# Testosterone-Dependent Interaction between Androgen Receptor and Aryl Hydrocarbon Receptor Induces Liver Receptor Homolog-1 Expression in Rat Granulosa Cells

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**SUMMARY**

Androgens play a major role in the regulation of normal ovarian function; however, they are also involved in the development of ovarian pathologies. These contrasting effects may involve a differential response of granulosa cells to the androgens, testosterone (T) and dihydrotestosterone (DHT). To determine the molecular pathways that mediate the distinct effects of T and DHT, we studied the expression of liver receptor homolog-1 (LRH-1), a gene differentially regulated by these steroids. We found that although both T and DHT stimulate androgen receptor (AR) binding to the LRH-1 promoter, DHT prevents T mediated stimulation of LRH-1 expression. T stimulated the expression of aryl hydrocarbon receptor (AHR) and its interaction with the AR. T also promoted the recruitment of the AR/AHR complex to the LRH-1 promoter. These effects were not mimicked by DHT. We also observed that the activation of extracellular regulated kinases by T is required for AR and AHR interaction. In summary, T, but not DHT, stimulates AHR expression and the interaction between AHR and AR leading to the stimulation of LRH-1 expression. These findings could explain the distinct response of granulosa cells to T and DHT and provide a molecular mechanism by which DHT negatively affects ovarian function.

## Introduction

Androgens are crucial for normal ovarian function and are involved in the development of ovarian pathologies (1-3). In primates (4, 5) and rodents (6-8), androgens promote the growth of small ovarian follicles via activation of the androgen receptors (AR) in the granulosa cells (3). In large antral follicles, androgens serve as substrates for aromatase, an enzyme that catalyzes the conversion of androgens to estrogens (9). Conversely, androgens increase follicular atresia (10, 11) and inhibit follicle stimulating hormone (FSH) induced granulosa cell proliferation (12, 13). Androgens are also thought to be responsible for the halt in follicle growth found in patients with polycystic ovarian syndrome (PCOS), a condition marked by hyperandrogenism (14). The mechanisms that control the balance between the beneficial and harmful effects of androgens in ovarian function remain unknown.

In rat granulosa cells, androgens greatly augment the stimulatory effect of FSH on estradiol production (15), aromatase expression (16), and tissue plasminogen activation production (17, 18). Notably, of the two classical androgens, testosterone (T) is more effective than dihydrotestosterone (DHT) in augmenting these actions of FSH (15, 16, 18). Differential effects of T and DHT have been reported for the regulation of the aromatase promoter in bovine granulosa cells (19) and, as we recently reported, for the expression of aromatase in rat granulosa cells (20). In addition, it is known that DHT, but not T, decreases ovulation (11), inhibits gonadotropin-stimulated steroidogenesis (21), and prevents the induction of luteinizing hormone (LH) receptor by FSH (22, 23). Moreover, DHT decreases cell cycle progression by inhibiting the expression of cyclin D2 (13) whereas T potentiates the stimulatory effect of FSH on cyclin D2 (24). Similarly, T treatment of rat granulosa cells stimulates the expression of the transcription factor liver receptor homolog-1 (LRH-1) but DHT treatment has no effect (20). The

molecular pathways that mediate the contrasting effects of T and DHT in granulosa cells remain to be investigated.

In this report, we examined the mechanisms involved in the differential response of granulosa cells to T and DHT using LRH-1 as the reporter gene. In the ovary, LRH-1 is expressed exclusively in granulosa cells of follicles at all stages of development (25, 26). We initially determined whether the AR is required for the stimulation of LRH-1 by T and the effect of T and DHT on the activation of the AR in granulosa cells. The differential effects of T and DHT on the expression of LRH-1 may be due to the recruitment of alternative cofactors by the AR (27). Therefore, we also tested the hypothesis that the recruitment of specific cofactors by the AR in the presence of T, but not in the presence of DHT, results in the activation and the expression of the LRH-1 gene.

## Materials and Methods

*Animals and granulosa cell culture* **-** Immature female Sprague Dawley rats were purchased (Charles River Laboratories Inc., Wilmington, MA) and housed in the Biological Resources Laboratory at the University of Illinois at Chicago. Animals were treated in accordance with the NIH Guide for Care and Use of Laboratory Animals, and all protocols were approved by the University of Illinois at Chicago Animal Care Committee. Primary granulosa cells were isolated from immature rats treated with E2 (1.5 mg/d for 3 d) and cultured for 96 hours as previously described (20). Culture media and treatments were renewed every 48 hours.

*RNA quantitation* – Cells were cultured in the presence of steroids for 96 hours prior to RNA isolation. Total RNA was isolated using TRIzol-Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Messenger RNA levels were quantified as previously

described (20) . The relative expression of target genes is expressed in reference to ribosomal L19 expression.

