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ONSET OF ANDROGEN ACTION IN MCF-7 HUMAN BREAST CANCER CELLS IS NOT ACCOMPANIED BY RECEPTOR DEPLETION

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Summary—Quantitative and qualitative changes in estrogen receptor follow addition of estradiol to estrogen responsive MCF-7 human breast cancer cells. We asked whether similar changes would accompany treatment of these cells with physiologically relevant concentrations of androgens. Androgen receptor sites were quantified by competitive protein binding assays on whole cells or extracts at various times following hormone addition. Both direct and exchange assays were employed. The androgen receptor in all of these experiments remained in a form which is completely exchangeable and approx 85% salt extractable. Quantity of receptor was unchanged (30,000 sites/cell, K_d 0.1 nM). Responsiveness to hormone treatment was demonstrated by antagonizing the estrogen dependent augmentation of cytoplasmic progesterone receptor in the MCF-7 cells with androgens. Thus, the androgen receptor was shown to be biologically active, but no time dependent quantitative or qualititative changes were observed during the first 6 h following androgen treatment.

INTRODUCTION

Intracellular events following association of steroids with specific receptor proteins are not completely understood. Processing of the nuclear estrogen receptor is one such possible regulatory event which has previously been described [1]. This phenomenon has been studied in the MCF-7 human breast cancer cell line [1–5] as well as rat uterine tissue [6]. It has been shown that during the first 3–5 h of exposure to estradiol, in temporal association with oestradiol-induced progesterone receptor, there is a progessive variable depletion of the occupied nuclear estrogen receptors in the MCF-7 cells which may reach 70% when receptor is measured by extraction and by an exchange assay [1]. This set of events has been termed "processing" and is not due to cycling of receptor to the cytoplasm. Further evidence for the importance of this phenomenon is derived from the failure of antiestrogens to induce extensive "processing", and the absence of estrogen induced "processing" in a hormone independent variant, R3 of MCF-7 cells [7].

Estrogen exposure leads to two independent events in MCF-7 cells. First, there is a depletion of estrogen receptors which is most apparent in cells with high concentrations of estrogen receptor. Increased density and insulin induce down regulation of estrogen receptors; further addition of estradiol causes little additional loss of estrogen receptor. Under both circumstances (high and low initial receptor content), estrogen addition is associated with several estrogen effects (e.g. progesterone receptor induction and increased proliferation) as well as important qualitative changes in receptor. These qualitative changes include a time dependent conversion of receptor to a form which is less extractable from the nucleus and less exchangeable [5]. This receptor form sediments more rapidly on sucrose gradients and is separable from newly translocated receptor by phosphocellulose chromatography [8]. This receptor form is still recognized by at least two monoclonal antibodies directed against ER.

To further understand processing, we have investigated this nuclear event in another hormone receptor system in the same human breast cancer cell line. We therefore asked whether "processing" of receptor was a general phenomenon for other classes of steroids. This study examines processing of the androgen receptor in the MCF-7 cell line.

EXPERIMENTAL

Chemicals

[^{3}]R1881 (87 Ci/mmol), [^{3}]R5020 (87 Ci/mmol), unlabeled R1881, and unlabeled R5020 were purchased from New England Nuclear Corp. (Boston, MA). 5 DHT and TA were obtained from Sigma Chemical

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The following abbreviations are used: R1881, methyltrienolone-17-β-hydroxy-17α-methyl-estra-4,9,11-triene-3-one; E_2, 17β estradiol (3,17β-dihydroxy-1,3,5(10)-estratriene; TA, triamcinolone acetonide (9α-fluoro16-hydroxyprednisolone 16,17-acetonide); R5020, progesterone, 17 methyl-19-nor-pregna-4,9-diene-3,20-dione; DHT, 5-dihydrotestosterone, 5α androstane-17β-ol-3-one; FCS, fetal calf serum; CCS, charcoaled calf serum; PBS, Dulbecco's phosphate-buffered saline (0.14 M NaCl – 2.6 mM KCl – 4.2 mM NaH_2PO_4 – 1 mM KH_2PO_4, pH 7.4); AR, androgen receptor; ER, estrogen receptor; PgR, progesterone receptor.

Co. (St. Louis, MO). Cortisol was purchased from Steraloids, Inc. (Wilton, NH).

