*Original Article*

# Androgens Upregulate Endometrial Epithelial Progesterone Receptor Expression: Potential Implications for Endometriosis

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**Samir N. Babayev, MD[1], Chan Woo Park, MD[1,2], Patrick W. Keller, BS[1], Bruce R. Carr, MD[1], Ruth A. Word, MD[1], and Orhan Bukulmez, MD[1]**

### Abstract

**Background:** Androgenic compounds have been implicated in induction of endometrial atrophy yet the mechanisms of androgen effects on human endometrium have not been well studied. We hypothesized that androgens may promote their endometrial effects via modulation of progesterone receptor (PR) expression. **Methods:** Proliferative phase endometrial samples were collected at the time of hysterectomy. We evaluated the effect of the potent androgen 5α-dihydrotestosterone (DHT) on endometrial PR expression by treating human endometrial explants, endometrial stromal cells, and Ishikawa cells with DHT. Ishikawa cells were also treated with DHT ± the androgen receptor (AR) blocker flutamide. The PR-B, total PR messenger RNA (mRNA), and PR protein expression were assessed. Expression of cyclin D1 and D2 was checked as markers of cell proliferation. **Results:** As expected, estradiol induced PR expression in isolated stromal cells, endometrial epithelial cells, and tissue explants. The DHT treatment also resulted in increased PR expression in endometrial explants and Ishikawa cells but not in stromal cells. Further, protein levels of both nuclear PR isoforms (PR-A and PR-B) were induced with the DHT treatment. Although flutamide treatment alone did not affect PR expression, flutamide diminished androgen-induced upregulation of PR in both endometrial explants and Ishikawa cells. Although estradiol induced both cyclin D1 and cyclin D2 mRNA, DHT did not induce these markers of cell proliferation. **Conclusion:** Androgens may mediate endometrial effects through upregulation of PR gene and protein expression. Endometrial PR upregulation by androgens is mediated, at least in part, through AR.

### Keywords

androgens, progesterone receptor, endometrium, endometriosis

## Introduction

Progesterone induces differentiation of stromal and epithelial cells in the endometrium to produce secretory and decidual changes. Two main nuclear isoforms of progesterone receptor (PR) mediate the genomic effects of progesterone. These 2 receptors, PR-A and PR-B, are produced from a single gene by 2 distinct promoters by translation initiation at 2 alternative sites. The PR-mediated progesterone signaling is antimitogenic in endometrial epithelial cells and, as such, mitigates the trophic effects of estrogen on eutopic normal endometrium and on ectopic implants of endometriosis.[1] Exploiting the effects of progesterone on endometrium, various progestins have been used in the medical management of endometriosis.[2]

Nevertheless, several studies indicate that progesterone action is impaired in endometriosis. Previous work has shown that PR-B is lost, PR-A is dramatically downregulated, and truncated PR isoforms are increased in endometriomas.[3,4] Part of the problem in the management of endometriosis with progestins is that they further downregulate already suppressed PRs in endometrial cells. Hence, progesterone resistance has been hypothesized to play a role in the initiation and the progression of endometriosis.[5]

Danazol is an oral derivative of testosterone with weak androgenic properties that suppresses hypothalamic–pituitary–gonadal axis and induces amenorrhea. Importantly, danazol was extremely effective in reducing endometriosis-associated pain but was poorly tolerated due to its androgenic

[1] Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, University of Texas Southwestern Medical Center, Dallas, TX, USA
[2] Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Cheil General Hospital and Women's Healthcare Center, Dankook University College of Medicine, Seoul, South Korea

**Corresponding Author:**
Orhan Bukulmez, Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390-9032, USA.
Email: orhan.bukulmez@utsouthwestern.edu

and anabolic properties.[6,7] There are limited data on the mechanisms of action of danazol and androgens in endometrium and endometriosis.

Androgens have been shown to increase PR expression in breast cancer cells, but it is unknown if similar mechanism exists in human endometrium, which has not been studied utilizing contemporary methodology.[8,9] The objective of this investigation, therefore, was to test the hypothesis that androgens may promote endometrial effects via the modulation of PR expression. To test this idea, cultured endometrial explants, human endometrial stromal cells (HESCs), and a human endometrial epithelial cell line, Ishikawa cells were used. We primarily utilized the potent androgen 5α-dihydrotestosterone (DHT) in experiments since it does not convert to estrogens or upregulate aromatase expression in endometrium unlike estradiol (E2) or aromatizable androgens such as testosterone and androstenedione.[10]

Cell cycle is regulated by the activity of cyclin-dependent kinases (CDKs). A critical point in the cycle progression through G1 phase is modulated by the expression of cyclin D1, which is associated with CDK4.[11] We also studied a proliferation marker from CDK family to compare potential proliferative effects of DHT versus E2.

## Materials and Methods

### Ishikawa Cell Culture

Ishikawa cells, a well differentiated endometrial carcinoma cell line,[12] were cultured in DMEM/F12 without phenol red media, containing 10% of fetal calf serum, 1% of antibiotic/antimycotic solution, and 0.25% of l-Glutamine (Thermo Fisher Scientific, Waltham, MA). Ishikawa cells were treated with DHT (Sigma-Aldrich, St. Louis, MO; 100 nM), danazol (Sigma-Aldrich, St. Louis, MO; 100 nM), flutamide (Sigma-Aldrich, St. Louis, MO; 1 μM), and 17β E2 (Sigma-Aldrich, St. Louis, MO; 3 nM) for 24 hours. Pure ethanol was used to dissolve all the treatment agents.