*Western blot analysis* - Cytosolic or nuclear extracts were isolated from primary rat granulosa cells as described previouly (20). Protein concentration was determined using BSA as the standard. The protein samples were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA) and processed by routine procedures. The primary antibodies, their species of origin, and the dilutions used were LRH-1 (rabbit, 1:500), Lamin B1 (rabbit, 1:500), β-actin (rabbit, 1:500), ARNT (rabbit,1:300), and AHR (mouse, 1:500) all from Abcam (Cambridge, MA), AR (rabbit, 1:500) from Millpore Copr (Billerica, MA), and MAPK44/42 (rabbit, 1:500) and phosphor-MAPK44/42 (rabbit, 1:500) from Cell Signaling Technology (Danvers, MA). The secondary antibodies used were anti-rabbit IgG-HRP (goat, 1:10,000) from Abcam, or anti-mouse IgG-HRP (goat, 1:10,000) from Jackson ImmunoResearch laboratory Inc. (West Grove, PA). Detection was performed with Immoblon western chemiluminescent horseradish peroxidase substrate (Millipore) or Supersignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL). Protein expression quantification was performed with ImageJ 1.45S software.

*RNA interference* - Short hairpin RNAs (shRNAs) under the control of the H1 promoter were used to specifically knockdown the expression of the AR, Ahr, Arnt, ERK1 and ERK2 genes. shRNA target recognition sequences used were shAR: ctg ctc cgc aga cat taa aga catc; shAhr: gga gct ctt ccc aga taat; shArnt: gga gct ctt agg aaa gaat; shERK1: aca gaa cat tcc taa atct; shERK2: gca gta tta tga ccc aagt; and shLUC (control): gcc tga agt ctc tga tta agt aca a. Oligonucleotides containing the target recognition sequence and its corresponding antisense sequence separated by a short spacer sequence were chemically synthesized (Integrated

DNA Technologies, Inc., Coralville, IA). These oligonucleotides were inserted into a lentivirus shRNA vector (Addgene, Inc., Cambridge, MA). Lentivirus stocks were generated in HEK293 cells (Invitrogen) cotransfected with the shRNA lentiviral vector along with the packaging and envelope plasmids psPAX2 and pMD2G (Addgene). Cell supernatants were concentrated by ultracentrifugation. Viral stocks were titrated in HEK293 cells aided by a GFP marker present in the shRNA plasmids. Viral stocks carrying shRNA were then added directly to the granulosa cells 2 hours after plating at a multiplicity of infection of 20, followed by an incubation for 96 hours in the presence of the treatments described in each figure. Culture media was changed 48 hours after plating but without the addition of virus.

*Plasmid and reporter constructs and cell transfection* - The promoter region -507 to +1 of the rat LRH-1 gene was cloned from genomic DNA using PCR and standard cloning techniques. PCR products were cloned into the pGL3 Basic luciferase reporter vector (Promega Corp., Madison, WI) by using *Xho*I and *Hind*III restriction sites. All cloning was confirmed by bidirectional sequencing. The region -507 to +1 was analyzed to predict transcription factor binding sites using online software (http://jaspar.cgb.ki.se and http://www.gene-regulation.com /pub/programs.html#match) as previously described (28). Reporter vectors with serial deletions of the 5' end of LRH-1 promoter were generated. These LRH-1 promoter luciferase reporter constructs were transfected into granulosa cells 48 h after plating using FugeneHD (Roche, Indianapolis, IN). 96 hours after the initiation of cultures, luciferase activity was assessed using the dual luciferase assay kit (Promega) and reported as relative luciferase activity (RLA). The AR response elements found at position -346 and -61 of the LRH-1 promoter were mutated using QuikChange II XL Site-Directed Mutagenesis (Agilent, Santa Clara, CA).

Expression plasmid encoding wild-type AHR or Y9F-AHR mutant were kindly provided by Dr. Gasiewicz and Dr. Bradfield. A FLAG sequence (gat tac aag gat gac gat gac aag) was fused to the 5' end of the WT-AHR and Y9F-AHR coding regions and subcloned into the pCDH vector (System Biosciences, Mountain View, CA). Viruses were produced in HEK293 cells as described for shRNAs. To confirm the expression and the DNA binding capacity of WT-AHR and Y9F-AHR, both proteins were overexpressed in HEK293 cells and the protein extracts were used to perform EMSA and Western blot.

*EMSAs* - Nuclear protein extracts from approximately $6x10^6$ granulosa cells cultured in 100-mm plates in the absence or presence of steroids were prepared as described previously (20). Double-stranded oligonucleotide probes containing the ARE346 or ARE61 labeled $\gamma^{32}$P-ATP was used. For competition experiments, increasing concentrations of unlabeled ARE oligonucleotide probes were used. For supershift experiments, 2 µl of the AR antibody (rabbit, PG21; Millipore Corp.) or 2 µl of a normal rabbit serum were added to the binding reaction 20 min before the addition of the labeled probe.

*Chromatin Immunoprecipitation (ChIP) assay* - Granulosa cells ($10x10^6$) were cultured in 100mm-plates and treated as indicated in the figure legend. ChIP assays were performed using the MAGNA ChIP kit (Millipore Corp) according to the manufacturer's protocol. For each 100-mm plate, immunoprecipitations were carried out using 6 µg of anti-AR antibody, anti-Ahr antibody or IgG. Immunoprecipitated DNA (ChIP DNA) and input DNA were amplified by PCR using primers spanning the ARE346 or ARE61 regions found in the LRH-1 promoter.

