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Estrogen and Antiestrogen Actions on Human Prostate Cancer: A Dissertation

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A Dissertation Presented

By

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ESTROGEN AND ANTIESTROGEN ACTIONS ON HUMAN PROSTATE CANCER

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ABSTRACT

Prostate cancer increases its incidence with age after men in their fifth decade as the ratio of estrogen to androgen rises. Epidemiological studies indicated that high levels of estrogens are associated with the high-risk ethnic groups for prostate cancer. Therefore, estrogens may be involved in prostatic carcinogenesis. It is widely believed that the actions of estrogens are mediated by estrogen receptors. However, expression of estrogen receptor in normal prostate and lesions of the gland was controversial. With the recent discovery of second estrogen receptor (ER-β), this issue became more complicated and it needs to be readdressed. In addition, the biological involvement of ER-β in human prostate remains to be investigated. In this study, we demonstrated that human normal prostate epithelial cells express ER-β but not ER-α, suggesting that estrogens act directly on these epithelial cells via ER-β. Using RT-PCR analysis, the transcripts of ER-β were detected in our primary human prostatic epithelial cell cultures that were derived from the ultrasound-guided peripheral zone biopsies and the cells express two estrogen-regulated genes such as progesterone receptor (PR) and pS2. Moreover, we had developed an ER-β antibody with fully characterizations and used it for immunohistochemistry. Results indicated that ER-β protein is expressed in the basal compartment of prostatic epithelium of the gland. Our findings lead to a new hypothesis that estrogens directly act on human prostatic epithelial cells to modulate its biological functions.

To investigate expression of ERs in prostate cancer, RT-PCR analysis was used. We found that all three human prostate metastatic cancer cell lines, DU145, PC-3 and LNCaP, express ER-β transcripts while ER-α mRNA expression only in PC-3 cells. Expressions of

PR and pS2 in these cell lines are various. LNCaP cells express both PR and pS2 mRNAs but DU145 cells with only PR and PC-3 cells with only pS2. Our immunohistochemical results on prostatic lesions revealed down-regulation of ER-β expression in high-grade of dysplasia and carcinoma of peripheral zone of the prostate compared to their low-grade lesions. This down-regulation in high-grade carcinoma was verified in transcriptional level by RT-PCR analysis on microdissected normal epithelium and lesion samples of the gland. In the metastasis, ER-β was found to be reactivated as we observed ER-β mRNA expression in prostate cancer cell lines.

Recent evidence suggests that ER-β may be antiproliferative factor for a protective effect against the mitogenic activity of estrogens in breast and androgens in prostate. Activation of the receptor may exhibit cell growth inhibition. We demonstrated that antiestrogens [ICI-182,780 (ICI) and 4-hydroxytamoxifen], raloxifene and phytoestrogen (resveratrol), but not estrogens (17β-estradiol and diethylstilbestrol), inhibit growth of DU145 cells which express only ER-β while PC-3 cells with both ERs showed growth inhibition in response to estrogen and antiestrogen treatments. In DU145 cells, the ICI-induced cell growth inhibition was prevented by blockade of ER-β expression using antisense oligonucleotide. It indicated that the inhibition is mediated via ER-β associated pathway. Using flow cytometry, we found that ICI-treatment could induce accumulation of cells at G_0-G_1 phase of cell cycle. Similarly, this G_0-G_1 cell accumulation was also induced by raloxifene in DU145 cells. For resveratrol, the treatment exhibited dual effects on cell cycle distribution in DU145 cells. In the early treatment, resveratrol induced cell cycle arrests at G_0-G_1 phase. The prolonged treatment leads to S-phase cell cycle arrest.

To study the molecular mechanism of this ER-β associated cell growth inhibition, real-time RT-PCR analysis was used to semi-quantitate the transcript levels of tentative ER-β regulated genes such as telomerase reverse transcriptase (TERT), survivin and thymidylate synthase (TS) in the treated cells compared to those in control. Results demonstrated that the treatment of ICI could down-regulate TERT and survivin mRNA expressions with dose-dependent fashion. As the ICI-treatment, resveratrol downregulated expression levels of TERT, survivin and TS in DU145 cells. Down-regulation of TS may be related to the S-phase cell cycle arrest observed in the prolonged treatment of resveratrol.

Taken together, our findings support the concept that ER-β participates in cell cycle regulation in normal and malignant prostatic epithelial cells. Presence of ER-β in basal cells of the prostate acini indicates that the direct actions of estrogens may be involved in the normal physiology of the gland. Loss of this receptor in primary prostate cancer and its re-expression in metastasis suggests the roles of ER-β in the cancer progression. Activation of the receptor by antiestrogen and phytoestrogen induced cell growth inhibition in prostate cancer cells. The mechanism may be mediated by reduction of cell survival factors and eventually decrease in cell viability and induction of cell cycle arrests.

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CHAPTER I

INTRODUCTION

A. Prostate cancer epidemiology

Prostate cancer is the most common solid malignancy and the second leading cause of cancer-related death in men in the United States. The American Cancer Society estimates that in the United States, approximately 198,100 new cases of prostate cancer will be diagnosed and 31,500 men will die of prostate cancer during the year 2001 and it will account for about 11% of male cancer-related deaths. This cancer is extremely rare before age 40 but the incidence increases with age. The rate of increase with age is greater than that for any other cancer; rates increase at approximately the 9th-10th power of age (Cook and Doll 1969). In addition to age, epidemiological data shows that race is also an important risk factor for prostate cancer. African-American men have the highest rates of prostate cancer in the world. The rate is about 50-70% higher in African-Americans than in Caucasian-Americans (Ross 1996, Paltz et al 2000, Farkas et al 2000, Hoffman et al 2001, Fowler et al 2001, Powell et al 2001). For Asian populations, native Chinese and Japanese showed the lowest prostate cancer rates (Ross 1996, Watanabe et al 2000, Powell et al 2001). The difference in incidence was thought to be different detection strategies for prostate cancer. However, after adjusting for these diagnostic biases, there are still significant differences among populations (Shimizu 1991, Watanabe et al 2000, Powell et al 2001). Even though the incidence rates in Chinese- and Japanese-Americans, compared to those in their homeland, increase after they immigrated to America, their incidence rates are still much lower than that in Caucasians and African-Americans (Shimizu 1991, McCredie et al 1994, Whittemore et al 1995, Angwafo 1998).

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Microscopic prostate cancer (latent cancer) does not show significant differences among ethnicities (Guileyardo et al 1980, Sakr et al 1995, Sakr et al 2000, Selman 2000). This asyndromatic cancer is frequently found in old men either at autopsy or after cryoprostatectomy for a pathological condition of the bladder. It is small (<0.5mm) and well differentiated (Rich 1935, Andrews 1949, Franks 1954, Sakr et al 2000). The prevalence of latent cancer suggests that prostate cancer initiation is likely due to endogenous factors. But the promotion step leading to clinical cancer is predominantly influenced by exogenous factors (Griffiths 2000). Endogenous estrogens (estrone and estradiol) and exogenous estrogens such as phytoestrogens or xenoestrogens may affect this promotion step. However, very little is known about the importance of estrogens in prostate cancer.

B. Prostatic carcinogenesis

i. Epidemiological and clinical evidence

Epidemiological studies on hormonal status among populations with different degrees of risk for prostate cancer demonstrated that high levels of estrogens were associated with the high-risk group of population and therefore this steroid hormone might be one of the risk factors (Hill et al 1984, Ross et al 1996, Henderson et al 1988, De Jong et al 1991, Bosland 2000). African men shows higher levels of estrogens, such as estrone (Ross et al 1986, Hill et al 1984) and estradiol (Hill et al 1984) than Caucasian men among older populations. In addition, De Jong et al found that serum level of estradiol were 15% higher in Caucasian-Dutch men than in the Japanese men (Je Dong et al 1991). Circulating estradiol levels were found to be 37% higher in pregnant African-American women versus European-American women; suggesting that African-American men are exposed to higher estrogen

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concentrations than European-American before birth (Henderson et al 1988, Bosland 2000). However, population-based case-control studies (Nomura et al 1988, Hsing & Comstock 1990, Comstocks et al 1993, Gann et al 1996, Dorgan et al 1998) found no significant differences in serum levels of estrogens between case patients and control subjects, except for Barrett-Connor et al (1990) who showed that the ratio increased linearly with an increasing serum level of estradiol.

The incidence of prostate cancer rises in aging men at a time when the ratio of estrogens to androgens is increasing up to 40%. Plasma levels of testosterone and other androgenic hormones were shown to decrease due to declining testicular function as men age (Vermeulen et al 1972, Gray 1991a, Gray 1991b, Kaufman et al 1998, Griffiths 2000). Concurrently, the prostatic epithelial cells but not stromal cells have lower levels of 5α-hydrotestosterone (DHT) (Krieg et al 1993) although the level in the whole prostate showed no change (Bartsch et al 2000) in accordance with the previous findings that DHT-forming index (Vmax /Km of 5α-reductase) decreased with age (Tunn et al 1988). In addition, an increase in serum estrogens was found in aging men since aromatization of adrenal androgens by peripheral adipose tissue was enhanced in the older men (Vermeulen et al 1972, Zumoff, 1982, Gray 1991a, Gray1991b, Kaufman et al 1998, Griffiths 2000). These endocrine changes at mid-life are referred to as andropause. In the aged prostate, cellular levels of estrogens (estradiol and estrone) are increased (Krieg et al 1993). Taken together, these observations indicate hormonal stimulations change in the aged prostate and suggest these changes, especially increased estrogenic stimulation, may be related to prostatic carcinogenesis.

ii. Animal models

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In animal models, administration of pharmacological doses of estrogens had been reported to induce squamous metaplasia, as a proliferative alteration in epithelium, in regressed prostates of castrated or hypophysectomized dogs (Leav et al 1978, Merk et al 1986). Levine and colleagues (Levine et al 1991) also found this estrogen-induced squamous metaplasia in men who had benign prostatic hyperplasia and underwent medical castration therapy with gonadotropin-releasing hormone agonist for more than 6 months. The metaplastic change is initiated by the proliferation of basal cells which subsequently differentiate into squamous cells (Leav et al 1978, Merk et al 1986, Levine et al 1991). However, administration of estrogen in castrated guinea pigs induced hypertrophy of secretory cells in the lateral prostate and increased the thickness of fibromuscular layer, possibly due to the increased cell proliferation of smooth muscle cells (Bruengger et al 1986, Tam et al 1991, Tam & Wong 1991, Ricciardelli et al 1994). Only the seminal vesicle showed basal cell hyperplasia in the estrogen-treated castrated guinea pigs (Tam et al 1991, Tam & Wong 1991). Recently, exogenous administration of synthetic estrogen (diethylstilbestrol) to intact mice elicited epithelial squamous metaplasia in prostate and the effects were not related to androgen deprivation which was mediated via hypothalamic-pituitary-gonadal axis (Risbridger et al 2001).

When Wistar rats were exposed to estrogens prenatally and/or neonatally, the rats developed squamous cell carcinoma in the prostate (Arai et al 1977, Vorherr et al 1979, Arai et al 1983). Prenatal (McLachlan et al 1975) and neonatal (Pylkkanen et al 1991) estrogen exposure in mice induced hyperplasia and dysplasia in prostates respectively. Recently, it had been reported that a 50% increase in free-serum estradiol in male murine fetuses via a maternal Silastic estradiol implant could induce prostate enlargement in the adult animals,

possibly due to the increased androgen sensitivity (Vom Saal et al 1997). The enhanced sensitivity to androgens in the adult prostate can be achieved with a low dose treatment of estradiol immature male rat at 20-22 days of age. This permanent alteration of the prostate by neonatal estrogen treatment was described as "estrogen imprinting" (Rajfer & Coffey 1978). Later, a detailed study on estrogen imprinting on rat prostate was conducted by Prins (1992) and demonstrated a decrease in weight, DNA contents and morphological changes in three prostatic lobes (ventral, lateral and dorsal) of neonatal estrogenized rats. The hypoplastic ventral and dorsal prostates showed an increase in interacinar stromal tissues, epithelial hyperplasia in disorganized acini, luminal sloughing, apparent lack of differentiation as well as a decrease in levels of androgen receptor with immunohistochemistry (Prins 1992) and estrogen receptor β with semiquantitative *in situ* hybridization and RT-PCR analysis (Prins et al 1998).

Treatment with estradiol in intact Noble rats caused massive atrophy in the prostate (Leav et al 1989, Ho & Yu 1995). This may be due to the suppression of LHRH stimulation of the pituitary gland by the estrogens. As the results, the treatment indirectly decreases testicular testosterone production. Since the prostate depends on androgens for its normal growth and function, reduction of androgen level can lead to regression of prostate. A combination of testosterone and estradiol induced prostate cancer in all Noble rats after 32 weeks of treatment (Noble 1977a, 1977b, Drago 1984, Leav et al 1989, Ho et al 1995, Bosland et al 1995). The cancers were derived from the periurethral, proximal ducts of the dorsolateral and anterior prostates (Leav et al 1989, Ho & Yu 1995, Bosland et al 1995). This can also be achieved in a shorter period of time by using an extremely high dose of testosterone in combination with estradiol (Wong et al 1998). In fact, long-term androgen

(testosterone or 5 alpha-dihydrotestosterone) treatment alone was also able to induce prostate cancer but the incidence was low (Noble 1977, Leav et al 1989, Bosland 1992, Ho & Yu 1995). Taken together, testosterone appears to be a weak carcinogen and the addition of estradiol can enhance these carcinogenic effects on the prostate, indicating the importance of estrogens in prostatic carinogenesis. Interestingly, the estradiol plus testosterone treatment-induced prostate cancers in Noble rats were confined to the periurethral, proximal ducts of the dorsolateral and anterior prostates but not in the periphery of the glands where dysplasia developed in the acini (Leav et al 1989, Ho & Yu 1995, Bosland et al 1995). The morphology of the acinar dysplasia is similar to human prostatic intraepithelial neoplasia (PIN) which is considered to be a precursor of prostatic adenocarcinoma (Leav et al 1989, Ho & Yu 1995, Bosland et al 1995, Bostwick & Montironi 1995, Shin & Ro 1995, Haggman et al 1997, Zlotta et al 1999, Alcaraz et al 2001). In addition to the Noble rats, it recently has been reported that treatment with testosterone plus estradiol could also induce atypical hyperplasia and carcinoma in a subset of mouse prostate tissues. This tissue has a recombinant rat urogenital mesenchyme and is also deficient of retinoblastoma gene product (Rb) (Wang et al 2000). The Rb-deficient prostate epithelia in these chimeric prostatic tissue recombinants only showed a mild hyperplasia (Wang et al 2000).

iii. Estrogen receptors (ERs) and estrogen actions in normal prostate

It is widely believed that the actions of estrogens are mediated by an ER. Binding of estrogens leads to conformational changes of the ER and the receptors recruit transcrptional factors. The whole complex then binds to specific regulatory regions of DNA, promoting the transcription of specific genes such as progesterone receptor and pS2 (Roberts 1988, Clarke 1990). Evidence also indicates that estrogen receptors can regulate transcription through

protein-protein interactions between the receptors and other transcription factors such as AP-1 and Sp-1 (Webb et al 1995, Paech et al 1997, Saville et al 2000). Ligand binding studies demonstrate both high affinity (type I) and low affinity (type II) estradiol binding sites in the rat (Swaneck 1982, Yu 1989) and in the human (Ekman 1983, Donnelly 1983) prostate. The type I binding site usually represents classical estrogen receptor (ERα) while the type II binding site is still poorly characterized. Our laboratory had demonstrated the hormonal regulation of the type II binding site in the rat prostate and the combined treatment of testosterone and estradiol in Noble rats elevated its level along with an increase in wet weight of the glands (Ho & Yu 1995). Inhibition of this type II site with a specific antagonist [2,6-bis((3-methoxy-4-hydroxyphenyl)-methylene)-cyclohexanone] in intact adult mice showed dose dependent reduction of prostatic weight (Markaverich & Alejandro 1998). Another estrogen receptor (ERβ) was recently cloned in rat prostate (Kuiper 1996) and also found in human testis (Mosselman 1996). This ER subtype also shows high affinity to estrogens similar to ERα. In sum, the normal prostate contains at least two high affinities (ERα and ERβ) and one low affinity (type II) estradiol binding sites.

Prior to discovery of ERβ, immunohistochemical and *in situ* hybridization data showed ER was expressed in the stroma of human prostate but not in epithelial cells (Seitz and Wernert 1987, Konishi 1993, Ehara 1995). Based on the cellular localization of ER, estrogens were thought to bind to stromal ER and exert its effects on epithelium only indirectly via paracrine mediators such as stromal growth factors (Chung 1993, Farnsworth 1996, Chung & Davis 1996) (Figure 1A). It was supported by the findings that the level of stromal basic fibroblast growth factor (bFGF) increased in the prostates of estrogen treated rats (Bacher et al 1993). Also, estrogen, via estrogen receptor mediated pathway, enhanced

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synthesis of epidermal growth factor (EGF) and insulin like growth factor –1 (IGF-1), mediating prostate enlargement in organ culture of rat fetal prostate (Gupta 2000). These growth factors have been shown to stimulate growth of prostatic epithelium (Marengo & Chung 1994, Udayakumar et al 1999). Administration of matrigel with EGF or bFGF orthotopically in ventral prostates of adult rats increased the size and is related to stimulatory growth of prostatic epithelium (Marengo & Chung 1994). Moreover, systemic administration of IGF-1 can induce growth of the rat prostate (Torring et al 1997) and this factor also was demonstrated to stimulate proliferation of epithelial cells from monkey prostate on extracellular matrix substrate (Udayakumur et al 1999).

However, the discovery of ERβ in the prostatic epithelial cells weakens this hypothesis. Estrogens may also have direct effects on epithelium of prostate (Figure 1B). Although the expression and cellular localization of the two ERs has been examined separately in the normal human prostate, the results remain inconclusive. Bohkhoff and colleagues found that ERα expression is restricted to stromal and basal cells and is undetectable in secretory luminal epithelial cells, and that none of stromal and epithelial compartments shows ERβ expression (Bonkhoff et al 1999). In contrast to their findings, a recent study on the human prostate (Royuela et al 2001) demonstrated that ERβ is only present in basal epithelial cells whereas ERα is expressed in stromal cells in normal prostate. The human prostate is comprised of different anatomical zones (peripheral, transitional and central zones) and they have different hormone receptor profiles determined by ligand binding assays (Bashirelahi et al 1983, Bowman et al 1986, Sciarra et al 1995). The ratio of androgen receptor to progesterone receptor was found to be higher in transitional and central zones than in peripheral zone of the human prostate (Bashirelahi et al 1983). Uneven

distribution of androgen receptor was also demonstrated in the human normal prostate showing higher levels in the peripheral zone compared to those in other zones (Bowman et al 1986). Additionally, the transitional zone contains higher levels of EGF and bFGF as well as androgens than peripheral zones. Different zones of the prostate show histological differences and the majority of prostate cancers are derived from the peripheral zone where PIN lesions are frequently found while benign hyperplasia frequently develops in the transitional zone (Greene et al 1995). In this study, we establish primary cultures of human normal epithelial cells from the peripheral zone of the prostate for RT-PCR analysis to determine the ERβ mRNA expression of in this particular cell type. We also develop ERβ specific antibody to immunolocalize this receptor and determine the ERβ expression in different zones of human normal prostate.

iv. Estrogen receptors and estrogen actions in prostate cancer

For human prostate cancer, expression of ER is still controversial. Some studies using immunohistochemistry and *in situ* hybridization have shown no cancerous epithelial cells expressing ER and demonstrated that ER mRNA/ER protein positive cells were limited to fibroblasts, myoblasts and smooth muscle cells (Wernert 1987, Ehara 1995, Hobisch et al 1997, Bodker et al 1999). In contrast, Konishi et al (Konishi 1993) detected a subset of prostate cancers expressing ER using immunohistochemistry. With highly sensitive RT-PCR analysis, ER transcripts were able to be detected in two prostate cancer cell lines (PC-3 and LNCaP) derived from metastases (Carruba 1994, Castagnetta 1995), except Hobisch et al (Hobisch et al 1997). Besides the question about ER expression in prostate cancer, the discovery of ERβ complicated the question with which receptor isoform is predominantly expressed. Because the DNA sequence recognized by the forward primer for RT-PCR in

Castagnetta's studies (Carruba 1994, Castagnetta 1995) was shared by both ERα and ERβ, careful primer-designs for RT-PCRs which can differentiate ERα and ERβ are needed to re-evaluate the expression of ERα and ERβ in these two cell lines and other prostate cancer cell lines. In addition, the ERα and ERβ specific antibodies should be developed for immunohistochemistry to re-examine the expression of ERα and ERβ in human prostate cancer tissues. This issue will be carefully addressed in our study. Although expressions of ERα and ERβ were studied with ERα and ERβ specific antibodies in human prostate cancer, the results remained inconclusive. Bonkhoff et al found detectable level of ERα but not ERβ in 11% high-grade prostatic intraepithelial neoplasia, 43% and 61% prostate cancer with Gleason grade 4 and 5 respectively, and 94% recurrent adenomcarcinoma after hormonal therapy (Bonkhoff et al 1999), whereas Royuela and colleagues (Royuela et al 2001) demonstrated that both ERα and ERβ expressed in epithelial compartment of prostate cancer. The stromal cells express ERα in the prostate but only a subset of prostate cancer showed ERβ expression in its stromal compartment (Royuela et al 2001). With ERα specific primer sets for RT-PCR analysis, three prostate cancer cell lines (LNCaP, ARCaP and C4-2) were studied and shown to express ERα mRNA as well as its splicing variants (Ye et al 2000). For the clinical specimens, Latil and colleagues (Latil et al 2001) demonstrated detectable levels of ERα and ERβ transcripts in both normal and cancer tissues and down-regulation of ERβ mRNA expression in one half of either localized or hormone-refractory tumors with real-time quantitative RT-PCR assay. Because of cellular heterogenicity of prostate tissues, the cellular localization of ERα mRNA expression and the down-regulation of ERβ mRNA still remained to be elucidated. Our study with immunohistochemistry using ERα and ERβ

specific antibodies and RT-PCR analysis on Laser-capture-microdissected normal and malignant prostate epithelial cells can provide more information about this issue.

C. Estrogen receptor beta (ERβ) and ERα actions

The human ER was first cloned in 1986 (Greene et al 1986, Green et al 1986). After 10 years, the second ER was discovered in 1996 when PCR products, generated using degenerate primers for conserved regions within the DNA- and ligand-binding domains of nuclear receptors (Enmark et al 1994), were used as probes to screen a rat prostate cDNA library (Kuiper et al 1996). The newly discovered ER was named as ERβ and the original ER was renamed as ERα (Kuiper et al 1996). The sequences of ERβ in different species such as human and mouse were also cloned (Mosselman et al 1996, Tremblay et al 1997). Comparison on the amino acid sequences of these two receptors in rat, mouse and human showed that ERβ is highly homologous to ERα. They share 95-97% homology in their DNA-binding domains and only 55-60% in the ligand-binding domains. The high variation was found in their N-terminal A/B domains only showed about 16% homology (Kuiper et al 1996, Mosselman et al 1996, Tremblay et al 1997). These differences may cause differential cognate ligand profiles and transactivational activities of these two receptors and the uniqueness in their biological functions.

Upon binding to ligands, estrogen receptors change their conformations and dimerize. The whole complex with other transcriptional factors interacts with specific DNA region as estrogen responsive element (ERE) to regulate gene expression. The ERE had been extensively studied in promoters of vitellogenin in Xenopus and chicken, and prolactin,

progesterone receptor as well as pS2 in mammals. It generally contains two repeats of core sequence (AGGTCA) as palindromes (see review in Nardulli & Shapiro 1993). With RT-PCR analysis on rat, tissue distribution of two receptors had been investigated and showed their expressions are quite different, i.e. moderate to high expression in uterus, testis, pituitary, ovary, kidney, epididymis, and adrenal for ERα and prostate, ovary, lung, bladder, brain, uterus, and testis for ERβ (Kuiper et al 1997). Tissues such as kidney and adrenal gland express predominantly or only ERα transcripts while ERβ predominantly in lung, spinal cord and prostate. Both receptors are highly expressed in ovary and uterus (Kuiper et al 1997). Cells co-expressed ERα and ERβ may form heterodimers upon binding to ligands. As expected, gel shift assays demonstrated that two mouse ERs dimerize with each other and bind to ERE (Pace et al 1997). The heterodimerization of human ERα and ERβ was also shown with mammalian two-hybrid system and the physical interaction of two receptors was verified with GST-pulldown assay and co-immunoprecipitation assay (Pettersson et al 1997, Ogawa et al 1998). In the presence of two ERs, they preferred forming heterodimer to homodimer demonstrated in gel shift assays with both ERs binding to ERE (Pettersson et al 1997), possibly also in ERα and ERβ co-expressed cells.