### Endometrial Explant Cultures

Proliferative phase endometrial samples were collected from 6 women of reproductive age with no evidence of endometriosis or submucosal fibroids, undergoing hysterectomy for benign reasons (eg, pelvic relaxation). The tissue collection protocol was approved by the institutional review board at University of Texas Southwestern Medical Center. Phenol red-free, serum-free DMEM/F12 medium (Thermo Fisher Scientific, Waltham, MA) with 1% antibiotic/antimycotic was used in all stages of tissue preparation and explant culture. The tissues obtained were first washed in medium and cut into uniform approximately 2 $mm^3$ pieces with a sterile scalpel blade. Then 3 to 4 tissue pieces were immediately transferred onto a triangle sieve covered with sterile filter paper in a 6-well plate. Explants were treated and incubated for 24 hours at 37°C in a humidified 95% air–5% $CO_2$ environment. Thereafter, explants were immediately placed into RNAlater solution (Thermo Fisher Scientific, Waltham, MA) and stored at −80°C. To isolate RNA, tissues were homogenized in TRIzol reagent (Thermo Fisher Scientific, Waltham, MA).

### Primary HESC Cultures

Endometrial stromal cells were isolated from tissues obtained from 3 premenopausal women (28-39 years old) using the selection criteria as mentioned above. Briefly, endometrial tissue was minced with a sterile surgical blade and digested in Hank balanced salt solution (Sigma-Aldrich, St. Louis, MO) containing collagenase B (1 mg/mL, 15 U/mg; Roche, Indianapolis, IN), deoxyribonuclease I (0.1 mg/mL, 1500 U/mg; Roche, Indianapolis, IN), penicillin (200 U/mL), and streptomycin (200 mg/mL) for 60 minutes at 37°C with agitation. The dispersed endometrial epithelial and stromal cells were separated by filtration through a 70-μm sterile cell strainer (BD Biosciences, Franklin Lakes, NJ). The filtered stromal cells were cultured in DMEM/F-12 containing fetal bovine serum (10%; Thermo Fisher Scientific, Waltham, MA) and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA) in a 95% air–5% $CO_2$ environment at 37°C. Cells were subcultured once before initiation of experiments. Consistent with previous investigations,[13,14] stromal cell purity was assessed as >98% by light microscopy. All experiments were performed in DMEM/F12 phenol red-free, serum-free medium (Sigma-Aldrich, St. Louis, MO) with routine additions of 1% antibiotic/antimycotic.

Treatments included vehicle (ethanol) and DHT (100 nM and 10 nM, dissolved in vehicle) for 24 hours. The final added volume of vehicle itself and all steroids dissolved in the vehicle were 1 μL/mL of serum-free medium (0.001% EtOH).

### Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction was used to determine relative levels of gene expression as described.[10] Briefly, reverse transcription reactions were conducted with 1 μg total RNA in a reaction volume of 20 μL. Each reaction contained 10 mM DTT, 0.5 mM dNTPs, 0.015μg/μL random primers, 40 U RNase inhibitor (Invitrogen #10777-019, Carlsbad, California), and 200 U reverse transcriptase. Reaction conditions were 10 minutes at 23°C, 60 minutes at 42°C, followed by 70°C for 15 minutes. Primer and probe sequences for amplifications were chosen using published complementary DNA sequences and the Primer Express program (Applied Biosystems, Foster City, California). Primers were chosen so that the resulting amplicons would cross an exon junction thereby eliminating false-positive signals from genomic DNA contamination. Taqman probes were used for the detection of *PR-B* amplicons, whereas SYBR Green was used for *GAPDH* and total *PR* (Table 1). Primer concentrations were 900 nM. Cycling conditions were 2 minutes at 50°C, followed by 10 minutes at 95°C, then 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. When SYBR Green was used, a preprogrammed dissociation protocol was used

**Table 1.** Sequences of Primers Used for Quantitative RT-PCR.

| Gene | | Primer Sequences |
|---|---|---|
| *GAPDH* | Forward | 5′-GGA GTC AAC GGA TTT GGT CGT A-3′ |
| | Reverse | 5′-CAA CAA TAT CCA CTT TAC CAG AGT TA-3′ |
| *PR-B* | Forward | 5′-CGG ACA CCT TGC CTG AAG TT-3′ |
| | Reverse | 5′-CAG GGC CGA GGG AAG AGT AG-3′ |
| | Probe | 6-FAM CGG CCA TAC CTA TCT CCC TGG 6-TAMRA |
| *Total PR* | Forward | 5′-AAT CAT TGC CAG GTT TTC GAA-3′ |
| | Reverse | 5′-GCC CAC TGA CAT GTT TGT AGG A-3′ |

Abbreviations: PR, progesterone receptor; RT-PCR, real-time polymerase chain reaction.

after amplification to ensure that all samples exhibited a single amplicon. Levels of messenger RNA (mRNA) were determined using the ddCt method (Applied Biosystems) and expressed relative to an external calibrator present on each plate. Cyclin D1 (CCND1[Hs00765553_m1]) and cyclin D2 (CCND2[Hs00153380_m1]) TaqMan gene expression assays were purchased from Applied Biosystems.

### Immunoblotting

Cells were lysed in RIPA buffer (50 mM Tris, 0.15 M NaCl, 0.1% SDS, 0.05% sodium deoxycholate (DOC), 1% Triton X-100, pH = 8.0) with protease inhibitors (Complete Mini, Roche #11836153001), centrifuged 10 minutes at 10 000*g*, and the supernatant/lysate was used for the Western blots.

Based on the BCA protein assay, 30 μg/lane was loaded onto 4% to 20% polyacrylamide gels. After electrophoretic separation, proteins were transferred to polyvinylidene difluoride (PVDF) membranes overnight at 4°C.