Cells and tissue culture

Monolayer cultures of MCF-7 human breast cancer cells [9] were grown in Richter's Improved Minimal Essential Medium (IMEM) [10] from Grand Island Biological Co. (GIBCO) [Grand Island, NY]. Either 5% FCS (Gibco, Grand Island, NY) or 5% CCS with 0.02 units/ml porcine insulin (Eli Lilly Co., Indianapolis, IN) was added to the medium. Charcoal-treated calf serum was prepared by treatment of 500 ml of serum with a charcoal pellet prepared from 100 ml of Dextran-coated charcoal slurry (2.5% untreated activated charcoal, 0.25% dextran in 10 mM Tris–HCl, pH 7) followed by a 4 h incubation in a shaker at 0°C. The procedure was repeated with a fresh charcoal pellet. After each incubation, the charcoal was removed by centrifugation. The serum was sterilized through a 0.20 μm Nalgene filter and stored at −20°C [11]. Cells were grown in a humidified incubator in 5% CO_2 at 37°C.

Whole cell binding experiments for androgen receptor

Direct and exchange assays. Cells in Richter's IMEM and 5% FCS were plated in Costar 6-well dishes (Cambridge, MA) at a density of 2–4 × 10^4 cells per well. At subconfluency, intact cells were incubated for 1 or 6 h at 37°C with varying concentrations of [^{3}H]R1881 and a 1500-fold excess of TA in the presence or absence of a 100-fold excess of unlabeled R1881. Following incubation, the cells were washed three times in PBS containing 1% CCS at 25°C. The washes were completed within approx 1 min time and under these circumstances essentially no dissociation occurs. Then 1 ml of 1 M NaOH was added to each well. The plates were placed in a gravity convection oven (Precision, Chicago, IL) at 60°C for 1 h. Samples were neutralized by adding 0.25 ml 4 M HCl. The contents of each well were transferred to scintillation vials containing 8 ml of Ready-Solv MP (Beckman, Palo Alto, CA). The number of cells was determined from a suspension of replicately plated wells harvested with PBS containing 0.02% EDTA using a Model ZF Coulter Counter (Coulter Electronics, Hialeah, FL). Scatchard analysis of the data was performed [12] using a computer assisted method [13]. An exchange assay was performed in a similar fashion except that intact cells were preincubated with 1 nM unlabeled R1881 for 1 and 6 h. Then, the cells were washed three times with medium and subsequently reincubated with varying concentrations of [^{3}H]R1881 and a 1500-fold excess of TA with or without a 100-fold excess of unlabeled R1881. Exchange proceeded for 5 h at 37°C. Then cells were washed and assayed as previously described.

Preparation of cytosol and nuclear extracts

Subcellular distribution of occupied androgen receptor was determined by treating the MCF-7 cells growing in 5% FCS at 37°C for 1 or 6 h with a single saturating dose of 1 nM [^{3}H]R1881 and a 1500-fold excess of TA. Nonspecific binding was determined by a parallel incubation with [^{3}H]R1881, TA, plus a 100-fold excess of unlabeled R1881. At the end of the incubation period, the medium was removed, and the intact monolayer was washed three times with PBS containing 1% CCS at 25°C.

Cells were then harvested, resuspended in ice-cold TEDG buffer (10 mM Tris–HCl, 1.5 mM EDTA, 0.5 mM diothiothreitol, and 10% (v/v) glycerol, pH 7.4) and homogenized in a glass Dounce homogenizer. Aliquots of the homogenate were analyzed for DNA by a method of enhancement of fluorescent dye complexed with DNA [14]. The remainder of the experiment was performed at 0–4°C. The homogenate was centrifuged at 800 ***g*** for 5 min, and the supernatant was centrifuged at 105,000 ***g*** for 60 min. The resultant supernatant was used as cytosol. The nuclear pellet was washed twice with TEDG buffer and resuspended in 1 ml of TEDG containing 0.6 M KCl (TEDGK buffer). The suspensions were then either homogenized with a glass Dounce homogenizer or sonicated with a Bronson Sonifier Cell Disrupter 350 (Shelton, CT) using two 10 s bursts at 50% duty cycle setting 2 with a 1 min cooling interval, followed by KCl extraction for 60 min. Both preparations were then centrifuged at 105,000 ***g*** for 30 min. Aliquots of the cytosol and nuclear extracts were counted in 8 ml of scintillation fluid. The remaining nuclear pellets were resuspended by sonication in 1 ml of TEDG buffer. Aliquots of the resuspended nuclear pellet were also counted in 8 ml of scintillation fluid.

