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Research Article

Androgens downregulate anti-Müllerian hormone promoter activity in the Sertoli cell through the androgen receptor and intact steroidogenic factor 1 sites†

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Abstract

Testicular anti-Müllerian hormone (AMH) production is inhibited by androgens around pubertal onset, as observed under normal physiological conditions and in patients with precocious puberty. In agreement, AMH downregulation is absent in patients with androgen insensitivity. The molecular mechanisms underlying the negative regulation of AMH by androgens remain unknown. Our aim was to elucidate the mechanisms through which androgens downregulate AMH expression in the testis. A direct negative effect of androgens on the transcriptional activity of the *AMH* promoter was found using luciferase reporter assays in the mouse prepubertal Sertoli cell line SMAT1. A strong inhibition of *AMH* promoter activity was seen in the presence of both testosterone and DHT and of the androgen receptor. By site-directed mutagenesis and chromatin immunoprecipitation assays, we showed that androgen-mediated inhibition involved the binding sites for steroidogenic factor 1 (SF1) present in the proximal promoter of the *AMH* gene. In this study, we describe for the first time the mechanism behind AMH inhibition by androgens, as seen in physiological and

pathological conditions in males. Inhibition of *AMH* promoter activity by androgens could be due to protein–protein interactions between the ligand-bound androgen receptor and SF1 or by blockage of SF1 binding to its sites on the *AMH* promoter.

Summary Sentence

Androgens act directly on prepubertal Sertoli cells to inhibit *AMH* promoter activity in the presence of the androgen receptor and intact SF1 sites.

Key words: testis, inhibition, testosterone, transcriptional regulation, steroid hormones/steroid hormone receptors, puberty.

Introduction

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), plays a crucial role in fetal sex differentiation by inducing the regression of the Müllerian ducts in the male. AMH is secreted to the circulation exclusively by immature Sertoli cells of the testis [1, 2], and has become a recognized biomarker for the study of testicular function during the prepubertal period in the male [3–5].

Early during fetal development, AMH expression is induced in Sertoli cells by SOX9 binding to a specific response element on the proximal region of the *AMH* gene promoter [6–8]. Other transcription factors, such as SF1 [6, 9–12], GATA4 [10, 13–15], and WT1 [16], further increase *AMH* expression by directly binding to response elements in the gene promoter or by protein–protein interactions. Even though its main action takes place in early fetal life, AMH is highly expressed by Sertoli cells throughout postnatal development until puberty, when AMH levels dramatically drop [17].

A negative correlation between serum testosterone and AMH levels during puberty in the male is seen under normal physiological conditions [18, 19] and in patients with precocious puberty, either central [18, 20] or gonadotropin-independent [18], which is indicative of a downregulation of AMH expression in response to androgens and not gonadotrophins. In addition to this, patients with androgen insensitivity syndrome (AIS), whose Sertoli cells cannot respond to androgens, exhibit high serum levels of AMH [21]. The lack of AMH downregulation has been corroborated in *Tfm* mouse [19], an experimental model of human AIS. Furthermore, studies on Sertoli cell specific knockout mice have clearly shown that the expression of the androgen receptor (AR) in Sertoli cells is needed for AMH downregulation to occur at puberty [22]. Nevertheless, the molecular mechanisms underlying the negative regulation of AMH by androgens remain unknown. Nuclear receptors, such as the AR, can activate or inhibit transcription of their target genes by interacting with specific DNA sequences, usually found within or around the promoter region of a responsive gene, or with other proteins present in the promoter of their target gene, upon ligand binding [23, 24].

In this study, we sought to elucidate the mechanisms through which androgens downregulate AMH expression in Sertoli cells.

Material and methods

Animals

Outbred CF-1 male of different ages and female pregnant mice (*Mus musculus*, CrlFcen: CF1, Mouse Genome Informatics) were obtained from the animal facility of the Faculty of Exact and Natural Sciences, University of Buenos Aires. Research using animals was performed in compliance with the Guide for the Care and Use of Laboratory Animals, 8th ed. Washington, DC: National Research Council, National Academies Press, 2011.

Plasmids

Constructs used in luciferase reporter assays, immunocytochemistry, and ChIP-qPCR assays are described in Supplementary Table S1.

Cell culture and transfections for luciferase reporter assays

SMAT1 cells, an immortalized prepubertal Sertoli cell line of mouse origin [25], were cultured in complete culture media (Dulbecco Modified Eagle Medium, Gibco 11995–065, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco 16000–044 or Natocor, Córdoba, Argentina), 2% MEM amino acids solution 50x (Gibco 11130051), 2% Amphotericin B (0.125 mg/ml, Sigma A-4888), and 1% Penicillin-Streptomycin (Gibco 15070–063, 5000 U/ml) and maintained at 37°C in a humidified atmosphere with 5% CO_2.

To study *AMH* regulation in response to androgens, SMAT1 cells were transiently transfected with 0.5 μg/well of luciferase reporter plasmids containing variants in length and mutants for the *AMH* promoter and/or 0.2 μg/well of hAR expression vector, by using the Lipofectamine 3000 (Invitrogen) method, following the manufacturer's instructions. The construct pMIR-*lacZ* (0.15 μg/well) was used as transfection control. Empty backbone plasmids were transfected accordingly to balance DNA amounts. Briefly, on day 1 cells were plated at 2×10^5 cells/well in 24 multi-well plates in complete culture media. On day 2, media was changed to 1% FBS-DMEM, 1% MEM, without antibiotics and antimycotic. After 2–3 h, media was changed to 1% charcoal-stripped FBS-DMEM, 1% MEM and the DNA-Lipofectamine solutions were added dropwise to each well in a 1:2 DNA-Lipofectamine-Optimem (Gibco, 11058021) solution: transfection media. After 4–5 h, transfection media was replaced by 10% charcoal-stripped media in DMEM, with MEM, antibiotics, and antimycotic. On day 3, cells were treated with vehicle (ethanol), testosterone (T, Steraloids), or 5α-dihydrotestosterone (DHT, Merck) 10^{-7}M for 24 h in serum free media. For antiandrogen treatment, cells were incubated with either vehicle (ethanol 10^{-7}M and DMSO 10^{-5}M), DHT (10^{-7}M) + DMSO (10^{-5}M), or DHT (10^{-7}M) + bicalutamide (10^{-5}M, Casodex, ICI 136–334, ICI Pharmaceuticals, Macclesfield, Cheshire, UK). Luciferase activity was measured with the Luciferase Assay System (Promega, E1500) using a Junior LB9509 luminometer (Berthold Technologies) and the Synergy HTX multimode reader with the Gen5 software version 3.02 (BioTek Instruments, Inc.). For the β-galactosidase activity, measurement extracts obtained for luciferase activity were used. Briefly, 20 μl of each sample was incubated with *buffer Z* 1x (Na_2HPO_4 60 mM, NaH_2PO_4 40 mM, KCl 10 mM, $MgSO_4.7H_2O$ 1 mM) with β-mercaptoethanol (0.35% v/v) and the reaction substrate O-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma Aldrich) for 90 min at 37°C. Reaction was stopped by addition of 44 μl of a Na_2CO_3 (3M) solution, and absorbance

