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Androgen Receptor Overexpression Induces Tamoxifen Resistance in Human Breast Cancer Cells

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Abstract

Although the androgen receptor (AR) is a known clinical target in prostate cancer, little is known about its possible role in breast cancer. We have investigated the role of AR expression in human breast cancer in response to treatment with the antiestrogen tamoxifen. Resistance to tamoxifen is a major problem in treating women with breast cancer. By gene expression profiling, we found elevated AR, and reduced estrogen receptor (ER) α mRNA in tamoxifen-resistant tumors. Exogenous overexpression of AR rendered ER α -positive MCF-7 breast cancer cells resistant to the growth-inhibitory effects of tamoxifen in anchorage-independent growth assays, and in xenograft studies in athymic nude mice. AR-overexpressing cells remained sensitive to growth stimulation with dihydrotestosterone. Treatment with the AR antagonist CasodexTM (bicalutamide) reversed this resistance, demonstrating the involvement of AR signaling in tamoxifen resistance. In AR-overexpressing cells, tamoxifen induced transcriptional activation by ER α that could be blocked by Casodex, suggesting that AR overexpression enhances tamoxifen's agonistic properties. Our data suggest a role for AR overexpression as a novel mechanism of hormone resistance, so that AR may offer a new clinical therapeutic target in human breast cancers.

Keywords

Androgen Receptor; Breast Cancer; Tamoxifen Resistance

Introduction

In the past tamoxifen (Tam) has been the most commonly employed hormonal agent in the treatment of human breast cancer, although aromatase inhibitors are now being used in the clinic. Unfortunately most patients who initially respond to these agents eventually develop tumors that are resistant to treatment. It is unlikely that simple loss of estrogen receptor (ER) α signaling is the driving force for the majority of hormonal resistance, as both Tam-

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resistant (Tam^R) tumors and Tam^R breast cancer cell lines frequently retain their ER α expression, and remain responsive to pure antiestrogens or aromatase inhibitors [1,2]. Modulation of signal transduction pathways that affect ER α activity [3], altered binding of receptor-regulating coactivators or corepressors [4], and increased cellular interactions between steroid hormone and growth factor signaling pathways are all proposed mechanisms for Tam resistance in ER α -positive breast cancer models [5,6]. For instance, it has been demonstrated that HER2 overexpression in ER α -positive MCF-7 human breast cancer cells renders them Tam^R [7], and markedly increased levels of EGFR and HER2 protein have been associated with acquired Tam^R in MCF-7 cells [8].

Although numerous studies have examined the role of ER α and progesterone receptor (PR) isoforms A and B in breast cancer and their correlations with other prognostic indicators [9], little is known about the possible role of the androgen receptor (AR) in hormone response in human breast tumors. We do know that AR is present in the majority of primary and metastatic invasive breast tumors, and is often co-expressed with ER α and PR in these tumors [10]. AR expression has been associated with a better outcome for untreated breast cancer patients [11]. Recent reports show that AR is a potential regulatory factor in breast cancer, and there is some evidence implicating AR in modulation of ER α genomic signaling [12]. For instance, estrogen induces a physical interaction between the AR amino-terminal domain and the ER α ligand-binding domain [13]. Growth factor regulation of AR transactivation has recently been postulated to have important functions in prostate cancer progression, especially after androgen withdrawal. HER2 kinase activation may even be required for optimal AR function at physiologically low androgen concentrations [14]. It has also been shown that ER α and AR complexes can regulate EGFR phosphorylation through c-Src signaling in human breast cancer cells, with effects on proliferation triggered by steroid hormones [15]. Furthermore, HER2 expression stabilizes AR protein and enhances AR binding to androgen responsive elements, suggesting important crosstalk between these two signaling molecules.

In the present study we examined whether AR levels could affect response to Tam in ER α -positive breast cancer cells, by engineering MCF-7 cells to overexpress AR. We demonstrate for the first time that AR overexpression confers Tam resistance and affects Tam-induced ER α signaling.