*Co-Immunoprecipitation assays* - Co-IP experiments were performed on whole-cell protein extracts obtained from $10x10^6$ rat granulosa cells cultured in 100mm-plates using Pierce Crosslink Immunoprecipitation Kit (Thermo Scientific, Cat. 26147) according to the

manufacturer's instructions. Antibodies used for IP were anti-AR, anti-AHR, normal rabbit serum, or normal mouse serum (IgG). The precipitated protein complexes were then used for Western blot analysis as described above.

*Nuclear Receptor Array* - PCR arrays were performed using the Rat Nuclear Receptors & Coregulators RT² Profiler™ PCR Array (cat. # PARN-056, Qiagen, Germantown, MD), which profiles the expression of 84 genes encoding nuclear receptors and their coregulators. The array was performed according to the manufacturer's protocol. Briefly, total RNA was isolated from granulosa cells treated with vehicle, T or DHT as described above, and cDNA was generated using the RT2 First Strand Kit included in the PCR Array kit; 5ug of RNA was used in each RT reaction. cDNA was loaded in the plate provided in the array kit and amplified using real-time PCR. The CT values were analyzed by PCR Array Data Analysis Web-based software (Qiagen).

*Statistical Analysis* - One-way or two-way analysis of variance (ANOVA) was used for the statistical analysis of data using the Prism software (Graph Pad Software, Inc., San Diego, CA). The tests used are indicated in the figure legends. Values were considered statistically significant at $p<0.05$.

## Results

***Testosterone-specific stimulation of LRH-1 in granulosa cells*** – Treatment of granulosa cells with increasing concentrations of T induced LRH-1 expression in a concentration dependent manner (Fig 1A). T also increased LRH-1 protein levels by sixfold (Fig.1B) and stimulated LRH-1 promoter activity by twofold (Fig.1C). In contrast, DHT had no effect on LRH-1 expression, protein levels or promoter activity.

Granulosa cells convert T into either estradiol (E2) or DHT by action of aromatase and 5α-reductase, respectively (25). To determine whether T effects are mediated by its conversion into E2 or DHT, we treated cells with E2, DHT or a combination of the two. The results suggested that E2 and DHT, alone or combined, had no effect on LRH-1 expression (Fig. 1D). To examine if the conversion of T to DHT or E2 affects LRH-1 expression, we treated cells with T in the presence of the 5α-reductase inhibitor, finasteride (5αRI) or the aromatase inhibitor, formestane (AroI). Remarkably, finasteride enhanced the stimulatory effect of T on LRH-1 expression by 57 percent; in contrast, formestane had no effect (Fig. 1E). We previously demonstrated that in granulosa cells finasteride and formestane block DHT and E2 synthesis, respectively (20). These results confirm the specific effect of T on LRH-1 expression and suggest that 5α-reductase activity may decrease LRH-1 expression by facilitating the conversion of T to DHT.

***The androgen receptor (AR) mediates T stimulation of LRH-1 expression -*** To examine if the AR mediates the stimulation of LRH-1 expression by T, granulosa cells were treated with hydroflutamine (HOF) or bicalutamide (CDX), two AR antagonists. We observed that both compounds prevented the stimulation of LRH-1 by T (Fig. 2A). To confirm this finding, AR expression was silenced using a virus carrying small hairpin RNA (shRNA) against the AR. We observed that the stimulation of LRH-1 gene expression (Fig. 2B) and promoter activity (Fig. 2C) in response to T was prevented by exposing granulosa cells to a virus carrying anti-AR shRNAs. These results suggest that the AR is required for T stimulation of LRH-1 expression.

After we established that the AR mediates the effects of T on LRH-1 expression, we examined whether the differential effects of T and DHT on LRH-1 may correlate with differences in AR expression or subcellular localization. We observed that treatment with T or

DHT increased the levels of AR in the nucleus, whereas the amount of AR protein in total cell lysates was not affected (Fig. 3A). These findings indicate that both T and DHT promote the translocation of the AR to the nucleus without altering AR protein levels.

Next, we examined if T and DHT regulate the transcriptional activity of the AR. For this purpose, a reporter construct consisting of three tandem repeats of the consensus AR response element (ARE) “GGTACAnnnTGTTCT” upstream of the cFos minimal promoter and the luciferase coding sequence (3xARE-Luc) was used. Granulosa cells were transfected with 3xARE-Luc and incubated in the presence of T or DHT. The results indicated that both T and DHT increased 3xARE-Luc activity (Fig. 3B), which suggests that both androgens can stimulate the transcriptional activity of the AR. We also examined the effect of DHT on the expression of Kit ligand (kitL), the gene for which is highly expressed in the ovary of DHT-treated mice (1). The results showed that DHT stimulates KitL expression by sixfold, despite the fact that DHT is unable to stimulate LRH-1 expression (Fig. 3C). Testosterone treatment also stimulated KitL expression but to a lesser extent (threefold) than DHT. These results suggest that both DHT and T have the potential to activate the AR and stimulate KitL expression in granulosa cells however, only T increases LRH-1 expression.

