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Non-canonical AR activity facilitates endocrine resistance in breast cancer

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Abstract

The role of androgen receptor (AR) in endocrine-resistant breast cancer is controversial and clinical trials targeting AR with an AR antagonist (e.g., enzalutamide) have been initiated. Here, we investigated the consequence of AR antagonism using *in vitro* and *in vivo* models of endocrine resistance. AR antagonism in MCF7-derived tamoxifen-resistant (TamR) and long-term estrogen-deprived breast cancer cell lines were achieved using siRNA-mediated knockdown or pharmacological inhibition with enzalutamide. The efficacy of enzalutamide was further assessed *in vivo* in an estrogen-independent endocrine-resistant patient-derived xenograft (PDX) model. Knockdown of AR inhibited the growth of the endocrine-resistant cell line models. Microarray gene expression profiling of the TamR cells following AR knockdown revealed perturbations in proliferative signaling pathways upregulated in endocrine resistance. AR loss also increased some canonical ER signaling events and restored sensitivity of TamR cells to tamoxifen. In contrast, enzalutamide did not recapitulate the effect of AR knockdown *in vitro*, even though it inhibited canonical AR signaling, which suggests that it is the non-canonical AR activity that facilitated endocrine resistance. Enzalutamide had demonstrable efficacy in inhibiting AR activity *in vivo* but did not affect the growth of the endocrine-resistant PDX model. Our findings implicate non-canonical AR activity in facilitating an endocrine-resistant phenotype in breast cancer. Unlike canonical AR signaling which is inhibited by enzalutamide, non-canonical AR activity is not effectively antagonized by enzalutamide, and this has important implications in the design of future AR-targeted clinical trials in endocrine-resistant breast cancer.

Introduction

The androgen receptor (AR) is a steroid hormone receptor that is important in the development of male-specific phenotype. AR has a well-established canonical function as a ligand-activated transcription factor involved in the regulation of target genes, but it also possesses non-canonical functions via the activation of cytoplasmic signaling cascades. These include the mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K)/Akt pathways (Zarif & Miranti 2016). AR signaling plays a critical role in the progression of prostate cancer

and inhibitors of AR are a mainstay for treating advanced stages of this disease. These anti-androgenic therapeutic agents may have broader clinical utility, as AR signaling has been implicated in the pathogenesis of other malignancies such as ovarian teratocarcinoma (Chung *et al.* 2014) and some subtypes of breast cancer (Hickey *et al.* 2012, Chia *et al.* 2015).

Breast cancer is a highly heterogeneous disease comprising several major immunohistochemical subtypes. About 70% of all cases are classified as estrogen receptor positive (ER+) and the remaining 30% estrogen receptor negative (ER−) breast cancers are sub-classified into two broad categories: HER2-enriched subtype that is human epidermal growth factor receptor 2 positive (HER2+) and triple-negative breast cancer (TNBC), which lacks expression of all three biomarkers (ER, progesterone receptor (PR) and HER2. AR is expressed across all subtypes of breast cancer and approximately 90% of ER+ (Niemeier *et al.* 2010), 60–75% of HER2+ (Micello *et al.* 2010, Niemeier *et al.* 2010) and 10–36% of TNBC (Micello *et al.* 2010, Niemeier *et al.* 2010) are immunopositive for AR.

The consequences of AR activity are subtype dependent. In the ER+ breast cancer, multiple *in vitro* studies have reported a growth inhibitory effect of 5 alpha-dihydrotestosterone (DHT), the most potent cognate ligand for AR, in endocrine-sensitive ER+AR+ cell line models of breast cancer (Lapointe *et al.* 1999, Greeve *et al.* 2004, Macedo *et al.* 2006), and this effect is reported to be mediated via antagonism of ER signaling (Lapointe *et al.* 1999, Lanzino *et al.* 2005, Macedo *et al.* 2006, Peters *et al.* 2009). These studies suggest that AR activity is tumor suppressive in ER+ breast cancer, a concept supported by clinical studies showing that AR expression is an independent prognostic marker of good outcome in ER+ breast cancers (Aleskandarany *et al.* 2016, Ricciardelli *et al.* 2018). In contrast, AR activity is associated with increased proliferation in the ER− breast cancer (Chia *et al.* 2011, Lehmann *et al.* 2011, Ni *et al.* 2011, Robinson *et al.* 2011)

Standard-of-care endocrine therapy for ER+ breast cancer includes selective ER modulators such as tamoxifen, aromatase inhibitors (AIs) that inhibit peripheral estrogen biosynthesis and selective ER degraders (SERDs) such as fulvestrant. While endocrine therapies have improved the survival of patients with early-stage ER+ disease, approximately one-third of women will eventually acquire resistance to endocrine therapy, leading to disease progression and death (EBCTCG 2005). The expression of AR is reported to be higher in endocrine-resistant breast cancer, but the role of AR in these tumors is controversial (De Amicis *et al.* 2010, Fujii *et al.* 2014).

AR activity has been reported to promote proliferation as growth suppression of endocrine-resistant cells was observed with the use of AR inhibitors such as bicalutamide and enzalutamide (De Amicis *et al.* 2010, Rechoum *et al.* 2014, Ali *et al.* 2015, Ciupek *et al.* 2015, D'Amato *et al.* 2016). These preclinical studies have contributed to the initiation of clinical trials with AR inhibitors including enzalutamide in endocrine-resistant breast cancer (ClinicalTrials.gov NCT Identifiers 02953860, 02007512 & 02580448). However, it is noteworthy that these studies largely utilized genetically modified MCF7 cell line models of endocrine resistance, which includes ectopic overexpression of AR (De Amicis *et al.* 2010) or knockdown of Rho GDP dissociation inhibitor (GDI) (Ciupek *et al.* 2015) in MCF7 cells to mimic tamoxifen resistance. MCF7 cells with ectopic overexpression of aromatase with or without ectopic AR overexpression were used as models of aromatase inhibitor resistance (Rechoum *et al.* 2014, Ali *et al.* 2015). There is only one study utilizing non-genetically modified cell line models of endocrine resistance, which demonstrated that enzalutamide inhibited *in vitro* and *in vivo* growth of tamoxifen-resistant (TamR) MCF7 cells (D'Amato *et al.* 2016). Critically, the effect of AR antagonism on an endocrine-resistant patient-derived xenograft (PDX), now considered the most clinically relevant preclinical disease model, is yet to be reported.

Here, we examined the consequences of antagonizing endogenous AR in breast cancer cell lines using siRNA-mediated knockdown or AR antagonist enzalutamide and investigated the effect of enzalutamide in a PDX model of endocrine resistance. We demonstrate that AR plays a functional role in endocrine-resistant breast cancer as knockdown of AR reversed the endocrine-resistant phenotype. This functional role of AR is, however, ascribed to its non-canonical activity, which was not inhibited by enzalutamide as the growth of cell line and PDX models was unaffected by the drug. These observations suggest that pharmacologically inhibiting AR may not be an effective therapeutic strategy for endocrine-resistant breast cancer.

