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14. ABSTRACT

CaP presents its greatest challenge to clinicians when it progresses to the hormone-independent state. Therapeutic methods which are effective regardless of androgen response, or even target androgen-independent CaP specifically, are of special medical and scientific interest. We have shown that estradiol (E2) can inhibit growth of hormone–independent CaP in vivo. Among the genes up-regulated by E2 are IFN-regulated genes. The LuCaP 35V xenograft does not grow in vitro; for this reason, this exploratory proposal was design to evaluate the responses of various CaP cell lines to E2 and IFNγ in vitro. Our results show that E2 did not inhibit growth of 5 prostate cancer cell lines in vitro. It is possible that inhibition by E2 is not a result of direct effects of E2 on tumor cells, and that the interaction with the host environment may be critical for this inhibition. Regarding E2 regulation of expression of IFN-regulated genes, our data suggest that E2 may not activate IFN pathways directly, However our results suggest that IFN-regulated genes may play a role in the growth inhibition caused by E2 in vivo, since DU 145 cells showed similar alterations in expression of these genes following E2 treatment as LuCaP 35V, but smaller in magnitude, and DU 145 growth was weakly inhibited by high doses of E2. In conclusions, the 5 CaP cell lines available to us did not respond to E2 treatment as do LuCaP 35V in vivo. Our results indicate the possibility that the observed effects of E2 on prostate cancer xenografts in vivo might be mediated via indirect effects through interactions of CaP cells with cells of the innate immune system or other indirect effects of E2 requiring interactions with the host environment.

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Task 2. Examination of effects of Interferon gamma on CaP cells *in vitro.*

- Determination of effects on proliferation (0-6 months).
- Determination of effects on apoptosis (0-6 months).
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- Examination of activation of STAT pathway (12-18 months).

INTRODUCTION

Prostate cancer, the most common malignancy in American men, presents its greatest challenge to clinicians when it progresses to the hormone-independent state. Despite substantial attention, the development of androgen independence in CaP is not well understood, and current treatment methods are of limited value. Progression to the androgen-independent state represents in essence the loss of the primary signaling pathway used to control recurrent CaP following radical prostatectomy. Accordingly, therapeutic methods which are effective regardless of androgen response, or even target androgen-independent CaP specifically, are of special medical and scientific interest.

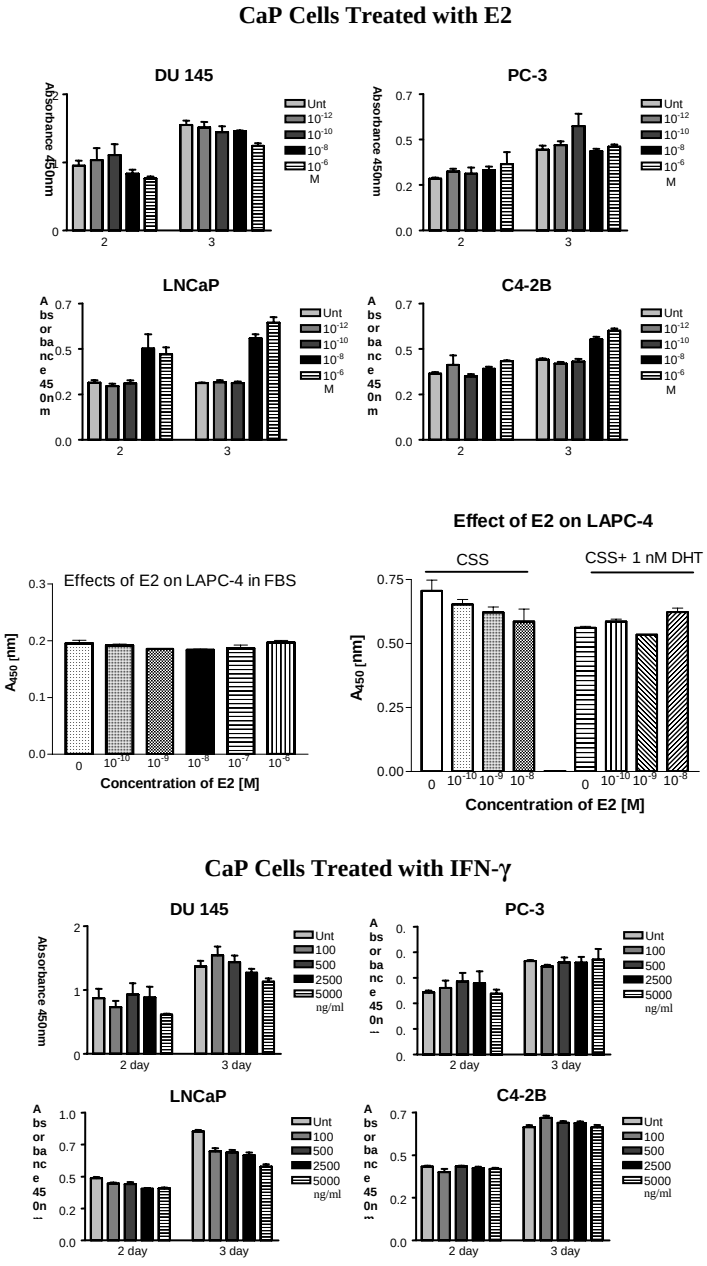
We have shown that estradiol (E2) can inhibit growth of hormone–independent prostate cancer in animal models. Expression of a variety of genes is upregulated by E2 treatment in the LuCaP 35V CaP xenograft. Among the immune-response-related genes altered by E2 treatment in CaP are those modulating cellular responses to interferons. This group was found to be significantly enriched in the set of genes up-regulated by E2 when tested by GSEA using an independently generated list of interferon-regulated genes. The increased expression of interferon–regulated genes is of particular interest due to the direct anti-tumor activities reported for these cytokines (1-8). Our results are in keeping with the results of up-regulation of IFN-regulated genes in LNCaP CaP cells following exposure to the estrogenic herbal preparation PC-SPES (9), and induction of IFNγ-regulated genes after E2 treatment in other tissues (10). In addition tamoxifen has been shown to enhance interferon-regulated gene expression in breast cancer cells (11). Specifically, IRF1, whose expression was increased 3-fold by E2 (qRT-PCR data), has been described as a negative regulator of proliferation (12) and exhibits tumor-suppressor activities in breast cancer cells (13). These published observations and our results are consistent with a model in which IFN and genes regulated by IFN modulate a component of the growth inhibitory activity of E2 toward androgen-independent CaP cells. The LuCaP 35V xenograft does not grow *in vitro*; for this reason, under this proposal we planned to evaluate the responses of various CaP cell lines to E2 and IFNγ *in vitro*. These studies should allow us to draw conclusions of two types: (1) what genes are consistently associated with growth inhibition of CaP by E2; and (2) the degree of congruence (in terms of gene expression) between the best *in vitro* model of this effect and the *in vivo* models we have described.

RATIONALE

The LuCaP 35V xenograft, which exhibits increased expression of IFN-regulated genes in response to E2 treatment, does not grow *in vitro*, and is therefore not amenable to detailed studies of signal transduction and phenotypic manipulations to determine the effects of altered gene expression. For this reason, under this exploratory proposal we evaluated the responses of various CaP cell lines to E2 and IFNγ *in vitro.* The studies were proposed to help establish whether increased expression of IFNγ and IFNγ-regulated genes associated with E2 treatment is an important aspect of the observed growth inhibition. Our results will help to determine the best *in vitro* model to use in a full hypothesis-driven proposal to examine the importance of the observed changes in inhibition of advanced prostate cancer.

RESULTS

Proliferation and Apoptosis:
We originally examined the effects of IFNγ and E2 on proliferation of four prostate cancer cell lines, LNCaP, C4-2B, PC-3 and DU 145. IFN-γ treatment had no significant effects on proliferation of the prostate cancer cells tested. When we evaluated effects of E2 on these cell lines, in contrast to our *in vivo* data with LuCaP 35 V, E2 stimulated the growth of LNCaP and C4-2B. However, this might be due to the mutated androgen receptor in these cells. E2 slightly inhibited the proliferation DU 145 after 2 and 3 days of treatment, but only at high concentrations (10^{-6} and 10^{-8} M). PC-3 cells were unaffected. We have obtained another prostate cancer cell line, LAP-C4, which expresses a wild-type androgen receptor. Our data show that E2 did not alter proliferation of LAPC-4 in either the presence or absence of androgens. There was a small decrease in CSS with 10^{-8} nM DHT, but it did not reach significance. Because no effects on cell number were seen, we did not evaluate apoptotic effects of E2 and IFN-γ on these cells.



Expression of Immune-related Genes by Real-time PCR
We have shown previously that E2 inhibits growth of LuCaP 35V and performed cDNA array analyses of phenotypic changes of LuCaP 35V associated with E2 treatment. GSEA analysis showed that interferon-regulated genes were enriched in E2-treated LuCaP 35V. Therefore under this proposal we have evaluated alteration of these genes after E2 and IFN-γ treatment *in vitro*. We performed real-time PCR to examine the expression pattern of immune-related genes in five prostate cancer cell lines and compared the results to changes in expression of these genes in E2-treated LuCaP 35V (Table 1).

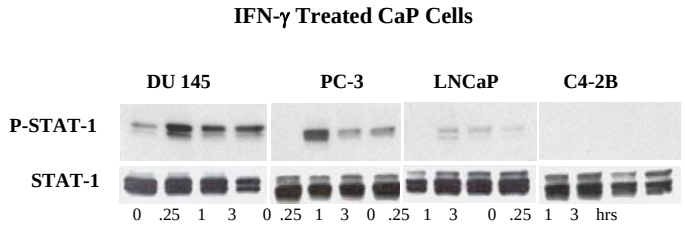
Table 1. Expression of IFN-regulated genes in CaP cells after IFNγ treatment. The results are presented as a fold change in comparison to untreated cells.

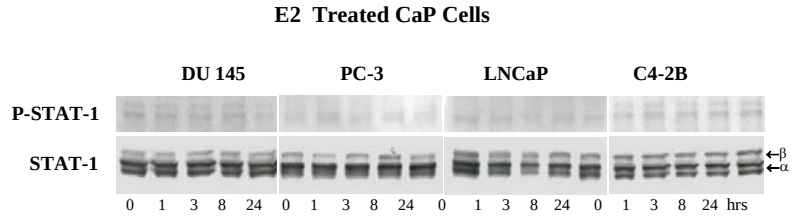
	LNCaP	PC-3	DU 145	C4-2B	LuCaP 35
BST2	6375.00	64600.00	50067.00	113.70	**2.50**
CD59	80960.00	1.54	1.94	9.30	**2.22**
IFITM3	6.69	2.14	7.48	0.71	**2.60**
IFITM1	152.94	59.86	11.60	7.60	**2.80**
IRF1	86.04	98.50	151.46	6.41	**1.53**
HLA-DRA	10862.82	117040.30	428.12	428.04	**6.35**
B2M	15.55	13.43	11.63	4.61	**2.85**

Table 2. Expression of IFN-regulated genes in CaP cells after E2 treatment. The results are presented as a fold change in comparison to untreated cells.

	LNCaP	PC-3	DU 145	C4-2B	LAPC-4	LuCaP 35
BST2	0.08	1.21	*2.16*	0.20	0.60	**2.50**
CD59	0.43	0.82	*1.33*	0.56	0.33	**2.22**
IFITM3	0.90	0.53	*1.75*	0.90	0.93	**2.60**
IFITM1	0.17	1.14	*1.25*	0.99	0.61	**2.80**
IRF1	0.17	0.96	*1.03*	0.21	0.94	**1.53**
HLA-DRA	0.05	0.00	*1.16*	0.24	3.09	**6.35**
B2M	3.14	0.77	*1.54*	2.31	0.60	**2.85**

Our results show that IFNγ increases expression of all of these genes in prostate cancer cell lines. However E2 decreased levels of these messages in the three androgen receptor-expressing cells lines, despite the fact that LNCaP and C4-2 have mutated androgen receptors while LAPC-4 expresses a wild-type androgen receptor. In PC-3 cells, some messages exhibited increased expression and some decreased. The greatest similarity to E2-treated LuCaP 35V was observed in DU 145 cells; however the magnitude of the changes was smaller.





Activation of JAK/STAT Pathway by E2 and IFN-γ Treatments

We observed activation of STAT-1 in LuCaP 35V treated with E2. Therefore we examined activation of STAT-1 in prostate cancer cells *in vitro* after treatment with E2 and IFN-γ. IFN-γ induced STAT-1 activation in DU-145, PC-3, and LNCaP, while E2 did not stimulate STAT-1 activation in any of the CaP cell lines tested.

KEY RESEARCH ACCOMPLISHMENTS

- Estradiol caused slight inhibition of proliferation of DU 145 prostate cancer cells at moderately high concentrations.
- Estradiol did not inhibit proliferation of PC-3 and LAPC-4 prostate cancer cells.
- Estradiol stimulated proliferation of LNCaP and C4-2B prostate cancer cells.
- INFγ did not inhibit proliferation of the prostate cancer cells tested.
- Treatment with E2 or INFγ did not alter expression of IFN-regulated genes to the same degree as E2 treatment of LuCaP 35V.
- E2 does not activate the STAT-1 pathway in prostate cancer cells *in vitro.*
- IFNγ activates the STAT-1 pathway in prostate cancer cells.

CONCLUSIONS

Our results show that E2 did not inhibit growth of 5 prostate cancer cell lines *in vitro,* while E2 treatment inhibit growth of 4 prostate cancer xenografts *in vivo*. Therefore we have not yet found an *in vitro* model capable of duplicating the E2 inhibition of proliferation observed *in vivo*. It is possible that inhibition by E2 is not a result of direct effects of E2 on tumor cells, and that the interaction with the host environment may be critical for this inhibition. Regarding E2 regulation of expression of interferon-regulated genes, our data suggest that E2 may not activate IFN pathways directly, since expression of evaluated genes was down-regulated by E2 in LNCaP and C4-2B cells. However these cells express mutated androgen receptors. We hypothesized that a wild-type androgen receptor might be required for these effects. Therefore in the extension period we have investigated the effects of E2 on LAPC4 prostate cancer cells, which express a wild-type androgen receptor. However, our data show that E2 did not inhibit proliferation of LAPC-4 *in vitro* and did not increase expression of interferon-regulated genes as does E2 treatment of LuCaP 35 V *in vivo*. However our results suggest that interferon-regulated genes may play a role in the growth inhibition caused by E2 *in vivo*, since DU 145 cells showed similar alterations in expression of these genes following E2 treatment as LuCaP 35V, but smaller in magnitude, and DU 145 growth was weakly inhibited by high doses of E2.

In conclusions, the five prostate cancer cell lines available to us did not respond to E2 treatment as do LuCaP 35V and 3 other xenografts *in vivo*. Our *in vitro* results indicate the possibility that the observed effects of E2 on prostate cancer xenografts *in vivo* might be mediated *via* indirect effects through interactions of CaP cells with cells of the innate immune system or other indirect effects of E2 requiring interactions with the host environment.

REPORTABLE OUTCOMES

Coleman IM, Kiefer JA, Brown LG, Pitts TEM, Brubaker KD, Nelson PS, Vessella RL, Corey E. Inhibition of Androgen-Independent Prostate Cancer by Estrogenic Compounds is Associated with Increased Expression of Immune-Related Genes. *Neoplasia*, in press (attached).

Corey E. Estrogen in Prostate Cancer: Friend or Foe? *Current Cancer Therapy Reviews*. Invited review. 2006 (attached).

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APPENDICES

1. Corey E. Estrogen in Prostate Cancer: Friend or Foe?
2. Coleman IM, Kiefer JA, Brown LG, Pitts TEM, Brubaker KD, Nelson PS, Vessella RL, Corey E. Inhibition of Androgen-Independent Prostate Cancer by Estrogenic Compounds is Associated with Increased Expression of Immune-Related Genes.

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Estrogen in Prostate Cancer – Friend or Foe?