***DHT inhibits testosterone stimulation of LRH-1*** – Knowing that inhibiting of the conversion of T into DHT potentiates the effect of T (Fig. 1) and that both T and DHT stimulate AR nuclear localization and transcriptional activity (Fig. 3), we explored the possibility that DHT may compete with T for binding to the AR and prevent LRH-1 expression. For this purpose, granulosa cells were incubated in the presence of 100 nM of T and increasing concentrations (1-100 nM) of DHT. The results showed that DHT inhibited T stimulation of LRH-1 mRNA expression in a concentration-dependent manner where 50 nM of DHT prevented the

stimulation of LRH-1 expression by 100 nM of T (Fig. 4A). Co-treatment with T and R1881, a non-metabolizable AR agonist, also resulted in the inhibition of T stimulation of LRH-1 (Fig. 4B). Noteworthy, the inhibitory effect of DHT on T-induced LRH-1 expression was markedly enhanced in the presence of aromatase and 5α-reductase inhibitors (Fig. 4C); however, both steroids synergized on the stimulation of 3xARE-Luc activity (Fig. 4D) and on the expression of KitL (data not shown). These findings suggest that DHT is able to displace T from the AR preventing the stimulation of LRH-1 expression.

***The LRH-1 promoter contains an androgen response element that responds selectively to testosterone -*** To determine the molecular mechanisms that mediate the effects of T on LRH-1 expression, the LRH-1 promoter region was analyzed to identify potential AR response elements (ARE). Two AREs were found within the promoter at 346 (ARE346) and 61 (ARE61) base pairs upstream of the transcription start site (29). To test the functionality of these elements, luciferase reporter constructs containing serial deletions of the LRH-1 promoter (LRH-1Luc) were made. T stimulated the activity of the reporter constructs containing the ARE346 element (507LRH-1Luc and 348LRH-1Luc), whereas the activity of the constructs lacking this element (266LRH-1Luc, -172LRH-1Luc and the empty pGL3 plasmid) was not affected by treatment with T. As expected, DHT had no effect on the activity of any of the reporter constructs tested (Fig. 5A). To confirm the participation of the ARE346 element in the regulation of LRH-1 by T, we created reporter constructs carrying mutations at ARE346, ARE61, or both sites. The mutation of ARE346 or both ARE346 and ARE61 eliminated T stimulation of LRH-1; whereas mutation of ARE61 alone had no effects (Fig. 5B). These findings suggest that the AR response element found at position -346 of the LRH-1 promoter may mediate the increase in LRH-1 expression induced by T in granulosa cells.

***Testosterone and DHT stimulate AR binding to the LRH-1 promoter -*** To determine whether the AR binds to the LRH-1 promoter, we performed electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) analyses. Nuclear extracts of T or DHT treated granulosa cells formed a shift band in the presence of a radiolabeled oligonucleotide probe containing the sequence of ARE346 (Fig. 6A). The formation of this band was prevented by the addition of an excess of unlabeled probe. The addition of an anti-AR antibody to the binding reaction caused the formation of a supershift band. In contrast, no shift bands were observed using a probe containing the sequence of ARE61 (data not shown).

ChIP performed in T or DHT treated granulosa cells revealed that there is an enrichment of the region containing the ARE346 element after chromatin precipitation using an anti-AR antibody (Fig. 6B). AR binding to the region containing ARE61 was not observed. Quantification of ChIP analyses indicated that both T and DHT increase recruitment of the AR to the LRH-1 promoter by greater than 3½-fold (Fig. 6B). These findings suggest that in the presence of T or DHT the AR is recruited to the promoter of the LRH-1 gene at or close to the ARE found at position -346.

***Cofactors differentially regulated by T and DHT in granulosa cells –*** The finding that both T and DHT stimulate AR recruitment to the LRH-1 promoter suggest that the ability of the AR to stimulate LRH-1 expression in the presence of T but not in the presence of DHT may rest on the recruitment of different cofactors in response to the two treatments. To address this possibility, a PCR array of nuclear receptors and co-regulators was carried out. This analysis revealed that T and DHT differentially regulate the expression of several transcription factors and nuclear receptors (Table 1). Of these factors, the expression of aryl hydrocarbon receptor (AHR) and nuclear receptor 1D1 were stimulated by T; whereas, nuclear receptor 1H3

expression increased in T-treated cells but decreased in DHT-treated cells. The expression of AHR nuclear translocator (ARNT), estrogen receptors 1 and 2, K(lysine)-acetyltransferase 5, nuclear receptors Nr0b2, Nr1d2, and Nr1i2, and nuclear coactivator 6 were decreased by DHT but not affected by T.

AHR and ARNT are known to interact with the AR (30); therefore, we evaluated the effect of T and DHT on their expression using real-time PCR and Western blotting. Confirming the PCR-array results, T stimulated the expression of *Ahr* mRNA by fourfold and *Arnt* mRNA levels by threefold whereas DHT had no effect (Fig. 7A). In addition, T stimulated the expression of AHR and ARNT protein levels but DHT had no effect (Fig. 7B). These findings suggest that T and DHT differently regulate the expression of AHR and ARNT, two factors known to interact with and regulate AR activity (30).