The actions of estrogen receptors on regulation of gene expression in molecular level were extensively reviewed by Kushner and his colleagues (Kushner et al 2000). At ERE, estrogen receptor uses two transactivation functions (AF-1 and AF-2 in N-terminal domain [NTD] and ligand binding domain [LBD] respectively) to recruit p160 coactivator proteins such as SRC-1, GRIP1 and p/CIP (Figure 2, Kushner et al 2000) that bind to an integrator molecule (CBP/p300). Crystallographic analysis indicated that nuclear receptor boxes of

p160 bind to hydrophobic cleft on the surface of LBD of α receptor (Shiau et al 1998). Crystal structures of LBD of ERβ with ligands had been generated and were similar to ERα. It forms hydrophobic cleft for binding of p160 coactivators (Pike et al 1999). The orientations of helix 12 of both receptors are related to the agonistic or antagonistic activity of ligands at ERE. The orientation affects the binding of p160 coactivator (Brzozowshi et al 1997, Shiau et al 1998, Pike et al 1999). In contrast to AF-2 in LBD, the activity of AF-1 in NTD of two receptors is ligand independent and is activated via phosphorylation (Kato et al 1995, Bunone et al 1996, Tremblay et al 1999). It binds to C-terminus of p160 coactivators such as GRIP1 and synergizes with the AF-2 (Webb et al 1998).

In addition to ERE, estrogen receptors had been demonstrated to also regulate genes with promoter containing activation protein-1 (AP-1) sites (Gaub et al 1990, Umayahara et al 1994, Webb et al 1995). Gaub et al found that ER transactivated ovalbumin gene promoter with AP-1 such as fos and jun proteins and showed that ER without DNA binding domain (DBD) was still functional in this transactivation. Data suggested that direct ER interaction with the target DNA is not required (Gaub et al 1990). Although ER can regulate IGF-1 gene expression, the 600bp of the promoter contained no conventional ERE was still activated by estrogens as target of estrogen regulation (Umayahara et al 1994). Interaction between ER and fos-jun complex was involved in this regulation, via facilitating the binding of the complex to AP-1 site in this promoter region (Umayahara et al 1994). However, ER without DBD showed no activation but the direct ER binding to target DNA was not involved (Umayahara et al 1994). In collagenase gene promoter, Webb and his colleagues found that ER with either tamoxifen or estradiol can activate this promoter at AP-1 site and demonstrated DBD dependent and independent pathways of ER actions at AP-1 site (Webb

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et al 1995). Tamoxifen induced activation is DBD dependent while estradiol induced one is DBD independent (Webb et al 1995, Kushner et al 2000).

Estrogen activation at AP-1 site was revealed to require the integrity of both AF-1 and AF-2 with the genetic dissection of ERα domain (Kushner et al 2000). The LBD of ERα can strongly activate AP-1 promoter in the presence of estrogens but not tamoxifen. Deletion or mutation of AF-2 abolished this activation. The DBD in this estrogen activation was not critical. Moreover, mutation of AF-1 in ERα also severely decreased this activation at AP-1 site (Kushner et al 2000). It indicated that the estrogen activation at AP-1 site is AF dependent and DBD independent as well as ligand specific (estrogen but not tamoxifen) (Gaub et al 1990, Kushner et al 2000). In this pathway (Figure 3), the AP-1 site recruits fos-jun complex which stimulates transcription by recruiting CBP/p300 and associated proteins such as p160 coactivators. The estrogens-ER complex triggers the p160 in the pre-existing complex at AP-1 site into a higher state of activity with both AF-1 and AF-2 and then enhances the CBP/p300 transcriptional activity (Kushner et al 2000).

In contrast, tamoxifen activation with ER at AP-1 site was DBD dependent and AF independent (Umayahara et al 1994, Kushner et al 2000). Direct ER binding to AP-1 site was not involved (Umayahara et al 1994). Deletion of DBD in ER showed no activation in the 600 bp promoter of IGF-1 (Umayahara et al 1994). ERβ in the presence of ICI-182,780, raloxifene or tamoxifen but not estrogens can transactivate the promoter with AP-1 site and the action is completely independent of AF-2 (Webb et al 1999, Kushner et al 2000). Moreover, the ERα without AF-1 also showed this transactivation as ERβ (Kushner et al 2000). It indicated that AF was non-essential in this DBD dependent activation. Based on the finding that ER can bind to N-CoR only in the presence of tamoxifen (Jackson et al 1997,

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Lavinsky et al 1998), Kushner and his colleagues proposed the mechanism for this DBD dependent and AF independent tamoxifen-ER activation at AP-1 site (Figure 4) (Kushner et al 2000). The complex of tamoxifen and ER at site where is away from AP-1 promoter binds to N-CoR and similar corepressors and recruits histone deacetylases (HDACs). It sequesters HDACs away from AP-1 site and sets free of the activity of histone acetylases in the fos-jun-p160-CBP/p300 complex at AP-1 site. As the results, it transactivates the promoter (Figure 4).

Tamoxifen with ER can also stimulate human quinone reductase (QR) gene expression via electrophilic responsive elements (EpRE) (Montano & Katzenellenbogen 1997, Montano et al 1998). EpRE motif in human QR is comprised 12-o-tetradecanoylphorbol-13-acetate (TPA) responsive element (TRE) and TRE-like element as well as AP-1 site. Although there was AP-1 site in human QR EpRE, ER regulation at EpRE is not related to AP-1 because TPA, a potent AP-1 activity inducer (Piette et al 1988), cannot transactivate QR transcriptional activity (Montano & Katzenellenbogen 1997, Montano et al 1998). Gel shift assay with rat glutathione-s-transferase-Ya EpRE which contained no AP-1 site showed that major EpRE-interacting and activating proteins are not AP-1 (fos-jun) (Nguyen et al 1992, Favreau et al 1993). Recently, hPMC2 [human homolog of Xenopus gene (XPMC2) which prevents mitotic catastrophe] was identified to directly bind to the EpRE and interact with ERs in yeast genetic screening and *in vitro* assays (Montano et al 2000). The interaction with ERβ is stronger than ERα (Montano et al 2000). Although the mechanism for this ER regulation at EpRE is unknown, hPMC2 may act as a negative cell cycle regulator by its regulation of QR transcriptional activity (Montano et al 2000). The enzyme activates the anticancer quinones which enhance cellular levels of reactive oxygen

species (ROS) (Qiu et al 1996). Then, the increased levels of ROS result in induction of p21 and influence the cell cycle progression (Qiu et al 1996).

D. Knockout mice models

Precise disruption or knockout of a particular gene in animals and examination of their phenotypes can provide great insight into the roles of the gene in development and normal physiology. ERα (Lubahn et al 1993, Dupont et al 2000) and ERβ (Krege et al 1998, Dupont et al 2000) single knockout (ERαKO and ERβKO) and double knockout (ERαβKO) (Couse et al 1999, Dupont et al 2000) mice were generated and all showed no lethality of ERα and ERβ mutations. Their phenotypes were distinct (Couse et al 1999, Dupont et al 2000, and see reviews in Couse & Korach 1999). Both sexes of ERαKO and ERαβKO are infertile while only ERβKO females are either infertile or subfertile with reduced litter size and males are fertile (Lubahn et al 1993, Krege et al 1998, Dupont et al 2000). In female reproductive tract, ERαKO develops hypoplastic uterus and vagina and no cyclic change (Lubahn et al 1993) but the genital tract of ERβKO is normal (Krege et al 1998). The lengths of oviduct, uterus horns and vagina of ERαβKO are normal while the diameter and thickness are smaller than those in wild-type females (Couse et al 1999, Dupont et al 2000). Ovaries of ERαKO adults are anovulatory and exhibit multiple hemorrhagic cysts and no corpora lutea (Lubahn et al 1993, Dupont et al 2000) but underwent normal pre- and neonatal development. The similar features of ovaries were found in ERαβKO females. Strikingly, all ERαβKO ovaries contain fully differentiated Sertoli cells and exhibit follicle transdifferentiation to structures resembling seminiferous tubules of the testis (Couse et al

1999, Dupont et al 2000). The ovaries of ERβKO mice show an increase in the number of atretic follicles (Krege et al 1998) but an independent study on ERαβKO mice found no change and the ovaries were macroscopically normal. In most of them, corpora lutea were scarce or absent (Dupont et al 2000). These findings indicated that the roles of both ERα and ERβ in proliferation of granulosa cells are crucial and that the presence of these two receptors are required to complete the folliculogenesis in ovary. In the early stages of folliculogenesis, these receptors apparently are dispensable and can partially compensate each other in the inactivation of either of these two receptors. It indicates that they show some degree of functional redundancy. In ERαKO and ERαβKO males, the testes are lack of germ cells in the seminiferous tubules and show a marked dilation of straight tubules and rete testis (Couse et al 1999, Dupont et al 2000). The male reproductive tracts of ERβKO mice are normal but the old animals develop hyperplasia in the prostate and the urinary bladder (Krege et al 1998). However, these abnormalities were not found in another study and it even showed no change in cell proliferation in prostates of 8 and 20 months old ERαβKO mice with labeling indices by Ki67 and BrdU immunostainings (Dupont et al 2000). In contrast to ovarian growth, ERα may play a critical role and be indispensable in testis development but not ERβ. Due to the contradictory results of prostate and bladder in old ERαβKO mice (Krege et al 1998, Dupont et al 2000), the precise roles of ERβ in male reproductive tract remain to be elucidated.

E. Estrogen therapy on prostate cancer

Hormonal therapy is the mainstay of treatment in patients with metastatic prostate cancer. Medical castration is commonly accomplished with luteinizing hormone-releasing hormone (LHRH) analogues but these drugs are extremely expensive. Diethylstibestrol (DES), a synthetic estrogen, has been shown to be a good alternative and cost effective therapy (Byar 1973, Pitts 1999, Seidenfeld et al 1999, Kitahara et al 1999, Bayoumi et al 2000). Theoretically, administration of DES causes suppression of LHRH stimulation of the pituitary gland and indirectly reduces production of testosterone in testis. The serum testosterone decreases to the anorchid level (Paulson 1984, Pitts 1999, Seidenfeld et al 1999). As the results, the androgen-dependent prostate cancer regresses. Later, the patients will develop androgen-independent cancer which is non-responsive to the treatments. However, DES may not be acting solely through the pituitary-gonadal axis as described above. DES can suppress androgen-independent cancer growth in previously orchiectomized patients who already had low level of serum testosterone. Several studies showed DES could directly act on prostate cancer cells (Schulz 1990, Brehmer 1972, Hartley-Asp 1985, Robertson 1996,). Robertson et al (Robertson 1996) demonstrated direct cytotoxic effects of DES in prostate cancer cells via an apoptotic mechanism and Hartley-Asp et al (Hartley-Asp 1985) showed DES induced metaphase arrest and inhibited microtubule assembly in prostate cancer cells. *In vitro* studies also showed prostate cancer cells are sensitive to 17β-estradiol (E$_2$) via an ER-associated pathway (Carruba et al 1994, Castagnetta et al 1995). LNCaP, an androgen-responsive prostate cancer cell line, shows a significant increase in cell proliferation by treatment of E$_2$. Although LNCaP cells expressed mutated androgen receptor (codon 868, threonine to alanine) which has increased affinity to estrogens (Trapman 1990, Veldscholte

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1990), the presence of ER by immunohistochemistry and RT-PCR analysis and reversion of E_2-induced growth by antiestrogen (ICI-182,780) suggested the biological response of LNCaP cells to E_2 is mediated via its own receptor (Castagnetta et al 1995). For the androgen-nonresponsive prostate cancer cell line (PC-3), the response to E_2 is totally different from LNCaP. Instead of growth stimulation, E_2 inhibited growth of PC-3 cells probably via an ER-associated pathway (Carruba 1994). We carried out the comprehensive study on the potential cell growth inhibition by estrogens such as DES and 17β-estradiol in different prostate cancer cells including DU145 cells which had not been examined before.

F. Antiestrogens and prostate cancer

In the early 80s, Noble (Noble 1980a and 1980b) demonstrated the estrogen-dependence of prostate cancer in Noble rat model. The tumor growth was inhibited by removal of estrogen treatment and by tamoxifen, a non-steroidal antiestrogen, with continuation of estrogen treatment. These findings prompted to the notion that using antiestrogens could be the first arm of treatment on prostate cancer. Clinical trials on tamoxifen with advanced stage prostate cancer patients were conducted (Glick et al 1982, Spremulli et al 1982). These studies showed tamoxifen to be a palliative treatment of the disease. This may be partially explained by the estrogenic effects of tamoxifen (Rayter et al 1994, Eells et al 1990, Kedar et al 1994). However, in an Eastern Cooperative Oncology Group trial, no patient responded to the treatment (Horton et al 1988). Moreover, the estrogenicity of tamoxifen may increase the risk of cardiovascular disease similiar to the side effects of DES treatment. Therefore, the potential for using antiestrogens as single arm or

adjuvant treatment in prostate cancer was not fully explored. Later, ICI-164,384 and ICI-182,780 as steroid antiestrogens were developed and exhibited no estrogenic activity (Wakeling et al 1991, Osborne et al 1995). These chemicals can help us to address this important issue that pure antiestrogens may be potential drugs for prostate cancer.

Experimental evidence suggests that their mechanisms of action may differ significantly from those of estrogens (Van Den Bemd et al 1999, Zou et al 1999, Barkhem et al 1998, Paech et al 1997) and therefore may yield more favorable outcomes. Secondly, a newly discovered ER subtype (ER-β) was found to be expressed at high levels in the epithelial compartments of the rat prostate (Kuiper et al 1997, Mosselman et al 1996, Lau et al 1998, Prins et al 1998, Chang and Prins 1999). Although ER-β shares high homology with the classical ER (ERα), the two ER subtypes may regulate different sets of cellular functions (Paech et al 1997, Montano et al 1998). Recent findings from an ERβ knockout mouse suggest that ERβ may suppress proliferation and prevent hyperplasia in the rodent prostate (Krege et al 1998). Taken together, these new findings raise an intriguing possibility that ERβ is expressed in normal and/or malignant human prostatic epithelial cell (PrEC) and plays a role in mediating estrogen action in these cell types.

CHAPTER II

HYPOTHESIS

Since ERβ was first identified in rat prostate and the presence of this receptor was confirmed by microdissected rat epithelial cells in our previous study (Lau 1998), we hypothesized that ERβ may also be expressed in normal and/or malignant human prostatic epithelial cell (PrEC). It may play a role in mediating estrogen action in these cell types. Alteration of this receptor expression may be involved in prostatic carcinogenesis and its expression level potentially correlates to the different stages of prostatic lesions. Although the usage of tamoxifen in treatment of prostate cancer had been tested and apparently failed the clinical trials which is possibly due to its partial estrogenicity, pure estrogen antagonists (ICI 164,384 and ICI 182,780), selective estrogen receptor modulators, such as raloxifene, and phytoestrogens may yield more favorable outcomes since experimental evidence suggests that their mechanisms of action possibly via ERβ may differ significantly from those of estrogens and tamoxifen. This has rekindled interests in using antiestrogens and/or as therapeutic for prostate cancer.

CHAPTER III

EXPRESSION OF ESTROGEN RECEPTOR (ER)- α AND ER-β IN NORMAL AND MALIGNANT PROSTATIC EPITHELIAL CELLS: REGULATION BY METHYLATION AND INVOLVEMENT IN GROWTH REGULATION

(Lau KM, LaSpina M, Long J, Ho SM. Cancer Research 60:3175-3182, 2000)

A. 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. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analyses were used to determine if transcripts of estrogen receptor (ER)-α and those of ER-β were expressed in our normal PrEC primary cultures, in a commercially available PrECs preparation (PrEC™, Clonetics), 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 ER-α and ER-β 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-β, but not those of ER-α, 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-β in normal

human PrECs. In contast, PrEC™ and BPH-1 cells expressed both ER-α and ER-β transcripts, and no PR nor pS2 mRNA in PrEC™ and only minimal level of PR mRNA in BHP-1. Among the three prostate cancer cell lines, LNCaP expressed ERβ mRNA along with transcripts of PR and pS2, DU145 expressed messages of ER-β and PR, and PC-3 cells exhibited ER-α, ER-β 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-α mRNA in LNCaP and DU145, and that of pS2 message in DU145. These findings provide the first experimental evidence that ER-α gene silencing in prostate cancer cells, and perhaps also in normal PrECs, is caused by DNA hypermethylation.

To evaluate the potential of using antiestrogens as prostate cancer therapies, we have assesesed the growth inhibitory action of estrogens [estradiol (E2) and diethylstilbestrol (DES)] and antiestrogens, [4-hydroxy-tamoxifene (4OH-TAM) and ICI-182,780 (ICI)] on PC-3 and DU-145 cells. In PC-3 cells, which express both ER subtypes, estrogens as well as antiestrogens are effective inhibitors. On the contrary, in DU145 cells, which express only ER-β, antiestrogens, but not estrogens, exhibit potency. By comparison, ICI is usually the more effective cell growth inhibitor. Importantly, the ICI-induced antiproliferative effect could be reversed by co-treatment of DU145 cells with an ER-β antisense oligonucleotide, hence lending additional support to a central role played by ER-β in antiestrogen action.

B. INTRODUCTION

Charles Huggins pioneered the use of the synthetic estrogen, diethylstibestrol (DES), in the treatment of advanced prostatic adenocarcinoma (PCa) in the early 40' (Huggins and Hodges 1941). 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 (Paulson 1985). However, recent investigations have demonstrated that DES exerts direct growth inhibitory effects on prostatic cancer cells via induction of mitotic arrest or apoptosis (Brehmer et al 1972, Hartley-Asp et al 1985, Schulze and Claus 1990, Robertson et al 1996). Unfortunately, due to serious adverse effects induced by the estrogenicity of DES (feminisation, exacerbation of heart failure, vascular complications, gynaecomastia, and impotence), the xenoestrogen has lost its attractiveness as a mainstay treatment for advanced PCa (Ahmed et al 1998). Clinical use of Tamoxifen (TAM), a nonsteroidal estrogen mixed agonist/antigonist, was introduced in the 80s as an alternative to DES in the treatment of PCa. It was better tolerated than DES, but only produced low response rates (Glick et al 1982, Spremulli et al 1982, Horton et al 1988, Bergan et al 1995, Bergan et al 1999). 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. First, pure estrogen antagonists (e.g. ICI-164,384 and ICI-182,780; Wakeling et al 1991) and Selective Estrogen Receptor Modulators (SERMs) such as raloxifene (Palkowitz et al 1997) have become available for clinical trials. Experimental evidence suggests that their mechanisms of action may differ significantly from those of estrogens (Van Den Bemd et al 1999, Zou et al 1999, Barkhem et al 1998, Paech et al 1997) and therefore may yield more favorable outcomes. Secondly, a newly

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discovered ER subtype (ER-β), was found to be expressed at high levels in the epithelial compartments of the rat prostate (Kuiper et al 1997, Mosselman et al 1996, Lau et al 1997, Prins and Birch 1997, Chang and Prins 1999). Although ER-β shares high homology with the classical ER (ER-α), the two ER subtypes may regulate different sets of cellular functions (Paech et al 1997, Montano et al 1998). Recent findings from an ER-β knockout mouse suggest that ER-β may suppress proliferation and prevent hyperplasia in the rodent prostate (Krege et al 1998). Taken together, these new findings raise an intriguing possibility that ER-β is expressed in normal and/or malignant human prostatic epithelial cell (PrEC) and plays a role in mediating estrogen action in these cell types.

Knowledge of the distribution of ERβ in normal and malignant human PrECs is limited at this time. A recent study reported a lack of ERβ expression in human prostate tissues (Bonkhoff et al 1999) while several preliminary reports noted expression of this receptor subtype in basal epithelial cells of the human (Lau et al 1999a, Sinisi et al 1999, Taylor et al 1999). In this study, we reported that 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 ERα and ERβ transcripts in our primary cultures of normal PrECs were compared to those found in a PrEC preparation obtained from a commercial source (PrEC™, Clonetics), in an immortalized PrEC cell line established from a benign prostatic hyperplasia specimen (BPH-1, Hayward et al 1995), and in three prostatic cancer cell lines (DU145, PC-3 and LNCaP). Expression levels of ER-α and ER-β in normal and malignant PrECs were then related to transcript expression levels of two estrogen-responsive genes (progesterone receptor [PR] and pS2) to

gain insights into the functionality of the ER subtypes. Additionally, we have compared the efficacy of two estrogens (DES and 17β-estradial [E2]) to those of two antiestrogens (4-hydroxy-Tam [4OH-TAM] and ICI-182,780 [ICI]) in inhibiting cell growth in PC-3 and DU145 cells. An ER-β antisense oligonucleotide (ODN) was then used to demonstrate that the antiestrogen-induced growth inhibitory effects on prostate cancer cells were mediated via an ER-β 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-α silencing in PrECs.

C. MATERIALS AND METHODS

i. Establishment of normal prostate epithelial cells (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 IRB (approval was granted to JL at the New England Medical Center, Boston, Massachusetts). 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 mid-portion of the core, and suspended in 5ml of culture medium (described below). The two remaining ends of the core were inked at the ends opposite the sectioned mid-portion piece, placed in 10% formalin, and processed for histology. Histological examination of the end pieces of a biopsy core allowed us to determine the histologic 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 Hank's balanced salt solution (HBSS) and cut into 5-7 smaller pieces. The pieces were suspended in 2ml 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), previously reported (Bright et al 1997), consisted of keratinocyte serum-free medium with 25 μg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, 2 mM L-

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glutamine, 10 mM HEPES buffer, P/S (100 U/ml Penicillin and 100 µg/ml Streptomycin), 5.5 µl/ml fungizone, 20 ng/ml cholera toxin and 1% heat-inactivated fetal bovine serum (FBS) was used to obtain enriched populations of PrECs. All culture reagents were obtained from Life Technologies (Grand Island, NY) except for FBS, which was purchased from Sigma (St. Louis, MO). The culture was incubated at 37 °C in a 5% CO_2 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 approximately 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 prostate epithelial cells (PrEC™) were purchased from Clonetics Co. (San Diego, CA). The PrEC™ cells were cultured in the PrEGM medium supplemented with SingleQuots™ (Clonetics Co., San Diego, CA) according to the manufacturer's recommended protocol.

ii. Maintenance of established prostatic cell lines

All culture reagents were obtained from Life Technologies (Grand Island, NY) and FCS from Sigma (St. Louis, MO) except otherwise specified. BPH-1 (Hayward et al 1995), 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 at San Francisco, California. This cell line was maintained in RPMI1640 with 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 4.5 g glucose/L and 1.5 g sodium bicarbonate/L (ATCC, 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 ATCC.

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 non-essential amino acids, P/S, 0.05 mM β-mercaptoethanol (Sigma Co), and 1% ITS+ [TM]. LNCaP were maintained in the same medium used for BPH-1 except that ITS+[TM] was left out from the medium. All cell cultures were incubated at 37°C under a 5% CO_2 atmosphere.

iii. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using RNA Stat-60 reagent (Tel-Test Inc., Friendwood, TX) according to protocols provided by the manufacturer. The quality of each total RNA sample was checked and controlled by the following steps: 1) measurement of optical density, 2) 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 ribosomal RNA signals, and 3) conducting a semi-quantitative RT-PCR for the 18S ribosomal RNA at low cycle numbers. One μg of total cellular RNA was reverse-transcribed using the GeneAmp RNA PCR kit (Perkin-Elmer, CT) and 2μl of the resulting cDNA was used in each PCR.