was measured at 420 nm by spectrophotometry. Relative luciferase units (RLU) were defined as the normalization of firefly readings against β-galactosidase activity. Percentage of change in response to androgen treatment was determined as the ratio between RLU of androgen treated/vehicle-treated cells for each transfection studied.

Immunohistochemistry and immunocytochemistry

Male mice were sacrificed by decapitation (1 and 5 days after birth) or cervical dislocation (9 days after birth and older). Testes were removed, fixed in Bouin's fixative between 4 and 16 h at 4°C, dehydrated using serial ethanol dilutions, xylene, and finally embedded in paraffin wax. Tissue sections (5 μm) were obtained and mounted on positively charged slides (Biogenex). Immunohistochemistry for the AR was performed. Slides were processed using the antigen retrieval technique by microwave, and AR was detected following a modified protocol from Saunder's lab [26]. Briefly, sections were deparaffinized with xylene and rehydrated in graded ethanol before washing in distilled water. For antigen retrieval, sections were microwaved two times (2.5 min each) at full power under a 0.01M sodium citrate buffer (pH 6.0). Slides were allowed to cool down in the buffer at RT and then they were washed twice in PBS (prepared from PBS 10x: NaCl 1.37 M, KCl 27 mM, Na_2HPO_4 100 mM, KH_2PO_4 18 mM, pH 7.4). For endogenous peroxidase blockage, slides were incubated for 30 min at RT with H_2O_2 3% and then incubated for another 30 min with PBS-BSA 4%. Slides were incubated with the primary antibodies overnight at 4°C in a humid chamber. Slides were then washed with PBS twice (10 min each). For signal revealing Super Sensitive IHC Detection System (Biogenex) was used. Samples were incubated 30 min at RT with a 1:50 dilution of MultiLink (HK268) (concentrated biotinylated anti-immunoglobulins in PBS with carrier protein and 0.09% sodium azide), followed by three PBS washes (5 min each). Sections were then incubated for 30 min at RT in a solution of 1:50 Peroxidase Label (HK320) (concentrated horse-radish peroxidase-conjugated streptavidin in PBS with carrier protein and 0.1% Proclin 300), followed by three 10-min PBS washes. The reaction was revealed with 3,3′-diaminobenzidine (DAB) and stopped with tap water. After the DAB reaction and wash with distilled water, slides were dehydrated using serial dilutions of ethanol and a final step of xylene and mounted for posterior analysis under an optic microscope.

For immunocytochemistry, SMAT1 cells were plated on 8-well Nunc LabTek chamber slide with cover (Thermo Scientific, Nalge Nunc International) at 1×10^5 cells per well, cultured, and transfected with the pSG5-hAR as described above. Cells were treated with either DHT or vehicle for 24 h and then fixed with ice-cold methanol for 10 min at RT, followed by two PBS washes. After the washes, immunodetection protocol was followed as described above, starting from the endogenous peroxidase block step.

For both immunodetection techniques, slides were analyzed under an Eclipse 50i microscope and images were acquired using a DS Fi1 digital camera and NIS Elements version 3.0 BR imaging software (Nikon Instruments). Immunohistochemistry and immunocytochemistry for the AR were performed using a rabbit polyclonal antibody for the AR (1:200 in blocking solution, Santa Cruz Biotechnology Cat# sc-816, RRID:AB_1563391, Supplementary Table S2) or normal rabbit IgG (1:200 in blocking solution, Santa Cruz Biotechnology Cat# sc-2027, RRID:AB_737197) as negative control (Supplementary Figure S1). Epididymal tissue present in testis sections was used as positive control for AR expression.

Cell culture and transfections for chromatin immunoprecipitation-qPCR assays

SMAT1 cells were plated in 25 cm^2 flasks (2×10^6 cells per flask) and transiently transfected with the pSG5-hAR vector following standard transfection protocol for Lipofectamine 3000 (see above). After 24 h treatment with DHT 10^{-7}M cells were washed and fixed with 36.5% formaldehyde (1% final concentration) for chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed using the HighCell# ChIP kit (Diagenode Inc.) following the manufacturer's instructions. Briefly, magnetic beads were previously washed with ice-cold ChIP buffer C1 and resuspended in said buffer (25 μl beads in 110 μl of ChIP buffer C1 per immunoprecipitation). Specific and control antibodies were added to each Eppendorf (2–4 μg per immunoprecipitation), and the beads were incubated at 4°C for at least 2 h on a rotating wheel at 40 rpm. Cells were trypsinized, washed twice with PBS, and collected in 500 μl of PBS; 36.5% formaldehyde was added to each tube to a final concentration of 1%, vortexed gently, and incubated for 8 min at RT to allow fixation to take place. Glycine was added to each tube (57 μl of a 1.25 M solution), vortexed gently, and incubated for 5 min to stop the fixation. The following steps were performed on ice. After centrifuging for 5 min at 4°C, supernatant was discarded, and the pellet was resuspended in 30 μl of the same solution. The cross-linked cells were then washed twice with ice-cold PBS. Ice-cold Lysis Buffer L1 was added to the pellets, cells were resuspended by pipetting, and incubated for 10 min at 4°C with gentle mixing. After centrifuging, the supernatant was discarded and ice-cold Lysis Buffer L2 was added to each cell pellet. Cells were then resuspended by pipetting and incubated for 10 min at 4°C with gentle mixing. After centrifuging, supernatant was discarded, and the pellet was resuspended in complete Shearing Buffer S1 by vortexing. After a 10-min incubation, chromatin was sheared by sonication for 10 cycles [30 s "ON", 30 s "OFF", each]. ChIP Buffer C1 was added to the sheared chromatin and spun for 10 min. The supernatant was collected to perform the IP. The antibody-coated beads are centrifuged, and the supernatant was discarded. The sheared chromatin was added to each tube of beads and a 1% of each sample was kept as input. The tubes were incubated at 4°C overnight under constant rotation on a rotation wheel at 40 rpm. After incubation, tubes were spun and washed three times with ice-cold ChIP Buffer C1. After washing with Buffer W1, pellets were centrifuged and the supernatant was discarded. All washes were done on a rotation wheel for 5 min at 4°C. DNA isolation Buffer (DIB, Diagenode kit) was added to both sample and input tubes. Tubes were incubated at 55°C for 15 min, spun, and incubated at 100°C for another 15 min. Tubes were then centrifuged and placed in ice for 1 min. Supernatants were transferred to clean tubes and stored at –20°C until used for qPCR.