Next, we examined if AHR activity is necessary for the upregulation of LRH-1 expression in response to T by using two AHR antagonists, α-naphthoflavone (Naph) and 6,2,4-trimethoxyflavone (TMF). We observed that T-induced LRH-1 expression was prevented in the presence of the inhibitors. The inhibitors had no effects on the stimulation of *Ahr* or *Arnt* expression by T (Fig. 7C). To confirm the effect of the AHR inhibitors and to determine the role of ARNT on the stimulation of LRH-1 expression, we used shRNA to decrease the expression of *Ahr* or *Arnt*. The results revealed that T is unable to stimulate LRH-1 expression in granulosa cells with reduced expression of either AHR or ARNT (Fig. 7D). Taken together these findings suggest that AHR and ARNT are both required for T induction of LRH-1 expression.

***The AR interacts with AHR and ARNT in a testosterone-dependent manner*** – The finding that lack of AHR abolished the effect of T on LRH-1 expression prompted us to investigate if

AHR interacts with AR in granulosa cells and to examine if this interaction is regulated by T. For this purpose, we immunoprecipitated lysates from granulosa cells treated with vehicle, T, or DHT using anti-AR or anti-AHR antibodies. Immunoprecipitates were analyzed by Western blotting using anti-AR, anti-AHR, or anti-ARNT antibodies. The results showed that the anti-AR antibody pulled down AHR and ARNT only in cells treated with T (Fig. 8A). Similarly, AR and ARNT were immunoprecipitated together with AHR in lysates of T-treated cells but not in vehicle or DHT-treated cells (Fig. 8B). These results suggest that T not only increases the expression of AHR but also stimulates the interaction between AR and AHR.

Knowing that AR binds to the LRH-1 promoter and that AR and AHR interact in a T-dependent manner, we sought to determine whether AHR is recruited to the LRH-1 promoter in an AR-dependent manner. ChIP analysis of the LRH-1 promoter indicated that only chromatin from T-treated cells immnunoprecitpitated with the anti-AHR antibody showed an enrichment of the LRH-1 promoter region containing the ARE346 element (Fig. 8C). To determine if the AR is required for AHR recruitment to the LRH-1 promoter, the same analysis was performed in cells transfected with control shRNA (shLuc) or with anti-AR shRNA (shAR). The results showed that enrichment of the LRH-1 promoter by the anti-AHR antibody was reduced in granulosa cells expressing lower levels of AR (Fig. 8C $4^{th}$ and $5^{th}$ columns). Quantification of these findings using real time PCR indicated that silencing of the AR caused a 90 percent decrease of the enrichment of AHR recruitment to the LRH-1 promoter when compared with shLuc transfected cells. These findings suggest that AHR is recruited to the LRH-1 promoter in the presence of T in an AR-dependent manner, further suggesting that AHR could function as an AR-cofactor in the regulation of LRH-1 expression.

Because the LRH-1 promoter lacks AHR/dioxin response elements (DRE; data not shown) and the mutation of the ARE346 response element is enough to completely prevent LRH-1 promoter activation by T (Fig 5B), the role of AHR on LRH-1 expression seems to occur in the absence of AHR interaction with DNA. To investigate this possibility, we overexpressed a wild-type (WT) or a DNA-binding mutant AHR in granulosa cells. Mutation of tyrosine 9 to phenylalanine (Y9F-AHR) in AHR abolishes the binding of this protein to all DRE and abrogates DRE-driven gene induction mediated by the AHR (31). We observed that both WT-AHR and Y9F-AHR were able to synergize with T to stimulate LRH-1 expression (Fig. 8D). Western blot and EMSA analysis confirmed the expression of both proteins and the ability of WT-AHR, but not Y9F-AHR, to bind DNA (Fig. 8D left panels). These findings suggest that AHR acts mainly as a cofactor of the AR and that the effects of AHR on LRH-1 expression are unlikely to be mediated by its interaction with DNA.

***ERK1/2 is needed for the stimulation of AHR and ARNT by testosterone*** – Extracellular regulated kinases (ERK) affect the transactivation potential of the AHR (32, 33); therefore, we examined if T regulates the activity of these kinases in granulosa cells. The results showed that T stimulates ERK1 and ERK2 phosphorylation; in contrast, DHT treatment had no effect. Moreover, inhibition of 5α-reductase and aromatase enhanced the stimulatory effect of T on ERK phosphorylation (Fig. 9A).

Next, we examined if ERK1/2 activity is required for the induction of LRH-1 expression by T. Because the two most commonly used inhibitors of ERK1/2 activation, U0126 and PD98059, are also potent antagonists of AHR (34-36), we examined if ERK1/2 activity is needed for the stimulation of LRH-1 by down-regulating the expression of these kinases using shRNA. We observed that the induction of LRH-1 expression by T was abolished in the presence of anti-

ERK1 and anti-ERK2 shRNAs (Fig. 9B). Moreover, we found that in cells transfected with anti-ERK1 and anti-ERK2 shRNAs the interaction between AR and AHR was abolished (Fig. 9C). These results suggest that normal expression levels of ERK1/2 are required for T to induce the interaction between AR and AHR and the expression of LRH-1 in ovarian granulosa cells.