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Abstract

Prostate cancer is an increasingly prevalent health problem among males, and the need for improved methods of treatment is great. In the 1940s estrogens were shown to be of benefit in prostate cancer, and their use continued for some 30 years, until the advent of LHRH agonists and similar drugs. At the time the mechanism of action of estrogens was thought to involve merely reduction in androgen levels, but new evidence, including expression of estrogen receptors by prostate epithelium and prostate, results showing a direct cytotoxic effect on prostate cancer, and preclinical data on inhibition of prostate cancer in intact female mice, suggests that estrogen exerts other effects on prostate cancer cells. Given that estrogens also decrease bone lysis caused by androgen suppression and may ameliorate cognitive side effects associated with low testosterone, estrogens show promise in treatment of androgen-independent prostate cancer. This review summarizes published reports of the effects on estrogens on prostate cancer in preclinical and clinical settings.

Key Words: prostate cancer, estrogen, estradiol

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Prostate cancer is an increasingly prevalent health problem among males, and the need for improved methods of treatment is great. For those 15-30% of radical-prostatectomy patients who experience recurrence of the disease, androgen-ablation therapy is currently the most commonly used treatment. However, nearly all patients treated by androgen ablation eventually experience recurrent androgen-independent prostate cancer, a fatal condition. Despite substantial attention, the development of androgen independence in prostate cancer is not well understood, and current treatment methods are of limited value. Accordingly, therapeutic methods which are effective regardless of androgen response, or even target androgen-independent prostate cancer specifically, are of special medical and scientific interest.

1. Estrogen and Prostate Cancer

The beneficial effect of estrogens in prostate cancer was established as early as 1941 by Huggins [1-3], who treated advanced prostate cancer patients by orchiectomy or estrogen administration. The basic observation was that testosterone promotes the growth of tumor cells with androgen receptors, and either treatment with estrogens [4,5] or castration causes a significant reduction in the biological availability of testosterone. The underlying hypothesis held that estrogen treatment reduced levels of testosterone to the same levels seen after castration**,** and that these effects were mediated primarily *via* suppression of the hypothalamo-hypophyseal axis [6,7]. However, in 1976 Mangan *et al* postulated that estrogens may also exhibit direct action on prostate cancer *via* its own estrogen receptors (ER) [8]. Estrogens, especially diethylstilbestrol (DES), were used for some 30 years to treat prostate cancer [9-14], although significant cardiovascular complications were associated with this treatment. The Veterans Administration Cooperative Urological Research Group (VACURG) (1967) evaluated the use of DES and concluded that hormonal therapy with DES should be withheld until symptoms of metastatic disease appeared, and that administration of DES at levels of 5 mg/day was associated with excessive risk of cardiovascular mortality [15,16]. In a further study, VACURGII compared different dosages of DES and concluded that 1 mg was as effective as 5 mg in controlling T3 M+ prostate cancer [17]. However, in 1988, even this level of DES was found to be associated with a high risk of cardiovascular complications, mainly in patients over 75 years of age [18]. A further shift in treatment patterns accompanied the development of synthetic GnHR analogs, which are now mainly used as a means of chemical castration. These developments essentially ended the era of DES treatment of prostate cancer [18-20].

Nevertheless, in 1986, in a retrospective study, de la Monte *et al.* reported that patients treated with estrogen survived somewhat longer than patients who had undergone surgical castration ($0.05<P<0.1$, [12]), but that they had significantly greater numbers of metastases ($P<0.001$) and greater overall tumor burdens. The relevant data were estrogen group (N=48), bilateral orchiectomy (N=8), and no hormonal treatment (N=33): survival (months) was 38.8 ± 4.1, 27.8 ± 7.4, and 25.4 ± 8.0; and metastatic sites were 11.4 ± 0.8, 6.9 ± 1.6, and 5.2 ± 8.0, respectively. It should be noted that the greater numbers and extent of metastases could be results of longer survival. In 1988 Byar *et al.* commented that no form of endocrine therapy had proven to be superior to 1 mg of DES daily [13]. DES diphosphate (DES-DP) exerted a direct cytotoxic effect on prostate cancer tumors, which could explain the favorable response observed in patients with metastatic and hormone-refractory prostate cancers [21]. These results indicated that there might be effects of estrogens on prostate cancer cells independent of the hypothalamo-hypophyseal suppression of androgens.

2. Estrogen Receptors

Nearly all established effects of estrogens are believed to be mediated by estrogen receptors (ER), although some exceptions should be noted [18,22,23]. Human ER complementary DNA was first characterized by Greene *et al.* [24]. The gene they studied is now called ERα, since a new subtype of ER, called ERβ, has recently been identified from a rat prostate cDNA library [25-27], and a human analog has been characterized [28]. ERβ possesses strong homology with ERα and exhibits high affinity for estradiol, suggesting that this receptor is an alternative molecule for mediation of estrogen action [29] (for reviews of estrogen receptors see [30-34]). The discovery of ERβ renewed interest in basic research involving estrogen pathways. It also led to the development of SERMs, molecules with differential agonist and/or antagonist activities, depending on the receptor involved, tissue type, and perhaps other variables [35]. New lines of evidence indicate that the two types of ER may transduce very different (or even opposing) signals, depending on ligand and tissue type (reviewed in [36]).

2.1. Expression of Estrogen Receptors in Primary Prostate Cancer

In past the presence of ER in prostatic tissues has been a controversial issue. A few groups reported detection of ER in prostate epithelium, stroma, and cancer cells [37-44], but others have reported the opposite [38,45-52]. In contrast to the inconclusive results of immunohistochemistry, radioligand-binding assays with normal prostate, benign prostatic hyperplasia, and prostate cancer have consistently demonstrated the presence of estradiol

binding sites [43]. Discrepancies regarding the expression of ER in these older reports may largely be attributable to the existence of two receptor types, since most were published before the discovery of ERβ.

Reagents with specificity for the ER subtypes have been available since 1997. Using these tools, ERα messages and protein have been found almost exclusively in prostatic stroma, with occasional isolated staining in basal epithelium [37,53,54]. ERβ messages were detected in basal and luminal cells of the prostate [55]. An early report indicated that ERβ was expressed in all lobes of rat prostate at high levels [56]. Studies in humans have shown expression of ERβ in prostatic epithelial cells [25] as well as prostate cancer cells [53,53,57-63] (also reviewed in [64]). In general, prostate epithelial cells and prostate cancer cells express ERβ; the levels of the message and/or protein appear to be down-regulated during disease progression, apparently recapitulating a pattern seen in colon and colorectal cancers [65,66]. The lower levels of ERβ in prostate cancer *vs*. normal prostatic epithelium are consistent with a proliferation-regulatory role for ERβ [67]. One hypothesis holds that ERβ in prostate cancer transduces a growth-inhibitory effect of estrogen on prostate cancer cells. In support of this hypothesis, a lower rate of cancer-related death in prostate cancer patients positive for ERβ *vs.* negative patients was observed, as well as an inverse correlation of ERβ expression with Gleason grade [68]. In this report the authors also identified an inactive ERβ variant truncated at the C-terminus (ERβcx) whose expression was increased in higher grade prostate cancer tumors [68].

2.2. Expression of Estrogen Receptors in Prostate Cancer Metastases

In contrast to the decreased expression of ERβ with prostate cancer progression, ERβ protein has been detected in a limited number of prostate cancer metastases [53,69]. In a larger study, we have shown that all metastases of prostate cancer examined showed some degree of nuclear ERβ immunoreactivity. Approximately half of the osseous and non-osseous metastases exhibited intense nuclear ERβ immunoreactivity in 50% or more of the tumor cells [70]. The mechanism whereby ERβ expression is enhanced in metastases *vs*. primary cancer has not yet been determined. The methylation pattern of the ERβ promoter may be altered in metastases [61,71]. Recently, an inverse correlation has been observed between methylation of CGI in the ERβ promoter and receptor expression in normal, hyperplastic, premalignant, and malignant prostate, and in lymph-node and bone metastases [72]. This is the first report indicating that

regulation of ERβ is reversible and tumor-specific. The presence of ERβ in androgen-independent metastatic prostate cancer cells suggests that these cells may be susceptible to inhibition by estrogenic compounds; hence ERβ may be a valid candidate for pharmacological targeting in treatment of metastatic prostate cancer. Renewed interest in the presence of ER in prostatic tissues and the potential benefits of estrogen therapy in prostate cancer is evidenced by the recent upsurge in reviews of this topic [64,73-78].

3. Effects of Estrogens on Prostate Cancer and Normal Prostate

3.1. Effects on Prostate Cancer in Vitro

To evaluate whether estrogenic compounds exhibit direct effects on prostate cancer cells, estradiol and DES were tested *in vitro* using available prostate cancer cell lines. Treatment with estradiol enhanced proliferation of LNCaP [79-81]. However, proliferative stimulation of LNCaP may be mediated by the mutated AR of LNCaP, which has increased affinity for estradiol [82]. In another study, proliferation of LNCaP cells was increased by estradiol but not DES, while the androgen-independent cell lines PC-3, 1-LN, and DU 145 were inhibited by DES treatment [83]. Estradiol caused dose-dependent inhibition of PC-3 proliferation in another study [84]. The novel estradiol analog 17alpha-20Z-21-[(4-amino)phenyl]-19-norpregna-1,3,5(10),20-tetraene-3,17beta-diol (APVE(2)) was shown to induce cell death in LNCaP, PC3, and DU145 prostate cancer cell lines [85]. Raloxifene, a mixed estrogen agonist/antagonist, was reported to cause apoptosis in androgen-dependent LNCaP cells as well as androgen-independent lines PC3, PC3M, and DU145 in androgen-free environments *in vitro* [86,87].

3.2. Effects on Prostate Cancer in Vivo

In keeping with early clinical results, administration of estrogenic compounds resulted in inhibition of prostate cancer in preclinical settings. Ellis *et al* reported that growth of the LuCaP 23.1 prostate cancer xenograft was inhibited in intact *vs.* ovariectomized female mice [88]. Therefore we set out to determine whether this effect could be observed with other prostate cancer xenografts and whether estradiol was responsible for the observed inhibition. Our results showed that growth of four different prostate cancer xenografts was inhibited in intact female mice *vs*. ovariectomized female mice. Estradiol supplementation to ovariectomized female mice resulted in growth inhibition similar to the inhibition in intact female mice [89]. Thus estradiol inhibits the growth of prostate cancer in an androgen-depleted environment. Moreover, we have recently shown that administration of estradiol or DES inhibits growth of androgen-independent

prostate cancer in castrated male mice as well. cDNA array analysis of the treated *vs.* untreated tumor*s* showed that multiple mechanisms were involved [90].

Multiple SERMs were also tested in preclinical settings to evaluate their effects on prostate and prostate cancer. Tamoxifen inhibited R3327 Dunning rat prostate cancer and Nb-2Pr-A tumors in Noble rats [91,92]. Raloxifene treatment of intact male rats resulted in regression of the ventral prostate and seminal vesicles [93]. In probasin/SV40 T antigen transgenic rats, raloxifene delayed prostate cancer development [94] and inhibited the growth of both androgen-sensitive and androgen-independent variants of the CWR22 CaP xenograft [95]. In contrast, raloxifene did not inhibit growth of PAIII rat prostate cancer model subcutaneous tumors. However, this treatment inhibited PAIII metastasis to the gluteal and iliac lymph nodes and lungs, and increased the survival of PAIII-bearing rats [96]. Trioxifene, another SERM, was shown to possess similar activities, inhibiting PAIII prostate cancer metastasis to the gluteal and iliac lymph nodes and lungs [97]. Toremifene treatment resulted in decreased premalignant and malignant lesions of the prostate in TRAMP transgenic mice [98].

3.3. Effects on Normal Prostate in Vivo

Despite the hypothesized tumor-supressor role of ERβ in prostate cancer and the observed inhibition of prostate cancer by estrogenic compounds, estrogens have also been implicated in stimulation of prostate proliferation and carcinogenesis. Estradiol increased growth of the prostate in castrated dogs [99] and caused stromal hyper-proliferation in other animal models. DES stimulated proliferation of prostate basal epithelium in mice [37,100]. A combination of estradiol and testosterone caused development of prostatic carcinomas in mice [101]. Exposure to high doses of androgen and estradiol together induced prostate growth, epithelial metaplasia, stromal hypertrophy, and a strong inflammatory reaction in stroma [100]. Even stronger enhancement of induction of prostatic dysplasia and carcinoma in Noble rats was observed by combined neonatal estradiol exposure and estradiol and testosterone treatment in adulthood [102]. For a review of the role of estrogens in development of prostate cancer see [103].

Implication of estradiol in development of prostate cancer is also supported by epidemiological studies. African Americans have higher incidence of prostate cancer *vs.* Caucasian American males and they also have higher levels of serum estrogens [104,105], while Japanese male have lower incidence of prostate cancer and lower levels of serum estrogens [104].

The differences in the effects of estradiol on cancer and normal prostate may be due at least in part to the presence of different subtypes of ER in stroma (ERα) *vs.* luminal epithelium (ERβ). The combination of estradiol and androgen was carcinogenic in normal mice, while only hyperplasia was observed in ERα-knockout mice [101]. Neonatal exposure to DES exerted detrimental effects on the reproductive tract *via* ERα [106]. The connection between ERβ and suppression of prostate-epithelium proliferation is supported by findings in ERβ-knockout mice (BERKO), which develop prostatic hypertrophy with aging [107]. Increased proliferation of prostatic epithelium in BERKO mice *vs*. wild-type mice was also observed [75,108], although conflicting results were reported in an earlier study [107]. Loss of ERβ expression was reported to be a common step in estrogen-dependent tumor progression [109].

4. New Era of Estrogen Use in Prostate Cancer

4.1. Transdermal Estradiol

Interest in the potential benefits of estrogen treatment in prostate cancer has recently revived. Cardiovascular side effects in prostate cancer patients associated with oral administration of estrogens result from hepatic metabolism of these compounds. The use of transdermal estrogen was shown to reduce or even negate the cardiovascular effects of oral estrogens [110]. In 1999 Henriksson *et al* tested the clinical performance of a new pharmaco-kinetically guided dosing regimen of parenteral estrogen in patients with advanced prostatic carcinoma. The aim was to accelerate endocrine effects while avoiding cardiovascular side effects [111]. Clinical effects of polyestradiol phosphate (PEP, Estradurin) administration were similar to orchiectomy, testosterone levels in serum were decreased by PEP, and no signs of increased cardiovascular morbidity were seen. Hedlund *et al* studied the effects of high doses of PEP on metastatic prostate cancer [112]. The effects of PEP in this trial were similar to those of flutamide in combination with triptorelin or bilateral orchiectomy; no differences were observed in time to biochemical or clinical recurrence or overall disease survival. Importantly no increase in cardiovascular mortality was associated with PEP administration. These results suggest that PEP has anticancer properties that are similar to those of other means of chemical androgen suppression without increased cardiovascular mortality.

The use of transdermal estradiol patches in prostate cancer was evaluated in a number of recent studies [113-118]. Transdermal administration of estradiol to patients with prostate cancer resulted in castrate levels of testosterone, and therapeutic responses equivalent to those

achieved with LHRH agonists were observed. Cardiovascular toxicity and other side effects were much less frequent than commonly seen with oral estrogens. Transdermal estradiol prevented andropause symptoms, improved quality-of-life scores, and increased bone density [113]. In another study prostate cancer patients progressing after primary hormonal therapy received transdermal estradiol; this treatment was well tolerated and produced a modest response rate, but was not associated with thromboembolic complications or clinically important changes in several coagulation factors [114]. Transdermal estrogen in low-dose (0.05 mg) and high-dose (0.1 mg) patches caused significant reductions in the overall severity of hot flashes, with reports of some mild side effects [115]. Transdermal estradiol administration increased bone mineral density, protected against thrombosis, increased arterial but not venous flow, and caused an initial decrease in arterial compliance in prostate cancer patients [113,116-118]. Moreover, short-term transdermal estradiol in comparison with standard androgen deprivation has been shown to improve lipid levels without deterioration of cardiovascular disease-associated inflammatory markers and may, on longer-term follow-up, ameliorate cardiovascular disease and improve mortality rates [119]. Results of these studies suggest that transdermal estradiol is a safe alternative means of administering estrogens. The androgen-suppressive effect of transdermal estradiol is approximately equivalent to that of current androgen-deprivation therapies, and in addition it alleviates some of the side effects associated with androgen withdrawal and provides significant economic advantages.

