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Characterization of androgen receptors in a well-differentiated endometrial adenocarcinoma cell line (Ishikawa)

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Abstract

Androgen receptors (AR) have been identified in the human endometrium, but their role in endometrial function and development towards endometrial receptivity remains poorly understood. In an effort to study the regulation and possible function in endometrial epithelium, we utilized the well-differentiated endometrial adenocarcinoma cell line, Ishikawa, as a model system. This cell line has proven to be stable, hormonally responsive, contains both estrogen and progesterone receptors, and has been shown to express endometrial proteins in a hormone responsive manner. In the present study, we demonstrate that Ishikawa cells also express AR, based on immunohistochemical staining, radioactive binding studies, RT-PCR and Northern blot analysis. The expression of AR is induced in Ishikawa cells by estrogens, similar to that reported for normal endometrium. Further, using an estrogen-responsive gene that has been characterized in this cell line, alkaline phosphatase, we show that androgens act as antiestrogens in diethylstilbestrol (DES) treated cells, inhibiting enzymatic activity in a dose-dependent manner. These data support a physiologic role for AR in the endometrium. Elevations in endometrial AR in certain clinical situations such as polycystic ovarian syndrome (PCOS) may amplify the effects of androgens on the endometrium leading to suspected defects in uterine receptivity, higher than expected infertility and high miscarriage rates observed in patients with this disorder.

Keywords: Androgens; Androgen receptor; Endometrium; Cell line; Implantation; Polycystic ovarian syndrome (PCOS)

1. Introduction

The endometrium is the lining of the uterus that undergoes cyclic regeneration each month in anticipation of implantation. As a steroid responsive tissue, much is known about the effects of estrogen and progesterone on the endometrium [1], which function through specific receptor proteins [2,3]. The secretory phase of the endometrial cycle is largely driven by progesterone from the corpus luteum and this sex steroid is required for normal implantation and pregnancy [4]. Much less is known about the role of androgens or the androgen receptor (AR) in the human endometrium, or about any potential role of the steroid and its receptor in defining uterine receptivity. Though no AR mutant mice have yet been generated, a role for androgens has been suggested in the female estrogen receptor null mutant (ERKO), since androgens have been shown to increase the wet weight of the uterus of ERKO animals [5].

Studies have described an increase in androgens during the menstrual cycle and in early pregnancy of women [6]. The AR, like their counterparts estrogen receptors (ER) and progesterone receptors (PR), are present in the nucleus of both stromal and epithelial cells in cycling endometrium [7,8] and appear to be up-regulated by estrogen [9]. Endometrial AR have been shown to be biologically functional, inducing prolactin secretion in stromal cell in vitro [10]. Little is known about the effect of hyperandrogenism on molecular events of implantation nor on other events in early pregnancy.

To investigate the role of AR in human endometrial epithelium, we have chosen the Ishikawa cell line, first developed by Nishida as a model system [11]. This

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well-differentiated cell line contains both a functional ER [12,13] and an estrogen-inducible PR [13,14]. In addition, we have also shown that the progesterone receptor is functional in these cells with the induction of specific progesterone responsive genes, such as the α1 integrin subunit [15]. This cell line has been shown to be very similar to normal endometrial epithelium in terms of its complement of cell adhesion molecules and has been used successfully to study the regulation of these molecules [16]. In the present study, we show that Ishikawa cells also contain AR localized to the cell nucleus that are inducible by estrogens. Using alkaline phosphatase as a marker of estrogen action, we also demonstrate that androgens function as anti-estrogens in this endometrial epithelial cell line. Such studies will likely provide a useful model to study the effects of androgens on the endometrium and lead to a better understanding of the role for androgens in the female reproductive tract in both health and disease.

2. Materials and methods

2.1. Cell culture

Ishikawa cells were cultured to confluence in 150-cm^2 flasks (Costar; Cambridge, MA) containing Dulbecco's Modified Eagles Medium/Ham's F-12 supplemented with charcoal-stripped fetal calf serum (pH 7.2), 200 mM L-glutamine, and penicillin/streptomycin at 37°C in 95% air/5% CO_2. Reagents were obtained from Sigma (St. Louis, MO) except where otherwise specified.