## Discussion

Our findings reveal a unique mechanism by which T and DHT may elicit differential responses in granulosa cells. The results suggest that T increases LRH-1 expression in an AR-dependent manner and stimulates, through the activation of ERK1/2, the interaction of between AR and AHR. The AR/AHR complex is in turn recruited to the promoter of the LRH-1 gene leading to increased expression of this transcription factor. In contrast, DHT has no effect on AHR expression, ERK1/2 activation, or LRH-1 expression (Fig. 10).

As the two major physiological androgens, T and DHT bind to the AR with high affinity (7, 15). This is supported by crystallographic studies showing nearly identical interactions of T and DHT with the AR ligand-binding pocket (15). However, the effects of T and DHT on gene expression are not always equivalent, as they differentially regulate the expression of a subset of AR target genes in the prostate (37-39) and the ovary (5, 15, 20, 40). The mechanisms of the differential response of ovarian granulosa cells to T or DHT have not been previously examined. Our results suggest that the negative effects of DHT on ovarian function may be explained by the displacement of T from the AR and the consequent decrease in the regulation of a specific set of ovarian genes.

Our findings suggest that in granulosa cells the AR uses AHR and ARNT as cofactors. AHR binds with high-affinity to a wide variety of hydrophobic environmental contaminants, such as dioxins (41). ARNT is an AHR interacting protein found in the nucleus that facilitates AHR regulation of gene expression (42). Both AHR and ARNT are expressed in granulosa cells of several species (43, 44). AHR knockout mice are subfertile due to folliculogenesis defects, impaired aromatase expression (45), and slow growth of early antral follicles (46). Follicle growth is also impaired in the absence of AR expression in granulosa cells (3). Because estradiol restores follicle growth in AHR-null animals (47) and T stimulates aromatase expression (20), the interaction between AR and AHR may support folliculogenesis by increasing estradiol synthesis and maintaining normal follicle growth.

Cofactor recruitment is a crucial regulatory step in AR signal transduction and function (48, 49). The interaction between AHR and AR modulates the transcriptional activity of both proteins (50); however, the factors/signals that regulate the interaction between AHR and AR remain unclear. Our findings suggest that, at least in granulosa cells, AHR and AR association takes place only in the presence of T and requires the activation of ERK1/2. T increases ERK1/2 activation not only in granulosa cells, as shown here, but also in Sertoli cells (51). The effectiveness of the different androgens on ERK1/2 activation has not previously been examined. Our results indicate that ERK1/2 activation occurs in granulosa cell cultured in the presence of T but not DHT. Similar findings have been described in Xenopus oocytes (29). In addition, T is also able to stimulate AHR expression in granulosa cells suggesting that it has a dual effect on the AR and AHR interaction. We propose that T regulates a set of specific genes needed for normal follicle growth by controlling the expression of AHR and its interaction with the AR.

The detrimental effect of DHT in ovarian gene expression may play a key role in the development of ovarian diseases associated with high levels of androgens such as PCOS. PCOS patients have increased 5α-reductase activity as evidenced by higher conversion rates of oral dehydroepiandrosterone into DHT (52), an augmented level of 5α-reduced steroids in urine, serum, and follicular fluid (53, 54), and high 5α-reductase activity in peripheral tissues (52) and granulosa cells (55). Moreover, the incidence of PCOS decreases in people carrying inactivating mutations of 5α-reductase (56, 57). These findings indicate that high levels of 5α-steroids, including DHT, may contribute to the pathophysiology of PCOS by preventing T actions in granulosa cells leading to a halt in follicle maturation.

In summary, we have found that T stimulation of AHR expression and AHR interaction with AR leads to the recruitment of both AR and AHR to the promoter region of the LRH-1 gene resulting in an increase in LRH-1 expression. Our findings can be used to explain the differential response of granulosa cells to T and DHT and provide a molecular mechanism by which DHT may negatively affect ovarian function.

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**Table 1: Cofactors differentially regulated by T and DHT in Ovarian Granulosa cells.** Cells were cultured in the presence of vehicle (control), T, or DHT for 96 hours prior to the determination of gene expression using a nuclear receptors & coregulators PCR array as described in the materials and methods section. The results are expressed as the ratio between control and T (T column), control and DHT (DHT column), or T and DHT (T/DHT column).

| *Gene* | *T* | *DHT* | *T/DHT* | *Accession Number* | *Name* |
|---|---|---|---|---|---|
| **Ahr** | **2.2** | **1.0** | **2.1** | NM_013149 | **Aryl hydrocarbon receptor** |
| **Arnt** | **1.6** | **0.1** | **21.5** | NM_012780 | **Aryl hydrocarbon receptor nuclear translocator** |
| **Esr1** | **0.6** | **0.4** | **1.7** | NM_012689 | **Estrogen receptor 1** |
| **Esr2** | **0.7** | **0.0** | **72.0** | NM_012754 | **Estrogen receptor 2** |
| **Kat5** | **1.5** | **0.5** | **3.3** | NM_001005872 | **K(lysine) acetyl transferase 5** |
| **Ncoa6** | **1.1** | **0.3** | **3.2** | XM_342552 | **Nuclear receptor coactivator 6** |
| **Nr0b2** | **1.4** | **0.3** | **5.4** | NM_057133 | **Nuclear receptor subfamily 0, group B, member 2** |
| **Nr1d1** | **2.3** | **0.8** | **3.1** | NM_145775 | **Nuclear receptor subfamily 1, group D, member 1** |
| **Nr1d2** | **1.1** | **0.2** | **5.5** | NM_147210 | **Nuclear receptor subfamily 1, group D, member 2** |
| **Nr1h3** | **3.1** | **0.0** | **233.4** | NM_031627 | **Nuclear receptor subfamily 1, group H, member 3** |
| **Nr1i2** | **1.7** | **0.4** | **5.0** | NM_052980 | **Nuclear receptor subfamily 1, group I, member 2** |