4.2. SERMs

The discovery of the second estrogen receptor and other advances in biomedical research led to the synthesis and use of selective estrogen receptor modulators (SERMs). These compounds bind differentially to ERα and ERβ and consequently exhibit tissue-specific effects [35,74,120-123]. Since ER is expressed in prostate cancer, certain SERMs have been evaluated in treatment of prostate cancer. Clinical trials with the SERMs tamoxifen and toremifene resulted in only weak positive effects on hormone-refractory prostate cancer. High doses of tamoxifen in 30 patients who had previously failed hormone therapy yielded only one partial response in PSA, while stabilization of the disease was observed in six patients. The tamoxifen treatment was well tolerated [124]. Toremifene, a derivative of tamoxifen that binds both estrogen receptors and exhibits antagonistic activities in breast cancer and agonistic effects in uterus, liver, and bone [125], was used in a Phase-II clinical study. The drug was well tolerated and toxicity was mild; however no objective responses were observed [126]. The overall conclusion from these trials was that these antiestrogens used in breast cancer treatment did not produce significant

objective responses in advanced hormone-refractory prostate cancer. In contrast to these results toremifene treatment caused a significant decrease in high-grade prostatic neoplasia (72% of 18 men), and the quality of life was not significantly affected by treatment [127]. In recent studies raloxifene and toremifene were shown to have promise in treatment of side effects associated with androgen deprivation [128,129] (see below).

4.3. Other Agents with Estrogenic Activities in Prostate Cancer

Other agents which possess estrogenic activities have also been evaluated for effects on prostate cancer, including estramustine, the herbal mixture PC-SPES, and phytoestrogens. Estramustine phosphate (EMP), is a steroid-derived compound used in treatment of advanced prostate cancer. EMP inhibits microtubule formation and exhibits estrogenic properties; however estramustine's mechanism of action against cancer is not well understood. Estramustine is currently being evaluated in combination with other chemotherapeutics [130-141]. PC-SPES, until recently was one of the most popular "alternative" estrogenic substances for treatment of prostate cancer. PC-SPES is a mixture of eight Chinese herbs with estrogenic activity, and it exhibits a number of inhibitory effects on prostate cancer *in vitro* and *in vivo* [142-146] (for reviews see [147-151]). Preliminary analysis of a clinical trial of PC-SPES and DES in patients with androgen-independent prostate cancer showed a decline in PSA of at least 50% in 40% of the patients receiving PC-SPES and in 24% treated with DES, with a median time to progression of 5.5 months in the PC-SPES group and 2.9 months in the DES group. Unfortunately this trial was closed prematurely due to withdrawal of PC-SPES from the market because contamination by DES and estradiol was detected in the PC-SPES preparation [152,153]. There is also a large body of literature documenting the effects of phytoestrogens on prostate cancer (for reviews see [154-158]).

5. Beneficial Effects of Estrogen on Side Effects Associated with Prostate Cancer Treatment

5.1. Effects on Bone

Osteoporosis was long considered to be dependent on androgens in men and estrogens in women. Early postmenopausal bone loss (type I osteoporosis) is caused by estrogen deficiency, and it has recently been shown that late postmenopausal bone loss (type II osteoporosis) may have the same cause. However, the etiology of bone loss in aging men is still unclear. Literature studies indicate that estrogens have much greater effects on bone mass than androgens [159-161]. A recent study indicated normal levels of testosterone but low levels of

estrogens in young men with osteoporosis, suggesting that estrogens play a role in osteoporosis in males [161]. Moreover evidence from a male deficient in ERα suggested the importance of estrogen in male bone metabolism, and several large epidemiologic studies have found that bone mineral density correlates better with serum estrogen than testosterone in aging men. Thus estrogen deficiency may lead to bone loss in men.

Advances in treatment of recurrent advanced prostate cancer have resulted in increasing numbers of patients that live long enough to experience complications from cancer treatment. Cancer treatment-induced bone lost (CTIBL) is a long-term treatment-associated complication of cancer therapies that directly or indirectly affect bone metabolism [162]. CTBIL is common in prostate cancer patients who receive chemical androgen deprivation or surgical castration, as these cause hypogonadism and increased bone turnover, significant bone loss, and increased risk of fractures [163]. Several studies have indicated that the incidence of fractures is higher in patients with prostate cancer on androgen deprivation compared to the general population [128,162-166]. Published results indicate that estrogen deficiency is a major cause of the increased fracture risk and decreased bone mineral density in these patients [167]. Transdermal estradiol therapy increases bone mineral density [113,118], and in a small study SERMs (raloxifene and toremifene) also increased bone mineral density in GnRH-treated patients [167]. Two recent studies showed beneficial effects of SERM treatment on bone health in prostate cancer patients undergoing androgen deprivation. Administration of raloxifene significantly increased bone mineral density of the hip and tended to increase bone mineral density of the spine in patients with non-metastatic prostate cancer [128], while toremifene caused suppression of bone turnover and increased bone mineral density [129].

5.2. Effects on Cognitive Function

Several recent studies suggest that both estrogens and androgens play important roles in modulation of mood and cognitive function [168-172]. Effects of gonadal steroids on memory have been reviewed recently [173]. Men with hypogonadism suffer decreases in cognitive function, while studies of androgen-replacement therapy suggest beneficial effects of replacement on cognitive functions. However, most studies to date have been small and require replication with greater numbers of patients to determine the clinical significance of these findings [173]. The effects of androgen deprivation on memory in prostate cancer patients have not been evaluated in detail. Recently Almeida *et al*. evaluated memory in patients with prostate cancer who had undergone chemical castration. Androgen-deprivation therapy was associated

with increased depression and anxiety scores, while disruption of the treatment resulted in better cognitive performance [174]. Immediate and delayed verbal memories were significantly worse in prostate cancer patients on androgen deprivation than in matched controls [175]. In the same study estradiol improved verbal memory performance in prostate cancer patients, suggesting that estradiol has the potential to reverse the neurotoxic effects on memory caused by androgen deprivation [175]. However, in another study, short-term estradiol administration did not result in any positive effects on memory in men on androgen deprivation *via* LHRH [176].

5.3 Effects on Immunity

Substantial evidence suggests that estrogens enhance immunity. The incidence of autoimmune disease was found to be 3-10 fold higher in women in comparison with men, and these increases are believed to be related to hormonal modulation of immunity [177]. This hypothesis is supported by the observation that physiological levels of estradiol are immunostimulatory, while testosterone at all concentrations suppresses immune responses. In animal models estradiol restored normal immune function which had been suppressed by trauma [178-180]. Moreover, allogeneic rejection of transplanted organs is substantially higher in intact female animals *vs*. ovariectomized female mice, and administration of estradiol to ovariectomized mice results in a much greater frequency of organ rejection [181]. The mechanisms whereby estrogens might mediate augmented immunity include increased T-cell and B-cell reactivity, augmented immunoglobulin synthesis, and upregulated generation of inflammatory cytokines [182]. Effects of estradiol on the immune response in prostate have also been reported. Administration of estradiol to male animals induced immune effector infiltration of the prostate [183]. Estradiol also induced production of inflammatory cytokines in the prostate prior to initiation of the inflammatory response [184].

The immune response of the body to tumor cells involves major histocompatibility class I molecules, which are expressed on most human cells; however some tumors express reduced levels of MHC class I in a possible mechanism of escape from immune surveillance [185-189]. We have recently found that estradiol treatment of androgen–independent prostate cancer xenografts increases expression of immune-related messages. These included MHC class I and II molecules as well as a number of interferon-regulated messages [90]. MHC class I and II molecules are also modulated by IFNγ [187,188,190]. According to this evidence, estradiol treatment enhances the immune response, and exerts direct effects on tumor cells as well, potentially resulting in decreased proliferation as well as enhanced killing of the tumor cells by

immune system. Therefore, the effects of estradiol treatment on the immune system in prostate cancer patients should be evaluated in sufficient detail to assess these potentially synergistic mechanisms.

6. Summary

A foe:

Estradiol has been implicated in carcinogenesis of the prostate. Oral estrogen treatment has also been associated with significant adverse side effects, including cardiovascular mortality.

A friend:

Estrogens have long been known to decrease the growth of prostate cancer *via* suppression of the hypothalamo-testis-androgen axis. Moreover, newer evidence from *in vitro* and preclinical studies of estradiol and SERMs indicates that estrogens may be effective even against androgen-independent cancer. Oral use of estrogen has been associated with significant negative effects, but parenteral estrogens ameliorate these effects and are a cost-effective alternative to androgen deprivation by GnRH. Finally, new data show that concurrent treatment with estradiol or other estrogenic compounds can minimize some of the side effects associated with standard androgen ablation and chemotherapy. The benefits include protective effects on bone, improvement of memory, and stimulation of the immune system.

A full description of the effects of estrogens on prostate cancer will await more definitive studies; however, new research supports the existence of direct inhibitory effects of estrogen on prostate cancer and the benefits of estrogen treatment even in cases of advanced disease. Additional evaluation and consideration of estrogen use in advanced prostate cancer are needed.

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Inhibition of Androgen-Independent Prostate Cancer by Estrogenic Compounds is Associated with the Increased Expression of Immune-Related Genes

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Abbreviations: CaP, prostate cancer; DES, diethylstilbesterol; PSA, prostate-specific antigen; SERM, selective estrogen receptor modulator; ERβ, estrogen receptor beta; E2, 17β-estradiol; SC, subcutaneous, SEM, standard error of mean; BrdU, 5-bromo-2-deoxyuridine; EGP, epithelial glycoprotein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMBS, hydroxymethylbilane synthase; HPRT1, hypoxanthine phosphoribosyltransferase 1; PSMB6, proteasome (prosome, macropain) subunit, beta type, 6; AR, androgen receptor; IFN, interferon;

Abstract

The clinical utility of estrogens for treating prostate cancer (CaP) was established in the 1940s by Huggins. The classical model of the anti-CaP activity of estrogens postulates an indirect mechanism involving the suppression of androgen production. However, clinical and preclinical observations present a challenge to this model. Collectively, these studies have shown that estrogenic compounds exert growth-inhibitory effects on CaP under low-androgen (castrate) conditions, suggesting additional modes whereby estrogens affect CaP cells and/or the microenvironment. Here we have investigated the activity of 17β estradiol (E2) toward androgen-independent CaP and identified molecular alterations in tumors exposed to E2 that indicate mechanisms of estrogen-mediated tumor inhibition. E2 treatment inhibited the growth of all four androgen-independent CaP xenografts studied (LuCaP 35V, LuCaP 23.1AI, LuCaP 49 and LuCaP 58) in castrated male mice. The molecular basis of growth suppression was studied by cDNA microarray analysis to identify alterations in gene expression after E2 treatment. Of particular interest are changes in transcripts encoding proteins that mediate the immune response (MHC class I and II genes, interferon-regulated factors). Our data show that estrogens have powerful growth-inhibitory effects on CaP *in vivo* in androgen-depleted environment, and identify novel mechanisms of estrogen-mediated anti-tumor activity. These results suggest that incorporating estrogens into CaP treatment protocols could enhance therapeutic efficacy even in cases of advanced disease.

Introduction

Despite substantial attention, the development of androgen-independent prostate cancer (CaP) is not well understood. Progression to the androgen-independent state represents resistance to suppression of the primary signaling pathway used to control recurrent CaP. Accordingly, evaluation of activities and mechanisms of new therapeutics that specifically target androgen-independent CaP growth are of special therapeutic interest.

Estrogens, particularly diethylstilbesterol (DES), were commonly used as initial treatment of advanced CaP for some 30 years [1-6]. Originally, it was believed that responses of CaP to estrogen therapy were mediated primarily *via* suppression of the hypothalamic-hypophyseal axis and the consequent reduction in testosterone levels [7-10]. However DES treatment was associated with significant side effects and the Veterans Administration Cooperative Urological Research Group (VACURG) (1967) recommended that hormonal therapy with DES be withheld until symptoms of metastatic disease appeared, and that administration of DES at levels of 5 mg/day was associated with excessive risk of cardiovascular mortality [11,12]. In a further study, VACURGII compared various dosages of DES and concluded that 1 mg /day is as effective as 5 mg /day in controlling T3 M+ CaP [13]. In 1988, however, even this level of DES was found to be associated with a high risk of cardiovascular problems, mainly in patients over 75 years of age [14]. The use of DES in treatment of CaP ended with the advent of luteinizing hormone releasing hormone analogs, which are now mainly used as a means of chemical castration.

Nevertheless, published work suggests that estrogens can inhibit the growth of CaP by mechanisms unrelated to androgen suppression; patients treated with estrogen appeared to survive somewhat longer than patients who had undergone surgical castration [3], administration of DES to patients with hormone–independent CaP suppressed PSA and prolonged survival more

effectively when compared with the anti-androgen flutamide [15], and Byar *et al.* [4] commented that no form of endocrine therapy had been proven superior to 1 mg of DES daily. The hypothesis of direct inhibitory effects of estrogen on CaP is supported by the observations that estrogen receptors are expressed in normal and neoplastic prostate epithelium [16-18], estrogens exhibit direct cytotoxic effects on CaP cells *in vitro* [19-23], and our own demonstration of growth inhibition of CaP by 17β estradiol (E2) in the androgen-free environment of ovariectomized female mice [24].

The discovery of a second estrogen receptor, ERβ, renewed interest in basic research involving estrogen pathways. Several reports have shown that ERβ is present in normal prostate epithelial cells as well as CaP, and the levels of ERβ message and/or protein appear to be down-regulated during disease progression [16-18,25]. A straightforward hypothesis holds that ERβ transduces a growth-inhibitory effect of estrogen on CaP cells. In support of this hypothesis, a lower rate of cancer-related death was observed in patients with CaP expressing ERβ *vs.* patients without ERβ [26], and an estrogenic compound operating *via* the ERβ receptor suppressed growth of DU 145 CaP cells [22,23]. In contrast to decreasing levels of ERβ with CaP progression, we have recently demonstrated that ERβ is expressed in a majority of CaP bone and soft tissue metastases [27], similar to another report on ERβ expression in a small number of CaP metastases [16]. Together, these studies suggest that estrogen action against prostate carcinoma could involve ERβ or potentially other direct modes of action such that prostate cancer growth may be restrained even in the androgen-independent state.

The current study was undertaken to determine whether estrogenic compounds can inhibit growth of androgen-independent CaP and to investigate phenotypic changes associated with the anti-tumor effects. Our results using human prostate cancer xenografts show that estrogenic

compounds clearly suppress androgen-independent growth of CaP in castrated hosts, calling into question the traditional view that estrogen's activity against CaP depends solely on androgen suppression. The results indicate that estrogens may be especially useful in treatment of androgen-independent CaP. We identified several novel molecular alterations resulting from tumor exposure to E2 that we hypothesize may contribute to E2-mediated tumor inhibition. Further studies are warranted to exploit the anti-tumor effects of E2 treatment in the context of advanced CaP.

Materials and Methods

Animal Studies

Xenografts: Androgen-sensitive, PSA-producing CaP xenografts LuCaP 35 [28], LuCaP 23.1 [29,30] and LuCaP 58 [31] (all originated from lymph node metastases), and androgen-insensitive neuroendocrine-type CaP xenograft LuCaP 49 (originated from an omental fat metastasis) [32] were used. The xenografts are maintained and propagated in Balb/c nu/nu intact male mice. The androgen-independent variants of LuCaP 35V and LuCaP 23.1 were developed from parental tumors upon re-growth after castration [28,31] and are maintained and propagated in castrated B17 Fox Chase SCID male mice (Charles River, Wilmington, MA).

Effects of E2 on Recurrent LuCaP 35 after Castration*:* All animal procedures were performed in compliance with the University of Washington Institutional Animal Care and Use Committee and NIH guidelines. In our first study LuCaP 35 tissue bits were implanted subcutaneously into SCID male mice. Tumor growth was monitored by measuring tumor volume twice a week. Serum was collected weekly for PSA determination. Animals were castrated when the tumors reached 200-400 mm^3. Animal with recurrent tumors (determined as two rising serum PSA values) were randomized into three groups of 10 animals each. Group-1 animals received placebo pellets.