Materials and methods

Reagents, hormones, and antibodies

17 β -estradiol (E), 4-hydroxytamoxifen (T), dihydrotestosterone (DHT), and heregulin- β (H) were obtained from Sigma (St. Louis, MO). Bicalutamide (Casodex, Cx) was purchased from LKT Laboratories Inc (St. Paul, MN). Antibodies used for immunoblotting were: anti-AR clone 441 and anti-Rho GDI from Santa Cruz Biotechnology (Santa Cruz, CA), anti-ER α 6F11 from Vector Laboratories Inc (Burlingame, CA), anti-PR from DAKO (Carpinteria, CA), anti-IRS1 and anti-cyclin D1 from Upstate (Lake Placid, NY), anti- β -actin from Sigma (St. Louis), and anti-total and phosphorylated forms of MAPK, HER2, and AKT from Cell Signaling Technology (Beverly, MA).

Breast tumor specimens and RNA expression microarray analysis

A cohort of frozen breast tumor specimens from nine patients who received Tam treatment was selected from the tumor bank of the Breast Center, Baylor College of Medicine, for use in a pilot RNA expression analysis. This study was approved by the Baylor College of Medicine Institutional Review Board in accordance with federal human research study guidelines. Within this cohort, metastatic tumors from five patients who developed recurrent

lesions while undergoing Tam treatment (resistant group), and four primary tumors that were collected at the time of initial diagnosis from patients who then remained disease-free for 93 to 123 months with a median follow-up of 106 months (Tam sensitive group) were examined using Affymetrix expression microarray as described previously [3]. An additional cohort of frozen breast tumor specimens with no clinical follow-up, but with ER values determined by ligand binding assay was used for immunoblot analysis of AR levels.

Cells and stable transfection

MCF-7 cells have been maintained in our laboratory for >20years [7]. Cells were grown in MEM (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; Summit Biotechnology, Fort Collins, CO), 200 units/mL penicillin, 200 µg/mL streptomycin, and 10 µg/mL insulin. Cells were incubated at 37°C in 5% CO₂. To generate MCF-7 cells stably overexpressing AR, pcR3.1-AR cDNA or empty pcR3.1 vector (Invitrogen) were transfected as described [16], and positive clones were identified using immunoblot analysis with the anti-AR 441 antibody from Santa Cruz Biotechnology.

Cell extracts and immunoblots

Cells were starved in phenol red and serum-free MEM for 48 hours, and then treated for 24–48 hours with different compounds as indicated. For rapid signaling studies, cells were starved as above followed by 5 min treatments with vehicle (C, ethanol), estrogen (E, 1nM), 4OH-Tam (100nM), heregulin-β (H, 20ng/ml) as indicated. In some rapid signaling experiments, cells were pretreated for 2 hours with 10µM Casodex, and then with 4OH-Tam for five minutes. After these varying treatments, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), and were then lysed immediately with 200µl of cell lysis buffer A (20mM Tris HCl, pH 7.4, 15 mM NaCl, 1mM β-glycerophosphate, 1mM sodium orthovanadate, and 10% glycerol plus 1:100 proteinase inhibitor cocktail III (Calbiochem, La Jolla, CA) per 100mm tissue culture plate. The cell lysates were cleared by centrifugation at 16,000 × g for 10 minutes at 4°C. Protein concentration was determined using the BCA Protein Assay kit (Pierce, Rockford, IL) according to the manufacturer's directions. Equal amounts of cell extracts were resolved under denaturing conditions by electrophoresis in 8% to 10% polyacrylamide gels containing SDS (SDS-PAGE) and transferred to nitrocellulose membranes by electroblotting (Schleicher & Schuell, Keene, NH). The blots were first stained with Ponceau S to confirm uniform transfer of all samples. After blocking of the transferred nitrocellulose membranes, they were incubated with primary antibodies, incubated with secondary antibodies for 1 hour at room temperature, and developed with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Tumor xenograft studies