2.2. Immunocytochemisty

Immunocytochemistry was performed on formaldehyde-fixed Ishikawa cells that had been cultured on chamber slides (Nunc, Naperville, IL, USA). After initial incubation with normal goat serum for 30 min at room temperature (4% normal goat serum), primary antibody directed against the androgen receptor (PG21; generously provided by Gail Prins, Ph.D., University of Illinois Chicago, Chicago, IL) was applied and incubated at 4°C overnight. After washing in phosphate-buffered saline (PBS; pH 7.2–7.4) three times each for 3 min, biotinylated goat anti-rabbit IgG conjugated to FITC (1:100 dilution) was applied to the chamber slides. Following a 30-min incubation at RT, slides were rinsed three times with PBS followed by double distilled water and mounted with coverslips. Photomicrographs were prepared using Kodak (Rochester, NY) TMZ 3200 ASA film in a Nikon Optiphot fluorescence microscope.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from Ishikawa cells cultured in the presence or absence of diethylstilbestrol (DES; 10^{-6} M) for 3 days using Tri Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. The total RNA was then reverse transcribed and cDNA was subjected to PCR using primers specific for androgen receptor and β-actin as an internal control. Total RNA (5 μg) was used for reverse transcription in a total volume of 20 μl using the Promega Reverse Transcription system. Reverse transcription mainly included an incubation period of 30 min at 42°C with oligo (dT) primer, followed by incubation for 5 min at 99°C to denature the enzyme. The RT product was then aliquoted equally into two tubes and PCR was performed in 50-μl volume using appropriate androgen receptor primers in one tube (sense 5′-AGATGGGCTTGACTTTCCCAGAAAG-3′; antisense 5′-ATGGCTGTCATTCAGTACTCCTGGA-3′) and β-actin primer were added in an another tube to serve as an internal control, so as to normalize the RNA used in the RT-PCR. PCR amplification of AR gave the predicted product size of 545 bp. PCR reaction mixture consists of 1 × PCR buffer, 2.0 mM $MgCl_2$, 200 μM of each dNTPs, 1.25 U Taq DNA polymerase and 50 pM of each forward and reverse primer. PCR amplification was carried out as follows: after an initial denaturation at 94°C for 10 min, 30 cycles consisting of 94°C (1 min), 55°C (2 min) and 72°C (3 min) were followed by 10 min of final extension at 72°C. The PCR products were electrophoretically resolved on 1.5% agarose gel, stained with ethidium bromide and photographed.

2.4. Biochemical measurement of androgen receptors

Androgen receptors were measured in cells that had been grown in culture medium alone or media treated with various hormones. Binding assays were performed in situ by the addition of 20 nM ^{3}H-R1881 (New England Nuclear, Boston, MA) in the presence or absence of a 100-fold excess of unlabelled R1881. Cells were washed twice with warm saline and then detached from culture flasks by light trypsin/EDTA digestion for 5 min (Gibco, Grand Island, NY).

Cells were then transferred to scintillation vials and radioassayed to determine the amount of bound ^{3}H-R1881. Specific binding was assessed as the difference in counts between the 'hot' and 'hot plus cold' wells and standardized to protein concentration using separate parallel cultures. Protein concentrations were determined using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). These binding assays were carried out in the control media or after treatment for

3 days with each of the following: 10^{-8} M 17β-estradiol (E_2) or diethylstilbestrol (DES), 10^{-6} M progesterone (P), testosterone (T), 5α-dihydrotestosterone (DHT), hydrocortisone (HC) or retinoic acid (RA). Hormones were drawn from a 1000 × stock solution in 100% ethanol (EtOH), while maintaining equal concentrations of EtOH in control flasks.

