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# Expression of Estrogen Receptor (ER)- $\alpha$ and ER- $\beta$ in Normal and Malignant Prostatic Epithelial Cells: Regulation by Methylation and Involvement in Growth Regulation<sup>1</sup>

Kin-Mang Lau, Mark LaSpina,<sup>2</sup> John Long, and Shuk-Mei Ho<sup>3</sup>

Division of Urology, Department of Surgery, University of Massachusetts Medical School, Worcester, Massachusetts 01655 [K-M. L., S-M. H.], Department of Biology, Tufts University, Medford, Massachusetts 02155 [K-M. L., M. L.] and Urological Clinic, New England Medical Center, Boston, Massachusetts 02111 [J. L.]

## ABSTRACT

The aim of the current study is to demonstrate normal and malignant prostatic epithelial cells (PrECs) as targets for receptor-mediated estrogenic and antiestrogenic action. Using an improved protocol, we have successfully isolated and maintained highly enriched populations of normal PrECs from ultrasound-guided peripheral zone biopsies, individually determined to be morphologically normal. Semiquantitative reverse transcription-PCR analyses were used to determine whether transcripts of estrogen receptor (ER)- $\alpha$  and those of ER- $\beta$  were expressed in our normal PrEC primary cultures, in a commercially available PrEC preparation (PrEC; Clontech), in an immortalized PrEC line established from a benign prostatic hyperplasia specimen (BPH-1), and in three prostatic cancer cell lines (LNCaP, PC-3, and DU145). Expression levels of ER- $\alpha$  and ER- $\beta$  transcripts were related to those of two estrogen-responsive genes [progesterone receptor (PR) and pS2], at the message levels, to gain insights into the functionality of the ER subtypes in PrECs. Interestingly, only transcripts of ER- $\beta$ , but not those of ER- $\alpha$ , were found in our primary cultures of normal PrECs, along with both PR and pS2 mRNA. These data strongly suggest that estrogen action was signaled exclusively via ER- $\beta$  in normal human PrECs. In contrast, PrEC (Clontech) and BPH-1 cells expressed both ER- $\alpha$  and ER- $\beta$  transcripts and no PR nor pS2 mRNA in PrEC and only a minimal level of PR mRNA in BPH-1. Among the three prostate cancer cell lines, LNCaP expressed ER- $\beta$  mRNA along with transcripts of PR and pS2, DU145 expressed messages of ER- $\beta$  and PR, and PC-3 cells exhibited ER- $\alpha$ , ER- $\beta$ , and pS2 mRNA. Thus, unlike normal PrECs, expression patterns of these genes in malignant PrECs are more variable.

Treatment of prostate cancer cells with demethylation agents effectively reactivated the expression of ER- $\alpha$  mRNA in LNCaP and DU145 and that of pS2 message in DU145. These findings provide experimental evidence that ER- $\alpha$  gene silencing in prostate cancer cells, and perhaps also in normal PrECs, are caused by DNA hypermethylation.

To evaluate the potential of using antiestrogens as prostate cancer therapies, we have assessed the growth-inhibitory action of estrogens (estradiol and diethylstilbestrol) and antiestrogens (4-hydroxy-tamoxifen and ICI-182,780) on PC-3 and DU-145 cells. In PC-3 cells, which express both ER subtypes, estrogens as well as antiestrogens are effective inhibitors. In contrast, in DU145 cells, which express only ER- $\beta$ , antiestrogens, but not estrogens, are growth inhibitors. By comparison, ICI 182,780 is the more effective cell growth inhibitor. Importantly, the ICI 182,780-induced antiproliferative effects were reversed by cotreatment of DU145 cells with an ER- $\beta$  antisense oligonucleotide, hence lending additional support to a central role played by ER- $\beta$  in mediating growth-inhibitory action of antiestrogens.

## INTRODUCTION

Charles Huggins pioneered the use of the synthetic estrogen, DES,<sup>4</sup> in the treatment of advanced PCa in the early 1940s (1). The action of DES was thought to be mediated via a blockade of the pituitary-testicular axis, which effectively lowered circulating levels of androgen and caused tumor regression (2). However, recent investigations have demonstrated that DES exerts direct growth-inhibitory effects on prostatic cancer cells via induction of mitotic arrest or apoptosis (3–6). Unfortunately, because of serious adverse effects induced by the estrogenicity of DES (feminization, exacerbation of heart failure, vascular complications, gynecomastia, and impotence), the xenoestrogen has lost its attractiveness as a mainstay treatment for advanced PCa (7). Clinical use of TAM, a nonsteroidal estrogen mixed agonist/antagonist, was introduced in the 1980s as an alternative to DES in the treatment of PCa. It was better tolerated than DES but only produced low response rates (8–12). It was therefore concluded that further investigation of TAM in advanced PCa treatment was not warranted. Of late, two recent developments have rekindled interests in using antiestrogens as therapeutics for PC: (a) pure estrogen antagonists (e.g., ICI-164,384 and ICI-182,780; Ref. 13) and selective estrogen receptor modulators, such as raloxifene (14), have become available for clinical trials. Experimental evidence suggests that their mechanisms of action may differ significantly from those of estrogens (15–18) and therefore may yield more favorable outcomes; and (b) a newly discovered ER subtype (ER- $\beta$ ) was found to be expressed at high levels in the epithelial compartments of the rat prostate (19–23). Although ER- $\beta$  shares high homology with the classical ER (ER- $\alpha$ ), the two ER subtypes may regulate different sets of cellular functions (18, 24). Recent findings from an ER- $\beta$  knockout mouse suggest that ER- $\beta$  may suppress proliferation and prevent hyperplasia in the rodent prostate (25). Taken together, these new findings raise an intriguing possibility that ER- $\beta$  is expressed in normal and/or malignant human PrECs and plays a role in mediating estrogen action in these cell types.

Knowledge of the distribution of ER- $\beta$  in normal and malignant human PrECs is limited at this time. A recent study reported a lack of ER- $\beta$  expression in human prostate tissues (26), whereas several preliminary reports noted expression of this receptor subtype in basal epithelial cells of the human (27–29). In this study, we reported the development of an effective method to obtain and culture “pure” or highly enriched populations of normal PrECs from needle biopsies of the peripheral zone of the human prostate. Expression levels of ER- $\alpha$  and ER- $\beta$  transcripts in our primary cultures of normal PrECs were compared with those found in a PrEC preparation obtained from a commercial source (PrEC; Clontech), in an immortalized PrEC cell line established from a BPH specimen (BPH-1; Ref. 30), and in three prostatic cancer cell lines (DU145, PC-3, and LNCaP). Expression

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<sup>2</sup> Present address: New England College of Osteopathy, Biddeford, ME 04005.