Membranes were blocked with TBST containing 5% nonfat powdered milk for 1 hour at 37°C and incubated with primary antibody of rabbit antihuman PR polyclonal antibody (Thermo Fisher Scientific, Waltham, MA, #RM-9102) 1:500 overnight at 4°C. The immunogen for this antibody was a recombinant peptide for amino acids 412-526 of the human PR thereby recognizing both nuclear PR isoforms. Thereafter, blots were incubated with a secondary antibody (goat antirabbit IgG-HRP conjugate [Bio-Rad, Hercules, CA, #170-6515], 1:10 000 at room temperature for 1 hour). Signals were detected by chemiluminescence using SuperSignal West Femto (Thermo Fisher Scientific, Waltham, MA, #34095).

For loading control calculations, the membrane was subsequently incubated with mouse anti-α-tubulin (Thermo Fisher Scientific, Waltham, MA, #32-2500) followed by a secondary antibody of goat antimouse IgG-HRP conjugate (Bio-Rad, Hercules, CA, #170-6516). Signals were detected by chemiluminescence using SuperSignal West Pico (Thermo Fisher Scientific, Waltham, MA, #34080). The digital enhanced chemiluminescence (ECL) images for quantification were obtained using a Fuji LAS 3000 imaging system. To quantify relative protein amounts, protein band density was calculated with MultiGauge software (Fuji Photo Film Co, Ltd, Tokyo, Japan) and normalized to density of the loading control (α-tubulin) which was invariant. Each blot was normalized to vehicle controls present on each blot which served as an external standard.

### Data Analysis

Data were expressed as mean ± standard error of mean. Student *t* test and 1-way analysis of variance were used as appropriate with $P < .05$ regarded as statistically significant.

## Results

### 5α-Dihydrotestosterone Induces PR Gene Expression in Dose- and Time-Dependent Manners in Endometrial Epithelial but Not Stromal Cells

We tested the hypothesis that androgens promote endometrial effects by the modulation of PR expression and determined the effects of DHT on endometrial PR expression. The HESCs and a human endometrial epithelial cell line, Ishikawa cells were used as a model for endometrial glandular cells. Although PR mRNA levels were unchanged by DHT treatment in stromal cells (Figure 1E), DHT increased mRNA expression of both total PR and PR-B in a dose- and time-dependent fashion in Ishikawa cells (Figure 1A-D). Regarding total PR expression, differences between vehicle versus 10 nM DHT ($P < .05$) and 10 nM DHT versus 100 nM DHT ($P < .05$) were significant (Figure 1A and B). Using a moderate concentration of 10 nM, DHT increased expression of total and PR-B as early as 6 hours with maximal effects at 48 hours (Figure 1C and D).

### Upregulation of PR mRNA Is Mediated via Androgen Receptor

To clarify whether DHT (100 nM)-induced upregulation of total PR and PR-B in Ishikawa cells is mediated through androgen receptor (AR), 2 approaches were taken. First, we tested whether pretreatment with an AR-specific inhibitor, flutamide blocked DHT-induced activation of PR gene expression. Second, we determined whether a nonaromatizable AR agonist (danazol 100 nM) mimicked DHT-induced effects on these cells. The DHT was more effective in induction of total PR than PR-B (Figure 2A and B). Importantly, flutamide completely blocked DHT-induced increases in total PR and PR-B mRNA (Figure 2A and B). Danazol did not increase PR-B or total PR mRNA levels in Ishikawa cells (Figure 2A and B).

### Validation of Findings at Protein Level in Ishikawa Cells

Effects of DHT and flutamide on PR were further validated using immunoblot analysis in Ishikawa cells (Figure 2C). An