Progesterone receptor assay

DCC Assay by single saturating dose of Scatchard analysis. MCF-7 cells were plated in 5% CCS plus 0.02 units/ml of insulin. On the following day, the medium was changed and E_2 (final concentration 10^{-9} M), DHT (final concentration 10^{-8} M) or E_2 plus DHT (10^{-9} M, 10^{-8} M) were added. DHT was used in these assays since R1881 binds to the progesterone receptor as well as the androgen receptor [16]. A fourth group of cells received vehicle only. Ethanol concentration was maintained below 0.1%. Media and steroids were changed every 48 h for 7 days. On the appropriate day, intact monolayers were washed at 25°C with PBS containing 1% charcoal-treated calf serum and harvested. Other progesterone receptor assays were performed on cells which had been plated and grown in 5% FCS. On the following day, the medium was changed and flasks were treated for 4 days with either DHT (final concentration 10^{-8} M) or vehicle. Media and steroids were changed after 48 h. A similar experiment utilizing cells grown in 5% FCS was performed except that DHT treatment was extended to 7 days. Similar cell numbers were present at the end of the experiments

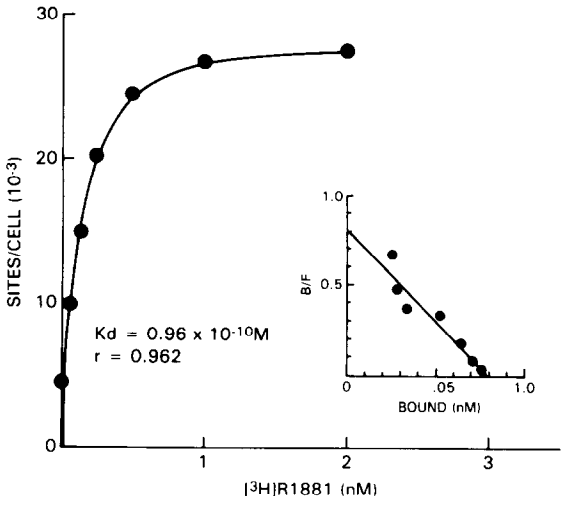

Fig. 1. Saturation curve for androgen receptor in whole cells of MCF-7. The inset shows the Scatchard analysis of [^{3}H]R1881 binding to MCF-7 whole cells. The saturation curve has been corrected for nonspecific binding.

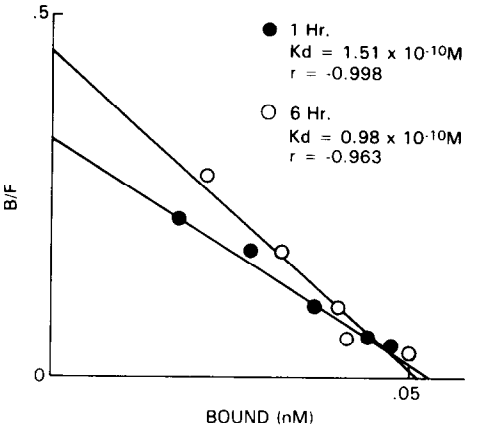

Fig. 2. Scatchard analyses of MCF-7 AR whole cell binding assay at 1 h (●) and 6 h (○).

in both the hormone-treated and control flasks grown in 5% FCS. Cytosols were prepared as previously described. Cytosol (100 μl) diluted to 2 mg protein/ml with TEDG buffer was incubated with 100 μl of either 9 nM [^{3}H]R5020 and 2×10^{-6} M cortisol with or without a 200-fold excess of unlabeled R5020 for single point assay or varying concentrations for Scatchard analysis for 19 h at 0°C. Then 1 ml of DCC (0.25% Norit A and 0.0025% Dextran in TED buffer) was added. The tubes were vortexed every 5 min for 15 min and then centrifuged at 800 *g* for 10 min. As described, 0.8 ml of supernatant was counted in 8 ml of scintillation fluid [1]. Binding data was analyzed as above.