**FIGURE LEGENDS**

**Figure 1**. **Testosterone but not dihydrotestosterone stimulates LRH-1 expression.**

**A**: LRH-1 mRNA expression in granulosa cells cultured for 96h in the presence of increasing concentrations of T or DHT ranging from 20 to 500 nM. **B**: LRH-1 and Lamin B (LAMB, loading control) protein expression in granulosa cells treated with vehicle (C), T (100 nM), or DHT (100 nM) for 96h. Top, representative Western blot, bottom quantification of three blots. **C**: LRH-1 promoter activity in cells treated with vehicle, T or DHT. RLA: relative luciferase activity. **D**: LRH-1 expression in cells incubated in the presence of 100 nM of T, DHT, E2 or DHT plus E2. **E**: LRH-1 expression in cells treated with vehicle (C) or T in the presence or absence of the 5α-reductase inhibitor finasteride (5αRI, 5 μM) or the aromatase inhibitor formestane (AroI, 5 μM). All experiments were repeated at least four times and the results are shown as the mean ± SEM. In A: ****p<0.001 and **p< 0.01 vs control (0 nM) (two- way ANOVA, Bonferroni test); In B, C, D and E: ***p<0.001, **p< 0.01, *P< 0.05 vs Control (C) (one- way ANOVA, Tukey test).*

**Figure 2**. **The androgen receptor mediates the stimulation of LRH-1 expression by T.**

**A**: LRH-1 expression in cells incubated in the presence of T and AR antagonists, hydroflutamine (HOF) or bicalutamide (CDX) both at 5 μM. **B**: LRH-1 expression and C: promoter activity in cells exposed to virus carrying shRNA anti-AR (shAR) or control (shLuc). Bars represent mean ± SEM n=3. ****p<0.001, **p<0.01, *p<0.05 vs Control (one-way ANOVA, Tukey test).*

**Figure 3**. **Testosterone and DHT stimulate androgen receptor nuclear translocation.**

**A**: Androgen receptor (AR) expression in the nuclear fraction or total cell lysates of granulosa cells treated with T or DHT. Lamin B (LAMB) was used as loading control. A representative blot is shown; the graphs on top represent the mean ± SEM of three different experiments. **B**: Activity of the AR reporter 3xARE-Luc in cells incubated in the presence of vehicle (C), T or DHT. **C**: KitL expression in cells cultured in the presence of T or DHT. Experiments were repeated at least three times. ***p<0.01, *p<0.05 vs Control (one-way ANOVA, Tukey test)*

**Figure 4**. **DHT or R1881 inhibit Testosterone stimulation of LRH-1 expression.**

**A and B**: LRH-1 expression in the presence of T (100 nM) and increasing concentrations of DHT (1-100nM) or R1881, an AR agonist (0.1-1μM). **C**: LRH-1 expression in cells treated with vehicle (C), T or T plus DHT in the presence of 5αRI (5 μM) and AroI (5 μM). **D**: 3xARE reporter activity in cells treated with vehicle (C), T, or T plus DHT in the presence of 5αRI and AroI. All experiments were repeated at least three times and the results are shown as the mean ± SEM *(***p<0.001, **p<0.01, p<0.05 vs Control; one-way ANOVA, Tukey test).*

**Figure 5**. **Testosterone stimulates LRH-1 promoter activity.**

**A**: Activity of luciferase reporters carrying serial deletions of the LRH-1 promoter in the presence of T or DHT. Empty pGL3 vector was used as control. **B**: Effect of T or DHT on the activity of the wild type (WT) 348bp LRH-1luc reporter or the mutants ARE346 (346mut), ARE61 (61mut) or both (346/61mut). All experiments were repeated at least four times and the

results are shown as the mean ± SEM *(for each construct ***p<0.005, **p< 0.01, *p< 0.05 vs Control; one-way ANOVA, Tukey Test).*

**Figure 6**. **Testosterone induces AR recruitment to the LRH-1 promoter.**

**A**: Left panel: EMSA performed using nuclear extracts from granulosa cells cultured with vehicle (C), T, Δ4A or DHT and a probe containing the ARE sequence found at position -346 of LRH-1 promoter. Middle panel: EMSA carried out in the presence of increasing concentrations of unlabeled probe. Right panel: Supershift analysis performed by adding normal antiserum (NS) or anti-AR antibody (αAR) to the EMSA reaction. **B**: Top: Primers used to amplify the region surrounding ARE346 or ARE61. Middle: Representative gel of PCR product resulting from the amplification of total (input) or anti-AR immunoprecipitated (αAR IP) chromatin obtained from cells cultured in the presence of vehicle (C), T, or DHT. Bottom, αAR IP/input ratio after real-time PCR quantification of total or αAR IP DNA. These experiments were repeated five times. In A, a representative gel is shown. In B, bars represent the mean ± SEM *(**p< 0.01 vs Control (C); one-way ANOVA,Tukey test).*

