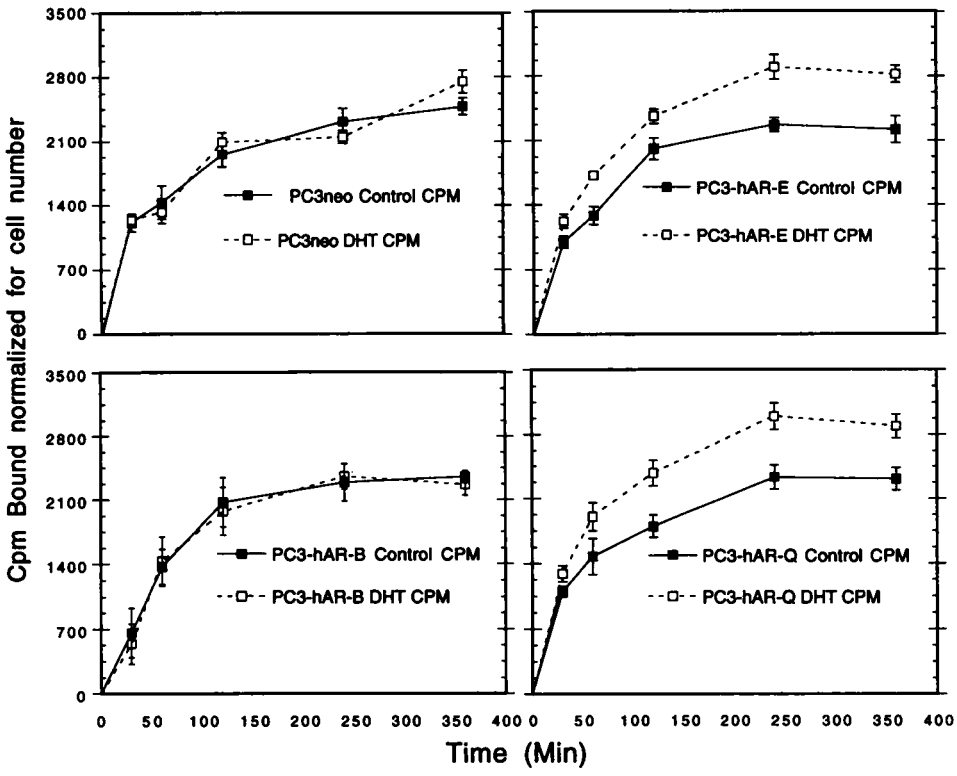

Fig. 5. Time course ^{125}I-labeled EGF binding to cultured prostate cancer cell lines at 4°C. Cells were cultured for 48 h with 5 nM DHT, washed, and then incubated with 20 pM ^{125}I-labeled EGF in binding buffer for selected periods. cpm were normalized for cell number. The assays were performed in triplicate. *Points*, mean; *bars*, SD.

cells (28). The role of an androgen response element, if any, in the transcriptional regulation of the genes encoding these two proteins remains unknown; thus, no direct mechanism for the control of EGFR transcription by androgen is apparent.

Androgens have been shown to increase EGFR protein levels in the androgen-sensitive ALVA 101 and LNCaP cell lines (11, 12). In the present study, ELISAs revealed elevated EGFR protein levels in the androgen-responsive PC3-hAR cell lines exposed to androgen. An induction of EGF binding in these same cell lines was also demonstrated by time course radioligand binding analysis. In combination with the increase in EGFR-EGF binding affinity, these data suggest that the enhanced proliferation of PC3-hAR cells cultured in the presence of EGF and DHT may be attributable to a DHT-mediated increase in EGFR number and binding affinity. The observed increase in overall EGF binding, combined with the 2-fold increase in receptor affinity, could result in an elevated mitogenic response in the PC3-hAR cells exposed to both EGF and DHT, due to the inherent signal amplification occurring in the EGFR signal transduction pathway. An additional explanation for the synergistic growth promoting effects of DHT and EGF might involve EGF/TGF-α-mediated stimulation of hAR activity. A study using transient expression systems in an AR-negative prostate cell line under steroid-depleted conditions has shown that a number of cytokines, including EGF, are capable of stimulating the expression of a reporter construct containing a tandem androgen response element (29). On the basis of these observations, it is possible that the activation of the hAR by EGF (29) and the up-regulation of the EGFR system by androgen suggested by our results, may both play roles in the growth-promoting activities of EGF and DHT. It will be of great interest to further elucidate the mechanisms of "cross-talk" occurring between the AR and EGFR systems.

