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# The EPI bioassay identifies natural compounds with estrogenic activity that are potent inhibitors of androgenic pathways in human prostate stromal and epithelial cells

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ABSTRACT

The reactive stromal phenotype is an important factor for prostate cancer progression and may be a new target for treatment and prevention. A new high efficiency preclinical protocol, the EPI bioassay, reflects the interaction of endocrine, paracrine and immune, (EPI) factors on induced androgen metabolism in human prostate reactive stroma. The bioassay is based on co-culturing human primary prostate stromal cells and LAPC-4 prostatic adenocarcinoma cells in a downscaled format of 96-well-plates for testing multiple doses of multiple target compounds. Metabolism of dehydroepiandrosterone (DHEA) with or without TGFβ1-induced stimulation (D+T) of the reactive stroma phenotype was assessed by increased testosterone in the media and PSA production of the epithelial prostate cancer cells. Using the non-metabolizable androgen R1881, effects from direct androgen action were distinguished from stromal androgen production from DHEA. Stromal cell androgenic bioactivity was confirmed using conditioned media from D+T-treated stromal cell monocultures in an androgen-inducible AR screening assay. We further showed that both agonists to estrogen receptor (ER), DPN (ERβ) and PPT (ERα), as well as estrogenic natural compounds including soy isoflavones attenuated D+T-induced PSA production. Studies with the pure ER agonists showed that activating either ERα or ERβ could inhibit both D+T-mediated and R1881-mediated PSA production with the D+T effect being more pronounced. In conclusion, natural compounds with estrogenic activity and pure ER agonists are very potent inhibitors of stromal conversion of DHEA to androgenic metabolites. More studies are needed to characterize the mechanisms involved in estrogenic modulation of the endocrine–immune–paracrine balance of the prostate microenvironment.
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## 1. Introduction

Within the prostate tissue microenvironment, multiple factors contribute to growth regulation and phenotype, including the stromal cell and epithelial cell composition, the stromal hormonal milieu, and the resident immune modulators. This laboratory has highlighted the role of the cytokine TGFβ1, in contributing to increased stromal metabolism of the steroid hormone DHEA to androstenedione and testosterone and the consequential upregulation of prostate specific antigen (PSA) in cocultured epithelial cells [1,2]. We herein hypothesize that estrogens and plant derived secondary metabolites with estrogenic activity, such as soy isoflavones can be natural inhibitors of steroid receptor activation, androgenic metabolism and/or paracrine effects involved in regulation of androgen metabolism *in vitro*.

While growth and PSA secretion in the prostate are androgen-regulated events, and the primary focus of cancer treatment is blocking the androgen pathways, the role of estrogen ($E_2$) in the prostate has become increasingly recognized as paradoxical as reviewed in Ricke et al. [3]. In aging male testosterone production gradually declines while estrogen production remains stable or either slightly increases, leading to increased estrogen/testosterone-ratio at the time of prostate cancer development and progression. In experimental models, exposure to $E_2$can amplify tumor formation in testosterone-induced rodent

*Abbreviations:* 6S, primary reactive stromal cell; ANOVA, analysis of variance; AR, androgen receptor; CC, coculture; CDS, charcoal dextran-treated serum; DAI, Diadzein; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; D+T, DHEA+TGFβ1; DMEM, Dulbecco's modified Eagles media; DPN, ERβ agonist; $E_2$, 17β-estradiol; ELISA, enzyme-linked immunosorbent assay; EPI, epithelial, paracrine, immune; EQ, equol; ER, estrogen receptor (α, or β); FBS, fetal bovine serum; GEN, Genistein; HSD, hydroxysteroid dehydrogenase; PPT, ERα agonist; PSA, prostate specific antigen R1881 (R); S.E.M., standard error of the mean T; TGFβ1, transforming growth factorβ-1.

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prostate cancer models [4,5]. On the contrary estrogen was used in early treatment protocols for prostate cancer to block androgen-driven pathways by indirect suppression of the hypothalamic/pituitary/gonadal axis rather than through tissue specific effects (reviewed in [6]). Downstream cellular effects through the ERα expressed in stromal cells can have an opposite effect as those downstream of the ERβ expressed in the epithelial cells, for example in regulation of proliferation. The ERα-mediated proliferative response to $E_2$ can lead to squamous metaplasia, while $E_2$ responses through ERβ are antiproliferative in a more general sense (reviewed in [7]).

There is controversy about efficacy and safety of the use of soy isoflavones in the treatment of menopausal complaints and breast cancer prevention. Soy isoflavones have also been assessed regarding cancer prevention in prostate [8,24]. A study in the UK correlated dietary-based urinary soy isoflavone levels to inverse prostate cancer risk, whereas no correlation between soy isoflavone levels in serum and breast cancer risk became apparent [9,10]. These studies point to a prostate cancer preventative activity of dietary soy isoflavones as also suggested by a recent publication [11]. From *in vitro* and *in vivo* studies it is known that genistein and soy isoflavones can affect a number of molecular mechanisms including regulation of gene expression and modulation of the epigenome [12,13]. However, only one study so far reports on the impact of soy isoflavone exposure in connection to PSA levels, the prototype marker for prostatic disease. While PSA levels in healthy subjects were not affected by isoflavones, they were found to be favorably affected in prostate cancer patients in four out of eight studies however, the molecular mechanism is not known [14].

In the study presented here, we aimed to shed light on how soy isoflavones may impact PSA production by human prostate cancer cells. As a prerequisite, the differential distinction between estrogenic effects via stromal ERα versus epithelial ERβ has been explored using specific estrogen receptor agonists in the EPI bioassay which replicates endocrine–immune–paracrine interactions in the prostate tissue microenvironment. Subsequently, the impact of the phytoestrogenic soy isoflavones, genistein and daidzein, as well as the daidzein metabolite equol, was investigated regarding their capacity to modulate both stromal conversion of DHEA and androgen-induced PSA secretion by epithelial cells.

## 2. Materials and methods

Test substances included the ER agonists, PPT (ERα subtype specific agonist) and DPN (ERβ subtype specific agonist) which were purchased from Tocris Biosciences (Bristol, United Kingdom). $E_2$, DHEA, DHT, Genistein (GEN), Diadzein (DAI) and Equol (EQ) and the dye Rhodanile Blue were obtained from Sigma Aldrich USA, R1881(R) was purchased from PerkinElmer Life And Analytical Sciences, Inc. and TGFβ1 from Invitrogen, Carlsbad, CA. ELISAs for testosterone and PSA were purchased from ALPCO (Salem, NH). Primary antibodies, α∼ERα α∼ERβ were purchased from Santa Cruz (Santa Cruz, CA), and α∼GAPDH, from Advanced ImmunoChemical Inc. (Long Beach, CA). Primary prostate stroma (6S) cells and LAPC-4 were procured as previously reported [2]. Additional lots of isolated primary stromal cells, 6B and 6C,were isolated from radical prostatectomy specimens in collaboration with Dr. Peter Pinto (at NCI-NIH) as described previously [15], and used in selected experiments (pure agonists) to validate stromal effects (data not shown).