Group-2 animals were supplemented with 17β-estradiol (E2) by SC implantation of slow-release Trocar pellets (E2 90-day release 100-125pg/ml, Innovative Research of America¸ Sarasota, FL), and group-3 animals were supplemented with DES pellets by SC implantation of slow-release Trocar pellets (DES 90-day release 0.01 mg pellet, Innovative Research of America). Animals were sacrificed when tumors exceeded 1000 mm^3, 90 days post-pellet implantation, or when the animals became compromised. Student's unpaired two-tailed t-test was used to analyze the differences between the groups.

***Effects of E2 on LuCaP 35V in Castrated Male Mice*:** In additional experiments to determine effects of E2 on proliferation and gene expression, we used the androgen–independent xenograft LuCaP 35V [28]. SCID male mice were castrated at 8 weeks of age and implanted with LuCaP 35V tumor bits at least two weeks after surgery. Tumor growth was monitored by tumor measurements twice a week using calipers, and tumor volume was calculated as LxHxWx 0.5236. Blood samples were collected weekly for determination of serum PSA levels (IMx Total PSA Assay, Abbott Laboratories, Abbott Park, IL). When tumors reached 200-400 mm^3 the animals were randomized into two groups. Group 1 was supplemented with E2 by SC implantation of slow-release Trocar pellets (60-day release 0.05 mg pellet, Innovative Research of America, Saratoga, FL). Group 2 was a control group, which received placebo pellets. Five animals from each group were sacrificed at days 1, 3, and 7 post-implantation of E2 pellets. One hour prior to sacrifice animals were injected intra-peritoneally with 80 mg/kg body weight BrdU (5-bromo-2-deoxyuridine, Sigma-Aldrich Co., St. Louis, MO) for evaluation of tumor cell proliferation. Tumors were fixed in formalin and embedded in paraffin. The ten remaining animals in each group were monitored for long-term assessment of tumor growth and PSA production after E2 treatment. Animals were sacrificed when tumors exceeded 1000 mm^3, 60 days post-pellet implantation, or when the animals became compromised. Tumors were frozen in liquid nitrogen

and stored at -80^{o} C and/or fixed with formalin and embedded in paraffin, and serum was collected for determinations of E2 levels (IMx estradiol immunoassay, Abbott Laboratories). Student's unpaired two-tailed t-test was used to analyze the differences between the groups, and the Log-rank test was used to evaluate differences in survival.

Effects of E2 on Growth of LuCaP 23.1 AI, LuCaP 49 and LuCaP 58 in Castrated Male Mice: To investigate whether the E2 inhibition of androgen–independent growth occurs with other CaP cells, not just LuCaP 35 lines, we set up similar experiments with three additional xenografts; LuCaP 35AI, LuCaP 49, and LuCaP 58. The experimental design was the same as for the study with LuCaP 35V. Tumor bits were implanted in castrated male mice (aiming for n=10 per group) at least two weeks after surgery, and tumor growth and PSA levels were monitored. Animals bearing each particular xenograft were randomized into two groups (tumors 200-400 mm^3). Group 1 was supplemented with E2 by SC implantation of slow-release Trocar pellets (60-day release 0.05 mg pellet, Innovative Research of America, Saratoga, FL). Group 2 was a control group, which received placebo pellets. Animals were sacrificed when tumors exceeded 1000 mm^3, 60 days post-pellet implantation, or when the animals became compromised. Tumors were frozen in liquid nitrogen and stored at -80^{o} C and/or fixed with formalin and embedded in paraffin. Student's unpaired two-tailed t-test was used to analyze the differences between the groups.

Proliferation and Apoptosis Assays

Samples of LuCaP 35V tumors treated with E2 for 1, 3, and 7 days and control tumors were fixed in formalin and embedded in paraffin. An anti-BrdU immunohistochemistry kit was used to assess the number of proliferating cells (Zymed, San Francisco, CA). Five-μm sections of paraffin-embedded tissues were used for the analysis as recommended by the manufacturer.

Apoptosis in tumors was assessed with a FragEL DNA fragmentation detection kit from Oncogene (La Jolla, CA) as recommended by the manufacturer. Positive nuclei or apoptotic cells were counted in five representative fields containing ~1000 cells, in three samples of treated and untreated tumors from each time point. Statistical analysis was performed using Student's t-test.

Cell Culture

Seven to nine hundred mm^3 LuCaP 35V tumors grown and passaged in castrated SCID mice were harvested for isolation of epithelial cells [28]. Isolated cells were rinsed three times and plated in 10% charcoal-stripped fetal bovine serum (Hyclone, Logan, UT) in phenol red-free RPMI-1640 (Invitrogen, Carlsbad, CA) overnight. LuCaP 35V cells were treated with 10^{-8}M E2 or vehicle (0.01 % EtOH) for 4 hours.

Western Blot Analysis

Following treatment with E2 or vehicle, nuclear and cytoplasmic fractions were prepared as previously published [33]. Proteins (25 μg/well) were separated on 12.5 % SDS-PAGE and transferred to PVDF membranes. Blots were blocked in a 1:1 solution of NaP-Sure blocker (Geno Technology Inc., St. Louis, MO) and Tris buffered saline + 0.1% Tween-20 (TBS-T) for 2 hrs, then probed with a rabbit polyclonal antibody against ER-β (Affinity BioReagents, Golden, CO) for 1 hour at room temperature. ER-β immunoreactivity was detected using a goat anti-rabbit secondary antibody conjugated with HRP (1:2000, Amersham, Piscataway, NJ). Blots were developed using the Amersham ECL.

EMSA

Nuclear extracts from LuCaP 35V treated with 10^{-8}M E2 or vehicle (0.01 % EtOH) for 4 hours (25 μg) were incubated with 50 fmol of dsDNA probes for 30 minutes at 37 °C in a buffer

containing: 20 mM Tris, pH 8, 10 mM NaCl, 3 mM EDTA, 0.05% Nonidet P-40, 2 mM DTT, 4% glycerol, 1 mM $MgCl_2$, 1μg poly dI-dC (Amersham). The binding consensus sequences used were the estrogen response element (ERE, GGATCTAGGTCACTGTGACCCCGGATC) and a mutated form of ERE (GGATCTAGTACACTGTGACCCCGGATC, Santa Cruz Biotechnology, Inc, Santa Cruz, CA). The double-stranded DNAs were end-labeled with [γ-^{32}P]ATP (Amersham) using T4 polynucleotide kinase (Promega, Madison, WI). For competition studies, 50 fmol unlabeled probe was added to the reaction. The protein-DNA complexes were separated in 4% non-denaturing polyacrylamide gels.

RNA Isolation

Tumors from animals treated with E2 for 60 days and control tumors were homogenized using an Omni TH homogenizer (Omni International, Warrenton, VA), and RNA was extracted using the TriPure Isolation Reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions. RNA quantity was determined based on A_{260}, and integrity of the RNA was confirmed by agarose gel.

cDNA Array Analysis

PEDB cDNA microarrays containing ~7,000 human prostate-derived cDNA clones were prepared on poly-l-lysine-coated glass microscope slides using a robotic spotting tool as previously described [34-36]. Equal amounts of total RNA from five tumors of LuCaP 35V (control), and E2-treated LuCaP 35V (treatment) were pooled and cDNA array experiments and analysis performed as previously described [37]. For individual experiments, every cDNA was represented twice on each slide, and the experiments were performed in triplicate with a switch in fluorescent labels to account for dye effects, producing six data points per cDNA clone per hybridization probe. Data were filtered to exclude poor quality spots, normalized, and include

clones whose expression was measurable in at least two out of the three arrays, reducing the initial list of 6720 clones to 5163 clones.

Gene Expression Analysis. To compare the overall expression patterns of the replicate LuCaP 35V (control), and E2-treated LuCaP 35V (treatment) arrays, $\log_2$ ratio measurements were analyzed using the SAM procedure [38] (http://www-stat.stanford.edu/_tibs/SAM/). A one-sample t-test was used to determine whether the mean gene expression of E2-treated LuCaP 35V vs. LuCaP 35V (control) differed significantly from zero. An FDR (false discovery rate) of less than 1% was considered significant. Clones differentially expressed with an FDR < 1% were stratified based on fold-change, and we chose to further evaluate only those with an average $\log_2$ (E2-treated/control) >0.58 or <-0.58, corresponding to a differential expression effect of 1.5-fold or greater. We assigned differentially-expressed genes to the following functional categories based on their annotations in the Gene Ontology database [39]: metabolism, immune/inflammatory response, proliferation/differentiation/apoptosis, signal transduction, structural/adhesion/motility, transcription regulation, translation-protein synthesis, transport, or other/unknown.

To determine whether phenotypic changes observed in the E2-treated tumors were enriched for genes in certain pathways, the cDNA array results were subjected to Gene Set Enrichment Analysis (GSEA) [40]. For this analysis, interferon-regulated, androgen-regulated, and estrogen-regulated gene sets were tested against our data. Interferon-regulated and estrogen-regulated gene sets were generated from SuperArray Bioscience Corporation GEArray pathway-focused gene lists (http://www.superarray.com), and the androgen-regulated gene set was generated based on results of DePrimo *et al.* [41]. To assess the statistical significance of the enrichment score (ES) observed in the data set for the three gene sets, we used permutation testing of the phenotype labels (*e.g.*, E2-treated vs. controls), generating a nominal (NOM) *p* value. A false discovery rate

(FDR) statistic was computed to adjust for gene set size and multiple hypothesis testing, with an FDR of less than 25% considered significant.

Quantitative RT-PCR

***qRT-PCR*:** First-strand cDNA synthesis was performed with 1.0 μg of pooled RNA from five animals of the E2 and control groups using oligo-dT_{18} primers according to manufacturer's instructions (Clontech, Palo Alto, CA). Real-time PCR was carried out on cDNA samples using the Platinum Quantitative PCR SuperMix-UDG reagent (Invitrogen, Carlsbad, CA) and performed on the Rotor-Gene 2000 (Corbett Research, NSW, Australia). PCR primers were designed to span an intron-exon boundary and avoid amplification of any known pseudogenes. Primers for genes evaluated are listed in Table 1. Two μl of cDNA was used per reaction with 200 nM primers, 0.5X Sybr Green 1 (Molecular Probes, Eugene, OR), and 5.5 mM $MgCl_2$. The PCR reaction parameters were as follows: 50˚C for 2 min and 95˚C for 2 min (one cycle), followed by 35 cycles at 95˚C for 10 sec and annealing/extension at either 65˚C or 69˚C for 30 sec; the final extension was 72˚C for 7 min. PCR reaction products were confirmed by agarose gel electrophoresis. Standard curves for each amplicon were generated from a four-fold dilution series of LNCaP cDNA run in duplicate (all standard curves had r values >0.99). Reactions were carried out in duplicate and expression levels were calculated from a standard curve.

***Normalization Strategy*:** The normalization scheme applied to the real-time PCR results was based on the method of Vandesompele *et al*. [42]. This method employs multiple internal control genes to identify the most stably expressed control genes in the samples of interest. The following genes were evaluated for use as internal control genes: *epithelial glycoprotein (EGP*), *glyceraldehydes-3-phosphate dehydrogenase (GAPDH), hydroxymethylbilane synthase (HMBS), hypoxanthine phosphoribosyltransferase 1 (HPRT1), and proteasome (prosome, macropain) subunit, beta type, 6 (PSMB6)*. Real-time PCR on pooled samples was performed in duplicate and

expression levels were calculated based on the standard curves as above. The average expression levels were imported into the geNorm program (http://allserv.rug.ac.be/~jvdesomp/genorm/) to determine the two most stably expressed internal control genes. Briefly, geNorm determines the gene-stability measure, or *M* value, as the average pair-wise variation between a particular internal control gene and all other control genes. The stepwise exclusion of the endogeneous control genes with highest *M* values resulted in the selection of *GAPDH* and *EGP* as the most stably expressed control genes. Normalization of real-time PCR data of the gene of interest was accomplished by dividing the raw expression levels by the geometric mean of the most stable endogeneous control.

Results

Inhibition of Androgen Independent CaP by E2 and DES

LuCaP 35 is an androgen-sensitive CaP xenograft, expressing PSA and wild-type AR, which recapitulates a response to androgen ablation and development of androgen-independent CaP similar to that observed in man [28]. Its growth in intact female mice is suppressed in comparison to ovariectomized female mice [24]. Therefore we have chosen this xenograft for the initial evaluation of the effects of estrogenic compounds in male mice. Surgical castration of intact male mice bearing LuCaP 35 CaP xenografts resulted in a reproducible time-dependent reduction in tumor volume and PSA serum levels. Recapitulating human disease, 88% of the tumors eventually recurred in the androgen-depleted environment, with a range in time to recurrence of 32-91 days (median 61.5 days, Figures 1A and 1B). Tumor recurrence was defined as two consecutive rising values of serum PSA. Without treatment, these androgen-independent tumors continued to grow and reached a size of ~1000 mm^3 by day 24-31 post-castration. Administration of E2 or DES inhibited the growth of recurrent LuCaP 35 tumors; at 104 days after castration the tumor volumes were E2: 134.3 ± 16.4 mm^3 (mean ± SEM), with PSA levels 1.82 ± 0.66 ng /ml;

and DES: 49.8 ± 12.1 mm^3, with PSA levels 3.20 ± 1.86 ng/ml. Tumor volumes and PSA levels decreased, and none of the tumors reached an estrogen-resistant state during the course of the study (90 days of treatment). PSA values closely followed tumor volume. Three animals from the E2- and DES-treated groups were monitored for an additional 60 days after expiration of the estrogen pellets. Tumor volumes and PSA serum levels in these animals started to increase during this period (Figure 1). The tumors in animals that were treated with E2 reached 587.6 ± 194.0 mm^3 ($p=0.0008$ from 90 days after pellet expiration) with concordant rises in PSA serum levels to 55.33 ± 21.18 ($p=0.003$, to the levels when pellets expired). Tumors in DES-treated animals started to increase in volume more slowly than E2-treated tumors after pellet expiration; tumor volumes increased 1.5-fold (79.43 ± 32.5 mm^3) but did not reach significance ($p=0.3075$), and PSA serum levels began to rise (17.23 ± 11.20 ng/ml, $p=0.0533$). As observed in our previous study in female mice, administration of E2 inhibited the growth of androgen-independent LuCaP 35V xenografts in castrated male mice as well. The tumor volume of LuCaP 35V-bearing animals treated with E2 increased minimally over the original volume during the 60-day period of the treatment (Figure 2A). However, the tumor size of LuCaP 35V in the control group increased from the time of enrollment until the time of sacrifice (day 25-35, tumor volume $\geq$1000 mm^3, Figure 2A) (at day 32, $p<0.0001$). PSA serum levels closely paralleled the tumor volumes (at day 28 $p= 0.0021$) (Figure 2B). Levels of E2 in the control group of castrated animals with LuCaP 35V (untreated) were below the limit of assay detection (<25 pg/ml). Levels of E2 at the time of sacrifice (60 days post-implantation of E2 pellets) were 127.1 ± 22.5 pg/ml in treated LuCaP 35V animals. Survival analysis, using tumor size ($\geq$ 1000 mm^3) as a death criterion, showed that E2 dramatically prolonged the survival of LuCaP 35V-bearing animals as determined by Log-rank test, $p<0.0001$ (Figure 2C).

Generalized Growth Inhibitory Effects of E2 toward Androgen-Insensitive CaP

Growth of the three additional CaP xenografts LuCaP 23.1 AI, LuCaP 49, and LuCaP 58 in the androgen-free environment was inhibited by E2 administration to varying degrees (Figure 3.) The tumor volume of LuCaP 23.1 AI treated with E2 decreased, with significant differences from untreated tumors after 7 days of treatment ($p=0.00089$), resulting in near- disappearance of the tumors by day 35. PSA serum levels closely followed the tumor volume. LuCaP 58 growth was also inhibited by E2 treatment, but to a lower extent; the tumor volume increased minimally over the original volume during the 60-day period of the treatment (Figure 2A) reaching significant inhibition *vs.* untreated tumors at day 7 ($p=0.0137$). LuCaP 49, a neuroendocrine CaP xenograft in which the androgen receptor is absent, was also inhibited byE2 administration, but the pattern of the inhibition was different than with the other 3 xenografts. No significant inhibition was observed for first 10 days of treatment, after that significant inhibition was reached (14 days, $p=0.0289$). E2-treated LuCaP 49 tumors continued growing but at a slower rate than the untreated tumors.