<sup>3</sup> To whom requests for reprints should be addressed, at Division of Urology, Department of Surgery, Rm S4-746, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655. E-mail: shuk-mei.ho@umassmed.edu.

<sup>4</sup> The abbreviations used are: DES, diethylstilbestrol; PCa, prostatic adenocarcinoma; TAM, tamoxifen; ER, estrogen receptor; E<sub>2</sub>, 17 $\beta$ -estradiol; PrEC, prostatic epithelial cell; BPH, benign prostatic hyperplasia; PR, progesterone receptor; 4OH-TAM, 4-hydroxy-TAM; ICI, ICI-182,780; ODN, oligodeoxynucleotide; FBS, fetal bovine serum; P/S, penicillin/streptomycin; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AR, androgen receptor.

levels of ER- $\alpha$  and ER- $\beta$  in normal and malignant PrECs were then related to the transcript expression levels of two estrogen-responsive genes (PR and pS2) to gain insights into the functionality of the ER subtypes. Additionally, we have compared the efficacy of two estrogens (DES and E<sub>2</sub>) to those of two antiestrogens (4OH-TAM and ICI) in inhibiting cell growth in PC-3 and DU145 cells. An ER- $\beta$  antisense ODN was then used to demonstrate that the antiestrogen-induced growth-inhibitory effects on prostate cancer cells were mediated via an ER- $\beta$  signaling mechanism. Finally, we provided the first experimental evidence in support of DNA methylation-mediated transcriptional inactivation of gene expression as the mechanism of ER- $\alpha$  silencing in PrECs.

## MATERIALS AND METHODS

**Establishment of Normal PrECs in Primary Cultures.** Tissue specimens used for generating primary cultures of normal PrECs were obtained from patients undergoing transrectal ultrasound-guided biopsies of the prostate for standard clinical indications. All patients contributing biopsy material were invited to participate in a prospective tissue acquisition study approved by the local Institutional Review Board (approval was granted to J. L. at the New England Medical Center). Written informed consent was obtained prior to biopsy from participating patients. From each participant, one biopsy core was obtained from the peripheral zone of the prostate, placed on a sponge pad soaked in sterile saline; a 1–2-mm section was excised from the midportion of the core and suspended in 5 ml of culture medium (described below). The two remaining ends of the core were inked at the ends opposite the sectioned midportion piece, placed in 10% formalin, and processed for histology. Histological examination of the end pieces of a biopsy core allowed us to determine the histological nature and the homogeneity of the core. Only specimens judged to be histologically normal, with no hyperplastic or neoplastic tissue contamination, were used to establish primary cultures of normal PrECs.

Each harvested tissue specimen was then washed three times with HBSS and cut into five to seven smaller pieces. The pieces were suspended in 2 ml of freshly prepared growth medium (see below) and transferred to a 60-mm Falcon culture dish (Becton Dickinson, Lincoln Park, NJ) coated with type I rat tail collagen (Collaborative Biomedical Products, Bedford, MA). An epithelial cell selection medium (the growth medium), reported previously (31), consisted of keratinocyte serum-free medium with 25  $\mu$ g/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, 2 mM L-glutamine, 10 mM HEPES buffer, P/S (100 units/ml penicillin and 100  $\mu$ g/ml streptomycin), 5.5  $\mu$ l/ml fungizone, 20 ng/ml cholera toxin, and 1% heat-inactivated FBS was used to obtain enriched populations of PrECs. All culture reagents were obtained from Life Technologies, Inc. (Grand Island, NY) except for FBS, which was purchased from Sigma Chemical Co. (St. Louis, MO). The culture was incubated at 37°C in a 5% CO<sub>2</sub> atmosphere without disturbance for 7 days to allow epithelial cells to grow out of the tissue pieces. Culture medium was then routinely replaced every 4 days until cell culture reached ~80% confluence. The cells were split once before they were used for RNA extraction.

In addition to primary cultures obtained from biopsy explants, a batch of normal human PrECs were purchased from Clonetics Co. (San Diego, CA). The PrEC cells were cultured in the PrEGM medium (Clontech) supplemented with SingleQuots (Clonetics Co.) according to the manufacturer's recommended protocol.

**Maintenance of Established Prostatic Cell Lines.** All culture reagents were obtained from Life Technologies and FCS from Sigma (St. Louis, MO), except otherwise specified. BPH-1 (30), a nontumorigenic, SV40-immortalized, highly differentiated human prostate epithelial cell line, was provided as a gift by Dr. Simon Hayward at the University of California (San Francisco, CA). This cell line was maintained in RPMI 1640 with 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 4.5 g glucose/l, and 1.5 g of sodium bicarbonate/L (American Type Culture Collection, Rockville, MD) plus 5% heat-inactivated FBS, ITS (insulin-transferrin-selenium mixture; Collaborative Biomedical Research, Bedford, MA), and P/S. Three human prostate cancer cell lines (DU145, PC-3, and LNCaP) were purchased from American Type Culture Collection. For routine maintenance, DU145 and PC-3 cells were

grown in DMEM/F-12 supplemented with heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 M nonessential amino acids, P/S, 0.05 mM  $\beta$ -mercaptoethanol (Sigma), and 1% ITS+. LNCaP cells were maintained in the same medium used for BPH-1 except that ITS+ was left out from the medium. All cell cultures were incubated at 37°C under a 5% CO<sub>2</sub> atmosphere.

**RNA Isolation and RT-PCR.** Total cellular RNA was isolated using RNA Stat-60 reagent (Tel-Test, Inc., Friendswood, TX) according to protocols provided by the manufacturer. The quality of each total RNA sample was checked and controlled by the following steps: (a) measurement of absorbance; (b) running of a denaturing RNA gel capable of detecting possible RNA degradation, as judged by the integrity and intensity of the 18S and the 28S rRNA signals; and (c) conducting a semiquantitative RT-PCR for the 18S rRNA at low cycle numbers. One  $\mu$ g of total cellular RNA was reverse transcribed using the GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT), and 2  $\mu$ l of the resulting cDNA were used in each PCR.