2.5. Northern blot analysis

Total RNA was isolated from Ishikawa cells, after treating them with hormones individually or in combination for a specified time, using Tri Reagent (Molecular Research Center, Cincinnati, OH). Cells had been cultured in controlled conditions or in the presence of E_2 (10^{-8} M), DES (10^{-8} M), DES + the estrogen receptor inhibitor, ICI 182,780 (ICI; 10^{-7} M), DES + 5α-DHT (DHT; 10^{-6} M), DES + DHT + ICI + the AR inhibitor hydroxyflutamide (HF; 10^{-8} M), DHT alone, DHT + HF, medroxyprogesterone acetate (MPA; 10^{-6} M), and MPA + DES. RNA concentrations were determined by spectrophotometry at 260 nm and stored at −70°C until use. Total RNA (20 μg per lane) was glyoxylated, followed by fractionated through 0.8% agarose gels and blotted onto nylon membrane by capillary action. RNA was cross-linked to the membrane by UV radiation (Stratalinker 1800, Stratagene, LaJolla, CA) using 12×10^4 μJ radiation. The membrane was prehybridized in aqueous solution (5 × SSC, 5% Denhardt's, 1% SDS and 100 μg/ml salmon sperm DNA) at 65°C for 2 h. An ~300-bp cDNA fragment encoding human androgen receptor, served as the template for synthesis of a labeled DNA probe using random priming method (Random Primed DNA labeling kit, Boehringer Mannheim Biochemicals, Indianapolis, IN). The membrane was subsequently hybridized with (α-^{32}P)dCTP (Amersham Pharmacia Biotech, NJ, USA) labeled AR probe for 16 h at 65°C. After hybridization, the membrane was washed twice at RT for 15 min in 2 × SSC and 0. 1% SDS, followed by another two washes, 15 min each, at 55°C, with 0.1 × SSC and 0.1% SDS. Autoradiography was performed using Hyperfilm (Amersham Pharmacia Biotech, NJ, USA) for 24–72 h at −70°C until the desired exposure was obtained. Further, the integrity and relative amount of RNA loaded into each lane was confirmed by using GAPDH ^{32}P-labeled cDNA probe as a constitutively expressed marker.

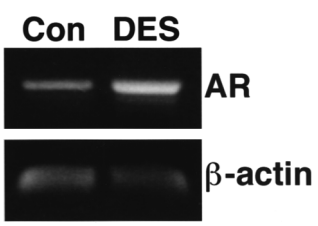

Fig. 1. RT-PCR analysis of androgen receptors (AR) mRNA using total RNA isolated from Ishikawa cells cultured under control conditions and following 3-day treatment with 10^{-8} M diethylstilbestrol (DES). AR specific amplicons were identified with the expected size of 545 bp. The β-actin specific primers were included to serve as an internal control and to normalize the RNA used in the RT-PCR.

2.6. Alkaline phosphatase activity

Alkaline phosphatase activity was measured in Ishikawa cells using a modified method of Littlefield et al. [17]. This simple bioassay has been successfully used in Ishikawa cells and provides a valid measure of estrogenic activity in these cells. Near confluent Ishikawa cells were cultured in the presence or absence of 10^{-8} M E_2 and with E_2 plus increasing doses of T or DHT using concentrations ranging from 10^{-4} to 10^{-10} M for 72 h in microtiter plates. Experiments were done in quadruplicate. At the end of the treatment phase, plates containing the cells were inverted and growth media removed. Wells were rinsed with PBS (pH 7.2) and the fluid removed with a brisk shake. After addition of 100 μl methanol, the microtiter plates were snap frozen in the −80°C freezer for at least 15 min. Plates were removed from the freezer and the methanol was aspirated. Using octapette, 100 μl of substrate solution (5 mM *p*-nitrophenyl phosphate (Sigma), 0.24 mM $MgCl_2$, and 1 M diethanolamine (pH 9.8)) was quickly added to each well. Plates were read on spectrophotometer at 3–5-min intervals at 405 nm until yellow color peaked.

3. Results

By RT-PCR we could demonstrate the presence of AR in Ishikawa cells that increased in response to DES treatment (Fig. 1). These observations are consistent with immunofluorescence of Ishikawa cells showing nuclear localization of AR and an increase in staining intensity after pretreatment with DES, using the polyclonal antibody PG21 (Fig. 2A and B). Binding assays using ^{3}H-R1881 in the presence or absence of 100-fold excess of unlabeled R1881 also further demonstrated increased binding normalized to cellular protein, in cells treated with estradiol. Co-treatment with E_2 + testosterone showed no decrease in specific binding activity (Fig. 2C).