### 2.1. Cell culture media

*Growth media*: The growth medium was based on DMEM/F12 (1/1) supplemented with 5% fetal bovine serum and a mixture of penicillin (100 units/mL)/streptomycin (100 μg/mL)/glutamine (292 μg/mL; Invitrogen).

*Treatment media*: The experimental medium consisted of a 1:1 mixture of medium F12 and medium 199 supplemented with 2% charcoal/dextran treated FBS and mixture of penicillin (100 units/mL)/streptomycin (100 μg/mL).

### 2.2. Economization of the co-culture of 6S and LAPC-4 cells to a 96-well-plate format

To study the impact of estrogenic compounds on the (reactive) stroma and/or on prostatic adenocarcinoma cells, we used a contact co-culture system consisting of either 6S, 6B or 6C human primary prostatic stroma cells and LAPC4 prostatic adenocarcinoma cells. As we previously reported, the stromal tissue compartment is induced to be "reactive" by a proinflammatory stimulus in the presence of 40 pM (1 ng/mL) of TGFβ1 [2]. Following reactive transformation the stroma responds with an increased capacity to metabolize DHEA to androstenedione and testosterone which in turn stimulate an increased secretion of PSA by the epithelial tissue compartment, the latter being measured by ELISA.

The protocol was a miniaturized version of the assay previously reported [2] and optimized to a 96-well-plate format for testing multiple natural compounds at multiple doses for anti-androgenic activity in this test system. The experimental procedures were as follows: 6S and LAPC4 cells were precultured to confluency in growth media. 10,000 stromal cells and/or 50,000 LAPC4 cells were plated using 100 μL each cell suspension in treatment medium per well in a 96-well-plate (total 200 μL). After 24 h, 100 μL medium per well of the 96-well-plate was removed (using a multipipette, which minimizes cell displacement) and carefully replaced by 100 μL of medium containing a 2× concentration solution of TGFβ1 at 80 pM/L (2 ng/mL) (final concentration in the test was 40 pM), except for 12 wells reserved for the controls which received fresh treatment medium.

On day 2, cultures were treated with the steroid hormones, including the solvent control (ethanol or DMSO), R1881 (1 nM; positive control) or DHEA (100 nM). The test compounds were also added to the other wells containing cells pretreated with TGFβ1 at doses between 0.1 and 10 μM if not otherwise stated. All experimental measurements were set up in quadruplicates. The addition of treatment components was optimized for the EPI bioassay. For DHEA treatment 50 μL of a 500 nM solution of DHEA (5× – final concentration – 100 μM) in incubation medium were added to all wells except for those eight intended to be used for the solvent control and the positive control R1881, which received 50 μL of treatment medium. Thereafter 2.5 μL of 100-fold concentrated test substances were administered, thereby ensuring that the last dilution step at least contained 10% incubation medium so that the amount of organic solvent did not exceed 0.1%, a dosage which has been shown to be safe regarding toxicity of the solvent in large scale cell culture experiments. The solvent for the ER agonists or $E_2$ was ethanol while DMSO was used as solvent for GEN, DAI and EQ. The treated co-cultures were incubated for three days. Thereafter 175 μL of the supernatant of each well was transferred to 96-well PCR plates (Applied Biosystems) preferentially for immediate for further analysis of both PSA and Testosterone by ELISA or alternatively for storage at −70 °C.

To be able to discriminate whether and to which degree estrogenic test substances indeed interfere with DHEA conversion similar experiments with a non-metabolizable androgen were performed. Experimentally this approach exactly mimicked the protocol described above except that no DHEA or TGF-β were added to the cultures instead was replaced by 1 nM of the pure AR agonist R1881 which directly induces PSA secretion from LAPC4 adenocarcinoma cells. A potential effect by any of the test substances under

these conditions should primarily be attributable to those steroid receptors which are expressed in LAPC4 cells namely AR and ERβ, and for 6S stromal cells – AR, ERα.

### 2.3. Western blot analysis of stromal and LAPC4 cell expression of ERα and ERβ

6S or LAPC4cells were plated in monocultures at a density of $5 \times 10^5$/well on 6-well plates, Cells were then grown in treatment media containing 2% CDS for 2 days, and treated with 10 μM R1881 or 40 pM TGFβ1 + 100 μM DHEA and allowed to culture for 4 more days. Protein was extracted from cells and analyzed by Western blot for expression of ERα and ERβ antibodies from Santa Cruz (Santa Cruz, CA), and GAPDH, from Advanced ImmunoChemical Inc. (Long Beach, CA) as reported previously [15].

### 2.4. Androgen receptor yeast expression assay

To assess that androgenic bioactivity was derived from reactive stroma without any influence of the epithelial tissue compartment an alternative determination of androgenic activity was made by analysing conditioned media from stromal cell monocultures in an androgen-inducible AR screening assay as previously described [16]. 6S stromal cells were plated in 24-well plates in the presence of 700 μl medium. Treatment was performed as described for the co-culture; however volumes were adjusted to the amount of medium used. Specifically cells were treated with 10 nM DHT (positive control), DMSO, 100 nM or 1uM DHEA with additional 40 or 100 pM TGFβ1. Thereafter, cultures were treated and processed as described for co-cultures. At the end of the culture period 600 μl of cell culture supernatants were collected, extracted with 2.4 ml ether. To monitor the extraction procedure a 10 nM solution of DHT was prepared in cell culture medium and processed analogous to test samples. Extracts were completely evaporated to dryness and dissolved in 60 μl of yeast basal medium. Androgenic activities were measured in triplicate thereby adding 20 μl each of the dissolved extract material to the AR yeast strain stably transfected with an AR construct as well as an expression plasmid carrying androgen-responsive sequences controlling the reporter gene *LacZ* encoding for β-galactosidase. Androgenic activities were measured against a DHT and DHEA standard ($10^{-11}$–$10^{-5}$ M each) used for calibration by induction of β-galactosidase hydrolysis of chlorophenol red β-D-galactopyranoside and read by spectrophotometer at 565 nm in a colorimetric assay.

### 2.5. Analytic measurements

PSA protein and steroid testosterone production were measured in conditioned media collected from the experiments using commercial ELISA kits (ALPCO, Salem, NH) according to manufacturers' instructions with one modification. The assay was tailored for analyzing50 μL of human serum. However, if PSA or testosterone is measured from cell culture supernatants, the kits tolerate 100 μL of supernatant without changing any other experimental steps of the test kits.