Intron-spanning primers were either obtained from published literature or designed using the Primer3 Output program.<sup>5</sup> Primer sequences for GAPDH, ER- $\alpha$  (primer set #1, exon 1–3), ER- $\beta$ , PR, and AR are given in Table 1. All PCR conditions were optimized for quantification of relative message contents under nonsaturating conditions. Preliminary experiments were conducted to ensure linearity for all semiquantitative procedures. Hot-start PCR using AmpliTaq Gold DNA polymerase (Perkin-Elmer) was used in all amplification reactions. The enzyme was activated by preheating the reaction mixtures at 95°C for 6 min prior to PCR. This protocol was chosen to minimize nonspecific product amplification. The routine PCR program was 30 cycles of 1 min at 94°C, 1 min at 60°C (annealing temperature), and 1 min at 72°C with the following modifications: (a) amplification for ER- $\beta$  cDNA used an annealing temperature of 58°C; (b) amplifications of ER- $\alpha$  cDNA and AR cDNA were carried out at an annealing temperature of 55°C; (c) cycle number for ER- $\alpha$  cDNA amplification was set at 35; and (d) GAPDH cDNA was amplified at 26 cycles. GAPDH cDNA levels served as a loading control. Amplification of the correct sequence was verified by direct DNA sequencing of each PCR product from at least two different samples.

To further confirm that primary cultures of normal PrECs and certain prostate cancer cell lines did not express ER- $\alpha$  mRNA (see "Results"), we used two additional pairs of primers (primer sets #2 and #3, Table 1) to amplify regions downstream of the coding sequence of ER- $\alpha$  (668 bp of exon 3–6 and 710 bp of exon 5–8, respectively). Together, these three pairs of pairs of ER- $\alpha$  primers (Table 1) covered most of the length of the coding sequence. They had been used successfully in our recent study on the expression of ER- $\alpha$  mRNA in ovarian cancer (32). If PCR at high cycle number failed to yield products from all three pairs of primers, we considered the sample to be devoid of ER- $\alpha$  cDNA.

**Treatment of DU145, PC-3, and LNCaP Cells with Demethylating Agents.** The three prostatic cancer cell lines were seeded at a density of 10<sup>4</sup> cells/ml medium in 25-cm<sup>2</sup> culture flasks, allowed to attach during a 24-h period, and exposed to two demethylating agents separately. The demethylating agents were added daily in aqueous solution. 5'-Azacytidine was added at final concentrations of 2.5 and 5  $\mu$ M and 5'-aza-2'-deoxycytidine at 0.5 and 0.75  $\mu$ M, respectively. Culture medium was changed every 4 days, and cells were subjected to a total of 8 days of demethylating agent treatment. At the end of the treatment period, the medium was removed, and cellular RNA was extracted for RT-PCR.

**Treatment of DU145 and PC-3 Cells with Estrogens/Antiestrogens.** Cells were seeded at a density of 5  $\times$  10<sup>3</sup> per ml into 24-well plates (Falcon; Becton Dickinson Labware, Lincoln Park, NJ) in a final volume of 1 ml of culture medium with 5% charcoal-stripped FBS. Twenty-four h after seeding, triplicate wells of cells were treated in with 1, 10, and 100  $\mu$ M of E<sub>2</sub>, DES, 4OH-TAM, or ICI. E<sub>2</sub>, DES, and 4OH-TAM were purchased from Sigma, and ICI was a generous gift from Zeneca Pharmaceuticals (Macclesfield, United Kingdom). Estrogens and antiestrogens were dissolved in absolute ethanol (Sigma) and added to the media daily. Cell cultures that were not treated with estrogenic compounds received absolute ethanol as a vehicle control. Total additive ethanol concentrations never exceeded 0.2% throughout the culture period. The cells were refed with freshly prepared medium every other day. At the end of a 4-day treatment period, cells in each well were trypsinized, and

<sup>5</sup> Internet address: <http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>.

Table 1 Primer sequences for the RT-PCR analysis

Target gene	Primer sequence	Location (nt) <sup>a</sup>	Expected size
<i>ER-<math>\beta</math></i>	<i>ER<math>\beta</math>-1</i> : 5'-TGA AAA GGA AGG TTA GTG GGA ACC-3'	230-253	528 bp
	<i>ER<math>\beta</math>-2</i> : 5'-TGG TCA GGG ACA TCA TCA TGG-3'	737-757	
<i>ER-<math>\alpha</math></i>	<i>ER<math>\alpha</math>-1</i> : 5'-TAC TGC ATC AGA TCC AAG GG-3'	41-60	650 bp
	<i>ER<math>\alpha</math>-2</i> : 5'-ATC AAT GGT GCA CTG GTT GG-3'	671-690	(exon 1-3)
	<i>ER<math>\alpha</math>-3</i> : 5'-GTG GGA ATG ATG AAA GGT GG-3'	742-761	668 bp
	<i>ER<math>\alpha</math>-4</i> : 5'-TCC AGA GAC TTC AGG GTG CT-3'	1390-1409	(exon 3-6)
	<i>ER<math>\alpha</math>-5</i> : 5'-GTG CCT GGC TAG AGA TCC TG-3'	1142-1161	710 bp
	<i>ER<math>\alpha</math>-6</i> : 5'-TGG TGC ATG ATG AGG GTA AA-3'	1832-1851	(exon 5-8)
<i>PR</i>	<i>PR-1</i> : 5'-GAT TCA GAA GCC AGC CAG AG-3'	1817-1836	533 bp
	<i>PR-2</i> : 5'-TGC CTC TCG CCT AGT TGA TT-3'	2330-2349	
<i>pS2</i>	<i>pS2-1</i> : 5'-GGA GAA CAA GGT GAT CTG CG-3'	52-71	236 bp
	<i>pS2-2</i> : 5'-CAC ACT CCT CTT CTG GAG GG-3'	268-287	
<i>AR</i>	<i>AR-1</i> : 5'-CTC TCT CAA GAG TTT GGA TGG CT-3'	2896-2918	342 bp
	<i>AR-2</i> : 5'-CAC TTG CAC AGA GAT GAT CTC TGC-3'	3214-3237	
<i>GAPDH</i>	<i>GAPDH-F</i> : 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3'	152-175	598 bp
	<i>GAPDH-R</i> : 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'	726-749	

<sup>a</sup> nt, nucleotide.

cell count was determined by direct counting using the trypan blue exclusion method. All treatment experiments were repeated at least three times to generate statistically relevant data.