Prereatment of Ishikawa cells with various other steroids also affected binding levels of ^{3}H-R1881 in these cells (Fig. 3). Specific binding, expressed as per cent of control, increased after treatment with DHT and T compared to control media treatment. DES and E_2, however, had the most striking effect with DES being more potent at elevating AR. Pretreatment with progesterone (P), HC, and RA had little, if any effect

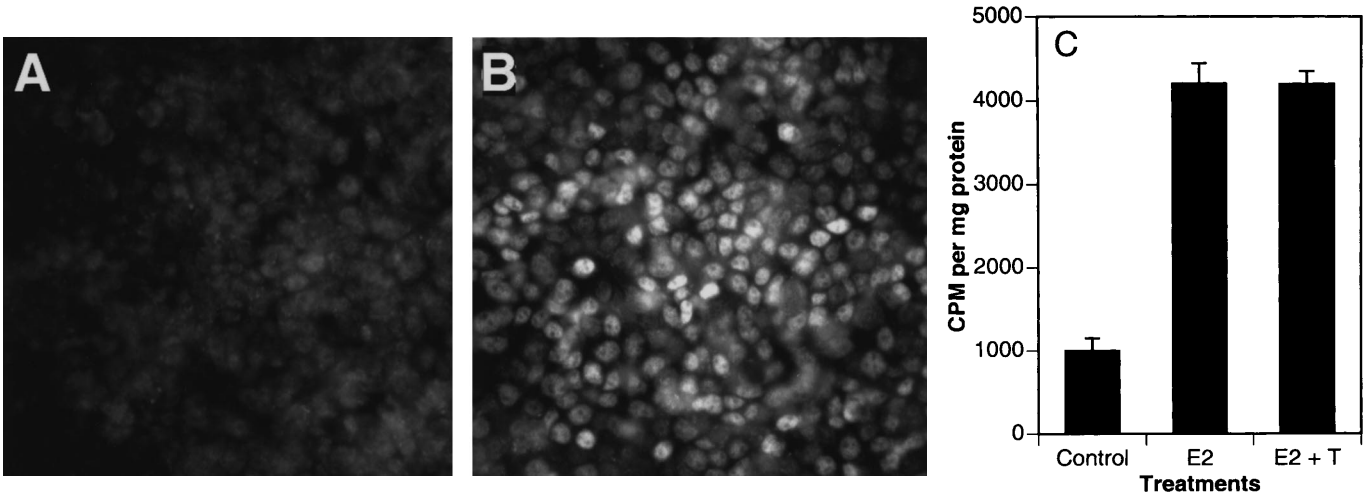

Fig. 2. Cellular localization of androgen receptors (AR) protein in Ishikawa cells by immunohistochemistry. Photomicrographs displaying immunofluorescent AR using a specific polyclonal antibody, PG21 that recognizes human AR in control cells cultured in charcoal stripped serum containing media without added hormones (A), showing low levels of intranuclear staining. Following treatment with 17β-estradiol (E_2: 10^{-8} M) for 3 days, there is increased localization of immunofluorescence staining of AR in the nuclei of these cells (B). Magnification: 200 ×. Radioactive binding assays using the synthetic androgen ^{3}H-R1881 plus or minus 100-fold excess of unlabeled R1881 shows a similar increase in specific binding of 17β-estradiol treated cells with little or no change with addition of testosterone (T: 10^{-6} M) (C). Binding studies were carried out in vivo as described in Section 2.

on specific binding. Since R1881 is known to cross-react with other steroid receptors (progesterone or glucocorticoid receptors), we also investigated the effect of various unlabeled (cold) steroids to compete with ^{3}H-R1881 binding (data not shown). While P does compete to a limited extent for ^{3}H-R1881 binding, HC had little, if any effect. Since progesterone receptors (PR) are known to be present in Ishikawa cells, and induced by estradiol [14], it is possible that the presence of PR may interfere with the measurement of AR using ^{3}H-R1881. However, since the binding assays compare favorably with the other more specific measures of AR used (e.g. RT-PCR, immunohistochemistry, and Northern blot analysis), the impact of such cross-reactivity appears minimal and does not alter the conclusions of the study. It is not known whether Ishikawa cells express the glucocorticoid receptor.

The effect of hormonal treatment on binding was also confirmed using Northern blot analysis. As shown in Fig. 4, both E_2 and DES increased AR message. This effect was inhibited by the co-treatment with its inhibitor ICI 182,780. DHT + DES also increased expression of the AR message and the combined treatment with both estrogen and androgen receptor inhibitors eliminated this effect. The rise in AR by DHT was only partially reduced by HF. Medroxyprogesterone acetate (MPA) had no effect by itself, but inhibited the rise in AR message in response to DES.