Immunoprecipitation for the AR was performed using a rabbit polyclonal antibody for the AR (Santa Cruz Biotechnology Cat# sc-816, RRID:AB_1563391) or normal rabbit IgG (Santa Cruz Biotechnology Cat# sc-2027, RRID:AB_737197) as negative control (Supplementary Table S2).

Resulting genomic DNA was diluted 1:50 and 3 μl from each sample were subjected to qPCR using FastStart Universal SYBR Green Master (Rox) (Roche Applied Science) in Step One Plus Real-Time PCR Systems (Life Technologies). The cycling program used included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 60°C for 1 min. Fluorescence was measured at the end of each extension step.

Following amplification, melting curves were acquired and used to determine the specificity of the qPCR products. Specific forward and reverse oligonucleotide primers were manually designed for both regions comprising the SF1 sites in the mouse *Amh* gene promoter (ensembl ENSMUSG00000035262, Ensemble release #83) and further analyzed using OligoAnalyzer 3.1 (Integrated DNA Technologies, Inc.). The sequences for the primers used were as follows: SF1–102bp, Fwd: 5′-GAAAGCCCTTTGAGACAGTCG-3′, Rev: 5′-GACGCCCCTATCAACACCA-3′, SF1–228bp, Fwd: 5′-GTAGTGGGGAGGGTGGATAC-3′, Rev: 5′-GCCTGAGCTGTCCTTGAGTAG-3′.

Percentage of input was obtained with the following formula: $2\bigwedge$(Ct Input—Ct ChIP), where Ct ChIP depicts de mean Ct value of the replicates for each ChIP reaction (with anti-AR or control IgG).

Statistical analysis

Relative luciferase units were defined as the normalization of firefly luciferase against β-galactosidase activity. Values are expressed as mean ± SEM for the percentage (%) RLU of androgen-treated vs vehicle-treated samples. Unpaired t-test (one tail) was used to assess hAR participation by comparing % (RLU androgen/RLU vehicle) of pSG5-hAR vs pSG5-transfected samples.

Percentage of change in response to androgen treatment was determined as the ratio between RLU of androgen-treated/vehicle-treated cells for each transfection condition, against a 100% theoretical value using a one sample t-test, where 100% indicates the ratio between androgen-treated and vehicle-treated RLU. A nonsignificant value was indicative of no androgen effect.

For antiandrogen treatment, RLU for each treatment was normalized to that of the vehicle treated, for each transfection condition. Normalized values were then analyzed using a one-way ANOVA and Dunn's multiple comparisons test was used to assess bicalutamide effect in DHT-treated cells.

After ChIP-qPCR, DNA extract enrichment in the regions encompassing SF1 sites was compared between experimental conditions (control IgG vs anti-AR) using an unpaired t-test.

A difference was considered statistically significant when the *P*-value was $P < 0.05$. All calculations were made using GraphPad Prism version 5.01 for Windows (GraphPad Software). All assays were done at least three times.

Results

Expression profile of the AR in mouse postnatal testis and SMAT1 cells

The already characterized clonal Sertoli cell line SMAT1, exhibiting *AMH* promoter activity [25, 27, 28], was used as experimental model in this work. Because this cell line derives from a 6.5-day-old mouse testis, and at this age the AR is weakly or not expressed in most Sertoli cells (Supplementary Figure S1), we evaluated AR expression in SMAT1 cells by immunocytochemistry (Figure 1). No endogenous expression of the AR was detected. After transfection with pSG5-hAR, AR expression was observed both in the cytoplasm and nucleus. After treatment with DHT (10^{-7} M) for 24 h, the receptor was localized mainly in the nucleus.

Androgens downregulate AMH promoter activity in Sertoli cells

Although AMH downregulation by androgens has been established as a landmark of pubertal onset, the mechanism underlying this downregulation is unknown. To test whether the androgen-dependent decrease in testicular AMH production occurs in a direct manner in Sertoli cells by a negative effect of androgens on the activity of the *AMH* promoter, the prepubertal mouse Sertoli cell line SMAT1 was transiently co-transfected with luciferase constructs containing 3078 bp of the wild-type human *AMH* promoter (pGL2B-5′*AMH*-3078), or with pGL2B (empty control), together with an expression vector for the human androgen receptor (pSG5-hAR) and incubated in culture media with either T or DHT 10^{-7}M, or vehicle (ethanol 10^{-7} M), for 24 h. The activity of the 3078 bp *AMH* promoter was significantly diminished by both T (63.25 ± 5.21% as compared to vehicle treatment, $P = 0.006$, n = 5) and DHT (45.15 ± 2.77%, $P < 0.001$, n = 14). However, this only occurred