Effects of E2 on Tumor Cell Proliferation and Apoptosis

To evaluate mechanisms mediating LuCaP 35V tumor reduction after E2 treatment, we measured incorporation of BrdU in untreated LuCaP 35V tumors compared with tumors from mice receiving E2 for 1, 3, and 7 days. The number of proliferating tumor cells decreased to $82.7 \pm 7.3\%$ of untreated tumors after 1 day (mean ± SEM), $65.7 \pm 4.2\%$ ($p=0.0063$) after 3 days, and $65.4 \pm 10.1\%$ ($p=0.0105$) after 7 days of E2 treatment (Figure 3). The rate of apoptosis in E2-treated and untreated tumors as measured by the TUNEL assay was not significantly different (data not shown).

Determination of E2-Mediated Alterations in Tumor Gene Expression by Microarray Analysis

Comparative analyses of cDNA microarray gene-expression profiles derived from LuCaP 35V xenografts treated with E2 compared to untreated controls identified 300 cDNAs whose expression levels were significantly associated with E2 treatment (False Discovery Rate <1%) and exhibited a difference in expression level of >1.5-fold. Consolidation of the redundant clones resulted in 233 unique genes, of which 129 were down-regulated and 104 up-regulated following E2 treatment (Tables 2 and 3). E2 treatment resulted in significant increases in the expression of several genes that are involved in immune-response (Table 2). These include MHC class I and MHC class II proteins, IFN-induced transmembrane protein 1 (IFITM1), IFN-induced transmembrane protein 3 (IFITM3), interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), interferon alpha-inducible protein 27 (IFI27), and interferon regulatory factor 1 (IRF1).

We have used GSEA to evaluate whether the phenotypic changes caused by E2 treatment in LuCaP 35V were associated with enrichment for interferon-, androgen-, and estrogen- regulated genes. Our analysis showed a significant enrichment of the interferon-regulated genes in the E2-treated LuCaP 35V tumors (NOM p-value < 0.001), which remained significant when adjusted for gene set size and multiple hypothesis testing (FDR=11.0%) (Figure 4A). A significant enrichment was also detected when the androgen deprivation down-regulated gene set was compared to our results (NOM *p*-value < 0.001); this enrichment also remained significant when adjusted for gene set size and multiple hypothesis testing (FDR=21.3%) (Figure 4 B.) Estrogen-regulated genes were also enriched in the phenotypic alterations after E2 treatment (NOM p-value <0.001), however these changes were not significant when adjusted for gene set size and multiple hypothesis testing (FDR = 54.5%). We hypothesize that this is due to the fact that changes in

expression of these genes occur in both up and down directions, as well as inclusion in the list of genes altered in breast cancer, which may not be relevant to this study (Figure 4C).

ERβ Localization and DNA Binding

ERβ (55kd) was detected by Western blot in nuclear extract but not in cytoplasm from LuCaP 35V and E2-treated LuCaP 35V (Figure 5A). E2 treatment increased levels of ERβ in the nucleus by approximately 30%. Using EMSA we showed that ERβ in the nucleus is able to bind to DNA. E2 treatment slightly increased levels of ERβ/DNA complexes (Figure 5B). Specificity of the interaction was demonstrated by disappearance of the specific band in control reactions with mutated xERE.

Determination of E2-Mediated Alterations in Tumor Gene Expression by quantitative RT-PCR

We performed quantitative RT-PCR (qRT-PCR) analysis to confirm the cDNA microarray results for selected genes of potential biological importance. All messages whose expression was determined to be up-regulated by cDNA array analysis were also increased by qRT-PCR in E2-treated LuCaP 35V (Figure 6A). We next examined whether the immune-response related genes found to be upregulated by E2 treatment of LuCaP 35V xenografts were also altered by E2 treatment in the other CaP xenografts. In LuCaP 58 the patterns of E2 alteration of expression of these genes was similar to those in LuCaP 35V. In contrast, in LuCaP 49, a neuroendocrine CaP xenograft whose growth suppression was less pronounced, the expression of the evaluated genes was minimally altered (Figure 6.). LuCaP 23.1 regressed almost completely after E2 treatment, and unfortunately there was insufficient tissue remaining for analysis. Gene expression changes in LuCaP 35 tumors treated with E2 or DES after castration were also evaluated. We found that the expression of genes related to immune-regulation was altered by E2 and DES treatment, as in

LuCaP 35V tumors. We continued to examine tumor gene expression levels after the expiration of the E2 pellets and found that levels of the E2-induced messages decreased, indicating dependence on the presence of E2 (Figure 7).

Discussion

Several studies dating to the 1980s have suggested that mechanisms other than androgen suppression may be involved in the estrogen-mediated inhibition of CaP growth. Estrogens appear to be slightly more effective in treating CaP than other means of androgen suppression [4]. Compounds with estrogenic activity are capable of exerting direct cytotoxic effects on androgen-independent CaP cells *in vitro* [19-23]. Our data obtained in the androgen-deficient environment of the female mouse [24] and in the present work show that estrogens have powerful growth-inhibitory effects on CaP *in vivo*.

In the present study we have shown that E2 and DES both inhibit the growth of androgen-independent CaP tumors in the androgen-depleted environment of castrated male mice. These data clearly demonstrate that E2 exhibits effects on CaP cells that are unrelated to suppression of the hypothalamic-hypophyseal axis and the subsequent decrease in testosterone. This novel observation prompted us to characterize the effects of E2 on androgen–independent CaP at the molecular level by profiling transcript alterations. While many of the genes differentially regulated by estrogen in this system are of unclear significance, others have quite plausible roles for contributing to the observed growth inhibition on the basis of their established functions. Among these are genes involved in signal transduction, cellular metabolism, and the control of transcription and translation. We also observed substantial changes in genes that function to regulate immune-responses; a mechanism that may contribute to the tumor growth inhibitory effects resulting from estrogen treatment.

Among the immune-response-related genes altered by E2 treatment in CaP are those modulating cellular responses to interferons. This group was found to be significantly enriched in the set of genes up-regulated by E2 when tested by GSEA using an independently generated list of interferon-regulated genes. The increased expression of interferon–regulated genes is of particular interest due to the direct anti-tumor activities reported for these cytokines [43-50]. Our results are in keeping with the results on up-regulation of IFN-regulated genes in LNCaP CaP cells following exposure to the estrogenic herbal preparation PC-SPES [51], and induction of IFNγ-regulated genes after E2 treatment in other tissues [52]. In addition tamoxifen has been shown to enhance interferon-regulated gene expression in breast cancer cells [53]. Specifically, IRF1, whose expression was increased 3-fold by E2 (qRT-PCR data), has been described as a negative regulator of proliferation [54] and exhibits tumor-suppressor activities in breast cancer cells [55]. These published observations and our results are consistent with a model in which IFN and genes regulated by IFN modulate a component of the growth inhibitory activity of E2 toward androgen-independent CaP cells.

E2 treatment significantly increased the expression of several MHC class I and II transcripts in the androgen-independent LuCaP 35V xenograft. Similarly, up-regulation of MHC class I transcripts has been observed in LNCaP cells upon PC-SPES exposure [51]. MHC class I molecules are expressed on most human cells and play a pivotal role in the immune response to viruses and tumor cells. Tumor cells often evolve mechanisms to modulate or escape immune surveillance through down-regulation of MHC class I molecules [56-60]. IFNγ treatment, like E2 treatment in our studies, has been reported to up-regulate the expression of MHC class I and II molecules in CaP cell lines [44,58,59]. According to this evidence, treatment of advanced CaP patients with E2 might result not only in direct inhibitory effects, but also in stimulation of T-cell

attack on the tumors by up-regulation of MHC proteins. Such a mechanism could not be directly tested in our studies which employed immune compromised SCID mice, but it represents an independent potential benefit of E2 treatment that could be exploited in the context of clinical therapies that employ vaccine or other immunomodulatory treatment strategies.

DES has been reported to be ineffective in inhibiting LuCaP 35 growth in intact male mice [61]. We also observed that E2 did not inhibit LuCaP 35 growth in intact male mice (data not shown). These results suggest that the phenotypic changes caused by E2 treatment are specific to an androgen-depleted environment. In contrast to our E2 data, raloxifene, an ER antagonist, has been reported to inhibit the growth of both androgen-sensitive and -independent CaP in vitro [20,21]. Raloxifene has also been reported to delay CaP development in probasin/SV40 T antigen transgenic rats [62], and inhibit the growth of both androgen-sensitive and androgen-independent variants of the CWR22 CaP xenograft [63]. Thus, the emerging picture of estrogenic effects on androgen-independent CaP is complex, possibly involving multiple mechanisms of which some may involve signal transduction by estrogen receptors, and others not. Additional preclinical studies are clearly warranted to deconvolute these effects.

A potential mechanism whereby E2 may cause alteration of the gene expression profile we have observed in CaP cells is signal transduction *via* ERβ that is expressed by CaP cells. It has been reported that the ERβ expression declines as CaP develops in the prostate gland, but we and other have shown that it reappears in lymph-node and bone metastases [27]. This apparent discrepancy is probably explained by the recent finding of reversible epigenetic regulation of ERβ in CaP metastases [64]. We have shown previously that the xenografts used in this study express ERβ [24]. In the present study we have shown that the androgen independent LuCaP 35V

xenograft expresses the ERβ protein in a form that is capable of DNA binding, and that ERβ levels in nuclei and DNA binding activities are increased upon E2 treatment. Together, these results suggest the possibility that the E2-mediated inhibition is at least by part transduced by ERβ signaling, but further studies are required to demonstrate a direct involvement of ERβ in these phenomena. One important aspect of preclinical testing involves the use of models that mimic the disease in patients. If it is eventually found that E2 is beneficial in advanced CaP and the effects are *via* ERβ, then evaluation of expression of ERβ in patient tumors could prove to be valuable in treatment decisions, much as is the case with HER2/Neu and herceptin treatment today.

The E2 inhibitory effects observed cannot be caused by suppression of the hypothalamic-hypophyseal axis reduction in testosterone levels since the tumors were grown in castrated male mice. However, our data do suggest that androgen receptor signaling may be at least partially involved in the inhibitory effects observed. All of the xenografts except LuCaP 49, express androgen receptor (data not shown), and inhibition of LuCaP 49, by E2 was less pronounced than in the other xenografts. Moreover, the GSEA showed that genes in an independently generated list of genes downregulated by androgen deprivation were significantly enriched in the phenotype of E2-treated LuCaP 35V with about half of genes downregulated by E2 and half upregulated by E2. For example, expression of heat shock protein 70, which is downregulated after castration [65], was upregulated by E2 treatment (Table 2). These results illustrate the complexity of these signaling networks. Further studies are needed to delineate the action of E2 on androgen receptor signaling in CaP cells.

The results reported here support multi-faceted roles for estrogen in inhibition of androgen-independent CaP growth. These observations extend the traditional view of estrogen activity

beyond suppression of circulating concentrations of androgens. Direct cellular effects and modulation of the immune response represent additional potential mechanisms that could be further exploited through combination therapies. Given that estrogens also decrease bone lysis caused by androgen suppression [66] and may ameliorate cognitive side-effects associated with low testosterone [67], the use of estrogens should be considered as a viable first-line treatment strategy for androgen-independent prostate cancer.

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Figure 1. Effects of Estradiol on Recurrent Growth of the LuCaP 35 Prostate Cancer Xenograft.

LuCaP 35 tumor bits were implanted into intact animals, and animals were castrated when tumors reached ~200-400 mm^3. Tumor volume was measured twice a week. Blood was drawn weekly for determination of PSA serum levels. At development of recurrent CaP, as determined by two subsequent increased PSA serum levels, animals were randomized into three groups. E2 and DES pellets were implanted into treatment animals; control animals received placebo pellets. Animals were sacrificed after tumors reached 1000 mg or 90 days post-implantation of the pellets. Three tumors from E2- and DES-treated animals were monitored for an additional 670 days after pellet expiration. Data were synchronized to the pellet implantation, and results are presented as mean ± SEM. A) Tumor Volume, B) Serum PSA Levels.

Figure 2. Effects of Estradiol on LuCaP 35V.

LuCaP 35V, an androgen-insensitive CaP xenograft, was grown in castrated male SCID mice. When tumors reached 200-400 mm^3 animals were supplemented with 60-day release E2 pellets as described in the Methods section. Data are presented as mean ± SEM. E2 inhibited growth of androgen-independent LuCaP 35V in castrated male mice, and caused significant increases in survival of treated animals. PSA levels closely followed the tumor volume. A) Tumor Volume, B) Serum PSA levels, C) Survival, D) Proliferation. E2 treatment decreased proliferation of LuCaP 35V at days 3 and 7 of treatment. LuCaP 35V grown in castrated male mice was treated with E2 for 1, 3, or 7 days. BrdU staining was used to detect proliferating cells. The percentage of positive nuclei was calculated based on counts of stained nuclei in five representative fields containing ~1000 cells from three samples of treated and untreated tumors from each time point. Data are presented as mean ± SEM. Statistical analysis was performed using Student's t-test.

Figure 3.

Effects of E2 Treatment on the Growth of CaP Xenografts in Androgen-Free Environment. LuCaP 23.1, LuCaP 49, and LuCaP 58 were implanted into castrated male mice and when tumors reached 200-400 mm^3 animal were divided into two groups per xenograft; 1) placebo, 2) E2 pellet. Tumor growth and PSA were monitored as described in the Methods section. Supplementation of E2 inhibited growth of all three xenografts. A) Tumor volume, b) PSA serum levels.

Figure 4. Measurements of ERβ Expression in LuCaP 35V Xenografts.

LuCaP35 cells were isolated from tumor bits and treated in vitro with E2 for 4 hours. ERβ was detected in nuclear extracts while cytoplasmic protein extracts were negative for ERβ. E2 increased the amount of ERβ in the nucleus by ~1.5-fold. B) Nuclear extracts of LuCaP 35V and LuCaP 35V treated with E2 *in vitro* for 4 hours were used for EMSA. ERβ /DNA complexes were detected in both samples, with increase amounts in E2-treated LuCaP 35V. Specificity of binding was demonstrated by competition with mutated ERE sequence (xERE).

Figure 5. Enrichment Plot of Gene Signatures in the E2-treated LuCaP 35V Data Set.

The plots show the locations of the interferon (A), androgen (B), estrogen (C) signature genes in the gene set ranked by the E2 phenotype. The running enrichment score (RES) as a function of position in the gene list is shown. The signal-to-noise ranks of all 2,584 genes in the gene set are shown, with low ranks indicating genes up-regulated by E2 treatment and high ranks indicating genes down-regulated by E2 treatment. The interferon signature genes are clearly overrepresented in the left side of the gene list, representing their enrichment in the genes significantly up-regulated by E2 treatment (FDR=11.0 %). The androgen signature genes are present on both sides

of the gene list, representing their enrichment in the genes significantly down-regulated as well as upregulated by E2 treatment (FDR = 21.3%). The estrogen signature genes are also clustered at both ends of the ranked list, representing up- and down-regulation by E2 treatment (FDR = 54.5%).

Figure 6. Quantitative RT-PCR Analysis of Expression of Immune-related Genes.

Sets of pooled samples (n=5) from control and E2-treated tumors were used for real-time PCR analyses. Data are presented as relative expression normalized to housekeeping gene as described in Method Section. Real-time analysis confirmed the results of cDNA array analysis of LuCaP 35V. Moreover immune-related genes exhibited similar alterations in LuCaP 58 upon E2 treatment. Alterations in these messages in LuCaP 49 were very small or undetectable, suggesting that other mechanisms are also involved in the E2 inhibition observed, and that expression of the AR may play a role in the altered expression of these messages. Results (mean ± SEM) are presented as mean ± SEM of the change factor over the untreated tumors.

Figure 7. Expression Changes in Immune-related Genes Following E2- or DES-Treatment of Androgen Independent CaP Xenografts.

