

Androgen Receptor Inhibits Estrogen Receptor- α Activity and Is Prognostic in Breast Cancer

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Abstract

There is emerging evidence that the balance between estrogen receptor- α (ER α) and androgen receptor (AR) signaling is a critical determinant of growth in the normal and malignant breast. In this study, we assessed AR status in a cohort of 215 invasive ductal breast carcinomas. AR and ER α were coexpressed in the majority (80-90%) of breast tumor cells. Kaplan-Meier product limit analysis and multivariate Cox regression showed that AR is an independent prognostic factor in ER α -positive disease, with a low level of AR (less than median of 75% positive cells) conferring a 4.6-fold increased risk of cancer-related death ($P = 0.002$). Consistent with a role for AR in breast cancer outcome, AR potently inhibited ER α transactivation activity and 17 β -estradiol-stimulated growth of breast cancer cells. Transfection of MDA-MB-231 breast cancer cells with either functionally impaired AR variants or the DNA-binding domain of the AR indicated that the latter is both necessary and sufficient for inhibition of ER α signaling. Consistent with molecular modeling, electrophoretic mobility shift assays showed binding of the AR to an estrogen-responsive element (ERE). Evidence for a functional interaction of the AR with an ERE *in vivo* was provided by chromatin immunoprecipitation data, revealing recruitment of the AR to the progesterone receptor promoter in T-47D breast cancer cells. We conclude that, by binding to a subset of EREs, the AR can prevent activation of target genes that mediate the stimulatory effects of 17 β -estradiol on breast cancer cells. [Cancer Res 2009;69(15):6131-40]

Introduction

Estrogen signaling is a key determinant of the growth and survival of normal and malignant breast epithelial cells, and this

underpins the widespread use of antiestrogens and aromatase inhibitors in the adjuvant treatment of breast cancer (1, 2). There is emerging evidence that androgen signaling also plays a key role in normal and malignant breast tissues (3). Although it is generally accepted that ovarian and adrenal androgens can influence breast cancer cell growth by aromatization to estrogens (4), androgens such as testosterone and its more active metabolite 5 α -dihydrotestosterone (DHT) can inhibit basal and 17 β -estradiol (E₂)-stimulated proliferation of breast cancer cells by an androgen receptor (AR)-dependent mechanism (reviewed in ref. 5). Androgens have been used as hormonal therapy for breast cancer, with an efficacy comparable with that seen with the widely used estrogen receptor α (ER α) antagonist, tamoxifen (6, 7). Indeed, it is possible that the greater therapeutic response of breast cancers to aromatase inhibitors compared with tamoxifen is due to a concomitant reduction in E₂ and an increase in androgen signaling (8). AR typically is present in a greater proportion of breast tumors (80-90%) than ER α (50-80%; reviewed in refs. 5, 9) and previous studies have indicated the potential for AR to predict disease progression (10, 11). In addition, we have reported that the level of the AR predicts both the likelihood and the duration of response to therapy with the synthetic progestin, medroxyprogesterone acetate (12), and that disease progression after medroxyprogesterone acetate therapy is associated with inactivating mutations in the AR gene (13). What is unclear, however, is the mechanism by which androgens influence hormonal sensitivity and disease progression in breast cancer and how best to use AR signaling to modulate the growth of breast cancer cells (9, 14). In this study, we show that the AR level is significantly associated with disease outcome in ER α -positive breast cancer. We further show that AR is a direct repressor of ER α signaling in breast cancer cells and show for the first time that this is due to an association of the AR with response elements of estrogen target genes.

Materials and Methods

Cell lines and tissues. MDA-MB-231, T-47D, and COS-1 cells (American Type Culture Collection) were maintained in RPMI 1640 supplemented with 5% fetal bovine serum (FBS). 293A cells (Invitrogen) were maintained in DMEM (high glucose) supplemented with 10% FBS, 0.1 mmol/L MEM nonessential amino acids, 2 mmol/L L-glutamine, and 1% penicillin-streptomycin. Tissue microarrays consisted of 215 invasive ductal carcinoma samples (15). Grading was according to the modified Bloom and Richardson system, and 157 of 215 samples were ER α positive based on a cut point of 10% positive tumor cells. All protocols were approved by the

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

A.A. Peters and G. Buchanan contributed equally to this work. L.M. Butler and W.D. Tilley codirected this project.

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Plasmids. Estrogen-responsive element (ERE)-tk-luc and ER α (pSG5-HGO) vectors were provided by Profs. Alessandro Weisz (Seconda Università di Napoli) and Pierre Chambon (College de France), respectively. pCMV-AR, AR Δ 553-622, and AR Δ 38-410, AR Δ 38-532 have been described (16–18). AR-23AQNAA27 and AR-C617Y variants were created in pCMV-AR and AR(1-707) using PCR megaprimer mutagenesis as described previously (19). Deletions were recreated in AR(1-707) by subcloning. pSG5-HA-AR(553-662) was created by PCR amplification of the AR DNA-binding domain (DBD) coding sequence using 5-TTGAATTCAGAGACCTGCCT-GATCTG-3' and 5-TTGATCCTCACATCCCTGCTTCATAACATT-3' and cloning into pSG5-HA. AR(1-707) was subcloned into the Gateway adenoviral expression vector pAD/CMV/V5-DEST (Invitrogen).

Transactivation assays. MDA-MB-231 or T-47D cells (1.75×10^4 and 1.5×10^4 per well, respectively, in 96-well plates) were transfected as described previously (16) with 100 ng ERE-tk-luc, 2.5 ng ER α (for MDA-MB-231 cells), and either an AR expression vector or a parental, control pCMV vector. The total amount of expression vector and DNA was kept constant in each transfection with pCMV and pBS(sk-). Cells were treated for 36 h in medium containing 5% DCC-FBS and steroid or vehicle control, lysed, and assayed for luciferase activity.

Immunohistochemistry, immunoblot, and immunoprecipitation. Immunohistochemistry was done using AR-U407 antiserum on 4 μ m sections of breast cancer tissue microarray blocks as described previously (20). Staining was scored in duplicate cores by visual appraisal of 100 cells in each of two fields of view and is presented as percent AR-positive cells. Images shown were obtained using a Nanozoomer slide scanner (Hamamatsu) and captured using NDPview software (Hamamatsu). Immunoblot analysis was done on (a) lysates of MDA-MB-231 cells (5×10^5 cells per well in 6-well plates) transfected with ER α (500 ng) or AR(1-707) expression vectors or pCMV, (b) lysates of cells transfected in 96-well plates, or (c) T-47D cells transduced with pAD-AR(1-707). Antisera used were AR(N-20), ER α (HC-20), cathepsin D (CTSD; H-75), β -actin (I-19; Santa Cruz Biotechnology), and progesterone receptor (PGR; hPRa3, provided by Prof. Christine Clarke, Westmead Millennium Institute, University of Sydney). Coimmunoprecipitation was done in T-47D or COS-1 cells transfected with AR and ER α expression vectors using AR(N-20) and ER α (HC-20) antisera. Chromatin immunoprecipitation assays were conducted using AR(N-20), ER α (H184), and normal rabbit IgG (Santa Cruz Biotechnology) antisera as described previously (21) on serum-starved T-47D cells (3×10^6 per 150 mm dish) treated for 45 min with 10 nmol/L DHT and/or 10 nmol/L E₂. Real-time PCR was done using either SYBR Green incorporation or FAM-labeled probes with primers to the following regions: KLK3/prostate-specific antigen (PSA) enhancer (Chr19: 56,045,991-56,046,075), CTSD enhancer 1 (Chr11: 1,750,279-1,750,879), PGR enhancer 1 (Chr11: 100,674,936-100,675,904), and nonspecific control (Chr20: 44,141,632-44,141,782). Input values were obtained from parallel samples that were not immunoprecipitated.