Intron-spanning primers were either obtained from published literature or designed using the Primer3 Output program (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). Primer sequences for GAPDH, ERα, ERβ, PR and AR are given in Table 1. All PCR conditions were optimized for quantification of relative message contents under non-saturating conditions. Preliminary experiments were conducted to ensure linearity for all semiquantitative procedures. Hot start PCR using AmpliTaq Gold DNA polymerase (Perkin-Elmer, CT) was employed in all amplification reactions. The enzyme was activated by pre-heating the reaction mixtures at 95°C for six minutes prior to PCR. This protocol was chosen to minimize non-specific 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: 1) amplification for ERβ cDNA used an annealing temperature of 58°C, 2) amplifications of ERα cDNA and AR cDNA were carried out at an annealing temperature of 55°C, 3) cycle-number for ERα cDNA amplification was set at 35, and 4) 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.

iv. Treatment of DU145, PC-3 and LNCaP cells with demethylating agents

The three prostatic cancer cell lines were seeded at a density of 10^4 cells per ml medium in 25 cm^2 culture flasks, allowed to attach during a 24h 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 µM and 5 µM and 5'aza-2'-deoxycytidine at 0.5 µM and 0.75 µM, respectively. Culture medium was changed every four 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 extracted for RT-PCR.

v. Treatment of DU145 and PC-3 cells with estrogens/antiestrogens

Cells were seeded at a density of 5 X 10^3 per ml into 24-well plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) in a final volume of 1 ml culture medium with 5% charcoal-stripped FBS. Twenty-four hours following seeding, triplicate wells of cells were treated in with 1µM, 10µM and 100µM of estradiol-17β (E$_2$), diethylstilbestrol (DES), 4'-hydroxytamoxifen (4OH-TAM) or ICI 182,780 (ICI). E$_2$, DES, and 4OH-TAM were

purchased from Sigma Co. (St. Louis, MO) and ICI was a generous gift from Zeneca Pharmaceuticals (Macclesfield, United Kingdom). Estrogens and antiestrogens were dissolved in absolute ethanol (Sigma Co., St. Louis, MO) and added to the media daily. Cell cultures 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 re-fed with freshly prepared medium every other day. At the end of a 4-day treatment period, cells in each well were trypsinized and cell count determined by direct counting using the Trypan blue exclusion method. All treatment experiments were repeated at least three times to generate statistically relevant data.

vi. Treatment of DU145 cells with ICI and ERβ antisense oligonucleotide (ODN)

DU145 cells (5 X 10^3 cells per well) were plated in 24 wells-plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ). After allowing 24 hrs for cell attachment, cell cultures were treated in triplicate with 1μM ICI in the presence of 2.5 μM ERβ antisense, sense or mismatch ODNs for 4 days. The ERβ antisense ODN, an 18-mer, was designed to recognize the first translation start site on the ERβ mRNA and its immediate 5'flanking region (Table 2). The nucleotide sequence of sense ODN is complementary to those of ERβ antisense ODN (Table 2). Based on the sequence of ERβ antisense ODN, 5 nucleotides were scrambled to generate a mismatch ODN which retains the same GC ratio of the ERβ 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 3 nucleotides were phosphorothioate-modified to increase their stability *in cellulo*. 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 obtained statistically relevant data.

vii. Statistics

Statistical analysis was performed by using Student SYSTAT software (Course Technology, Inc., Cambridge, MA). Data was 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.

D. RESULTS

i. Expression of AR, ERβ, PR and pS2 mRNA, but not ERα transcripts, in normal PrEC in primary cultures

Five primary cultures of normal PrECs (N4#6, N3#5, N3#4, N2#3, 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 (Figure 4) demonstrated that our normal PrEC cultures retained high levels of androgen receptor (AR) mRNA expression, which usually disappeared in late passage normal PrEC primary cultures or in established PrEC (Grant et al 1996). Interestingly, all five cultures of normal PrECs expressed uniform levels of ERβ RNA, and transcripts of the estrogen responsive genes, PR and pS2. In contrast, expression of ERα 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 SV-40 immortalized prostatic epithelial cell line, expressed both ERα and ERβ, but no PR or pS2 transcripts in PrEC™ and only minimal level of PR mRNA in BPH-1.

ii. Expression of ERβ, ERα, PR and pS2 mRNA in prostatic cancer cell lines

All three prostatic cancer cell lines, DU145, PC-3 and LNCaP, express ERβ mRNA (Figure 4a,c). In contrast, ERαmRNA 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.

iii. Expression of ERα variant in prostate cell lines

When RT-PCR analyses were conducted for ERα 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 co-amplified (Figure 4a, b). Sequencing analysis (data not shown) revealed that this smaller PCR product was derived from an ERα mRNA variant which had whole exon 2 deleted. We recently reported the co-existence of this ERα mRNA variant with wild-type transcripts in normal and malignant human ovarian surface epithelial cells (Lau et al 1999b).

vi. Demethylation reactivates ERα and pS2 mRNA expressions in DU145 cells and ERα expression in LNCaP cells

Prior to exposure to demethylating agents, ERα and pS2 transcripts were not detected in RNA samples prepared from DU145 cells (Fig. 4a,c and Fig. 5). After the 8-day treatment with 5'-aza-cytidine (2.5µM and 5µM) or 5'-aza-2'deoxycytidine (0.5µM and 0.75µM), DU145 cells regained expression of both transcripts (Fig. 5). 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 ERα mRNA expression (data not shown).

v. 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 ERβ mRNA, was adversely effected by the antiestrogens, ICI and 4OH-TAM (Figure 6a & 6b). A dose-dependent inhibition of cell numbers was observed in cultures exposed to ICI for 4 days when compared to control cultures treated with vehicle (absolute ethanol). A 40% reduction ($p < 0.001$) in the cell numbers was achieved with an ICI dose of 1μM. A similar growth inhibitory response was observed when DU145 cells were treated with 4OH-TAM. However, cell number reduction achieved with 1μM of 4OH-TAM was only around 25% ($p < 0.001$). In contrast, exposure of DU145 cells to estrogens (E2 and DES) did not affect cell growth in 4-day exposure experiments (Figure 6c & 6d).

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 1μM or 10μM of ICI, or with 1μM of 4OH-TAM ($p < 0.001$, Figure 7a & b). Furthermore, exposure of PC-3 cells to E2 at 1μM or 10μM concentrations also induced inhibition of cell growth ($p < 0.05$ and $p < 0.01$, respectively; Figure 7c). Interestingly, treatment with DES at the various concentrations did not elicit statistically significant cell growth inhibition in PC-3 cells (Figure 7d).

vi. Reduction of ICI-induced cell growth inhibition by ERβ antisense ODN.

Treatment of DU145 cells were with ICI at 1μM induced a 40% reduction in cell number (Figure 7). Co-treatment of DU145 cells with ICI and an ERβ antisense ODN led to restoration of cell number ($p < 0.001$, Figure 8) while co-treatments with an ERβ sense ODN or a mismatch ODN (Table 2) did not reverse the ICI-induced effects. These data support the

notion that the ICI-induced cell growth inhibition in DU145 cells is mediated via an ERβ

signaling mechanism.

E. DISCUSSION

The roles played by estrogens in the neoplastic transformation of PrEC as well as in PCa progression and treatment remain controversial. Exposure of human or rodent to estrogens induces a proliferative lesion, squamous metaplasia, in their prostates (Sugimura et al 1988, Yonemura et al 1995, Triche and Harkin 1971, Levine et al 1991) while prolonged treatment of Noble rats with androgen plus estrogen causes a high incidence of prostate adenocarcinoma in the dorsolateral prostates of the treated animals (Noble 1980, Drago 1984, Leav et al 1995, Bosland et al 1995). Paradoxically, DES, TAM and other estrogens have been used as treatment regimens for advanced metastatic PCa (Huggins and Hodges 1941, Ahmed et al 1998, Glick et al 1982, Spremulli et al 1982, Horton et al 1988, Bergan et al 1995, bergan et al 1999, Klotz et al 1999, Smith et al 1998). 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 (Brehmer et al 1972, Hartley-Asp et al 1985, Schulze and Claus 1990, Robertson et al 1996, Landstrom et al 1996). Precisely how estrogens/antiestrogens elicit these actions remain uncertain.

Traditionally, the actions of estrogens/antiestrogens are thought to be mediated via the classical ER, the α-subtype, which has been localized to the stromal compartment and basal epithelial cells of human and rodent prostates (Prins and Birch 1997, Bonkhoff et al 1999, Wernert et al 1988, Ehara et al 1995, Kirschenbaum et al 1994, Hiramatsu et al 1996). Since ER-α is not expressed in the normal glandular epithelium of rat or human prostate (Hartley-Asp et al 1985, Lau et al 1997, Bonkhoff et al 1999, Ehara et al 1995,

Kirschenbaum et al 1994, Hiramatsu et al 1996, Rohlff et al 1998), it is widely believed that the action of estrogen/antiestrogen on normal PrECs is indirect, likely mediated via estrogen-induced stromal factors. However, following the discovery of ER-β (Kuiper et al 1997) and its localization to the epithelial compartment of rodent prostates (Kuiper et al 1997, Lau et al 1997, Couse et al 1997) a distinct possibility has been raised that estrogen/antiestrogen could influence PrEC function via an ER-β signaling pathway. However, at present, information on ER-β in human PrECs is limited. Only one recent study (Bonkhoff et al 1999) have evaluated the expression pattern of ER-β transcripts and proteins in human prostatic tissues and found non-detectable levels in both normal and diseased tissues. In contrast, the present study unequivocally demonstrated expression of ER-β mRNA in highly enriched or pure human PrEC cultures established from peripheral zone biopsies. Furthermore, since ER-α message was undetectable, but transcripts of two estrogen dependent genes, PR and pS2, were expressed in these cultures, these data strongly suggest that ER-β is the cellular mediator of estrogen action in normal human PrECs. Of interest to note is that both ERα and ERβ mRNA, but not PR or pS2 transcripts were expressed in a PrEC preparation purchased from a commercial source (PrEC™, Clonetics) and in the immortalized PrEC line, BPH-1, with only minimal level of PR transcripts. The discrepancies between ER subtype, PR and pS2 expression in our primary PrEC cultures and those observed in PrEC™ and BPH-1 could be due to the tissue of origin of these cell cultures/lines. In this regard, PrEC™ cultures (Clonetics) are routinely prepared from whole prostates and BPH-1 was derived from a benign hyperplastic specimen (Hayward et al 1995) while 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 (Carruba et al 1994, Catagnetta et al 1995) observed ER-α expression in human prostate cancer cell lines, including LNCaP, PC-3 and DU-145, while others (Hobisch et al 1998) did not. Similarly, observations on ER-α expression in prostate cancer specimens were equally controversial. Bonkhoff and co-workers recently reported that ER-α expression was infrequent in low-to-moderate grade adenocarcinoma but common in high grade and metastatic cancers (Bonkhoff et al 1999). Conversely, Konishi and associates noted the presence of ER-α immuno-positivity in well-differentiated adenocarcinomas but not in poorly differentiated specimen (Konishi et al 1993). The latter observation was supported by two additional studies that reported no ER-α expression in lymph node and distant metastases (Hobisch et al 1998, Castagnetta and Carruba 1998). These issues become more convoluted when expression pattern of ER-β is taken into consideration. A lack of ER-β expression in human prostate tissues was reported by Bonkhoff and co-workers (Bonkhoff et al 1999) while several preliminary reports noted expression of this receptor subtype in dysplastic and cancerous tissues (Lau et al 1999a, Sinisi et al 1999, Taylor et al 1999). Findings in the present study revealed that ERβ mRNA was expressed in all three cancer cell lines (PC-3, DU145 and LNCaP) although, in accordance with the literature, the ERα message was only detected in PC-3 cells (Carruba et al 1994). Unlike primary PrEC cultures, which uniformly expressed both PR and pS2 transcripts, PR mRNA expression was only detected in DU145 and LNCaP cells while pS2 transcripts found in PC-3 and LNCaP cells. Hence, despite uniform expression of ER-β, the expression patterns of ER-α, PR and pS2 among these prostatic cancer cell lines was

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variable. Since transcription of PR and pS2 is well recognized to be regulated by estrogen (Brown et al 1984, Stack et al 1988, Savouret et al 1991) the loss of expression of these two genes in some prostatic cancer cell lines suggest a deregulation of estrogen signaling in these cells. A similar phenomenon has been observed in ovarian cancer cell lines which express both ER subtype but no PR (Lau et al 1999b).

Up till now, it remains unknown as to why prostatic epithelium expresses only ER-β and not ER-α. In the present study, we demonstrated that treatment of DU145 and LNCaP cells with demethylating agents reactivated ERα expression in these cells. These data provide the first experimental evidence in support of DNA methylation-mediated gene silencing (Zingg et al 1997, Jones and gonzalgo 1997) as a mechanism of ER-α inactivation in PrECs. In breast cancers, it had been shown that hypermethylation of the promoter region of ER-α was associated with loss of expression of this receptor subtype in hormone refractory cancers (Ottaviano et al 1994, Ferguson et al 1995, Martin et al 1995, Lapidus et al 1998). Although due to 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-α silencing in the normal prostatic epithelium. Interestingly, loss of pS2 expression in DU145 cells might also be linked to hypermethylation-mediated transcriptional inactivation since exposure of this cell line to demethylating agents revived pS2 expression. The pS2 gene encodes an 84 amino acid, cystein rich, secretary protein, which is widely expressed in estrogen sensitive tissues (Stack et al 1988). Its expression in prostate cancer specimens has been shown to be associated with

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premalignant changes and neuroendocrine differentiation (Bonkhoff et al 1995, Colombel et al 1999).

Different variants of ER-α transcripts are often found to co-exist with the wild type transcript in normal and malignant tissues (Hirata et al 1995, Leygue et al 1996). 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 (Carruba et al 1994). In the current study, we found an exon 2 deletion variant in PC-3, BPH-1 and PrEC™ 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 (Ahmed et al 1998). In contrast, TAM, a non-steroidal anti-estrogen, is better tolerated but produces little objective responses in multiple trials (Glick et al 1982, Spremulli et al 1982, Horton et al 1988, Bergan et al 1995, Bergan et al 1999). With the discovery of ER-β as a new estrogen signaling pathway and the availability of pure anti-estrogen such as ICIs (Wakeling et al 1991), 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 (E2 and DES) an two antiestrogens (4OH-TAM and ICI) in inhibiting cell growth in two androgen refractory prostate cancer cell lines, PC-3 that we found express both ER subtypes and DU145 that express only ER-β. Our results demonstrated all four estrogenic/antiestrogenic compounds, at relatively low doses, exerted

antiproliferative effects on PC-3 cells, with antiestrogens exhibiting greater potencies. On the contrary, DU145 responded only to antiestrogens. In both cases, ICI was found to be more potent than 4OH-TAM as a growth inhibitor. Importantly, the antiestrogen-induced growth inhibitory response in DU145 cells was reversible by co-treatment with an ER-β antisense ODN, although the efficiency of the antisense to block ERβ protein synthesis in the treated cells was not demonstrated in our study. Further experiments were warranted to verify the involvement of this ICI-induced growth inhibition in DU145 cells. Taken together, these findings raise several significant implications. First, 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 ERβ by Gustafsson (Reynolds 1999). Secondly, since 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 (England and Jordon 1997). Finally, our data have provided the first demonstration that estrogen/antiestrogen action in prostatic cancer cells could signal via an ER-β pathway. Since ER-β selective ligands have recently been reported (Montano et al 1999), this development raises the likelihood of using receptor subtype ligands as cancer therapeutics in the future.

Webb and his colleagues found that estrogen receptors with either tamoxifen or estradiol can transactivate the promoters with AP-1 site and demonstrated that tamoxifen induced DNA binding domain (DBD) dependent activation while the estrogen-induced activation is DBD independent (Webb et al 1995, Kushner et al 2000). ERβ in the presence

of ICI-182,780, raloxifene or tamoxifen but not estrogens can transactivate the promoter with AP-1 site and the action is completely independent of AF-2 (Webb et al 1999, Kushner et al 2000). In the DBD dependent activation of AP-1 site, the potency of ICI is higher than that of TAM and estrogens showed no effects on this activation (Webb et al 1995, Kushner et al 2000). In our study, ICI inhibited about 40% of cell growth while TAM induced 20% cell growth inhibition, indicating the higher potency of ICI on cell growth inhibition in DU145 cells as compared to TAM. In addition, no effects on cell growth was found in the estrogen treatments. Therefore, the ICI may transactivate AP-1 site via DBD dependent pathway to modulate the cell growth in DU145 cells.

In summary, this paper has demonstrated that human normal PrECs express exclusively ER-β 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-α gene silencing in prostate cancer cells, and perhaps also in normal cells, may be caused by DNA-hypermethylation. Overall, data from this paper lend support to the notion that ER-β, plays a central role estrogen/antiestrogen signaling in normal and malignant human PrECs.

CHAPTER IV

COMPARATIVE STUDIES OF ESTROGEN RECEPTOR-BETA, -ALPHA AND ANDROGEN RECEPTOR IN NORMAL HUMAN PROSTATE GLANDS, DYSPLASIA, AND IN PRIMARY AND METASTATIC CARCINOMA.

A. ABSTRACT

An antibody specific for estrogen receptor-β (ER-β), was used to immunolocalize the receptor in histologically normal prostate, dysplasias (prostatic intraepithelial neoplasia-PIN), primary carcinomas and in metastases to lymph nodes and bone. Comparisons were made between ER-β, estrogen receptor -α (ER-α) and androgen receptor (AR) immunostaining in these tissues. Concurrently, transcript expression of the three steroid hormone receptors was studied by RT-PCR analysis on laser microdissected (LCM) samples of normal prostatic acini, dysplasias and carcinomas.

In normal prostate, ER-β immunostaining was exclusively localized in the nuclei of basal cells and to a lesser extent stromal cells. ER-α staining was only present in stromal cell nuclei. AR immunostaining was variable in basal cells but strongly expressed in nuclei of secretory and stromal cells.

Overall, prostatic carcinogenesis was characterized by a loss of ER-β expression at the protein and transcript levels in high-grade dysplasias, its reappearance in grade 3 cancers, and its diminution/absence in grade 4/5 neoplasms. In contrast, AR was strongly expressed in all grades of dysplasia and carcinoma. Since ER- is thought to function as an inhibitor of

prostatic growth, androgen action, presumably mediated by functional AR and unopposed by the receptor, may have provided a strong stimulus for aberrant cell growth. With the exception of a small subset of dysplasias in the central zone and a few carcinomas, ER-stained cells were not found in these lesions.

The majority of bone and lymph node metastases contained cells that were immunostained for ER-β. Expression of ER- in metastases may have been influenced by the local microenvironment in these tissues. In contrast, ER-α stained cells were absent in bone metastases and rare in lymph nodes metastases. Irrespective of the site, AR positive cells were found in all metastases. Based on our recent finding of ER-β mediated growth inhibition of prostate cancer cells *in vitro* (Lau et al 2000), the presence of ER-β in metastatic cells may have important implications for the treatment of late stage disease.

B. INTRODUCTION

Cellular differentiation and proliferation of prostatic epithelium has long been considered to be primarily mediated by androgens. In this regard, the majority of prostate cancers are initially responsive to antiandrogenic therapies but eventually become refractory to this form of treatment (Grayhack et al 1987, Kozlowski et al 1991). The first medical antiandrogenic therapy used to treat men with prostate cancer employed estrogens, primarily acting indirectly at the hypothalamic level to down- regulate circulating levels of androgens, with resultant degenerative effects on neoplastic cells (Huggins and Hodges 1941). Paradoxically, despite its use as an antiandrogen, pharmacological doses of estrogens can also induce a marked proliferative alteration of prostatic epithelium termed squamous metaplasia, in the glands of a variety of mammals including humans (Helpap and Stiens 1975, Leav et al 1978, Merk et al 1986, Mawhinney and Neubauer 1979, Levine et al 1991). The metaplastic change is initiated by the proliferation of basal cells which subsequently differentiate into squamous cells (Leav et al 1978, Merk et al 1986, Levine et al 1991). Importantly, it has been shown that estrogens alone can directly induce squamous metaplasia in the regressed prostates of castrated or hypophysectomized dogs (Leav et al 1978, Merk et al 1986).

Despite the proliferative effects of estrogens on basal cells which are the purported progenitor cells of prostatic glandular epithelia (De Marzo et al 1998), the role that the hormone may play in the abnormal growth of the gland remains undefined. Results from a number of epidemiological (Ho et al 1997, Bosland 2000) and experimental studies (Ho et al 1997, Bosland 2000, Santti et al 1994, Pykkanen et al 1996, Leav et al 1988, Bosland et al 1995) have suggested that estrogens may be involved in this process.

Effects of estrogens on target tissues are now known to be mediated by ligand-specific transcription factor receptor proteins termed estrogen receptor -α and -β (ER-α and ER-β) (Kuiper et al 1996, Mosselman et al 1996, Tremblay et al 1997, Bhat et al 1998). The two isoforms have highly homologous in DNA–binding domains but significant differences in acid amino sequences are found in the N-terminal, hinge region, ligand binding and F domains (Mosselman et al 1996). Both receptors are present in many of the same tissues but differences in organ and tissue distribution as well as in levels of expression has been reported for the two isoforms (Mosselman et al 1996, Enmark et al 1997, Kuiper et al 1997). In this regard, ER-β mRNA was found to be predominant over the ER-α isoform in the rat prostate (Kuiper et al 1996) and it was also present in human gland albeit in lesser amounts than in the testis (Mosselman et al 1996). ER-β and ER-α were also shown to bind to the same ligands with different affinity (Kuiper et al 1997). In addition, following binding to estrogens and antiestrogens, the two ER-isoforms utilize different enhancer elements such as estrogen responsive element (ERE) and AP1 sites in promoter regions of gene (Paech et al 1997). Taken together, these two studies suggest that ER- and - may mediate diverse downstream effects (Paech et al 1997, Enmark and Gustafsson 1999, Gustafsson 1999).

Although the precise biological function of the two ER-isoforms in the prostate is currently undefined (Chang and Prins 1999), in one study, ER-β knockout mice have been reported to develop age–related prostatic hyperplasia, which suggests that the receptor may act to inhibit abnormal growth of the gland (Krege et al 1998). In support of this concept, Poelzl et al (2000) have recently reported that ER-β, but not the α isoform, specifically interacts with MAD2 the cell cycle spindle assembly checkpoint protein. Moreover, Lau et al

(2000) demonstrated that antiestrogens downregulate cell proliferation in human prostate cancer cells that only express the ER- isoform. ER-β has also been found to be involved in mediating estrogen/antiestrogen induction of quinone reductase via its interaction with the electrophile/antioxidant response element (EpRE) in the promoter region of the gene (Montano et al 1998). It has therefore been proposed that the receptor may be involved in regulating the expression of antioxidant enzymes and thus play a role in protecting cells against oxidative injury Chang and Prins 1999, Montano et al 1998).

Prior to the discovery of ER-β, ER-α antibodies or *in situ* hybridization were used to study estrogen receptor localization in normal, hyperplastic and carcinomatous human prostate tissues (Wernert et al 1988, Schulze et al 1990, Ehara et al 1995). Most of these reports showed that ER-α was predominately localized in the stroma of normal and hyperplastic prostates with the occasional detection of the receptor in basal cells and glandular epithelia. With the exception of one recent study (Bonkhoff et al 1999), ER-α immunostaining was not detected in primary or metastatic prostate cancers. To date, there is only one published report of ER-β localization in the normal human prostate (Taylor and Azzawi 2000). Similarly, one study has reported ER-β protein expression in human breast tissues using an antibody developed by the investigators (Fuqua et al 1999).

In the current investigation, we developed a novel antibody directed against the F domain of ER-β, a region that has no homology with the α receptor (Mosselman et al 1996). We demonstrate that this antibody does not cross react with ER- . This reagent was used to immunolocalize the receptor in morphologically normal glands from the 3 anatomic zones of the prostate, dysplasia (also termed prostatic intraepithelial neoplasia -the purported

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precursor of carcinoma) (McNeal 1993, Bostwick 1995), and in primary and metastatic carcinoma. Laser–capture microdissection/reverse transcription polymerase chain reaction (LCM/RT-PCR) was also used to study transcript expression of the receptor in dysplastic lesions and in grade 3 and 4/5 carcinomas. Results from the studies of ER-β were compared with the concomitant investigation of androgen receptor (AR) and ER-α expressions at both immunohistochemical and transcript levels. In this manner, changes in the expression of any of the three receptors could be evaluated as to how they may relate to the development and progression of prostatic carcinoma. Differences in receptor expression between grades 3 and 4/5 were emphasized in our study since it has recently reported that the percentage of grade 4/5 carcinoma in a prostatic neoplasm is highly predictive of disease progression (Stamey et al 1999).