### 2.6. Rhodanile Blue staining for cell number assessment

As the cells in this model are grown in contact coculture it was difficult to distinguish stromal from epithelial cell activities, especially for any possible toxicity from treatments. To assess the relative cell number of LAPC4 adenocarcinoma cells, a microplate assay for selective measurement of growth of epithelial tumor cells in direct coculture with stromal cells was applied. Following removal of the cell culture medium for ELISA analysis, co-cultures were immediately and preferentially stained for epithelial cells. Cultures were fixed with 50 μL of freshly made 5% glutaraldehyde for 20 min. (a 10× solution can be used for initial fixation if remaining culture media exists to minimize cell detachment). Culture dishes were handled and pipetted carefully, so as to minimize LAPC4 cells sloughing off plate. Following removal of the fixative cells were gently washed 3 times with tap water, blotted on a paper towel, and the plate was allowed to dry completely. Rhodanile Blue [17] was prepared fresh for each use, dissolved at 0.2% in distilled water and pre-centrifuged to remove stain crystals. Cells were stained for 15 min with 50 μL dye per well. The staining solution was discarded and plates were washed at least three times with epithelial cells remaining stained while background staining of the stromal cells diffused (use microscope to check each wash). After drying the washed plates completely, Rhodanile Blue was eluted from stained cells by adding 100 μL per well of ethanol. Plates were read and evaluated using a spectrophotometer at 550 nm and the Softmax Pro program (Molecular Devices, Sunnyvale, CA.) This procedure enabled the semiquantitative measurement of growth of epithelial tumor cells in the presence of respective stroma cells.

### 2.7. Statistics

All experiments were performed on quadruplicate samples with at least three independent repeats. Results are presented as mean and standard deviation (STDEV). Statistical analysis was performed by ANOVA (one-way) followed by Bonferroni *post hoc* test in order to determine significant differences ($p \leq 0.05$). Probability designations are as follows: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ (for all effects compared to solvent control). $^{+}p \leq 0.05$, $^{++}p \leq 0.01$, $^{+++}p \leq 0.001$ (for all effects compared to non-estrogen treated positive control either D + T or R1881 only).

## 3. Results

Soy isoflavones genistein, diadzein and equol were compared to estradiol and the pure estrogen receptor agonists PPT and DPN for their ability to reduce androgenic activity in prostate cocultures. The purpose of the study was to investigate the role of reactive stroma in this process and to validate the EPI bioassay for its ability to elaborate these mechanisms. PSA secretion by LAPC4 prostatic adenocarcinoma cells in coculture was used as a measure of androgen activity and was induced directly by R1881 or indirectly by TGFβ1-induced reactive stromal cells treated with DHEA.

### 3.1. LAPC-4 cell growth measured in cocultures

To assess the potential differences of LAPC-4 cell growth from treatment on the biochemical measurements of PSA and testosterone, LAPC-4 cell numbers in contact coculture with stromal cells, was semiquantitatively measured by Rhodanile Blue stain using a protocol which preferentially stains the epithelial cells with minimal to no background staining from the stromal cells (Fig. 1). Representative photomicrographs of co-cultures are shown from the treatments with solvent control, (Fig. 1A), DHEA (D) (Fig. 1B), and DHEA + TGFβ1 (D + T) (Fig. 1C). Absorbance of eluted stain represents comparative measurements of the numbers of LAPC-4 cells treated with R1881, DHEA, D + T, and D + T plus increasing doses of $E_2$, PPT, DPN (Fig. 1D). No significant changes in eluted stain absorbance were observed between control and any of the treatments during the three day experimental period, reflecting no changes in growth ofLAPC4 cells with steroid or ER agonist treatment.

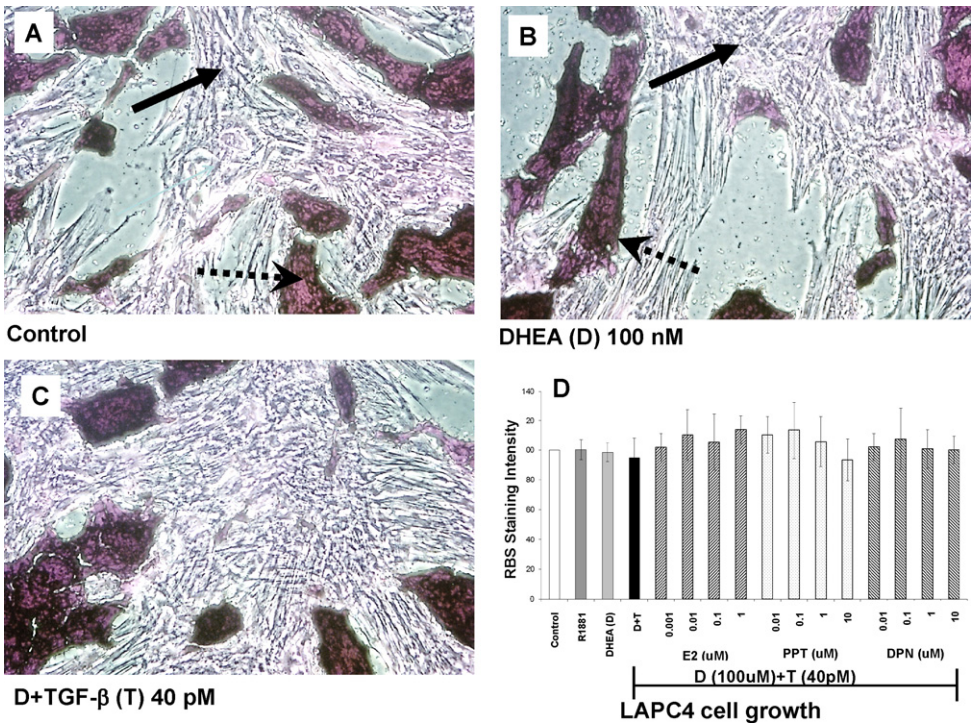


**Fig. 1.** Growth of LAPC-4 cells in co-culture: 6S stromal prostate cells and LAPC-4 cells were co-cultured, fixed and stained with Rhodanile Blue as described in Section 2. Representative figures are shown for control and treatment conditions (A–C). Following de-staining of the stromal cell compartment (solid arrows) a dark red staining of epithelial cell clusters persists (dashed arrows). Absorbance of dye eluted by ethanol extraction represents a semiquantitative measure of LAPC-4 prostate cancer cell number (D). Graph represents 3 experiments performed in quadruplicate.