Immunofluorescence. Tissue sections (2 μ m) were dewaxed, rehydrated, and blocked with 30% hydrogen peroxide. After antigen retrieval in citrate buffer (pH 6.5), sections were incubated with 5% blocking serum for 30 min and then overnight with ER α (1:50; Santa Cruz Biotechnology) and AR (1:50; DAKO) primary antibodies in a humid chamber at 4°C. Secondary antibodies Alexa Fluor 594 (AR, 1:400; Invitrogen) and Alexa Fluor 488 (ER α , 1:400; Invitrogen) were incubated for 1 h each at room temperature. The sections were then counterstained with 4',6-diamidino-2-phenylindole and mounted with special fluorescence mounting medium (DAKO). Slides were viewed using an Olympus IX71 fluorescent microscope and images were obtained with an Olympus DP70 cooled digital color camera at $\times 20$ magnification.

Adenoviral transduction. Viral pAD-LacZ and pAD-AR(1-707) stocks were prepared in 293A and purified by CsCl gradient centrifugation. T-47D cells (2×10^4 per well in 24-well plates) were transduced with virus at a multiplicity of infection of 15 for 16 h. Medium was replaced with RPMI 1640 supplemented with 5% FBS or 5% DCC-FBS and 1 nmol/L E₂ or vehicle. Viable cells were assessed by trypan blue exclusion.

Electrophoretic mobility shift assay. Nuclear extracts from COS-1 cells untransfected or transfected with AR or ER α expression vectors were incubated alone or in combination with the appropriate synthetic complementary oligonucleotides (vitellogenin A2 ERE 5'-AGCTTTCTA-GAAGGTC ACAG TGACCTACTAGT-3' and PSA/KLK3 PSA androgen-responsive element (ARE) 5'-AGCTTCTTGCAGAACA GCA AGTGC-TAGCTG-3') labeled with [α^{32} P]dATP (200,000 cpm) and where appropriate with AR(N-20) and ER α (G-20)X antisera (Santa Cruz Biotechnology). Bands were resolved by electrophoresis on 5% polyacrylamide (acrylamide/bisacrylamide, 29:1), nondenaturing gels in 0.5 \times TGE (12.5 mmol/L Tris, 95 mmol/L glycine, 0.5 mmol/L EDTA), according to previous methodology (22).

Molecular modeling and molecular dynamic simulation. Molecular modeling was done as described previously (23) with the YASARA Dynamics Program (version 6.2.4; ref. 24). Briefly, AR-DBD (PDBid 1R4I) and ER α -DBD (PDBid 1HCQ; ref. 25) crystal structures were superimposed and the ARE was mutated *in silico*, so that it accorded with the consensus ERE. Molecular dynamic simulations (7.6 Å cutoff with a particle Ewald mesh approximation used for longer-range electrostatic forces) were done in a cell with periodic boundaries extending 10 Å outside the target structure in all three axes filled with water molecules subject to simulated annealing energy minimization. Snapshots taken at 50 ps intervals for 500 ps were overlaid with those of the AR-DBD/ARE.

Statistical analysis. For the Kaplan-Meier plots and the Cox proportional hazards model, statistical analyses were done using the Statistical Package for the Social Sciences version 13.0, with relapse and/or death from breast cancer as the endpoints. All other statistical analyses were done using GraphPad Prism version 5.02 and statistical significance accepted at $P \leq 0.05$. Comparisons between multiple groups were analyzed using one-way ANOVA with Tukey's post-hoc test.

Results

AR expression in breast cancer is related to overall survival in ER α -positive but not ER α -negative disease. To assess the prognostic value of AR expression in breast cancer, we undertook immunohistochemical analysis of AR in a cohort of 215 invasive ductal breast carcinomas of known ER α status (Supplementary Table S1). Moderate to intense AR immunoreactivity was observed in the nuclei of tumor cells, with a mean percent AR positivity of 62.8%, a median of 75%, and a range of 0% to 96%. Weak cytoplasmic staining was also observed in some tumors. To assess the potential effect of AR on ER α function, we divided the cohort into ER α positive (157 cases) and ER α negative (58 cases). The median AR immunostaining in ER α -positive cases (84.0%) was significantly greater than in ER α -negative disease (19.75%; $P < 0.0001$), although the range of immunostaining in both groups was almost identical (Supplementary Table S1; Fig. 1A and B). Kaplan-Meier product limit analysis with the median percent AR positivity in tumor cells for the entire cohort (75%) as a cut point showed that AR is significantly associated with overall survival in ER α -positive disease (Fig. 1C; $P = 0.002$) but not ER α -negative disease (Fig. 1D; $P = 0.32$). In ER α -positive breast cancer, multivariate Cox regression analysis indicated a 3.0-fold increased risk of relapse and a 4.6-fold increased risk of cancer-related death for patients with lower than the median percent AR positivity in tumor cells (Supplementary Tables S2 and S3).

Evidence for functional interactions between AR and ER α in breast cancer cells. To determine whether AR has the potential to influence ER α activity in individual breast cancer cells, we first used dual-labeling immunofluorescence of both receptors to show that AR and ER α are coexpressed in normal breast epithelial cells, although AR is expressed in a greater percentage of epithelial cells

than ER α , and are colocalized in a high percentage (80-90%) of breast tumor cells (Fig. 2A; $n = 5$). Given that AR and ER α are coexpressed in breast tumor cells, we then assessed whether AR affects ER α transactivation function in breast cancer cells by ectopically expressing both receptors as well as a luciferase-linked ER α -responsive reporter gene in the AR- and ER α -negative MDA-MB-231 human breast cancer cell line. A dose-dependent inhibition of ER α activity was observed following transfection of increasing amounts of wild-type AR in the presence of the activating ligand, DHT (Fig. 2B).