MCF-7 vector control transfected cells and MCF-7-AR11 stable transfectants were established as xenografts in ovariectomized 5 to 6-week-old BALB/c athymic nude mice (Harlan Sprague-Dawley, Madison, WI) implanted with 320 pg/ml of estradiol in cholesterol using sialastic tubing (Dow Corning, Midland, MI) as previously described [3]. Mice were inoculated subcutaneously with 5×10^6 cells. When tumors reached ~200 mm³ (about 14 days) animals were randomly allocated to continue E (n=6–7 per group), or to estrogen withdrawal (estrogen tubes removed) (n=5–7 per group), or to estrogen withdrawal plus Tam (n=5–9 per group). Tam was given at 500µg/animal subcutaneously in peanut oil, 5 days/week for ~30–40 more days. Tumor growth was assessed and tumor volumes were measured as described previously, and tumor graphs show sizes starting at randomization [22]. Animal care was maintained in accordance with institutional guidelines. To address the main question of whether AR-overexpressing tumors grew faster than vector control tumors in the absence of Tam and whether they were more resistant to Tam, we calculated the time to tumor doubling as the time in weeks from randomization to two-fold increase in total

tumor volume over baseline. Time to tumor doubling is a direct way to assess differences in tumor growth rate, without explicitly modeling tumor growth which requires a common model structure across all groups. Survival methods were used to present the data and were computed by the Kaplan-Meier method and compared by the generalized Wilcoxon test, which is more appropriate when, as is common in animal experiments, the assumption of proportional hazards is likely to be violated.

Anchorage-independent growth assays

Cells were starved for 2 days in phenol red-free MEM supplemented with 5% charcoal-stripped FBS, 200units/mL penicillin, and 200µg/mL streptomycin. Soft agar assays were performed in six-well plates using 5×10^3 cells per well. After 14 days, the colonies with >50 cells from triplicate assays were counted. Data shown are the mean colony numbers of three wells, and are representative of two independent experiments.

Transient transfection studies

Cells were maintained in phenol red-free MEM serum-free medium for two days, then one day prior to transfection, were plated in 24-well plates in phenol red-free MEM supplemented with 5% charcoal-stripped FBS (Hyclone, Logan, UT). Cells were transfected using Eugene 6 reagent (Roche, Indianapolis, IN) following the manufacturer's protocol. Each well was transfected with 0.5µg of ARE luciferase reporter or ERE₂-tk-luciferase reporter plasmids [17,18], and 50ng of β-galactosidase expression vector as a transfection internal control standard. Cells were treated with 1nM estrogen, 1nM DHT, 100nM Tam, 10µM Casodex, or ethanol vehicle control for an additional 18–24 hours as indicated. Then the cells were washed twice with PBS and harvested into 1X reporter lysis buffer (Promega, Madison, WI), and luciferase activities were assayed using the dual luciferase assay system (Promega, Madison, WI) following the manufacturer's instructions. Experiments were performed in triplicate.

qRT-PCR

cDNA was synthesized from 1µg of total RNA using Superscript II Reverse Transcriptase from Invitrogen (Carlsbad, CA). Random primers were used for cDNA synthesis in a 20µl reaction per the manufacturer's protocol. cDNA was analyzed by real time PCR using the Applied BioSystems 7500 Fast system. Briefly, the cDNA reaction was diluted 1 to 5 with RNase-free water and 5 µl of this dilution was added to each PCR reaction. Each 25µl reaction also contained 0.4µM forward primer, 0.4µM reverse primer, 0.1µM probe, 1X PCR buffer, 0.125mM dNTP, 5mM MgCl₂, 0.5X ROX and 0.025units/µl Taq Polymerase (Invitrogen, Carlsbad, CA). Each reaction was performed in triplicate. Samples were incubated at 95°C for 30 sec and then subjected to 40 cycles of PCR (95°C for 5 seconds and 60°C for 30 seconds). Primer and probe sequences are: ERα forward primer 5'-CAG GAA CCA GGG AAA ATG-3', ERα reverse primer 5'-AAC CGA GAT GAT GTA GCC AGC-3', ERα TaqMan probe 5'-AGA GGG CAT GGT GGA GAT CTT CGA CA-3', AR forward primer 5'-CCTGGCTTCCGCAACTTAC-3', AR reverse primer 5'-AGCCCCATCCAGGAGTACTG-3' and AR TaqMan probe 5'-CGTGGACGACCAGATGGCTGTCA-3'.