RESULTS

In order to determine the number of androgen receptors in the MCF-7 cell line, saturation and Scatchard analysis of [^{3}H]R1881 binding to intact cells was performed. Figure 1 shows that there are 27,700 high affinity ($K_d = 0.96 \times 10^{-10}$ M) androgen receptor binding sites per cell. Saturation of these sites was achieved using 1 nM [^{3}H]R1881 in the presence of 1500-fold excess of TA. These findings are consistent with the results of studies previously performed in our laboratory and by other investigators [15–17].

Time-dependent changes in androgen receptor levels were first examined using the whole assay. In the ER system, a minimal loss of competable ER binding was found when the cells were labeled with [^{3}H]E_2 *in vivo* for 1 and 6 h provided that estrogen was added to the cells whose ER concentration was set at a lower level by conditions of higher density and the presence of insulin. If instead these intact cells were preincubated for 1 and 6 h with unlabeled E_2, and this was subsequently exchanged for [^{3}H]E_2, a 50–70% loss of competable ER binding was observed [8]. Therefore, intact MCF-7 cells were labeled with [^{3}H]R1881 for 1 and 6 h. Scatchard analysis (Fig. 2) shows that androgen receptor levels are similar (28,800 and 30,528) at these time points. Exchangeability of receptor was then examined by pretreating the intact cells with unlabeled R1881 and subsequently exchanging the unlabeled hormone with [^{3}H]R1881. Scatchard analysis in Fig. 3 shows that cells pretreated for 1 and 6 h and exchanged over 5 h with [^{3}H]R1881 have approximately the same competable AR content (23,000 sites per cell) as to that of controls assayed by direct labeling with [^{3}H]R1881 for 1 to 6 h. The apparent binding affinity was unaltered by exchange conditions

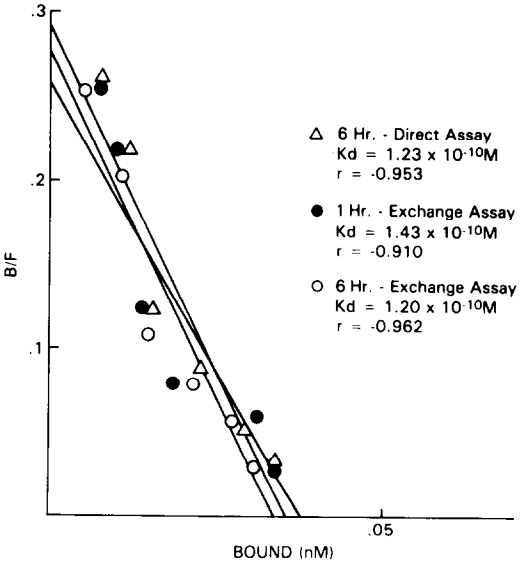

Fig. 3. Scatchard analyses of [^{3}H]R1881 binding to MCF-7 whole cells. A 6 h direct assay (△) is compared to cells pretreated with unlabeled R1881 for 1 (○) and 6 (○) h and exchanged over 5 h for [^{3}H]R1881. The plot for the 1 h direct assay is not shown.

Table 1. The subcellular distribution of androgen receptor in MCF-7 cells following *in vivo* labeling with [^{3}H]R1881

Extraction technique	*In Vivo* incubation (h)	Specific [^{3}H]R1881 Binding (fmol/mg DNA)			
		Cytosol	Nuclear Extracts	Nuclear Pellet	Total Cellular
Dounce homogenization	1	178	618	95	891
Dounce homogenization	6	117	684	97	898
Sonication	6	157	567	76	800

($K_d = 1.2–1.4 \times 10^{-10}$ M). Therefore, unlike ER which is both lost and converted to a less exchangeable form, the androgen receptor maintains complete exchangeability in the androgen treated intact cell. Next, we examined whether any time dependent changes in nuclear androgen receptor KCl extractability occurred. In doing so, exchange assays of nuclear KCl extracted receptor were avoided since other investigators have encountered multiple problems in methodology [18]. Therefore, we labeled intact cells with 1 nM [^{3}H]R1881 for 1 and 6 h and measured KCl extractable prelabeled nuclear androgen receptor. The results in Table 1 show the subcellular distribution of AR following *in vivo* labeling for 1 or 6 h with [^{3}H]R1881. The 6 h nuclear pellets were either Dounce homogenized or sonicated. In contrast to the findings of a loss of ER from the nuclear compartment with Dounce homogenization and KCl extraction, no significant difference was seen in the KCl extractable nuclear androgen receptor content. In fact, after 1 or 6 h of hormone treatment the number of high affinity binding sites did not vary significantly in any of the subcellular fractions, and less than 14% of the nuclear androgen receptor was resistant to salt extraction under the conditions used. These data support the conclusion that the androgen receptor remains in a form which is not only completely exchangeable but is also nearly completely salt extractable.