**Treatment of DU145 Cells with ICI and ER- $\beta$  Antisense ODN.** DU145 cells ( $5 \times 10^3$  cells/well) were plated in 24-well plates (Falcon; Becton Dickinson Labware). After allowing 24 h for cell attachment, cell cultures were treated in triplicate with  $1 \mu\text{M}$  of ICI in the presence of  $2.5 \mu\text{M}$  ER- $\beta$  antisense, sense, or mismatch ODNs for 4 days. The ER- $\beta$  antisense ODN, an 18-mer, was designed to recognize the first translation start site on the ER- $\beta$  mRNA and its immediate 5' flanking region (Table 2). The nucleotide sequence of sense ODN is complementary to those of ER- $\beta$  antisense ODN (Table 2). On the basis of the sequence of ER- $\beta$  antisense ODN, five nucleotides were scrambled to generate a mismatch ODN that retains the same GC ratio of the ER- $\beta$  antisense ODN (Table 2). Both the sense and the mismatch ODNs served as controls for the antisense ODN. In all three ODNs, the first and the last three nucleotides were phosphorothioate modified to increase their stability *in cellulo*. The number of viable cells in each well was determined by direct counting using the trypan blue exclusion method after a 4-day treatment period. At least three individual experiments were performed to obtain statistically relevant data.

**Statistics.** Statistical analysis was performed by using Student SYSTAT software (Course Technology, Inc., Cambridge, MA). Data were analyzed by one-way ANOVA, followed by the Tukey post-hoc test, and a 95% confidence limit was used for all comparisons among treatment groups.

## RESULTS

**Expression of AR, ER- $\beta$ , PR, and pS2 mRNA, but not ER- $\alpha$  Transcripts, in Normal PrECs in Primary Cultures.** Five primary cultures of normal PrECs (N4#6, N3#5, N3#4, N2#3, and N2#2) were established in our laboratory from ultrasound-guided peripheral zone biopsies over a period of 18 months. The biopsy cores were all judged upon histological examination to contain only normal prostatic tissue with no BPH or cancerous foci contamination. The primary cell cultures were all early passages (second or third), cobblestone in appearance, with no visible fibroblast contamination. Semiquantitative RT-PCR analyses (Fig. 1) demonstrated that our normal PrEC cultures retained high levels of AR mRNA expression, which usually disappeared in late-passage normal PrEC primary cultures or in established PrECs (33). Interestingly, all five cultures of normal PrECs expressed uniform levels of ER- $\beta$  RNA and transcripts of the estrogen-responsive genes, *PR* and *pS2*. In contrast, expression of ER- $\alpha$  mRNA was noticeably absent in all five cultures, even when high cycle number PCR (>42 cycles) was used to amplify the cDNA. Interestingly, PrEC (Clonetics Co.), a commercially prepared normal PrEC culture, and BPH-1, a SV40 immortalized prostatic epithelial cell line, expressed both ER- $\alpha$  and ER- $\beta$ , but no PR or pS2 transcripts in PrEC (Clontech), and only a minimal level of PR mRNA in BPH-1.

**Expression of ER- $\beta$ , ER- $\alpha$  PR, and pS2 mRNA in Prostatic Cancer Cell Lines.** All three prostatic cancer cell lines, DU145, PC-3, and LNCaP, express ER- $\beta$  mRNA (Fig. 1, *a* and *c*). In contrast, ER- $\alpha$  mRNA was expressed only in the PC-3 cells. Interestingly, PR transcripts were detected only in DU145 and LNCaP cells and not in PC-3 cells. Messages of pS2 were found in PC-3 and LNCaP cells but not in DU145 cells. In accordance with reports in the literature, AR mRNA expression was only noted in LNCaP cells.

**Expression of ER- $\alpha$  Variant in Prostate Cell Lines.** When RT-PCR analyses were conducted for ER- $\alpha$  mRNA semiquantification in PrEC (Clonetics Co.), BPH-1, or PC-3 cells, we noticed that, in addition to the expected PCR product, a smaller PCR product was coamplified (Fig. 1, *a* and *b*). Sequencing analysis (data not shown) revealed that this smaller PCR product was derived from an ER- $\alpha$  mRNA variant that had the entire exon 2 deleted. We reported recently the coexistence of this ER- $\alpha$  mRNA variant with wild-type transcripts in normal and malignant human ovarian surface epithelial cells (33).

**Demethylation Reactivates ER- $\alpha$  and pS2 mRNA Expressions in DU145 Cells and ER- $\alpha$  Expression in LNCaP Cells.** Prior to exposure to demethylating agents, ER- $\alpha$  and pS2 transcripts were not detected in RNA samples prepared from DU145 cells (Fig. 1, *a* and *c*, and Fig. 2). After the 8-day treatment with 5'-aza-cytidine ( $2.5$  and  $5 \mu\text{M}$ ) or 5'-aza-2'-deoxycytidine ( $0.5$  and  $0.75 \mu\text{M}$ ), DU145 cells regained expression of both transcripts (Fig. 2). Interestingly, the absence of AR mRNA expression in DU145 cells was not reversed by treatment with demethylating agents. Exposure of LNCaP cells to demethylating agents also reactivated ER- $\alpha$  mRNA expression (data not shown).

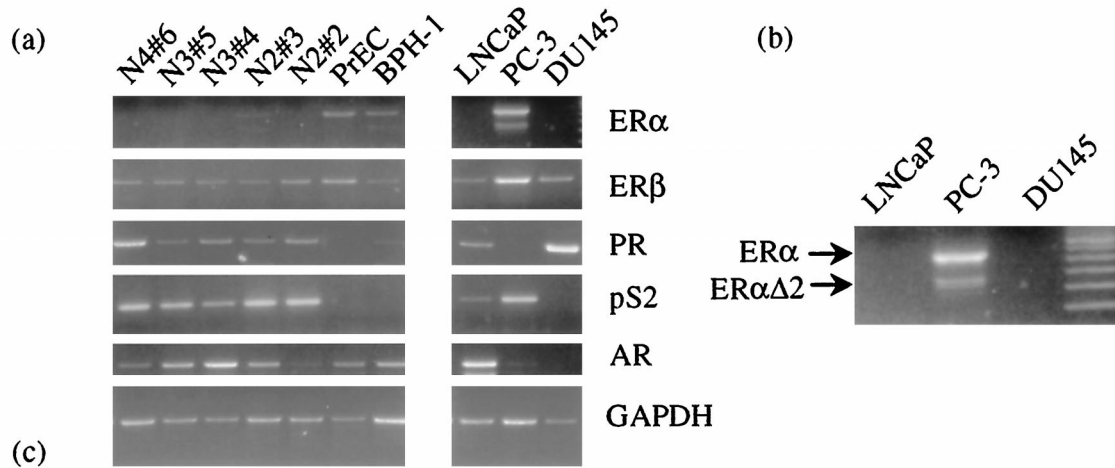
**Effect of Antiestrogens and Estrogens on Cell Growth of DU145 and PC-3 Cells.** Cell growth analyses showed that the growth of DU145 cells, which only expressed ER- $\beta$  mRNA, was adversely effected by the antiestrogens ICI and 4OH-TAM (Fig. 3, *A* and *B*). A dose-dependent inhibition of cell numbers was observed in cultures exposed to ICI for 4 days when compared with control cultures treated with vehicle (absolute ethanol). A 40% reduction ( $P < 0.001$ ) in the cell numbers was achieved with an ICI dose of  $1 \mu\text{M}$ . A similar