A possible modulation of an EGFR/TGF-α autocrine growth loop by androgen in the ALVA 101 cell line has been reported (11). A similar androgen-induced increase in growth factor production could account for the mitogenic effect detected in the androgen-sensitive PC3-hAR cells cultured in the presence of DHT alone under serum-free conditions. We tested for this by analyzing the EGF and TGF-α protein levels in the cell lysate and culture media of androgen-exposed cells. No fluctuation was detected in the concentration of these growth factors in cells cultured with androgen. From these results we conclude that DHT exposure does not potentiate an EGF/TGF-α-based autocrine growth loop in the PC3-hAR cells via an up-regulation of cytokine production. However, we cannot exclude the possibility that another growth factor that acts through the EGFR pathway might be controlled directly or indirectly by androgens in these cell lines. The family of growth factors related to EGF continues to expand, with several previously uncharacterized EGFR ligands reported to be detectable in the conditioned media of the DU145 prostate cancer cell line; these proteins may represent isoforms of known EGF ligands not recognized in our ELISAs or RIAs, or may be novel growth factors (30). It is important to note that cell-associated TGF-α levels were decreased in the PC3-hAR cells as compared to the PC3neo controls. Such a decrease in growth factor production may result in a reduction of the endogenous EGF/TGF-α autocrine growth loop in the PC3-hAR cells. This alteration in TGF-α production may account for the ability of exogenous EGF to stimulate growth in the PC3-hAR cells although having no effect on the PC3neo cell line because the latter cells may produce sufficient endogenous cytokines to maximally stimulate the EGFR pathway. In addition, an attenuated EGF/TGF-α autocrine pathway may serve to explain why the PC3-hAR cells grow more slowly than the PC3neo controls in SFM. However, whether the alterations in TGF-α production in the PC3-hAR cells or the growth characteristics of these cells in SFM is attributable to specific or nonspecific actions of the ectopically expressed hAR is presently unclear.

Using radioligand binding analysis, we detected a reproducible and statistically significant 2-fold increase in EGFR-EGF-binding affinity in the two androgen-sensitive PC3-hAR cell lines. Although glucocorticoids have been shown to modulate high affinity EGFR-binding site populations in HeLa cells (31), and phorbol esters have been demonstrated to control EGFR binding via a MAP kinase-controlled phosphatase (32), this is the first time an androgen-medi-

ated EGFR affinity change has been shown in prostate cancer cells. Previous studies involving the LNCaP prostate cancer cell line reported no modulation of EGF binding by androgen (12, 33). The LNCaP cell line is androgen sensitive and displays altered steroid binding and response traits due to a point mutation in the AR (34, 35). PC3 cell lines expressing the normal human androgen receptor were utilized in the present study (16). The normal and mutated ARs might possess differences in DNA-binding specificity, conformation upon DNA binding, or transcriptional activity upon interaction with a given androgen response element. These factors determine what target genes will be transactivated in a given cell and might account for the observed differences in the control of EGFR-binding characteristics by androgen.

The androgen refractory behavior exhibited by the PC3-hAR-B cell line is intriguing, as the ability of these cells to respond to EGF suggests that the EGF-signaling pathway is extant. Although comparable levels of AR expression were detected in all three of the PC3-hAR cell lines, the possibility remains that the transfected *AR* gene has undergone mutation in this cell line. Alternatively, a more distal component of the AR-signaling pathway may be mutated or its normal pattern of expression perturbed, perhaps due to an insertion of the transfected DNA segment. Further analysis is in progress to establish the molecular processes underlying the androgen-insensitive phenotype of this cell line.