To our knowledge, our report is the first where antibodies specific for ER-β, ER-α and AR were used together with LCM /RT-PCR to compare the expression of these receptors in normal human prostate, dysplastic lesions and carcinoma.

We find that within the epithelial compartment of normal human prostate, ER- is predominately localized in basal cells and to a lesser extent stromal cell nuclei. In contrast, ER- was rarely detected in basal cells but was strongly expressed in stromal cell nuclei.

Expression of the   receptor was diminished in high-grade dysplasia and grade 4/5 carcinoma of the peripheral zone. A similar trend was found at the transcript level in microdissected tissues. The majority of metastases to bone and lymph nodes however contained ER- immunopositive carcinoma cells. In contrast to ER-β, ER- expression at both protein and transcript levels was absent in all dysplasias but present in a few carcinomas of the peripheral zone. The   receptor was however expressed in metastases to two lymph

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nodes and in the majority central zone dysplasias. AR expression remained consistently strong in all grades of dysplasias and primary carcinomas as well as in metastases.

In summary, we report that a downregulation of ER-β expression occurs during prostatic carcinogenesis. This change may contribute to a loss in growth control processes mediated by the β receptor which could amplify the effects of persisting proliferative stimuli such as those mediated by AR.

The presence of ER-β as the predominant ER subtype in most metastases, together with our recent findings that antiestrogens binding to the receptor inhibit proliferation of prostate cancer cells (Lau et al 2000), may be useful in devising new ligand-specific treatments for late stage disease.

C. MATERIALS AND METHODS

i. Generation of the GC17 polyclonal antibody:

The composition of the immunizing peptide used to generate the GC17 rabbit anti ER- antibody was selected with aid of the computer programs Protean (DNASTAR, Inc., Madison, WI) and Peptool (BioTools, Inc., Edmonton, AB, Canada). A peptide sequence in the F domain of the human ER- receptor (amino acids 449-465) was selected, as there is no homology with estrogen receptor alpha (ERα) at this region (Mosselman et al 1996, Gustafsson 2000) . The peptide was custom synthesized by Research Genetics (Huntsville, AL) with a format of 4-branch Multiple Antigenic Peptide. Each rabbit (male NZW, 5-6 lbs) was first inoculated with 0.5 mg peptide antigen with complete Freund's adjuvant, and then boosted with 0.25 mg peptide plus Incomplete Freund's adjuvant at day 14, day 21 and every two weeks afterward until a satisfactory serum titer was obtained. A direct Enzyme-Linked ImmunoSorbent Assay (ELISA) was used to assess the immune responses to the peptide antigen (Harlow and Lane 1988).

ii. Methods used to test the specificity of the GC-17 antibody:

a) Competitive inhibition ELISA assay

The wells of an ELISA plate, PRO-BINDtm (Becton-Dickenson Labware, Franklin, NJ) were coated with a recombinant protein composed of the entire ER-β sequence (PanVera, Madison, WI) at a concentration of 5μg/ml. The GC17 antibody (1:6000) was then pre-incubated with the immunizing peptide at concentrations ranging from 4mg-4μg/ml at room temperature for 30 mins. In addition, 4mg of a control peptide encompassing sequences in the N-terminal region of ER-β (Research Genetics) was preincubated with the GC17 antibody (1:6000) at room temperature for 1 h. The resulting antigen/antibody complexes

were then incubated with the bound recombinant ER-β protein on the ELISA plate at 37°C for 2 h. Alkaline phosphatase conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA) was used to recognize the GC17 antibody which bound to recombinant ER-β protein on the plate. The whole complexes were visualized by incubation with p-Nitrophenyl phosphate in 2-amino, 2-methyl, 1,3-propanediol buffer pH 9.6. Results were quantified by optical density using the Microplate reader 550 (Bio-Rad, Richmond, CA). The entire assay was done four times.

b) Competitive Immunohistochemistry

Lau et al (2000) have recently demonstrated that DU145 and LNCaP cells, both derived from a metastatic prostate cancer, express abundant ERβ mRNA but not ERα message. These cells were used to compliment and confirm that the GC-17 antibody reagent specifically detected ER-β but not ER-α by immunohistochemical staining. Using the GC-17 antibody and the anti-ER-α antibody (NCL-ER-6F11, Novacastra, Newcastle, UK) at the same dilutions as for tissue sections (see below), we carried out immunohistochemical studies on 10% formalin-fixed cytospins of DU145 and LNCaP cells that had been routinely processed, embedded in paraffin, sectioned at 5 μm and mounted on SuperFrost Plus™ slides (VWF Scientific, West Chester PA).

We performed peptide competition studies at the immunohistochemical level that approximated the conditions used in the ELISA assays described above. GC-17 antibody, at a dilution of 1:6000, was incubated with the immunizing ER-β peptide at concentrations of 400 and 40μg at room temperature for 1h. In addition, competitive studies were conducted using ER-α recombinant peptide (400 and 40μg, Affinity Bioreagents Inc., Golden, CO). on

DU145 cells. Incubation conditions and time were identical to those used for the ER-β peptide competition studies. Deparaffinized sections of DU145 and LNCaP cells and human prostate tissue were then incubated with these mixtures at room temperature for 1h. Competition studies, done on prostate tissues, were identical to those performed on DU145 cells, except the peptide and antibody mixtures were incubated overnight and then applied to sections for 24h at room temperature. All of the remaining immunohistochemical and other staining procedures were identical to those used for tissue sections (see immunohistochemical procedures).

c) Western Blot Analysis

Four human normal prostate tissues from radical prostatectomies and one normal human testis tissue were used in this analysis. In addition, we used normal prostate epithelial cells (Clonetics, Walkersville, MD) and DU145 cells (ATCC, Rockville, MD) for these studies. Recombinant proteins, ER-α (RP310) and short form of ER-β (RP311) (Affinity Bioreagents Inc., Golden, CO) as well as long form of ER-β (PanVera, Madison, WI), were included as controls. Tissues or cells were homogenized in radioimmunoprecipitation (RIPA) buffer containing 50mM Tris-HCl pH 7.4, 1% Nonidet P-40 (Amaresco, Solon, OH), 0.5% sodium deoxycholate, 0.1% SDS, 1mM phenoylmethylsulfonyl fluoride (PMSF) in isopropanol, 1mM activated sodium orthovanadate and 2X Complete™ proteinase inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). Twenty-five μg of tissue protein extracts, 0.5μg of recombinant ER-α protein or 0.5μg of recombinant ER-β protein were mixed with 2X SDS loading buffer (125mM Tris buffer pH 6.8, 20% glycerol, 2% SDS, 2% β-mercaptoethanol and 1μg/ml bromophenol blue) and electrophoresized onto a 10% SDS-

polyacrylamide gel under reducing condition. The separated proteins were transferred onto a PolyScreen® PVDF transfer membrane (NEN, Boston, MA). The membrane was incubated for 1 hr in blocking buffer (PBS with 5% nonfat dry milk). The primary antibodies were applied at 1:6000 for GC17 ER-β antibody or 1:5 for 1D5 ER-α antibody (Biogenex, Mountainview, California) in PBS-T (PBS with 0.05% Tween-20) buffer with 0.1% bovine serum albumin for overnight at room temperature. After washing 5 times with PBS-T buffer, the membrane was incubated with horseradish peroxidase-linked donkey anti-rabbit IgG antibody (Amersham Pharmacia Biotech, Piscataway, NJ) for GC17 or goat anti-mouse IgG antibody (NEN, Boston, MA) for 1D5 at 1:2500 for 1 h. The signals were visualized with chemiluminescence ECL detection system (NEN, Boston, MA) and autoradiography. All reagents were purchased from Sigma (St. Louis, MO) unless specified.

iii. Prostate tissues:

a) Formalin fixed radical prostatectomy specimens:

Tissues studied were from 50 radical prostatectomy specimens collected by JEM at Stanford University Medical School, during the years 1995 -1999. Patients ranged in age from 46-73 years of age and none had received any treatment prior to their undergoing prostatectomy. Prostates were fixed in 10% buffered formalin for 24 h then sectioned transversely. Tissues were dissected fixed in 10% buffered formalin for 3 h, routinely processed, and embedded in paraffin. A histopathological diagnosis was made by JEM on a hematoxylin and eosin stained (H&E). The criteria used in the grading of the carcinomas were those described by Stamey et al (1999). The slide, together with the corresponding paraffin block, was then sent to IL where immunohistochemical studies were carried out. At least one section from each case was stained with H&E and reviewed by IL to assure that it

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matched the tissue components in the original slide. Paraffin sections were cut at 6μm mounted on SuperFrost™ Plus slides. Sections were left unbaked until used for immunohistochemical studies.

Among the 50 cases selected for study, 26 contained areas of carcinoma. Five of these were clear cell carcinomas of the transition zone while all the remaining cancers were found in the peripheral zone. All of the peripheral zone cancers were composed of mixtures of grade-3 and 4/5 carcinomas. In contrast, all of the clear cell carcinomas were predominately grade-3 neoplasms. Twenty of the peripheral cancer specimens also contained varying amounts of low/moderate to high-grade dysplastic lesions. Dysplasia of the peripheral zone was found in the absence of carcinoma in 6 of the 50 total cases we studied. Additionally, 7 of the 50 cases, were low/moderate grade dysplasias of the central zone that did not coexist with cancer. Among the 50 cases, two specimens each of lesion-free normal peripheral, central and the transition zone were included in our study. Among the cases studied, 15 examples of benign prostatic hyperplasia (BPH) were either commingled with other lesions (Ho et al 1997) or occurred separately (Leav et al 1978).

b) Bone and lymph node metastases:

In addition to the prostatectomy cases, archived paraffin blocks containing bone metastases were obtained from 7 patients of MET, treated at University of Massachusetts Medical Center. The patient's ages ranged from 59-74 and they were all clinically stage D2 at the time of diagnosis. All received antiandrogen treatment as follows: 1)Four patients were orchectomized. One of these patients was given the LH/RH agonist Lupron (TAP Pharmaceuticals inc, Deerfield IL) and the AR competitive inhibitor Eulixin (Flutamide-Schering corp. Kenilworth, NJ) for 3 months, one treated with Eulixin for 24 months and the

remaining two were not given any further antiandrogenic therapy. 2)Three patients were not orchectomized. Two were treated with Lupron for 8 months and the other with Lupron and Eulixin for 3 months. Following these antiandogenic therapies for the periods noted above, it was determined that all seven patients were failing therapy. At those time points, biopsies of suspected bone metastases were obtained from the iliac crest of each patient. These samples were immediately fixed in 10% buffered, routinely processed, embedded in paraffin, 6μm sections placed on SuperFrost™ Plus slides, and stained with H&E. Replicate sections of these lesions were used for immunohistochemical studies.

In addition, we also studied 5 archived examples of metastases to regional lymph nodes. Two cases were obtained from Department of Pathology at University of Massachusetts Medical School and the remaining 3 were from University of Florida Medical School (a generous gift from Dr William Murphy). The patients were 60-85 years of age. Regional lymph nodes (external iliac and pelvic) were obtained from all patients during radical prostatectomy. Only one patient had received any treatment prior to surgery (Lupron).

c) Frozen tissues for LCM/RT-PCR

Eighteen separate specimens, derived from radical prostatectomies, were placed in cassettes containing TBS (Triangle Biomedical Sciences, Durham, NC) and quick-frozen in liquid nitrogen by JEM and then shipped in dry ice to IL. Approximately fifteen minutes elapsed from the surgical removal of the gland to the initiation of freezing. Formalin-fixed and paraffin embedded tissue sections, immediately adjacent to the quick frozen specimens, were also taken for subsequent immunohistochemical studies (see below).

For diagnostic purposes and lesion selection, tissues were first cryostat sectioned and then fixed briefly in 70% ethanol and stained with H&E. The frozen tissue blocks were then

stored at –70°C until they were used for microdissection. Prior to LCM, sections from these cases were found to contain varying amounts of grade three and 4/5 carcinoma as well as dysplastic and normal glands.

iv. Procedures:

a) Immunohistochemical procedures

The followings are the primary antibodies and the dilutions used in our studies:

Anti-estrogen receptor beta (ERβ), rabbit polyclonal antibody GC17, diluted at 1:6000; anti-estrogen receptor alpha (ERα), mouse monoclonal antibody NCL-ER-6F11, diluted at 1:50 (Novocastra, Newcastle upon Tyne, UK); anti-androgen receptor (AR), rabbit polyclonal antibody, diluted to 22.7µg/ml (Upstate Biotechnologies, Lake Placid, NY); anti-Mib5/Ki67, mouse monoclonal antibody, diluted at 1:50 (Immunotech, Westbrook, ME) and anti-high molecular weight cytokeratin (HMWC), mouse monoclonal antibody 34βE12, diluted at 1:50 (Enzo Diagnostics, Farmingdale, NY). Immunostaining for Prostatic Specific Antigen (PSA) was done with a Nexus Immunostainer (Ventana, Tuscon Ariz.) using prediluted reagents.

Five µm thick sections were cut and mounted on SuperFrost™ Plus slides. Sections were left unbaked until immediately prior to use at which point they were baked for 1 h at 60°C. After baking, sections were deparaffinized through three changes of xylene and rehydrated through graded alcohols into water. Heat induced epitope retrieval (HIER) was performed by boiling sections in citrate buffer pH 6.0 (pH 6.2 for ERβ) for 15 min on a laboratory hotplate. After boiling, sections were removed from the hotplate, allowed to cool at room temperature (RT) for 20 min, and were then rinsed thoroughly with water (sections stained for PSA did not require HIER). Sections were then placed in 3% hydrogen peroxide

for 15 min at RT to block endogenous peroxidase, washed with water, and placed in PBS (Sigma, St.Louis, Mo). Sections were then incubated with Power Block (Biogenex) nonspecific blocking reagent for 10 min at RT to reduce nonspecific staining, washed with water, and placed in PBS. Sections were then incubated with normal goat serum at 1:50 (Vector, Burlingame, CA) for 15 min at RT. The goat serum was then shaken off and sections were incubated with primary antibodies overnight at 4°C. After overnight incubation, each section received 20 sec of washing with PBS, 20 sec of washing with Biogenex Optimax Detergent Wash Solution followed by 10 min of washing in PBS on a rotator. Solutions were changed for every eight slides. Following washing, sections were incubated with either Biogenex Mutlilink secondary antibody at a dilution of 1:20 for 20min at RT or DAKO (Carpinteria, CA) ready to use secondary antibody for 10 min at RT. Sections were again washed according to the protocol described above. Sections were then incubated with either Biogenex Streptavidin-conjugated horseradish peroxidase at a dilution of 1:20 for 20 min at RT or DAKO ready to use streptavidin-conjugated alkaline phosphatase for 10 min at RT. Sections were again washed as previously described. Immunostaining was visualized using either Biogenex liquid 3,3-diaminobenzidine (Biogenex) or DAKOs New Fuchsin as the chromogen. Following development, sections were rinsed in water, lightly counterstained with 10% Harris Modified Hematoxylin.

Positive controls for GC-17 included DU145 cells (see above and results section) and tissue sections of prostate which were previously shown to be consistently stained with the antibody. Positive tissue controls for ER-α were human breast cancers, shown to contain numerous immunostained cells. Morphologically normal human prostate sections served as positive controls for AR as well as for HMWC and MIB5/Ki–67 stains. For all reagents,

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negative controls were performed by substituting the primary antibody with a class-matched isotype.

b) Laser capture microdissection and RT-PCR

In all instances, immunohistochemical studies for ER-β were performed on the paraffin tissue sections which were adjacent to the frozen sections used for microdissection and RT-PCR analysis. Frozen sections were cut on a cryostat at 5μm placed on precleaned glass slides (Fisher Scientific, Pittsburgh PA) and immediately fixed in 70% ethanol for 5 s. The sections were then briefly dipped in distilled water, stained with 10% Harris hematoxylin for 15 s, dipped in distilled water, then successively placed in 70% ethanol for 30 s, briefly immersed in 1% eosin then placed in 95% ethanol 1 min, two changes of 100% ethanol 1 min each, and two changes of xylene 30 s each. After air-drying for approximately 30 min tissues were microdissected using a Pixcell 2 Laser microdissection unit (Arcturus, Mountainview, CA). Eight to ten normal acini were microdissected from each of 3 different cases. Similarly, 10-20 dysplastic glands were dissected from 4 separate cases of high-grade lesions, and approximately the same numbers of neoplastic glands were obtained from 5 cases of grade 3 and six different examples of grade 4/5 carcinomas. RNA was extracted from each sample and then separately subjected to RT-PCR analysis. Total cellular RNA was separately isolated using RNA Stat-60 reagent (Tel-Test Inc., Friendwood, TX) according to protocols provided by the manufacturer. The total isolated cellular RNA was reverse-transcribed using the GeneAmp RNA PCR kit (Perkin Elmer, CT) in total 20μl reaction mixture and 2μl of the resulting cDNA was used in PCR on ER-α, AR and GAPDH and 3μl for PCR on ER-β. Hot start PCR using AmpliTaq Gold DNA polymerase (Perkin-Elmer, CT) was employed in all amplification reactions. The enzyme was activated by pre-heating the reaction mixtures at

95°C for 6 mins prior to PCR. The PCR programs were 45 cycles for GAPDH and 55 cycles for ER-α, AR and ER-β of 1 min at 94°C, 1 min at 60°C (annealing temperature) and 1 min at 72°C. This protocol was chosen to minimize non-specific product amplification. The primer sequences for ER-α, AR and GAPDH were described in our previously study (Lau et al 2000). The primer set for ER-β was newly designed and the forward primer is 5'-GATGAGGGGAAATGCGTAGA-3' and the reverse primer is 5'-CTTGTTACTCGCATGCCTGA-3'.

D. RESULTS

i. Specificity of the GC-17 antibody:

a) Competitive ELISA

Pre-incubation of GC-17 with the immunizing peptide (C-terminus of ER-β, ERBC) successfully suppressed binding to the recombinant protein (Fig. 9). The suppression occurred in a concentration–dependent manner when compared to the control where the antibody was not pre-incubated with the immunizing peptide. In contrast, pre-incubation of GC-17 with the control N-terminus peptide of ER-β (ERBN) did not significantly suppress binding when compared with the control, indicating that the antibody was not cross–reactive with this region of the ER-β protein.

b) Competitive immunochemistry

Strong nuclear immunostaining was detected in sections of DU145 and LNCaP cells which served as positive controls for the peptide competition studies (Fig. 10, panel A). Pre-incubation of GC-17 with either 400μg/ml or 40μg/ml of the immunizing peptide ERBC totally abolished nuclear staining in sections of these cells, when compared with positive controls where the peptide was omitted (Fig. 10, panel B). Identical results were obtained with sections of human (Fig. 10, panels C&D). In contrast, preincubation of GC17 with the recombinant ER-α protein failed to block ER-β immunosatining of DU145 cells by the antibody (data not shown). These studies confirmed that GC17 does not cross react with ER-α and supports data from our Western Blot findings (see below). Thus, both the competitive ELISA and competitive immunohistochemistry showed GC-17 to be highly specific for binding to the ER-β protein.

In addition, DU145 and LNCaP cells, that only express ER-β (Lau et al 2000), were negative when immunostained with the ER-α (NCL-ER-6F11) antibody. Positive staining of prostate tissues with the ER-α antibody was restricted to stromal cells (see immunohistochemistry of normal prostate below).

c) Western Blot Analysis

Using Western blot analysis, GC17 was demonstrated to specifically recognize two recombinant ER-β proteins and show no cross-reactivity to ER-α protein (Fig. 11, panels A and B). The size of recombinant ER-β protein (RP311) from Affinity Bioreagents Inc. (Golden, CO) is approximately 53kDa and it represents a short form of ER-β protein using a reported initiator codon (corresponding to 43-530 amino acids) (Kuiper et al 1996, Mosselman 1996, Tremblay et al 1997). The long form of ER-β recombinant protein (PanVera, Madison, WI), containing 530 amino acids, was also recognized by GC17 antibody and showed a 59kDa band in the blot. A previous study demonstrated that the native long form of ER-β protein in the cells is approximately 63kDa (Bhat et al 1998). Interestingly, GC17 recognized an approximately 63kDa protein in human normal testis and prostate tissues, suggesting that the long form may be the natural ER-β protein in human tissues (Fig. 11b and 11c). The size difference between long form of recombinant ER-β protein and native protein in cells may be related to the occurrence of post-translational modifications in cells and tissues. The level of ER-α protein in human normal testis and prostate tissues was undetectable with the 1D5 human antibody (Fig. 11a) for 30s exposure to X-ray film. However, very weak signal of ER protein can be detected only when the blot was exposed to a X-ray film for over 10 mins.

ii. Immunohistochemistry of prostate tissues:

a) Normal prostate

In morphologically normal ducts and acini, nuclear ER-β expression was consistently densely localized in nuclei of basal cells as defined by HMWC staining in replicate sections (Fig. 12, panels A & B). Strong nuclei staining was absent in secretory cells but frequently observed in stromal cells. Occasionally nuclear membrane staining for the receptor was also evident in a few luminal cells (Fig. 12, panels A & B). ER-α immunostaining was not present in secretory cells of normal ducts and acini but individual scattered -receptor positive basal cells were observed in less than 10% of all sections studied. The receptor was however consistently found in stromal cell nuclei, especially in periglandular locations.

Pronounced nuclear staining for AR was a constant finding in secretory and stromal cell nuclei. In agreement with a past study (Leav et al 1996), variable immmunostaining for AR was also observed in individual basal cells of normal glands. No difference was found in the cellular localization of the three steroid hormone receptors when the peripheral, transition, and central zones of the prostate were compared. Moreover, the same localization of the steroid hormone receptors, found in the three normal zones, was also seen in foci of BPH.

The most consistent immunolocalization for the three receptors in basal cells was found within periurethral ducts.

b) Dysplastic lesions

Immunohistochemical findings in dysplastic and carcinomatous lesions are summarized in Table 1.

In the peripheral zone, a consistent pattern of ER-β expression was found in dysplastic lesions. A secretory cell localization for nuclear ER-β expression was commonly observed in low to moderate grade dysplasitic lesions (Fig. 12, panel C). The majority of both basal and dysplastic secretory cells in these lesions, contained moderate to strongly stained nuclei (Fig. 12, panel C). A marked diminution to total absence of ER-β immunostained nuclei was a consistent feature in almost all dysplastic cells in the high grade lesions we studied (Fig. 12, panel D). Staining was however present in residual basal cells within these high-grade lesions. Thus, a loss of ER- staining in high-grade dysplasias paralleled a decline in receptor-positive basal cells. In contrast to normal cells, the cytoplasm of dysplastic cells in lesions of all grades but especially in high grade lesions were frequently stained by the GC-17 reagent, a feature not seen when the primary antibody was omitted from the incubation or with the use of any of the other antireceptor reagents. Similarly nuclear membrane of cells in high grade dysplastic cells also frequently stained with the GC-17 antibody (Fig. 12, panel D). ER- positive dysplastic cells were not present within any of the peripheral zone lesions we studied.

In marked contrast, ER- stained cells were detected in 5 of 7 (71%) of dysplasias in the central zone (Fig. 12, panel E). There was however great variation in the numbers (10-90%) of immunopositive cells in any given central zone lesion. In replicate sections, 6 of these lesions contained dysplastic cells that were also positive for ER-β staining.

As previously reported (Leav et al 1996), AR was strongly expressed in the majority (>95%) of dysplastic cells irrespective of the origin or grade of the lesion.

c) Grade 3 and 4/5 carcinomas.