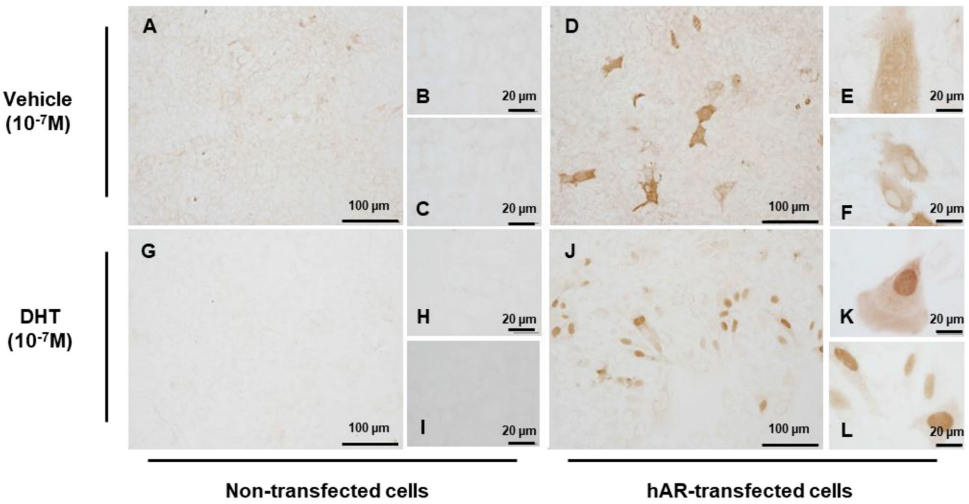

Figure 1. Expression of the androgen receptor in the Sertoli cell line SMAT1. Androgen receptor expression in SMAT1 cells nontransfected and transfected with the pSG5-hAR construct was studied by immunocytochemistry. Nontransfected cells (A–C, G–I) showed no endogenous expression of the AR, with or without DHT (10^{-7}M) treatment. In contrast, hAR expression was detected in transfected SMAT1 cells (D–F, J–L) with and without DHT (10^{-7}M) treatment. Scale bar: 100 μm (A, D, G, J), 20 μm (B, C, E, F, H, I, K, L) (n = 3).

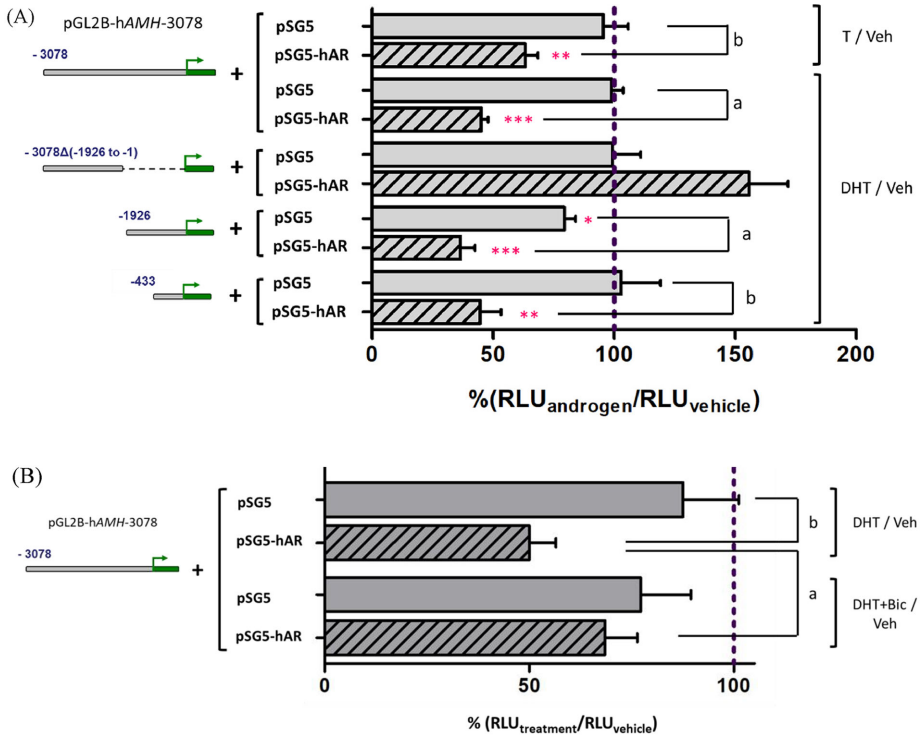

Figure 2. Length variants *AMH* promoter activity in SMAT1 cells in response to androgen treatment for 24 h. (A) Values are expressed as mean ± SEM relative luciferase units (RLU) of androgen treated/vehicle treated. All treatments (T, DHT, and vehicle) were done at 10^{-7}M. Unpaired t-test (one tail) was used to assess hAR participation by comparing pSG5-hAR vs pSG5 conditions. a: $P < 0.01$, b: $P < 0.0001$. Androgen effect: % change for each column was evaluated using a one sample t-test against a theoretical value of 100%, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. A value of 100% indicates no changes in response to androgen treatment. Solid bars: pSG5 transfected, striped bars: pSG5-hAR transfected. (B) Treatment with the antiandrogen bicalutamide partially reverses AMH promoter activity inhibition by androgens. SMAT1 cells co-transfected with luciferase constructs containing 3078 bp of the wild-type human *AMH* promoter (pGL2B-5′*AMH*-3078), or with pGL2B (empty control), together with an expression vector for the human androgen receptor (pSG5-hAR) or pSG5 (empty control) were incubated in culture media with either vehicle (ethanol 10^{-7}M and DMSO 10^{-5}M), DHT (10^{-7}M) + DMSO (10^{-5}M), or DHT (10^{-7}M) + bicalutamide (10^{-5}M) for 24 h. A partial reversal of *AMH* promoter activity inhibition was observed after treatment with the antiandrogen bicalutamide (one-way ANOVA, Dunn's multiple comparisons test between DHT and DHT + bicalutamide-treated conditions in the presence of the hAR, $P < 0.01$, n = 8). Values are expressed as mean ± SEM relative luciferase units (RLU) of treatment/vehicle. a: $P < 0.05$, b: $P < 0.01$. Bic: bicalutamide. Solid bars: pSG5 transfected, striped bars: pSG5-hAR transfected.

in the presence of the hAR (pSG5-hAR vs pSG5 condition for T: $P = 0.015$ and DHT: $P < 0.001$ treatment) (Figure 2). Because there is a hemi-estrogen response element in the *AMH* promoter [29], all following assays were done in the presence of DHT to avoid T conversion to estradiol, which could interfere with the results.