A constitutively active AR inhibits endogenous ER α transactivation in, and proliferation of, breast cancer cells. We next determined whether AR signaling can inhibit endogenous ER activity to affect E₂-dependent growth of breast cancer cells. We used an AR variant, AR(1-707), that lacks the ligand-binding domain and exhibits strong constitutive activity on exogenous androgen-responsive promoters (18). Transfection of increasing amounts of AR(1-707) expression vector into T-47D human breast cancer cells resulted in a dose-dependent decrease in endogenous ER signaling (Fig. 3A). Transduction of T-47D cells with adenovirus expressing AR(1-707) resulted in a significant reduction in the number of viable cells after 3 and 6 days growth in medium containing 5% FBS compared with LacZ control or mock-transduced cells (Fig. 3B). Immunoblot analysis of T-47D cells transduced with AR(1-707) revealed a concomitant decrease in protein levels of the PGR, an E₂-regulated protein (Fig. 3B). Moreover, compared with mock treatment or transduction of LacZ, AR(1-707) inhibited E₂-induced proliferation of T-47D cells at a level comparable with treatment with the ER α antagonist, tamoxifen (Fig. 3C). Immunoblot analysis done on the same samples at day 3 revealed that both tamoxifen and AR(1-707) abolished E₂-mediated induction of PGR α and PGR β but not of CTSD (Fig. 3D).

AR(1-707) requires DNA binding, but not transactivation capacity, to inhibit ER α activity. To determine how the AR(1-707) variant might inhibit ER α activity, we created a series of deletion and mutation constructs that affect the transcriptional output of the AR variant on an androgen-responsive reporter gene (Fig. 4A) and tested their capacity to inhibit ER α activity in transfected MDA-MB-231 cells. Increasing the amount of AR(1-707) transfected caused a potent dose-dependent decrease in ER α activity (Fig. 4B). An effect of AR(1-707) on ER α protein levels does not appear to be responsible for reduced ER α -induced activity (Fig. 4B). Cell- and promoter-specific activity of the AR is driven by two autologous activation functions in the receptor NH₂-terminal transactivation domain, Tau-1 and Tau-5 (Fig. 4A). Deletion of the minimal domain of Tau-1 (residues 157-361), the complete Tau-1 (residues 38-410), or both Tau-1 and Tau-5 (38-535) had minimal effects on the capacity of AR(1-707) to inhibit ER α activity (Fig. 4C). Similarly, mutation of the AR NH₂-terminal transactivation domain ²³FQNLF²⁷ peptide, which in the full-length AR engages directly with the conserved AF-2 surface in the ligand-binding domain following ligand binding (the AR N/C interaction; ref. 26) and is necessary for AR transcriptional competence *in vivo*, did not affect inhibition of ER α activity by AR(1-707) (Fig. 4C). These results suggest that inherent transactivation capacity of the AR, or interaction of the AR-FQNLF peptide with AF-2 in ER α , does not contribute to inhibition of ER α by AR(1-707). In contrast, when the DBD of AR(1-707) was deleted (Δ 553-662), or DNA-binding capacity was disrupted by the C617Y variant, the constitutive variant was no longer capable of inhibiting ER α activity (Fig. 4D). The loss of an

inhibitory effect of the AR variants on ER α activity was not due to decreased levels of variant AR protein (Fig. 4D), implying that DNA-binding competence of AR(1-707) is necessary for inhibition of ER α activity.

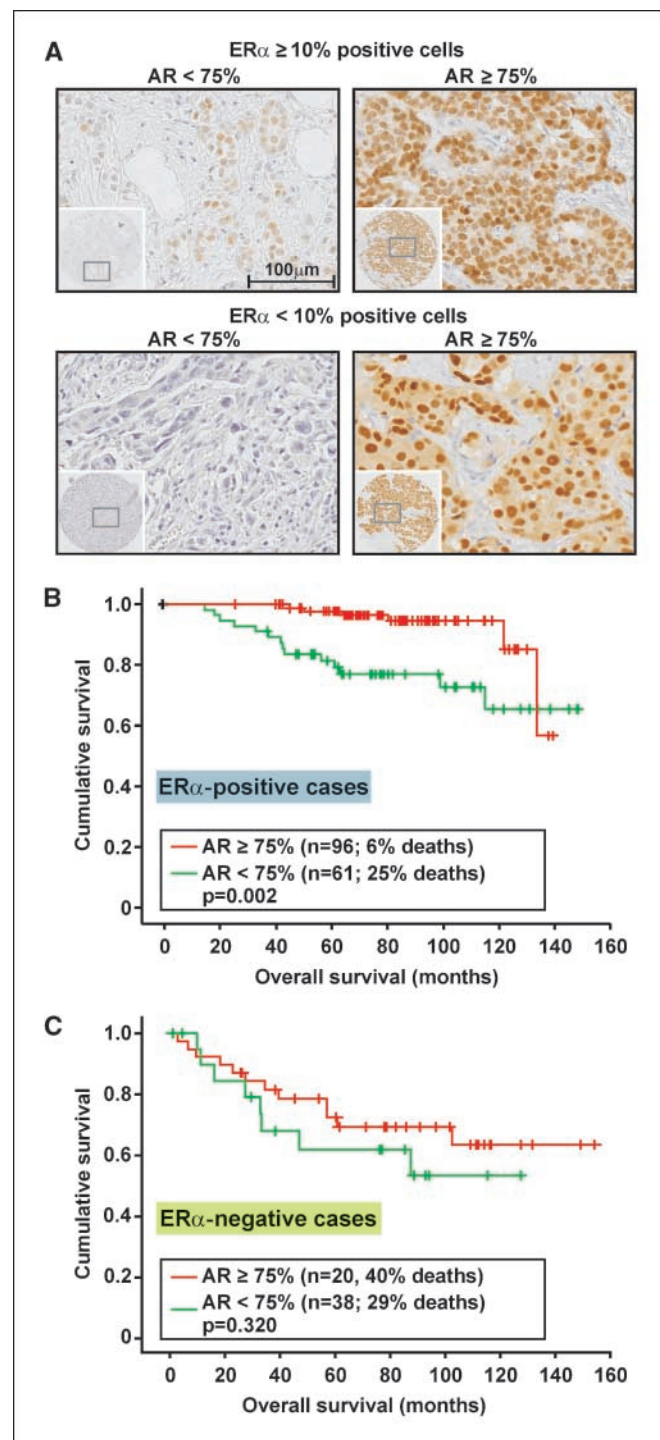


Figure 1. AR expression in breast cancer influences overall survival in ER α -positive but not ER α -negative disease. A, examples of $\geq 75\%$ AR-positive cells and <75% positive cells (AR-U407 antibody) in ER α -positive and ER α -negative breast cancer samples on tissue microarrays. Original magnifications, $\times 5$ (inset) and $\times 20$ (main image). B and C, Kaplan-Meier product limit analysis of overall survival in ER α -positive and ER α -negative breast cancer patients, respectively. Patients were dichotomized by the median percent AR-positive nuclear area (75%).