RESULTS

Exogenous overexpression of AR in MCF-7 cells confers Tam resistance *in vitro*

To identify genes whose expression was associated with the development of Tam resistance, we previously employed expression microarray analysis to compare primary tumors from patients treated with Tam who did not recur during extended follow-up vs. metastatic tumors

from patients that progressed during adjuvant Tam treatment. We have begun to systematically examine individual genes identified in this study for their contribution to the problem of hormone resistance, and have already discovered a novel mechanism of resistance from this cohort study involving mitogen-activated protein kinase (MAPK) phosphatase 3 [3]. Analysis of this cohort also identified AR as being more highly expressed in the resistant metastatic tumors compared with the tamoxifen-sensitive primary tumor group (Fig. 1A, $P=0.0017$; the median level of AR RNA assayed by expression microarray was 2.1-fold higher in the resistant group [data not shown]). We confirmed levels of AR mRNA in these tumors using qRT-PCR (Fig. 1B), showing that AR levels tended to be higher, but this did not reach statistical significance using this quantitative assay ($P=0.11$), but ER α levels were significantly lower ($P<0.05$) in the Tam^R cohort of tumors. Lower levels of ER α in our small Tam^R metastatic tumor cohort are consistent with several clinical reports of associations between lower ER α in metastatic patients compared with the primary tumor, and with poorer survival [19–21]. In addition, *in vitro* derived Tam^R lines have been shown to express lower levels of ER α [22]. To examine the levels of AR protein that were generally expressed in breast tumors relative to ER levels (expressed as fmols by ligand binding assay), we employed immunoblot analysis of 10 random tumors (Fig 1C). Many of these tumors (#5, 7, and 10) expressed relatively high levels of AR (3.1X, 1.9X, and 1.7X respectively) of AR protein compared to the levels of AR in the MCF-7 control cell line. ER α protein levels in these tumors are also shown for comparison. The results suggest that there are indeed tumors which express relatively high levels of both AR and ER α .

To examine whether AR overexpression could affect response to Tam in breast cancer cells, we genetically engineered a model by stably transfecting MCF-7 cells with either an empty pcR3.1 vector plasmid (vector control cells), or a plasmid encoding the human full-length AR cDNA in pcR3.1 which also expresses the neomycin resistance marker. Cells were selected in G418 antibiotic for plasmid expression. Resulting stable clones were screened for AR levels using immunoblot analysis. Two MCF-7-AR-overexpressing clones (Fig. 1D, clones AR11 and AR30) showed elevated levels of AR compared with vector control-transfected MCF-7 cells, similar to the range of AR levels seen in some tumors (Fig. 1C). Since it has been reported that AR expression can interfere with ER α activity in some breast cancer cells [13], we then examined levels of ER α using immunoblot analysis. The MCF-7-AR11 and AR30 clones maintained ER α expression; showing that AR overexpression did not interfere with ER α protein levels in our cells. Immunoblotting with an antibody to β -actin was used as loading control in this experiment.

To assess whether ectopic AR overexpression might affect anchorage-independent growth of these cells, cells were starved in phenol red-free medium supplemented with charcoal-stripped serum, then growth was assessed in soft agar in the presence of estrogen (E) or 4OH-Tam (T) for 14 days (Fig. 1E). Estrogen treatment increased the number of soft agar colonies both in the MCF-7 vector control transfected cells, and in MCF-7-AR11 and AR30 overexpressing cells, compared to vehicle (C) treated cells. Treatment with Tam (T) had little effect on the number of colonies in MCF-7 vector control cells (compare vector C vs. vector 4OH-Tam-treated; $P=0.62$). In contrast, colony numbers of MCF-7-AR11 and AR30 AR-overexpressing cells were enhanced by Tam treatment (AR11 was marginally significant [$P=0.06$], whereas AR30 was highly significant $P=0.02$). The MCF-7 AR-overexpressing cells also formed larger colonies (data not shown), and contained twice as many colonies as the MCF-7 vector cells in the presence of Tam. These results suggest that AR overexpression might affect cellular responsiveness to Tam. This effect was not due to changes in AR levels, as neither estrogen nor Tam treatment affected AR levels in either vector control or AR-overexpressing cells (immunoblot data not shown).