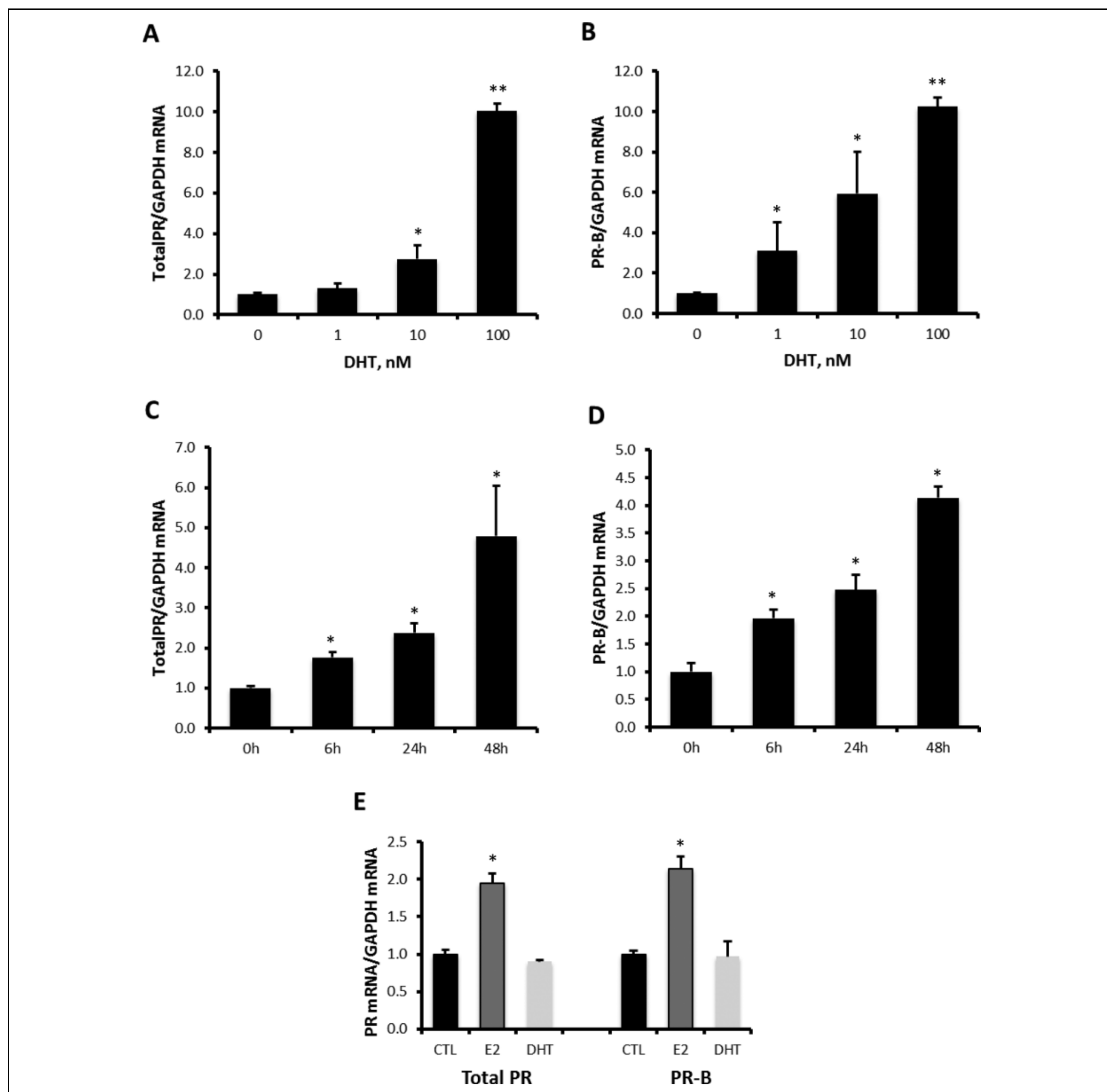


**Figure 1.** 5α-Dihydrotestosterone (DHT) dose- and time-dependently increases total progesterone receptor (PR) and PR-B messenger RNA (mRNA) in Ishikawa cells. Ishikawa cells were treated with DHT for the indicated dose and time. Ishikawa cells were treated with different doses of DHT for 48hours and analyzed for total PR (A) and PR-B (B) mRNA. The DHT (10nM)-induced increases in total PR (C) and PR-B (D) mRNA as a function of time. (E) In human endometrial stromal cells treated for 48 hours PR mRNA levels were unchanged by DHT treatment (10 nM). 17β estradiol (E2) is at 10 nM. Error bars represent standard error of mean (SEM). n = 3 in each group. *$P < .05$; **$P < .01$ compared with controls (0 nM vehicle, A and B), 0 hour (C and D).

abdominal wall endometrioma in which PR-B and PR-A were highly expressed was used as a positive control. The PR isoforms were poorly expressed in control epithelial cells, but both isoforms were induced dramatically by E2. Importantly, DHT also induced both PR-B and PR-A proteins in these cells. Flutamide alone had no effect, whereas DHT combined with E2 seemed to have synergistic effects, and flutamide inhibited DHT induction of both PR-B and PR-A proteins. Induction of truncated isoforms of PR was noted although function of these truncated isoforms expressed in some hormone-responsive cancers has remained elusive.[15] Quantification of immunoreactive PR proteins (normalized