Materials and methods

Cell culture and reagents

MCF7 cells were sourced from the Michigan Cancer Foundation. Derivatives of MCF7 cells that are resistant

to tamoxifen (TamR) or long-term estrogen deprived (LTED) were obtained from Myles Brown (Dana-Farber Cancer Institute) (Bailey *et al.* 2015). T47D cells were obtained from the American Type Culture Collection. All cell lines have been verified through short tandem repeat profiling, and mycoplasma contamination was excluded. MCF7, TamR and T47D cell lines were cultured in RPMI 1640 media (Thermo Fisher) supplemented with 10% fetal bovine serum (GE Healthcare), 20mM HEPES (Thermo Fisher) and 0.28IU/mL insulin (Novo Nordisk). Base media for TamR cells was also supplemented with 5μM 4-hydroxytamoxifen (Sigma). TamR cells used in experiments were grown in base media without tamoxifen. The LTED cell line was maintained in phenol-red free RPMI 1640 media (Thermo Fisher) supplemented with 10% steroid-depleted FBS (Thermo Fisher), 20mM HEPES (Thermo Fisher) and 0.28IU/mL insulin (Novo Nordisk). All cells were cultured in a humidified incubator at 37°C with 5% CO_2 and were used at <10 passages post revival. The AR antagonist enzalutamide (Selleck) was re-suspended as a 40mM stock solution in DMSO and DHT (Sigma) was re-suspended as a 10μM stock solution in ethanol.

siRNA knockdown

Transient AR knockdown was performed using two different siRNA oligos: AR siRNA-A (siA) 5′-GGAACUCGAUCGUAUCAUU-3′ (#4390824, Thermo Fisher) and AR siRNA B (siB) 5′-CAGUCCCACUUGUGUCAAA-3′ (Sigma) (Wang *et al.* 2011). Cells were transfected with nonsense (NS) siRNA (#4390843, Thermo Fisher) as a control. Transfection of these siRNAs was carried out using Lipofectamine RNAiMAX (Thermo Fisher), via reverse transfection, as per manufacturer's instructions, using a final siRNA concentration of 10nM in 2mL of media in 6cm² culture dishes. For downstream proliferation assays, cells were trypsinized and then seeded at the appropriate cell numbers 2 days post transfection.

Western blotting analysis

Cell pellets were lysed in RIPA buffer supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher). Protein lysates (20μg) were resolved by using 4–15% mini-Protean TGX pre-cast gels (Bio-Rad) and transferred to 0.45μm Immobilon-FL polyvinylidene fluoride membrane (Millipore). Membranes were immunoblotted with primary antibodies from Cell

Signaling at a dilution of 1 in 1000; anti-AR (#5153), anti-ER (#8644), anti-PR (#3153) anti-FOXA1 (#23738), anti-Akt (total; #2920), anti-Akt (phospho-serine 473; #9271), anti-FOXO3a (#2497) and GAPDH (#5174), and anti-cyclin A (#sc-596, 1:200, Santa Cruz). These antibodies were detected using IRDye 680RD Donkey anti-mouse (#926-68072, LI-COR) or IRDye 800CW Donkey anti-Rabbit (#926-32213, LI-COR) secondary antibodies at a dilution of 1 in 20,000 on the Odyssey CLx Imaging System (LI-COR). Densitometric analyses were carried out using ImageJ.

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using the RNeasy Plus Mini kit (Qiagen). RT-qPCR analysis was performed using the TaqMan Gene Expression Assays (Thermo Fisher) on the 7900HT Fast Real-Time PCR System (Thermo Fisher) or the iQ SYBR Green system (Bio-Rad) on the CFX384 Touch cycler (Bio-Rad) as per manufacturers' instructions. TaqMan probes used are as follows: *AR* (Hs00907244_m1), *ESR1* (Hs01046816_m1), *CTGF* (Hs00170014_m1), *CXCL8* (Hs00174103_m1), *PGR* (Hs01556702_m1) and *GAPDH* (Hs03929097_g1). Primers used include *SEC14L2* (forward: GCCGAATCCAGATGACTATTTTCT, reverse: GATGTTGTCAATGTCCTTTTGCTT), *FKBP5* (forward: AAAAGGCCAAGGAGCACAAC, reverse: TTGAGGAGGGGCCGAGTTC) and *GAPDH* (forward: TGCACCACCAACTGCTTAGC, reverse: GGCATGGACTGTGGTCATGAG). Expression level of the gene of interest was normalized to that of *GAPDH*. Relative fold change was computed based on the (2ΔΔCT) method and data are represented as relative to the control treatment group.

Proliferation assays

Cell proliferation was measured using Countess Automated Cell Counter and AlamarBlue cell viability assays (Thermo Fisher). Overall, 50,000 cells were seeded into 6cm² dishes in 2mL media and treatment commenced the following day and cells were quantified 4 days post treatment. Experiments were performed in three independent replicates. Cell viability assays were performed according to manufacturer's instructions in 96-well plate format. 2–4000cells were seeded into each well in 100μL media and treatment commenced the following day. Growth media was replaced with 200μL of fresh media containing 1× concentration of the indicated treatment every 2–3 days and cells were analyzed 3–6 days

post treatment. Technical repeats (6–8) were performed for each condition and experiments were performed in three independent replicates.

Colony-forming assays

Assays were performed in 12-well plates seeded at a density of ~3000 cells per well. Treatment was added 24 h later and cells were harvested after 6–9 days of treatment. Cells were stained with 0.05% w/v crystal violet (Sigma), 1% formaldehyde (Thermo Fisher), 1× PBS and 1% methanol in distilled water. Images were scanned using the Perfection V800 Photo Scanner (Epson) with a DPI of 1200 dots per inch. Area occupancy was analyzed using an ImageI plugin, ColonyArea (Guzman *et al.* 2014), and the results for treatment groups were expressed relative to controls. Experiments were performed in three independent replicates, each comprising three technical replicates for each condition.

ChIP-PCR

Chromatin immunoprecipitation (ChIP) was performed in TamR cells as previously described (Holmes *et al.* 2016) using anti-ER (sc-543x) and anti-IgG (sc-2027) (Santa Cruz). ER binding to the distal and proximal *CXCL8* gene promoters was assessed by RT-qPCR using iQ SYBR Green Supermix according to manufacturer's instructions (#1708885, Bio-Rad) (Fu *et al.* 2016). ER occupancy at the *CXCL8* promoter regions was expressed as fold enrichment over IgG, normalized to the intergenic controls and then expressed as fold change relative to the control group.

Immunofluorescent staining

TamR cells grown on poly-L-lysine-coated coverslips were treated with vehicle or 10 µM enzalutamide for 48 h in the absence or presence of 10 nM DHT. Coverslips were fixed in ice-cold methanol overnight at −20°C. Cells were immunostained with primary antibodies against AR (#5153, Cell Signaling), ER (#M7047, DAKO) and alpha-tubulin (#T6199, Sigma). Fluorescent secondary antibodies were used to detect the primary antibodies (1:250) and DNA was counterstained with 4,6-diamidino-2-phenylindole (1:250, DAPI, Sigma). Coverslips were mounted onto glass slides with Prolong Gold anti-fade reagent (Thermo Fisher) and microscopy was carried out using Automated Upright Microscope System (Leica). DAPI staining was used to define nuclear regions and

fluorescent nuclear ER signals were quantified to assess changes in nuclear ER in the different conditions.

Microarray and gene set enrichment analysis

Gene expression profiling was performed on the TamR cells 48 h post transfection with NS control or AR siRNA-A using three biological replicates. Microarray hybridization was performed on the Affymetrix PrimeView Human Gene Expression Array. CEL files were further processed in the R software (v 3.4.3) environment using affy (Gautier *et al.* 2004) and limma (Ritchie *et al.* 2015) packages from Bioconductor (Gentleman *et al.* 2004). The CEL files and normalized expression matrix can be downloaded from Gene Expression Omnibus (GEO, accession number 115270). Normalized log2 probe signals were computed using robust multi-array average (RMA) (Irizarry *et al.* 2003). The subsequent differential expression vectors between AR-knockdown and control samples were set for each gene at a *q*-value threshold of 0.05. Probes were then annotated using the Affymetrix Archived NetAffx Annotation Files for PrimeView. Differentially expressed genes were matched against hallmark signatures from the Molecular Signature Database (MSigDB) using the Gene Set Enrichment Analysis (GSEA) portal (http://software.broadinstitute.org/gsea/index.jsp) (Subramanian *et al.* 2005) and the degree of overlap between our differentially expressed genes and the signatures were reported.