Casodex (bicalutamide, Cx) is an AR antagonist with antiproliferative effects in AR-expressing prostate cancer cells [23], but its reported effects in breast cancer cells vary [24]. Casodex alone had little effect on the growth of any of our MCF-7 transfectants under control conditions (Fig. 1E). As a positive control, dihydrotestosterone (DHT, D) treatment increased soft agar colonies in all cells, and was nearly equivalent to estrogen-stimulated growth. Adding Tam reduced DHT-stimulated growth (DT), suggesting that the actions of AR might be affected by antagonizing ER α in our cells. Casodex in combination with Tam (CxT) reduced soft agar growth below Tam treatment alone in all cells ($P=0.02$ for vector and AR11 cells, and <0.01 for AR30 cells). These results suggest that Casodex might restore Tam sensitivity in MCF-7-AR overexpressing cells, and that even in cells with lower levels of AR, proliferation might be responsive to combination treatment with Tam and Casodex.

Tamoxifen induces agonistic effects in AR-overexpressing cells

Mammalian two-hybrid analyses have demonstrated that the amino-terminal region of AR can interact with the ER α ligand-binding domain, and that the two receptors can interfere with each others' genomic transcriptional activities in the presence of agonists for both receptors [13]. We therefore examined whether AR or ER α transcriptional activities were altered in AR-overexpressing cells, using luciferase reporter transactivation assays. Luciferase reporter plasmids containing either consensus androgen-responsive element (ARE) sequences or estrogen-responsive element (ERE) sequences were transiently transfected into MCF-7 vector-alone, MCF-7-AR11, and AR30 cells in the absence or presence of various hormones. As expected, after exposure to DHT (D), AR activity was increased in all cell lines, with higher increases seen in the two AR-overexpressing lines. In contrast, Tam treatment (T) had little (vector control cells, $P=0.05$) or no effect on AR transcriptional activity (AR11 and AR30 cells, $P=0.35$ and 0.26 , respectively; Fig. 2A). Combination treatment with D+T did not alter the levels of DHT-stimulated activity (data not shown) suggesting that ER α may not be involved in AR-mediated transactivation in this assay.

ER α transcriptional activity was assessed in Fig. 2B. Treatment with estrogen (E) induced ER α activity in all lines. ER α activity was not induced in MCF-7 vector control cells after Tam treatment, but Tam induced an increase in ER α transcriptional activity in AR-overexpressing cells (AR11 and AR30, $P<0.01$), suggesting that Tam may be acting as an agonist in this context. Casodex treatment (Cx) had little effect by itself on ER α transcriptional activity, but was able to abrogate the high Tam-induced agonistic activity in the AR30-overexpressing cells ($P<0.01$), but not in the AR11 clone ($P=0.25$) which exhibited less Tam agonist activity. Thus, Casodex may be able to inhibit high Tam agonist activity in AR-overexpressing cells.

Exogenous overexpression of AR in MCF-7 cells confers Tam resistance *in vivo*

To analyze the endocrine sensitivity of AR-overexpressing cells *in vivo*, we examined the ability of MCF-7-AR11 and vector control cells to form tumors in ovariectomized athymic nude mice. We established xenografts in mice supplemented with estrogen for 14 days, and then mice were randomly assigned to continued estrogen treatment (Fig. 3A), or to estrogen withdrawal in the absence (Fig. 3B) or presence (Fig. 3C) of Tam. Time 0 weeks reflects the time at randomization. Survival analysis of time to tumor doubling was used to analyze these experiments as described in the Materials and Methods section. Survival analyses comparing the time to tumor doubling found no significant difference between vector and AR-transfected tumors in the estrogen-treated group ($P=0.14$, Fig 3A). In contrast, the growth of AR-overexpressing tumors was increased in both the estrogen-withdrawal and the Tam-treated groups; these results were borderline significant ($P=0.055$, $P=0.058$, respectively Figs. 2B and C). Thus, these *in vivo* data reiterate what we observed in the

anchorage-independent soft agar assays, showing that AR overexpression conferred resistance to anti-hormonal treatments.