Table 2 Oligonucleotide sequences for the antisense ODN experiments

Target gene	Sequences	Location <sup>a</sup>	Size
ER- $\beta$	Antisense 5'-C <sup>b</sup> A <sup>b</sup> T <sup>b</sup> CAC AGC AGT GCT A <sup>b</sup> T <sup>b</sup> A <sup>b</sup> -3'	-15 to +3	18 bp
	Sense 5'-T <sup>b</sup> A <sup>b</sup> T <sup>b</sup> AGC CCT GCT GTG A <sup>b</sup> T <sup>b</sup> G <sup>b</sup> -3'		
	Mismatch 5'-G <sup>b</sup> A <sup>b</sup> T <sup>b</sup> CTC AGC ACG GCA A <sup>b</sup> A <sup>b</sup> T <sup>b</sup> -3'		

<sup>a</sup> The first base of translation-initiating site is +1.

<sup>b</sup> This base was phosphorothioate modified.



N4#6	N3#5	N3#4	N2#3	N2#2	PrEC	BPH-1	LNCaP	PC-3	DU145	Sample
-	-	-	-	-	+	+	-	+	-	ER $\alpha$
+	+	+	+	+	+	+	+	+	+	ER $\beta$
+	+	+	+	+	-	-/+	+	-	+	PR
+	+	+	+	+	-	-	+	+	-	PS2
+	+	+	+	+	+	+	+	-/+	-	AR

Fig. 1. Results of RT-PCR analyses of ER- $\alpha$ , ER- $\beta$ , PR, pS2, and AR mRNAs among normal and malignant prostatic epithelial cells. Total RNAs were extracted and reverse transcribed. The resultant cDNAs were subjected to PCR analyses under optimized conditions. The amplified products were run into 2% agarose gel with ethidium bromide. Three individual experiments were performed. *a*, representative fluorographs for ER- $\alpha$ , ER- $\beta$ , PR, pS2, AR- and GAPDH RT-PCR analyses. *b*, an excerpt from the ER- $\alpha$  fluorograph, enlarged to illustrate the exon 2 deletion variant of ER- $\alpha$  (ER $\alpha$  $\Delta$ 2) in PC-3 cells. *c*, a summary of the data from RT-PCR analyses performed in three individual experiments. +, detectable level of expression; -, undetectable level of expression.

growth-inhibitory response was observed when DU145 cells were treated with 4OH-TAM. However, cell number reduction achieved with 1  $\mu$ M 4OH-TAM was only ~25% ( $P < 0.001$ ). In contrast, exposure of DU145 cells to 1  $\mu$ M estrogens ( $E_2$  and DES) did not

affect cell growth in 4-day exposure experiments (Fig. 3, C and D). However, in a separate series of experiments, DU145 cells were treated with 10  $\mu$ M DES, and we observed a significant reduction in cell number (data not shown). Furthermore, we had conducted competition experiments to determine whether  $E_2$  could reverse the growth-inhibitory effects induced by ICI and 4OH-TAM. DU145 cells were treated with 10<sup>-6</sup> M ICI or 4OH-TAM in the absence or presence of 10<sup>-8</sup>, 10<sup>-7</sup>, 10<sup>-6</sup> M  $E_2$ .  $E_2$  at all three concentrations was unable to reverse the ICI- or 4OH-TAM-induced cell growth inhibition (data not shown).

When PC-3 cells, which expressed transcripts of both ER subtypes, were exposed to antiestrogens (ICI and 4OH-TAM), a 25–30% reduction in cell growth was noted in cultures treated with 1 or 10  $\mu$ M of ICI or with 1  $\mu$ M 4OH-TAM ( $P < 0.001$ ; Fig. 4, A and B). Furthermore, exposure of PC-3 cells to  $E_2$  at 1 or 10  $\mu$ M concentrations also induced inhibition of cell growth ( $P < 0.05$  and  $P < 0.01$ , respectively; Fig. 4C). Interestingly, treatment with DES at the various concentrations up to 10<sup>-6</sup> M did not elicit statistically significant cell growth inhibition in PC-3 cells (Fig. 4D). However, in a separate experiment, PC-3 cells were treated with 10  $\mu$ M DES, and we observed a significant reduction in cell number (data not shown). Additionally, we had conducted competition experiments to determine whether DES could reverse the growth-inhibitory effects induced by ICI and 4OH-TAM. PC-3 cells were treated with 10<sup>-6</sup> M ICI or 4OH-TAM in the absence or presence of 10<sup>-8</sup>, 10<sup>-7</sup>, or 10<sup>-6</sup> M of DES. DES was able to reverse the ICI-induced, but not 4OH-TAM-induced, cell growth inhibition in a dose dependent manner (data not shown).