To verify that the observed inhibition was dependent on AR presence, SMAT1 cells co-transfected with luciferase constructs containing 3078 bp of the wild-type human *AMH* promoter (pGL2B-5′*AMH*-3078), or with pGL2B (empty control), together with an expression vector for the human androgen receptor (pSG5-hAR) or pSG5 (empty control) were incubated in culture media with either vehicle (ethanol 10^{-7}M and DMSO 10^{-5}M), DHT (10^{-7}M) + DMSO (10^{-5}M), or DHT (10^{-7}M) + bicalutamide (10^{-5}M) for 24 h. A partial reversal of *AMH* promoter activity inhibition was observed after treatment with the antiandrogen bicalutamide (Figure 2B).

The proximal region of the AMH promoter is involved in the downregulation by androgens

Once direct action of androgens on the *AMH* promoter activity was established, we sought to elucidate which region of the *AMH* promoter could be involved. SMAT1 cells were transiently co-transfected with luciferase constructs containing length variants of the wild-type human *AMH* promoter, or with pGL2B (empty control), together with pSG5-hAR, and incubated in culture media with either DHT or vehicle 10^{-7} M for 24 h. Androgen treatment downregulated the activity of the *AMH* promoter constructs containing the proximal 433 bp (44.7 ± 8.6%, $P = 0.001$, n = 6) or 1926 bp (36.5 ± 6.0%, $P < 0.001$, n = 5), but not when the construct carried only distal sequences of the *AMH* promoter comprising –3078 to –1926 bp (155.8 ± 16.0%, $P = 0.014$, n = 4). As with the whole promoter, the inhibitory effect was only seen in the presence of the hAR (pGL2B-5′*AMH*-433: $P = 0.005$ and pGL2B-5′*AMH*-1926: $P < 0.001$) (Figure 2A). Unexpectedly, when the –1926 to –1 region of the AMH promoter was missing, androgens seemed to increase promoter activity. This could be due to movement of the two already known activating NFκB and AP2 sites [27] closer to the transcription start site.

Intact SF1 sites in the AMH promoter are necessary for the negative regulation by androgens

Recognition sequences for several known transcription factors are present in the *AMH* promoter. Because the *AMH* promoter lacks known inhibitory androgen response elements, we tested whether the AR-dependent inhibition could be due to an interaction of the AR with *AMH* trans-activating factors, such as GATA4, AP1, and SF1, all of which have sites in the proximal promoter of the *AMH* gene