Endocrine-resistant patient-derived breast cancer xenograft (PDX) model

PDX (Gar15-13) was generated under the Human Research Ethics Committee (HREC)-approved protocol at the St Vincent's Hospital (SVH) (Protocol HREC/16/SVH/29) and *in vivo* experiments were approved by the Garvan Animal Ethics Committee (Protocol 15/25). The patient has consented to the use of her tissue for research-related purposes. This PDX was established from a metastatic ER+PR−AR+ liver biopsy obtained from a patient with ER+ breast cancer who had disease progression on an aromatase inhibitor. Consistent with its aromatase inhibitor-resistant phenotype, this model did not require estradiol supplementation for growth. Patient tumor tissues were implanted into the 4th inguinal mammary gland of female NOD-SCID-IL2γR−/− (NSG) mice (Australian BioResources) as previously described (Oakes *et al.* 2012). Animals were anesthetized with isoflurane, and analgesia was achieved with ketoprofen and bupivacaine. The PDX tumor was

concordant with the patient tissue (ER+ PR–AR+). For the therapeutic experiments, a single PDX tumor was divided and implanted into a cohort of NSG recipient mice. Tumor-bearing mice were randomized using the online GraphPad Software to receive either vehicle or enzalutamide when tumors reached 150–200 mm³ (https://www.graphpad.com/quickcalcs/randomize1.cfm). Enzalutamide was re-suspended in 25% TWEEN®80 (Sigma) and sonicated using Bioruptor (Diagenode) at medium setting for 5 min at 30 s on/off intervals to achieve a homogenous suspension and administered at a dose of 20 mg/kg/day by oral gavage (Moilanen *et al.* 2015). Tumor volumes were calculated using the formula (length × wide × wide)/2. Mice were culled when the tumors reached ethical endpoint of 1000 mm³.

Immunohistochemistry (IHC) and quantification

Antibodies used for IHC include anti-AR (#SC-27190, 1:1000, Santa Cruz), anti-ER (M7047, 1:300, Agilent), anti-Ki67 (M7240, 1:400, Agilent) and anti-SEC14L2 (#271902, 1:1000, Santa Cruz). These primary antibodies were detected with biotinylated IgG secondary antibodies (Agilent, 1:400), using streptatvidin-HRP (Agilent) for amplification of signal followed by the addition of 3,3'-diaminobenzidine (Sigma) substrate for visualization of signal. Images were scanned using NanoZoomer (Hamamatsu). Proliferation indices were determined by the proportion of Ki-67 positive cells for each tumor by manual counting of >1000 cells from three random 80× magnification fields (Dowsett *et al.* 2011). Quantification of staining intensity was performed using ImageJ from at least three representative images at 80×.

Statistical analyses

Statistical analyses were performed on GraphPad Prism version and the type of statistical analysis performed for each experiment is described in the figure legends.

Results

AR knockdown reduces the proliferation of ER+AR+ endocrine-resistant cells

We first evaluated possible changes in the expression of steroid hormone receptors and the critical pioneer factor FOXA1 (Hurtado *et al.* 2011) in the endocrine-resistant cells and western blots were performed to compare ER, AR, PR and FOXA1 levels in the endocrine-sensitive (ES) MCF7
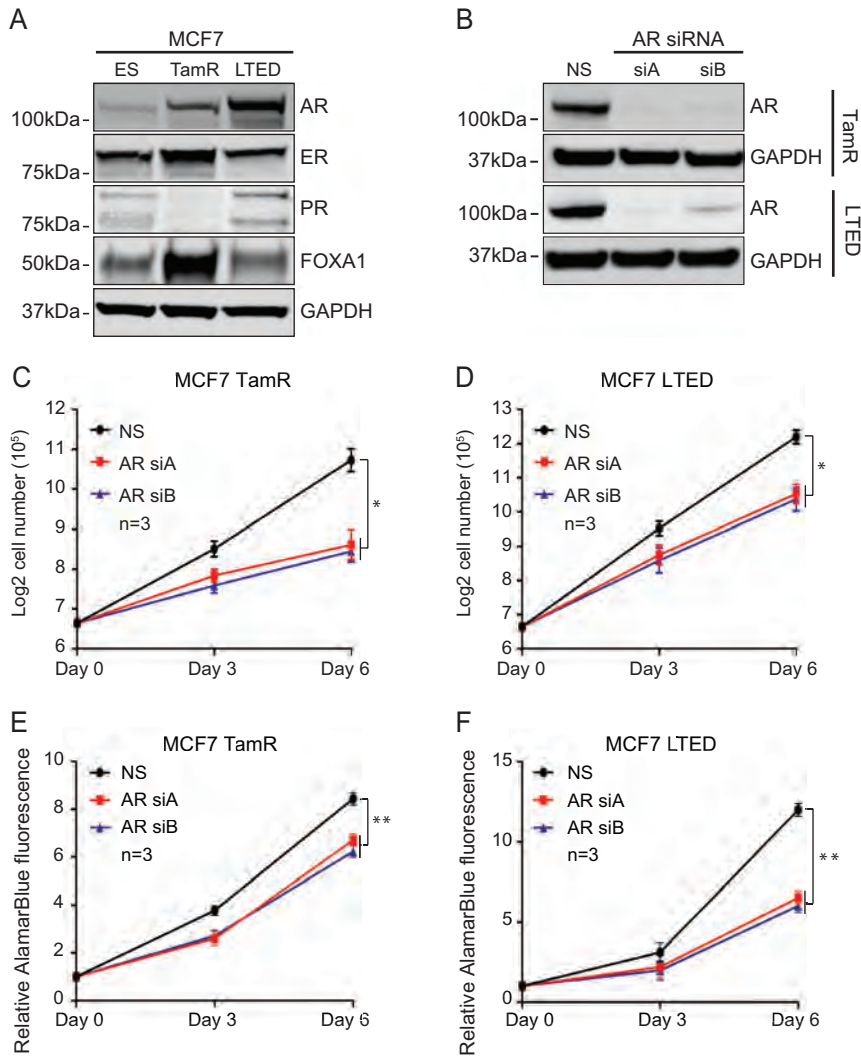
cells and the endocrine-resistant derivatives (Fig. 1A). ER expression was retained in the endocrine-resistant models and was slightly increased in the TamR cells compared to the ES and LTED cells. AR expression was increased in both endocrine-resistant models relative to ES cells, with higher levels in the LTED model (Fig. 1A). In the TamR line, PR expression was lost and FOXA1 expression increased, consistent with previously published data (Fu *et al.* 2016).

To assess the effect of AR loss on proliferation of these endocrine-resistant cells, we performed transient knockdown of AR using two specific AR siRNAs (AR siA and siB). Both resulted in reduction of AR transcript and protein levels as demonstrated by RT-qPCR (Supplementary Fig. 1A and B, see section on supplementary data given at the end of this article) and immunoblotting, respectively (Fig. 1B). AR knockdown significantly reduced proliferation of TamR and LTED cells relative to NS siRNA-transfected controls, as determined by cell counting and Alamar Blue viability assays (Fig. 1C, D, E and F). Collectively, these observations support a mitogenic role for AR in endocrine-resistant breast cancer.