### 3.2. Direct vs indirect induction of PSA secretion

As indicated above a direct (pure androgen, R1881) and an indirect mechanism (stromal metabolism of DHEA to androgens) were compared for induction of PSA secretion from LAPC4 cells. Following treatment, cell culture supernatants were collected from cultures treated with solvent control, D, D + T or R1881, and assayed using PSA ELISA. As shown in Fig. 2A PSA secretion was increased by 4.7 fold in DHEA-treated co-cultures compared to control. When stromal cells were induced to be reactive by TGFβ1 24 h prior to

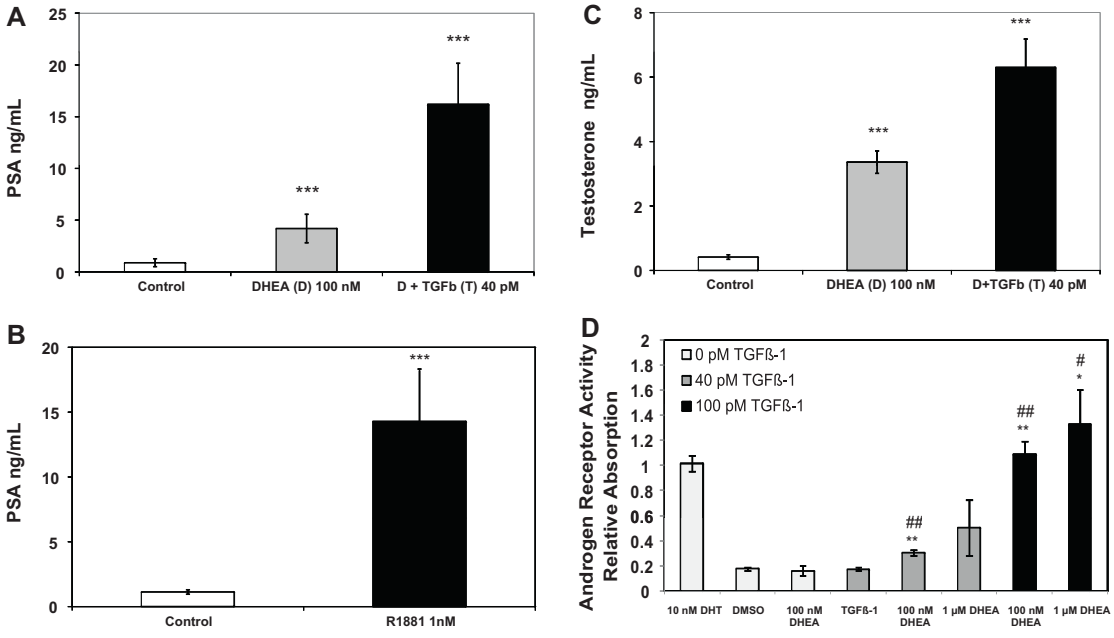


**Fig. 2.** PSA and testosterone production in miniaturized stroma-epithelial cocultures following co-administration of DHEA + TGFβ1 and PSA production in response to R1881. Cell culture supernatants were collected from contact co-cultures treated with solvent control, D, D + T or R1881, and assayed using ELISA for PSA or testosterone. PSA production by LAPC4 cells was inducible by androgens derived from stromal conversion of DHEA and further induced by TGFβ1 (A) or by the pure AR agonist R1881 (B). Testosterone production in contact cocultures was increased in presence of DHEA plus 40 pM TGFβ1 over DHEA alone (C). $^{*}p \leq 0.05$, $^{**}0.01 \leq p \leq 0.001$, $^{***}p \leq 0.001$. Androgenic activity was also detected in a yeast AR reporter assay by measuring activity of conditioned media from stromal cell monocultures treated with 100 nM and 1 μM DHEA and 40 pM and 100 pM TGFbeta. (D) All experiments represented were performed three times using quadruplicate samples per data point.

treatment with DHEA, there was an additional 4 fold increase in PSA production in a cocultured epithelial cells. PSA secretion was also stimulated directly in these cocultures by the androgen receptor agonist R1881. A dosage of 1 nM induced an approximately 10 fold increase in PSA secretion into the cell culture medium (Fig. 2 B).

Conditioned media was also assayed for testosterone using ELISA. As can be seen from Fig. 2C cocultures treated with DHEA produced 3.4 ng/mL testosterone which was increased to 6.3 ng/mL in D + T treated cultures. This indicates the increase in PSA production is associated with increased testosterone production found in same samples. These findings confirm our previously reported results (4, 8) but this time were acquired from this novel, convenient, cost-and time saving EPI bioassay.

To measure androgenic activity produced by the stromal tissue compartment independent of any epithelial influence, we assayed conditioned media from stromal cells in monoculture using an androgen receptor yeast reporter assay (Fig. 4D). 6S stromal cells were treated with 10 nM DHT (positive control), DMSO, DHEA with additional 40 or 100 pM TGFβ1. Absorbance is expressed relative to DHT. No activity is expressed in treatments with DMSO control, DHEA or TGFβ1 alone. Cells treated with the combination of DHEA + TGFβ1 show a dose responsive increase in androgenic activity, with either increasing doses of DHEA or of TGFβ1. This result indicates that reactive stromal activity at least to a large degree is independent from reciprocal epithelial influences.

### 3.3. PSA expression and testosterone formation in the co-cultures treated with ER agonists

To test whether and how estrogens modulate PSA production in the above mentioned experimental model, estrogenic effects were evaluated in a receptor selective manner. In addition to either D + T or R1881 we treated cocultures with the pure ER agonists PPT (ERα) and DPN (ERβ) in comparison to estradiol (Fig. 3A). As can be seen all estrogenic ligands tested inhibited DHEA/TGFβ1(Fig. 3A) as well asR1881-induced PSA secretion (Fig. 3B) in a dose dependent manner. The agonists were tested up to 10 μM while highest dose of $E_2$ was 1 μM. PPT, DPN and $E_2$ at 1 μM inhibited D + T-induced PSA production by 74, 85 and 92% respectively ($p < 0.01$) PPT, DPN and $E_2$ at 1 μM inhibited R1881-induced PSA production by 65, 63 and 90% respectively. These results were replicated using additional primary stromal cell lots (data not shown).