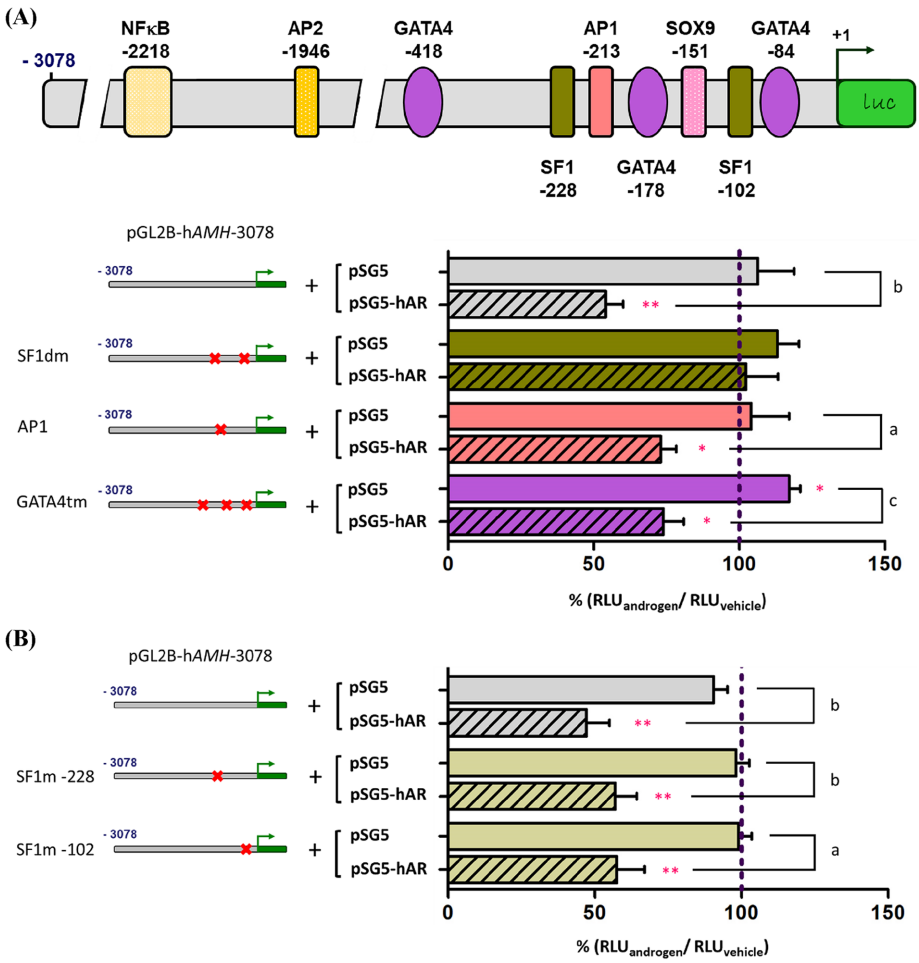

Figure 3. Mutated full-length *AMH* promoter activity in SMAT1 cells in response to androgen treatment for 24 h. (A) Values are expressed as mean ± SEM for the % (RLU of androgen treated/vehicle treated). Unpaired t-test (one tail) was used to asses hAR participation by comparing ratios of % (RLU androgen/RLU vehicle) of pSG5-hAR vs pSG5 conditions. a: $P < 0.05$, b: $P < 0.01$, c: $P < 0.001$. Androgen effect: % change for each column was evaluated using a one sample t-test against a theoretical value of 100%, $^{*}P < 0.05$, $^{**}P < 0.01$. SF1dm: 5′*AMH*-luc with SF1 sites at –102 and –228 bp mutated, GATA4tm: 5′*AMH*-luc with GATA4 sites at –84, –178, and –418 bp mutated, AP1: 5′*AMH*-luc with AP1 site at –213bp mutated. A value of 100% indicates no changes in response to androgen treatment. All treatments (DHT and vehicle) were done at 10^{-7}M. Solid bars: pSG5 transfected, striped bars: pSG5-hAR transfected. (B) Activity of *AMH* promoter with mutated SF1 sites in SMAT1 cells in response to androgen treatment for 24 h. Values are expressed as mean ± SEM for the % (RLU androgen/RLU vehicle). Unpaired t-test (one tail) was used to asses hAR participation by comparing ratios of %(RLU androgen/RLU vehicle) of pSG5-hAR vs pSG5 conditions. a: $P < 0.01$, b: $P < 0.001$. Androgen effect: % change for each column was evaluated using a one sample t-test against a theoretical value of 100%, $^{**}P < 0.01$. SF1m-102: 5′*AMH*-luc with the SF1 site at –102 mutated; SF1m-228: 5′*AMH*-luc with the SF1 site at –228 mutated. All treatments (DHT and vehicle) were done at 10^{-7}M. Solid bars: pSG5 transfected, striped bars: pSG5-hAR transfected.

(Figure 3A). The inhibition induced by DHT was conserved when the sites for GATA4 at –84, –178, and –418 (GATA4tm, 73.8 ± 7.0%, $P = 0.034$, n = 4) and AP1 at –213 (AP1m, 73.0 ± 5.3%, $P = 0.015$, n = 4) were mutated, but not when the two sites for SF1 at –102 and –228 were abolished (SF1dm, 102.2 ± 11.1%, $P = 0.224$, n = 4) (Figure 3A). Furthermore, when both SF1 sites were mutated, the activity of the *AMH* promoter was reduced, and did not further decrease in response to DHT (Supplementary Figure S2).

Next, we sought to determine whether one of the SF1 sites or both were involved in the downregulation of *AMH* promoter activity by DHT. We performed luciferase assays in SMAT1 cells transfected with *AMH* promoter carrying independent mutations for each of the SF1 sites. After a 24-h treatment with DHT 10^{-7}M, downregulation of *AMH* promoter activity was observed when either the proximal (57.57 ± 9.37, $P = 0.006$, n = 6) or the distal (57.06 ± 7.23, $P = 0.002$, n = 6) SF1 site was mutated. This only occurred in the presence of the hAR (SF1 –102 m: $P = 0.001$, SF1 –228 m: $P < 0.001$) (Figure 3B). These results indicate that both recognition sequences for SF1 are involved in the downregulation of *AMH* promoter activity in response to DHT in SMAT1 cells.

To further test this hypothesis, ChIP-qPCR assays were performed using SMAT1 transfected with the pSG5-hAR and treated with DHT for 24 h. ChIP was done with a polyclonal antibody against the AR or the control IgG, followed by qPCR using specific primers for the SF1 sites on the endogenous mouse *Amh* promoter. Extracts were enriched in both the proximal SF1 region at –102 bp (AR: 0.222 ± 0.058% vs control IgG: 0.043 ± 0.015%, $P = 0.020$, n = 3) and the distal SF1 region at –228 bp (AR: 0.547 ± 0.182% vs control IgG: 0.052 ± 0.024%, $P = 0.027$, n = 3) of the *Amh* promoter, indicating that both SF1 sites are involved in the downregulation of the *Amh* promoter activity in the presence of the AR

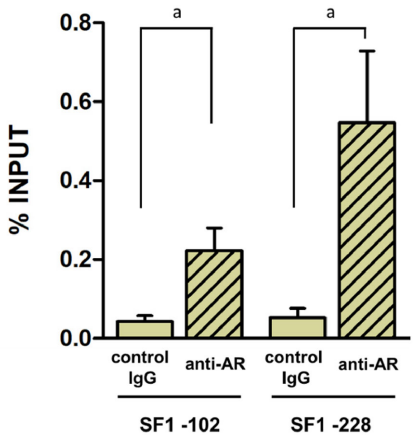

Figure 4. Involvement of SF1 sites in the downregulation of *AMH* promoter activity by androgens. Enrichment of *Amh* promoter regions encompassing SF1 sites as shown by ChIP-qPCR. Unpaired t-test, one tail, $^{*}P < 0.05$, n = 3.

after a 24-h DHT treatment, in agreement with the luciferase assays (Figure 4).

Discussion

Clinical and experimental evidence has shown downregulation of testicular AMH secretion by androgens during puberty [18, 19]. However, the underlying molecular mechanisms remain unknown. With this study we sought to shed some light on the androgen-mediated regulation of *AMH* gene expression in Sertoli cells. Here we show a novel direct inhibitory effect of androgens on Sertoli cell *AMH* promoter activity, which requires the AR and the proximal promoter region of the *AMH* gene, particularly that comprising the 433 bp upstream of the transcription start site. Furthermore, we provide evidence for the participation of SF1 sites in the downregulation of *AMH* promoter activity by androgens.

Our study focuses on the role androgens play in the regulation of the Sertoli cell immaturity marker, AMH, and on the mechanism underlying the clinical observations regarding AMH downregulation around the onset of puberty in the male. A critical player in this mechanism is the AR. Its expression ontogeny in the testis has been thoroughly studied, showing similar expression patterns in rodents [19, 26], monkeys [30], and humans [31–33]. In agreement with previous findings, we found consistent expression of the AR in peritubular myoid cells from day 1 onwards. In Sertoli cells, weak AR expression was detected only in the nucleus of a few Sertoli cells on day 5, which then extended to all of them through day 9 (Supplementary Figure S1). SMAT1 cells do not have endogenous expression of the AR (Figure 1); therefore, they are a useful model to assess receptor-mediated androgen effect by comparing AR transfected to nontransfected cells.