LuCaP 35 tumor bits were implanted into intact animals, and animals were castrated when tumors reached ~200-400 mm^3. At the time of development of recurrent CaP, animals were randomized into three groups. E2 and DES pellets were implanted into treatment animals; control animals received placebo pellets. Animals were sacrificed after tumors reached 1000 mg, 90 days after pellet implantation (E2, DES), or 60 days after pellet expiration (E2+60, DES+60). RNA was extracted and qRT-PCR performed as described in the Methods section. The results show that E2 and DES treatment increased the expression of immune-related messages in a similar manner. Gene expression changes were dependent on the presence of estrogenic compounds, since after pellet expiration levels of these messages decreased, in some cases nearly to the levels

observed in untreated animals. Data (mean ± SEM) are presented as relative expression normalized to housekeeping gene as described in Method Section.

Table 3. Genes down-regulated in E2-treated LuCaP 35V *vs.* untreated LuCaP 35V

HUGO	NAME	GENBANK	ENTREZ GENE	AVE FOLD Δ	GENE LIST
	METABOLISM				
	METABOLISM - CARBOHYDRATE				
UGDH	UDP-glucose dehydrogenase	BC022781	7358	-2.0	
GALNT7	UDP-N-acetyl-alpha-D-galactosamine	BM976847	51809	-1.8	
GPI	glucose phosphate isomerase	AI124792	2821	-1.8	
RPN1	ribophorin I	CD644128	6184	-1.8	AR
SORD	sorbitol dehydrogenase	BC025295	6652	-1.6	AR
GRHPR	glyoxylate reductase/hydroxypyruvate reductase	BE728720	9380	-1.5	
ACLY	ATP citrate lyase	BI869432	47	-1.5	
	METABOLISM - LIPID/STEROL				
RODH	3-hydroxysteroid epimerase	AF223225	8630	-9.5	
FACL3	fatty-acid-Coenzyme A ligase, long-chain 3	AK023191	2181	-3.0	
TMEPAI	transmembrane, prostate androgen induced RNA	NM_199170	56937	-2.6	AR
PPAP2A	phosphatidic acid phosphatase type 2A	CR617429	8611	-2.5	
EBP	emopamil binding protein (sterol isomerase)	CN395741	10682	-2.2	AR
DHCR24	24-dehydrocholesterol reductase	BC011669	1718	-2.1	AR
PIGF	phosphatidylinositol glycan, class F	BQ006858	5281	-2.1	
CERK	ceramide kinase	NM_182661	64781	-1.5	
	METABOLISM - PROTEIN				
HMGCS2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	NM_005518	3158	-2.9	AR
MME	membrane metallo-endopeptidase	AL833459	4311	-2.3	
KLK3	kallikrein 3, (prostate specific antigen)	CF140712	354	-2.3	AR, IFN
ODC1	ornithine decarboxylase 1	BU153337	4953	-1.9	AR
GOT2	glutamic-oxaloacetic transaminase 2, mitochondrial	AK098313	2806	-1.7	
ACY1L2	aminoacylase 1-like 2	AK094996	135293	-1.7	
GBDR1	putative glialblastoma cell differentiation-related	BC004967	10422	-1.7	
ADAM23	a disintegrin and metalloproteinase domain 23	AF052115	8745	-1.7	
ALDH1A3	aldehyde dehydrogenase 1 family, member A3	BX538027	220	-1.6	AR
KLK2	kallikrein 2, prostatic	NM_005551	3817	-1.6	AR
GOT1	glutamic-oxaloacetic transaminase 1, soluble	CR616132	2805	-1.5	AR
	METABOLISM - OTHER				
NDUFS3	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa	AF100743	4722	-2.1	
ACPP	acid phosphatase, prostate	AI547266	55	-2.1	AR
DTYMK	deoxythymidylate kinase	AA427388	1841	-2.1	
DCXR	dicarbonyl/L-xylulose reductase	BM795570	51181	-1.6	
RRM1	ribonucleotide reductase M1 polypeptide	AK122695	6240	-1.6	
AK3	adenylate kinase 3	AW014145	205	-1.6	
NME1	non-metastatic cells 1, protein (NM23A) expressed in	NM_000269	4830	-1.6	E2
	PROLIFERATION / DIFFERENTIATION / APOPTOSIS				
CCDC5	coiled-coil domain containing 5	AI142429	115106	-2.0	
TPT1	tumor protein, translationally-controlled 1	AU119000	7178	-1.7	
MAD2L1	MAD2 mitotic arrest deficient-like 1	BC005945	4085	-1.6	
PCNA	proliferating cell nuclear antigen	AA953221	5111	-1.6	
CCNG2	cyclin G2	CR598707	901	-1.6	
MCM3	MCM3 minichromosome maintenance deficient 3	BQ213935	4172	-1.5	
	SIGNAL TRANSDUCTION				
FKBP5	FK506 binding protein 5	BU618502	2289	-2.7	AR
RACGAP1	Rac GTPase activating protein 1	AB040911	29127	-2.2	
STMN1	stathmin 1/oncoprotein 18	BM543057	3925	-2.0	
CAMKK2	calcium/calmodulin-dependent protein kinase kinase 2, beta	NM_006549	10645	-2.0	AR
MAP2K1	mitogen-activated protein kinase kinase 1	L05624	5604	-1.9	IFN
RAB27A	RAB27A, member RAS oncogene family	U38654	5873	-1.9	
GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	BE300778	10399	-1.8	
MAP2K4	mitogen-activated protein kinase kinase 4	NM_003010	6416	-1.7	
SLC9A3R2	solute carrier family 9, isoform 3 regulatory factor 2	BU540416	9351	-1.7	
TM4SF3	transmembrane 4 superfamily member 3	NM_004616	7103	-1.6	
APPBP1	amyloid beta precursor protein binding protein 1, 59kDa	BC041323	8883	-1.6	
CCL2	chemokine (C-C motif) ligand 2	BU532858	6347	-1.6	
RAN	RAN, member RAS oncogene family	BG775164	5901	-1.5	
	STRUCTURAL / ADHESION / MOTILITY				
DKFZP761D0211	hypothetical protein DKFZp761D0211	CR619764	83986	-2.1	
COL1A1	collagen, type I, alpha 1	CV799740	1277	-2.1	
HMMR	hyaluronan-mediated motility receptor	CR601287	3161	-2.0	
COL2A1	collagen, type II, alpha 1	CX119275	1280	-1.8	
TSPAN-1	tetraspan 1	CA454232	10103	-1.7	
Postn	periostin, osteoblast specific factor [*Mus musculus*]	BC031449	50706	-1.7	
LCP1	lymphocyte cytosolic protein 1	BC015001	3936	-1.7	
MYBPC1	myosin binding protein C, slow type	BF516586	4604	-1.6	
SMOC1	SPARC related modular calcium binding 1	CD049369	64093	-1.6	
NUP93	nucleoporin 93kDa	CR612078	9688	-1.6	
SYNPO2	synaptopodin 2	AL833547	171024	-1.5	
CKAP5	cytoskeleton associated protein 5	CR623748	9793	-1.5	
CXCR4	chemokine (C-X-C motif) receptor 4	BF591711	7852	-1.5	
	TRANSCRIPTION REGULATION				
NKX3-1	NK3 transcription factor related, locus 1	BX102941	4824	-3.3	
SPDEF	SAM pointed domain containing ets transcription factor	BG328411	25803	-2.5	
TOP2A	topoisomerase (DNA) II alpha 170kDa	AW172827	7153	-2.3	E2
CREB3L4	cAMP responsive element binding protein 3-like 4	AF394167	148327	-2.3	
H2AFZ	H2A histone family, member Z	BU178992	3015	-1.9	
RFC3	replication factor C3, 38kDa	BC000149	5983	-1.9	
CDK2AP1	CDK2-associated protein 1	BU608264	8099	-1.8	
SMARCA2	SWI/SNF rel., matrix assoc., actin dep. reg. of chromatin, subfamily a, member 2	BM671383	6595	-1.6	
SMC2L1	SMC2 structural maintenance of chromosomes 2-like 1	BC032705	10592	-1.5	
SNRPB	small nuclear ribonucleoprotein polypeptides B and B1	BX363533	6628	-1.5	
RAD51C	RAD51 homolog C	AW270829	5889	-1.5	
HIRIP3	HIRA interacting protein 3	NM_003609	8479	-1.5	
	TRANSLATION - PROTEIN SYNTHESIS				
GOLPH2	golgi phosphoprotein 2	AW591201	51280	-2.6	
RPS2	ribosomal protein S2	CR610190	6187	-2.3	
RPL4	ribosomal protein L4	BM451248	6124	-2.2	
NAG	neuroblastoma-amplified protein	NM_015909	51594	-2.1	
LOC388817	Peptidylprolyl isomerase A-like	BM972350	388817	-2.1	
LRIG1	leucine-rich repeats and immunoglobulin-like domains 1	BC014276	26018	-2.0	
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	BC020477	1915	-1.9	
RPS8	ribosomal protein S8	BQ218087	6202	-1.9	
RAI14	retinoic acid induced 14	AY317139	26064	-1.8	
RPL6	ribosomal protein L6	BC071912	6128	-1.8	
RPL9	ribosomal protein L9	BQ961538	6133	-1.8	
RPL10A	ribosomal protein L10a	BQ941098	4736	-1.7	
EEF1B2	eukaryotic translation elongation factor 1 beta 2	BX353697	1933	-1.7	
RPS6	ribosomal protein S6	BG029552	6194	-1.6	
RPL26	ribosomal protein L26	BG925676	6154	-1.6	
RPL31	ribosomal protein L31	CN269893	6160	-1.6	
RPL5	ribosomal protein L5	BM721056	6125	-1.6	
NACA	nascent-polypeptide-associated complex alpha polypeptide	BU164695	4666	-1.6	
RPL13A	ribosomal protein L13a	BQ229130	23521	-1.6	
EIF3S6IP	eukaryotic translation initiation factor 3, subunit 6 interacting protein	BX424780	51386	-1.6	
RPL11	ribosomal protein L11	BU902342	6135	-1.6	
RPS3A	ribosomal protein S3A	BM463771	6189	-1.5	
RPS15A	ribosomal protein S15a	CN351294	6210	-1.5	
RPLP0	ribosomal protein, large, P0	BG575128	6175	-1.5	
RPS13	ribosomal protein S13	CA843734	6207	-1.5	
RPL10	ribosomal protein L10	BM423499	6134	-1.5	
RPS4X	ribosomal protein S4, X-linked	BQ959684	6191	-1.5	
	TRANSPORT				
DBI	diazepam binding inhibitor	BQ940531	1622	-2.5	
VPS45A	vacuolar protein sorting 45A	AK023170	11311	-2.2	
HBE1	hemoglobin, epsilon 1	AA115963	3046	-2.0	
SLC39A6	solute carrier family 39, member 6	BC008317	25800	-1.7	
RAB3B	RAB3B, member RAS oncogene family	BF792558	5865	-1.7	
KPNA2	karyopherin alpha 2	U09559	3838	-1.6	
TOMM40	translocase of outer mitochondrial membrane 40 homolog	BQ883428	10452	-1.6	
SLC16A1	solute carrier family 16, member 1	AK000641	6566	-1.6	AR
SLC25A3	solute carrier family 25, member 3	BC068067	5250	-1.5	
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	CR591449	506	-1.5	
	OTHER / UNKNOWN				
KIAA0114	KIAA0114 gene product	BI850303	57291	-2.3	
BRP44	Brain protein 44	BQ287816	25874	-2.2	
THAP5	THAP domain containing 5	NM_182529	168451	-2.0	
HN1	hematological and neurological expressed 1	CN363269	51155	-2.0	
KIAA0460	KIAA0460 protein	AB007929	23248	-2.0	
PRAC	small nuclear protein PRAC	BU942850	84366	-1.8	
SURF4	surfeit 4	CR602588	6836	-1.7	

Table 2. Genes up-regulated in E2-treated LuCaP 35V *vs.* untreated LuCaP 35V

HUGO	NAME	GENBANK	ENTREZ GENE	AVE FOLD Δ	GENE LIST
	METABOLISM				
	METABOLISM - CARBOHYDRATE				
Lyzs	lysozyme [Mus musculus]	M21050	17105	2.9	
SIAT1	sialyltransferase 1	NM_173217	6480	2.7	
EXT1	exostoses 1	BQ021387	2131	1.8	
	METABOLISM - LIPID/STEROL				
UGT2B15	UDP glycosyltransferase 2 family, polypeptide B15	AF180322	7366	3.7	
SORL1	sortilin-related receptor, L(DLR class) A repeats-containing	AK096577	6653	2.4	
PSAP	prosaposin	CR617297	5660	1.9	
APOE	apolipoprotein E	BG715607	348	1.8	
CLN2	ceroid-lipofuscinosis, neuronal 2, late infantile	AF017456	1200	1.8	
	METABOLISM - PROTEIN				
FOLH1	folate hydrolase (prostate-specific membrane antigen) 1	BC025672	2346	3.6	
SQSTM1	sequestosome 1	BQ220165	8878	1.8	
DDC	dopa decarboxylase	CA488364	1644	1.8	
MAOA	monoamine oxidase A	NM_000240	4128	1.5	
	METABOLISM - OTHER				
SOD2	superoxide dismutase 2, mitochondrial	BU527631	6648	1.9	
VKORC1	vitamin K epoxide reductase complex, subunit 1	NM_024006	79001	1.7	
TBC1D14	TBC1 domain family, member 14	AL833868	57533	1.5	
	IMMUNE RESPONSE				
CD74	CD74 antigen	CA437013	972	5.1	
HLA-DRA	major histocompatibility complex, class II, DR alpha	BG757515	3122	3.4	
HLA-F	major histocompatibility complex, class I, F	AK096962	3134	3.0	
LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein	BQ883924	3959	2.6	
HLA-DQB1	major histocompatibility complex, class II, DQ beta 1	L34104	3119	2.5	
HLA-C	major histocompatibility complex, class I, C	X67818	3107	2.4	
HLA-B	major histocompatibility complex, class I, B	AK124160	3106	2.3	IFN
HLA-A	major histocompatibility complex, class I, A	AK027084	3105	2.2	IFN
IFITM3	interferon induced transmembrane protein 3	BQ441207	10410	2.1	
BST2	bone marrow stromal cell antigen 2	BQ053580	684	2.0	IFN
B2M	beta-2-microglobulin	BM453762	567	1.9	AR, IFN
CD59	CD59 antigen p18-20	BM550387	966	1.8	
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	BI670242	3434	1.8	IFN
IRF1	interferon regulatory factor 1	CR594837	3659	1.8	IFN
IFI27	interferon, alpha-inducible protein 27	BM998410	3429	1.5	IFN
	PROLIFERATION / DIFFERENTIATION / APOPTOSIS				
NDRG4	NDRG family member 4	AB021172	65009	2.8	
BCCIP	BRCA2 and CDKN1A interacting protein	BQ421346	56647	1.7	
BIRC3	baculoviral IAP repeat-containing 3	BC037420	330	1.7	AR
TMBIM1	transmembrane BAX inhibitor motif containing 1	AK130380	64114	1.6	
AGR2	anterior gradient 2 homolog	BQ685832	10551	1.6	AR
UNC13B	unc-13 homolog B	NM_006377	10497	1.6	
TM4SF13	transmembrane 4 superfamily member 13	AK093487	27075	1.6	
NPM1	nucleophosmin	CN404150	4869	1.6	
NDRG1	N-myc downstream regulated gene 1	CR600627	10397	1.5	AR
KIAA0971	KIAA0971 protein	CD671614	22868	1.5	
	SIGNAL TRANSDUCTION				
HSPA1A	heat shock 70kDa protein 1A	CR605852	3303	7.3	
IFITM1	interferon induced transmembrane protein 1	BQ219055	8519	2.8	IFN
LY6E	lymphocyte antigen 6 complex, locus E	U42376	4061	2.2	
STAT1	signal transducer and activator of transcription 1, 91kDa	BG678000	6772	1.9	IFN
ARHGAP5	Rho GTPase activating protein 5	BG260763	394	1.8	
OGT	O-linked N-acetylglucosamine (GlcNAc) transferase	U77413	8473	1.7	
RALGPS1A	Ral guanine nucleotide exchange factor RalGPS1A	AB002349	9649	1.6	
FKBP4	FK506 binding protein 4, 59kDa	CD613711	2288	1.5	
SH3KBP1	SH3-domain kinase binding protein 1	AY423734	30011	1.5	
NUDT4	nudix-type motif 4	NM_019094	11163	1.5	
	STRUCTURAL / ADHESION / MOTILITY				
MYLK	myosin, light polypeptide kinase	BC062755	4638	3.9	AR
MYH3	myosin, heavy polypeptide 3, skeletal muscle, embryonic	CK824450	4621	1.8	
SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	AL547671	6678	1.8	
INA	internexin neuronal intermediate filament protein, alpha	CR591335	9118	1.6	
CLDN4	claudin 4	BC000671	1364	1.5	
LAMB2	laminin, beta 2	AI754927	3913	1.5	
	TRANSCRIPTION REGULATION				
ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	BM973065	3397	2.7	
HIST1H2AC	histone 1, H2ac	BC050602	8334	2.3	
PMF1	polyamine-modulated factor 1	BC050735	11243	2.0	
NONO	non-POU domain containing, octamer-binding	BG171743	4841	1.9	
ZNFX1	zinc finger, NFX1-type containing 1	AB037825	57169	1.7	
NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive	NM_006599	10725	1.7	
NOLC1	nucleolar and coiled-body phosphoprotein 1	BE908347	9221	1.7	
TRIM22	tripartite motif-containing 22	AW080955	10346	1.7	AR, IFN
GPBP1	GC-rich promoter binding protein 1	AL161991	65056	1.6	
ADAR	adenosine deaminase, RNA-specific	U18121	103	1.5	IFN
	TRANSLATION - PROTEIN SYNTHESIS				
HSP90AA2	heat shock protein 90kDa alpha, class A member 2	BC001695	3324	2.1	
DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1	BC002352	3337	1.9	
GOLPH4	golgi phosphoprotein 4	AA447271	27333	1.8	
DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	BQ221194	3301	1.8	
EIF4A2	eukaryotic translation initiation factor 4A, isoform 2	BT009860	1974	1.7	
RPL23AP7	ribosomal protein L23a pseudogene 7	X92108	118433	1.6	
UBC	ubiquitin C	AK129749	7316	1.5	AR
	TRANSPORT				
SELENBP1	selenium binding protein 1	BC009084	8991	2.9	
APBA2	amyloid beta (A4) precursor protein-binding, family A, member 2	BC082986	321	2.6	
FLJ39822	hypothetical protein FLJ39822	CA390853	151258	2.0	
SLC12A2	solute carrier family 12, member 2	AF439152	6558	2.0	
FLJ39822	hypothetical protein FLJ39822	AC019197	151258	1.9	
C6orf29	chromosome 6 open reading frame 29	AY358457	80736	1.9	
ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	NM_001677	481	1.7	
ATP6V1A	ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A	BC012169	523	1.7	
FLJ10618	hypothetical protein FLJ10618	AL049246	55186	1.5	
NPC2	Niemann-Pick disease, type C2	CR608935	10577	1.5	
NAPA	N-ethylmaleimide-sensitive factor attachment protein, alpha	BC007432	8775	1.5	
ATP6AP2	ATPase, H+ transporting, lysosomal accessory protein 2	BI491181	10159	1.5	
SLC25A26	solute carrier family 25, member 26	AJ580932	115286	1.5	
	OTHER / UNKNOWN				
MUC13	mucin 13, epithelial transmembrane	AK000070	56667	3.9	
SAMD9L	sterile alpha motif domain containing 9-like	BC038974	219285	3.8	
	Transcribed locus	CD103928		2.8	
	Transcribed locus, strongly similar to XP_496055.1 PREDICTED: similar to p40	AW452111		2.3	
C1orf43	hromosome 1 open reading frame 43	BQ900746	25912	1.9	
C1orf80	chromosome 1 open reading frame 80	BC015535	64853	1.8	
SERINC3	serine incorporator 3	BI518460	10955	1.8	
FAM73A	Family with sequence similarity 73, member A	AU131144	374986	1.6	
ITM2B	integral membrane protein 2B	CR745752	9445	1.6	