As absolute values measured from the two experimental approaches to induce PSA production (D + T- *vs* R1881-induced) differed in their magnitude it was difficult to obtain a comparison about the relative potency of estrogens in the two experimental approaches. This is important because androgens produced following treatment of cells with DHEA + TGFβ1 most likely were produced by the stromal metabolism of DHEA and function predominantly in a paracrine manner in the epithelium [2]. On the contrary, PSA production in response to R1881 to a large degree represents a direct receptor effect, either on stromal or epithelial

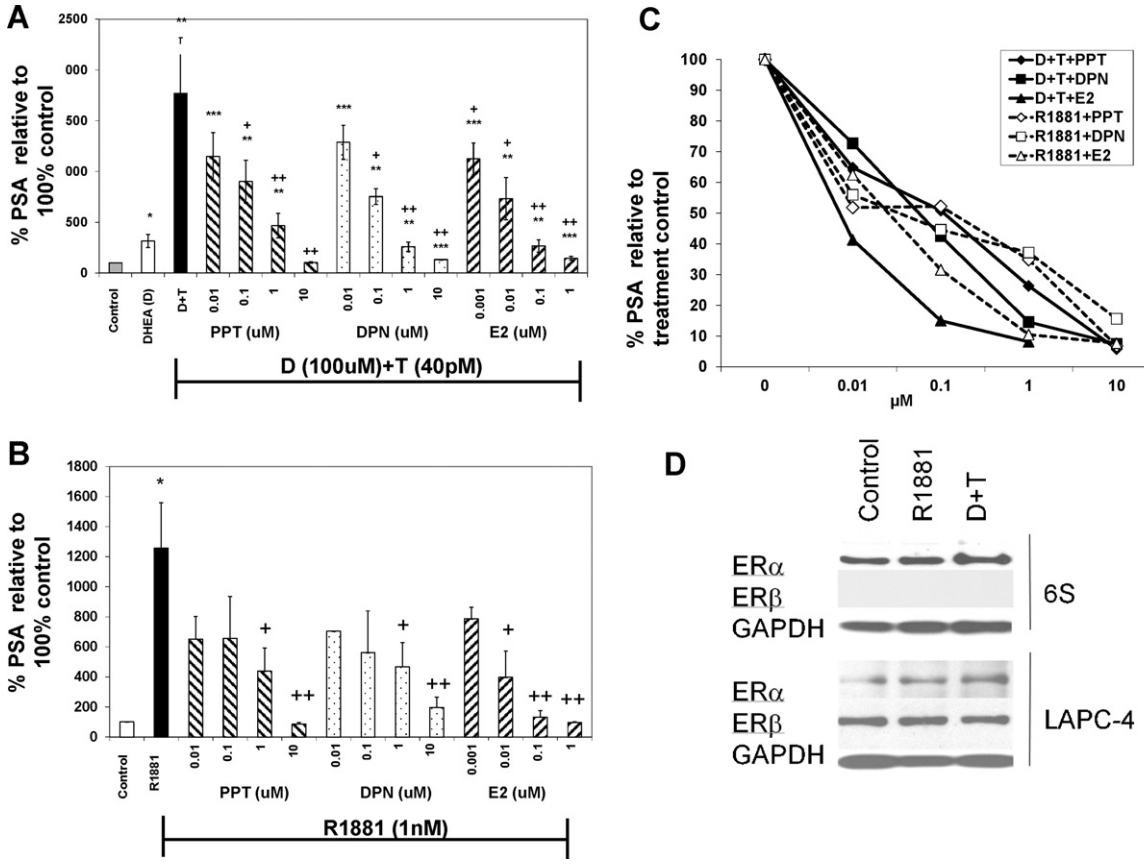


**Fig. 3.** Estrogenic ligands inhibit PSA secretion in co-cultured 6S prostatic stroma and LAPC4 adenocarcinoma cells. Treatment of co-cultures with estradiol or the pure estrogen receptor agonists PPT (ERα) and DPN (ERβ) decreased D + T- (A) or R1881 (R)- (B) induced PSA production. (C) Comparative graph shows similar trend of PSA inhibition for all agonist treatments whether induced by D + T or R1881(R). (D) Expression of ERα and ERβ are shown in Western Blots of 6S and LAPC-4 cells treated with R1881 or D + T as compared to control. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ (for all effects compared to solvent control). $^{+}p \leq 0.05$, $^{++}p \leq 0.01$, $^{+++}p \leq 0.001$ (for all effects compared to non-estrogen treated positive control either D + T or R1881 only). Graphs represent data from experiments that were performed three times using quadruplicate samples per data point.

cells, and does not include either induction of reactive stroma or metabolism of DHEA. To overcome the difficulty of comparing the relative magnitude of the response of treatment with estrogenic ligands in D + T *vs* R1881-induction of PSA, respective controls were set at 100% and all other values measured as a percent change from the positive controls (D + T or R1881). Using this procedure it appears that $E_2$ is more potent in both steroid experiments than the ERα agonist PPT (Fig. 3C) or the ERβ agonist DPN but there is not a strong distinction between treatments of D + T treated and R1881 for each of the agonists.

Expression for ERα and ERβ in both 6S and LAPC4 cells was determined using Western blot for respective receptors (Fig. 3D). While ERα and ERβ were present in LAPC-4 cells, the stromal cells expressed only the ERα. There was no detectible ERβ in the stromal cells by Western blot or by qPCR (data not shown). The slight increase of ERα expression by D + T treatment in both cell types is an interesting result that is beyond the scope of this project and can be further explored.

Treatment with PPT, DPN and $E_2$ also inhibited testosterone production in cocultures treated with D + T in a dose-dependent manner (Fig. 4A).The agonists were tested up to 10 μM while highest dose of $E_2$ was 1 μM. PPT, DPN and $E_2$ at 1 μM inhibited D + T-induced testosterone production by 19, 41 and 38% ($p < 0.001$ respectively). PPT and DPN at 10 μM inhibited testosterone up to 67 and 62% ($p < 0.001$ respectively). The relative inhibitory effect was very similar at each dose by all agonists as determined by calculating data relative to the positive control (D + T) (Fig. 4B).

### 3.4. Soy isoflavones inhibit PSA production by LAPC4 cells in stromal cocultures

Co-cultures of human 6S stroma cells and LAPC4 adenocarcinoma cells were prepared in 96-well plates as described in Section 2, pretreated with 40 pM TGFβ1 and incubated with 100 nM DHEA plus the soy isoflavones genistein (GEN), daidzein, (DAI) or the daidzein metabolite, equol (EQ) at 0.1, 1 or 10 μM. In cultures treated with D + T, PSA expression was roughly 4 fold over DHEA alone ($p \leq 0.001$). The isoflavones showed a significant and dose dependent inhibition of D + T-induced PSA production in the LAPC4 cells (Fig. 5A). GEN and DAI were almost equally potent in this