**Reduction of ICI-induced Cell Growth Inhibition by ER- $\beta$  Antisense ODN.** Treatment of DU145 cells with ICI at 1  $\mu$ M induced a 40% reduction in cell number (Fig. 3). Cotreatment of DU145 cells with ICI and an ER- $\beta$  antisense ODN led to restoration of cell number ( $P < 0.001$ ; Fig. 5) whereas cotreatments with an ER- $\beta$  sense ODN or a mismatch ODN (Table 2) did not reverse the ICI-induced effects. These data support the notion that the ICI-

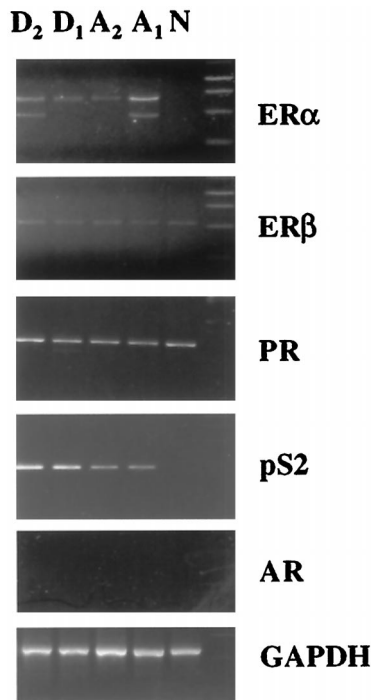


Fig. 2. Reactivation of ER- $\alpha$  and pS2 mRNA expression in DU145 cells by demethylating agents. Cells were treated with either 2.5  $\mu$ M ( $A_1$ ) or 5  $\mu$ M ( $A_2$ ) 5'-azacytidine or 0.5  $\mu$ M ( $D_1$ ) or 0.75  $\mu$ M ( $D_2$ ) 2'-deoxy-5'-aza-cytidine for 8 days. Cells without treatment were used as control (N). At the end of treatment, total RNAs were extracted and subjected to RT-PCR analyses as described in Fig. 1.

## Treatment of antiestrogens on DU145 cells

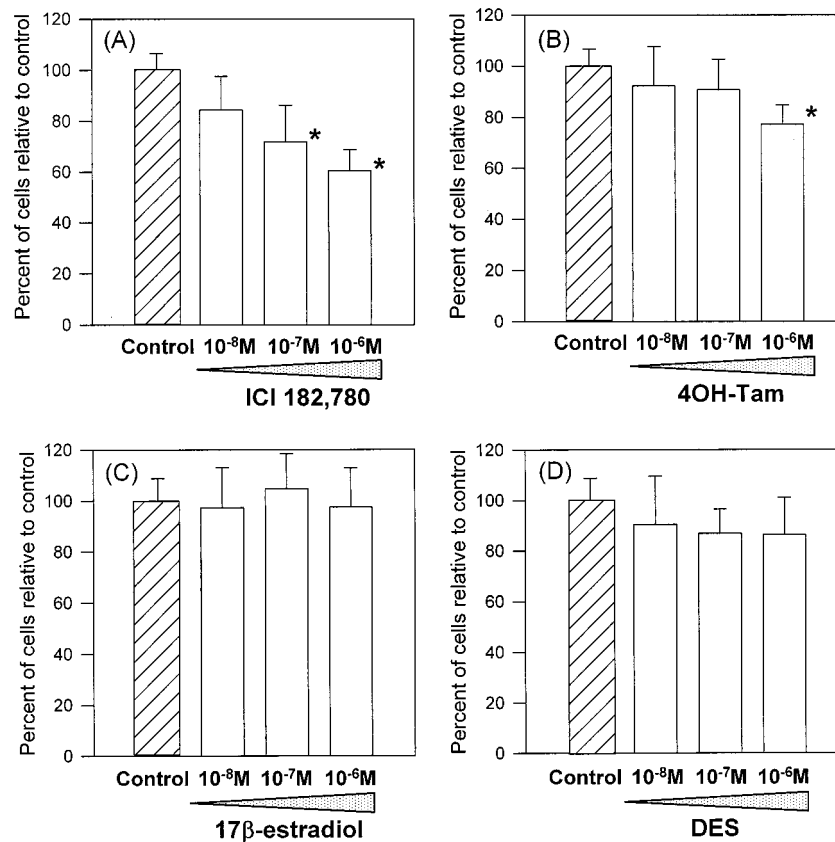


Fig. 3. Effects of antiestrogens and estrogens on cell growth of DU145 cells. Cells ( $5 \times 10^3$  cells/well) were plated in triplicate wells onto a 24-well plate. After 24 h for cell attachment, cells were treated for 4 days with antiestrogens (A, ICI and B, 4OH-TAM) or estrogens (C,  $E_2$  and D, DES) at various concentrations as indicated. Cells treated with vehicle (absolute ethanol) were used as control. The number of viable cells at the end of 4 days of treatment was determined by the trypan blue exclusion method. Three individual experiments were performed. Columns, means; bars, SD;  $n = 9$ ; \*,  $P < 0.001$  compared with control (▨).

induced cell growth inhibition in DU145 cells is mediated via an ER- $\beta$  signaling mechanism.

## DISCUSSION

The roles played by estrogens in the neoplastic transformation of PrECs as well as in PCa progression and treatment remain controversial. Exposure of humans or rodents to estrogens induces a proliferative lesion, squamous metaplasia, in their prostates (34–37), whereas prolonged treatment of Noble rats with androgen plus estrogen causes a high incidence of PCa in the dorsolateral prostates of the treated animals (38–41). Paradoxically, DES, TAM, and other estrogens have been used as treatment regimens for advanced metastatic PCa (1, 7–12, 42, 43). In addition to acting as chemical castration agents, both estrogen and antiestrogen are believed to exert direct growth-inhibitory effects on prostatic cancer cells via induction of apoptosis or cell cycle arrest (3–6, 44). Precisely how estrogens/antiestrogens elicit these actions remains uncertain.

Traditionally, the actions of estrogens/antiestrogens are thought to be mediated via the classical ER, the  $\alpha$  subtype, which has been localized to the stromal compartment and basal epithelial cells of human and rodent prostates (22, 26, 45–48). Because ER- $\alpha$  is not expressed in the normal glandular epithelium of rat or human prostate (4, 21, 26, 46–49), it is widely believed that the action of estrogen/antiestrogen on normal PrECs is indirect, likely mediated via estrogen-induced stromal factors. However, after the discovery of ER- $\beta$  (19) and its localization to the epithelial compartment of rodent prostates (19, 21, 50), a distinct possibility has been raised that estrogen/antiestrogen could influence PrEC function via an ER- $\beta$  signaling pathway. However, at present, information on ER- $\beta$  in human PrECs is limited. Only one recent study (26) has evaluated the