AR knockdown restores characteristics of classical ER signaling in MCF7 TamR cells

We next performed gene expression profiling to identify transcriptomic changes associated with transient knockdown of AR in TamR cells. Efficient AR knockdown was confirmed at both the transcript and protein levels prior to gene expression profiling (Fig. 2A and B). Loss of AR was associated with increased levels of ER protein (Fig. 2B) and immunofluorescence staining indicated an increase in nuclear ER protein in these cells (Supplementary Fig. 2A and B). Notably, AR expression demonstrated a diffuse and predominantly cytoplasmic pattern under the control (NS) conditions (Supplementary Fig. 2A). The predominant cytoplasmic localization of AR and nuclear localization of ER in ES, TamR and LTED cells under basal growth conditions were confirmed via immunoblotting of ER and AR in cytoplasmic and nuclear fractions (Supplementary Fig. 3).

Transcriptional profiling identified 3313 differentially expressed genes at a false discovery rate <0.05, with 1605 and 1708 genes upregulated and downregulated respectively. GSEA indicated that genes upregulated by AR knockdown were enriched in pathways involved in ultra-violet response, PI3K-Akt signaling, p53 and estrogen response (Fig. 2C). Conversely, pathways involved in E2F, G2/M checkpoint, androgen response and estrogen
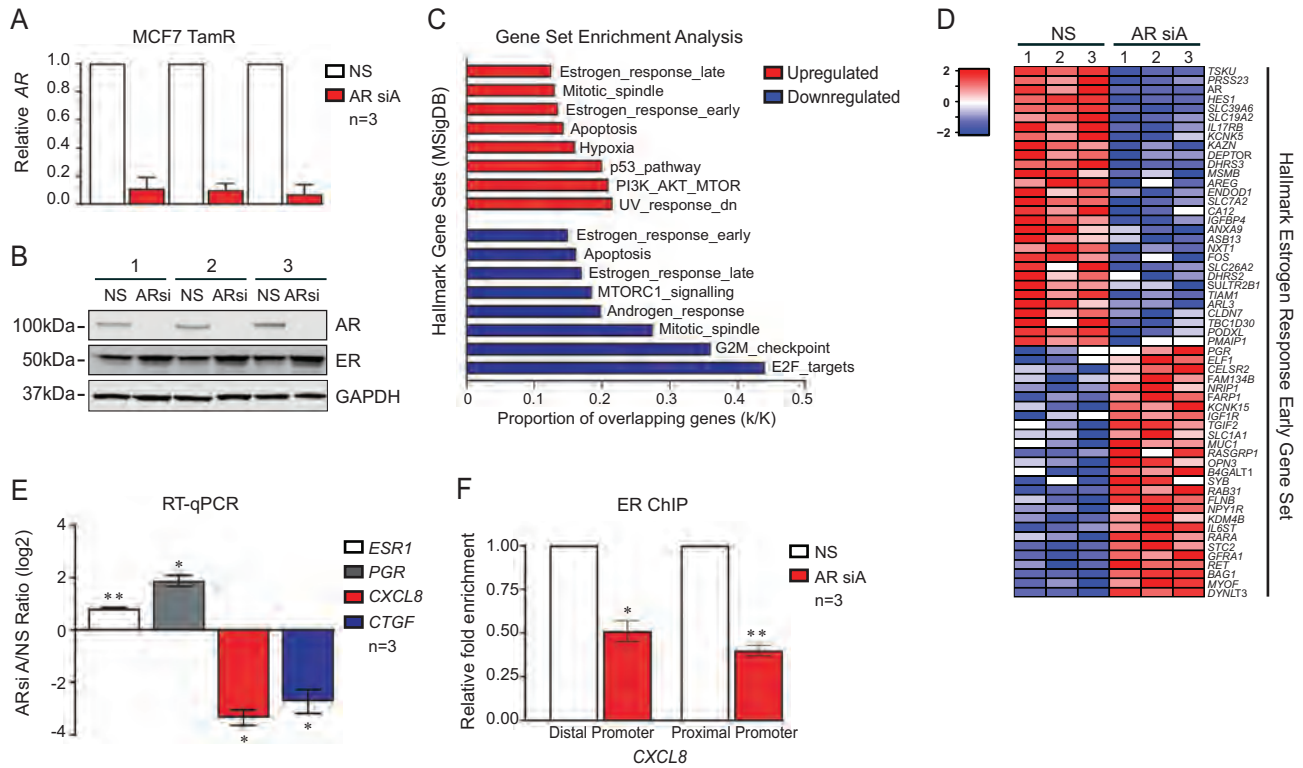
Figure 1

AR knockdown reduces proliferation of ER+AR+ endocrine-resistant cells. (A) Expression of steroid hormone receptors including AR, ER, PR and FOXA1 in endocrine-sensitive (ES), tamoxifen-resistant (TamR) and long-term estrogen-deprived (LTED) MCF7 cell lines by immunoblotting. (B) The efficiency of AR siRNA-A (siA) and siRNA B (siB) on AR protein in the endocrine-resistant cell lines was compared to nonsense (NS) siRNA 4 days post transfection by immunoblotting for AR and GAPDH (loading control). The effect of AR loss on proliferation was assessed using cell counting and AlamarBlue assay 3 and 6 days post transfection in TamR (C and E) and LTED (D and F) cells. Cell numbers in log2 scale are presented in (C) and (D). *$P < 0.05$, **$P < 0.01$ using Student's *t*-test. Error bars = s.e.m. from three biological replicates.

response were enriched in the downregulated gene set (Fig. 2C). A heat map of the top 50 differential genes revealed C-X-C motif chemokine ligand 8 (*CXCL8*) and connective tissue growth factor (*CTGF*) as the top two genes downregulated with the loss of cytoplasmic AR (Supplementary Fig. 4). These genes have recently been reported to promote tamoxifen resistance and are highly regulated by ER in MCF7 TamR cells (Fu *et al.* 2016).

To identify the estrogen response genes affected by AR knockdown, we overlapped the differential gene set with the hallmark Estrogen Response Early gene set (Broad Institute) (Fig. 2D). AR is a component of this hallmark gene set, and was downregulated as expected. Notably, transcript levels of classical ER-regulated genes such as progesterone receptor (*PGR*), insulin-like growth factor 1 receptor (*IGF1R*) and retinoic acid receptor-alpha (*RARA*) were increased in AR-deficient TamR cells. This is consistent with the increase in *ESR1* mRNA