A transition from ER-β positive to negative staining of cells was observed in all 21 cases where cancer was found in the peripheral zone that paralleled the progression of the grade 3 carcinomas to the less differentiated grade 4/5 neoplasms. ER-β positive cells were found in 13 of 15 (87%) grade 3 carcinomas of the peripheral zone. The spectrum of expression ranged from examples where all nuclei in an individual neoplastic gland were strongly stained (Fig. 13, panel A) to instances where receptor immunostaining was weak and/or found in few cells within a given microscopic field. The latter examples were most often located in areas where a transition to higher-grade carcinoma occurred (Fig. 13, panel B). Unlike their counterparts in the peripheral zone, the vast majority of cells comprising grade 3 clear cell carcinomas in the transition zone were devoid of ER-β immunostaining. In two of five cases, scattered receptor- positive neoplastic cells were however found in a minority of glands (Fig. 13, panel C).

Almost complete absence of ER-β nuclear staining was seen in all but 3 of 15 (20%) grade 4/5 carcinomas (Fig. 13; panel C). In the majority of cases however staining of the nuclear membrane was frequently apparent. In the 3 cases, positive cells represented only 10% or less of the total cancer cells in a given lesion and the staining intensity was usually diminished.

Immunostaining for ER-α was detected in only two (7.6%) of the total twenty-six cases of primary carcinoma we studied. In these 2 instances, a few (< 10%) weakly positive cells were found in both grades 3 and 4/5 carcinomas. Staining for the receptor was consistently absent in all clear cell carcinomas of the transition zone.

Irrespective of grade, strong nuclear AR immunostaining was a constant feature in the vast majority cells (>95%) comprising cancers of the peripheral zone. Nuclear AR

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immunostaining was also present in almost all grade 3 clear cell carcinomas but it was less intense than found in peripheral zone carcinomas. Interestingly, the occasional few cells that were ER-β positive in these cancers were found to be negative for AR expression in replicate sections.

No change in the location or intensity of immunostaining for the three receptors in the stroma was evident in sections that contained carcinoma.

d) Metastatic lesions

Nuclear ER-β immunostaining was present in metastatic carcinoma cells in bone lesions from all but one of the seven cases (Fig. 14, panel A). The intensity of signal did however vary from strong to weak staining within cells comprising the lesions of individual case and/or among the cases. In three instances, carcinoma cells were surrounded by a prominent desmoplastic response in which ER-β staining was frequently found in the nuclei of fibroblasts. Nucleated hematopoietic marrow cells were also positive for the receptor while mature red cells were negative, a finding that served as a positive and negative internal tissue control for ER-β immunostaining in these lesions.

In contrast to the finding of ER-β immunostaining in foci of all but one case of bone metastasis, no staining was observed for ER- α in these lesions. Despite the fact that all of seven patients, including the one that lacked ER-β expression had been given antiandrogenic therapy, nuclear AR staining was observed in most metastatic cells in the bone biopsies. However AR staining was also consistently present in the cytoplasm as well as in the nucleus of metastatic cells, a finding observed in bone and lymph node lesions from all patients who received antiandrogenic therapy (see below).

PSA immunostaining was found in metastatic bone lesions in four cases but it tended to be scant especially when compared to lesions in lymph nodes from patients who did not receive antiandrogenic therapy (see following discussion).

Among the five cases of lymph node metastases, two contained a majority of neoplastic cells (>50%) that were uniformly strongly stained for ER-β (Fig. 14, panel B). In one case immunostaining for the receptor was absent in the metastatic cells (Fig. 14, panel C) while in the remaining two cases a mix of negative and positively stained neoplastic cells were found. In one of the two cases, where strong receptor expression was detected, the patient had been treated with the LH/RH agonist Lupron prior to surgery. Lymphocyte nuclei were consistently stained for the receptor and served as an internal positive tissue control in the two cases where no receptor was detected in metastatic cells within the lymph nodes (Fig. 14, panel B).

ER-α immunostained carcinoma cells were found in 2 of 5 cases of lymph node metastases. Both patients were untreated prior to surgery. In one of these cases, ER-β staining was absent in metastatic cells (Fig. 14, panel C). In one case, numerous α receptor positive cells (>50%) were mixed with those in which receptor expression was absent (Fig. 14, panel D). Very weak staining for ER-α was identified in a few cells (<10 %) in the other case.

In all five cases, AR immunostaining was present in the vast majority (>95%) of metatastatic cells (Fig. 14, panel E). Nuclear AR expression was always strong in cancer cells and with the exception of the case where the patient received Lupron, cytoplasmic staining was not present in any of the metastatic cells. Strong PSA immunostaining was

present in the cytoplasm of most metastatic cells in all cases including the one where the patient had received antiandrogenic therapy.

iii. LCM/RT-PCR analysis:

An example of a LCM specimen used in our study is seen in Fig. 15 (panels A&B)

Our findings with RT-PCR analysis for ER-β mRNA on LCM lesions approximated the results of immunohistochemical studies done on paraffin sections immediately adjacent to the frozen specimens used for RT-PCR as well as on other cases (Fig. 15, panel C). ER-β transcripts were detected in 2 of the 3 samples of normal prostatic acini. In contrast, receptor message was found in only 1 of 4 microdissected samples of high-grade dysplasias. Sixty percent of grade 3 carcinomas contained ER- transcripts while receptor message was detected in 2/6 (30 %) of the grade 4/5 cancers. In 2 cases where ER-β message was present in grade 3 lesions, grade 4/5 carcinomas sampled within the same section, lacked receptor mRNA expression. In close agreement with our immunohistochemical findings, AR mRNA was present in all normal glands, dysplasias, grade 3 cancers and all but one of the grade 4/5 carcinomas. ER-α transcripts were not detected in any of the microdissected specimens we studied.

E. DISCUSSION

In this study we developed and comprehensively demonstrated the specificity of a novel antibody, prepared against the F domain of ER-β. Using this reagent, together with antibodies for AR and ER-α, we immunolocalized the 3 receptors in normal human prostate, preneoplastic lesions, and carcinoma and in metastases. LCM/RT-PCR was used to assess the expression of the 3 receptors at the transcript level.

Using the GC-17 antibody reagent, we now show that ER-β is predominately immunolocalized in basal cells and to a lesser extent in stromal cells of the morphologically normal human prostate. In addition nuclear membrane localization of ER-β was occasionally observed in secretory cells. Our current studies have also confirmed past reports (Wernert et al 1988, Schulze et al 1990) that ER- α is detected in stromal cells and rarely in basal cells of the normal gland. As previously reported (Leav et al 1996, Bonkhoff et al 1993, Gleave and Chung 1995), we found that AR was predominately localized in the nuclei of differentiated secretory cells and variably in basal cells of the normal acinar /duct unit as well as in stromal cells. The finding of all three receptors in stromal cells is compatible with the concept that they transduce steroid hormone signals to epithelial cells by paracrine mechanism (Gleave and Chung 1995). In this regard, Hall et al (1999) have reported that ER- functions as a transdominant inhibitor of ER- transcription and that it acts to decrease overall cellular sensitivity to estradiol. Therefore the isoform, when colocalized with the receptor in stromal cells, may play a critical role in regulating paracrine signaling from these cells to epithelia. However the presence of ER-β as virtually the sole ER subtype in basal cells, the purported precursor of secretory cells (De Marzo et al 1998), suggests that estrogens, acting

through the receptor, may directly modulate the growth of these cells. In this context, it should also be noted that AR is variably expressed in basal cells (Leav et al 1996, Bonkhoff et al 1993). Thus, proliferative signals mediated by AR in basal cells or by ER-α and AR in stromal cells may be opposed by the purported growth-inhibitory action of ER- (Chang and Prins 1999, Krege et al 1998, Poelzl et al 2000, Lau et al 2000) localized in basal cells.

Prior to the discovery of ER-β (Leav et al 1978, Merk et al 1986) we reported that the separate administration of androgens and estrogens to hypophysectomized and castrated dogs induced marked proliferation of basal cells. Additionally, each hormone was found to direct distinct pathways of cell differentiation culminating in either squamous cells with estrogens or prototypic glandular cells with androgens (Merk et al 1986). Thus, while paracrine stimulatory signals emanating from stromal cells likely contributed to these effects, the direct action of the hormones mediated through their cognate receptors in basal cells may have also played a role. In either case results from these studies indicate that basal cells are major targets for the effects of steroid hormones in the prostate.

Mindful of these findings it was therefore of particular interest to trace the expression of ER- , normally localized in basal cells, and AR during prostatic cancer development and progression.

The transition from normal to low / moderate dysplastic glands in the peripheral zone was marked by the appearance of ER-β homogeneously immunostained nuclei in secretory as well as basal cells with no changes in the localization of the other receptors.

The expression of ER- was diminished in high-grade dysplasias when compared to normal glands and lower grade lesions. Since the receptor is predominately localized in basal cells in the normal gland, this finding is consistent with the reported depletion of these

receptor positive cells in the majority of high-grade dysplasias (Bostwick 1995). ER-β staining was present in the majority of grade 3 carcinomas of the peripheral zone but was greatly diminished or absent in most grade 4/5 carcinomas. Interestingly nuclear membrane staining was evident in many cells in high grade dysplasias and grade 4/5 carcinomas. The precise meaning of this finding is currently undefined but appears to represent the localization of ER- that occurs with neoplastic progression and could reflect alteration in receptor function. Recently, the promoter of human ER- gene has been cloned and showed regions of CpG islands (Li et al 2000). The diminution of ER- expression in high-grade dysplasias and grade 4/5 cancers may be therefore related to the alteration of DNA methylation pattern in CpG islands of the promoter, resulting in down-regulation of the receptor at the transcriptional level.

While the underlying mechanism(s) for these findings in dysplasias awaits further investigation, based on the proposed antiproliferative function of the receptor (Chang and Prins 1999, Krege et al 1998, Poelzl et al 2000, Lau et al 2000), the presence of ER-β in secretory cells of low/moderate grade lesions may represent a transient-abortive attempt to counter growth of these cells. In contrast, the attrition of receptor positive basal cells in the high-grade dysplasias may signify a continuing loss of growth inhibitory function mediated by ER- in these precursor lesions. The "reappearance" of the receptor in most grade 3 carcinomas and its possible effects on cancer growth is perplexing but may be related to the more favorable prognosis reported for these well differentiated neoplasms (Stamey et al 1999).

Interestingly, in contrast to grade 3 carcinomas of the peripheral zone, expression of the receptor was present only in a few scattered cells that comprised clear cell cancers of

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the transition zone. The differences in   receptor expression between the two grade 3 cancers likely reflect a distinct biology inherent in neoplasms with the clear cell phenotype.

While absent from peripheral zone lesions, ER-α staining was however found in dysplastic cells in most lesions of the central zone. This finding may reflect suspected biological differences (McNeal et al 1988a) as well as distinct pathways of hormone responsiveness between the zones of the human prostate that could play a role in the pathogenesis of these lesions. Interestingly, the central zone is rarely the site of origin for prostate cancer (McNeal et al 1988b).

Our findings of RT-PCR analysis for ER-   mRNA expression on microdissected specimens approximated results from our immunohistochemical studies, indicating that receptor expression was downregulated at both the transcriptional and translational levels in high-grade dysplastic and 4/5 grade neoplastic lesions. Our findings in prostate therefore differ from those reported for human colon cancer where Folley et al (2000) demonstrated that a selective loss of ER-β protein but not receptor message expression occurs in these neoplasms. These authors attributed the loss of ER- beta protein in colon cancers to be from posttranscriptional modifications of the receptor.

In our study ER-α expression was limited to only a small subset of dysplastic lesions in the central zone and to a very few primary carcinomas in the peripheral zone. Our findings therefore differed from those of Bonkhoff et al (1999) who found immunostaining for the receptor in high-grade dysplasias and grade 4/5 carcinomas. Using *in situ* hybridization these authors also reported that a high percentage of dysplasias and carcinomas in their study contained cells that expressed ER-α message. These results again differed from our findings

as we did not detect ER-α message in any dysplasias or primary carcinomas we studied using the highly sensitive and specific LCM/RT-PCR analysis.

Unlike ER-β, AR was consistently strongly expressed at both the transcript and immunohistochemical levels, irrespective of grade, across the spectrum of preneoplastic lesions and carcinomas we studied. These results are in agreement with our past studies of AR in dysplasias [41] and those of others in both primary and metastatic carcinomas (Culig 2000). Importantly, results from a past study (Ruizeveld de Winter 1994) demonstrated the presence of structurally intact AR that functionally binds androgen in the majority of both hormone–dependent and independent carcinomas. Our current findings, together with those cited above, suggest that AR is expressed and functional in the majority of high-grade dysplasias and primary prostate cancers. It is therefore possible that continued androgen mediated stimulation of dysplastic and tumor cells, coupled with the apparent loss of ER-expression, may enhance carcinogenic progression as well as the growth of established prostatic carcinomas.

One of the most intriguing findings in our study was the high percentage of cases (83%) where the expression of ER-β was present in prostatic carcinoma cells metastatic to bone and regional lymph nodes. In marked contrast, ER- staining was absent in all but 2 cases where receptor positive cells were found in lymph node metastases.

While 58% of the patients with metastatic lesions in our study had been given antiandrogen therapy, positive staining for ER-β was also present in cancer cells within the lymph nodes of three individuals that had not received this treatment. The presence of ER-β in metastatic cells from patients who did or did not receive antiandrogenic treatment suggests

that blocking of androgen action was not a primary cause for the expression of the receptor in these sites.

It is tempting to speculate that the new tissue microenvironment in which these metastatic cells are found may have provided local factors that influenced the expression of ER-β and to a lesser extent ER-α in these sites. In this context, it has been reported that bone fibroblasts produced growth factors that induced human prostate cancer growth (Yoneda 1998). Alternatively, the metastatic process may, in some undefined manner, have favored the spread of cells with either ER isoform from the primary cancer.

In summary, we demonstrate that a consistent pattern of lost ER-β expression at both the transcriptional and translational levels occurs during prostatic carcinogenesis and tumor progression. This may signify the loss of an important role the receptor would normally play in inhibiting growth of the prostate that could contribute to neoplastic development. The continued expression of presumably functional AR throughout these processes, as well as other undefined factors, may therefore exert persistent unopposed growth stimulus acting on these cells.

The cause of ER expression and the effects that it may have on the growth of metastatic cells remains to be defined. The presence of the β isoform in these cancer cells may however have important ramifications for the treatment of patients with late stage disease. In this regard, we recently reported that estrogens as well as antiestrogens are potent growth inhibitors of human prostate cancer lines that express both estrogen receptor isoforms (Lau et al 2000). In contrast, the growth of cells such as DU145, which only express ER-β, was markedly inhibited by the antiestrogen ICI 182,807. These findings together with results

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from our current studies may therefore be helpful in devising new ligand-specific strategies

for treating patients with metastatic prostate cancer.

CHAPTER V

ICI-182,780-, RALOXIFENE- AND RESVERATROL-INDUCED CELL GROWTH INHIBITIONS IN PROSTATE CANCER ARE ASSOCIATED WITH DOWN-REGULATIONS OF TELOMERASE REVERSE TRANSCRIPTASE, SURVIVIN, AND THYMIDYLATE SYNTHASE mRNA EXPRESSIONS.

A. ABSTRACT

Recent evidence suggests that ER-β may be an antiproliferative factor for a protective effect against the mitogenic activity of estrogens in breast and androgens in prostate while ER-α is involved in the estrogen-induced mitogenesis of breast epithelial and uterine cells. We previously demonstrated that ICI-induced cell growth inhibition in DU145 cells is mediated via ER-β actions. In this study, we investigated the mechanism of this inhibition in DU145 cells. Flow cytometry data indicated that ICI-treatment could induce the accumulation of cells at G_0-G_1 phase of cell cycle. With raloxifene, the treated cells also showed this accumulation along with significant cell growth inhibition in DU145 cells. Using real-time RT-PCR analyses on thymidylate synthase (TS), telomerase reverse transcriptase (TERT), and survivin to semi-quantitate their transcript levels, this G_0-G_1 cell accumulation by ICI treatment is associated with the ICI-induced down-regulation of TERT and survivin expressions. These down-regulations are dose-dependent and they appear to be regulated by ER-β since the ICI-induced inhibition is mediated via ER-β actions. It had been reported that the alterations in TERT and survivin decrease cell viability and prevent cells from entering cell cycle. Therefore, the ICI-induced alterations of these gene expressions may be a key factor in the observed cell growth inhibition in DU145 cells.

In addition, Rv treatment also exhibits inhibitory effects on cell growth of DU145 cells. A time course study showed that Rv had dual effects on the cell cycle distribution of the treated cells. In the early treatment (3 and 6h), there was accumulation of cells at G_0-G_1 phase. However, the treated cells were arrested at S and G2/M phases after 12 and 24h exposure to Rv. The transcript levels of TERT and survivin were also reduced by the Rv-treatment, leading to prevent cell from entering cell cycle and decrease in cell viability. In contrast to ICI-treatment, 10^{-6}M Rv reduced TS transcript level in DU145 cells. The down-regulation of TS may be related to the S and G_2/M cell cycle arrests observed after longer exposure to Rv which resulted in growth inhibition of DU145 cells.

Taken together, our findings support the concept that ER-β participates in cell cycle regulation in normal and malignant prostatic epithelial cells. Activation of the receptor by antiestrogen and phytoestrogen induced cell growth inhibition and the mechanism may be mediated by reduction of cell survival factors, leading to decrease in cell viability, and induction of cell cycle arrests.

B. INTRODUCTION

Estrogens modulate sexual development and reproductive functions in addition to effecting the cardiovascular and central nervous systems and bone. Their effects are mediated by at least two estrogen receptors, ER-α and ER-β (see reviews in Gustafsson 1999 and Pettersson and Gustafsson 2001). Although they share high level (86%) of amino acid homology in their DNA binding domains, the N-terminal and ligand binding domains are different (23% and 58% homology respectively) and these domains contain transactivation functional regions for recruiting coregulator proteins, suggesting that their biological functions may be different (Mosselman et al 1996, Gustafsson 2000, Pettersson and Gustafsson, 2001). The transactivation activities of these two receptors are promoter dependent. At an activating protein 1 (AP-1) responsive element, 17β-estradiol increases reporter activity with ER-α but inhibits it with ER-β. However, with the antiestrogens, the activity decreases with ER-α and increases with ER-β (Paech et al 1997). In contrast, transactivation with two receptors at classical estrogen responsive element is similar between 17β-estradiol and antiestrogens (Paech et al 1997). In addition, tissue distributions of these two receptors are different. In normal rat, ER-α expressed moderate to high levels in uterus, pituitary, ovary, kidney, epididymis, adrenal gland and cortical bone while high expression of ER-β was found in prostate, ovary, lung, bladder, brain, uterus, testis and cancellous bone (Kuiper et al 1997, Onoe et al 1997, Ehrlich 2000). However, the functions of ER-β in different tissues remain to be elucidated.

Expression of ER-β has been studied in various types of human cancers such as breast, colon, ovary and prostate (Vladusic et al 1998, Hu et al 1998, Roger et al 2001, Mann

et al 2001, Foley et al 2000, Campbell-Thompson et al 2001, Pujol et al 1998, Lau et al 1999, Rutherford et al 2001, Lau et al 2000, Latil et al 2001, Leav et al 2001). In colon cancer, ER-β was found to be down-regulated when compared to its level in normal tissue (Foley et al 2000, Campbell-Thompson et al 2001). With newly developed antibodies to ER-β, we and others demonstrated that reduced expression of this receptor in proliferative preinvasive mammary tumors (Roger et al 2001) and in high grade of prostate cancers (Leav et al 2001). Since the reduction of ER-β expression is associated with increased Ki-67 staining that is a cell proliferative marker, it has been suggested that the receptor subtype may have a protective effect against the mitogenic activity of estrogens in premalignant lesions of the breast (Roger et al 2001). This concept is supported by our finding that sole ER-β is immunolocalized in basal cells of human normal prostatic acini which constitute the proliferative epithelial cell compartment in the gland and are purported precursor of secretory epithelial cells. In addition, ER-β knockout mice have been reported to develop age-related prostatic hyperplasia, which suggests that the receptor may act to inhibit abnormal growth of prostate (Krege et al 1998). In this regards, ER-β was found to specifically interact with MAD2 protein which functions as a cell cycle spindle assembly checkpoint protein (Poelzl et al 2000). Taken together, these findings suggested that ER-β may be an antiproliferative factor that curbs cell proliferation as part of a negative feedback system for cell cycle regulation. Reduced expression of this receptor in cancers may result in the loss of this negative feedback function, leading to uncontrolled cell growth.

In contrast to the down-regulation of this receptor in high-grade primary prostate cancers, we found reactivation of ER-β expression in metastatic prostate cancer cells in

lymph node and bone (Leav et al 2001). All these bone metastases were obtained from patients who had failed antiandrogen therapy. Although the mechanism involved in this reactivation is unknown, we can take advantage of this phenomenon to design new treatment for hormone refractory prostate cancer. Thus since ER-β may be an antiproliferative factor, the activation of the receptor by ligand binding may inhibit the growth of advanced stage of prostate cancer. In our previous study, we demonstrated that antiestrogens such as 4-hydroxytamoxifen and ICI-182,780 (ICI) inhibited growth of androgen-independent prostate cancer cells (DU145 and PC-3) (Lau et al 2000). With RT-PCR analysis and immunocytochemistry, DU145 cells was shown to express sole ER-β (Lau et al 2000, Leav et al 2001). This ICI-induced cell growth inhibition was considered to be mediated via ER-β since the blockade of ER-β by antisense oligonucleotide can reverse the inhibitory effects of antiestrogens (Lau et al 2000). These findings suggest antiestrogens may activate ER-β in DU145 cells and hence induce antiproliferative effects.

Selective estrogen receptor modulators and phytoestrogens had been demonstrated to bind effectively to ER-β (Kuiper et al 1997, Couse et al 1997) but their biological effects on prostate cancer remain to be elucidated. In this study, we explored the potential use of raloxifene (Ral) and resveratrol (Rv) on inhibiting the growth of prostate cancer cells. In addition, mechanisms of the effects of these compounds in advanced prostate cancer need to be investigated. This provides invaluable information in further development of target-specific drugs for this heretofore-incurable disease.