expression pattern of ER- $\beta$  transcripts and proteins in human prostatic tissues and found nondetectable levels in both normal and diseased tissues. In contrast, the present study unequivocally demonstrated expression of ER- $\beta$  mRNA in highly enriched or pure human PrEC cultures established from peripheral zone biopsies. Furthermore, because ER- $\alpha$  message was undetectable but transcripts of two estrogen-dependent genes, *PR* and *pS2*, were expressed in these cultures, these data strongly suggest that ER- $\beta$  is the cellular mediator of estrogen action in normal human PrECs. Of interest to note is that both ER- $\alpha$  and ER- $\beta$  mRNA, but not *PR* or *pS2* transcripts, were expressed in a PrEC preparation purchased from a commercial source (PrEC; Clontech) and in the immortalized PrEC line, BPH-1, with only a minimal level of *PR* transcripts. The discrepancies between ER subtype, *PR*, and *pS2* expression in our primary PrEC cultures and those observed in PrEC (Clontech) and BPH-1 could be attributable to the tissue of origin of these cell cultures/lines. In this regard, PrEC cultures (Clontech) are routinely prepared from whole prostates, and BPH-1 was derived from a benign hyperplastic specimen (30), whereas our primary cultures were established from ultrasound-guided peripheral zone biopsies.

Issues relating to whether ER, and which subtype, is expressed in cancerous PrECs remain unsettled. Several investigators (51, 52) observed ER- $\alpha$  expression in human prostate cancer cell lines, including LNCaP, PC-3, and DU-145, whereas others (53) did not. Similarly, observations on ER- $\alpha$  expression in prostate cancer specimens were equally controversial. Bonkhoff *et al.* (26) reported recently that ER- $\alpha$  expression was infrequent in low-to-moderate grade adenocarcinoma but common in high-grade and metastatic cancers. Conversely, Konishi *et al.* (54) noted the presence of ER- $\alpha$  immunopositivity in well-differentiated adenocarcinomas but not in poorly

## Treatment of antiestrogens on PC-3 cells

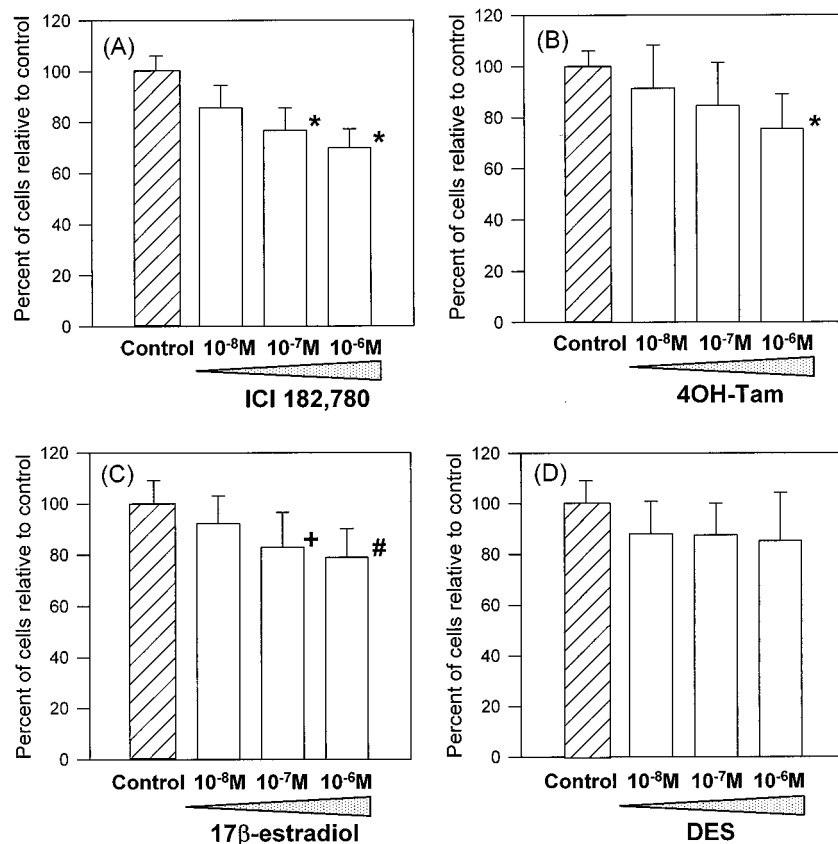


Fig. 4. Effects of antiestrogens and estrogens on cell growth of PC-3 cells. Cell growth assay was described in Fig. 3. A, ICI. B, 4OH-TAM. C, E<sub>2</sub>. D, DES. Columns, means; bars, SD;  $n = 9$ ; \*,  $P < 0.001$ ; #,  $P < 0.01$ ; +,  $P < 0.05$ ; compared with control (▨).

differentiated specimen. The latter observation was supported by two additional studies that reported no ER- $\alpha$  expression in lymph node and distant metastases (53, 55). These issues become more convoluted when the expression pattern of ER- $\beta$  is taken into consideration. A lack of ER- $\beta$  expression in human prostate tissues was reported by Bonkhoff *et al.* (26), whereas several preliminary reports noted expression of this receptor subtype in dysplastic and cancerous tissues

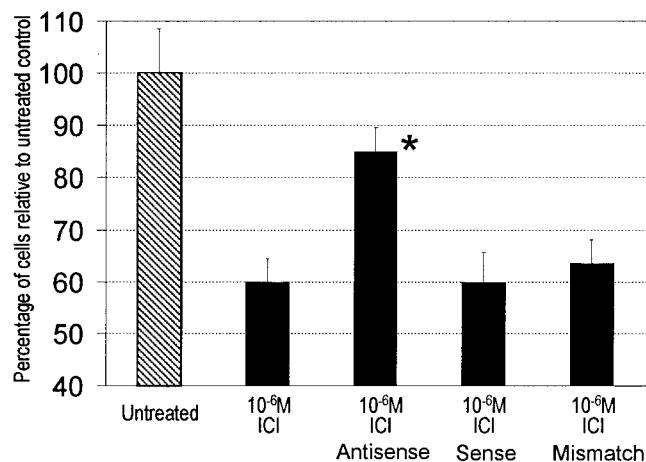


Fig. 5. Reversal of ICI-induced DU145 cell growth inhibition by ER- $\beta$  antisense ODN. Cells were treated with ICI at  $10^{-6}$  M in the absence or presence of  $2.5 \mu\text{M}$  of the antisense, sense, or mismatch ODNs for 4 days. Cell numbers in "untreated" cultures received no ICI treatment and were arbitrarily assigned a value of 100% (first column on the left). Cell numbers in cultures treated with ICI and ICI plus ODNs were expressed as percentages of cell numbers in the "untreated" cultures. Columns, means; bars, SD;  $n = 16$ . \*, significant difference between ICI-treated cultures and those that received ICI plus the antisense ODN at  $P < 0.001$ .