The inhibitory effect of androgens on AMH testicular production could be the result of an indirect effect of androgens on Sertoli cell production of AMH. Indeed, other cell types present in the testes, such as the peritubular myoid cells, show strong AR expression and could, therefore, respond to testosterone or DHT and indirectly modulate AMH production in Sertoli cells. However, the luciferase reporter assays performed in this study with the transfected Sertoli cell line, SMAT1, show a striking negative effect of androgens in the transcriptional promoter activity of *AMH* in the absence of other cell types. This indicates that a direct negative effect of androgens on *AMH* expression can be achieved in immature Sertoli cells. This negative effect on *AMH* promoter activity requires the presence of androgens, testosterone or DHT, and the AR (Figure 2A). These observations are in line with and provide insight into the molecular mechanisms underlying the differential androgen-mediated regulation of AMH in the testis. Indeed, during fetal and early postnatal life, i.e. until days 4–7 in the mouse and the first 12 months of life in humans, AMH is not downregulated despite the existence of high intratesticular levels of testosterone and high AR expression in peritubular myoid cells [19, 32, 33]. Furthermore, AMH remains high in boys <1 year of age with precocious puberty whose testes produce high testosterone levels [20]. The lack of AR expression in Sertoli cells makes them physiologically insensitive to androgens in those periods but when the AR expression appears in Sertoli cells and intratesticular testosterone levels are high, either timely at puberty [18] or precociously [18, 20], AMH is downregulated.

Our findings of direct inhibitory effect of androgens on *AMH* gene expression provide the molecular explanation for a poorly known regulatory mechanism, i.e. androgen-inhibited genes. Indeed, while upregulation of gene expression by androgens has been widely studied, leading to the description of androgen responsive elements (ARE) on target gene promoters, the molecular mechanisms underlying inhibition by androgens remain elusive in most cases. Amongst the regulatory options involving the AR and its target genes, some require that the AR interacts with an ARE or with other transcription factors to exert its regulatory role. *Maspin*, which encodes a serine protease inhibitor with a tumor-suppressing role in the mammary gland that is downregulated in primary prostate cancer cells and lost in metastatic cells, has an ARE in its proximal promoter (–277 to –262 region) involved in the inhibition of its promoter activity [34, 35]. In the MCF-7 breast cancer cell line, DHT acts as a transcriptional downregulator of the *Ccnd1* gene after AR binding to a specific ARE in the proximal promoter, together with the recruitment of the transcription factors DAX1 and HDAC1 [36]. Androgen receptor interaction with other transcription factors resulting in downregulation of a target gene without direct binding to an ARE occurs in the case of the gene encoding the neurotrophin receptor p75 [37], which lacks typical AREs. Here, the AR interacts with the transcription factor AP1, thus interfering with its trans-activating capacity on the *p75* promoter.

Our results regarding *AMH* promoter downregulation in the absence of AREs and in the presence of AR suggest that an interaction between this receptor and other trans-activating elements, or their binding sites, may be taking place. In fact, our findings in luciferase assays with the *AMH* promoter constructs carrying mutations in the sites for different transcription factors show that inhibition in response to androgens is lost only when the two SF1 sites are mutated (Figure 3A). In the absence of androgen activity, SF1 trans-activates the *AMH* promoter [6, 9–12]. Here we show that when both SF1 sites are mutated, the activity of the *AMH* promoter is reduced, and does not further decrease in response to DHT (Supplementary Figure S2). Conversely, the negative effect of androgens persists when sites for other trans-activating factors, like GATA4 and AP1, are mutated (Figure 3A), thus ruling out involvement of these factors in androgen action on AMH expression. In this study, we also show that the ligand-bound AR diminishes the *AMH* promoter activity induced by SF1 acting on any of its two sites, as evidenced by the assays performed with the independent mutants for the SF1 binding sequences (Figure 3B). Involvement of the two SF1 sites in androgen-dependent inhibition of *AMH* promoter activity is further supported by our ChIP-qPCR results, which showed enrichment for both

regions containing the SF1 sites separately, when immunoprecipitated with an antibody for the AR (Figure 4). Due to experimental limitations we could not show direct evidence of an interaction between SF1 and the AR in SMAT1 cells in our study. Indeed, co-immunoprecipitation assays were performed, but Western immunoblot against SF1 was not informative due to the presence of the heavy IgG chain band at 50 kDa, which coincides with the estimated molecular weight for SF1 (53 kDa). Nevertheless, the ChIP-qPCR assay provides strong evidence, since it specifically involves AR interaction with the regions containing the SF1 sites. This interaction could be direct, implying that the AR binds to SF1 sites itself, or indirect, if the AR interacts with site-bound-SF1 on the *AMH* promoter.

In addition to the aforementioned transcriptional or genomic mode of action, an increasing body of evidence suggests that androgens can exert rapid, nongenomic effects [38]. Nongenomic steroid activity typically involves the rapid induction of conventional second messenger signal transduction cascades, including increases in free intracellular calcium, and activation of protein kinase A (PKA), protein kinase C (PKC), and mitogen-activated (MAP) kinase. Second messenger induction by nongenomic steroid action is insensitive to inhibitors of transcription and translation. Commonly, these effects are observed to occur within seconds to minutes and are considered to be too rapid to involve changes in transcription and protein synthesis. Our experimental design does not allow us to rule out the occurrence of nongenomic mechanisms in the downregulation of *AMH* promoter activity by androgens. Another limitation of our experimental design stems from the use of plasmids carrying promoter fragments that lack epigenetic marks, which could cause methylation-regulated events to be missed.

Evolutionary conserved binding elements for SF1 [9, 11] and GATA4 [10, 11] have been described in the *AMH* proximal promoter of several species, suggesting a common mechanism in the regulation of AMH expression in mammals [27, 39]. Regarding SF1, this transcription factor is involved not only in basal AMH expression in the fetal testis [6, 9, 11] but also in the positive response to FSH via the classical cAMP pathway [28]. Whether an interplay between SF1-mediated AMH activation induced by FSH and androgen-induced downregulation of AMH involving SF1 exists was not addressed in this work.

