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Research Article

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Ligand Binding Prolongs Androgen Receptor Protein Half-Life by Reducing its Degradation

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Abbreviations: AR, androgen receptor; ARE, androgen response element; cDNA; complementary DNA; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; ER, estrogen receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCs, granulosa cells; IPO7, importin 7; mRNA, messenger RNA; NFκB, nuclear factor κB; RNA-seq, RNA sequencing; PCR, polymerase chain reaction; siRNA, small interfering RNA.

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Abstract

Androgens are important in female reproduction, but the molecular actions of androgens in female reproductive tissues are not fully understood. We investigated the androgen-responsive transcriptome in human and mouse granulosa cells (GCs) and surprisingly found that the gene-regulation activity of androgen receptor (AR) in these cells is negligible. We then investigated extranuclear actions of AR and found that in human and mouse GCs, as well as in prostate cancer cells, dihydrotestosterone (DHT) dramatically increases the half-life of its own receptor protein. Using the human granulosa-like KGN cells, we show that this effect is not the result of increased *AR* gene transcription or protein synthesis, nor is it fully abrogated by proteasome inhibition. Knockdown of PTEN, which contributes to degradation of cytoplasmic AR, did not diminish AR accumulation in the presence of DHT. Using immunofluorescence cellular localization studies, we show that nuclear AR is selectively protected from degradation in the presence of DHT. Knockdown of importin 7 expression, a potential regulator of AR nuclear import, does not affect DHT-mediated nuclear accumulation of AR, suggesting importin 7-independent nuclear import of AR in GCs. Further, DNA binding is not required for this protective mechanism. In summary, we show that ligand binding sequesters AR in the nucleus through enhanced nuclear localization independent of DNA binding, thereby protecting it from proteasome degradation in the cytoplasm. This phenomenon distinguishes AR from other sex steroid receptors and may have physiological significance through a positive feedback loop in which androgen induces its own activity in male and female reproductive tissues.

Key Words: androgen, granulosa, proteasome, prostate, ovary, transcriptome

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Androgens play an important role in female reproduction. Disorders of androgen excess in women, such as the highly prevalent polycystic ovary syndrome and others, carry a high burden of infertility. Androgen deficiency due to global androgen receptor (AR) knockout in animal models leads to reduced female fertility and early cessation of ovarian function [1-4]. Moreover, mouse studies of tissue-specific deletion of AR have pinpointed the granulosa cell (GC) compartment as the site where androgen activity is required to preserve fertility [5, 6]. Studies in rodents and primates show that the potent AR ligand dihydrotestosterone (DHT) enhances follicle development in vitro, predominantly by regulating preantral to antral follicle maturation [5, 7, 8]. Similarly, chronic in vivo exposure to dehydroepiandrosterone (DHEA) increases the antral follicle count in sheep [9]. Although the role of androgens in promoting follicle development has not been directly proven in humans, small studies suggest that this may be the case [10-12]. In fact, approximately 25% of fertility practices worldwide prescribe DHEA to women with diminished ovarian reserve to enhance follicular recruitment for in vitro fertilization [13-15]—a practice that may be most effective in women with lower baseline DHEA levels [16]. In addition, there is moderate evidence supporting the use of testosterone in poor responders to ovarian stimulation in assisted reproduction [17, 18]. However, despite our advanced understanding of the physiologic effects of androgen-related disorders in females, the molecular actions of androgens within the ovary, and particularly within the GC remain to be elucidated.

AR-regulated transcriptome has been defined in prostate cancer cells [19, 20], where it is a major therapeutic target, but to date very few direct gene transcription targets of AR have been reported in GC. Gene expression differences induced in the mouse ovary by global AR knockout [4] and in rhesus monkey follicles by treatment with androgens [21] include important folliculogenesis factors, consistent with in vitro studies showing that androgen treatment results in increased GC proliferation [22-24] and reduced apoptosis [11, 25]. However, no studies prove these genes to be direct transcriptional targets of AR through functional *cis*-acting androgen response elements (AREs). This paucity of knowledge regarding genomic effects of AR in the ovary is surprising, given its demonstrated role in follicle development. Regulation of noncoding RNA may be equally important: For instance, transcription of antiapoptotic microRNA *miR125b* is induced by androgens in GCs [25], leading to increased GC survival, and this may explain much of the observed follicle-promoting effect of androgens.

Rapid nonenomic actions of AR have been described in most detail in the prostate. We and others have shown that nonclassical extranuclear activity of AR involves transactivation of the epidermal growth factor receptor and activation of the mitogen-activated protein kinase phosphorylation cascade [26, 27]. It is particularly important in prostate cancer, where it enhances growth factor signaling leading to increased cell survival, proliferation and invasion [28, 29]. Nongenomic androgen signaling in GCs is likely to play a role as well: In fact, our laboratory has previously shown that AR induces the protein expression of follicle-stimulating hormone receptor in GCs through a posttranscriptional mechanism [25]. In addition, we have reported 2 noncoding transcripts induced by AR in GCs that in turn regulate cell apoptosis (miR-125b [25]) and ovulation (miR-101 [30]). Thus, nongenomic signaling and transcription of noncoding RNAs by androgens may work in concert to regulate GC function.

Like many other steroid hormone receptors, ARs are primarily cytoplasmic when unliganded. When androgens bind to ARs, they are imported into the nucleus through interactions with cytoplasmic and nuclear factors, particularly importin 7 (IPO7) [31] and STAT 5 [32]. Transcriptionally active nuclear AR is recycled into the cytoplasm, where it becomes available for ligand binding again or is degraded by the proteasome [33]. This is a distinct property of AR compared with the estrogen receptor (ER), which is rarely recycled and is destined for proteasome-mediated degradation once bound by its ligand [33, 34]. Studies show that cytoplasmic AR can be trapped by interacting with PTEN, which may reduce AR nuclear import and enhance its degradation [33], although many androgen-dependent prostate cancer cell lines are characterized by loss or inactivation of PTEN [35]. Importantly, most of these observations were made in prostate cells.

Here we investigated both nuclear and extranuclear actions of AR in GCs using mouse primary GCs and the human immortalized GC-derived KGN cell line. Unexpectedly, 18 hours of DHT treatment did not result in significant changes in gene expression. We therefore examined nongenomic effects of DHT in these cells and found that the most striking effect of androgen treatment was upregulation of AR protein abundance through reduced degradation. We further explored the mechanisms by which this occurs and found that nuclear retention of ligand-bound AR sequesters it from degradation in the cytoplasm.

Materials and Methods

Laboratory Animals and Dihydrotestosterone Treatments

Mice were maintained in an experimental animal facility according to the protocol for the Care and Use of Laboratory Animals, approved by the University Committee on Animal

Resources at the University of Rochester. C57BL/6J mice (males, aged 13-16 weeks, and females, aged 18 weeks) were injected into the peritoneum with 29 µg of DHT suspended in a mixture of 90% sesame oil and 10% ethanol in a 100-µL volume, and humanely killed 18 hours later. Ventral prostates or ovaries were dissected in sterile phosphate-buffered saline, ground in TRK lysis buffer, and RNA was extracted using an E.Z.N.A. RNA extraction kit (Omega).

Cell Culture and Treatments

For all experiments using cultured cells, KGN cells (RRID:CVCL_0375) were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen 11330-057) with 10% fetal bovine serum (FBS), C4-2 cells (ATCC CRL-3314) were grown in RPMI1640 (Invitrogen 11875-119) with 10% FBS, and HEK-293 cells (ATCC CRL-1573) were grown in high-glucose DMEM (Invitrogen 11965-084) with 10% FBS at 37 °C and 5% CO_2. Human AR expression plasmid was constructed by amplifying AR complementary DNA (cDNA) from pCMV-hAR (RRID:Addgene_89078) and ligating it into the polylinker region of the pcDNA3.1 vector between the NheI (5′) and BamHI (3′) restriction sites. The DNA-binding mutant AR expression plasmid pCDNA3-FLAG-AR-R614H [36] was obtained from Dr Shu-Yuan Yeh (University of Rochester). HEK-293 cells were grown in 24-well plates and transfected with 100-ng of either wild-type or DNA-binding mutant AR expression plasmid and 400-ng of pcDNA3.1 vector using jetPRIME (Polyplus 114-01, 1 µL/well). Cells were washed with phosphate-buffered saline and maintained in serum-free media for 24 hours prior to DHT treatments. Working concentrations of the treatments were 25-nM dihydrotestosterone (Steraloids A2570-000) in ethanol, 5-µM Bay-11-7082 (Sigma B5556) in dimethyl sulfoxide (DMSO), 5-µM actinomycin D (Sigma A1410) in DMSO, 50-µM cycloheximide (Fisher 0970100) in ethanol, 10-µM bortezomib (Sigma 5.04314) in DMSO, and 50-nM bafilomycin A1 (Sigma B1793) in DMSO. The final v/v content of vehicle was 0.1%. All samples contained the same final amounts of all vehicles within each experiment.