C. MATERIALS AND METHODS

i. Cell growth assay

DU145 (American Type Culture Collection, Manassas, VA) are routinely maintained in DMEM/F12 medium supplemented with heat inactivated fetal bovine serum (FBS), 2mM L-glutamine, 1mM sodium pyruvate, 0.1 M non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin (P/S), and 0.05 mM β-mercaptoethanol (Sigma Co., St. Louis, MO). Cells were seeded at $2X10^3$ cells per well in 96 wells-plates as day 0. The cells were allowed to attach for overnight in the culture medium as above except using 5% charcoal stripped FBS and the medium was changed at day 1. The charcoal stripping removes majority of steroids in serum. The medium also contains no phenol red, which had been reported to have estrogenic properties. The cells were exposed to ICI, Ral, or Rv at 0.1, 1 and 10 µM final concentration. Cells treated with empty vehicle (100% ethanol) were used as a negative control. Freshly prepared medium with tested compounds was re-fed the cells once at day 3. At the end of a 4-day treatment, cell numbers was assessed by direct cell count with the Trypan blue exclusion method. For each treatment, triplicate wells were used and three separated experiments were done. Statistical analysis will be performed using SPSS program (SPSS, Chicago, IL). Data were analyzed by one-way ANOVA, followed by the Tukey post-hoc test, and a 95% confidence limit will be used for all comparisons among treatment groups.

ii. Determination of DNA content by flow cytometry

Cells were seeded at $5X10^5$ cells per $75cm^2$ culture flasks as day 0. The cells were allowed to attach for 24h and the culture medium, as above except using 5% charcoal stripped FBS, was changed at day 1. After 12h, the cells were exposed to ICI, Ral, or Rv at

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10 μM final concentration with freshly prepared medium supplemented with 5% charcoal stripped FBS for 24h. Cells treated with empty vehicle (100% ethanol) were used as control. Two millions of control or treated cells were fixed with 2 ml 70% ethanol for at least 24h at −20°C. The cells were stained in propidium iodide (PI) staining solution containing 1:1 dilution of 20μg/ml PI in Hanks balance salt solution (Ca^{2+} and Mg^{2+} free) and 1mg/ml RNase A for 30 min at 37°C. The stained cells were subjected to FACScan Analyzer (Becton Dickinson Immunocytometry Systems, San Jose, CA) in our flow cytometry core facility. In addition, a time-course experiment was performed on DU145 cells with Rv treatment. Cells were prepared as above at different time points used (3, 6, 12, and 24h for Rv and 1, 2, 3, and 4 days for ICI).

iii. Analysis of DNA synthesis by bromo-deoxyuridine (BrdU) incorporation

Cells were seeded at 5×10^5 cells per $75 cm^2$ culture flasks as day 0. The cells were allowed to attach for 24h and the culture medium, as above except using 5% charcoal stripped FBS, was changed at day 1. After 12h, pulse-chase experiments with BrdU were performed and the cells were exposed to Rv at 10 μM final concentration with freshly prepared medium supplemented with 5% charcoal stripped FBS for 12, 18 and 24h. The cells treated with empty vehicle (100% ethanol) were used as control for different time points of treatment. After 6h of Rv treatment, BrdU was added to the medium to achieve a final concentration of 10 μM. At the end of 12, 18 or 24h Rv treatment, 2×10^6 cells were trypsinized and washed twice with 1% bovine serum albumin (BSA) (Sigma) in phosphate buffered saline (PBS). The cells were suspended in PBS on ice and fixed with ice-cold 70% ethanol. 2N hydrochloric acid with Triton X-100 was slowly added to the fixed cells for DNA denaturing. After centrifuge, the cell pellet was resuspended in 0.1M sodium

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tetraborate pH 8.5 to neutralize the acid and the cells were kept in 0.5% Tween 20/1% BSA/PBS before immunofluorescence staining with FITC-conjugated anti-BrdU (Becton Dickinson, San Jose, CA). The cells were incubated with the antibody for 30 min at room temperature and washed once with 0.5% Tween 20/1% BSA/PBS. The BrdU labeled cells were pelleted and resuspended in PBS with 50μg/ml RNase A (Promega) and 20μg/ml PI at 30°C. The stained cells were analyzed with a FACScan Analyzer with laser excitation at 488nm.

iv. Real-time quantitative RT-PCR analysis

Cells were seeded at $5X10^5$ cells per $75cm^2$ culture flasks as day 0. The cells will be allowed to attach for 24h and the culture medium, as above except using 5% charcoal stripped FBS, was changed at day 1. After 12h, the cells were exposed to ICI or Rv at 0.1, 1 and 10 μM final concentration with freshly prepared medium supplemented with 5% charcoal stripped FBS for 24h. Cells treated with empty vehicle (100% ethanol) were used as control. Triplicate was set up for each treatment. Total RNA was isolated by TRI reagent (Sigma) following manufacturer's recommended protocols. SYBR Green technology was used for real-time quantitative RT-PCR analysis. One μg of total RNA was reverse-transcribed using the GeneAmp RNA PCR kit (Perkin-Elmer, CT) and a SYBR Green PCR Master Mix with AmpliTaq Gold enzyme (PE Biosystems, Foster City, CA) was employed. Two μl of resultant cDNA was amplified in triplicate in a 25 μl reaction mixture containing 12.5 μl of 2X SYBR Green PCR Master Mix, and 10 pmol of each primer. The intron-spanning primers were designed using the Primer3 Output program from Whitehead Research Institute, Massachusetts Institute of Technology, Cambridge, MA. The primers for survivin, Telomerase reverse transcriptase (TERT), and thymidylate synthase (TS) were

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shown in Table 4. The primer sets had been tested on genomic DNA and show no PCR product to confirm that they are intron-spanning. Since the formations of primer-dimers and non-specific PCR products significantly affect the accuracy of the assay, the melting curve profile was obtained and the carefully optimized PCR condition and AmpliTaq Gold enzyme (Perkin Elmer) for Hot-start PCR was used. The mRNA expression of these ER-β regulated genes was normalized by the expression of housekeeping gene (GAPDH) for all the comparisons. The integrity of total RNA samples also was determined by the analysis on this housekeeping gene.

D. RESULTS

i. Inhibition of DU145 cell growth by ICI, Rv and Ral

Cell growth analyses showed that the growth of DU145 cells, which only expressed ERβ (Lau et al 1999, Leav et al 2001), was adversely effected by ICI, Ral and Rv. The inhibitory effect of ICI on DU145 cell growth had been reported in our previously study (Lau et al 1999). Both Ral and Rv induce a dose-dependent cell growth inhibition (Figure 16). A 60% reduction ($p < 0.001$) in the cell numbers was achieved with an Rv dose of 10 μM (Figure 16A). A similar growth-inhibitory response was observed when DU145 cells were treated with Ral (60% reduction, $p < 0.001$) (Figure 16B). At 1 μM, Rv induced approximate 20% inhibition ($p < 0.05$) on DU145 cell growth while 40% ($p < 0.001$) for Ral. For the Ral treatment, the concentration at 0.1 μM can also induce statistically significant inhibitory effects on DU145 cell growth ($p < 0.001$).

ii. Induction of cell cycle arrest by ICI, Ral and Rv

The cell cycle distributions, which are based on the DNA content of cells, of control and treated cells were presented in Table 5. After 24h treatment of 10 μM ICI, the percentage of DU145 cells at G_0-G_1 phase of cell cycle increased ($p \leq 0.001$) when compared to those of control. Similarly, treatment of Ral also induced cell cycle arrest at G_0-G_1 phase ($p \leq 0.001$). In contrast, the percentage of Rv-treated cells at S ($p \leq 0.001$) and G_2M ($p \leq 0.01$) phases showed statistically significant increase.

Cell cycle distributions in DU145 cells at various time points after exposure to ICI and Rv were analyzed (Figures 17A and 17B). After the exposure to Rv for 3 and 6 h, the percentage of cells at G_0-G_1 phase was higher than that of the control cells. There was a

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reduction of the Rv-treated cells at S phase and even no cell was found in the G_2M phase. However, at 12h Rv treatment, the majority of cells were demonstrated at the S phase while the percentage of cells at G_0-G_1 phase was the same as the control cells and still no cells in the G_2M phase. After 24h Rv treatment, the cells at S and G2M phases predominates the cell populations in the culture (Figure 17A). In contrast, the treatment of ICI induced accumulation of G_0-G_1 cells throughout the 4 days exposure (Figure 17B). With the slightly increase in the percentage of cells at G_0-G_1 phase in the control cells with time, the degree of induction of G_0-G_1 cell accumulation by ICI treatment decreased.

To further investigate the effects of Rv on DU145 cells, the pulse-chase experiments with BrdU incorporation were performed (Table 6). After 12h Rv treatment, the percentage of cells at the mid S phase was higher than that of the control cells. As time progress (18h), the cells at the late S phase showed high percentage in the cell population of culture with Rv treatment when compared to those in the control. At 24h, the high percentages of cells at three (early, mid, and late) S phases was found in the Rv treated cell population.

In summary, treatment of ICI and Ral on DU145 cells induced the accumulation of G_0-G_1 cells while Rv treatment increased the percentage of G_0-G_1 cells in population in the early phase of the treatment and arrested the cells at S-phase of the cell cycle in the prolonged treatment.

iii. Effects of ICI and Rv treatments on thymidylate synthase, TERT, and survivin mRNA expression

To examine the effects of ICI and Rv on gene expressions of TS, TERT, and survivin, real-time RT-PCR analysis was used to semi-quantitate their transcript levels in treated cells relative to the control. Treatment of ICI for 24h in DU145 cells reduced TERT and survivin

mRNA expressions at three different concentrations (10^{-5}, 10^{-6} and 10^{-7}M) (Figure 18B and 18C). These downregulations were dose-dependent. In contrast, the level of TS in the ICI-treated cells was comparable to those in the control (Figure 18A). For Rv, treatments for 24 h at 10^{-6} and 10^{-7}M downregulated TERT and survivin in DU145 cells (Figure 18E and 18F) while the treatments at these two doses induced a trend of reduction in transcript level of TS (Figure 18D). Interestingly, 10^{-5}M Rv treated cells expressed comparable transcript levels of TS and TERT to those in the control (Figure 18D and 18E). The level of survivin mRNA in these treated cells did not further reduce at 10^{-5}M Rv treatment compared to those at 10^{-6}M, although the reduction by 10^{-5}M Rv treatment was still statistically significant when compared to those in the control (Figure 18F).

E. DISCUSSION

We previously reported that antiestrogens such as ICI and 4-hydroxytamoxifen exhibit growth inhibitory effects on DU145 cells that express sole ER-β. This antiestrogen-induced cell growth inhibition can be reversed by the blockade of ER-β with antisense oligonucleotide, suggesting that it may be mediated via ER-β actions (Lau et al 2000). Potentially, the findings may lead to development of new treatment for hormone-refractory metastatic prostate cancer where ER-β expression was found as DU145 cells (Leav et al 2001). In this study, we revealed the mechanism of this inhibition and demonstrated that ICI can induce accumulation of cells at G_0-G_1 phase of cell cycle. It suggests ICI treatment reduces cell viability as well as prevents cell cycle entry which results in inhibition in cell proliferation. This may be related to our findings that the treatment of ICI in DU145 cells downregulated both TERT and survivin expressions.

Telomerase is a ribonucleoprotein complex containing template RNA, telomerase-associated protein and reverse transcriptase subunit (TERT) responsible for de novo telomere synthesis and addition of telomeric repeats to existing telomere (Greider 1995). TERT was found to be a rate-limiting determinant of telomerase activity (Meyerson et al 1997, Xu et al 1999). Its activity had been demonstrated to be in high level in the majority of human cancers and negative in many normal tissues. Interestingly, telomerase activity had also been detected in the normal basal layer of epidermis, where most proliferation occurs (Yasumoto et al 1996, Harle-Bachor et al 1996) and in the normal oral mucosa that is highly proliferative tissue (Kannan et al 1997). In addition, using isogeneic samples of cultured and uncultured human uroepithelial cells and transitional cell carcinomas, telomerase activity had been demonstrated to associate with cellular proliferation and not cellular transformation in human

uroepithelial cells. The activity was detected in proliferating mammary and prostate epithelial cells (Belair et al 1997). These findings supported the concept that telomerase activity is a cell proliferation factor. Disruption of telomere maintenance by alterations in TERT expression and telomerase activity limits cellular lifespan in human cancer cells (Hahn et al 1999). Recent studies have shown that downregulation of TERT by antisense oligonucleotide in DU145 cells can decrease cell viability and inhibit cell growth (Schindler et al 2001). These indicate TERT expression is essential to the growth of DU145 cells and the ICI-induced reduction of TERT mRNA may contribute to the growth inhibitory effects of this compound on DU145 cells.

Survivin is a member of the family of inhibitors of apoptosis (IAP) and localized to the components of the mitotic apparatus (Li et al 1998). It is involved in both inhibition of apoptosis and control cell division (Reed and Bischoff 2000). Interference with survivin expression/function using antisense or a dominant negative mutant caused spontaneous apoptosis in keratinocytes (Grossman et al 1999). In addition to induction of apoptosis, the antisense to survivin in HeLa cells decreased cell viability and the number of proliferating cells at G_2/M phase, resulting in inhibition of cell growth (Ambrosini et al 1998). Overexpression of survivin was found in various cancers including prostate but its level was undetectable in almost all normal tissues. The high level of survivin in cancer cells leads to prolong cell viability and may contribute to cancer by facilitating the insurgence of mutations (Thompson et al 1995, Ambrosini et al 1997). Although survivin is an inhibitor of apoptosis, its expression is correlated to proliferation index but not apoptosis index in hepatocellular carcinoma (Ito et al 2000). It promotes cell proliferation by interacting with cycle-dependent kinase 4 and releasing p21 from this kinase (Ito et al 2000). Moreover, survivin had been

suggested to initiate cell cycle entry (Suzuki et al 2000). Overexpression of survivin can accelerate S phase shift and induce resistance to G_1 cell cycle arrest through activation of cdk2/cyclin E complex leading to retinoblastoma protein phosphorylation (Suzuki et al 2000). Therefore, the down-regulation of survivin by ICI treatment may prevent cell entering into cell division. However, our flow cytometry data indicate no significant induction of apoptosis by ICI even though the level of survivin was reduced. It may be due to the facts that DU145 cells express mutated p53 protein (Isaacs et al 1991) and survivin is involved in p53-dependent apoptosis (Grossman et al 2001).

In this study, we demonstrated that Rv exhibited cell growth inhibitory effects on prostate cancer cells in agreement with the previous reports (Hsieh and Wu 1999, Mitchell et al 1999). These studies showed down-regulation of prostate-specific antigen by Rv treatment in LNCaP cells and suggested that Rv can modulate androgen-stimulatory growth of the cells (Hsieh and Wu 1999, Mitchell et al 1999). However, growth of DU145 cells is androgen-independent and Rv-induced growth inhibition in this cell line is not mediated via androgen receptor associated pathway. In addition, it has been reported that Rv induced apoptosis in LNCaP cells while the 4-days treatment in DU145 cells showed a trend of accumulation of cells at S phase (Hsieh and Wu 1999). Our flow cytometry data showed that Rv-treatment in DU145 cells can induce statistically significant cell cycle arrest at S phase after 24h exposure. In the time course study, Rv induced accumulation of cells at G_0-G_1 phase in the early treatment (3 and 6h). The prolong treatments (12 and 24h) cause DU145 cells arrested at S and G_2/M phases. Rv-treatment apparently exhibits dual effects on cell cycle distribution in DU145 cells.

Treatment of Rv in DU145 cells also down-regulated expression of survivin. Since survivin initiates cell cycle entry (Suzuki et al 2000), the accumulation of G_0-G_1 cells in the early treatment may be due to Rv-induced prevention of cells into cell cycle. Interestingly, the higher dose (10^{-5}M) of Rv-treatment did not further reduce the level of survivin transcripts and the degree of reduction was similar to those by 10^{-6} and 10^{-7}M dose. After 24h exposure to 10^{-5}M Rv, a large portion of the cell population was arrested at S and G_2/M phases. Expression of survivin shows cell cycle periodicity and these cells express high level of survivin (Li et al 1998). It increases by 6 folds in S phase and by more than 40 folds in G_2/M phase (Li et al 1998). The increased amount of S and G_2/M cells together with high levels of survivin in 10^{-5}M Rv-treated cell populations apparently prevented the further down-regulation of the gene by Rv. A similar phenomenon was also found for TS in Rv-treated DU145 cells. Previous studies have reported that the activity of TS is highest during S phase (Navalgund et al 1980, Ayusawa et al 1986, Mirjolet et al 1998). TS is a key enzyme in nucleotide metabolism and catalyzes the methylation of deoxyuridine monophosphate to deoxythymidine monophosphate in the presence of the cofactor N^5,N^{10}-methylenetetrahydrofolate. This reaction is the sole *de novo* source of thymidylate for DNA synthesis (Pinedo et al 1988). Inhibition of TS by fluorouracil can induce S phase arrest in colon (Backus et al 2001) and also prostate cancer cells (Cattaneo-Pangrazzi et al 2000). Down-regulation of TS mRNA and protein by antisense oligonucleotide also inhibits cell proliferation (Ferguson et al 1999). Berg et al (2001) have recently shown that the inhibition is due to accumulation of cells at G_2/M phase. The down-regulation of TS by Rv-treatment may therefore also contribute to the accumulation of cells at S and G_2/M phases and inhibit cell growth in DU145 cells. The Rv-induced DU145 cell growth inhibition may also have

been due to the reduced expression of TERT in the treated cells as was the case for the ICI-treatment. In consistent with this concept, Schindler et al (2001) have reported that the disruption of telomere maintenance decreases cell viability and inhibits cell growth. Recently, Rv was found to directly interact with DNA polymerases α and δ and inhibit DNA synthesis (Stivala et al 2001). With this current finding, the enzyme activities of these two isoforms of DNA polymerases warrant further study in Rv-treated DU145 cells.

We found that ICI-treatment can down-regulate TERT and survivin mRNA expressions in DU145 cells and Rv can modulate the levels of TS, TERT and survivin transcripts in these cells which expressed only ER-β. Together with our finding that ICI-induced cell growth inhibition is mediated via ER-β actions (Lau et al 2000) and that Rv and ICI binds to estrogen receptors (Gehm et al 1997, Bowers et al 2000), these genes may be regulated via ER-β. In concert with our findings, TS, TERT, and survivin were down-regulated by tamoxifen in colon cancer cells which also only express ER-β (Nakayama et al 2000). In fact, telomerase activity is believed to be under hormonal control in prostate (Meeker et al 1996). Moreover, estrogen can up-regulate TERT mRNA expression in MCF-7 and human ovarian epithelial cells which express estrogen receptors. However, in the cells without estrogen receptor such as MDA-MB231 and HeLa cells, estrogen showed no effect on expression of this gene (Kyo et al 1999, Misiti et al 2000). Similarly, TS expression can be induced by estrogen and even tamoxifen in MCF-7 cells (Aitken and Lippman 1985, Aitken et al 1985) for the G_1/S progression (Xie et al 2000). These findings supported that they may be ER-β regulated genes.

In conclusion, our findings support the concept that ER-β may be antiproliferative factor in agreement with the recent report that ER-β inhibits proliferation and invasion of breast cancer cells (Lazennec et al 2001). Activation of the receptor with antiestrogens and phytoestrogens inhibits prostate cancer cell growth and the mechanism may be mediated by reduction of potentially ER-β-regulated cell survival factors such as TERT, survivin and TS, leading to decrease in cell viability and induction of cell cycle arrests.

CHAPTER VI

CONCLUSION

In this study, we provide evidence that normal human prostatic basal epithelial cells express ER-β but not ER-α. It suggests that estrogens in human prostate act directly on the epithelial cells via their own receptor, instead of the paracrine regulation by ER-α in the stromal cells. These findings warrant further study in the biological involvements of ER-β in human prostate physiology.

To investigate ER-β expression in human clinical prostate samples, we had developed an ER-β antibody and characterized this antibody extensively to demonstrate its specificity. Results indicated that ER-β expression is down-regulated in high-grade dysplasia compared to the low-grade lesions. The down-regulation was also found in high-grade prostate cancers. Recently, reduced expression of this receptor was demonstrated in proliferative preinvasive mammary tumors (Roger et al 2001). Since the reduction of ER-β expression is associated with increased Ki-67 staining that is a cell proliferative marker, it has been suggested that the receptor subtype may have a protective effect against the mitogenic activity of estrogens in premalignant lesions of the breast (Roger et al 2001). This concept is supported by our finding that sole ER-β is immunolocalized in basal cells of human normal prostatic acini which constitute the proliferative epithelial cell compartment in the gland and are purported precursor of secretory epithelial cells. In addition, ER-β knockout mice have been reported to develop age-related prostatic hyperplasia, which suggests that the receptor may act to inhibit abnormal growth of prostate (Krege et al 1998). In this regards, ER-β was found to specifically interact with MAD2 protein which functions as a cell cycle spindle

assembly checkpoint protein (Poelzl et al 2000). Taken together, these findings suggested that ER-β might participate in cell cycle regulation that curbs cell proliferation as part of a negative feedback system for cell cycle regulation. Reduced expression of this receptor in primary cancers may result in the loss of this negative feedback function, leading to uncontrolled cell growth.

However, ER-β is widely expressed in the metastatic prostate cancer using our newly developed antibody for immunohistochemistry. It is in agreement with the findings of RT-PCR analysis on prostatic carcinoma cell lines. DU145 and LNCaP cells was shown to express exclusively ER-β while PC-3 cells expressed both ER-α and ER-β transcripts. Mechanisms for this reactivation of ER-β in metastatic cancers and its biological significances in prostatic metastasis remain unknown. Signoretti and Loda (2001) speculated that metastatic prostate cancers seem to display a basaloid phenotype. Many genes expressed by the basal cells, such as bcl-2 (McDonnell et al 1992, McDonnell et al 1997, Colombel et al 1993), Her-2-neu (Signoretti et al 2000) and prostate stem cell antigen (Reiter et al 1998), are reactivated in metastatic cancers and ER-β may be one of them (Signoretti and Loda 2001). Role of ER-β in metastatic prostate cancer cells remains unknown and should be a focus of further investigation.

The positivity of androgen-independent metastatic prostate cancer for ER-β could lead to development of new therapy for this late-stage of disease since this receptor may be an antiproliferative factor. Two androgen-independent prostate cancer cell lines, DU145 and PC-3 cells, were found to exhibit dose-dependent cell growth inhibition by antiestrogen (ICI and 4OH-TAM) treatments. Moreover, blockade of ER-β using antisense oligonucleotide

reduced the ICI-induced inhibition in DU145 cells, suggesting that the cell growth inhibition by ICI is mediated via ER-β actions. Based on our findings, ICI was proposed to treat the patients with advance prostate cancers and the clinical trial will be performed in University of Massachusetts Medical Center, Worcester, Massachusetts in collaboration with AstraZeneca Pharmaceuticals LP (Wilmington, DE).

Selective estrogen receptor modulators and phytoestrogens had been demonstrated to bind effectively to ER-β (Kuiper et al 1997, Couse et al 1997) but their biological effects on prostate cancer remain to be elucidated. In this study, we explored the potential use of raloxifene (Ral) and resveratrol (Rv) on inhibiting the growth of prostate cancer cells and found that both effectively inhibit DU145 cell growth and the inhibition is dose-dependent. Treatments of ICI and Ral induced cell cycle arrest at G_0-G_1 phases. The G_0-G_1 phases cell cycle arrest by ICI is associated with the down-regulation of survivin and telomerase reverse transcriptase (TERT). These down-regulations are dose-dependent and they appear to be regulated by ER-β since the ICI-induced inhibition is mediated via ER-β actions. It had been reported that the down-regulation of TERT and survivin decrease cell viability and prevent cells from entering cell cycle (Schindler et al 2001). Therefore, the ICI-induced alterations of these gene expressions may be a key factor responsible for holding cells in G0-G1 phases of cell cycles in DU145 cells.

Rv has duel effects on cell cycle distribution depending on the duration of treatments. After 3 or 6 hrs treatment of Rv, the cells were arrested at G_0-G_1 phases. The prolonged treatment of Rv induced S-phase cell cycle arrest. The transcript levels of TERT and survivin were also reduced by the Rv-treatment, leading to prevent cell from entering cell cycle and decrease in cell viability. In contrast to ICI-treatment, 10^{-6}M Rv reduced TS

transcript level in DU145 cells. The down-regulation of TS may be related to the S and G_2/M cell cycle arrests observed after longer exposure to Rv which resulted in growth inhibition of DU145 cells.

In conclusion, ER-β plays significant roles in the normal physiology of human prostate and the genesis and progression of the cancer. This receptor may be antiproliferative factors that curbs cell proliferation as part of a negative feedback system for cell cycle regulation in the organ. Down-regulation of the receptor is involved in prostatic carcinogenesis. In addition, ER-β in advanced prostate cancer may be potential target of new drug design for this heretofore-incurable disease.