and ER protein levels post AR knockdown (Fig. 2B). Using RT-qPCR, we validated the increases in *ESR1* (0.8 log2-fold change, $P < 0.01$) and *PGR* mRNA levels (1.8 log2-fold change, $P < 0.05$), as well as reductions in both *CXCL8* (−3.35 log2-fold change, $P < 0.05$) and *CTGF* (−2.7 log2-fold change, $P < 0.05$) mRNA levels post AR knockdown (Fig. 2E). The decrease in *CXCL8* transcripts post AR knockdown was associated with a significant reduction (~50%) in ER chromatin binding at both the distal and proximal promoter regions (Fig. 2F). Transient AR knockdown in LTED cells also resulted in an increase in ER protein as well as *ESR1* and *PGR* mRNA levels (Supplementary Fig. 5A and B). Collectively, these data suggest that AR in endocrine-resistant MCF7 cells contributes to endocrine-resistant phenotype by inhibiting canonical ER signaling events. This led us to hypothesize that ablation of AR may restore sensitivity to tamoxifen.
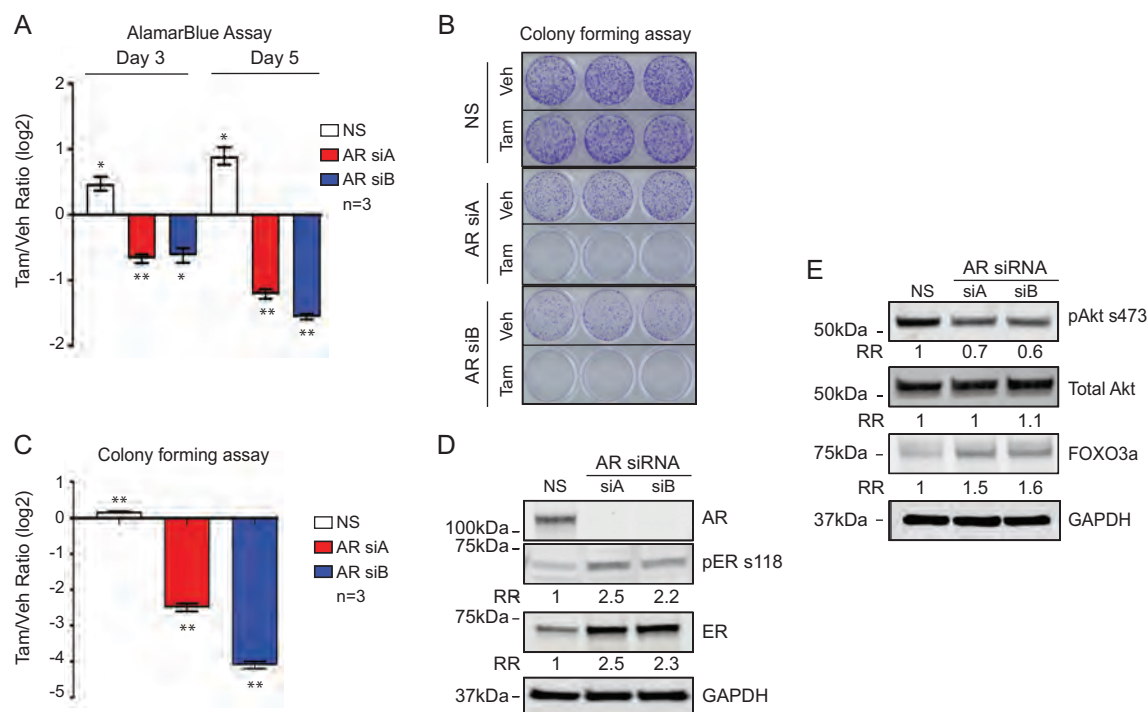
Figure 2

AR knockdown restores characteristics of classical ER activity. The effect of AR knockdown on gene expression using microarray profiling and on ER chromatin binding at selected sites in MCF7 TamR cells was assessed. The efficiency of AR siA vs that of NS 2 days post transfection was assessed using RT-qPCR (A) and immunoblotting (B). Each reaction was performed in three technical replicates and error bars = s.d. (C) Differentially expressed genes identified from microarray profiling were subjected to MSigDB hallmark GSEA. The top nine enrichment groups for the up- and downregulated genes, with q values of <0.001 are presented. (D) The differential gene set induced by the transient knockdown of AR was overlapped with that of the MSigDB hallmark Estrogen Response Early signature. (E) RT-qPCR was used to validate changes in the selected genes *ESR1*, *PGR*, *CXCL8* and *CTGF* identified in the expression profiling. Changes in these genes post AR knockdown are presented as log2-fold change of the expression of each gene in AR siRNA-transfected cells relative to NS siRNA-transfected cells. (F) ER binding at distal and proximal *CXCL8* promoters post AR knockdown was assessed using chromatin immunoprecipitation (ChIP). Data is represented as fold change relative to NS-transfected cells. *$P < 0.05$, **$P < 0.01$ using Student's *t*-test. Error bars = s.e.m. from three biological replicates.

AR knockdown restores tamoxifen sensitivity in TamR cells

Next, we evaluated the effect of tamoxifen on proliferation following AR loss. Tamoxifen increased the viability of NS-transfected cells relative to vehicle controls ($P < 0.05$, Fig. 3A), consistent with the mitogenic effect of tamoxifen on MCF7 TamR cells (Jeselsohn *et al.* 2017). In contrast, tamoxifen significantly reduced viability of AR-deficient cells compared to vehicle treatment ($P < 0.05$, Fig. 3A). Colony-forming assays recapitulated these findings, whereby fewer colonies were observed in tamoxifen-treated cells following transfection with AR siA- or siB relative to vehicle ($P < 0.01$, Fig. 3B and C). Tamoxifen did not significantly impact the colony formation of NS-transfected cells. Given that phosphorylation of ER at serine-118 was predictive of response to tamoxifen in early-stage breast cancer (Murphy *et al.* 2004), we assessed

if AR knockdown altered phosphorylation of ER at this site. AR knockdown in TamR cells increased phosphorylation of ER at serine-118, but it occurred proportionately to the observed increase in total ER (Fig. 3D). We then investigated the effect of AR loss on the PI3K/Akt signaling pathway given that it was significantly enriched among genes differentially expressed in the absence of AR (Fig. 2C) and inhibition of this pathway has been shown to augment canonical ER activity and enhance the efficacy of an ER-targeted therapy in ER+ tumors (Bosch *et al.* 2015). AR knockdown reduced phosphorylation of Akt at serine-473 and increased the expression of downstream Forkhead box transcription factor 3a (FOXO3a), which is negatively regulated by the Akt signaling pathway (Guo & Sonenshein 2004). These observations suggested that the loss of AR in TamR cells was sufficient to re-sensitize these cells to tamoxifen and that this effect may be attributed to inhibition of the PI3K/Akt signaling pathway.
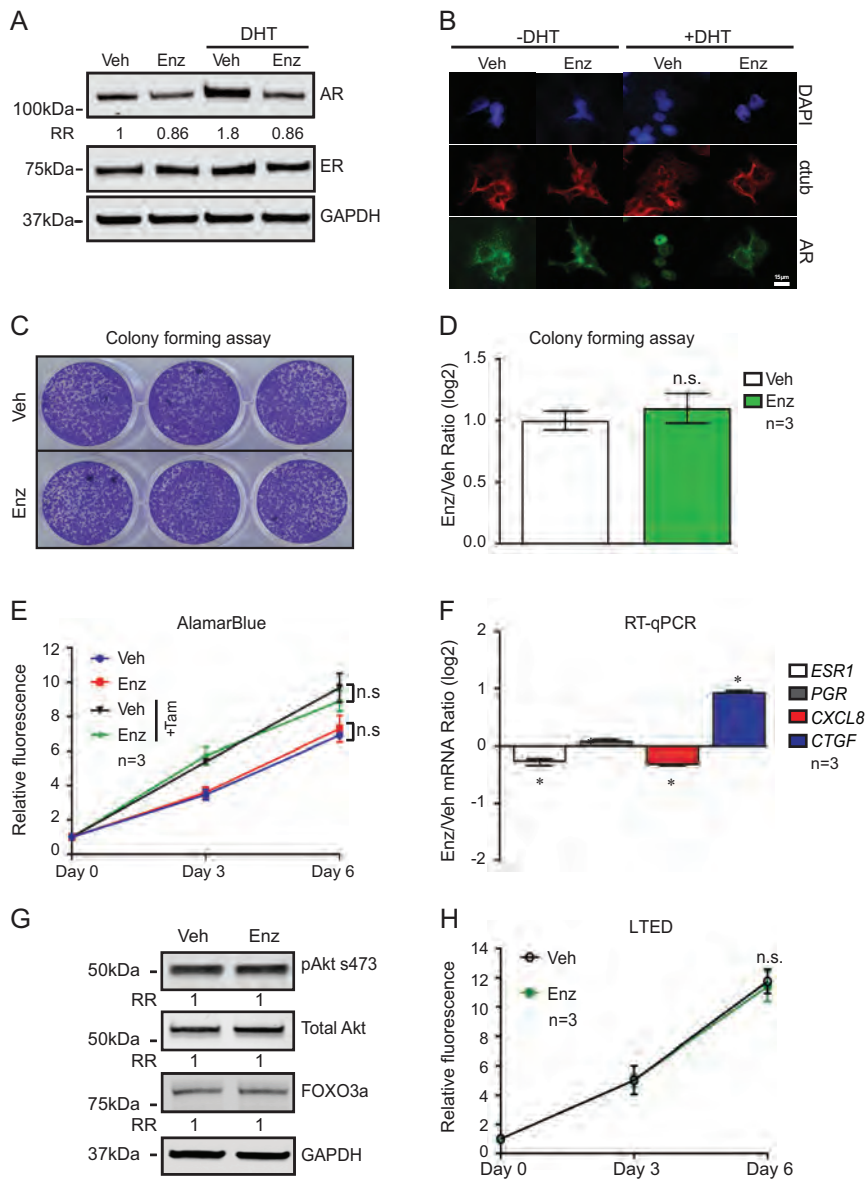
Figure 3