RNA Sequencing

Ovaries from DHT-treated mice were transferred to DMEM/F12, where GCs were extruded by the poke-and-press method [37]. The cells were pelleted and RNA was isolated using RNeasy Plus Mini Kit (Qiagen) per the manufacturer's recommendations. KGN cells were grown in a 12-well plate to approximately 50% confluency, then serum starved for 24 hours before treatment with 25-nM DHT or ethanol for 12 hours and RNA isolation as described earlier. RNA library preparation, sequencing, and primary data analysis was performed by the Genomics Research Center at University of Rochester. The total RNA concentration was determined with the NanoDrop 1000 spectrophotometer (NanoDrop) and RNA quality assessed with the Agilent Bioanalyzer (Agilent). The TruSeq Stranded mRNA Sample Preparation Kit (Illumina) was used for next-generation sequencing library construction per the manufacturer's protocols. Briefly, mRNA was purified from 200-ng total RNA with oligo-dT magnetic beads and fragmented. First-strand cDNA synthesis was performed with random hexamer priming followed by second-strand cDNA synthesis using dUTP incorporation for strand marking. End repair and 3′ adenylation was then performed on the double-stranded cDNA. Illumina adaptors were ligated to both ends of the cDNA and amplified with polymerase chain reaction (PCR) primers specific to the adaptor sequences to generate cDNA amplicons of approximately 200 to 500 base pairs in size. The amplified libraries were hybridized to the Illumina flow cell and single end reads were generated for each sample using an Illumina sequencer. Raw reads generated from the Illumina basecalls were demultiplexed using bcl2fastq version 2.19.1. Quality filtering and adapter removal were performed using FastP version 0.20.0 with the following parameters: "--length_required 35 --cut_front_window_size 1 --cut_front_mean_quality 13 --cut_front --cut_tail_window_size 1 --cut_tail_mean_quality 13 --cut_tail -y -r." Processed/cleaned reads were then mapped to the Homo sapiens reference genome (GRCh38+Gencode-31 Annotation) or the Mus musculus reference genome (GRCm38+Gencode-M22 Annotation) using STAR_2.7.0f with the following parameters: "—twopass Mode Basic --runMode alignReads --outSAMtype BAM SortedByCoordinate—outSAMstrandField intronMotif --outFilterIntronMotifs RemoveNoncanonical –outReads UnmappedFastx." Genelevel read quantification was derived using the subread-1.6.4 package (featureCounts) with a GTF annotation file (Gencode 31 or Gencode M22) and the following parameters: "-s 2 -t exon -g gene_name." Differential expression analysis was performed using DESeq2-1.22.1 with a *P* value threshold of .05 within R version 3.5.1 (https://www.R-project.org/). Heat maps were generated using the pheatmap package were given the rLog transformed expression values. All RNA sequencing (RNA-seq) data are available on Gene Expression Omnibus (NCBI GEO accession: GSE158218).

Quantitative Polymerase Chain Reaction

RNA samples from KGN cells, prostate tissue, C4-2 cells, or primary GCs were reverse-transcribed and amplified in the same

reaction using the qScript XLT 1-Step RT-qPCR ToughMix kit (QuantaBio) and species-specific ROX-containing TaqMan primers (Applied Biosystems, Hs with capital letters for human and Mm with lower case letters for mouse: Hs01003372_m1 [VCAM1], Hs00219060_m1 [ERRFI1], Hs00263492_m1 [PLAT], Mm00434658_m1 [Klkb1, a.k.a. PSA], Mm00438070_m1 [Ccnd2], Mm00448533_m1 [Pxn], Mm00442688_m1 [Ar], Hs00171172_m1 [AR], Hs02576345_m1 [KLK3, a.k.a. PSA], Mm99999915-g1 [glyceraldehyde 3-phosphate dehydrogenase; Gapdh], and Hs02786624_g1 [GAPDH]). RNA expression was normalized to GAPDH using the ΔΔCT method and expressed relative to control within each experiment.

Luciferase Assays

KGN and C4-2 cells were grown in 24-well plates to approximately 50% confluency and transfected using JetPrime with 400 ng of AR reporter plasmid MMTV-luc (pGL4.36[luc2P/MMTV/Hygro] Vector, Promega) and 40 ng of β-galactosidase reference plasmid. For studies of the DNA binding domain AR mutant, 20 ng of wild-type, mutant, or vector control plasmid were transfected into HEK-293 cells along with reporter and transfection control plasmids as described earlier. After 24 hours, cells were serum starved for 8 hours, then treated with 25-nM DHT or ethanol vehicle, with or without 5-μM Bay-11-7082 or DMSO vehicle for 24 hours. Luciferase activity was assayed using the Dual Light kit (Invitrogen t1004) and quantified using a luminometer. Raw counts of luciferase activity were adjusted for transfection efficiency using the β-galactosidase assay in the same kit.

Knockdown Experiments

Small interfering RNA (siRNA) (Dharmacon) was transfected using DharmaFECT3 (3 μL/mL) in Opti-MEM at final amounts of 40 pmol/mL PTEN (L-003023-00-0005) and 60 pmol/mL IPO7 (L-012255-00-0005). Nontargeting siRNA (D-001910-01-05) was added as negative control in all experiments. Serum-containing media were added after 24 hours. The total duration of knockdown was 5 days in all experiments.

Western Blots

After treatments as indicated, cells were lysed directly in the culture plates with lysis buffer containing 50-mM Tris base, 150-mM NaCl, 5-mM EDTA, 1% Triton X-100, and protease and phosphatase inhibitor cocktail (Halt, Thermo 1861281). Cell lysates were boiled for 5 minutes in sample buffer containing 5% β-mercaptoethanol, then separated in sodium dodecyl sulfate–polyacrylamide gels (7.5% for AR, 12.5% for GAPDH, 10% for all other probes, or 4%-15% gradient gels) and transferred onto polyvinylidene fluoride membranes in 20% methanol. Blots were blocked for 1 hour at room temperature with 5% milk in Tris-buffered saline Tween, then probed overnight at 4 °C with antibodies for AR (Cell Signaling Technology catalog No. 5153, RRID:AB_10691711, 1:1000), GAPDH (Cell Signaling Technology catalog No. 2118, RRID:AB_561053, 1:2000), IPO7 (Abcam catalog No. ab99273, RRID:AB_10672198, 1:1000), and PTEN (Cell Signaling Technology catalog No. 9559, RRID:AB_390810, 1:1000). Band density was quantified using ImageJ and normalized to GAPDH.

Immunofluorescence

Cells were seeded on poly-L-lysine–treated glass coverslips to approximately 25% confluency. After indicated treatments, cells were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, and blocked with 2% bovine serum albumin. The samples were incubated overnight in a humidity chamber at 4 °C with rabbit anti-AR antibody (Cell Signaling Technology catalog No. 5153, RRID:AB_10691711, 1:200), then for 2 hours at room temperature with fluorescein goat–antirabbit immunoglobulin G (Molecular Probes catalog No. F2765, RRID:AB_221635, 1:500). Samples were mounted in Duolink In Situ Mounting Medium with DAPI (4', 6-diamidino-2-phenylindole; Sigma DUO82040).