Table 1. Primer Sequences

ABREVIATION	NAME		PRIMER SEQUENCES	POSITION		ANNELING TEMPERATURE	SIZE	ACCESSION #
GAPDH	Glyceraldehyde dehydrogenase	5'	TGC ACC ACC AAC TGC TTA GC	556	575	65	86	NM_002046
		3'	GGC ATG GAC TGT GGT CAT GAG	642	622			
EGP	Epithelial glycoprotein	5'	GCT GGA ATT GTT GTG CTG GTT ATT TC	1019	1044	65	152	NM_002354
		3'	TGT GTC CAT TTG CTA TTT CCC TTC TTC	1171	1145			
CD74	CD74 antigen (invariant polypeptide of major histocompatibility complex class II antigen-associated)	5'	GTG CGA CGA GAA CGG CAA CTA TC	704	726	69	218	NM_001025159
		3'	GAA GAC CGC CTC TGC TGC TCT C	901	922			
HLA II DRA	Major histocompatibility complex class II DR alpha	5'	CCC AGA GAC TAC AGA GAA CGT GG	714	736	69	265	NM_019111
		3'	GGG CTG GAA AAT GCT GAA GAT GAC	979	956			
HLA 1F	Major histocompatibility complex class I F	5'	GTT GCC CAC CAC CCC ATC TCT G	628	649	65	371	NM_018950
		3'	GCT CTT CTT CCT CCA CAT CAC AG	977	999			
IFI-TM-1	Interferon induced transmembrane protein 3 (1-8U)	5'	CGT CGC CAA CCA TCT TCC TGT C	530	509	69	246	NM_003641
		3'	TTC ACT CAA CAC TTC CTT CCC CAA	284	307			
HLA DQB 1	Major histocompatibility complex class II DQ beta 1	5'	GCC TTA TCA TCC ATC ACA GGA GTC	797	820	65	223	NM_002123
		3'	GTC ACA GCC ATC CGC CTC AAG G	999	1020			
IFI-TM3	Interferon induced transmembrane protein 3 (1-8U)	5'	GTC CAA ACC TTC TTC TCT CCT GTC	250	273	69	264	NM_021034
		3'	CGT CGC CAA CCA TCT TCC TGT C	514	493			
BST2	Bone marrow stromal cell antigen 2	5'	GAG GTG GAG CGA CTG AGA AGA GA	406	428	69	204	NM_004335.
		3'	GTT CAA GCG AAA AGC CGA GCA GG	610	588			
β-2M	Beta-2-microglobulin	5'	GAG TAT GCC TGC CGT GTG AAC CA	349	371	69	313	NM_004048
		3'	ACC TCT AAG TTG CCA GCC CTC CT	640	662			
CD 59	CD59 antigen p18-20	5'	CTG CTG CTC GTC CTG GCT GTC T	149	170	69	370	NM_000611
		3'	GCT CTC CTG GTG TTG ACT TAG GG	497	519			
IFN-TR1	Interferon-induced protein with tetratricopeptide repeats 1	5'	CTG AAA ATC CAC AAG ACA GAA TAG C	5	29	69	377	NM_001001887
		3'	GTC ACC AGA CTC CTC ACA TTT GCT	359	382			
IRF-1	Interferon regulatory factor 1	5'	GTA CCG GAT GCT TCC ACC TCT CAC C	524	545	69	105	NM_002198
		3'	GCT GGA ATC CCC ACA TGA CTT CCT C	605	629			
IFI-27	Interferon alpha-inducible protein 27	5'	GTT GTG ATT GGA GGA GTT GTG G	226	247	65	193	NM_005532
		3'	GAG AGT CCA GTT GCT CCC AGT	399	419			
ERβ	Estrogen receptor beta	5'	GCT AAC CTC CTG ATG CTC CTG TCC	1784	1807	65	204	NM_001437
		3'	AGC CCT CTT TGC TTT TAC TGT CCT CT	1988	1963			

FIGURE 1

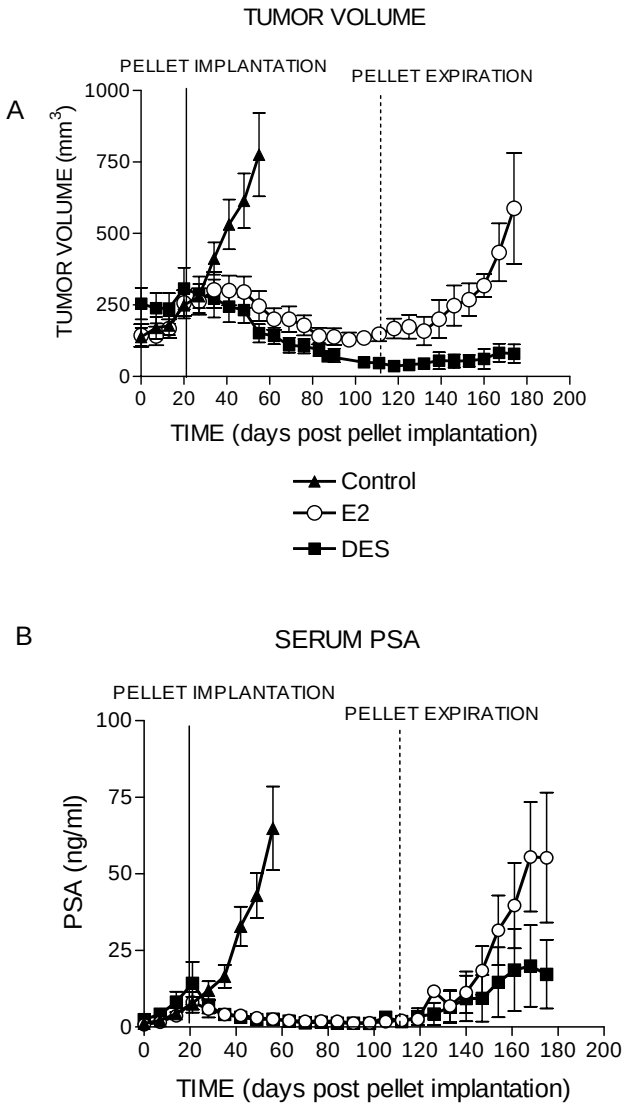

A
TUMOR VOLUME
PELLET IMPLANTATION
PELLET EXPIRATION
TUMOR VOLUME (mm3)
1000
750
500
250
0
0 20 40 60 80 100 120 140 160 180 200
TIME (days post pellet implantation)
Control
E2
DES
B
SERUM PSA
PELLET IMPLANTATION
PELLET EXPIRATION
PSA (ng/ml)
100
75
50
25
0
0 20 40 60 80 100 120 140 160 180 200
TIME (days post pellet implantation)

FIGURE 2

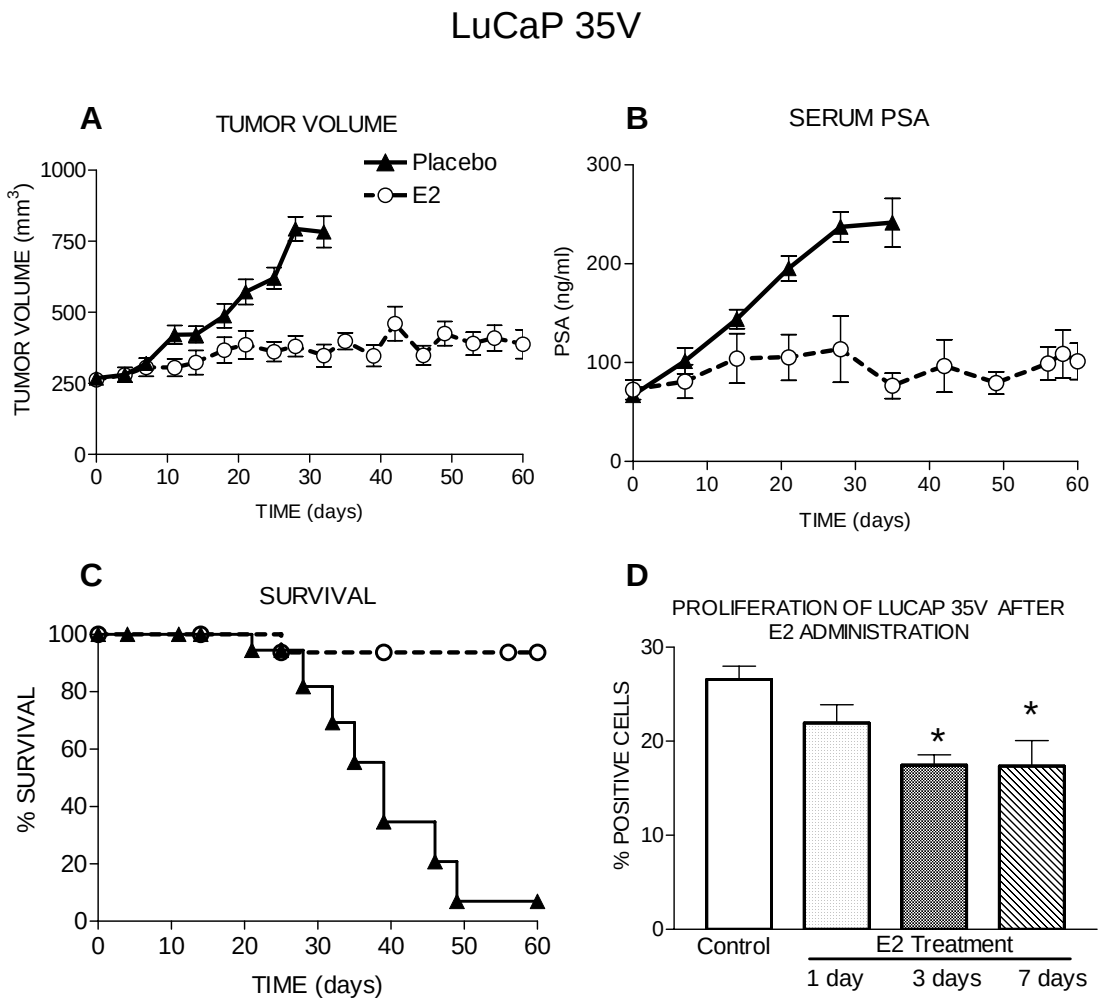

LuCaP 35V
A
TUMOR VOLUME
Placebo
E2
TUMOR VOLUME (mm3)
1000
750
500
250
0
0
10
20
30
40
50
60
TIME (days)
B
SERUM PSA
PSA (ng/ml)
300
200
100
0
TIME (days)
C
SURVIVAL
% SURVIVAL
100
80
60
40
20
0
TIME (days)
D
PROLIFERATION OF LUCAP 35V AFTER E2 ADMINISTRATION
% POSITIVE CELLS
30
20
10
0
*
*
Control
E2 Treatment
1 day
3 days
7 days