AR knockdown re-sensitizes MCF7 TamR cells to tamoxifen. The effect of AR knockdown on the response of MCF7 TamR cells to tamoxifen was evaluated using AlamarBlue and colony formation assays. (A) The viability of MCF7 TamR cells was assessed using AlamarBlue assay in response to 3 and 5 days treatment with vehicle (Veh) or 5 μM tamoxifen (Tam) after transfection with NS, AR siA or AR siB RNA for 2 days. Data is presented as the fold change of Tam-treated cells to Veh-treated cells in log2 ratio. (B) 6-day colony-forming assay assessing the response of MCF7 TamR to Tam as per the treatment and transfection conditions in (A). (C) Quantification of the colony area coverage using ImageJ with data presented as the fold change of tamoxifen to Veh-treated cells in log2 ratio. (D) Cell lysates extracted from MCF7 TamR cells transfected with NS, AR siA or AR siB for 2 days, were immunoblotted for AR, phospho-ER at serine-118 (pER s118), ER and GAPDH. (E) Cell lysates in (D) were immunoblotted for phospho-Akt at serine 473 (pAKT s473), total Akt, FOXO3a and GAPDH. Densitometry was performed using ImageJ and data is represented relative to control cells (RR; relative ratio). *$P < 0.05$, **$P < 0.01$ using Student's *t*-test. Error bars = s.e.m. from three biological replicates.

Pharmacological inhibition of AR does not phenocopy the effect of AR knockdown

We then determined if pharmacological inhibition of AR with enzalutamide, which works in part by blocking nuclear localization of ligand-bound AR (Tran *et al.* 2009), could recapitulate the effect of AR knockdown. We first assessed the effect of enzalutamide, in the presence or absence of DHT, on AR expression and cellular localization. Under basal growth conditions, without DHT supplementation, enzalutamide reduced AR expression by ~14% relative to vehicle-treated cells (Fig. 4A). As expected, DHT-induced stabilization of AR protein, which increased by 1.8-fold relative to vehicle treatment. This DHT-mediated AR stabilization effect was completely abrogated with enzalutamide treatment. The expression of ER was not significantly altered in response to enzalutamide (Fig. 4A). Using immunofluorescence analysis, enzalutamide also inhibited DHT-induced AR nuclear translocation, consistent with its mechanism of action (Fig. 4B).

In contrast to siRNA-mediated loss of AR, functional antagonism with enzalutamide had no effect on the colony formation of TamR cells relative to vehicle treatment (Fig. 4C and D). Similarly, treatment with enzalutamide had no effect on the viability of TamR cells, and it did not re-sensitize TamR cells to the inhibitory effects of tamoxifen (Fig. 4E). In contrast to the effect of transient AR knockdown in these cells (Fig. 2E), enzalutamide increased *CTGF* (1 log2-fold change, $P < 0.05$), modestly decreased *ER* (-0.28 log2-fold change, $P < 0.05$) and *CXCL8* levels (-0.34 log2-fold change, $P < 0.05$) (Fig. 4F) but had no effect on the expression of *PGR*. Enzalutamide also did not inhibit the PI3K/Akt pathway, as there were no changes observed in the phosphorylation of Akt at serine-473 or in the expression level of FOXO3a (Fig. 4G). Furthermore, enzalutamide did not reduce the viability of LTED cells, suggesting its lack of efficacy was not limited to one form of endocrine resistance (Fig. 4H). These results demonstrate that pharmacological inhibition of AR does not recapitulate the effect of AR knockdown in endocrine-resistant breast cancer cells *in vitro*.
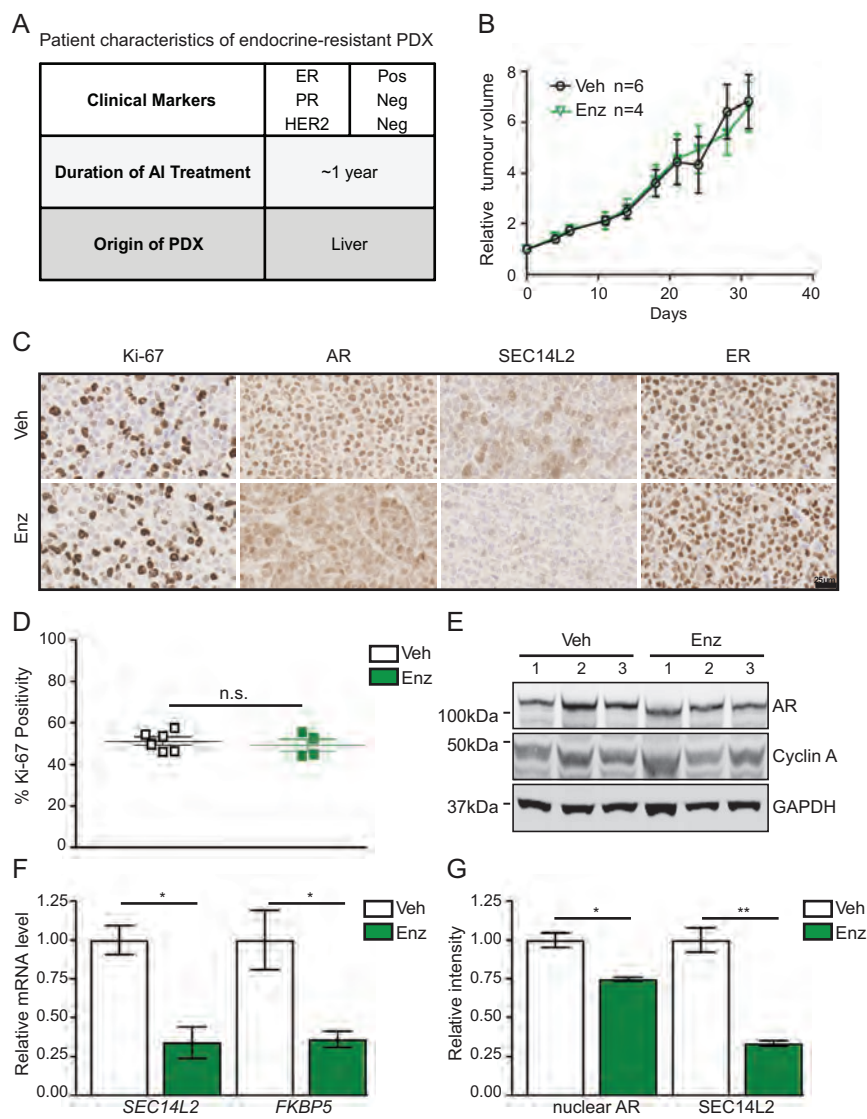
Figure 4