Statistical Analysis

All data are presented as mean ± SEM. Statistical analysis was performed in GraphPad Prism 3 using a 2-tailed *t* test for single-variable comparisons, and analysis of variance for comparisons of multiple variables with P less than .05 indicating statistical significance. To calculate the Kd for the DHT dose-response curve, the data were expressed as percentage stimulation and fitted to a nonlinear model with one binding site. For experiments following AR protein degradation over time, an exponential one-phase decay nonlinear regression model was used to fit a curve to the data, and curves were compared using 2-way analysis of variance. The half-life of AR was estimated based on the best fit curve.

Results

Androgen-induced Androgen Receptor Transcriptional Activity Is Limited in Granulosa Cells

To evaluate changes in mRNA expression induced by androgens in mouse GCs, we harvested ovaries from mature mice (n = 5 per treatment) 18 hours after intraperitoneal

injection of DHT (29 μg) or vehicle, extruded granulosa cells as previously described, and isolated mRNA (Supplementary Fig. 1A, [38]). To confirm that this steroid delivery method results in adequate gonadal exposure to DHT for gene transcription, we subjected mature male mice (n = 3) to the same treatment and analyzed gene expression in the ventral prostate using quantitative PCR (qPCR). This was necessary because there are no known, validated direct transcriptional targets of AR in mouse GCs. As expected, the mRNA levels of androgen-responsive genes cyclin D2 (*ccnd2*), paxillin, and androgen receptor were increased 3- to 4-fold in prostate tissue after DHT treatment of males (Supplementary Fig. 1C, [38]). Since DHT reached the prostate in males, we therefore concluded that the intraperitoneal DHT injection had likely reached the ovaries in females, and proceeded with RNA-seq and analysis of the GC mRNA samples. Surprisingly, no genes were significantly differentially expressed in the GCs obtained from the DHT-treated compared with the vehicle-treated female mice (Supplementary Fig. 1B [38]).

We hypothesized that the lack of differences in gene expression seen in mice could be a false-negative result due to high experimental variability implicit in animal studies and/or due to the steroid not reaching the ovaries in sufficient concentrations to induce gene transcription. To address these possibilities, we assessed the androgen-regulated transcriptome in human granulosa-derived immortalized KGN cells. Serum-starved KGN cells were treated with 25-nM DHT or ethanol for 12 hours in 5 independent experiments, then mRNA was extracted and analyzed using RNA-seq. After adjusting for multiple hypotheses testing, and only in paired comparisons to eliminate variability due to sample collections on different dates, we found that 173 genes were differentially expressed in DHT-treated compared with vehicle-treated cells (Fig. 1A). Of these, 125 genes were upregulated by DHT, but the largest fold change was only 1.37 (37% increase in expression). Similarly, among the 48 genes that were downregulated by DHT, the largest magnitude of fold change was only 0.87 (13% reduction in expression). To confirm these findings, we measured the expression of the 3 most robustly DHT-induced genes in these samples by qPCR analysis of the same samples. Consistent with the results of RNA-seq, VCAM1, ERRFI1, and PLAT were induced only 20% to 50% by DHT treatment in our experiments (Fig. 1B). Gene ontology enrichment analysis of the gene set differentially regulated by DHT revealed no statistically significant results in biological processes or cellular components (Supplementary Table 1A [38]). Among gene ontology molecular functions, fibronectin binding was enriched among DHT-induced genes, and PANTHER pathways analysis revealed that the p53 pathway was differentially induced by DHT (Supplementary Table 1A [38]). There were only 5 genes represented in each of these enriched categories, and the maximum fold change was 1.26, as listed in Supplementary Table 1B [38].

Although the data listed earlier are statistically significant, it is unlikely that these small-magnitude changes in gene expression are functionally meaningful. To compare the transcriptional activity of AR on a potent ARE in GCs with prostate cancer cells, where AR is known to induce biologically significant gene transcription changes, we transfected KGN cells and prostate cancer-derived C4-2 cells with the AR transcription reporter plasmid MMTV-luciferase and treated the cells with 25-nM DHT or ethanol for 24 hours. While luciferase activity was induced approximately 300-fold by DHT in C4-2 cells, only a 2-fold increase in luciferase activity was achieved in KGN cells (Fig. 1C). It was reported previously that inhibition of nuclear factor κB (NFκB) can unmask gene transcription by ER in KGN cells [39]; to address the possibility that AR activity can be similarly affected, we included the NFκB inhibitor Bay-11-7082 in these experiments. However, we did not observe any differences in luciferase activity in the presence or absence of the inhibitor. Taken together, these data suggest that in GCs, AR is only minimally transcriptionally active on genomic and heterologous promoters, and that the majority of its actions are likely mediated by other mechanisms.

Androgen Treatment Increases Androgen Receptor (AR) Protein Expression in Granulosa Cells Independent of *AR* Gene Transcription

In light of our gene transcription data showing very low AR transcriptional activity in GCs, we then turned to exploring nongenomic effects of androgens in these cells. We measured the phosphorylation of extracellularly regulated kinase 1/2, Src, EGFR, LKB1, AKT, and CREB by Western blot (data not shown) and found no differences in DHT-treated KGN or primary mouse GCs. However, AR protein itself was consistently upregulated in our DHT-treated KGN samples taken from the same experiment that was used for RNA-seq (Supplementary Fig. 2A, [38]). Unlike the gene expression changes that we reported earlier, the increase in AR protein level was dramatic, with an approximately 3-fold induction after 12 hours of DHT treatment. Similarly, in primary mouse GCs treated in vitro with DHT for 12 hours, AR protein level and nuclear localization were significantly increased (Supplementary Fig. 2B [38]). Notably, immunofluorescence of AR expression in cultured primary mouse GCs showed abundant cell membrane–associated AR (outlined with arrows in Supplementary Fig. 2B [38]), which was less apparent after treatment with DHT.

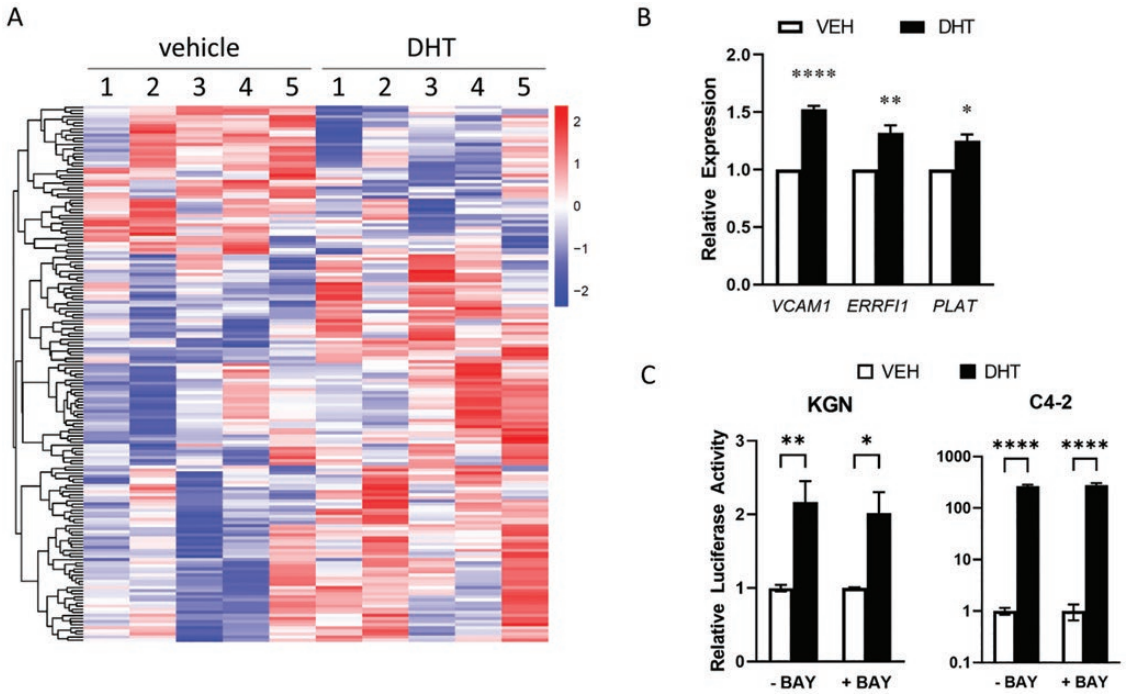

Figure 1. A, RNA sequencing heat map of differentially expressed genes from KGN cells after 12 hours of 25-nM dihydrotestosterone (DHT) treatment (n = 5 per group, individual samples indicated). B, Gene expression measured by quantitative polymerase chain reaction of the same samples as in A (n = 5 per group). Data are relative to control and analyzed as paired samples. C, β-Galactoside–adjusted luciferase activity in cultured cells (n = 3) transfected with MMTV-luciferase and β-galactosidase, and treated as indicated (BAY: nuclear factor κB inhibitor Bay-11-7082, 5 μM). Data are means ± SEM relative to control within each group. **P* less than .05. ***P* less than .01. *****P* less than 10^{-4}.