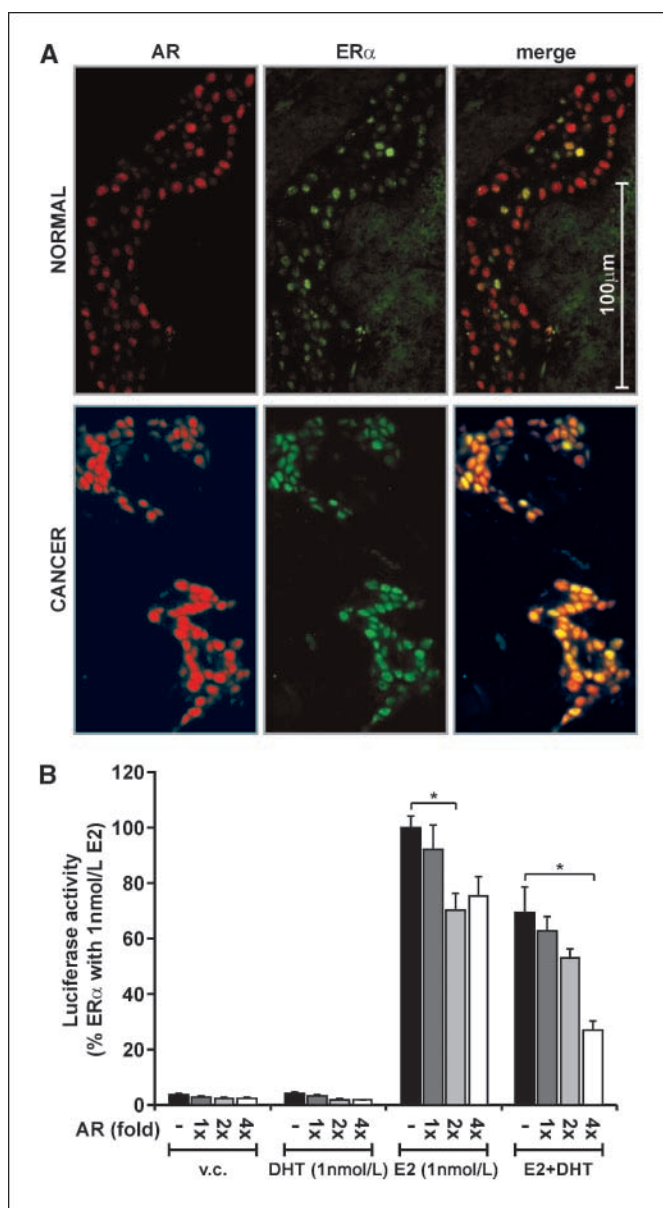


Figure 2. Functional interactions between AR and ER α signaling in breast epithelial cells. **A**, colocalization of AR and ER α in human breast tissues. Samples of normal (top; $n = 5$) and malignant (bottom; $n = 5$) breast tissues were analyzed by dual-label immunofluorescence using AR- and ER α -specific primary antibodies and Alexa Fluor 594-labeled (AR; red) and Alexa Fluor 488-labeled (ER α ; green) secondary antibodies. Colocalization of the two receptors is indicated in the merged image. **B**, inhibition of ER α activity by AR in human breast cancer cells. MDA-MB-231 human breast cancer cells were transfected with an ER α expression vector and the ERE-tk-luc luciferase reporter construct along with 0 \times , 1 \times , 2 \times , or 4 \times molar excess of AR expression vector and treated for 36 h with vehicle control (v.c.) or ligands as indicated and assayed for luciferase activity. Data in this and subsequent figures represent mean \pm SE activity from six individually transfected wells and presented as a percentage of activity induced by 1 nmol/L E $_2$ in the presence of ER α . *, $P \leq 0.05$, ANOVA.

Inhibition of ER α activity by full-length AR requires DNA binding but not constitutive interaction between these proteins. Unlike DHT, the selective AR modulator medroxyprogesterone acetate induces AR activity but does not favor formation of the AR N/C interaction (27). We found that full-length AR activated by DHT or medroxyprogesterone acetate (albeit at a higher con-

centration) had an almost identical capacity to mediate inhibition of ER α activity (Fig. 5A). This result implies that the AR N/C interaction is not necessary for inhibition of ER α activity by full-length AR. A similar finding was obtained using the AR-E895Q variant, which is also capable of transcriptional activity but contains a N/C disrupting mutation in the core of AF-2 (data not shown). Analogous to AR(1-707), deletion of the DBD from the full-length AR completely abrogated DHT-induced inhibition of ER α activity (Fig. 5B). Consistent with these findings, overexpression of the AR-DBD was sufficient to inhibit ER α activity without the requirement for ligand (Fig. 5B). Using immunoprecipitation, we were not able to detect a robust interaction between endogenous ER α and AR in T-47D human breast cancer cells (Fig. 5C) or between ER α and AR(1-707) in transfected COS-1 cells (data not shown). Similar to previous reports (28), we were able to show a weak interaction between full-length AR and ER α when these proteins were overexpressed in COS-1 cells (data not shown). Collectively, these data suggest that the DBD of AR is essential and sufficient for inhibition of ER α activity and that a direct interaction between these proteins probably is not a major contributing mechanism.

AR can interact with ERE. Comparison of the DNA binding logos defined by recent genome-scale studies (29–32) reveals an important distinction between an ERE and an ARE (Fig. 6A). Namely, an ARE is much less constrained in sequence homology than an ERE, which implies that the AR is capable of binding to a greater diversity of response elements than ER α . To test whether AR can bind to an ERE to interfere with ER α transactivation of a target gene, we first used an electrophoretic mobility shift assay to show binding of the AR to both a consensus ARE and a consensus ERE. Specificity of AR binding to the vitellogenin ERE was confirmed by supershift analysis with a specific AR antibody (Fig. 6B). The converse was not observed, with ER α binding confined to the consensus ERE. In reactions containing both AR and ER α , supershift analysis found no evidence for AR/ER α heterodimer formation (Fig. 6B), suggesting that AR binds to the consensus ERE as a homodimer. Using *in silico* molecular dynamic simulation, we assessed whether it is possible to exclude an interaction of the AR with an ERE. We created a dynamic simulation of the AR-DBD homodimer bound to the consensus ERE by successively mutating the PSA-ARE in the solved AR-DBD homodimer crystal structure (Fig. 6C, left). The minimal similarity of these AR/PSA-ARE and AR/ERE simulations suggests that the AR-DBD can form a stable interaction with the consensus ERE in a conformation almost identical to that formed on an ARE. In contrast, molecular modeling virtually excludes the possibility of an AR/ER α heterodimer forming on an ERE (Fig. 6C, right), which is in agreement with the electrophoretic mobility shift assay results. In particular, our model predicts a clash between AR-A594 and ER α -P222 residues that oppose conserved arginines (AR-R605/ER α -R233) at the dimer interface and makes a heterodimer on DNA unlikely. Indeed, the ER α DBD homodimer interface is thought to be intrinsically weak (33).