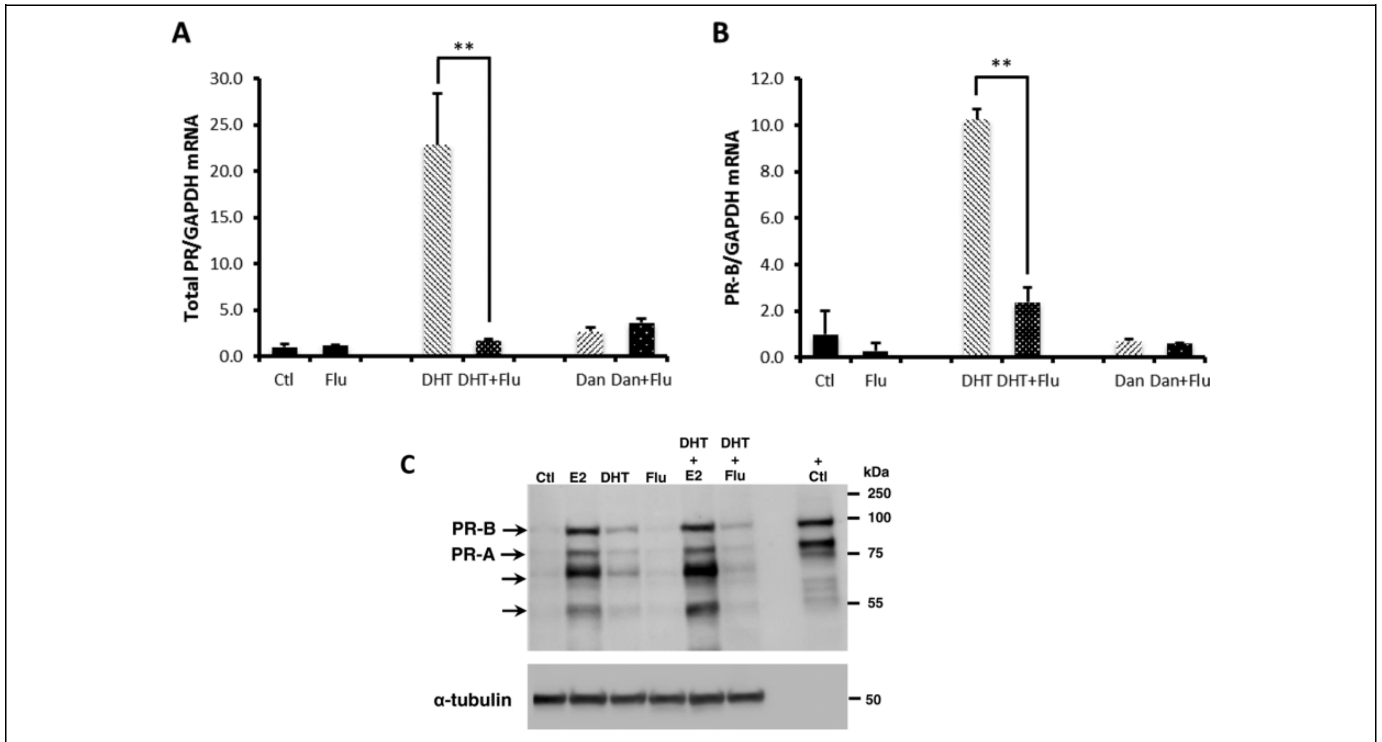


**Figure 2.** Upregulation of progesterone receptor (PR) messenger RNA (mRNA) is mediated via androgen receptor (AR). Ishikawa cells were treated with 2 different AR agonists (5α-dihydrotestosterone [DHT] 100 nM and danazol 100 nM) and AR antagonist (flutamide 1 μM) for 24 hours and analyzed for total PR (A) or PR-B (B) mRNA and PR immunoblotting (C). Positive control for immunoblotting was abdominal wall endometrioma in which PR-B and PR-A were highly expressed. Note that α-tubulin was not highly expressed in tissue relative to cells in culture. Ctl (control) indicates the treatment with 0.001% ethanol; + Ctl, positive control; E2, 17β estradiol; Flu, flutamide; →, truncated forms of PR. Error bars represent standard error of mean (SEM). n = 3 in each group. *$P < .05$; **$P < .01$ compared with controls.

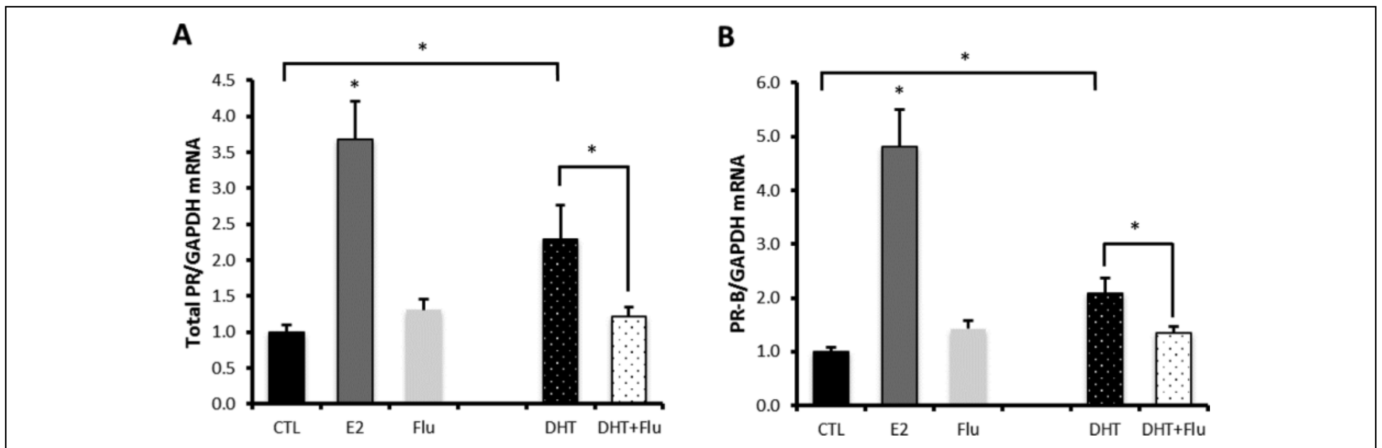


**Figure 3.** 5α-Dihydrotestosterone (DHT) increased total progesterone receptor PR (A) and PR-B (B) messenger RNA (mRNA) in endometrial explants. CTL (control) indicates the treatment with 0.001% ethanol. Human endometrial explants were treated with DHT (100 nM) versus E2 (10 nM) versus DHT (100 nM) + flutamide (100 μM) for 24 hours. E2, 17β estradiol; Flu, flutamide. Error bars represent standard error of mean (SEM). n = 3 in each group. *$P < .05$; **$P < .01$ compared with controls.

to α-tubulin) in 2 experiments indicated that DHT-induced PR-B 1.9-fold and PR-A 1.4-fold. The DHT treatment augmented E2-induced PR expression by 19% to 30%. Results were identical between 2 blots and in agreement with DHT-induced PR mRNA (Figure 1). Thus, immunoblotting was not repeated a third time for statistical analysis. Nevertheless, a type 1 error is acknowledged in interpreting the protein results.

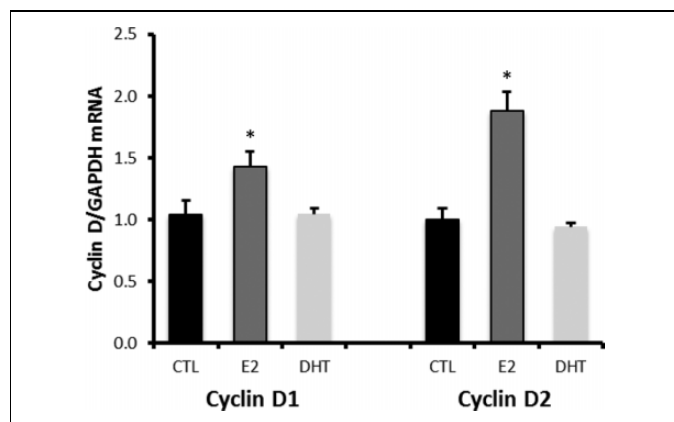


**Figure 4.** We compared the effects of estradiol and 5α-dihydrotestosterone (DHT) on expression of cyclin D1 and D2 as markers of cell proliferation. Ishikawa cells were treated for 24 hours. Although estradiol (3 nM) induced both cyclin D1 and D2, DHT (100 nM) did not. CTL (control) indicates the treatment with 0.001% ethanol; E2, 17β estradiol. Error bars represent standard error of mean (SEM). n = 3 in each group. *$P < .05$ compared with controls.