(A) Indirect action

Epithelium

Stroma

Growth and differentiation factors

ER

Estrogens

(B) Direct and indirect actions

Epithelium

ER

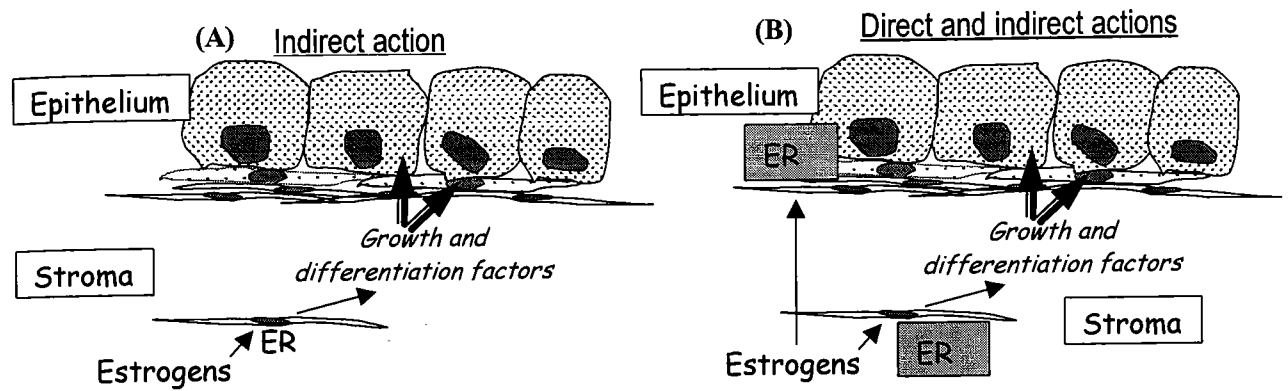*Growth and differentiation factors*

Stroma

Estrogens

ER

Figure 1. Hypotheses of estrogen actions on prostate (A) Indirect action: Estrogens bind to ER in stromal cells and the complexes transactivate gene expressions of growth and differentiation factors which act on basal and glandular epithelial cells. (B) Direct and indirect actions: Estrogen actions are mediated via ERα in stromal cells as described above. In addition, estrogens directly act on epithelial cells via ERβ associated pathway.
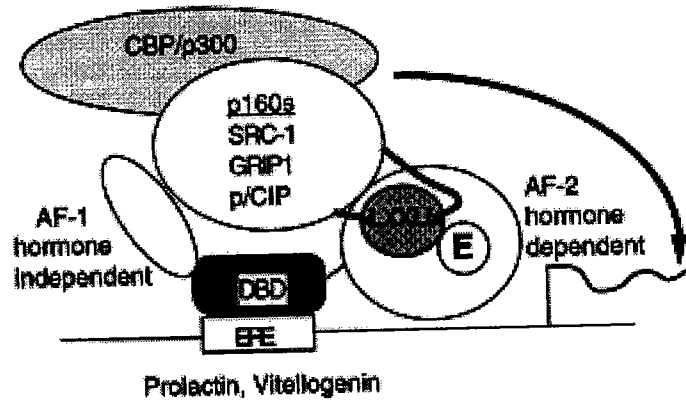
ER activates at EREs by recruiting coactivators

CBP/p300

p160s
SRC-1
GRIP1
p/CIP

AF-1
hormone
Independent

AF-2
hormone
dependent

DBD

E

ERE

Prolactin, Vitellogenin

Figure 2. ER acts at an ERE by binding both DNA and coactivators (Kushner et al 2000).
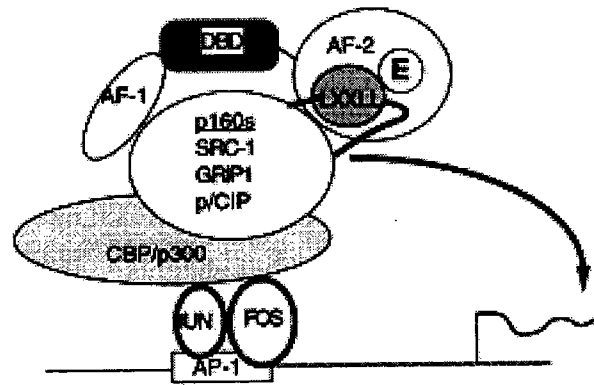
Figure 3. DBD independent and AF dependent pathway for estrogen-ER activation of AP-1. Jun/Fos recruit CBP-p160s, and ER binds to the coactivators and triggers them to higher activity (Kushner et al 2000).

ER-SERM induction of AP-1 by diversion of HDACs
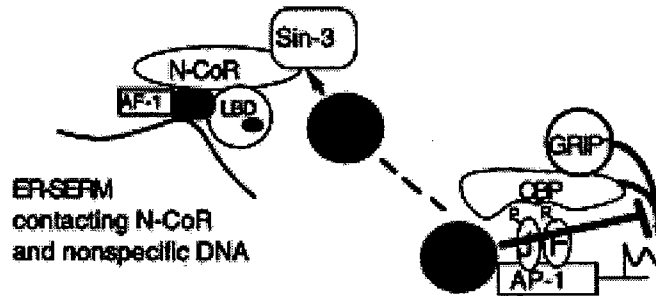
Figure 4. DBD dependent and AF independent pathway for tamoxifen-ER activation at AP-1. ER-tamoxifen located away from the AP-1 site (for example, on non-specific DNA) binds a complex of N-CoR and HDACs through the DBD-hinge-LBD. This draws away HDACs that are associated with the AP-1 site and allows unopposed activity of HATs in coactivators recruited by Jun/Fos (Kushner et al 2000).
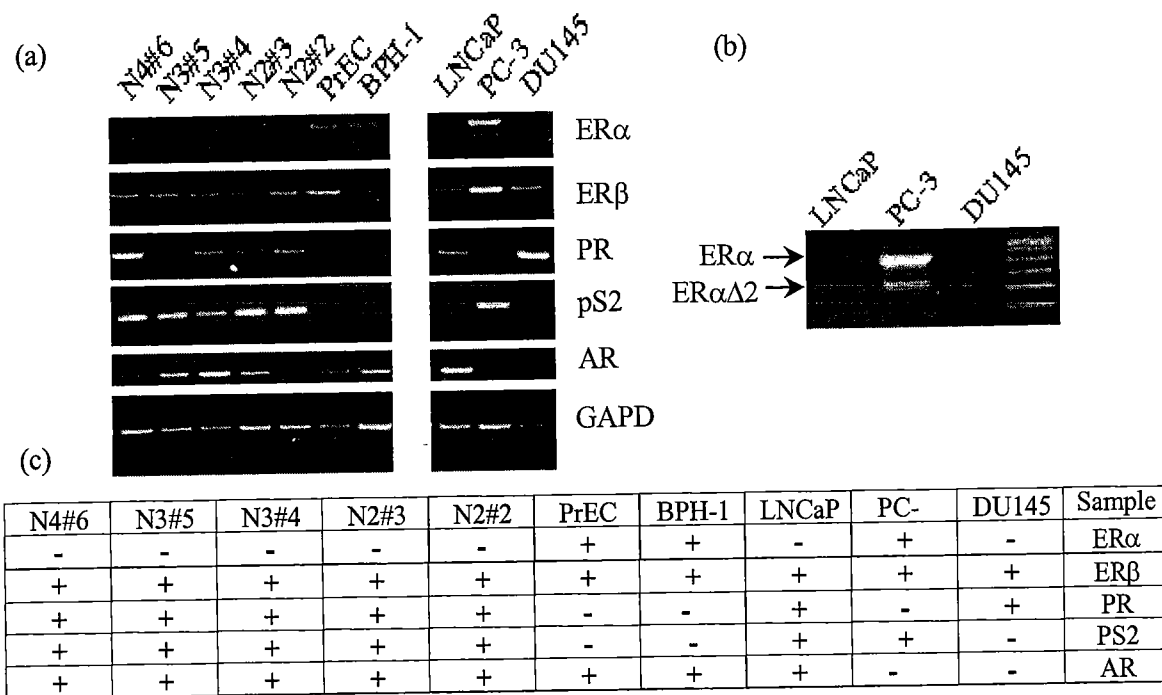
(a)

(b)

(c)

N4#6	N3#5	N3#4	N2#3	N2#2	PrEC	BPH-1	LNCaP	PC-	DU145	Sample
-	-	-	-	-	+	+	-	+	-	ERα
+	+	+	+	+	+	+	+	+	+	ERβ
+	+	+	+	+	-	-	+	-	+	PR
+	+	+	+	+	-	-	+	+	-	PS2
+	+	+	+	+	+	+	+	-	-	AR

Figure 4. Results of RT-PCR analyses of ERα, ERβ, 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 eithidium bromide. Three individual experiments were performed. (A) Representative fluorographs for ERα, ERβ, PR, pS2, AR and GAPDH RT-PCR analyses. (B) An excerpt from ERα fluorograph, enlarged to illustrate the exon 2 deletion variant of ERα (ERαΔ2) 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).
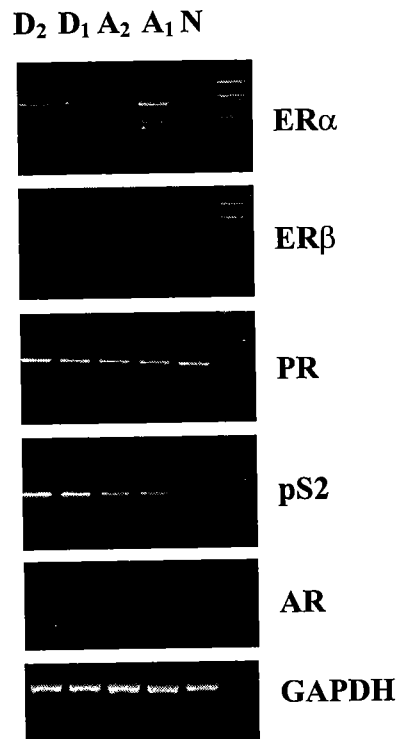
Figure 5. Reactivation of ERα and pS2 mRNA expressions in DU145 cells by demethylating agents. Cells were treated with either 2.5 μM (A1) or 5 μM (A2) 5'-aza-cytidine, or 0.5 μM (D1) or 0.75 μM (D2) 2'-deoxy-5'-aza-cytidine for 8 days. Cells without treatment were used as control (N). At the end of treatment, total RNA was extracted and subjected to RT-PCR analyses as described in Figure 1.
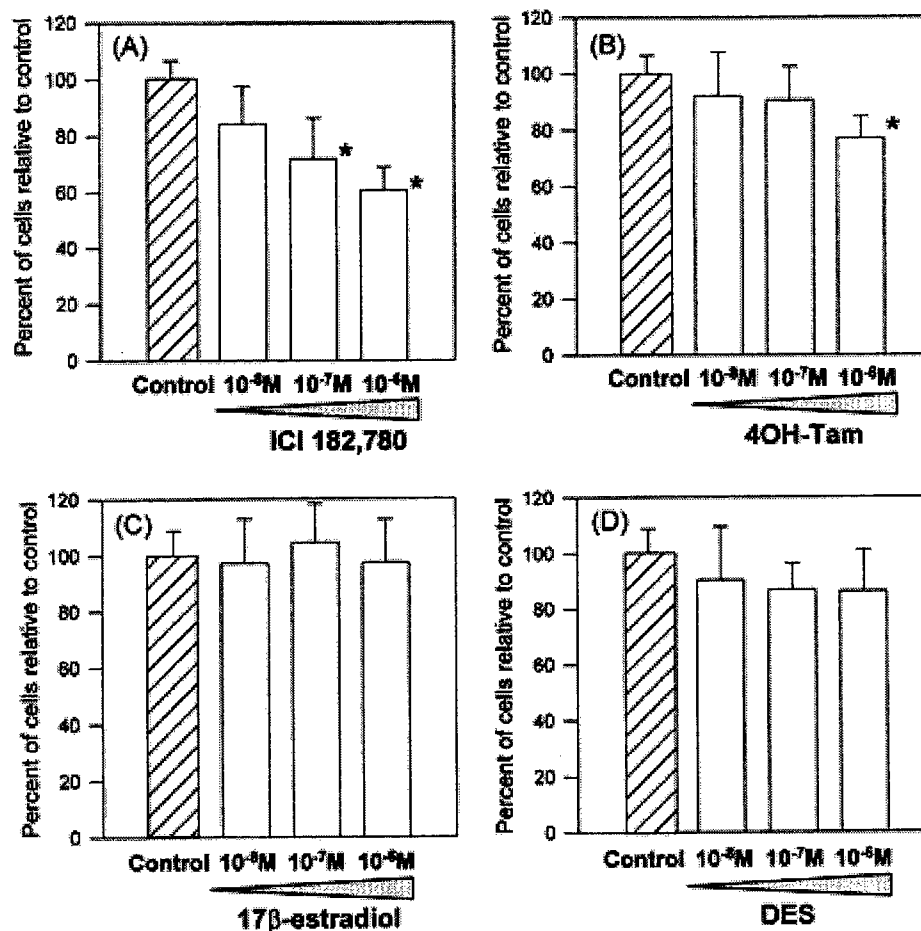
Treatment of antiestrogens on DU145 cells

Figure. 6. Effects of antiestrogens and estrogens on cell growth of DU145 cells. Cells (5 x 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 (▨).

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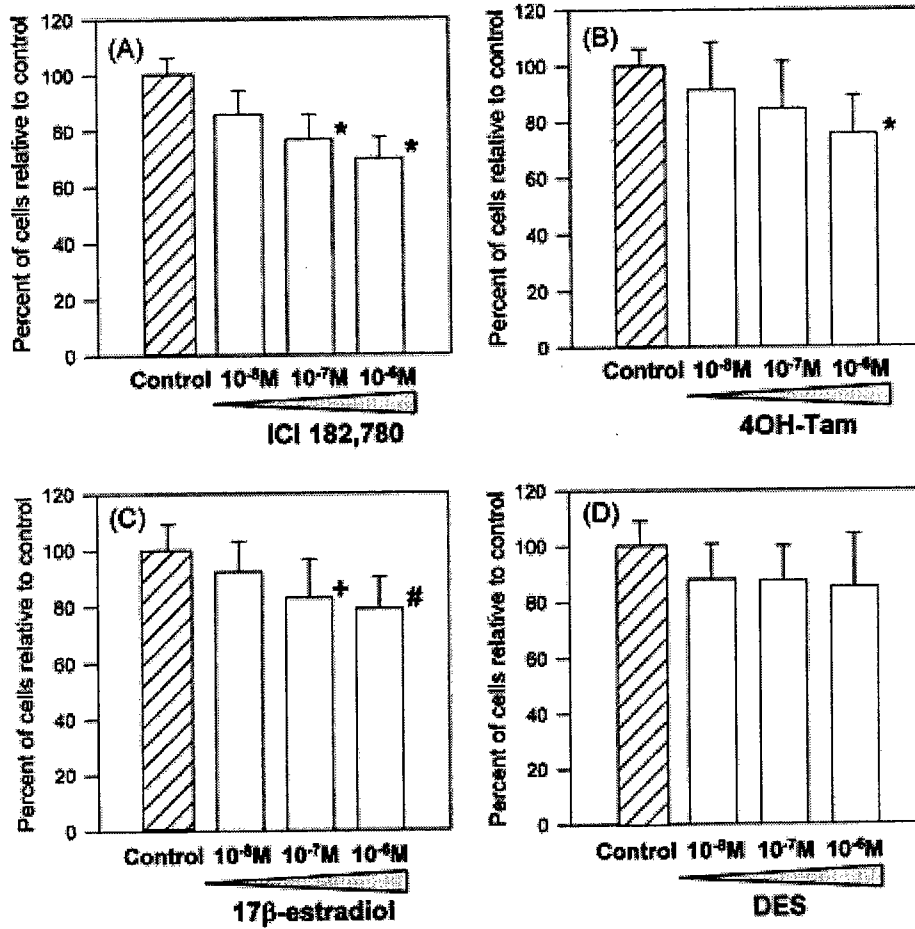**Treatment of antiestrogens on PC-3 cells**

Figure 7. Effects of antiestrogens and estrogens on cell growth of PC-3 cells. Cell growth

assay was described in Fig. 6 . *A,* ICI. *B,* 4OH-TAM. *C,* E_2. *D,* DES. *Columns,* means; *bars,*

SD; $n = 9$; *, $P < 0.001$; #, $P < 0.01$; +, $P < 0.05$; compared with control (▨).

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Percentage of cells relative to untreated control
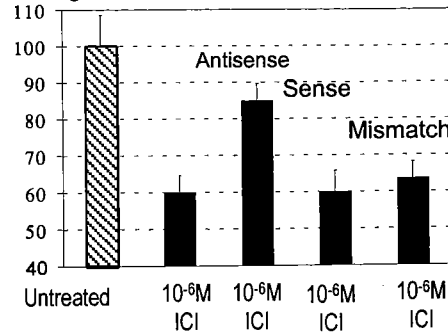
Figure 8. Reversal of ICI-induced DU145 cell growth inhibition by ERβ antisense ODN.

Cells were treated with ICI at 10^{-6} M in the absence or presence of 2.5 μM of the antisense,

sense or mismatch ODNs for 4 days. Cell numbers in "untreated" cultures received no ICI

treatment were arbitrarily assigned a value of 100% (first histogram 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. Histograms= means, bars=S.D., n = 16. * denotes

significant difference between ICI treated cultures and those received ICI plus the antisense

ODN at $p < 0.001$.
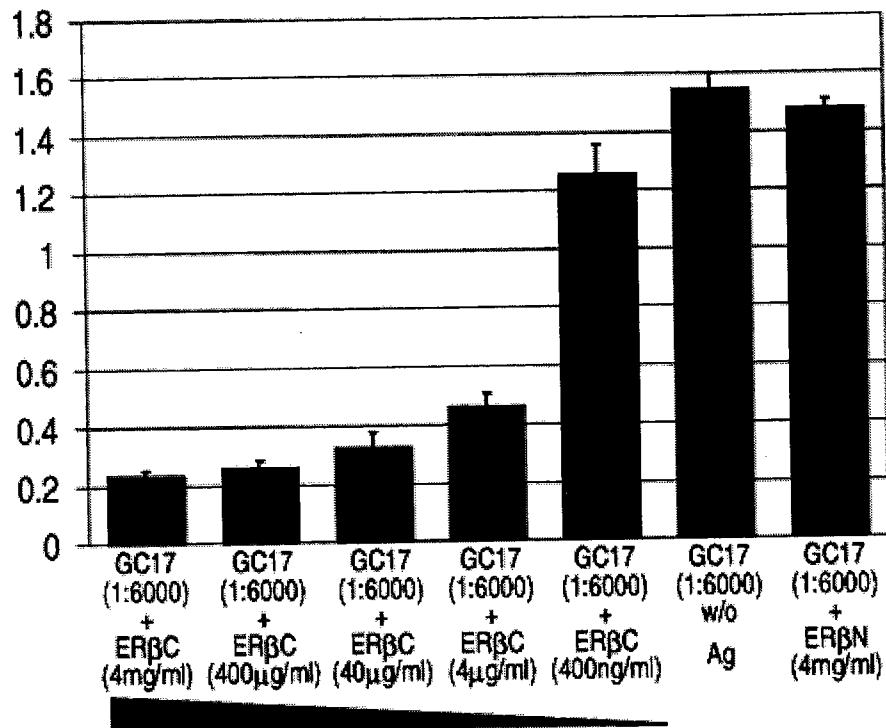
Figure 9. Competitive ELISA. This graphic representation illustrates the concentration-dependent competition of the immunizing peptide (ERβC) with the GC-17 antibody. In addition, preincubation with 4 mg/ml of the control N-terminus peptide (ERβN) and GC-17 failed to compete out the binding to the recombinant ER-β peptide, demonstrating the specificity of the antibody. The GC-17 antibodies, bound to recombinant ER-β protein on ELISA plates, were recognized by alkaline phosphatase-conjugated anti-rabbit IgG antibodies. The whole complexes were visualized by incubation with *p*-nitrophenyl phosphate. The optical density at 405 nmol/L was measured. The entire assay was done four times. The column represents the mean value of optical density at 405 nmol/L of four measurements and the bar represents the SD. w/o, GC-17 preincubated without peptide antigen.

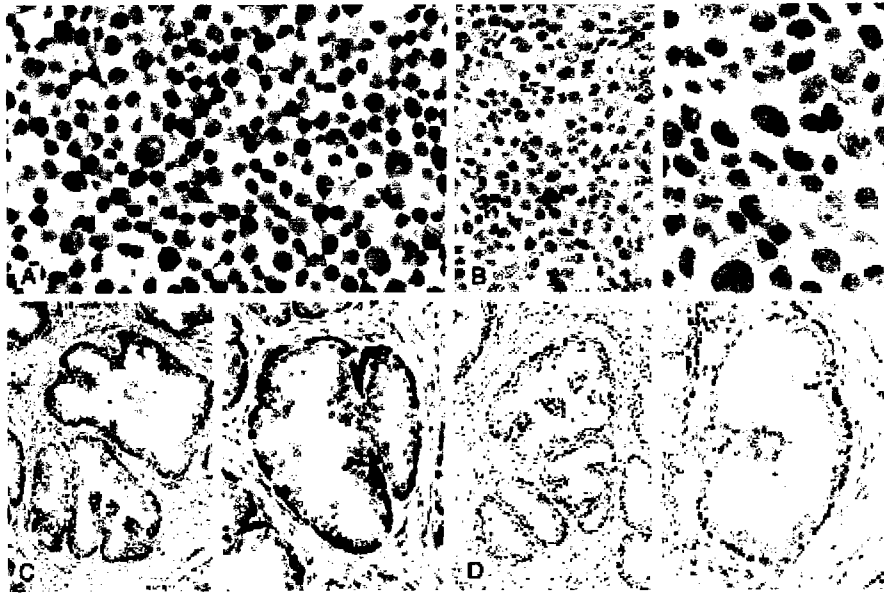**Figure 10.** Competitive ER-β immunostaining DU145 cells (**A** and **B**) and normal prostate

(**C** and **D**). **A:** DU145 cells immunostained in the absence of competing peptide are shown.

Note the strong nuclear staining in the majority of cells (original magnification, x265). **B:**

After incubation of GC-17 with 40 μg of the immunizing peptide, there was almost a total

absence of cells with positively stained nuclei (compare with **A**) [original magnifications:

x115 (**left**), x350 (**right**)]. **C:** Tissue section of normal prostate. In the absence of competing

peptide strong immunostaining of cells in the basal layer of glands is seen in this section of

prostate (see also Figure 4, A and B) [original magnifications: x115 (**left**), x230 (**right**)]. **D:**

A replicate section of the prostate illustrated in **C** after preincubation of GC-17 with 40 μg of

immunizing peptide. Note the absence of immunostaining in these sections [original

magnifications: x90 (**left**), x280 (**right**)]. For details of the procedures used in these studies

see Materials and Methods. All sections were counterstained with 10% Harris hematoxylin.
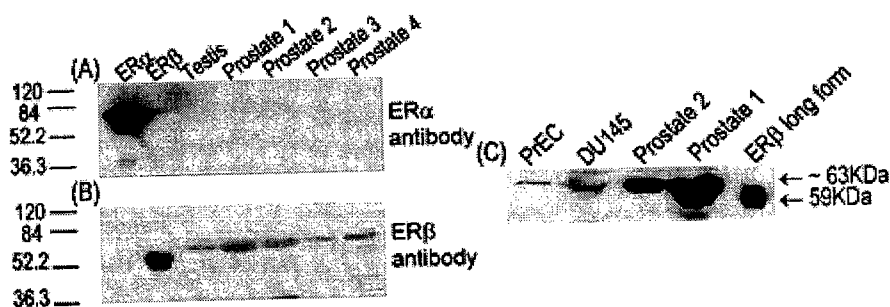
Figure 11. Western blot analysis. These autoradiographs illustrate the binding ability and specificity of GC-17 to ER-β protein and its lack of cross-reactivity with ER-α protein. **A** and **B:** Shown from **left** to **right** are the ER-αrecombinant protein, short form of ER-β recombinant protein, and tissue lysates from human testis and normal prostate (1 to 4). **C:** From **left** to **right** are cell lysates of PrEC and DU145 cells, tissue lysates from human normal prostate (1 and 2), and long form of ER-β recombinant protein. The recombinant proteins and cell or tissue lysates were separated with SDS-PAGE gel and the separated proteins were transferred onto PolyScreen polyvinylidene difluoride transfer membrane. The blot was incubated with the ER-α monoclonal antibody (**A**) or GC-17 ER-β polyclonal antibody (**B** and **C**) and the complexes were visualized by the chemiluminescence ECL detection system. Note that the antibody detects only the ER-α protein using ER-α monoclonal antibody and GC-17 ER-β polyclonal antibody does not detect the ER-α protein but clearly identifies single bands for both forms of ER-β recombinant proteins and for the cell or tissue lysates. These bands correspond to the reported size of the short and long forms of the receptor (see Results).