FIGURE 3

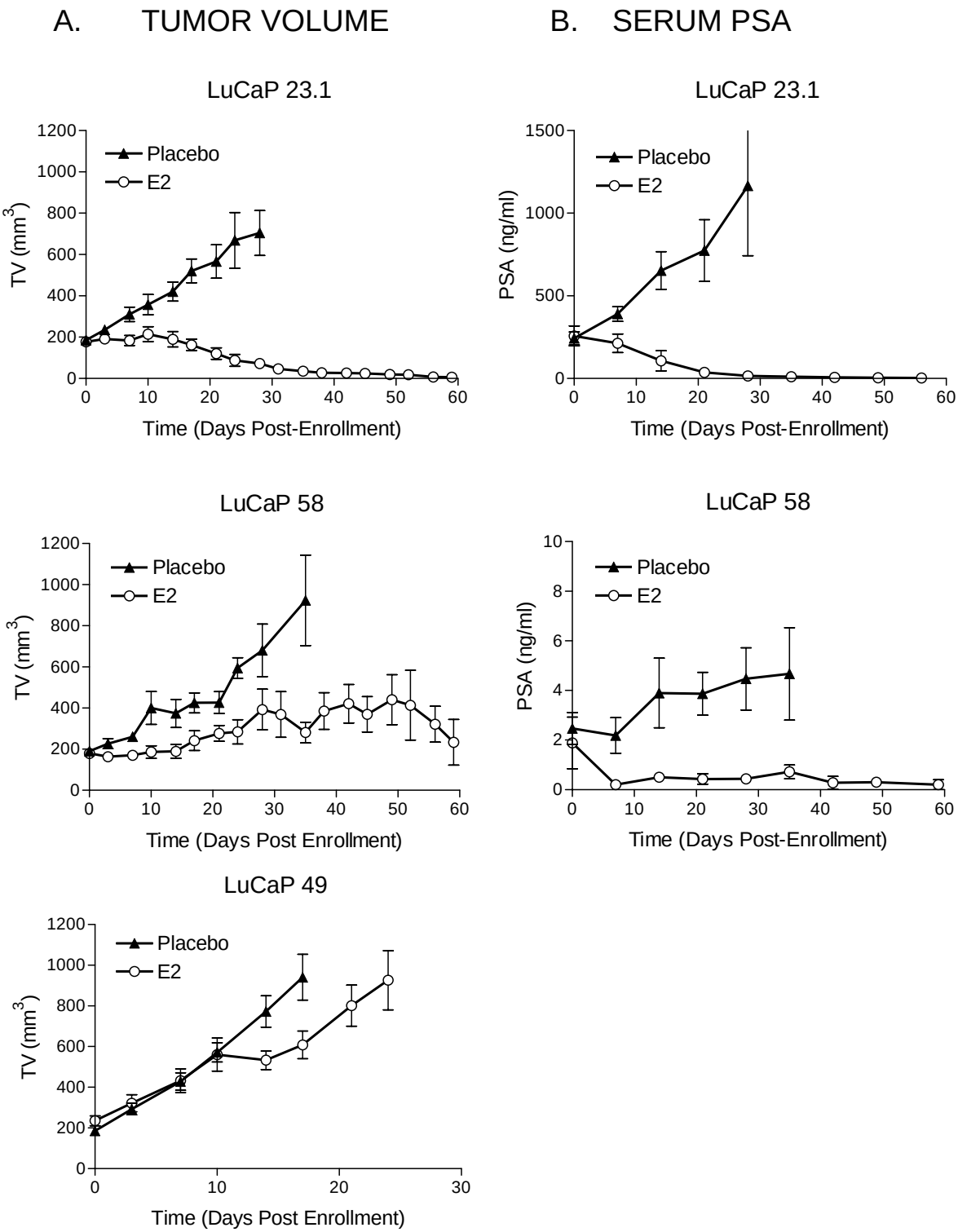
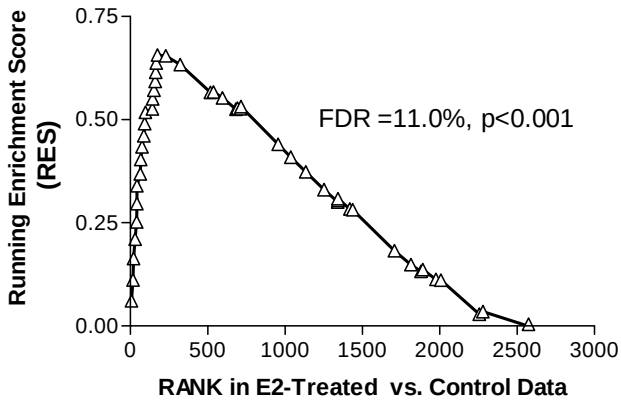
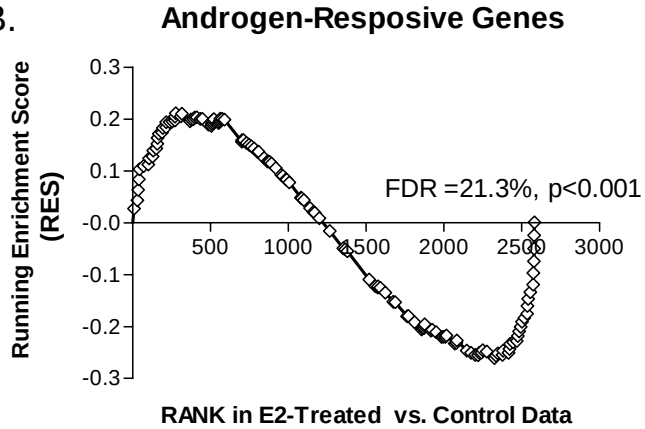

A. TUMOR VOLUME
B. SERUM PSA
LuCaP 23.1
TV (mm3)
Placebo
E2
Time (Days Post-Enrollment)
LuCaP 23.1
PSA (ng/ml)
Placebo
E2
Time (Days Post-Enrollment)
LuCaP 58
TV (mm3)
Placebo
E2
Time (Days Post Enrollment)
LuCaP 58
PSA (ng/ml)
Placebo
E2
Time (Days Post-Enrollment)
LuCaP 49
TV (mm3)
Placebo
E2
Time (Days Post Enrollment)

FIGURE 4

A.


Interferon-Responsive Genes
Running Enrichment Score (RES)
0.75
0.50
0.25
0.00
FDR =11.0%, p<0.001
0
500
1000
1500
2000
2500
3000
RANK in E2-Treated vs. Control Data

B.


Androgen-Resposive Genes
Running Enrichment Score (RES)
0.3
0.2
0.1
-0.0
-0.1
-0.2
-0.3
FDR =21.3%, p<0.001
500
1000
1500
2000
2500
3000
RANK in E2-Treated vs. Control Data

C.

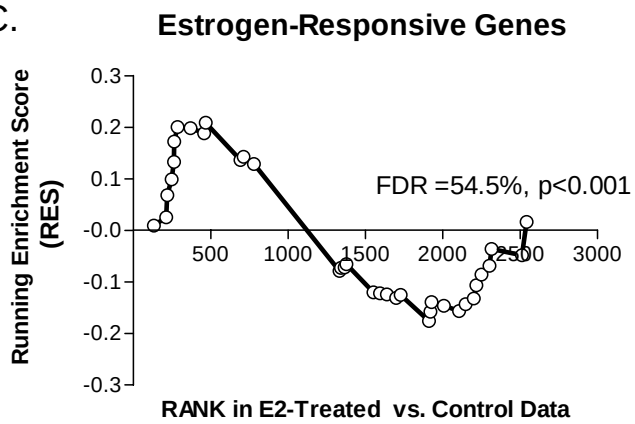

Estrogen-Responsive Genes
Running Enrichment Score (RES)
0.3
0.2
0.1
-0.0
-0.1
-0.2
-0.3
FDR =54.5%, p<0.001
500
1000
1500
2000
2500
3000
RANK in E2-Treated vs. Control Data

FIGURE 5

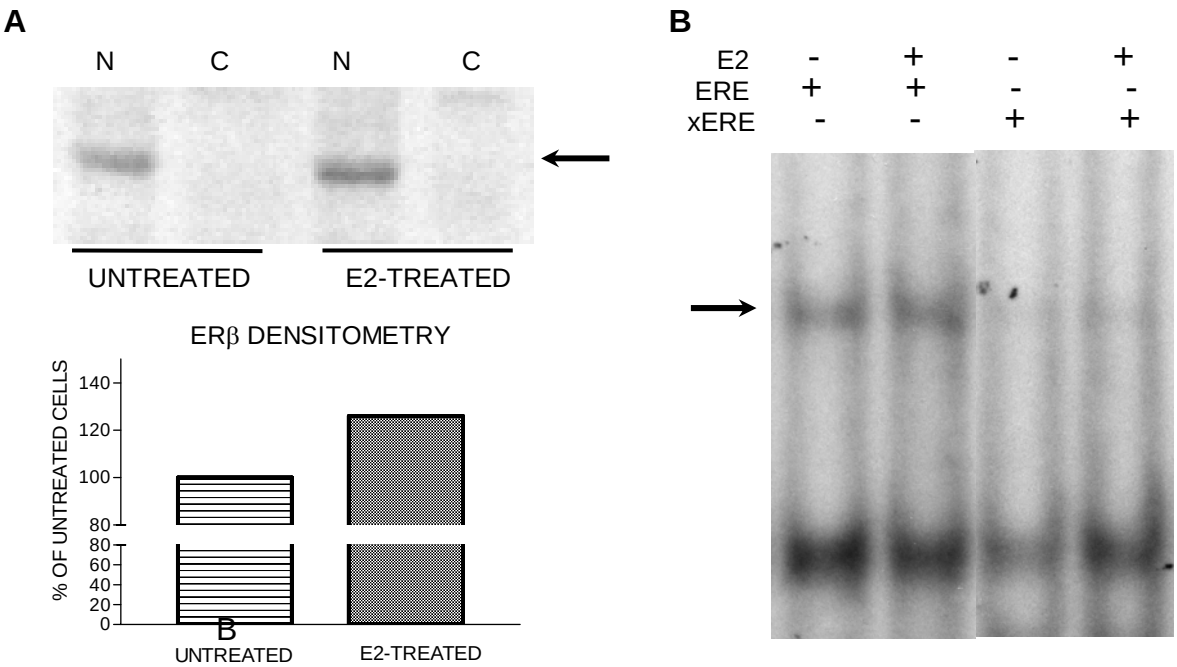

A
N
C
N
C
UNTREATED
E2-TREATED
ERβ DENSITOMETRY
% OF UNTREATED CELLS
140
120
100
80
80
60
40
20
0
B
UNTREATED
E2-TREATED
B
E2
ERE
xERE
-
+
-
+
+
+
-
-
-
-
+
+

FIGURE 6

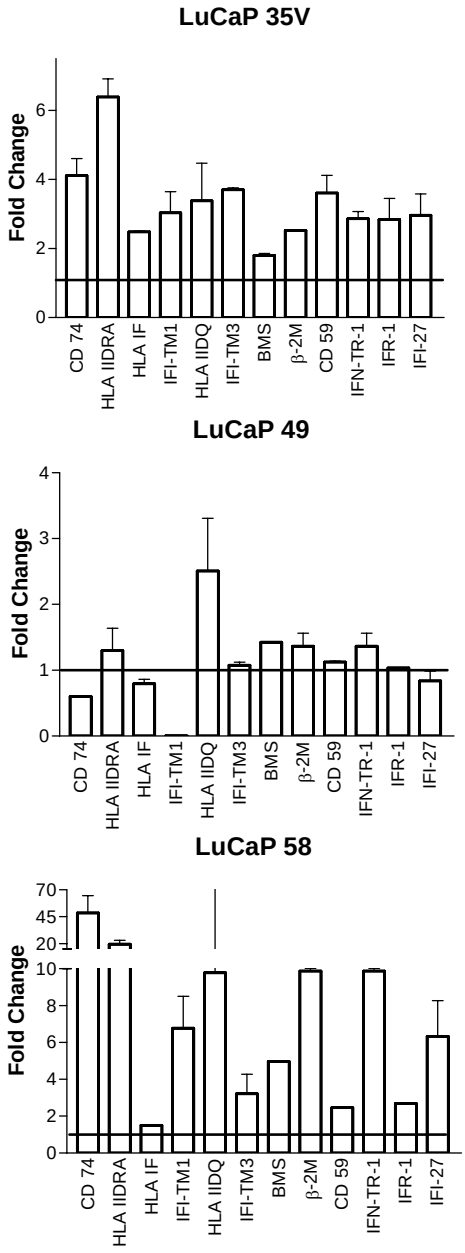
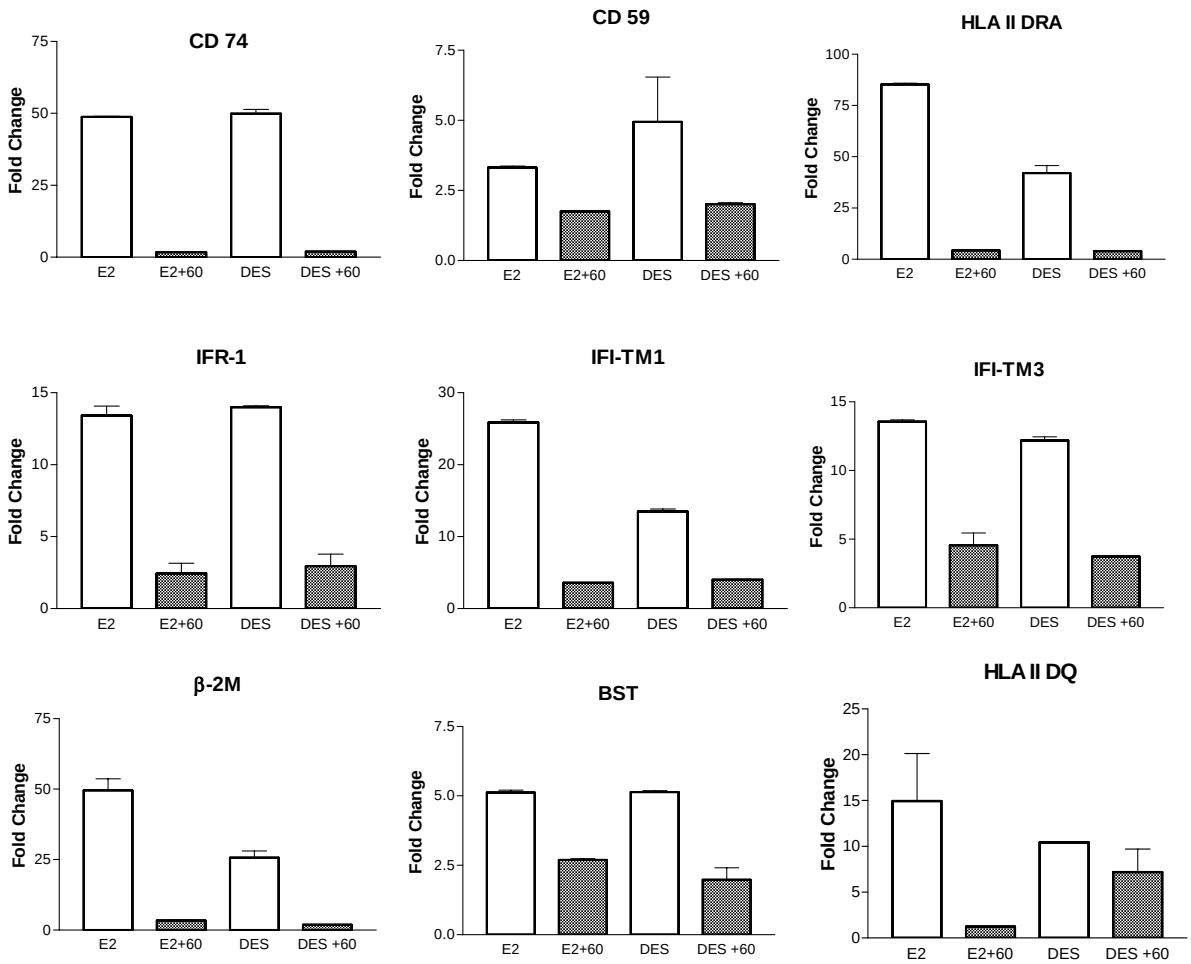

LuCaP 35V
Fold Change
0
2
4
6
CD 74
HLA IIDRA
HLA IF
IFI-TM1
HLA IIDQ
IFI-TM3
BMS
β-2M
CD 59
IFN-TR-1
IFR-1
IFI-27
LuCaP 49
Fold Change
0
1
2
3
4
CD 74
HLA IIDRA
HLA IF
IFI-TM1
HLA IIDQ
IFI-TM3
BMS
β-2M
CD 59
IFN-TR-1
IFR-1
IFI-27
LuCaP 58
Fold Change
0
2
4
6
8
10
20
45
70
CD 74
HLA IIDRA
HLA IF
IFI-TM1
HLA IIDQ
IFI-TM3
BMS
β-2M
CD 59
IFN-TR-1
IFR-1
IFI-27

FIGURE 7


CD 74
Fold Change
75
50
25
0
E2
E2+60
DES
DES +60
CD 59
Fold Change
7.5
5.0
2.5
0.0
E2
E2+60
DES
DES +60
HLA II DRA
Fold Change
100
75
50
25
0
E2
E2+60
DES
DES +60
IFR-1
Fold Change
15
10
5
0
E2
E2+60
DES
DES +60
IFI-TM1
Fold Change
30
20
10
0
E2
E2+60
DES
DES +60
IFI-TM3
Fold Change
15
10
5
0
E2
E2+60
DES
DES +60
β-2M
Fold Change
75
50
25
0
E2
E2+60
DES
DES +60
BST
Fold Change
7.5
5.0
2.5
0.0
E2
E2+60
DES
DES +60
HLA II DQ
Fold Change
25
20
15
10
5
0
E2
E2+60
DES
DES +60