### *5α-Dihydrotestosterone Modestly Induces PR mRNA in Human Endometrial Explants*

Cultured explants were treated with vehicle versus DHT at 100 nM. E2 (10 nM) treatment was used as a positive control for PR induction. In explants, as expected, E2 induced total PR and PR-B ≈4-fold. Interestingly, DHT also induced modest, but significant, increased expression of total PR and PR-B mRNA (≈2-fold) compared with vehicle-treated controls (Figure 3A and B). Flutamide blocked DHT-induced PR expression in endometrial explants (Figure 3). Only moderate induction of PR with androgens in endometrial explant cultures as compared with Ishikawa cells is likely due to fewer number of epithelial cells relative to nonresponsive stromal cells in these tissues.

### *5α-Dihydrotestosterone Does Not Induce Cyclin D1 and D2 Expressions*

We compared the effects of E2 and DHT on expression of cyclin D1 and D2 as markers of cell proliferation in Ishikawa cells. Although E2 induced both cyclin D1 and D2 mRNA as expected, DHT did not show such effects (Figure 4).

## Discussion

Per conventional teaching, estrogen receptor (ER) and PR coregulate each other.[5] The ER binds to specific DNA sequence-termed estrogen response elements on PR promoter and thereby increases PR expression. Hence the endometrium has to be primed with estrogen, to respond to progesterone and also induce withdrawal bleeding. Progesterone, on the other hand, downregulates both ER and PR. Estradiol induces endometrial proliferation and angiogenesis acting through ER and mediators like vascular endothelial growth factor and transforming growth factor-α.[16]

The potential effects of androgens on endometrium and endometriosis have been elusive. It is known that endometrium expresses AR.[17] There have been various observations on the effects of androgens on endometrium. An earlier study reported the effects for progesterone and testosterone treatment in HESCs in culture focusing mainly on decidualization.[18] The authors reported that “testosterone inhibited PR gene expression with progesterone treatment.” It is not clear whether the authors meant progesterone cotreatment or testosterone alone decreased PR expression. Our data suggest that DHT does not influence PR expression in HESC. The differences in results can be explained by the fact that the authors used testosterone treatment on HESCs cultured for a very extended period of up to 9 days. In addition, it is not clear whether the endometrial samples were collected in follicular or luteal phase. It is possible that all these samples were from luteal phase since Noyes criteria for endometrial dating were done for every specimen. The authors also did not treat HESCs with E2 as a positive control for PR expression, and therefore, the proper response of HESCs used in that study cannot be predicted from the results. Hence, it is not known if responses of HESCs in prolonged culture to steroid hormones are similar to those in vivo or cells in short-term primary culture. Nevertheless, in agreement with previous reports, androgens did not increase PR expression in HESCs.

It has been demonstrated that maximal endometrial AR expression can be detected during the follicular phase[19]; therefore, we utilized only follicular phase samples. A possible explanation why we did not observe PR upregulation with DHT in HESCs is likely related to the inherent differences between endometrial epithelial and stromal compartments. There is no doubt that the transcriptional machinery and chromatin structure differ in stromal cells relative to glandular epithelium. In fact, unlike HESC, it is very challenging to achieve and maintain pure epithelial cell cultures from human endometrium. Thus, in this study, human endometrial explants comprised of both stromal and glandular compartments were utilized to show modest DHT-induced increases in PR expression. Ishikawa cells were simply used as a model for the epithelial component of endometrium showing robust increases in PR expression with DHT treatment. It is well known that Ishikawa cells express AR.[19] Our data, therefore, are compatible with the notion that the component of the human endometrium responsible for increased PR expression with DHT treatment is the glandular epithelium.

As mentioned, danazol was primarily approved for the treatment of endometriosis.[20] As many medical management modalities, danazol’s maximum effectiveness is seen when the amenorrhea is induced at high systemic doses that result in bothersome androgenic side effects.[20] Hence, the beneficial effects of danazol, like leuprolide acetate, have been linked to central suppression leading to ovarian suppression. However, one study including patients planned for endometrial ablation and comparing pretreatment with GnRH agonist, leuprolide acetate to danazol concluded that danazol was more effective in assuring the success of endometrial ablation by suppressing endometrial proliferation than leuprolide acetate.[21] It has also been shown that danazol treatment induces marked hypotrophy of endometrial

mucosa while treatment with GnRH agonist buserelin results in weakly proliferative or inactive endometrial mucosa.[22] Danazol has been reported to induce endometrial atrophy regardless of the systemic dose used (200-800 mg daily doses).[23] Danazol has also been shown to be effective for endometriosis-associated pain when used via vaginal route at low doses.[24,25] These studies suggest that danazol may have additional direct effects on endometrium. Considering danazol's androgenic properties, it can be speculated that androgenic properties may play a role in induction of endometrial atrophy with danazol, which has not been studied. Danazol is still considered a weak androgen with some PR agonistic properties, which might confound our results.[26] Therefore, we chose to primarily focus on the effects of the most potent androgen DHT to better define androgen effects on endometrial PR expression while also treating cells with danazol.