The functionality of AR in Ishikawa cells was assessed further by using the estrogen-induced alkaline phosphatase enzyme as a biomarker. This enzyme has been shown increase in this cell line in a dose-dependent fashion in response to estrogen. We have also shown that progesterone inhibits this rise, consistent with its role as an anti-estrogen (unpublished observation). As shown in Fig. 5, the estrogen-induced increase in the alkaline phosphatase enzyme activity was diminished by both T and DHT in a dose-dependent manner. It is noted, however, that DHT was more potent than T

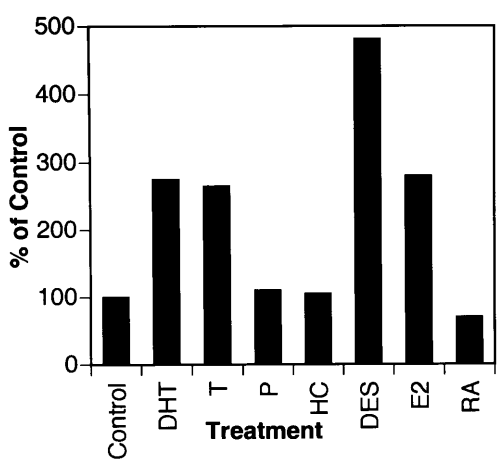

Fig. 3. Comparison of specific androgen receptor binding to the synthetic androgen ^{3}H-R1881 in Ishikawa cells treated with various steroid hormones for 3 days. Ishikawa cells were treated with no hormones (control), 5α-dihydrotestosterone (DHT: 10^{-6} M), testosterone (T: 10^{-6} M), progesterone (P: 10^{-6} M), hydrocortisone (HC: 10^{-6} M), diethylstilbestrol (DES: 10^{-8} M), estradiol-17β (E_2: 10^{-8} M) or retinoic acid (RA: 10^{-6} M) for 3 days. Specific binding in the presence of ^{3}H-R1881 plus or minus 100-fold excess of cold R1881 between pooled triplicate samples was expressed as per cent of control, using the specific binding in control samples as the basis for comparison.

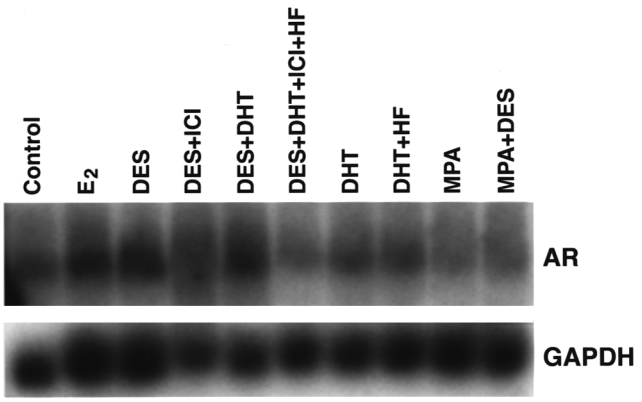

Fig. 4. Northern blot analysis demonstrating the regulation of human androgen receptor (AR) in Ishikawa cells. A single transcript of AR was identified (top panel) by Northern blot analysis. Total RNA was prepared from Ishikawa cells, untreated (control) or treated for 3 days with E_2 (10^{-8} M), diethylstilbestrol (DES: 10^{-8} M), DES + the estrogen receptor inhibitor ICI 182,780 (ICI: 10^{-7} M), DES + 5α-DHT (DHT: 10^{-6} M), DES + DHT + ICI + the AR inhibitor hydroxyflutamide (HF: 10^{-8} M), DHT alone, DHT + HF, medroxyprogesterone acetate (MPA: 10^{-6} M) and MPA + DES. Note the apparent increase in mRNA abundance following estrogen or DES treatment is blocked using the antagonist. DES plus DHT also displayed increased AR message but there was diminished AR expression in the presence of the synthetic progestin, MPA. Equal loading of RNA was confirmed by examination of GAPDH (lower panel).