We further evaluated this effect in KGN cells treated with a range of DHT doses for 24 hours, measured AR protein using band densitometry relative to GAPDH, and fitted the data to a nonlinear curve using the one site—a total binding model. AR protein was induced by DHT with a calculated Kd of 0.17 nM (Fig. 2A). When KGN cells were treated with a saturating dose of 25-nM DHT over a range of time, the increase in AR protein level became apparent on a Western blot after 2 hours of treatment and continued to rise over 24 hours (Fig. 2B), reaching nearly maximum induction at 12 hours. To confirm the universality of this effect in GCs, we measured AR protein expression in mouse primary GCs (Fig. 2C) and saw a similar induction over 24 hours. Furthermore, DHT enhanced AR expression similarly in C4-2 prostate cancer cells (Fig. 2D), indicating that this induction of AR expression is not unique to GCs.

Our observation that AR protein level rises as early as 2 hours after the start of DHT treatment suggested that this may not be due to induction of AR gene transcription, which would likely require a longer time. To further investigate this possibility, we measured *AR* mRNA using qPCR in KGN cells after 24 hours of treatment with 25-nM or 100-nM DHT. We observed no change in *AR* mRNA level after treatment with either DHT dose (Fig. 3A). Similarly, in cultured C4-2 cells treated with DHT (Supplementary Fig. 3A [38]), cultured primary GCs treated with DHT (Supplementary Fig. 3B [38]), and in GCs from mice injected with DHT (Supplementary Fig. 3C [38]), there was no increase in *AR* mRNA, whereas *PSA* mRNA increased as expected in C4-2 cells after DHT treatment. It is possible that, although the steady-state level of mRNA is constant, its turnover rate is increased, leading to increased protein translation. To test whether this mechanism contributes to AR protein synthesis in the presence of DHT, we blocked mRNA synthesis with actinomycin D for up to 12 hours. As expected, there was a reduction in *AR* mRNA level after actinomycin D treatment that reached approximately 50% at 12 hours (Fig. 3B) due to degradation of the mRNA. AR protein level in relation to GAPDH remained approximately the same after actinomycin D treatment (Fig. 3C). However, when DHT was added together with actinomycin D, the AR protein level increased even while *AR* mRNA levels declined (see Fig. 3B and 3C). The DHT-induced rise in AR protein was essentially the same magnitude whether mRNA synthesis was blocked or not, indicating that DHT effects on AR protein levels do not require de novo production of *AR* mRNA.

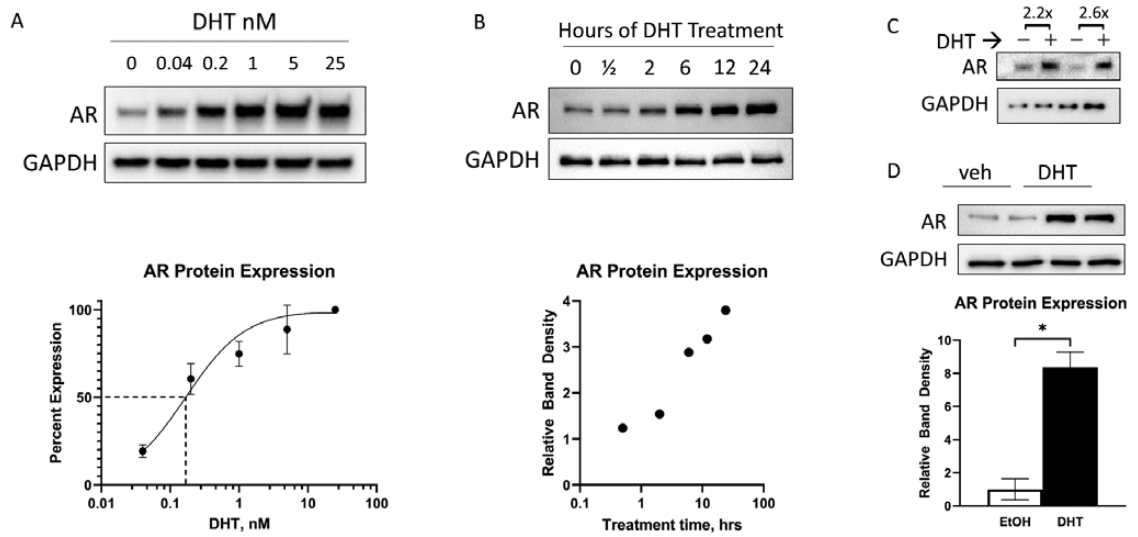

Figure 2. A, Western blot of androgen receptor (AR) expression in KGN cells treated with indicated concentrations of dihydrotestosterone (DHT) for 24 hours (top) and quantification of band density (bottom) fitted to a one-site binding model (n = 4). B, Western blot and quantification of AR expression in KGN cells treated with 25-nM DHT for indicated times. Experiment was repeated 3 times with similar results. C, Western blot of AR expression in mouse primary granulosa cells cultured with or without 25-nM DHT, as indicated, for 24 hours. D, Western blot and quantification of AR expression in C4-2 cells treated with vehicle (veh) or 25-nM DHT for 24 hours.