Up to this point, no evidence for post translocational changes in the AR had been found. Since processing had been shown to parallel biological responsiveness in the ER system, it was imperative to determine whether or not the cells were responsive to androgen treatment. Previous data has suggested that androgen effects on growth in MCF-7 cells or other mammary tumors [15] occurring at supraphysiologic concentrations (10^{-6} M) of hormone were possibly mediated through an interaction with the estrogen receptor [19, 20]. Recently, MacIndoe reported that androgens (10^{-8} M) prevent the estrogen-dependent augmentation of cytoplasmic progesterone receptor in MCF-7 cells [21,22].

Figure 4a shows the effects of 7 days of treatment with E_2 (10^{-9} M), DHT (10^{-8} M), and E_2 (10^{-9} M) plus DHT (10^{-8} M) upon the cytosolic progesterone receptor content of MCF-7 cells in 5% CCS. Cells treated with vehicle or DHT alone contain less than 0.16 pmol progesterone receptor/mg DNA. Cells treated with an optimal concentration of E_2 (10^{-9} M) showed an 11-fold induction of progesterone receptor (1.87 pmol/g DNA) over basal levels. The cells treated with E_2 plus DHT demonstrated stimulation of progesterone receptor induction to a lesser degree (60%) with only a 7-fold induction (1.15 pmol/mg DNA) over basal levels.

In Fig. 4b, progesterone receptor levels were determined by assaying cells grown for 4 days in 5% FCS

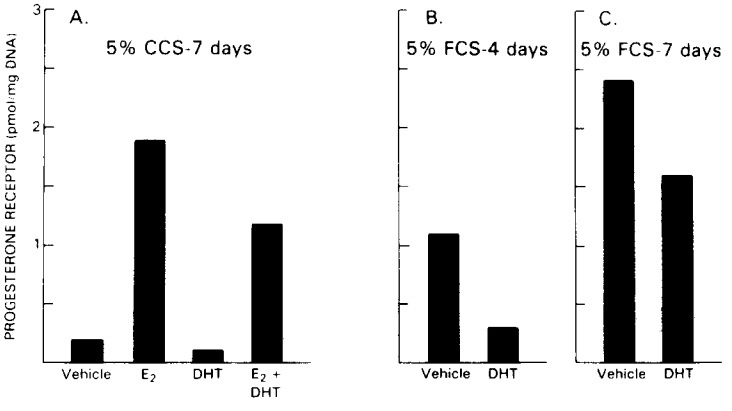

Fig. 4a. PgR content of MCF-7 cells grown in 5% CCS following 7 days of treatment with vehicle E_2 (10^{-9} M), DHT (10^{-8} M), or E_2 (10^{-9} M) plus DHT (10^{-8} M).

Fig. 4b. PgR content of MCF-7 cells grown in 5% FCS following 4 days of treatment with vehicle or DHT (10^{-8} M).

Fig. 4c. PgR content of MCF-7 cells grown in 5% FCS following 7 days of treatment with vehicle or DHT (10^{-8} M).

with vehicle or DHT (10^{-8} M). Endogenous E_2 in media containing 5% FCS ranges between 0.03–0.06 nM [23]. The basal level of progesterone receptors in cells grown in 5% FCS for 4 days was 1.1 pmol/mg DNA. This level was similar to that previously determined in MCF-7 cells grown for 4 days in 5% CCS and 0.01–0.1 nM E_2 [7]. Under these conditions of less effective stimulation of progesterone receptor induction, androgens antagonize estrogenic action with greater efficacy. In another experiment using identical conditions progesterone receptor on days 3 and 4 was 1.5 and 1.1 fmol/mg DNA in the absence of DHT. In the presence of 10^{-8} M DHT progesterone receptor was reduced to 0.72 and 0.30 pmol/mg DNA on days 3 and 4.