To assess the potential for AR to bind to EREs *in vivo*, we interrogated known androgen and estrogen target promoter regions by chromatin immunoprecipitation assays. AR, but not ER α , was found to occupy the androgen-responsive PSA/KLK3 enhancer in a ligand-dependent manner (Fig. 6D). ER α and AR were found to occupy the estrogen-responsive CTSD promoter only when both ligands (E $_2$ + DHT) were added to the cultures (Fig. 6D). In contrast, we detected occupancy of both AR and ER α to the PGR promoter in the absence of ligands and increased recruitment of both proteins in the presence of E $_2$ but not DHT (Fig. 6D).

Recruitment to the PGR promoter supports the effect of AR on E_2 -induced PGR expression shown in Fig. 3B and D, albeit the latter was mediated by a constitutively active AR variant. These findings are consistent with the enhanced ability of AR to inhibit E_2 -induced PGR expression compared with CTSD (Fig. 3D). The above results suggest that the AR can be recruited to specific EREs and that the interaction is likely dependent on priming of that response element by E_2 /ER α .

Discussion

In this study, we provide important new insights into the interplay between AR and ER α signaling in breast cancer. Our findings provide evidence that inhibition of breast cancer growth by androgens is mediated primarily through the AR rather than by

indirect mechanisms such as hormone metabolism, nongenomic steroid signaling, or actions of the unliganded receptor as have been suggested previously (34, 35). This is evident first from analysis of AR in clinical breast cancer and its relationship to survival. In ER α -positive disease, AR was an independent predictor of disease outcome, with AR percent positivity in tumor cells of <75% being associated with a significantly reduced relapse-free and overall survival. Furthermore, DHT was required in our *in vitro* studies for inhibition of ER α by the full-length AR but not for inhibition by a strong constitutive truncated AR variant. An important finding in the current study is that ER α activity could be inhibited equally by wild-type AR or AR deletion variants with minimal intrinsic (~9%) transcriptional capacity, which implies that the effect of androgens on breast cancer cells derives primarily from inhibition of ER α signaling rather than via activation of

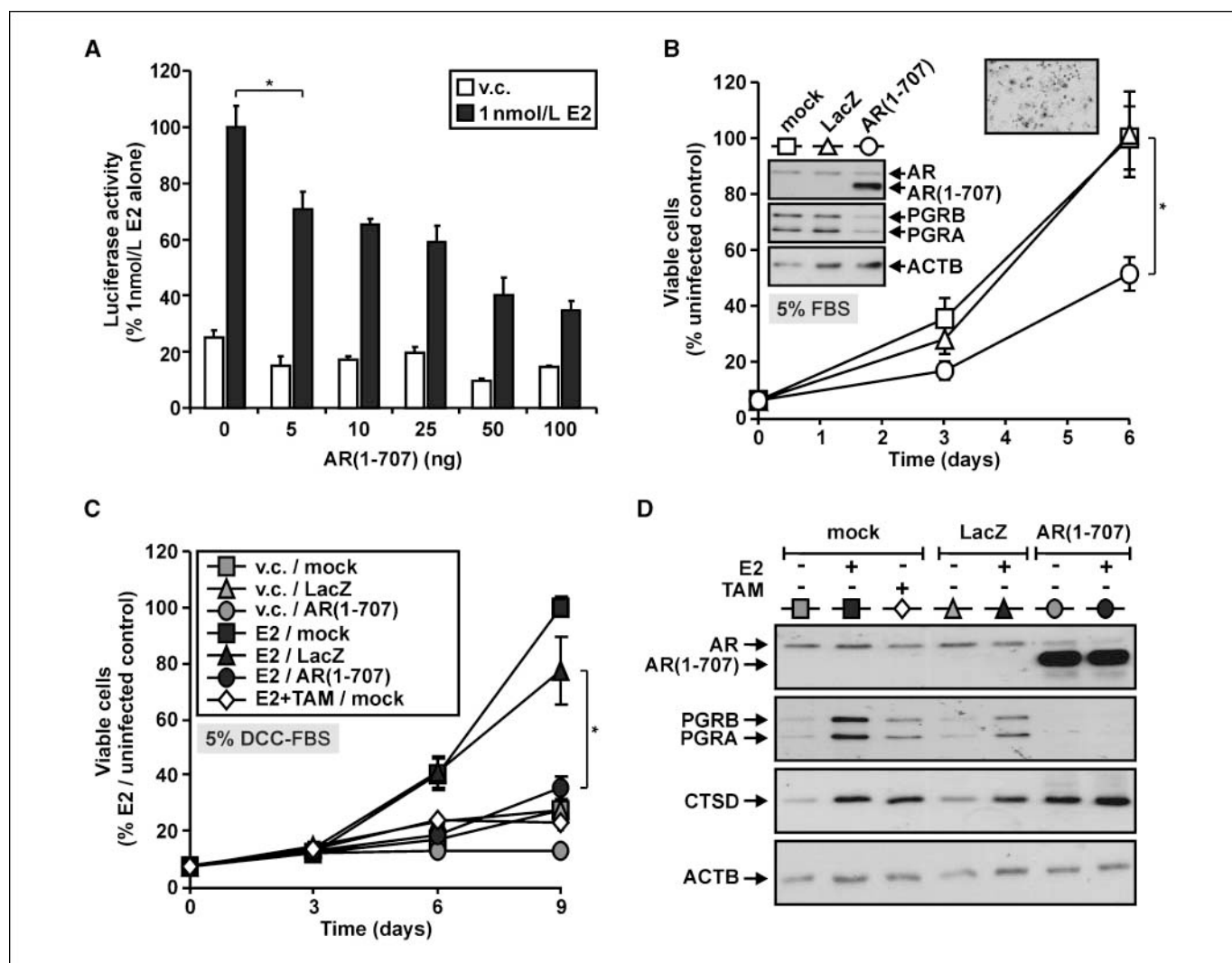


Figure 3. Inhibition of ER activity and E_2 -induced growth of T-47D cells by constitutively active AR. A, inhibition of E_2 activity by AR(1-707). T-47D cells were transfected with the ERE-tk-luc reporter and increasing amounts of AR(1-707), treated for 36 h as shown, and assayed for luciferase activity. Data are presented as percent of activity with 1 nmol/L E_2 in cells without AR(1-707). B and C, inhibition of cell growth by AR(1-707). T-47D cells were mock treated or transduced with adenovirus expressing LacZ or AR(1-707) (multiplicity of infection of 15) and grown in (B) 5% FBS or (C) 5% DCC-FBS with vehicle control, 1 nmol/L E_2 , or 1 nmol/L E_2 and 1000 nmol/L tamoxifen (TAM). Viable cells were counted manually using trypan blue exclusion. Mean \pm SE percent viable cells in triplicate wells compared with mock treatment at (B) 6 or (C) 9 d. B, insets, LacZ expression 4 d after infection and an immunoblot at 6 d. D, immunoblot analysis from cells treated as in C for 3 d shows AR, AR(1-707), PGR isoforms, CTSD, and β -actin (ACTB). *, $P \leq 0.05$, ANOVA.