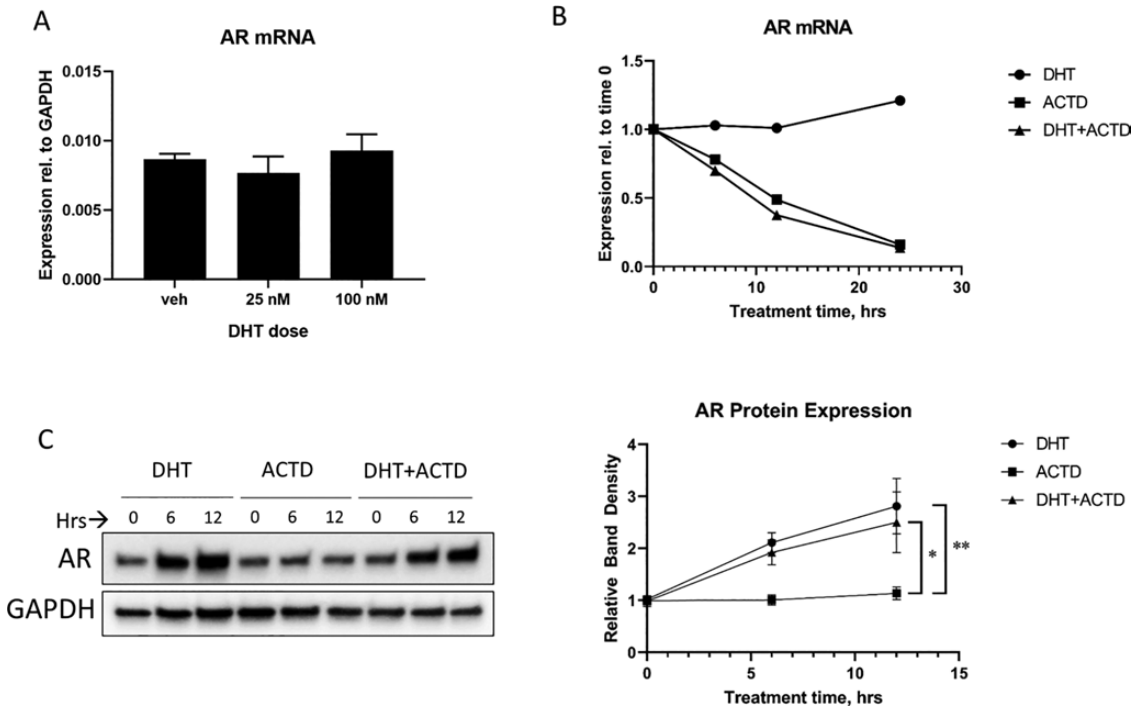

Figure 3. A, Androgen receptor (*AR*) messenger RNA (mRNA) expression measured by quantitative polymerase chain reaction (qPCR) in KGN cells after treatment with indicated doses of dihydrotestosterone (DHT) for 24 hours. B, *AR* mRNA expression measured by qPCR in KGN cells treated with 25-nM DHT and/or 5-μM actinomycin D (ActD) as indicated for 6 to 24 hours. Data are relative to time 0 and not adjusted to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) because of GAPDH degradation with actinomycin D treatment. C, Western blot of AR protein expression in KGN cells treated as indicated for up to 12 hours (left) and quantification of band density (right, n = 3) with best-fit curves analyzed by analysis of variance. **P* less than .05. ***P* less than .01.

Androgen Receptor Protein Half-Life Is Increased in the Presence of Dihydrotestosterone

Having established that AR gene transcription is not involved in DHT-induced AR protein accumulation, we then investigated whether this happened due to enhanced translation of AR or its reduced degradation. To isolate the natural degradation of AR, we treated KGN cells with cycloheximide to block protein synthesis, and observed

that AR protein levels decreased to approximately 25% of initial level after 24 hours, with a half-life of approximately 7 hours (Fig. 4). When DHT was added together with cycloheximide, AR protein was degraded significantly slower, with a half-life of approximately 23 hours. We performed the same experiment in C4-2 cells (Supplementary Fig. 4 [38]) and observed that DHT extended AR half-life in these cells from approximately 12 hours to more than 30 hours. We therefore concluded that DHT increases AR protein half-life by reducing its degradation, not by enhancing its protein synthesis.

Dihydrotestosterone Extends Androgen Receptor Half-Life Independently of Proteasome or PTEN Activity

Previously, it was reported that AR is degraded mainly by the proteasome [33], and that PTEN can interact with AR in the cytoplasm, reducing its nuclear import and increasing its proteasomal degradation [33]. To test the role of the proteasome in the DHT-induced extension of AR half-life, we treated KGN cells with cycloheximide to block new protein synthesis, and examined the effect of proteasome inhibitor bortezomib on the degradation timeline of AR in the presence or absence of DHT. As expected, AR half-life was increased from approximately 6 to 16 hours when the proteasome was inhibited with bortezomib (Fig. 5). However, when DHT was present in addition to bortezomib, AR half-life was further extended dramatically, beyond 48 hours. These data show that DHT action is additive to the inhibition of the proteasome pathway to extend AR half-life in GCs. Similarly, in C4-2 cells the half-life of AR was increased from approximately 16 to 39 hours by proteasome inhibition, and far more beyond 48 hours with the addition of DHT (Supplementary Fig. 5 [38]). Therefore, in the prostate cells as well as in GCs, inhibition of the proteasome does not fully explain the activity of DHT in prolonging the half-life of AR.

To investigate a potential role of the lysosome in AR protein degradation, we used bafilomycin A1, a validated lysosome inhibitor [40], in KGN and C4-2 cells treated with cycloheximide to block new protein synthesis. Shown in Supplementary Fig. 6 [38], AR protein was degraded at the same rate in the presence or absence of bafilomycin A, while the addition of DHT significantly delayed its degradation, as in our prior experiments. This finding is consistent with prior reports of the proteasome as the main contributor to AR protein degradation.

As mentioned, PTEN has been reported to directly bind to AR to inhibit its nuclear translocation and shorten its half-life [33]. To examine the role of PTEN in AR protein

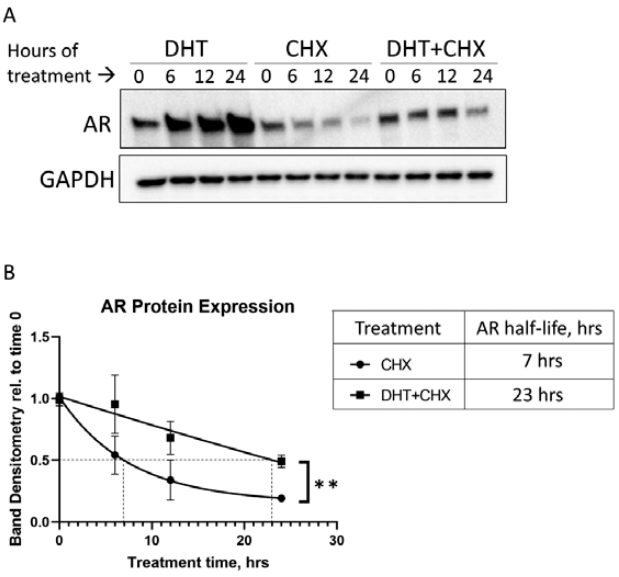

Figure 4. A, Western blot of androgen receptor (AR) expression in KGN cells treated with 25-nM dihydrotestosterone (DHT) and/or 50-μM cycloheximide (CHX) as indicated for 6 to 24 hours. B, Quantification of band density from A (n = 3) fitted to a one-phase decay model and analyzed by analysis of variance. Protein half-life was estimated based on the curves as shown by the dotted lines. ***P* less than .01.

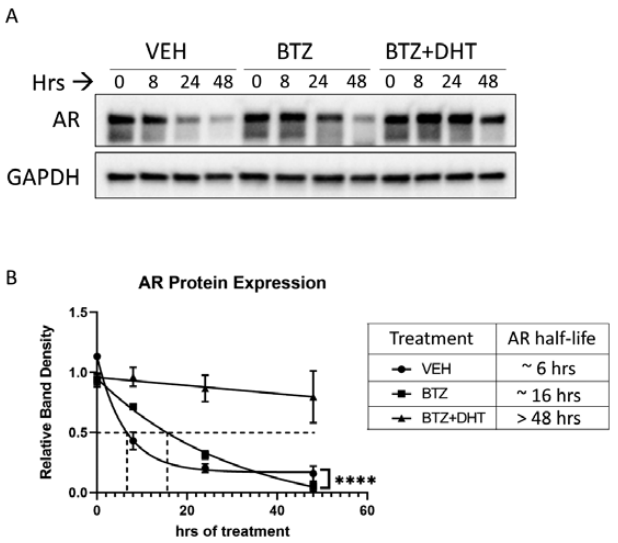

Figure 5. A, Western blot of androgen receptor (AR) expression in KGN cells treated with 50-μM cycloheximide (CHX—all samples), 10-μM bortezomib (BTZ), and/or 25-nM dihydrotestosterone (DHT) as indicated for up to 48 hours. B, Quantification of band density from A (n = 3) fitted to a one-phase decay model and analyzed by analysis of variance. Protein half-life was estimated based on the curves as shown by the dotted lines. *****P* less than 10^{-4}.

stability in GCs and in the observed impact of DHT, we depleted PTEN expression in KGN cells using siRNA and studied the degradation of AR after blocking protein synthesis with cycloheximide. Even though PTEN protein expression was dramatically reduced by siRNA, the AR protein degradation timeline was unaffected and still

significantly extended by adding DHT (Fig. 6). This suggests that, unlike previously reported in prostate cells, in GCs PTEN does not play a major role in regulating AR protein expression, and that DHT extends the half-life of AR through a PTEN-independent pathway.