Figure 4c demonstrates the effect of DHT (10^{-8} M) treatment for 7 days on the induction of progesterone receptor in cells grown only in 5% FCS. Progesterone receptor levels in the cells at 7 days are 2.4 pmol/mg DNA, and DHT (10^{-8} M) is shown only to partially antagonize this induction (1.6 pmol/mg DNA). These data are qualitatively and quantitatively similar to those reported by MacIndoe [21,22] and verify that our observations on alteration in receptor are being made in association with androgen action through the androgen receptor.

Figure 5 shows the Scatchard analyses of PgR content in these treatment groups. No difference in binding affinity is detected in these assays ($K_d = 4.8$–4.5×10^{-10} M) and are consistent with those of other investigators examining progesterone receptor [24]. Thus, androgen responsiveness is demonstrated in MCF-7 cells which previously have shown the absence of quantitative or qualitative changes in androgen receptors during the first 6 h following androgen treatment.

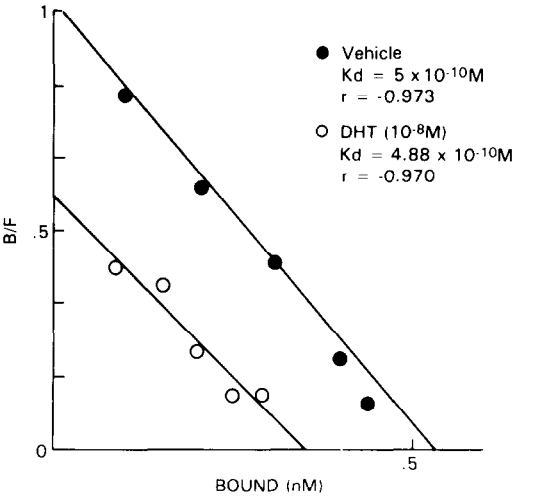

Fig. 5. Scatchard analyses of PgR content of MCF-7 cells grown for 7 days in 5% FCS with vehicle (●) or 10^{-8} M DHT (○).

DISCUSSION

Androgen action in breast cancer may be studied in hormone dependent tumor models. Pharmacologic doses of androgens can induce regression or reduce the rate of appearance of DMBA-induced rat mammary tumor [25, 26] whereas the Shionogii carcinoma 115 is an androgen-dependent mouse mammary adenocarcinoma, and failure of tumor growth occurs if androgens are withheld [27, 28, 29]. Since androgen effects on breast cancer models vary, further study is needed to understand their role in human breast cancer.

We have studied the set of events that follow binding of androgens to their receptor in the MCF-7 cell line. Previously, estradiol treatment of MCF-7 cells was shown to lead to a time dependent progressive depletion of approx 70% of E_2 filled nuclear receptor and was thought to represent a net loss of estrogen receptor [1]. More recently, it has been shown that estrogen exposure leads to at least two independent events in these cells. Depletion of estrogen receptor is most apparent in cells with a high concentration of estrogen receptor. Therefore, if increased density and insulin induce down regulation of the estrogen receptor, addition of estradiol to these cells causes little additional loss of estrogen receptor [8]. However, under both growth conditions, a second event characterized by qualitative changes in nuclear localized receptor occurs. Similarly, our studies show that treatment with androgen leads to no loss of AR, but in contrast, the AR remains in a completely exchangeable and approx 85% extractable form.

MacIndoe *et al.* reported that androgens prevented the estrogen-dependent augmentation of cytoplasmic progesterone receptor in MCF-7 cells [21,22]. These studies provide evidence for androgen responsiveness in the MCF-7 cell line. In our cells, androgens antagonize at least 40% of the estrogenic stimulation of progesterone receptor induction. Further antagonism may not have been observed since the PgR induction was 11–15-fold over basal progesterone levels in cells grown only in 5% CCS for 7 days. Other studies examining this antagonism of PgR induction differ in that the basal levels of PgR are higher, and the receptor induction is less than 10-fold during a similar treatment interval. Androgens may be more effective in antagonizing PgR induction under these conditions [21, 22].

In summary, we have demonstrated that androgen action in the MCF-7 cell line is not accompanied by receptor depletion. Further investigation may define a "processed" receptor characterized by physical or chemical properties other than exchangeability or salt extractability.

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