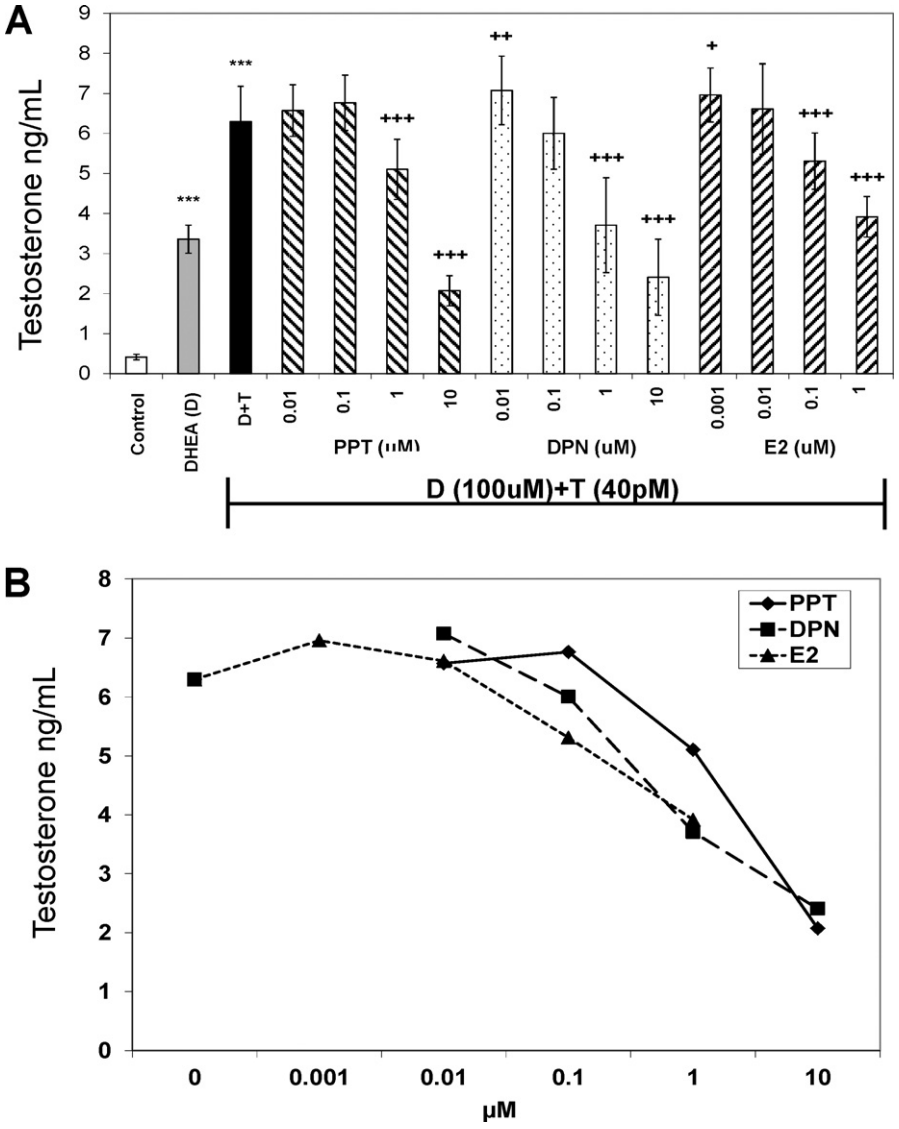


**Fig. 4.** Testosterone production in PPT, DPN or $E_2$ treated cocultures. Treatment of co-cultures with estradiol or the pure estrogen receptor agonists PPT (ERα) and DPN (ERβ) attenuated D + T testosterone production in dose responsive manner (A) with comparative inhibition from PPT *vs* DPN and E2 showing no significant differences in activity expressed in (B). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ (for all effects compared to solvent control). $^{+}p \leq 0.05$, $^{++}p \leq 0.01$, $^{+++}p \leq 0.001$ (for all effects compared to non-estrogen treated positive control either D + T or R1881 only). Graphs represent 3 experiments performed in quadruplicate.