Dihydrotestosterone Protects Androgen Receptor From Degradation Through Increased Nuclear Retention

It is well known that ligand binding induces nuclear translocation of AR in androgen-sensitive tissues, but to date this has not been characterized in GCs. This is especially of interest given the aforementioned minimal effects of DHT on AR-mediated transcription. To investigate the subcellular localization of AR in GCs and whether it plays a role in regulating AR half-life, we examined the localization of AR in KGN cells using immunofluorescence. At baseline, AR was observed in the cytoplasm and the nucleus of the cells (Fig. 7). DHT treatment resulted in marked translocation of AR into the nucleus (top panels). Blocking protein synthesis with cycloheximide significantly depleted AR both in the cytoplasm and in the nucleus between 12 and 24 hours (middle panels); however, when DHT was added together with cycloheximide, the cytoplasmic portion of AR was primarily degraded, whereas nuclear AR expression remained stable between 12 and 24 hours (bottom panels).

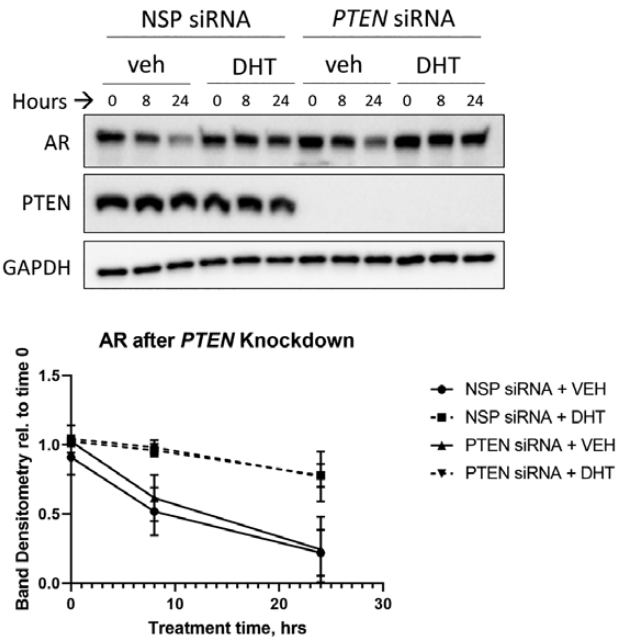

Figure 6. Top: Western blots of androgen receptor (AR) and PTEN expression in KGN cells treated with 50-μM cycloheximide (CHX—all samples), with or without 25-nM dihydrotestosterone (DHT) as indicated for up to 24 hours, after transfection with small interfering RNA (siRNA) targeting PTEN or nonspecific (NSP) siRNA. Bottom: quantification of band density (n = 3) fitted to best-fit curves (VEH: vehicle).

These data demonstrate that, as in other androgen-sensitive tissues, AR in GCs translocates into the nucleus on ligand binding. Additionally, the nuclear portion of AR is selectively protected from degradation in the cytoplasm.

Next, we aimed to determine whether increased nuclear import or reduced nuclear export is the primary mechanism behind DHT-mediated nuclear localization and protection of AR protein. It was previously shown in prostate cells that IPO7 is an important cytoplasmic factor that dissociates from AR on ligand binding, allowing for its nuclear import [31]. We therefore examined the role of IPO7 in DHT-regulated AR protein localization in KGN cells. Contrary to our expectations, when IPO7 was knocked down in KGN cells, AR localization did not change (Fig. 8), despite a dramatic depletion of IPO7 protein levels. Further, IPO7 knockdown did not significantly affect AR degradation dynamics when the cells were treated with cycloheximide, in the presence or absence of DHT. Therefore, IPO7 does not appear to play a major role in AR protein expression and localization in GCs.

DNA Binding Does Not Affect Androgen Receptor Protein Degradation

We have shown that ligand-bound AR is protected from degradation at least in part through increased nuclear localization. To test whether AR's ability to bind to DNA is required for this effect, we examined the degradation dynamics of an AR mutant that is severely deficient in DNA binding. The mutant AR demonstrated reduced transcriptional activity compared with the wild-type protein in a luciferase reporter assay (Fig. 9A), while its responsiveness to DHT was still present, consistent with an unaltered ligand binding domain but reduced DNA binding. When transfected into HEK-293 cells, which do not express endogenous AR, the DNA-binding AR mutant was degraded at the same rate as the wild-type AR when protein synthesis was blocked with cycloheximide (Fig. 9B). In the presence of DHT, both the wild-type and mutant AR protein levels increased between 24 and 48 hours, and when cycloheximide was added together with DHT, the degradation of the 2 proteins was similarly delayed at 24 hours. Thus, the inability of AR to bind to DNA did not affect the degradation dynamics of AR, and ligand binding still had the protective effect on AR protein stability.

Discussion

Androgens play an important role in the ovary, demonstrated by the negative effects of AR deletion in GCs on follicular growth and the reproductive lifespan in mice.

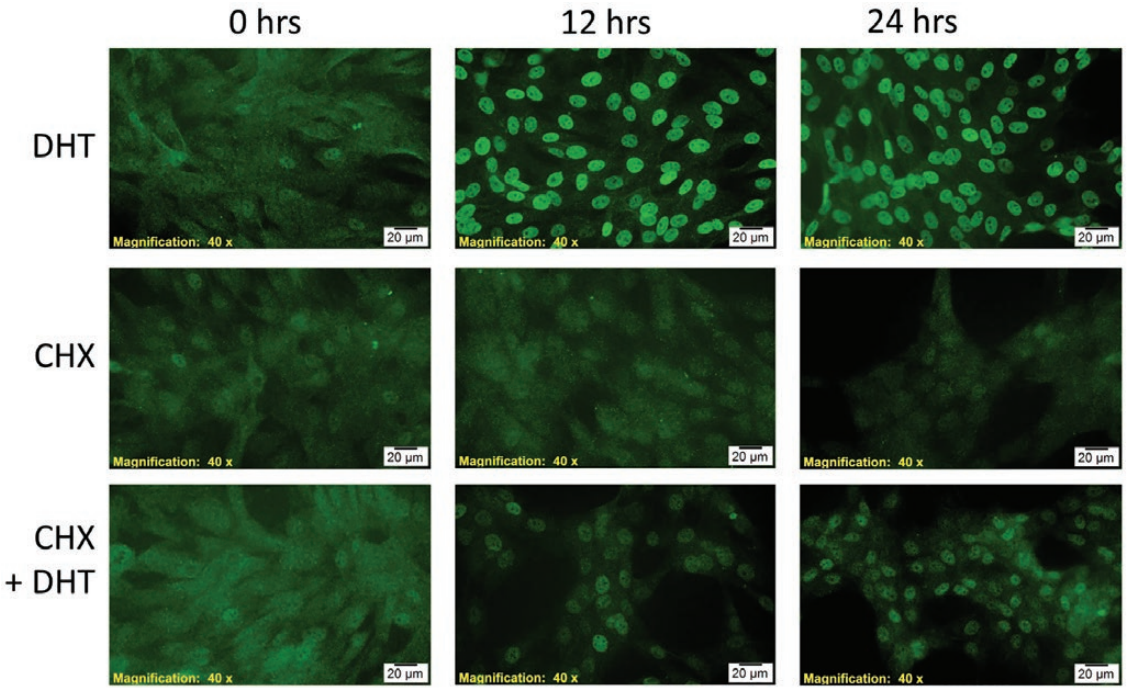

Figure 7. Immunofluorescence of androgen receptor (AR) expression in KGN cells treated with 25-nM dihydrotestosterone (DHT) and/or 50µM cycloheximide (CHX) for 12 to 24 hours. Primary antibody was diluted 1:200 and secondary 1:500. Exposure time was 500 ms for all samples under 40× magnification.