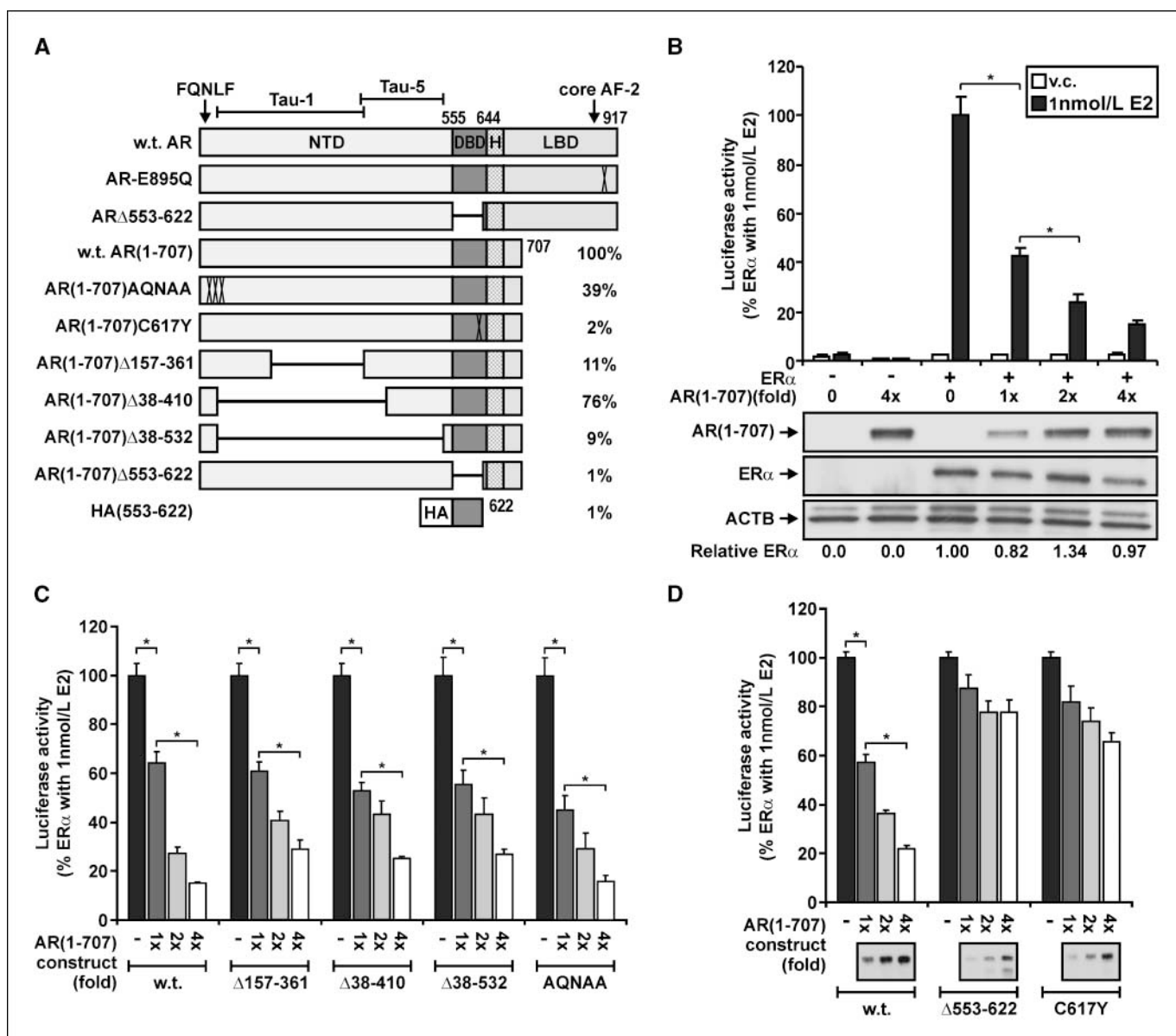


Figure 4. Inhibition of ERα activity by AR(1-707) requires DNA binding but neither the ²³FQNLFF²⁷ peptide nor autologous transcriptional functions. **A**, schematic of the AR and variants delineating the NH₂-terminal transactivation domain (NTD), hinge (H), domains for DNA-binding (DBD) and ligand-binding (LBD), and transactivation functions Tau-1 and Tau-5. Percentages represent relative activity on an ARE in MDA-MB-231 cells. **B**, inhibition of ERα activity by AR(1-707). MDA-MB-231 cells were transfected as Fig. 2. Data are presented relative to ERα alone with 1 nmol/L E₂. Immunoblot shows AR, ERα, and β-actin levels in parallel transfected cells. ERα levels relative to β-actin were calculated from densitometry. **C**, inhibition of ERα activity by AR(1-707) requires neither an intact ²³FQNLFF²⁷ peptide nor AR transactivation functions. Cells were transfected with AR variants and assayed as Fig. 2B. **D**, inhibition of ERα activity by AR(1-707) requires the DBD. Cells were transfected and assayed as in Fig. 2B. Immunoblot assays show AR expression in cell lysates. *, *P* ≤ 0.05, ANOVA.

AR-regulated target genes. In support of this hypothesis, inhibition of E₂-induced proliferation of human breast cancer cells by both tamoxifen and the constitutive truncated AR was accompanied by an identical effect on E₂-regulated targets, that is, a decrease in the level of PGR but not of CTSD. This latter effect is consistent with recruitment of AR to the promoter of *PGR* but not *CTSD* in the presence of DHT alone as determined by chromatin immunoprecipitation analyses. Given the apparent higher redundancy in the AR DNA sequence recognition (see Fig. 6A), molecular modeling indicates that there are no specific energy constraints that would theoretically preclude binding of AR to either the PGR or CTSD enhancer sequences (data not shown). The differential recruitment

of AR to these two promoters is not unexpected, considering the well-documented role of pioneer and other collaborating transcription factors in directing binding of nuclear receptors to particular canonical and noncanonical DNA elements and in stabilizing those interactions. Collectively, these data provide compelling evidence that binding of AR to ERα-regulated genes can occur *in vivo* and indicates a mechanism by which AR may regulate ERα function in breast cancer cells.

The common evolutionary origin of AR, ERα, and other nuclear receptors provides several avenues through which their function could be reciprocal, including formation of homodimers and heterodimers, recognition of similar nucleotide motifs, and

recruitment of numerous common transcriptional coregulators in the modulation of chromatin structure and gene transcription. In the current study, we did not show an interaction between AR and ER α either in whole-cell extracts or on DNA. In support of this, our modeling studies virtually preclude the possibility of ER α /AR heterodimerization on EREs. Although a previous study has shown the potential for ER α /AR heterodimerization (36), it was estimated that this interaction involved <10% of the cellular pool of ER α and AR, suggesting that this mechanism is unlikely to explain the marked effect of AR on ER α activity. An alternative means of antagonism could involve squelching of limited transcriptional coregulators (35, 37, 38), but this appears to be an unlikely mechanism as transcriptionally inert ARs deleted of essentially all known transactivation/coregulator recruitment domains retained the capacity to effectively inhibit ER α , whereas those that retained these regions but deleted of the DBD had no effect.