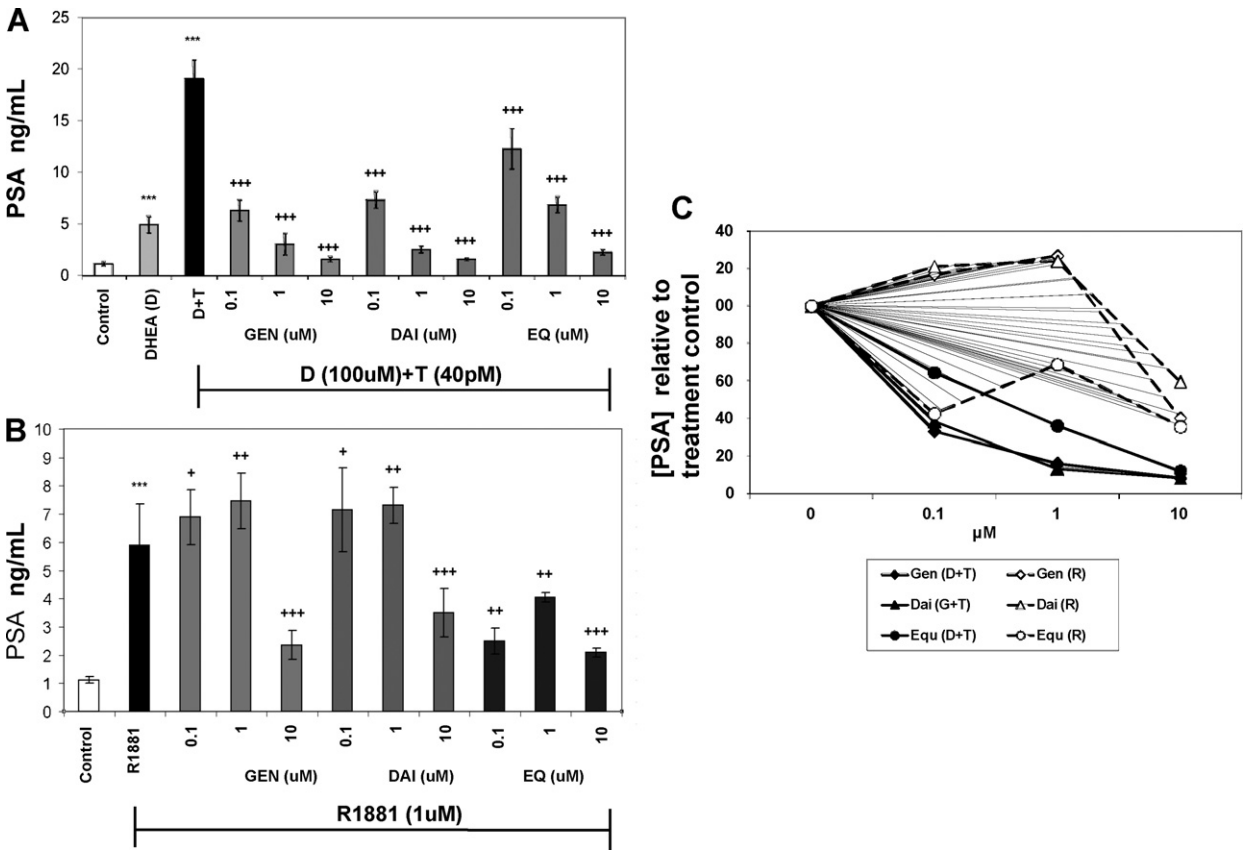


**Fig. 5.** Impact of the isoflavones genistein (GEN), daidzein (DAI) and of equol (EQ) on PSA secretion in co-cultured 6S prostatic stroma and LAPC4 adenocarcinoma cells. Treatment of co-cultures with GEN, DAI and EQ strongly suppressed D+T-induced PSA production-(A). In R1881 treated cultures, (B) a biphasic response pattern was detectable in GEN and DAI treatments where PSA production was increased at low concentrations and inhibited at the highest dose of GEN or DAI tested. Treatment of co-cultures with EQ at all doses tested suppressed R1881-induced PSA production (B). For GEN and DAI a clear dissociation of potency regarding the inhibition from DHEA and TGFβ1-induced PSA *vs* R1881-induced PSA production became apparent. This dissociation was by far less pronounced for EQ (C). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ (for all effects compared to solvent control). $^{+}p \leq 0.05$, $^{++}p \leq 0.01$, $^{+++}p \leq 0.001$ (for all effects compared to non-estrogen treated positive control either D+T or R1881 only). Graph represents 3 experiments performed in quadruplicate.

assay with inhibition at 0.1, 1 and 10 μM by GEN at 67, 84, 92% and by DAI at 62,87, and 92%respectively, compared to D+T ($p \leq 0.001$ for all points). EQ, the daidzein metabolite, appeared to be slightly less potent with inhibition at 36, 64 and 88% at 0.1, 1 and 10 μM respectively ($p \leq 0.001$ for all points).

On the contrary, in cultures treated with R1881, EQ inhibited PSA production at 58% ($p \leq 0.01$), 32 ($p \leq 0.01$), and 65% ($p \leq 0.001$) compared to R1881 alone (Fig. 5B). GEN and DAI inhibited R1881-induced PSA production only at 10 μM doses with 60% and 41% inhibition respectively ($p \leq 0.001$ for all points). At the lower doses of 0.1+1.0 μM GEN and DAI ($p \leq 0.05$; $p \leq 0.01$ respectively) augmented the R1881-induced increase in PSA production pointing to a potentially additive effect on PSA production.

This data is also represented in Fig. 5C to show the relative differences between isoflavone inhibition of D+T- *vs* R1881-induced PSA as was calculated as described above for the pure agonist treatments. This analysis illustrates greater inhibition is provided by the isoflavones in the D+T group in the context of stromal mediated DHEA metabolism and distinguishes the secondary androgenic effect from the direct androgenic effect (R1881).

## 4. Discussion

In an *in vitro* reconstruction of prostate microenvironment we investigated the influence of soy isoflavones and estrogenic agonists on stromal androgen metabolism and induced epithelial PSA secretion. The EPI bioassay is a novel down-scaled coculture protocol that is economical in time and cost for identifying natural compounds. It can address the multiple mechanisms that interact on tissue microenvironmental level including direct effects on alteration of androgen metabolism and activity or indirect effects on androgen metabolism, interfering with paracrine interactions and reversing the progression of reactive stromal in the prostate stromal–epithelial microenvironment. Our results provide evidence that estrogens and plant-derived secondary metabolites with estrogenic activity, particularly soy isoflavones, are natural inhibitors of androgenic metabolism and/or paracrine effects involved in regulation of androgen metabolism *in vitro*. The Rhodanile Blue staining of the cocultures allowed normalization of ELISA data with relative measurements of cell numbers [17]. The growth results of LAPC4 cells treated with steroid hormones and ER agonists presented in Fig. 1D are similar to data from earlier studies showing no change in growth with these hormones in these cells during the observed time period [18].

Estrogens and natural compounds with estrogen-like activities (so-called phytoestrogens) have been implicated with the treatment and/or prevention of prostate cancer. There is unequivocal evidence of prostate cancer chemoprevention in prostate rodent cancer models in regard to exposure to soy isoflavones [19–23]. Epidemiological and preclinical evidence suggests that soy isoflavones may have the potential to reduce prostate cancer risk as evidenced by two recent meta-analyses aiming to associate soy consumption with prostate cancer risk [24,25]. Additional evidence linking isoflavone consumption to reduced prostate cancer risk includes a positive association of urinary isoflavone excretion with reduced prostate cancer risk [10] and decreased serum DHT and free

testosterone levels in, healthy men supplemented with soy isoflavones for 3 months decreases in [26]. However, data on the influence of dietary isoflavone exposure and PSA levels [14] or prostate cancer metastasis are inconsistent since mouse xenograft models with human prostate cancer cells yielded inconsistent results [27,28].

Importantly, mechanistic data are missing particularly regarding the roles of estrogens in prostate cancer prevention. It is well established that most phytoestrogens preferentially bind to and activate the ERβ [29], although investigations in animal models using either ER knock-out mice or pure ER agonists suggest that soy isoflavones also trigger ERα-dependent mechanisms [30,31,32]. We hypothesize that one of the driving forces in prostate cancer progression is local androgen production by metabolism of endogenous DHEA in the tumor-associated stroma following reactive transformation of the stromal cells. We have shown that within TGFβ-1 induced reactive stroma cells, the expression of 3β- and 17β-hydroxysteroid dehydrogenases (3β- and 17β-HSD) is up-regulated thereby increasing androgen production from DHEA within this tissue compartment [33]. Recently we have reported a nongenomic mechanism involving the TGFβ receptor where red clover isoflavones decreased TGFβ1-induced up-regulation of aggregates of 3β-HSD, therefore altering the HSD activity [34]. While phytoestrogens can inhibit HSD expression [35] little if anything is known whether estrogens interfere with the induction of reactive stroma in prostate tissues.

We have demonstrated here that all estrogenic compounds tested inhibited DHEA + TGFβ1-induced testosterone production and PSA secretion into the conditioned media of the cocultured cells. This finding holds for $E_2$, PPT and DPN as pure agonists for ERα and ERβ as well as particularly for the soy isoflavones genistein and daidzein including the major daidzein metabolite EQ. Results were verified in cocultures using additional lots of primary stromal cells (6B and 6C) with similar results (data not included). Inhibition of production of testosterone by the cocultures is one means by which ER agonists may inhibit PSA production. This inhibitory response is similar to that in previous reports where the ER antagonist ICI 180,172 [36] or red clover extracts inhibited AR-driven pathways [2].