**Figure 7. Testosterone induces aryl hydrocarbon receptor (AHR) and AHR nuclear translocator (ARNT) expression.**

**A**: AHR and ARNT mRNA expression in granulosa cells treated with vehicle (C), T or DHT. **B**: Representative blot showing AHR and ARNT protein levels in cells incubated in the presence of vehicle (C), T or DHT. β-actin (BACT) was used as loading control. Graphs show the mean ± SEM of three different experiments. **C**: LRH-1, AHR, or ARNT expression in cells cultured in the presence of vehicle (C) or T plus the addition of the AHR antagonists: α-naphthoflavone (NAF) (10μM) or 6,2′,4′-trimethoxyflavone (TMF) (10nM). **D**: LRH-1, AHR, or ARNT

expression in granulosa cells infected with virus carrying control shRNA (shLuc), anti-AHR shRNA (shAhr), or anti-ARNT shRNA (shArnt). All the experiments were repeated at least four times and the results are shown as the mean ± SEM *(*p< 0.05 vs. Control and DHT; Columns with different letters differ significantly: a-b, p<0.05, a-c and b-c p<0.01, (one-way ANOVA, Tukey test).*

**Figure 8**. **Testosterone induces AR-AHR-ARNT complex formation.**

**A**: AHR, ARNT, and AR expression in immunoprecipitated (IP) fractions obtained using an anti-AR antibody (αAR) or normal rabbit serum (IgG) and total lysate of cells treated with vehicle (C), T or DHT. **B**: AR, ARNT and AHR expression in immunoprecipitated (IP) fractions obtained using an anti-AHR antibody (αAHR) or normal mouse serum (IgG) and total lysate of cells treated with vehicle (C), T or DHT. **C**: LRH-1 promoter enrichment in genomic DNA immunoprecipitated using an anti-AHR antibody (αAhr). DNA was obtained from cells cultured in the presence of vehicle (C), T, DHT T plus shLuc, or T plus shAR. The graph represents the mean ± SEM of three different experiments. *(*p<0.05 vs Control; one-way ANOVA, Tukey test).* **D**: LRH-1 expression in granulosa cells infected with an empty virus or viruses carrying DNA encoding for wild-type AHR (WT) or Y9F-AHR (Mut). EMSA was performed using total lysate of HEK293 cells transfected with empty, wild-type AHR (WT), or Y9F-AHR (Mut) expression vectors and a DRE probe. WT* indicates a reaction in which binding was competed with an excess of unlabeled probe. GC: nuclear extract of granulosa cells was used as positive control for AHR binding. WB: Western blot against the FLAG epitope on total lysate obtained from HEK293 cells transfected with wild-type AHR (WT), or Y9F-AHR (Mut) expression vectors. All

experiments were repeated at least three times. The graph represents the mean ± SEM of three different experiments. *(*p<0.05 vs Control; one-way ANOVA, Tukey test).*

**Figure 9**. **ERK1 and ERK2 are required for the interaction of AHR and AR and for LRH-1 expression induced by testosterone.**

**A**: Top, Representative blot showing phosphorylated ERK1/2 (P-ERK1/2) and total ERK1/2 (t-ERK1/2) in granulosa cells cultured with vehicle (C), T or DHT in the presence or absence of 5αRI or AroI. Graphs show the mean ± SEM of the ratio P-ERK/t-ERK from three different experiments *(*p<0.05 vs. Control; one-way ANOVA, Tukey test).* **B**: LRH-1 expression in cells cultured in the presence of vehicle (C) or T infected with virus carrying control shRNA (shLUC), or shRNAs against ERK1 and ERK2 (shERK1/2). Bars represent the mean ± SEM, n=4 *(**p<0.01 vs Control, two-way ANOVA, Tukey test).* **C**: AHR and AR expression in immunoprecipitated (IP) fractions obtained using an anti-AR antibody (αAR) and total lysate of cells treated with vehicle (C) or T in the presence or absence of virus carrying control shRNA (shLuc) or shRNAs against ERK1 and ERK2 (shERK1/2). The blots are representative results of three different experiments.

**Figure 10**. **Potential mechanism of LRH-1 up-regulation by testosterone in rat granulosa cells.** Both T and DHT induce the binding of the AR to the promoter region of the LRH-1 gene. T specifically stimulates the expression of AHR and its association with the AR. The interaction between AHR and AR requires the activation of ERK1/2 by T. In contrast, DHT does not stimulate AHR or ERK1/2 activity; consequently, it has no effect on the expression of LRH-1. However, DHT stimulates Kit Ligand (KitL) expression more efficiently than T. Solid lines

indicate stimulation. Dashed lines indicate no effect. The thickness of the lines represent the extent of the effect.