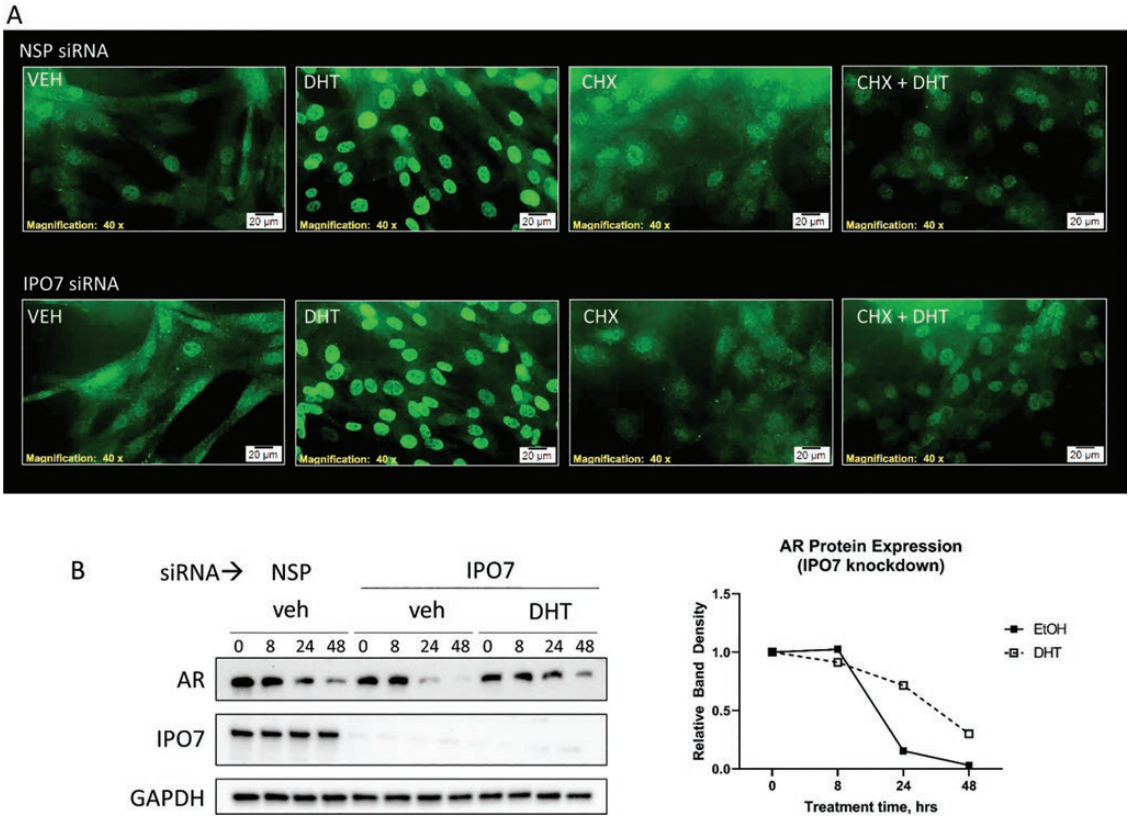

Figure 8. A, Immunofluorescence of androgen receptor (AR) expression in KGN cells treated with 25-nM dihydrotestosterone (DHT) and/or 50-µM cycloheximide (CHX) for 24 hours after transfection with small interfering RNA (siRNA) targeting importin 7 (IPO7) or nonspecific (NSP) siRNA. B, Western blots of AR and IPO7 expression and quantification of AR in KGN cells treated as indicated. Data are representative of 2 experiments. For Westerns, all conditions included treatment with cycloheximide.

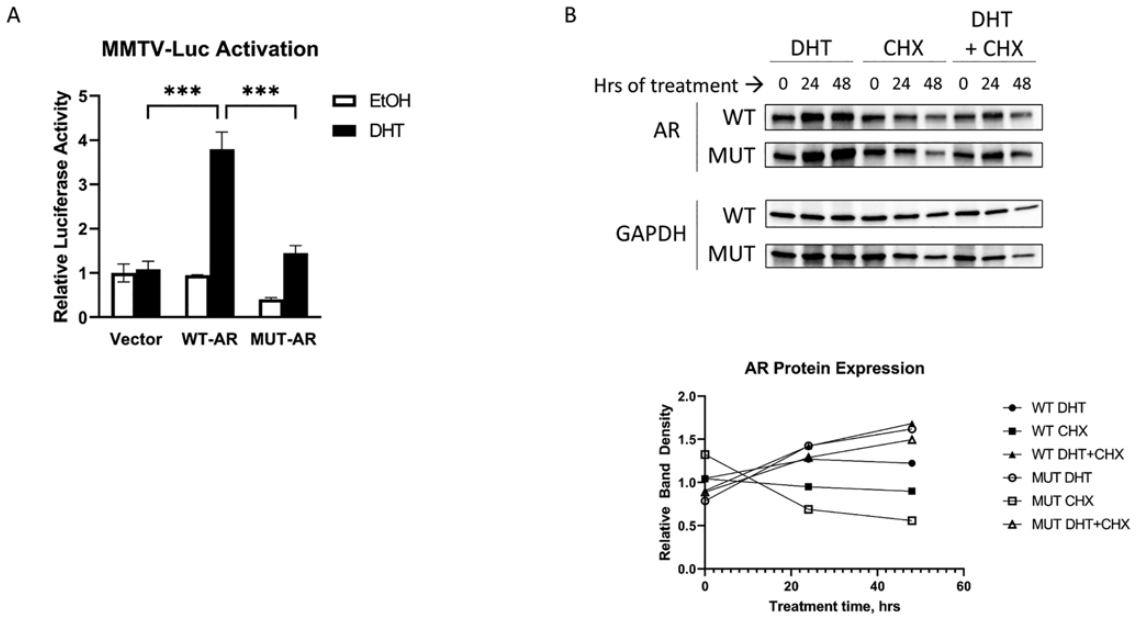

Figure 9. A, Luciferase reporter assay of DNA-binding mutant androgen receptor (AR) transcriptional activity. HEK-293 cells were transfected with the empty vector control, wild-type AR, or mutant AR plasmids, then treated for 18 hours with 25-nM dihydrotestosterone (DHT). B, Protein degradation study of the DNA-binding AR mutant. HEK-293 cells were transfected with wild-type (WT) or DNA-binding mutant (MUT) AR and treated with 25-nM DHT and/or 50-μM cycloheximide (CHX) for 24 hours, before Western blotting for AR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Data are representative of 2 experiments.

We therefore examined genomic and nongenomic effects of DHT in GCs. Surprisingly, both in mouse GCs and cultured human granulosa-derived KGN cells, the AR-regulated transcriptome is negligible to nil. No significant gene expression differences were found in GCs of mice 18 hours after intraperitoneal injection of DHT. The same treatment regimen produced significant induction of androgen-responsive genes in the male prostate, indicating that the hormone was capable of reaching target tissues at an effective concentration to induce transcriptional signaling. A very small number of genes were differentially expressed in DHT-treated KGN cells after paired-samples analysis, which eliminates experimental variation due to collecting and processing the samples on different dates. Without the pair factor, only 2 genes were differentially expressed in these samples. However, even after using the pair factor model, no gene was induced by more than 37% or downregulated by more than 13% after 12 hours of DHT treatment. Concerted small changes in the expression of functionally related genes can drive biological changes, so we performed gene set enrichment analysis on the genes differentially regulated by DHT. We found that only 8 DHT-induced genes fell into 2 statistically enriched categories (see Supplementary Table 1 [38]) of molecular function and signaling pathways, while there was no enrichment among biological processes or cellular components. These very small differences, while statistically significant after adjusting for multiple hypotheses testing, are unlikely to be of biological significance. Accordingly, while transfection of AREs driving luciferase expression in prostate cancer cells resulted in an approximately 300-fold increased luciferase production, only a 2-fold luciferase induction was detected when the same response element was expressed in KGN cells (see Fig. 1C). Thus, our studies in primary and cultured mouse and human GCs, respectively, suggest that androgen actions in GCs are not due to widespread dramatic changes in transcription. Our findings, although surprising, are supported by a conspicuous lack of any published studies to date specifically examining the direct androgen-regulated gene transcriptome in GCs. There are notable changes in gene expression in the ovaries of mice lacking ovarian AR [4], but no data confirming that any of those genes are direct transcriptional targets of AR, leaving the possibility that these changes are due to indirect effects of chronic loss of AR expression. It is possible that the lack of transcription is due to an absence of necessary transcription coregulators in GCs. In one study focusing on estrogen-regulated gene transcription in GCs, pharmacologic inhibition of NFκB with Bay-11-7082 appeared to unlock the transcriptional activity of ER in KGN cells. However, the same treatment had no effect on AR-mediated transcription in our experiments (Fig. 1C). In short, we undertook an exhaustive search for transcriptional targets of AR in GCs and found no convincing evidence that AR has significant gene-transcription activity in these cells. We infer from the lack of mRNA

changes that the actions of AR likely go through non-mRNA pathways, such as transcription of microRNAs and nongenomic signaling. Our laboratory has previously reported that micro-RNAs miR125b [25] and miR101 [30] are induced by androgens in GCs and mediate nongenomic effects of AR, including enhanced follicle-stimulating hormone receptor protein expression and the mRNA levels of *Runx1*, a factor involved in ovulation. We now believe that noncoding RNAs may be responsible for the majority of androgen and AR transcriptional actions in GCs. Importantly, both of the RNA-seq experiments in the present study were not designed to detect noncoding RNAs, but mRNA expression only. Total RNA-seq in androgen-stimulated GCs should be undertaken to discover other important noncoding targets of AR.