Tamoxifen induces expression of ER α -regulated genes in AR-overexpressing cells

Elevated levels of epidermal growth factor receptor and the HER2 receptor have been demonstrated in Tam^R cell lines [25] and Tam^R breast cancer patient samples [26]. Thus we next addressed whether increased HER2 signaling might be involved in AR-associated resistance. However, unlike other *in vitro* derived models of resistance, we did not observe upregulation of either total or phosphorylated HER2 using immunoblot analysis in vector control and MCF-7-AR11 cells (Fig. 4A). In this experiment short term treatment with heregulin was used as a positive control to stimulate HER2 and AKT phosphorylation. Short term treatment with Tam also did not alter HER2 phosphorylation, or its downstream signaling molecule AKT, in either line. These results suggest that AR-associated resistance might employ novel mechanisms distinct from those described by several other groups involving ER α -growth factor receptor crosstalk in HER2-positive breast cancer cells [27].

Because Tam treatment is known to alter estrogen-regulated gene expression in breast cancer cells [28] and we saw enhanced Tam agonist activity in the AR-overexpressing cells, we next used immunoblot analysis to examine the effect of Tam on the expression of endogenous estrogen-regulated proteins (Fig. 4B). As expected, estrogen increased levels of the estrogen-regulated IRS1 and cyclin D1 proteins in both vector control and AR-overexpressing cells. However, we found that Tam also induced expression of these two proteins, but only in the MCF-7-AR11 cells. As a control, we examined levels of ER α ; receptor levels were decreased by estrogen and stabilized by Tam treatment as has been shown by others [29]. β -actin levels were used as a loading control. Thus, Tam is acting as an agonist to induce known estrogen-regulated, endogenous gene expression in AR-overexpressing cells.

Active mitogen-activated protein kinase (MAPK p42/44) has also been correlated with resistance in a number of different systems [30,31], and it has been reported that AR can mediate the activation of MAPK [32]. Using immunoblot analysis we discovered that short-term treatment with Tam did not affect MAPK phosphorylation in MCF-7-AR30 cells, though there was a modest effect in vector control cells (Fig. 4C); heregulin (H) treatment was used as a positive activation control. Both heregulin (Fig. 4C) and epidermal growth factor (EGF, Fig. 4D) increased pMAPK levels in AR-overexpressing cells (AR30) more than in vector control cells, suggesting a potential role for enhanced c- ErbB2 family signaling to MAPK in the overexpressing cells. Which c-ErbB2 family member other than HER2 mediating these effects is currently under investigation.

We conclude that AR might be playing a novel, but yet to be defined role in the emergence of resistance to Tam in breast cancer cells, as evidenced in soft agar and xenograft growth studies, and that AR overexpression may be associated with other biomarkers of clinical resistance, including elevated levels of cyclin D1 and IRS-1.

Discussion

The clinical success of the aromatase inhibitors and the sequencing of these agents with Tam have greatly enhanced our management of hormone-dependent breast cancer [33]. These ER-targeted therapies are initially useful in many breast cancer patients, but resistance eventually develops and disease recurs. An emerging hypothesis is that ER action remains important in the problem of resistance, perhaps due to ER crosstalk with growth factor receptors such as c-ErbB1 or HER2, or possibly with other intracellular signaling networks [3]. This is suggested by the usefulness of various inhibitors to the c-ErbB2 family of

receptors in the preclinical and adjuvant setting [27,34]. However, not all the data from clinical trials support this concept [35]. We have begun to examine individual gene candidates for their potential role in resistance, using pilot data obtained from a small microarray study designed to identify gene expression differences in tumors from patients recurring while on Tam treatment [3]. Herein we describe the role of overexpression of one such candidate, the AR, in the development of Tam resistance, using an ER α -positive breast cancer model. We show that AR overexpression conferred resistance using the preclinical assays of anchorage-independent growth in soft agar, and Tam-resistant growth in xenografts. The relative resistance of AR-overexpressing cells to estrogen withdrawal seen in the xenograft experiments also suggests that unliganded AR overexpression might predict response to aromatase inhibitors, a hypothesis that we will explore in the future. Regardless, our results strongly suggest that AR may be involved in a resistant phenotype since the AR antagonist Casodex was shown to reverse resistance and decrease Tam agonist activity.