Endometriosis is associated with robust inflammatory response via nuclear factor κ B (NF-κB) activation as reviewed.[27,28] In fact, inflammation is accepted to be one of the core elements in pathophysiology, leading to more vascularization, adhesive disease, deep infiltration, pain, organ dysfunction, and perhaps fertility problems. It has been shown in other systems that the NF-κB activation can be suppressed by sole over expression of PR-B since PR-B can be constitutively active.[29] Hence upregulation of PR-B may have anti-inflammatory effects. Many of the effects of using progestin suppression in endometriosis-associated pain were linked to both menstrual suppressive properties and anti-inflammatory properties of progestins especially in chronic pelvic pain. The concept of progesterone resistance in endometriosis has been suggested and studied.[5,30] As mentioned, per conventional knowledge, high-dose progestin use may also lead to downregulation of PRs. Inflammation itself may also lead to perturbations of PR isoform expression in endometriosis as reported.[3,4] One prospective randomized trial found that the continuous use of combined oral contraceptive pills (OCPs) can be as effective as leuprolide acetate when used in patients with chronic pelvic pain due to endometriosis.[31] This may be due to the fact that ethinylestradiol in combined OCPs may help to enhance the effects of progestins by avoiding PR downregulation. It may also be related to the weak androgenic effects of some progestins used in OCPs. Combined OCPs may not be the best choice for patients with hypertension older than 35 years and those with increased risk for thromboembolism.[32]

This in vitro investigation should be considered a pilot proof of concept study to assess the effects of potent androgens on endometrium. The DHT as the most potent AR ligand is produced via local action of 5α-reductase, and it cannot be aromatized further. We have shown that aromatizable androgens, testosterone, and androstenedione induce aromatase expression in endometrium most possibly through conversion to E2. The DHT lacked such an effect and did not induce aromatase in endometrium.[10] Hence DHT was used to test our hypothesis.

In our study, DHT is demonstrated to increase PR expression along with its constitutively active PR-B isoform in human endometrial tissues and cells, and the cellular component that is responsible for PR induction seems to the endometrial glandular/epithelial cells rather than endometrial stroma. This effect can be mediated via AR. Although E2 induces PR expression with increased cell proliferation as evidenced by increased cyclin D1 and D2 expressions, DHT-associated induction of PR was not associated with such an effect. The DHT effects on endometrial glandular and epithelial cells may be of relevance to prevention of endometrial hyperplasia since endometrial stroma is reduced relative to epithelial gland proliferation as the condition advances to endometrial cancer.

Of note, especially total PR mRNA expression was not significantly increased with danazol in Ishikawa cells, and the potential suppressive effect of flutamide cotreatment on total PR expression on danazol exposed cells was not observed (Figure 2A and B). The DHT and danazol were used in equimolar concentrations. Therefore, danazol, as a weak AR agonist, showed subtle effects on total PR expression. Although, it is believed that, antiandrogens such as cyproterone acetate and flutamide function largely as AR antagonists, they can exhibit partial agonistic properties with ARs and other members of the steroid receptor family.[33] Emerging data indicate that antiandrogens often do not function as silent antagonists and can sometimes lack antagonist actions or even act as partial AR agonists.[34-36] Perhaps, this context-dependent partial agonistic feature of flutamide becomes more robust when used with another weak androgen agonist danazol, which might explain its failure to antagonize effects of danazol on total PR expression.

Certainly, more mechanistic studies are needed to investigate how PR expression is increased via androgens in human endometrium. To our knowledge, it is not known if PR promoter has androgen response element. Although AR seems to be instrumental in PR induction, the intermediate mediators for such effects are not known and need to be further investigated. It is clear that higher concentrations of DHT is associated with more intense PR expression, and clinically, this finding may bring the same pitfalls as danazol in terms of androgenic side effects, if used systemically. However, PR-inducing effects of strong nonaromatizable AR ligands can be exploited by investigating relevant candidate compounds. If the intermediate mediators are known, candidate compounds may be developed to induce PR expression without intense androgenic side effects. In addition, sequential and short-term use of androgenic compounds along with continuous progestins can be considered to alleviate progesterone resistance while minimizing androgenic side effects in patients with endometriosis who cannot be treated with combined OCPs or who cannot tolerate high-dose progestins.

## Declaration of Conflicting Interests

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## References

1. Patel B, Elguero S, Thakore S, Dahoud W, Bedaiwy M, Mesiano S. Role of nuclear progesterone receptor isoforms in uterine pathophysiology. *Hum Reprod Update*. 2015;21(2):155-173.