A novel aspect of the current study was the finding that AR can bind to a consensus ERE *in vitro*, and to a subset of endogenous ER α regulatory sites *in vivo*, and that the AR DBD is necessary and sufficient for inhibition of ER α activity by the AR. Although not always recognized, there is nonetheless considerable data indicating that crosstalk between response elements might be a common phenomenon within the nuclear receptor superfamily. For example,

ER α interacts with peroxisome proliferator-activated receptor- γ and inhibits its activity by binding to the consensus peroxisome proliferator-activated receptor response element (39). The orphan nuclear receptor hERR α 1 has been shown to bind to the ERE in the E₂-regulated *CTSD* gene promoter (40). On the other hand, the thyroid hormone receptor can potentiate ER α activity in a mechanism independent of thyroid hormone receptor/DNA interactions (41) but can bind to the ERE in the PGR promoter (42). For each nuclear receptor, the consensus response element has historically been defined by binding sequences usually in the form of two palindromic/inverted hexameric half sites separated by a three-nucleotide spacer, adjacent to genes transcriptionally responsive by that receptor. However, for the AR and ER α , there is evidence for interactions with sequences that do not conform to the defined consensus response element (43, 44). The AR is also unique in that it can bind to response element half-sites arranged as both inverted and direct repeats (45, 46). Compared with other nuclear receptors, the AR makes additional stabilizing interactions at the AR-DBD dimer interface, which increases its relative affinity for nonconsensus response elements (47). These concepts have recently been tested by unbiased genome-scale capture of steroid receptor binding sites in intact cells (29–31). Those studies have shown that the consensus response elements for AR and ER α are

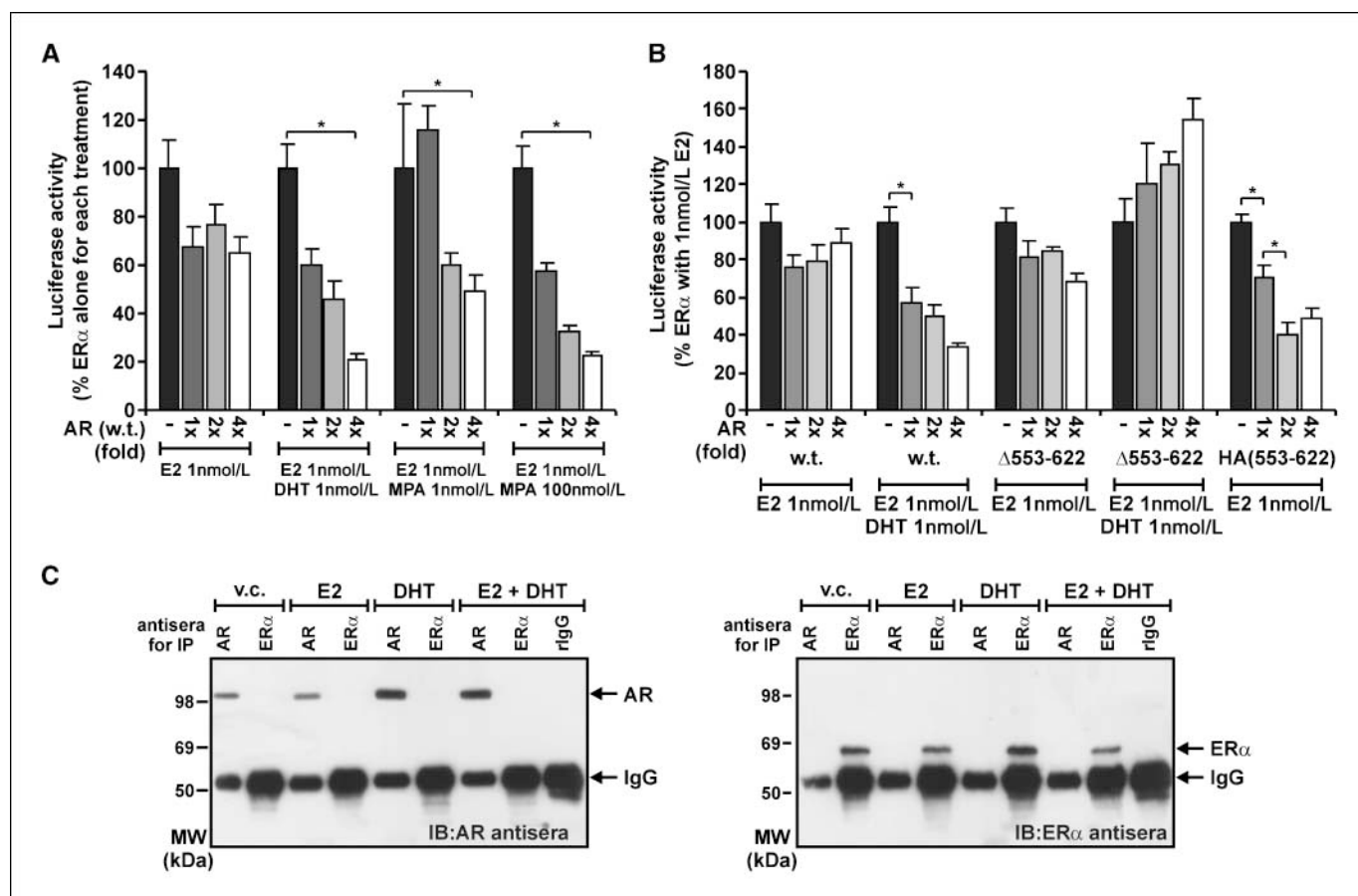


Figure 5. Inhibition of ER α activity by the full-length AR requires ligand activation of the AR and an intact DBD but neither the AR N/C interaction nor a strong interaction between AR and ER α . **A**, inhibition of ER α by AR requires ligand activation but not the AR N/C interaction. MDA-MB-231 cells were transfected as in Fig. 2. Data are presented as percent of activity induced by ER α alone for each treatment. **B**, AR DBD is necessary and sufficient for inhibition of ER α by the full-length AR. MDA-MB-231 cells were transfected as in Fig. 2. **C**, immunoprecipitation of endogenous ER α and AR in T-47D cells does not reveal a strong interaction between these proteins. T-47D cells were cultured as shown and immunoprecipitated with AR(U407), ER α (HC-20), or IgG antisera. Immunoblot was done with either AR(N-20) (left) or ER α (HC-20) (right) antisera. *, $P \leq 0.05$, ANOVA.