Having shown that androgen actions in GCs are not likely to be mediated by any direct transcriptional activity of AR, we examined nongenomic effects of DHT in GCs. Intriguingly, we found a significant amount of AR associated with the cell membrane in primary mouse GCs (see Supplementary Fig. 2B [38]). While membrane-associated AR has been extensively studied in prostate cancer cells by our laboratory and others, and is known to initiate important nongenomic signals through the mitogen-activated protein kinase cascade [26-29], this is the first report to our knowledge of plasma membrane AR localization in GCs. Moreover, DHT treatment resulted in diminished appearance of membrane AR, suggesting that membrane-associated AR may be responding to ligand binding.

While investigating any nongenomic effects of DHT in GCs, we found that AR half-life is dramatically extended in the presence of its ligand. This effect is apparent not only in mouse and human GCs, but also in cultured human prostate–derived C4-2 cell line, suggesting that it is a ubiquitous phenomenon. Prior studies in various prostate cancer cell lines have reported that AR protein levels are higher in the presence of DHT [41, 42]. These studies have shown a moderate, though not consistent, increase in AR protein in LNCaP cells treated with DHT, a dramatic AR protein increase in DHT-treated HeLa cells, and a significant AR protein increase in C4-2 cells after 24 hours of treatment with even picomolar concentrations of DHT [41]. Importantly, ours is the first study to specifically focus on potential mechanisms of AR protein stabilization by DHT, and is the first to report this effect in GCs, in which this effect is more dramatic than in most prostate cancer cells, and where, as we demonstrate in this manuscript and elsewhere [25, 30], nongenomic androgen effects may be more significant than direct transcriptional effects on mRNA expression. Several other mechanisms regulating AR gene expression have been reported, including differences in polyglutamine repeats within the N-terminal transcription activation region [43], Cdk1-dependent phosphorylation of AR at serine-81 [41], and upregulation and downregulation of the *AR* mRNA by androgens [42, 44, 45]. The effect of DHT on *AR* mRNA expression is cell specific. Some studies suggest that androgens may moderately suppress *AR* mRNA transcription in prostate cancer [44-46] but stabilize the mRNA molecule [42], whereas this effect is absent in breast cancer cells [42]. In our experiments, DHT did not affect the steady-state mRNA levels of *AR* in either GCs or human prostate cancer cells, with the exception of mouse prostate tissue, in which *AR* mRNA was significantly induced after in vivo treatment with DHT. Differences in the landscape of transcription coregulators in various tissues and across species likely account for these differences in the effect of DHT on *AR* mRNA. Importantly, inhibition of transcription had no significant effect on DHT-mediated enhancement of AR protein expression, indicating that this phenomenon is mainly posttranscriptional.

Consistent with prior studies showing that AR is degraded primarily by the proteasome [33, 47], inhibition of proteasome activity with bortezomib in our experiments approximately doubled AR half-life from approximately 7 to approximately 15 hours. It is possible that bortezomib did not fully block proteasome activity. However, the addition of DHT together with bortezomib further extended the half-life beyond 48 hours, suggesting that DHT action is additive to proteasome inhibition.

Previous studies identified several factors that may affect AR protein stability. Among these, we focused on PTEN and IPO7 and evaluated the role of these factors in DHT-induced AR protein accumulation. Most androgen-dependent prostate cancer cell lines are characterized by loss of PTEN [35], and restoration of PTEN activity can reduce androgen-stimulated proliferation of these cells [48]. Studies in LNCaP prostate cancer cells have suggested that PTEN can contribute to cytoplasmic retention of AR, making it more available for proteasomal degradation [33]. We confirmed that PTEN is not expressed in C4-2 cells (data not shown), therefore the observed effect of DHT is not through interaction with the PTEN pathway. However, PTEN is expressed in GCs, therefore we investigated whether PTEN plays a role in AR protein dynamics in GCs. Knockdown of PTEN expression in KGN cells did not have the expected effect of increasing AR protein half-life in our experiments, suggesting that PTEN is not necessary to maintain AR protein in GCs. Further, the effect of DHT was unchanged by loss of PTEN, confirming that DHT extends AR half-life through a PTEN-independent mechanism in GCs.

IPO7 has been shown to bind cytoplasmic unliganded AR and release it for nuclear import when ligand binding occurs [31]. In our experiments, AR localization to the

nucleus was unchanged even when the level of IPO7 was dramatically reduced both in GCs and prostate cells, pointing to likely redundancies in the AR nuclear import machinery. These findings contrast with a previous study showing that IPO7 plays an important role in cytoplasmic retention of unliganded AR, when ectopically expressed in Cos7 cells [31]. These discrepancies further emphasize the importance of cellular context when interpreting such findings, and the need for more study of AR in GCs specifically. Notably, knockdown of IPO7 also did not abrogate the effect of DHT on AR protein stability in our experiments. Further, DNA binding does not appear to be required for AR to be protected from degradation in the presence of its ligand (see Fig. 9), suggesting that DHT-induced nuclear retention of AR does not rely on its ability to bind DNA. In summary, we investigated several factors previously reported to regulate AR stability in various prostate cell types and found that DHT-induced AR protein stabilization is mostly independent of these pathways.

Our examination of AR localization showed that cytoplasmic AR is degraded even in the presence of DHT, whereas nuclear AR protein accumulates despite blocking protein synthesis. We therefore conclude that ligand-bound AR is sequestered from degradation in the cytoplasm by enhanced nuclear retention. This is distinctly opposite from the ER, which has a reduced half-life when bound by its ligand [34]. Unlike AR, ER is not recycled after nuclear import, and is destined for proteasomal degradation once it is ligand bound. In the ovarian microenvironment, where locally produced androgens and estrogens exert paracrine and autocrine signaling, this difference in how AR and ER respond to their ligands is likely to be of functional importance. Our findings suggest that even a transient exposure to androgens may have a lasting effect in GCs and likely in other androgen-responsive tissues, whereas continuous estrogenic stimulation may be needed to achieve its full effects.

In conclusion, we present the first detailed examination of AR protein stability and report that both in prostate and GCs, AR half-life is dramatically increased in the presence of its ligand, independently of many previously described pathways regulating AR. This effect is more pronounced in GCs than in prostate cells, and in both cell types it appears to rely on nuclear sequestration of AR. However, we also report that gene transcription by AR in GCs is probably negligible, compared with its robust gene regulation in prostate cells, and therefore the effects of androgens in GCs may be mediated in large part by nuclear AR-mediated changes in noncoding RNAs. We acknowledge that, while the mechanism described in this study is novel in GCs, the functional significance of this phenomenon remains unknown. Further, we investigated factors potentially contributing to this observation and ruled out a significant role of IPO7 or the necessity for AR to bind DNA, but the precise mechanism by which ligand binding leads to nuclear retention of AR remains unknown. More research is needed to illuminate the pathways through which androgens affect GCs, pertinent to disorders related to androgen imbalance in the ovary, such as polycystic ovary syndrome and diminished ovarian reserve.

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Disclosures: The authors have nothing to disclose.

Data Availability: All data generated or analyzed during this study are included in this published article or in the data repositories listed in "References."

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