The AR is a nuclear receptor which plays a central role in prostate carcinogenesis, and several lines of evidence have implicated it as a regulatory factor in breast cancer as well. We know that the AR is expressed in approximately 60% of primary breast tumors [10]. Interestingly, Buchanan et al. [36] have shown that decreased AR levels and mutated ARs were associated with resistance to the synthetic progestin medroxyprogesterone acetate in metastatic breast cancer. The mechanism of this reported progestin resistance is not known, since medroxyprogesterone acetate can bind with high affinity to the AR as well as the progesterone receptor [37]. Androgen ligands can have inhibitory or stimulatory effects on the growth of breast cancer cells [24,38], again highlighting a potential role for androgen-bound AR in the growth of breast cancer. Most recently, it has been demonstrated that the pure steroidal antiestrogen fulvestrant can repress AR levels and transcriptional activity [39], but it is not known whether ER α is involved in this effect. Androgens have been used in the past as a hormonal therapy for advanced breast cancer, with an efficacy comparable to that of Tam [40], an important finding which suggests that both the AR and ER α pathways regulate breast tumor progression.

AR is capable of activating cell signaling pathways in response to ligands, but can also be activated through signaling pathways independent of androgens in prostate cancer, which can contribute to the progression of hormone-independent disease [41,42]. AR protein levels and activity can be affected by HER2 signaling, but the downstream kinase that regulates AR has not been identified [14]. We did not see activation or amplification of HER2 in our engineered AR overexpression model. We also did not see obvious changes in the levels of another c-ErbB2 family member (epidermal growth factor receptor, data not shown), though future studies will have to explore this idea more thoroughly. Since we saw enhanced activation of MAPK using either heregulin or EGF treatment in AR-overexpressing cells, we will focus on growth factor receptors upstream of these signaling molecules in future experiments. In MCF-7 cells, it has been shown that ER α and AR complexes can regulate c-ErbB2 signaling through c-Src engagement [15]. Thus it is a possibility that crosstalk between AR and other cytoplasmic signaling pathways might impact AR-mediated resistance, similar to the crosstalk that has been demonstrated for ER α .

What about the genomic effects of AR overexpression in breast cancer cells? In AR-overexpressing cells we observed enhanced Tam agonist activity on ER α at ERE sites, which could be blocked by Casodex treatment. Although this is an understudied area, there is good evidence that ER α and AR are capable of interacting *in vivo* [13,43]. Thus, since the primary p160 coactivator binding site in AR is in the hormone-independent AF-1 domain [13], unliganded AR may bring coactivators to the ER α complex; we will test this possibility in future experiments. Although AR antagonists such as Casodex are frequently used in prostate cancer, Casodex has not been adequately tested in human breast cancer patients, but

our results suggest that this agent might be useful in combination with ER-targeted therapies.

In conclusion, we hypothesize that the AR may assist Tam-dependent stimulation of ER α action through activation of cell signaling pathways that facilitate this ER α activation. If AR and ER α do physically interact, their cooperation on genomic targets could also play a role in breast tumor progression and hormonal resistance. There are a number of possibilities for likely mechanisms by which overexpression of AR could induce resistance. One intriguing possibility is that AR and ER α interact in the presence of Tam, and are recruited to ER-responsive gene promoters. AR overexpression could provide unique assistance to ER α action either by participating in the displacement of corepressor proteins, by recruiting coactivators, or even acting as a coactivator itself. For example, cyclin D1 is a well-characterized endogenous target of ER α transcriptional activity [44]. Another model of acquired Tam resistance did not reveal changes in cyclin D1 levels with Tam treatment [45], as we report here in our MCF-7-AR overexpressing cells. The major antiproliferative effect of Tam has been related to the ability of Tam to inhibit cyclin D1 expression [46]. Furthermore, cyclin D1 overexpression is a predictor of poor response to Tam in postmenopausal breast cancer patients [47]. AR overexpression could abrogate the ability of Tam to inhibit cyclin D1 levels. Alternatively, it is tempting to speculate that AR and ER α could collaborate to regulate cyclin D1 gene expression, thereby promoting cell cycle progression in the presence of Tam. Emerging data mapping AR and ER α genome-wide binding sites will be informative in this regard. Our combined results suggest that AR may provide a new target for therapeutic augmentation of combination hormonal therapies in breast cancer.