2. Cheong YC, Smotra G, Williams AC. Non-surgical interventions for the management of chronic pelvic pain. *Cochrane Database Syst Rev*. 2014;(3):CD008797.
3. Bukulmez O, Hardy DB, Carr BR, Word RA, Mendelson CR. Inflammatory status influences aromatase and steroid receptor expression in endometriosis. *Endocrinology*. 2008;149(3): 1190-1204.
4. Attia GR, Zeitoun K, Edwards D, Johns A, Carr BR, Bulun SE. Progesterone receptor isoform A but not B is expressed in endometriosis. *J Clin Endocrinol Metab*. 2000;85(8):2897-2902.
5. Kim JJ, Kurita T, Bulun SE. Progesterone action in endometrial cancer, endometriosis, uterine fibroids, and breast cancer. *Endocr Rev*. 2013;34(1):130-162.
6. Chalmers JA, Shervington PC. Danazol treatment and follow-up of patients with endometriosis. *J Int Med Res*. 1977;5(suppl 3):72-74.
7. Dmowski WP, Scholer HF, Mahesh VB, Greenblatt RB. Danazol—a synthetic steroid derivative with interesting physiologic properties. *Fertil Steril*. 1971;22(1):9-18.
8. Liberato MH, Sonohara S, Brentani MM. Effects of androgens on proliferation and progesterone receptor levels in T47D human breast cancer cells. *Tumour Biol*. 1993;14(1):38-45.
9. Schmidt WN, Katzenellenbogen BS. Androgen-uterine interactions: an assessment of androgen interaction with the testosterone- and estrogen-receptor systems and stimulation of uterine growth and progesterone-receptor synthesis. *Mol Cell Endocrinol*. 1979; 15(2):91-108.
10. Bukulmez O, Hardy DB, Carr BR, et al. Androstenedione up-regulation of endometrial aromatase expression via local conversion to estrogen: potential relevance to the pathogenesis of endometriosis. *J Clin Endocrinol Metab*. 2008;93(9):3471-3477.
11. Choi YJ, Anders L. Signaling through cyclin D-dependent kinases. *Oncogene*. 2014;33(15):1890-1903.
12. Hata H, Holinka CF, Pahuja SL, Hochberg RB, Kuramoto H, Gurpide E. Estradiol metabolism in Ishikawa endometrial cancer cells. *J Steroid Biochem*. 1987;26(6):699-704.
13. Varma VA, Melin SA, Adamec TA, et al. Monolayer culture of human endometrium: methods of culture and identification of cell types. *In Vitro*. 1982;18(11):911-918.
14. Handwerger S, Harman I, Zeitler P. *In Vitro Methods for Studying Secretion*. Amsterdam, Netherlands: Elsevier Science Publications; 1987.
15. Dai Q, Shah AA, Garde RV, et al. A truncated progesterone receptor (PR-M) localizes to the mitochondrion and controls cellular respiration. *Mol Endocrinol*. 2013;27(5):741-753.
16. Schmitt FC, Soares R. TGF-alpha and angiogenesis. *Am J Surg Pathol*. 1999;23(3):358-359.
17. Higuchi T, Kanzaki H, Iwai M, Narukawa S, Fujita J, Mori T. Expression of messenger ribonucleic acid for gonadal steroid receptors in the human pelvic peritoneum. *Fertil Steril*. 1995;63(1):52-57.
18. Iwai M, Kanzaki H, Fujimoto M, et al. Regulation of sex steroid receptor gene expression by progesterone and testosterone in cultured human endometrial stromal cells. *J Clin Endocrinol Metab*. 1995;80(2):450-454.
19. Apparao KB, Lovely LP, Gui Y, Lininger RA, Lessey BA. Elevated endometrial androgen receptor expression in women with polycystic ovarian syndrome. *Biol Reprod*. 2002;66(2):297-304.
20. Selak V, Farquhar C, Prentice A, Singla A. Danazol for pelvic pain associated with endometriosis. *Cochrane Database Syst Rev*. 2007;(4):CD000068.
21. Shamonki MI, Ziegler WF, Badger GJ, Sites CK. Prediction of endometrial ablation success according to perioperative findings. *Am J Obstet Gynecol*. 2000;182(5):1005-1007.
22. Fedele L, Marchini M, Bianchi S, Baglioni A, Bocciolone L, Nava S. Endometrial patterns during danazol and buserelin therapy for endometriosis: comparative structural and ultrastructural study. *Obstet Gynecol*. 1990;76(1):79-84.
23. Floyd WS. Danazol: endocrine and endometrial effects. *Int J Fertil*. 1980;25(1):75-80.
24. Ferrero S, Tramalloni D, Venturini PL, Remorgida V. Vaginal danazol for women with rectovaginal endometriosis and pain symptoms persisting after insertion of a levonorgestrel-releasing intrauterine device. *Int J Gynaecol Obstet*. 2011; 113(2):116-119.
25. Razzi S, Luisi S, Calonaci F, Altomare A, Bocchi C, Petraglia F. Efficacy of vaginal danazol treatment in women with recurrent deeply infiltrating endometriosis. *Fertil Steril*. 2007;88(4):789-794.
26. Kokko E, Janne O, Kauppila A, Ronnberg L, Vihko R. Danazol has progestin-like actions on the human endometrium. *Acta Endocrinol (Copenh)*. 1982;99(4):588-593.
27. Bukulmez O. Endometriosis and the role of reproductive medicine. *Minerva Ginecol*. 2009;61(4):299-318.
28. Soares SR, Martinez-Varea A, Hidalgo-Mora JJ, Pellicer A. Pharmacologic therapies in endometriosis: a systematic review. *Fertil Steril*. 2012;98(3):529-555.
29. Williams KC, Renthal NE, Condon JC, Gerard RD, Mendelson CR. MicroRNA-200a serves a key role in the decline of progesterone receptor function leading to term and preterm labor. *Proc Natl Acad Sci U S A*. 2012;109(19):7529-7534.
30. Reis FM, Petraglia F, Taylor RN. Endometriosis: hormone regulation and clinical consequences of chemotaxis and apoptosis. *Hum Reprod Update*. 2013;19(4):406-418.
31. Guzick DS, Huang LS, Broadman BA, Nealon M, Hornstein MD. Randomized trial of leuprolide versus continuous oral contraceptives in the treatment of endometriosis-associated pelvic pain. *Fertil Steril*. 2011;95(5):1568-1573.
32. Lidegaard O, Edstrom B, Kreiner S. Oral contraceptives and venous thromboembolism: a five-year national case-control study. *Contraception*. 2002;65(3):187-196.
33. Berrevoets CA, Umar A, Brinkmann AO. Antiandrogens: selective androgen receptor modulators. *Mol Cell Endocrinol*. 2002; 198(1-2):97-103.
34. Kemppainen JA, Lane MV, Sar M, Wilson EM. Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and antihormones. *J Biol Chem*. 1992;267(2):968-974.
35. Nguyen TV, Yao M, Pike CJ. Flutamide and cyproterone acetate exert agonist effects: induction of androgen receptor-dependent neuroprotection. *Endocrinology*. 2007;148(6):2936-2943.
36. Wong C, Kelce WR, Sar M, Wilson EM. Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. *J Biol Chem*. 1995;270(34): 19998-20003.