Our data demonstrate that expression of the AR in PC3 cells exposed to androgen is sufficient to initiate a biological response in the form of increased EGFR expression, elevated EGFR-EGF-binding affinity, and enhanced EGF-induced cell growth in a serum-free environment. This work represents the most direct experimental demonstration of androgenic control of a peptide growth factor pathway in a prostate cancer cell line. In combination with the published observations of similar phenotypic characteristics in LNCaP and ALVA 101, our results support and extend the hypothesis that the proliferative action of androgen stimulation in AR-expressing prostate cancer cells involves peptide growth factor pathways. Although previous investigations have reported the existence of an EGFR/TGF-α autocrine loop controlled by androgens, we detected no change in EGF or TGF-α protein levels in the PC3-hAR cell lines cultured with androgen. In addition, our proliferation studies show that DHT alone in SFM was capable of enhancing PC3-hAR cell growth. Together these results suggest that prostate cancer cell growth regulation by androgens may involve other additional, and as yet uncharacterized, growth factors or auto-stimulatory signaling events (10).

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REFERENCES

1. Sandberg, A. A. Endocrine control and physiology of the prostate. Prostate, *1:* 169–184, 1980.
2. Griffiths, K., Davies, P., and Eaton, C. L. Cancer of the prostate: endocrine factors. *In:* J. R. Clarke (ed.), Oxford Reviews of Reproductive Biology, pp. 192–210. London: Oxford Publications, 1987.
3. Traish, A. M., and Wotiz, H. H. Prostatic epidermal growth factor receptors and their regulation by androgens. Endocrinology, *121:* 1461–1467, 1987.
4. Eaton, C. L., Davies, P., and Philips, M. E. A. Growth factor involvement and oncogene expression in prostatic tumors. J. Steroid Biochem., *30:* 341–345, 1988.
5. Gelman, E. P. Oncogenes and growth factors in prostate cancer. J. Natl. Inst. Health Res., *3:* 62–64, 1991.
6. Samsbury, J. R. C., Farndon, J. R., Sherbert, G. V., and Harris, A. L. Epidermal growth factor receptors and oestrogen receptors in human breast cancer. Lancet, *1:* 364–346, 1985.
7. Habib, F. K., and Chisholm, G. D. The role of growth factors in the human prostate. Scand. J. Nephrol. Suppl., *126:* 53–58, 1991.
8. Derynck, R. Transforming growth factor-α and -β and their potential roles in neoplastic transformation. Cancer Treat. Res., *47:* 177–195, 1989.
9. Connolly, J. M., and Rose, D. P. Production of epidermal growth factor and transforming growth factor-α by the androgen-responsive LNCaP human prostate cancer cell line. Prostate, *16:* 209–218, 1990.
10. Hofer, D. R., Sherwood, E. R., Bromberg, W. D., Mendelsohn, J., Lee, C., and Kozlowski, J. Autonomous growth of androgen-independent human prostatic carcinoma cells: role of transforming growth factor α. Cancer Res., *51:* 2780–2785, 1991.
11. Liu, X., Wiley, H. S., and Meikle, A. W. Androgens regulate proliferation of human prostate cancer cells in culture by increasing transforming growth factor-α and epidermal growth factor/TGF-α receptor. J. Clin. Endocrin., *77:* 1472–1478, 1993.
12. Schuurmans, A. L. G., Bolt, J., and Mulder, E. Androgens stimulate both growth rate and epidermal growth factor receptor activity of the human prostate tumor cell line LNCaP. Prostate, *12:* 55–63, 1988.
13. Lippman, M. E., Dickson, R. B., and Bates, S. Autocrine and paracrine growth regulation of human breast cancer. Breast Cancer Res. Treat., *7:* 59–70, 1986.
14. Koga, M., and Sutherland, R. L. EGF partially reverses the inhibitory effects of antiestrogens on T47-D human breast cancer cell growth. Biochem. Biophys. Res. Commun., *146:* 738–745, 1987.
15. Kaighn, M. E., Narayan, K. S., Ohnuki, Y., Lechner, J. F., and Jones, L. W. Establishment and characterization of a human carcinoma cell line (PC3). Invest. Urol., *17:* 16–23, 1979.