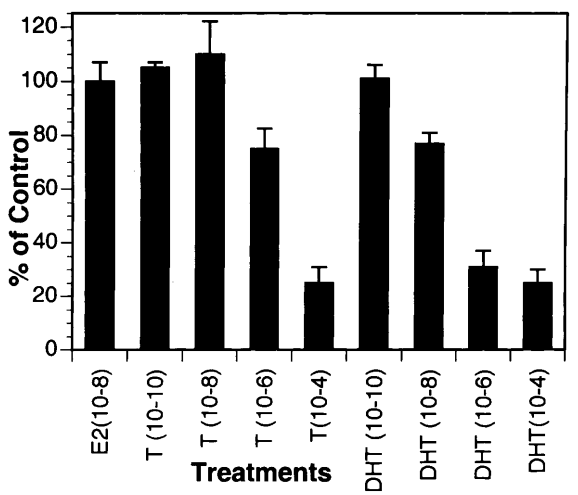

Fig. 5. The dose-dependent effect of two androgens on the estrogen-induced alkaline phosphatase activity in Ishikawa cells. Cells were cultured in the presence of 10^{-8} M estradiol (E_2) with or without increasing doses of testosterone (T) or 5α-dihydrotestosterone (DHT: 10^{-4} to 10^{-10} M for 72 h. Alkaline phosphatase activity was measured as described in Section 2. The mean rise in alkaline phosphatase activity by E_2 was designated as 100%. The effect of E_2 alone or the co-treatment with T or DHT was presented as % of control ± S.E. Note the dose-dependent decrease in alkaline phosphatase activity in androgen-treated cells.

with inhibitory activity seen at 10^{-8} M compared to T that was not inhibitory until a concentration of 10^{-6} M was reached.

4. Discussion

Ishikawa cells were first described by Nishida, as well differentiated adenocarcinoma cells expressing both ER and PR [11]. Littlefield and colleagues have demonstrated that these cells maintain estrogen-responsiveness using the induction of alkaline phosphatase [17,18] as a model to study estrogenic effects of anti-estrogens [19,20] and phytoestrogens [21]. On the other hand, we and others have previously demonstrated that these cells contain a functional PR [13,14] that can be induced by estrogen [14,20].

In the current study we show that AR are also present in Ishikawa cells. Like normal endometrium, Ishikawa cells have ER and an estrogen inducible AR. Immunohistochemical localization of AR in human endometrium has previously been reported [8,22]. In addition, human endometrial AR appear to be increased by the action of estrogen in both the endometrium and leiomyomata in women [9]. In Ishikawa cells, we also found that the progestin MPA downregulates AR, similar to the action of P on PR in human endometrium [3] and in Ishikawa cells [14]. In fact, DHT and T appeared to have a modest positive effect on AR binding activity and on the expression of AR message by Northern blot analysis. This is different from that observed in other hormone responsive tissues. Studies in mouse mammary gland, for example, show an inhibitory effect of estrogens on AR expression [23].

The identification of estrogen-inducible AR in human endometrium and in the Ishikawa cell line has broad implications for both health and disease in women. Preliminary studies from our laboratory have found elevated endometrial AR in the endometrium of women with polycystic ovarian syndrome (PCOS) and chronic anovulation and hyperandrogenism [24]. Based on the current study, the unopposed estrogen and increased serum androgen levels in these patients may account for the elevation in AR that was seen in their endometrium. The elevation in both endometrial AR and serum androgens may possibly explain the poor reproductive performance and high miscarriage rate often seen in these women, even once ovulatory status has been achieved [25–27]. As demonstrated in Ishikawa cells, the marked rise in AR could accentuate the already high androgen levels and result in an exaggerated androgen response in this target tissue.

There is surprisingly little data available regarding the role of androgens as anti-estrogens in the endometrium. In the present study, we have demonstrated that both T and DHT display anti-estrogenic properties, using alkaline phosphatase as a marker of estrogen action. This activity appears to be selective; while androgens reduce alkaline phosphatase activity, no reduction in estrogen-induced AR was noted. Progesterone, on the other hand, is a potent antiestrogen and effec-

tively prevents this estrogen-induced rise in AR expression. It will be interesting to evaluate the range of genes that are turned on (or off) in response to androgen action in this endometrial cell line and to compare these responses seen to those in other hormone dependent tissues such as breast or prostate.

In summary, Ishikawa cells maintain a full complement of endometrial receptors for estrogen, progesterone and androgens. This report, which examines the presence and regulation of AR in these cells, is further evidence to support the use of this model for the study of human endometrial epithelium. These results, which show differential regulation of AR by estrogens and progestins also suggests that further study of AR in the endometrium of women is warranted. The anti-estrogenic effects of androgens in estrogen-primed Ishikawa cells indicate that women with PCOS might have clinically significant alterations in their endometrium. The action of androgens, that has been associated with poor reproductive outcome in women, could be due in part to abnormalities in the level of endometrial AR or other genes in the endometrium of these women.

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