AR pharmacological inhibition does not recapitulate effects of AR knockdown in MCF7 TamR cells. The effect of enzalutamide (Enz) treatment on MCF7 TamR cells in relation to growth and ER signaling was assessed. The effect of 10 µM Enz on AR expression and subcellular localization was evaluated using immunoblotting and immunofluorescence. Cells were treated with enzalutamide for 48 h in the presence or absence of 10 nM DHT prior to harvest for immunoblotting for AR, ER and GAPDH (A) and immunofluorescence with DAPI (blue) and antibodies against cytoplasmic marker alpha-tubulin (αtub; red) and AR (green) (B). Densitometric analysis was performed using ImageJ and data is represented relative to vehicle (Veh)-treated cells in the absence of DHT (RR; relative ratio). (C) Colony-forming assays evaluating the effect of 9-day Veh or Enz treatment on MCF7 TamR cells. (D) Quantification of the colony area coverage using ImageJ and data is presented as the fold change of Enz to Veh treatment. (E) AlamarBlue assessing the effect of 10 µM Enz on the viability of MCF7 TamR cells at 3 and 6 days post treatment in the absence or presence of 5 µM tamoxifen (Tam). Data is presented as relative to day 0. (F) RT-qPCR was performed to determine the effect of 48 h 10 µM Enz treatment on *ESR1*, *PGR*, *CXCL8* and *CTGF* mRNA levels in MCF7 TamR cells relative to Veh-treated cells. Data is presented as log2 ratio. (G) Cell lysates from MCF7 TamR cells treated with Veh or 10 µM Enz for 48 h were immunoblotted for phospho-Akt at serine 473 (pAKT s473), total Akt, FOXO3a and GAPDH. (H) AlamarBlue assessing the effect of 10 µM Enz on the viability of MCF7 LTED cells at 3 and 6 days post treatment. *$P < 0.05$, n.s. = not significant, using Student's *t*-test. Error bars = s.e.m. from three biological replicates.

Pharmacological inhibition of AR does not affect *in vivo* tumor growth of an endocrine-resistant ER+ PDX model

Lastly, we evaluated the efficacy of enzalutamide on the Gar15-13 PDX *in vivo*. This model was established from a postmenopausal patient who had tumor recurrence 1 year into adjuvant therapy with the aromatase inhibitor anastrozole (Fig. 5A). Enzalutamide did not affect the basal growth of these tumors relative to vehicle (Fig. 5B), concordant with our *in vitro* findings with TamR and LTED cells. Moreover, AR was mainly nuclear in this PDX model, unlike the endocrine-resistant cells. The proportion of Ki-67-positive cells and the protein levels of cyclin A, markers of proliferation

and cells in S-phase respectively, were similar in both treatment arms (Fig. 5C, D and E). Antagonism of canonical AR transcriptional activity with enzalutamide *in vivo* was evident by reduced mRNA expression of AR target genes SEC14 Like Lipid Binding 2 (*SEC14L2*) and FK506 binding protein 5 (*FKBP5*) (Fig. 5F), and further supported by reduced protein levels of nuclear AR and cellular SEC14L2 (Fig. 5C, G, and Supplementary Fig. 6). In contrast, ER expression was unchanged and remained predominantly nuclear in localization (Fig. 5C) while PR remained negative in enzalutamide-treated tumors (Supplementary Fig. 6). Collectively, these results demonstrate that antagonizing canonical AR activity may not be the optimal treatment option for endocrine-resistant breast cancer.

Figure 5

Enzalutamide does not inhibit the growth of an endocrine-resistant PDX. The effect of antagonizing AR using enzalutamide (Enz) on an ER+AR+ endocrine-resistant PDX was assessed. (A) This PDX model was established from the liver metastasis of a patient with ER+PR+HER2− breast cancer who relapsed after 1 year of adjuvant aromatase inhibitor treatment. (B) PDX-bearing mice were treated with either vehicle (Veh) or 20 mg/kg Enz when tumors reached ~150–200 mm^3. Tumors were harvested when they reached the ethical endpoint of 1000 mm^3 and data is presented as fold change of tumor volumes at harvest from baseline tumor volumes. (C) IHC staining was performed to determine the effect of treatment on Ki-67, AR, SEC14L2 and ER. Representative images at 80× are presented. Scale bar = 25 μm (D) Proliferation index of tumors treated with Veh or Enz were determined. This was based on the quantification of proportion of cells positive for Ki-67 in >1000 cells from at least three random high-magnification fields. (E) Protein lysates extracted from Veh- or Enz-treated tumors were immunoblotted for AR, cyclin A and GAPDH. (F) Intensity of nuclear AR and total SECL14L2 was performed using ImageJ and data is represented as intensity of Enz ($n = 6$) relative to Veh ($n = 4$). (G) RT-qPCR was performed to determine the effect of Enz treatment ($n = 3$) on *SECL14L2* and *FKBP5* relative to Veh treatment ($n = 3$). *$P < 0.05$, **$P < 0.01$ and n.s. = not significant using Student's *t*-test. Error bars = s.e.m.

Discussion

AR has been implicated in the development of endocrine-resistant breast cancer but the mechanistic basis remains unclear. In this study, we investigated the consequences of transient knockdown and pharmacological antagonism of endogenous AR using a combination of endocrine-resistant ER+AR+ cell line and PDX models. Results from our *in vitro* siAR experiments to knockdown AR in cell lines support the hypothesis that AR can facilitate endocrine resistance. However, we found that AR is predominantly located in the cytoplasm of endocrine-resistant cells under normal growth conditions, which implicates non-canonical signaling rather than canonical, nuclear AR activity. In support of this, treatment with an AR antagonist, enzalutamide, did not phenocopy the effect of AR

knockdown despite demonstrable inhibitory effects on canonical, ligand-induced AR transactivation events. Likewise, enzalutamide failed to inhibit the *in vivo* growth of an endocrine-resistant PDX model, although it inhibited AR nuclear localization and transcriptional activity. Non-canonical activity of AR and other steroid hormone receptors, including ER, have been described previously (Bjornstrom & Sjoberg 2005, Zarif & Miranti 2016, Leung & Sadar 2017), but the clinical significance of this type of AR signaling in breast cancer remains unclear. These findings suggest that the role of AR in endocrine-resistant breast cancer is complex and may be modulated by conditions that impinge upon the ability of AR to signal in a non-canonical manner.