(27–29). Findings in the present study revealed that ER- $\beta$  mRNA was expressed in all three cancer cell lines (PC-3, DU145, and LNCaP), although in accordance with the literature, the ER- $\alpha$  message was only detected in PC-3 cells (51). Unlike primary PrEC cultures, which uniformly expressed both PR and pS2 transcripts, PR mRNA expression was only detected in DU145 and LNCaP cells whereas pS2 transcripts were found in PC-3 and LNCaP cells. Hence, despite uniform expression of ER- $\beta$ , the expression patterns of ER- $\alpha$ , PR, and pS2 among these prostatic cancer cell lines was variable. Because transcription of PR and pS2 is well recognized to be regulated by estrogen (56–58), the loss of expression of these two genes in some prostatic cancer cell lines suggests a deregulation of estrogen signaling in these cells. A similar phenomenon has been observed in ovarian cancer cell lines that express both ER subtype but no PR (33).

Until now, it remains unknown as to why prostatic epithelium expresses only ER- $\beta$  and not ER- $\alpha$ . In the present study, we demonstrated that treatment of DU145 and LNCaP cells with demethylating agents reactivated ER- $\alpha$  expression in these cells. These data provide the first experimental evidence in support of DNA methylation-mediated gene silencing (59, 60) as a mechanism of ER- $\alpha$  inactivation in PrECs. In breast cancers, it had been shown that hypermethylation of the promoter region of ER- $\alpha$  was associated with loss of expression of this receptor subtype in hormone-refractory cancers (61–64). Because of the fragile nature of PrECs in primary culture, we had not subjected them to demethylating agent treatment; it is reasonable to assume that the same mechanism transcriptional inactivation operates in ER- $\alpha$  silencing in the normal prostatic epithelium. Interestingly, loss of pS2 expression in DU145 cells might also be linked to hypermethylation-mediated transcriptional inactivation because exposure of this cell line to demethylating agents revived pS2 expression.

The *pS2* gene encodes an 84-amino acid, cystein-rich, secretory protein, which is widely expressed in estrogen-sensitive tissues (57). Its expression in prostate cancer specimens has been shown to be associated with premalignant changes and neuroendocrine differentiation (65, 66).

Different variants of ER- $\alpha$  transcripts are often found to coexist with the wild-type transcript in normal and malignant tissues (67, 68). These variants, produced by alternative splicing, are whole exon deletion variants that may have "outlaw functions." In PC-3 cells, a previous study has demonstrated the expression of an exon 4 deletion variant (51). In the current study, we found an exon 2 deletion variant in PC-3, BPH-1, and PrEC (Clontech) cells. Whether ER transcript variants have functional or regulatory roles in prostatic cells is a topic of future investigation.

Therapies for metastatic prostate cancers are limited. In addition to androgen ablation therapies, estrogens/antiestrogens have been used, singularly or in combination with other modalities, for treatment of the disease. DES is an effective treatment therapy; however, its estrogenicity induces significant adverse effects in patients and have resulted in termination of its use (7). In contrast, TAM, a nonsteroidal antiestrogen, is better tolerated but produces little objective responses in multiple trials (8–12). With the discovery of ER- $\beta$  as a new estrogen signaling pathway and the availability of pure antiestrogen such as ICI (13), it becomes appropriate to address the issue of whether pure antiestrogens could be considered in the treatment of prostate cancer. In this study, we have compared the efficacy of two estrogens ( $E_2$  and DES) and two antiestrogens (4OH-TAM and ICI) in inhibiting cell growth in two androgen-refractory prostate cancer cell lines, PC-3 cells that we found express both ER subtypes, and DU145 cells that express only ER- $\beta$ . Past studies have shown that DES, when given at high concentrations (10–30  $\mu$ M range), exerts colchicine-like action, inhibits tubulin assembly, and induces apoptosis in prostate cancer cells (69, 70). Because the focus of the current study is to elucidate receptor-mediated action of DES, we have chosen to use concentrations not higher than  $10^{-6}$  M. Our results demonstrated all four estrogenic/antiestrogenic compounds, at relatively low doses, exerted antiproliferative effects on PC-3 cells, with antiestrogens exhibiting greater potencies. Furthermore, competition experiments showed that DES could reverse the effect of ICI, thus suggesting that estrogens and antiestrogens may share similar cellular mediators in this cell line. In contrast, DU145 cells responded only to antiestrogens with regard to cell growth inhibition. In both cases, ICI was found to be more potent than 4OH-TAM as a growth inhibitor. Furthermore, competition experiments demonstrated that  $E_2$  did not reverse the ICI-induced or 4OH-TAM-induced growth inhibition. This finding suggests that estrogens have little impact on the antiestrogenic action in DU145 cells. Importantly, the antiestrogen-induced growth-inhibitory response in DU145 cells was reversible by cotreatment with an ER- $\beta$  antisense ODN. Taken together, these findings raise several significant implications: (a) it is apparent that the estrogen/antiestrogen-induced antiproliferative action on prostatic cancer cells is ER subtype dependent. It supports the prediction for the antiproliferative action of ER- $\beta$  as previously discussed (71); (b) because ICI consistently expresses a higher potency, it may be better suited to be used in prostate cancer treatment. In clinical trials for breast cancer treatment, this compound has demonstrated high efficacy and low toxicity (72); and (c) our data have provided the first demonstration that estrogen/antiestrogen action in prostatic cancer cells could signal via an ER- $\beta$  pathway. Because ER- $\beta$  selective ligands have been reported recently (73), this development raises the likelihood of using receptor subtype ligands as cancer therapeutics in the future.

In summary, this study has demonstrated that human normal PrECs express exclusively ER- $\beta$  and likely signal via this receptor subtype

for estrogen/antiestrogen action. On the contrary, prostatic cancer cells exhibit a more variable pattern of ER subtype expression, and their responses to individual estrogen or antiestrogen will depend on the ER subtype(s) expressed in the cells. Significantly, we provide the first experimental evidence that ER- $\alpha$  gene silencing in prostate cancer cells, and perhaps also in normal cells, may be caused by DNA hypermethylation. Overall, data from this study lend support to the notion that ER- $\beta$  plays a central role in estrogen/antiestrogen signaling in normal and malignant human PrECs.

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