16. Hansen, N., Chang, C., Chodak, G. W., and Rukstalis, D. B. Modulation of hormone sensitivity in PC3 cells by transfection of a normal human androgen receptor. American College of Surgeons Surgical Forum, *43:* 745–748, 1992.
17. Kokontis, J., Kiyoshi, I., Hiipakka, R., and Liao, S. Expression and function of normal and LNCaP androgen receptors in androgen-sensitive human prostatic cancer cells. Receptors, *1:* 271–279, 1991.
18. Miller, C. L., Feldhaus, A. L., Rooney, J. W., Rhodes, L. D., Sibley C. H., and Singh, H. Regulation and a possible stage specific function of Oct-2 during pre-B-Cell differentiation. Mol. Cell. Biol., *11:* 4885–4894, 1991.
19. Kokontis, J., Takakura, K., Hay, N., and Liao, S. Increased androgen receptor activity and altered c-*myc* expression in prostate cancer cells after long-term androgen deprivation. Cancer Res., *54:* 1566–1573, 1994.
20. Goldstein, A., and Barrett, R. W. Ligand dissociation constants from competition binding assays: errors associated with ligand depletion. Mol. Pharmacol., *31:* 603–609, 1987.
21. Bharucha, D. B., and Tager, H. S. Analysis of glucagon-receptor interactions on isolated canine hepatocytes. J. Biol. Chem., *265:* 3070–3079, 1990.
22. Carroll, R. J., and Ruppert, D. Transformation and Weighting in Regression, New York: Chapman and Hall, 1988.
23. Box, G. E. P., and Cox, D. R. An analysis of transformations. J. R. Stat. Soc. B, *26:* 211–246, 1964.
24. Hudson, L. G., Santon, J. B., and Gill, G. N. Regulation of epidermal growth factor gene expression. Mol. Endocrinol., *3:* 400–408, 1989.
25. Ischii, S., Xu, Y. H., Stratton, R. H., Roc, B. A., Merlino, G. T., and Pastan, I. Characterization and sequence of the promoter region of the human epidermal growth factor receptor gene. Proc. Natl. Acad. Sci. USA, *82:* 4920–4924, 1985.
26. Xu, Y. H., Richert, N., Ito, S., Merlino, G. T., and Pastan, I. Characterization of epidermal growth factor receptor gene expression in malignant and normal human cell lines. Proc. Natl. Acad. Sci. USA, *81:* 7308–7312, 1984.
27. Kageyama, R., Merlino, G. T., and Pastan, I. A transcription factor active on the epidermal growth factor receptor gene. Proc. Natl. Acad. Sci. USA, *85:* 5016–5020, 1988.
28. Hou, X., Johnson, A., and Rosner, M. R. Identification of an epidermal growth factor receptor transcriptional repressor. J. Biol. Chem., *269:* 4307–4312, 1994.
29. Culig, Z., Hobisch, A., Cronauer, M., Radmayr, C., Trapman, J., Hittmair, A., Bartsch, G., and Klocker, H. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor 1, keratinocyte growth factor and epidermal growth factor. Cancer Res., *54:* 5474–5478, 1994.
30. MacDonald, A., Chisholm, G. D., and Habib, F. K. Production and response of a human prostatic cancer line to transforming growth factor-like molecules. Br. J. Cancer, *62:* 579–584, 1990.
31. Fanger, B. O., Viceps, A., Madore, D., and Cidlowski, J. A. Regulation of high and low affinity epidermal growth factor receptors by glucocorticoids. Arch. Biochem. Biophys. *235:* 141–149, 1984.
32. Griswold-Prenner, I., Carlin, C. R., and Rosner, M. R. Mitogen-activated protein kinase regulates the epidermal growth factor receptor through activation of a tyrosine phosphatase. J. Biol. Chem., *268:* 13050–13054, 1993.
33. MacDonald, A., and Habib, F. K. Divergent responses to epidermal growth factor in hormone sensitive and insensitive human prostate cancer cell lines. Br. J. Cancer, *65:* 177–182, 1992.
34. Veldscholte, J., Berrvoets, C. A., Ris-Staplers, C., Kuiper, G., Jenster, G., Trapman, J., Brinkman, A. O., and Mulder, E. The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. J. Steroid Biochem., *41:* 665–669, 1992.
35. Horoszewicz, J. S., Leong, S. S., Kawinski, E., Karr, J., Rosenthal, H., Chu, T. M., Mirand, E. A., and Murphy, G. P. LNCaP model of human prostatic carcinoma. Cancer Res., *43:* 1809–1818, 1983.