Figure 12. A and **B:** Immunolocalization of ER-β in the normal human prostate using the GC-17 antibody. Note the strong staining of cells in the basal layer and its virtual absence in the nuclei and cytoplasm of secretory cells (best seen in **B**). Some nuclear membrane staining was however evident in a few secretory cells (**A** and **B**). Nuclear staining is also evident in stromal cells. Original magnifications: x100 (**A**), x400 (**B**). **C:** ER-β immunostaining in low/moderate-grade dysplasia. Moderate to strong expression of the receptor is evident in dysplastic secretory cells. Note also the strongly stained cells in the basal layer (original magnification, x400). **D:** ER-β-immunostained section of high-grade dysplasia. Nuclear staining is almost totally absent in this lesion. In some dysplastic cells light staining of

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nuclear membranes is evident. This area of the lesion is almost totally devoid of receptor-stained basal cells. A few stained basal cells are however evident in the **bottom left** corner of the lesion. Note the presence of two positively stained basal cells in a portion of a normal gland on the **right** (original magnification, x250). **E:** ER-α immunostaining in dysplasia of the central zone. The majority of dysplastic cells are immunostained for the receptor in this lesion. There was however great variation in the percentage of positive cells found in these central zone lesions. Intraluminal pseudo-gland formation seen here was common in dysplasias of the central zone (original magnification, x200). All sections were counterstained with 10% Harris hematoxylin.
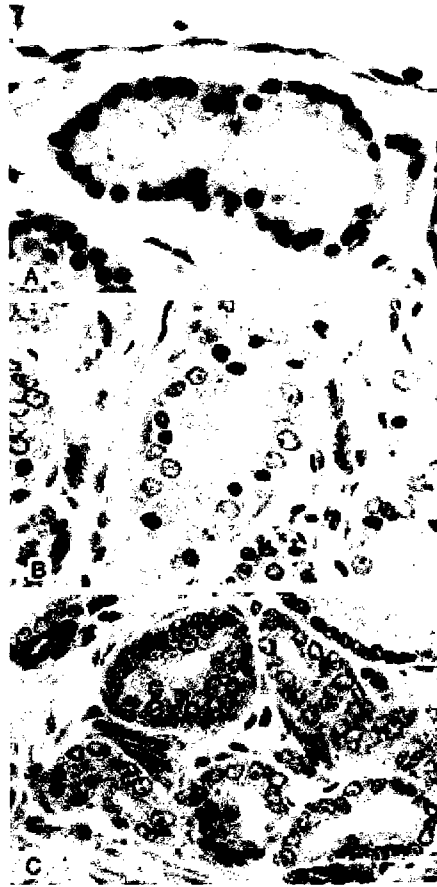
Figure 13. A: ER-β staining in grade 3 carcinoma. Strong nuclear immunostaining is evident in this grade 3 carcinoma. Light cytoplasmic staining of neoplastic cells is also present. Although positive immunostaining for the receptor was found in the majority of grade 3 cancers there was variation in the percentage of stained cells in any given lesion. This was especially the case in areas of transition from grade 3 to grade 4/5 carcinoma (see **C**) (original magnification, x400). **B:** ER-β immunostaining of a clear cell carcinoma in the transition zone. Immunostaining for the receptor is absent in the majority of cells in this grade 3 clear-cell carcinoma. Scattered among the negatively stained cells are cells with small nuclei that are immunopositive for the receptor. The occurrence of these positive cells was uncommon in clear cell carcinomas. In most of these cancers all of the cells were

unstained for the receptor. Interestingly, in replicate sections, these ER-β-positive cells were negative for AR immunostaining whereas the reverse was true for the majority of cells that were negative for the β receptor (original magnification, x400). All sections were counterstained with 10% Harris hematoxylin. **C:** ER-β staining in an area of transition from grade 3 to grade 4/5 carcinoma. The majority of nuclei in this cancer are unstained. A few positively stained nuclei are however seen in two grade 4/5 glands (**bottom left**) and in a single cell in a grade 3 gland (**bottom right**). Light cytoplasmic staining is evident in most cells. Nuclear membrane staining is also present in many of these cells. Cytoplasmic staining was common in all grades of dysplasia and carcinoma. Nuclear membrane immunostaining was however only a feature of high-grade dysplasias and grade 4/5 carcinoma (original magnification, x400).
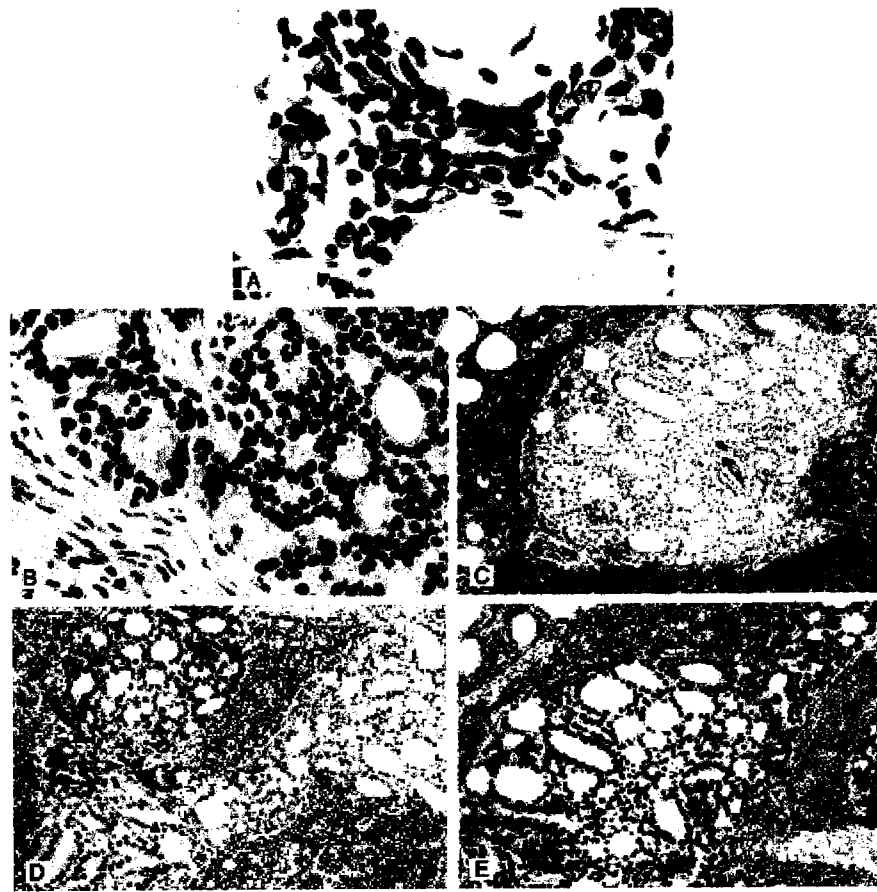
Figure 14. **A:** ER-β immunostaining in a prostatic carcinoma metastatic to bone. Note the strong nuclear immunostaining for the β receptor in this metastatic lesion. The neoplastic cells are localized between spicules of bone. Strong to moderate staining was present in the majority of metastases to bone (original magnification, x400). **B:** ER-β immunostaining in a prostatic carcinoma metastatic to an internal iliac lymph node. Strong nuclear immunostaining is evident in this metastatic lesion. Strong PSA immunostaining of these cells were found (not illustrated). Note that the nuclei of several stromal cells in this lymph node are also positively stained for the receptor (original magnification, x400). **C:** ER-β immunostaining of a prostatic carcinoma metastatic to a lymph node. In this example the

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metastatic cells were unstained for the receptor. Cells in this same cancer were however immunostained for ER-α and AR (see **D** and **E**). As was the case for the metastasis illustrated in **B** these cells were strongly PSA-positive. Note that lymphocytes are strongly stained for the receptor, which was a consistent finding and provided an internal positive control for immunostaining with the β antibody (original magnification, x100). **D:** ER-α immunostaining of the same lesion illustrated in **C**. Immunopositive cells are seen scattered throughout this metastatic lesion. In one other case of lymph node metastasis a very few positive cells (<10%) were present. ER-β was however strongly expressed in that metastatic lesion. Note the absence of ER-α immunostaining of lymphocytes, a consistent finding that was in contrast to ER-β staining in these cells (original magnification, x100). **E:** Representative section of AR immunostaining in metastatic lymph node lesions. Strong nuclear staining was uniformly found in the majority of cells that comprised these metastatic lesions. Cytoplasmic staining occurred only in metastases from the patient who had received anti-androgenic therapy. Lymphocytes were lightly stained for AR (original magnification, x100). All sections were counterstained with 10% Harris hematoxylin.
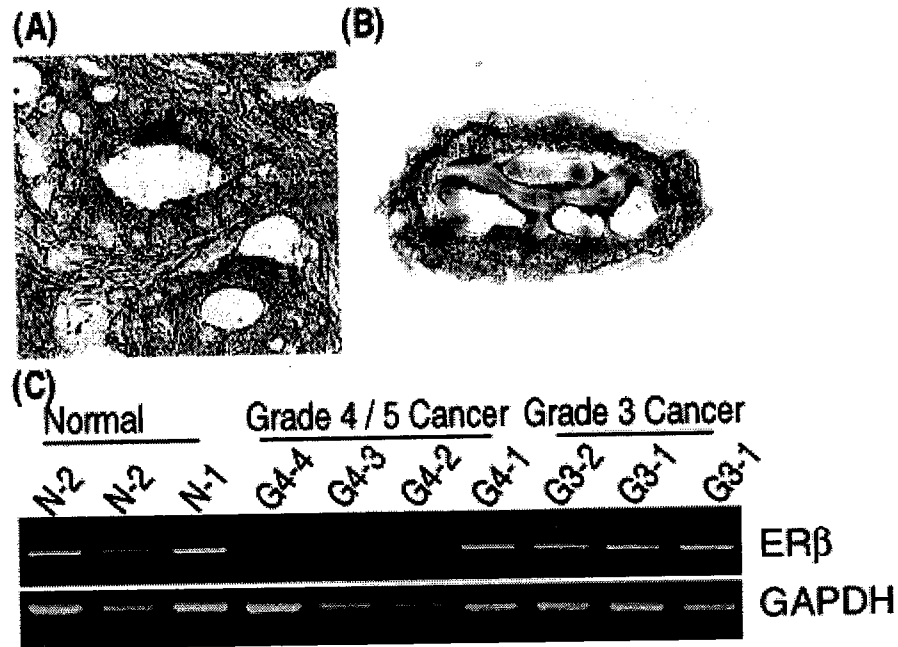
Figure 15. A and **B:** LCM dissection of a grade 3 carcinoma. **A:** The grade 3 neoplastic gland that was microdissected is seen in the center of this microscopic field (original magnification, x325). **B:** The microdissected lesion is seen on the transfer cap (original magnification, x325). Both sections were lightly stained with 10% Harris hematoxylin. **C:** Results of RT-PCR analysis of ER-β mRNA on microdissected human normal prostate acini (N-1 and N-2) and grade 3 (G3–1 and G3–2) and 4/5 (G4–1 to G4–4) carcinomas. Total RNAs were extracted from the microdissected tissues on transfer cap and reverse-transcribed. The resultant cDNAs were subjected to PCR analyses. The amplified products were run into 2% agarose gel with ethidium bromide. Representative fluorographs for ER-β and GAPDH RT-PCR analyses are shown.
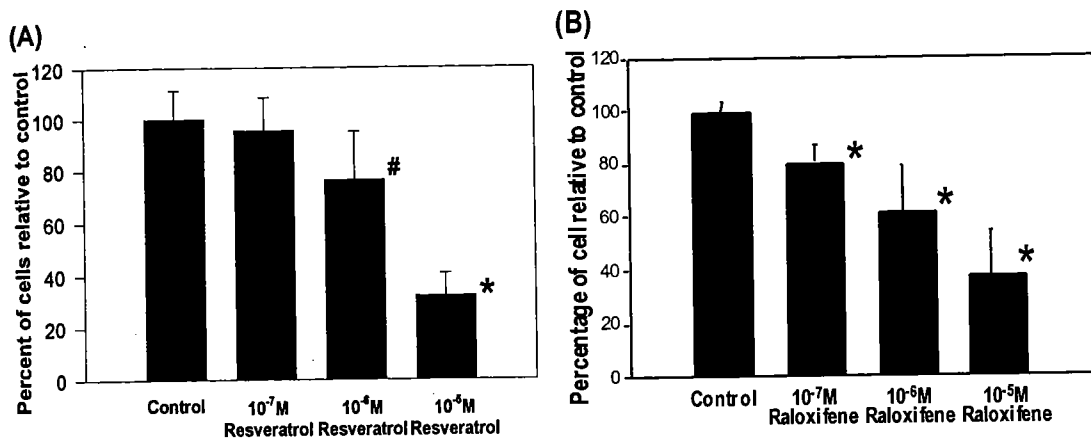
Figure. 16. Rv- and Ral-induced cell growth inhibition in DU145 cells. (A) Inhibitory effects of Rv on growth of DU145 cells. (B) Inhibitory effects of Ral on growth of DU145 cells. Cells were seeded at $5X10^3$ per ml of culture medium in 24 wells culture plates. 24 hours after plating, the cells were treated with 10^{-7}, 10^{-6} and 10^{-5}M of Rv. Control cultures are treated with vehicle alone (0.1% ethanol as final concentration). At the end of a 4-day treatment, cell numbers were assessed by direct cell count with the Trypan blue exclusion method. *Columns* = group means; *error bars* = S.D.; n = 18 cultures; # denotes significant difference at $p < 0.05$ and * at $p < 0.001$ when compared to untreated control.
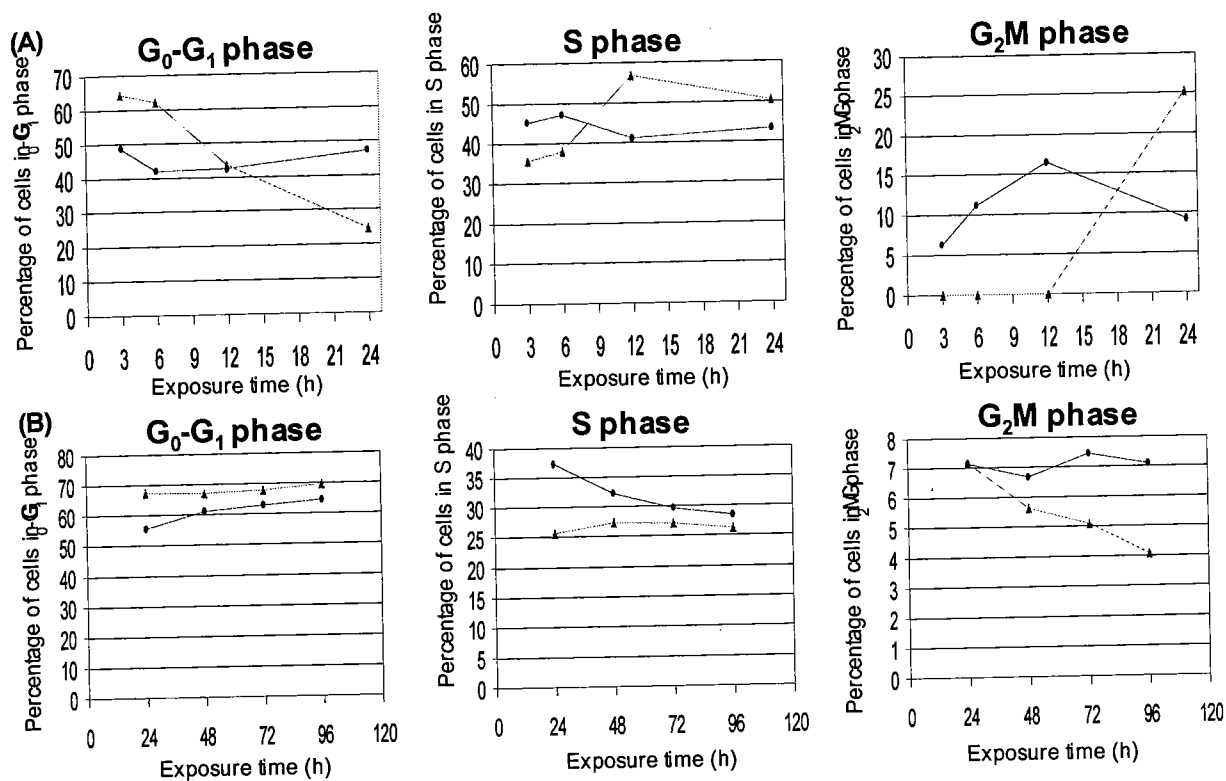
Figure 17. Cell cycle distributions in DU145 cells at various time points after exposure to Rv (A) and ICI (B). DU145 cells were treated with 10 µM Rv for 3, 6, 12, and 24h and ICI for 24, 48, 72, and 96h (7). The cells with 0.1% ethanol as final concentration were used as controls for each time point (,). At various time points, cells were fixed, stained with PI, and subjected to flow cytometry for determination of cell cycle distribution.
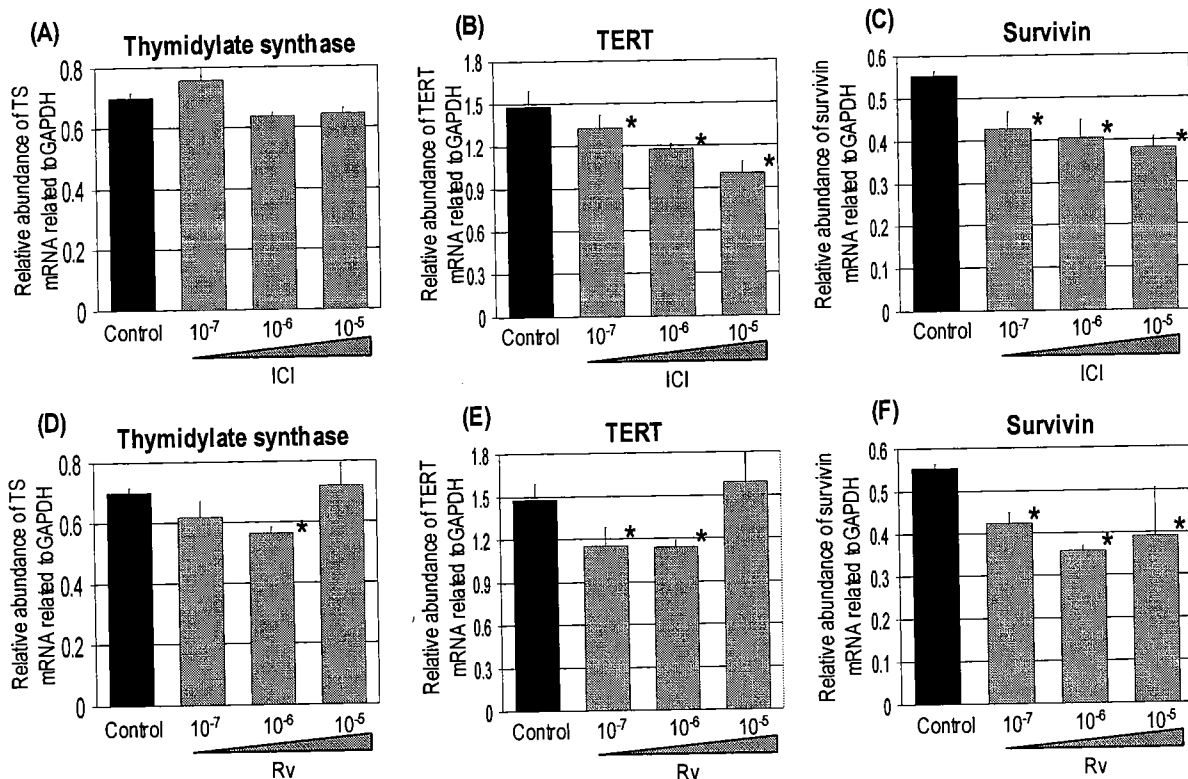
Figure 18. Results of Real-time RT-PCR analyses of thymidylate synthase (TS), TERT and survivin on DU145 cells treated with 10^{-5}, 10^{-6} and 10^{-7}M ICI (A-C) and Rv (D-F) for 24 h. The signals of TS, TERT and survivin were normalized by the signal of GAPDH and the relative abundance was used for all the comparisons. *Columns* = means; *error bars* = S.D.; *n* = 3; * denotes significant difference at $p < 0.05$ when compared to control.

Table 1: Primer sequences for the RT-PCR analysis

Target gene	Primer sequence	Location	Expected Size
ERβ	ERβ-1:5'TGA AAA GGA AGG TTA GTG GGA ACC3' ERβ-2:5'TGG TCA GGG ACA TCA TCA TGG3'	nt.230-253 nt.737-757	528 bp
ERα	ERα-1:5'TAC TGC ATC AGA TCC AAG GG3' ERα-2:5'ATC AAT GGT GCA CTG GTT GG3'	nt.41-60 nt.671-690	650 bp
PR	PR-1:5'GAT TCA GAA GCC AGC CAG AG3' PR-2:5'TGC CTC TCG CCT AGT TGA TT3'	nt.1817-1836 nt.2330-2349	533 bp
pS2	PS2-1:5'GGA GAA CAA GGT GAT CTG CG3' PS2-2:5'CAC ACT CCT CTT CTG GAG GG3'	nt.52-71 nt.268-287	236bp
AR	AR-1:5'CTC TCT CAA GAG TTT GGA TGG CT3' AR-2:5'CAC TTG CAC AGA GAT GAT CTC TGC3'	nt.2896-2918 nt.3214-3237	342 bp
GAPDH	GAPDH-F:5'CCA CCC ATG GCA AAT TCC ATG GCA3' GAPDH-R:5'TCT AGA CGG CAG GTC AGG TCC ACC3'	nt.152-175 nt.726-749	598 bp

Table 2: Oligonucleotide sequences for the antisense ODN experiments

Target gene	Sequences	Location[@]	Size

ERβ
  Antisense
  Sense
  Mismatch
	5'C*A*T* CAC AGC AGG GCT A*T*A*3'
	5'T*A*T* AGC CCT GCT GTG A*T*G*3'
	5'G*A*T* CTC AGC ACG GCA A*A*T*3'
		-15 to +3	18 bp

* This base was phosphorothioate-modified.
@ The first base of translation-initiating site is +1.

Table 3: **Immunohistochemical findings in dysplasias and carcinomas**

Lesion	ER-α	ER-β	AR
Dysplasia / Peripheral zone			
Moderate grade*	-	+	+
High grade	-	-	+
Dysplasia /Central zone*	+	+	+
Carcinoma/ Peripheral zone			
Grade 3	⁻/+	+	+
Grade 4/5	⁻/+	⁻/+	+
Carcinoma/ Transition zone	-	-	+
Metastatic carcinoma			
Bone	-	+	+
Lymph nodes	⁻/++	+	+

* Data reflects positive staining in dysplastic and basal cells. Staining restricted to residual basal cells in high-grade dysplasias. + = Positive staining in >50% of cases; -/++ = Positive staining in 40% of cases; -/+ = Positive staining in< 20% of cases. See text for estimates of the % of positively stained cells in each lesion.

Table 4: Primers for Real-Time RT-PCR analysis on survivin, TERT, thymidylate synthase (TS), and GAPDH

Genes	Primer sequences	Size
1. Survivin	F: 5'-GATGACGACCCCATAGAGGA-3' R: 5'-GCACTTTCTTCGCAGTTTCC-3'	187bp
2. TERT	F: 5'-AACTTGGCGGAAGACAGTGGT-3' R: 5'-CAAGACCCCAAAGAGTTTGC-3'	241bp
3. Thymidylate synthase	F: 5'-TCCCGAGACTTTTTGGACAG-3' R: 5'-CAAGCGCACATGATGATTCT-3'	206bp
5. GAPDH	F: 5'-GGAAGCTCACTGGCATGGC-3' R: 5'-TAGACGGCAGGTCAGGTCCA-3'	70bp

Table 5: Percentages of cells at different phases of cell cycle after treatment of ICI, Ral, or Rv at 10 μM for 24h

Treatment for 24h	G_0-G_1*	S*	G_2M*
Control	56.61 ±0.37	33.78 ±0.31	9.61 ±0.22
ICI	66.52 ±0.06	25.83 ±0.41	7.64 ±0.46
Ral	63.83 ±0.65	27.89 ±0.36	8.28 ±0.30
Rv	26.25 ±1.72	50.54 ±0.74	23.21±2.24

* Mean of percentages for 3 separated experiments ± standard deviation

Table 6. Results of pulse-chase experiments with BrdU incorporation for treatments of Rv at various time points.

Duration of treatment	Total S phase (%)	Early S phase (%)	Mid S phase (%)	Late S phase (%)
Control - 12h	43.74	13.63	8.37	22.40
Rv - 12h	66.54	15.60	29.16	22.30
Control - 18h	56.31	43.12	10.51	3.59
Rv -18h	67.68	28.03	11.72	29.74
Control - 24h	49.34	24.63	13.99	11.26
Rv - 24h	69.35	33.83	18.76	18.62

CHAPTER VII

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