The loss of proliferative capacity observed with knockdown of endogenous AR in the endocrine-resistant cell line models supported previous findings that AR

plays a role in the proliferation of breast cancer cells (De Amicis *et al.* 2010, Rechoum *et al.* 2014, Ciupek *et al.* 2015). However, our data provide further mechanistic insight by revealing that this effect is likely due to the non-canonical signaling activity rather than canonical nuclear activity of AR. AR knockdown also reduced the proliferation of ES MCF7 cells, both in maintenance and estrogen-supplemented media conditions (Yeh *et al.* 2003, D'Amato *et al.* 2016) and importantly, these studies provided evidence of functional non-canonical AR activity in breast cancer cells. Yeh *et al.* (2003) observed that AR-null MCF7 cells had defective MAPK activity that was restored by overexpression of a truncated form of AR lacking the ligand-binding domain. D'Amato *et al.* (2016) revealed that estrogen induced AR nuclear translocation and binding to non-canonical DNA response elements, whereas androgen recruited AR to canonical DNA response elements. While we observed AR to be predominantly in the cytoplasm of cells, some nuclear protein was evident. Treatment with enzalutamide reduced nuclear AR but had no impact on cell proliferation, suggesting that the limited nuclear AR present in our models was not playing a significant mitogenic role. Hence, our data and that of others support the hypothesis that non-canonical activity of the AR has mitogenic effects on endocrine-sensitive and -resistant forms of MCF7 cells.

In addition to inhibiting the growth of TamR cells, AR knockdown also restored features of classical ER signaling and sensitivity to the anti-proliferative effect of tamoxifen. This effect of AR loss on ER signaling in TamR cells has not been reported previously and appears to be distinct from the effect of AR loss in ES MCF7 cells, which reduced classical activation of ER under estradiol-stimulated conditions (Yeh *et al.* 2003). This dichotomous effect of AR on ER signaling in an endocrine-sensitive vs -resistant state could be ascribed to the different expression levels of AR or key nuclear co-factors that influence AR and ER function. In ES MCF7 cells, low endogenous levels of AR could facilitate ER activity (Yeh *et al.* 2003, D'Amato *et al.* 2016), but the relatively higher levels of AR in TamR cells inhibited classical ER activity in our study. Lanzino *et al.* (2005) demonstrated that overexpression of AR in MCF7 cells suppressed classical ER signaling via the sequestration of an AR-associated protein of 70 kDa (ARA70), a co-factor of ER and AR. Increased expression of FOXA1 in the TamR cells may also play a role as this transcription factor is known to be important for ER and AR signaling in breast cancer cells (Augello *et al.* 2011) and FOXA1 regulates ER activity in ES and endocrine-resistant breast cancer (Ross-Innes *et al.* 2012).

The increase in classical ER signaling events and restoration of sensitivity to tamoxifen induced by loss of cytoplasmic AR in the TamR cells mirrors the effect observed with inhibition of PI3K/Akt in ES MCF7 cells (Bosch *et al.* 2015). This suggests a functional link between the non-canonical activity of AR and the PI3K/Akt signaling pathway in contributing to the acquisition of endocrine resistance. The exact mechanism by which AR influences PI3K/Akt pathway is unknown but a direct interaction between AR and epidermal growth factor receptor (EGFR) has been reported (Ciupek *et al.* 2015). Hence, it is possible that perturbation of this AR–EGFR interaction impacts on growth factor signaling and the downstream PI3K/Akt pathway (Ciupek *et al.* 2015).

Although AR knockdown reduced proliferation of endocrine-resistant cells and restored sensitivity to tamoxifen, this was not recapitulated by pharmacological inhibition of AR with enzalutamide. Similarly, enzalutamide did not affect the growth of an estrogen-independent PDX model derived from a patient who progressed on an aromatase inhibitor. This lack of therapeutic efficacy cannot be attributed to insufficient dosing because enzalutamide effectively antagonized canonical AR signaling as evidenced by reduced AR nuclear expression and transcriptional activity. Our results do not concur with a previous study (D'Amato *et al.* 2016) in which enzalutamide re-sensitized MCF7 TamR cells to tamoxifen *in vitro* and significantly suppressed growth of TamR xenografts *in vivo* when given as a monotherapy. Differences in the dose of enzalutamide used could account for these divergent results. Enzalutamide has been reported to suppress growth of AR-negative breast cancer cell lines *in vitro* when used at >10 µM concentrations (Thakkar *et al.* 2016), implicating off-target effects of enzalutamide in breast cancer cell lines. In support of this, we demonstrate that enzalutamide significantly reduced the viability of an ER+ AR-null T47D derivative cell line at doses >10 µM (Supplementary Fig. 7). Notably, D'Amato *et al.* (2016) reported that enzalutamide suppressed colony formation of TamR cells at a minimal dose of 20 µM. Likewise, the growth-suppressive effect of enzalutamide on TamR xenografts *in vivo* was achieved with 50 mg/kg, which is greater than the 10–30 mg/kg doses commonly used in prostate cancer models (Evans *et al.* 2011, Lin *et al.* 2013, Moilanen *et al.* 2015). We used a dose of 20 mg/kg in our *in vivo* experiments to minimize the off-target effects of enzalutamide, and there was demonstrable anti-androgenic effect in tumor cells within our PDX. Collectively, these data strongly support the concept that canonical AR signaling does not promote growth of endocrine-resistant breast cancer.

Observations from previous AR-targeted clinical studies also support the notion that canonical AR signaling does not promote growth of endocrine-resistant breast cancer and that the use of AR antagonists is ineffective in the endocrine-resistant setting. Two clinical trials have assessed the efficacy of AR antagonists in breast cancer, and both have reported no clinical benefit from AR inhibition. The first study trialed the first generation AR antagonist flutamide in non-selected patients with metastatic breast cancer (Perrault *et al.* 1988). The second and more recent study evaluated the efficacy of androgen biosynthesis inhibitor abiraterone acetate in ER+ patients who had progressed on a non-steroidal aromatase inhibitor (O'Shaughnessy *et al.* 2016), a similar clinical context to the patient from which our PDX model was generated from.

In contrast, multiple clinical studies have reported that the use of AR agonists to activate canonical AR activity in endocrine-resistant breast cancer is associated with tumor suppression and a clinical benefit rate of 38–50% (Birrell *et al.* 1995, Boni *et al.* 2014, Overmoyer *et al.* 2015, Kono *et al.* 2016). The agents used to activate AR in these studies include testosterone (Boni *et al.* 2014), testosterone analog fluoxymesterone (Kono *et al.* 2016, medroxyprogesterone acetate, which is a synthetic progestin with androgenic activity {Birrell, 1995 #1794), and a selective AR modulator that induces a selected spectrum of AR activity (Overmoyer *et al.* 2015). In agreement, activation of AR with DHT in the MCF7 TamR cells reduced growth of these cells and transient knockdown of AR abolished this DHT-induced growth inhibition, which suggests that the expression and canonical activity of AR is critical for AR agonist-induced growth inhibition (Supplementary Fig. 8).

A caveat of our study is the use of a single endocrine-resistant PDX model derived from a liver metastasis. Despite this, our observations are valuable as PDX models are considered the most clinically relevant and resistance to aromatase inhibition, a feature of this model, is frequently fatal. Further investigation using additional PDX models of tamoxifen and AI resistance, as well as models derived from different metastatic deposits, are warranted. While our *in vivo* data strongly support the concept that canonical, nuclear AR signaling does not promote endocrine-resistant breast cancer, it would be informative to test pharmacological degradation of AR *in vivo* to mimic the siRNA-mediated knockdown we performed in endocrine-resistant cells *in vitro*. Novel AR degraders are in development for the treatment of castrate-resistant prostate cancer {Salami, 2018 #2071} and may help resolve the role of AR in endocrine-resistant breast cancer.

In summary, our study provides evidence that non-canonical AR activity facilitates an endocrine-resistant phenotype in breast cancer, and this activity cannot be inhibited pharmacologically with the AR antagonist enzalutamide. The lack of efficacy associated with the use of enzalutamide in our study has implications for current and future clinical trials that aim to specifically target AR in endocrine-resistant breast cancer.

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