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Abbreviations

AR	Androgen receptor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERα	Estrogen receptor alpha
PR	Progesterone receptor
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction

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A

B

E **Fig. 1.**

A, AR RNA levels as measured by expression microarray in Tam Sen and Tam Res cohorts. **B**, quantitation of AR and ER α RNA relative to control actin levels in the same cohorts of patients. **C**, immunoblot analysis of AR levels in 10 tumors, with MCF-7 cells as a control. AR levels are expressed relative to MCF-7 AR levels. ER α levels measured by ligand binding assay are also shown. **D**, immunoblot analysis of AR and ER α expression in vector control-transfected MCF-7 cells (Vector), and two AR-transfected MCF-7 clones (AR11 and AR30). β -actin was used as a loading control. **E**, anchorage-independent growth of vector control, MCF-7-AR1, and MCF-7-AR30 cells was assessed by soft agar colony formation assay in control conditions (C, ethanol), or in the presence of 1nM estrogen (E), 100nM 4OH Tam(T), 1nM DHT (D), 10nM Casodex (Cx), or combinations. The colony number was assayed after growth under the respective conditions for 14 days. Graphs represent the mean colony number of three dishes.

Fig. 2.

A, ARE luciferase reporter assay of vector control, AR11, and AR30 cells treated for 24 hours with control vehicle (C), 1nM dihydrotestosterone (D), or 100nM 4OH-Tam (T). Each column represents the mean luciferase activities from three independent experiments normalized to β -galactosidase activity. **B**, ERE luciferase reporter assay of vector control, AR11, and AR30 cells treated for 24 hours with control vehicle (C), 1nM estrogen (E), 100nM 4OH Tam (T), 10 μ M Casodex (Cx), or Tam plus Cx (CxT). Each column represents the mean luciferase activities from three independent experiments.

Fig. 3.

A–C, MCF-7 vector control-transfected and MCF-7-AR11 cells were injected into athymic nude mice supplemented with estrogen for 14 days and then randomly assigned to either continue estrogen treatment (A), or have their estrogen pellets removed (B), or have their pellets removed and begin treatment with Tam (C). For the vector control groups, there were 6 animals for the estrogen-treated group, and 5 animals each for the no-treatment and Tam groups. For the MCF-7-AR11 groups, there were 9 animals for the Tam-treated group, and 7 animals each for the no-treatment and estrogen-treated groups. “Survival” was defined as the number of animals with tumors not yet doubled in size. $P=0.14$ estrogen treatment, 0.055 estrogen withdrawal, and 0.058 Tam treatment groups.

Fig. 4.

A, immunoblot analysis of whole cell extracts using antibodies against p-HER2, total HER2, p-AKT, or total AKT. Serum-starved vector control and MCF-7-AR11 cells were treated with 100 nM Tam for 15 minutes or left untreated. Treatment with 10ng/ml heregulin (H) was used as a positive control. **B**, immunoblot analysis of whole cell extracts using antibodies against IRS-1, cyclin D1, and ER α . Serum-starved vector control and MCF-7-AR1 cells were treated for 48 hours with vehicle control (C), 10nM estradiol (E), or 100nM Tam (T). β -actin antibody was used as a loading control. **C**, immunoblot analysis of whole cell extracts using antibodies against p-MAPK or total MAPK. Serum-starved vector cells and MCF-7-AR30 cells were treated with 100nM Tam (T) for varying times (min) or heregulin (H). **D**, immunoblot analysis of vector control and AR30 whole cell extracts using antibodies to p-MAPK or total MAPK after treatment for 5 minutes with ethanol control (C), estrogen (E2), Tam, or 50nM EGF.