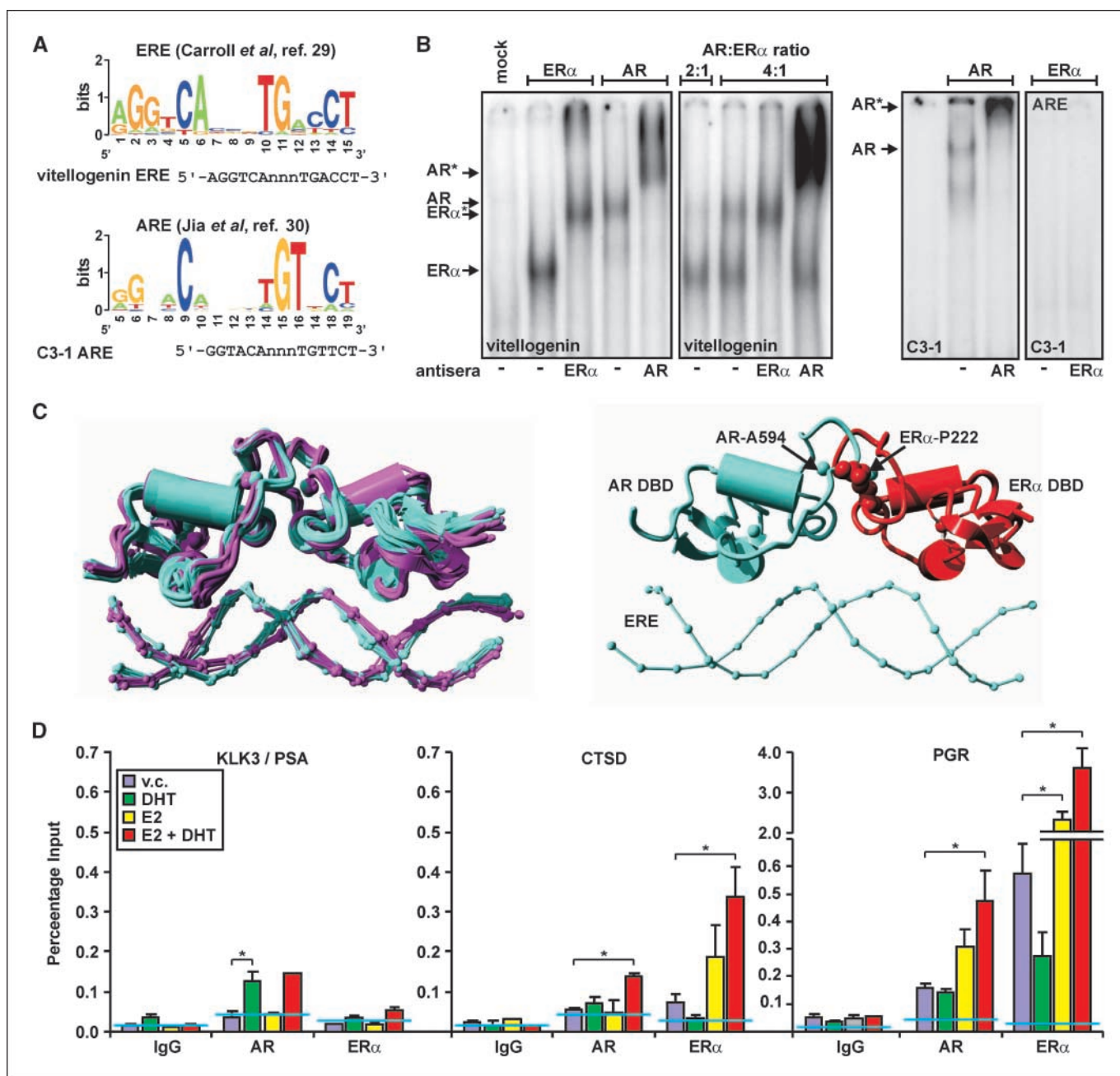


Figure 6. Interaction of the AR with ERE *in vitro*. **A**, logos of ARE and ERE from genomic-scale studies reveals that recognition of DNA by AR is less constrained than ER α . Vitellogenin and C3-1 elements used in **B** are shown. **B**, nuclear extracts from COS-1 cells transfected with AR or ER α were used alone or combined at 2:1 or 4:1 ratios in electrophoretic mobility shift assays with vitellogenin ERE or C3-1 ARE oligonucleotides. *, ER α (G-20) or AR(N-20) antisera were included. **C**, *in silico* modeling predicts binding of AR-DBD homodimers to EREs and that AR/ER α DBD heterodimers are unlikely. *Left*, accumulated molecular dynamic snapshots of an AR DBD homodimer on the vitellogenin ERE (magenta) or PSA-ARE (cyan), which suggest stable/productive engagement of both; *right*, DBD monomers of AR (cyan) and ER (red) superimposed on the vitellogenin ERE predicts that residues opposing AR-R605/ER α -R233 will preclude formation of a productive heterodimer. **D**, chromatin immunoprecipitation assays using AR, ER α , and IgG antisera in T-47D cells treated as shown. Enriched chromatin was assayed by real-time PCR for AR binding sites in the KLK3/PSA enhancer, ER α binding sites in the CTSD enhancer, and ER α binding sites in the PGR promoter. Data are presented as a percent of input and represent mean \pm SD for triplicate PCRs. Horizontal blue lines, maximum values for the nonspecific control site. *, $P \leq 0.05$, ANOVA. Images in panel A are reproduced with permission from *Cell* (top) and *PLoS ONE* (bottom).

more redundant than previously thought and that the AR, in particular, shows much greater flexibility in binding to variable sequences than would have been predicted previously (30, 31). Our data thereby support the notion that the inherently greater plasticity in response element recognition by the AR is permissive for binding to a subset of EREs. We hypothesize that binding of the AR to an ERE will interfere with the cyclic recruitment of ER α and

its coregulators at these sites (48), thereby preventing the estrogen-dependent progression of those loci to an active transcriptional regulatory sequence. A key question arising from our studies is whether AR binds preferentially to subsets of ER α -regulated genes based on the nature of their ERE and potentially adjacent sequence elements. This could be addressed by demarcating the ER α cistrome into classes that are affected by AR and those that are not,

using a genome-wide analysis of AR and ER α binding loci, and superimposing that data on target gene expression related to the breast cancer phenotype.

In summary, we have identified a previously unrecognized mechanism for the specific and direct inhibition of ER α activity by the AR in breast cancer cells. Our findings have important implications for understanding how the balance between these two pivotal hormone signaling pathways is critical for the growth and survival of breast cancer cells. Alternative therapeutic strategies targeting the AR signaling pathway in breast cancer could be particularly beneficial for women who relapse while being treated with conventional estrogen ablation therapies or are adversely affected by long-term, systemic estrogen depletion with aromatase inhibitors. Furthermore, our finding that AR is an independent predictor of outcome in ER α -positive breast cancer suggests that routine measurement of AR and inclusion in prognostic algorithms such as Oncotype Dx (49) may be warranted to improve disease management.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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