The precise mechanism whereby the activation of ERβ or ERα by their selective agonists or the soy isoflavones leads to decreases in testosterone and PSA production in these cocultures is still under investigation. However, a few clues can be obtained from the co-culture system in which PSA production and secretion was stimulated in two different ways: (a) by direct stimulation with the non-metabolizable androgen R1881 which can activate AR of both stromal and LAPC4 cells, and (b) by indirect stimulation from co-culturing stromal and epithelial cells with D + T. DHEA does not activate the AR of LAPC-4 cells [36] therefore any effects are mediated by the stroma where TGFβ1 stimulates conversion of the prohormone DHEA to androgens.

No significant differences were seen between inhibition of D + T-induced or R1881-induced PSA by treatment with PPT *vs* DPN (or $E_2$) (Fig. 3C), indicating that this inhibition was predominated by the steroid receptor response. There were significant differences in PSA inhibition between D + T-induced and R1881-induced cultures by treatment with GEN and DAI (Fig. 5C) with the D + T effect being more pronounced. This indicates that the isoflavone effect included a steroid receptor response plus additional paracrine factors that may be involved in TGFβ-induced induction or maintenance of reactive stroma.

In the light of these two experimental approaches a number of effects can be discriminated pointing to different modes of action. First, apparently both ERα and ERβ are necessary to obtain the response, since both ER subtype agonists were able to attenuate PSA production. Second, the ERβ agonist DPN inhibited stromal testosterone production in the cocultures although the stromal cells do not express ERβ. Third and finally, the most striking result from our study is the observation that the soy isoflavones were very potent inhibitors of PSA production induced by D + T, whereas GEN and DAI showed a biphasic response pattern in the presence of R1881, as low doses (0.1 and 1 μM) were co-stimulatory to R1881-induced PSA production while the high dose (10 μM) evoked a strong inhibitory response. This also suggests an ERβ selective response, as soy isoflavones preferentially interact with ERβ [18]. Genistein and daidzein strongly inhibited DHEA/TGFβ1-induced PSA production compared to R1881-induced PSA production, while co-treatment with EQ inhibited PSA secretion and showed less DHEA metabolism than genistein and daidzein. Interestingly, all doses of EQ strongly inhibited R1881-induced PSA production.

ERβ is selectively expressed in LAPC4 cells (Fig. 3D) suggesting the response to ERβ agonists arise from the epithelial tissue compartment. Both by Western blot (Fig. 3D) and qPCR (data not shown) we failed to identify ERβ expression in the 6S prostate stroma cells, although in other studies ERβ was detectable in stromal cell cultures derived from benign prostate hyperplasia [37]. ERβ has been found in human fetal tissues in the stroma [38], but ERβ expression has not been detected in adult prostate stroma in situ [39] or rat prostate stroma [40].

The stromal cells express ERα [41] and are principal in the TGFβ1-stimulated conversion of DHEA to androgens as seen in the positive androgenic results from the yeast AR reporter assay of stromal conditioned media (Fig. 2D). This suggests that the decrease in androgen production and PSA secretion in response to estrogenic compounds or isoflavones either involves ERα function, as seen in the effect following PPT treatment, or is mediated through other unknown non-receptor mediated mechanisms. This is a likely option for soy isoflavones as numerous ER-independent activities have been described including the inhibition of the steroidogenic enzymes. It is known that isoflavones inhibit 3β- and 17β-HSDs at μM concentrations [35,42], and can be more potent inhibitors of 3β- than 17β-HSD activities [43]. EQ was found to be a much weaker enzyme inhibitor than genistein in testicular cells [43], which is similar to our finding of weaker activity of EQ in the prostate co-culture system.

The interaction between ERα and ERβ within stromal and epithelial cells is a fertile ground for future studies. It is difficult to resolve the attenuation of the epithelial PSA production by the pure ERα agonist PPT, targeting the stromal ERα, which is indicative of an ERα dependent process. The molecular basis of this is as yet unknown, particularly as neither co-treatment with the ER antagonist fulvestrant nor with the AR antagonists casodex or flutamide reversed the effects of DPN or PPT in these cocultures (data not shown). Added to these previous results from our laboratory that treatment of LAPC4 or LNCaP monocultures with fulvestrant or casodex resulted in similar inhibition of PSA secretion [44]. That study found that ERβ could be co-immunoprecipitated with AR and postulated an interaction or "cross-talk" between the AR and ERβ. However, we also reported that treatment with DPN and PPT did not inhibit androgen-induced PSA in the LAPC4 monocultures [44] further indicating the importance of the stromal cells mediating the agonists effects. Use of the ERα specific antagonist, MPP or raloxifen, or yet to be identified ERβ-specific antagonists will help unravel these mechanisms. Finally, these inhibitory activities may be due to downstream effects of activation of the ERα or ERβ. In a rat mammary model treated with genistein, several downstream proteins were identified including a time dependent increase of annexin A2, Gelsolin, and Fetuin B and decrease of VEGF-R2 and EGFR [45]. Similar work in human prostate will be helpful.

In summary, both natural (estradiol, GEN, DAI or EQ) and synthetic (PPT or DPN) agonist activation of ERα or ERβ interfered with AR pathways of stromal testosterone production and

epithelial PSA secretion. Using this novel test system we concluded the effects of these estrogenic agents on PSA production and testosterone metabolism involved two androgenic pathways. One was a direct androgen (R1881) where any effects were likely mediated through interactions with the AR and the second involved paracrine stromal products induced by androgen. This study also highlighted that the soy isoflavones were more potent inhibitors in D + T treated samples than the ER agonists, indicating that stromal-mediated inhibitor effects were additive to the receptor effects. This bioassay can identify natural or synthetic agents that affect steroid metabolism in the prostate. This model may also identify paracrine factors involved in modulation of induction of reactive stroma providing insights into targets and mechanisms for cancer prevention and provides alternatives to animal testing. Reactive stroma-induced DHEA metabolism may be an important early player in prostate physiological balance between androgens and estrogens since it can be metabolized towards the androgenic or estrogenic pathway. Hypothetically, if isoflavones are a present feature of the diet (such as in some Asian diets) and thus the prostate microenvironment, their presence may diminish the conversion of DHEA towards androgenic ligands. We found when the source of the androgen signal was D + T-treated stromal cells, there were greater inhibitory effects, suggesting these agents can also interfere with steroid metabolism enzymes or maintenance of reactive stroma or may reverse TGFβ1 effects that maintain reactive stroma. These mechanisms addressing interactions between steroid hormone, paracrine and inflammatory disciplines represent a very important and complex area of research for human prostate physiology where additional downstream effects of ERα and ERβ remain to be elaborated.

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