Based on our findings, at least three mechanisms can be initially proposed to explain how the AR acts on the *AMH* gene promoter to inhibit its transcriptional activity (Figure 5). One mechanism could involve sequestration of SF1 by the AR that would prevent the former from binding to its sites on the *AMH* promoter. SF1 would then not be able to exert its positive effect, resulting in diminished *AMH* promoter activity. This alternative, however, is discarded by our ChIP-qPCR results, which provide evidence for the need of interaction with the SF1 sites. A second alternative could be competition between the AR and SF1 for its sites on the *AMH* promoter. Binding of the AR to SF1 sites would prevent SF1-mediated upregulation

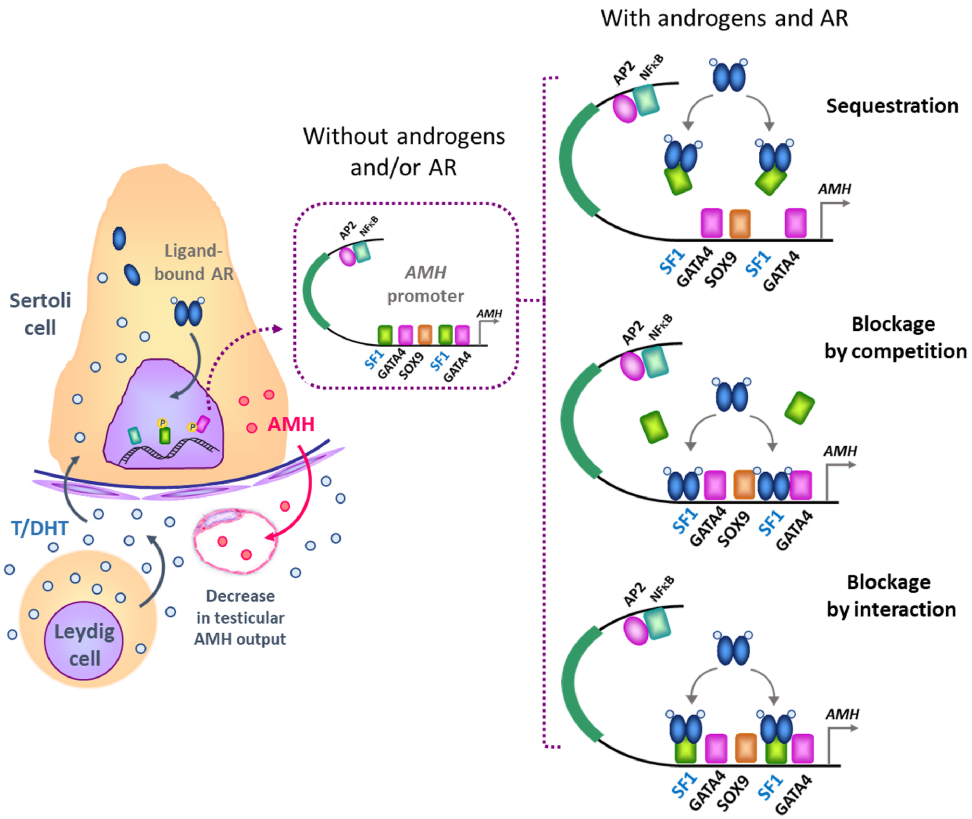

Figure 5. Proposed molecular mechanism for the androgen receptor-mediated inhibition of the *AMH* gene promoter activity in the presence of androgens in Sertoli cells. In the absence of androgens or androgen receptor (AR), SF1 is able to bind to its sites on the *AMH* promoter and exert its trans-activating effect on the transcriptional activity of the gene. When androgens and their receptor are present in the Sertoli cell, the ligand-activated AR could either sequestrate SF1 by a protein–protein type interaction, preventing SF1 from exerting its stimulatory effect (sequestration), compete with and block the SF1 transcription factor for and from its binding site, impeding its action on the *AMH* gene promoter (blockage by competition), or interact with site-bound-SF1 and prevent it from exerting its stimulatory effect on *AMH* promoter activity enabling androgen-mediated downregulation instead (blockage by interaction).

and allow androgen-mediated inhibition of *AMH* promoter activity. A third option could entail AR binding to site-bound SF1. This interaction could alter SF1 conformation or its interaction with other trans-activating elements, thus not allowing SF1 to upregulate *AMH* promoter activity and instead allowing its androgen-dependent inhibition.

This is the first study to show a direct downregulation of the ligand-bound AR on the *AMH* promoter activity in the Sertoli cell, thus shedding light on the mechanism underlying the decline of testicular AMH secretion in response to the elevation of androgens in normal and precocious puberty and also explaining the lack of AMH downregulation in human conditions with, or in experimental mouse models of, androgen insensitivity.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplementary Figure S1. Immunohistochemistry for the androgen receptor in the postnatal mouse testis, including negative control (A) and positive control (G). Arrows: peritubular myoid cells, arrowheads: Sertoli cells, stars: epididymal tissue. Scale bar: 100 μm (A–G), 20 μm (H–L).
Supplementary Figure S2. *AMH* promoter activity assessment by luciferase assays in the presence of SF1 site mutations. Relative luciferase units (RLU) were defined as the normalization of firefly luciferase against β-galactosidase activity and determined for vehicle-treated and DHT (10^{-7}M)-treated conditions. SF1dm: *5′AMH*-luc with SF1 sites at –102 and –228 bp mutated; Veh: vehicle.
Supplementary Table S1. Plasmids used in this study.
Supplementary Table S2. Antibodies used in this study.

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We thank Mariana Cruz for helping with mouse manipulation, Eduardo Dascal for histology processing, Mercedes Astarloa for preparation of DNA for ChIP experiments, Dr Olli Jänne (University of Helsinki, Finland) for the androgen receptor plasmids, and Drs. Raquel Fueyo Arévalo and Marian Martínez (Institut de Biología Molecular de Barcelona, Spain) for discussion of ChIP results.

Author contributions

All authors participated in the design of the experiments. CR, NdC, and RAR generated the AMH plasmids. NYE performed the experiments. NYE, HFS, and RAR analyzed the data. NYE and RAR drafted the manuscript. All authors approved the final version.

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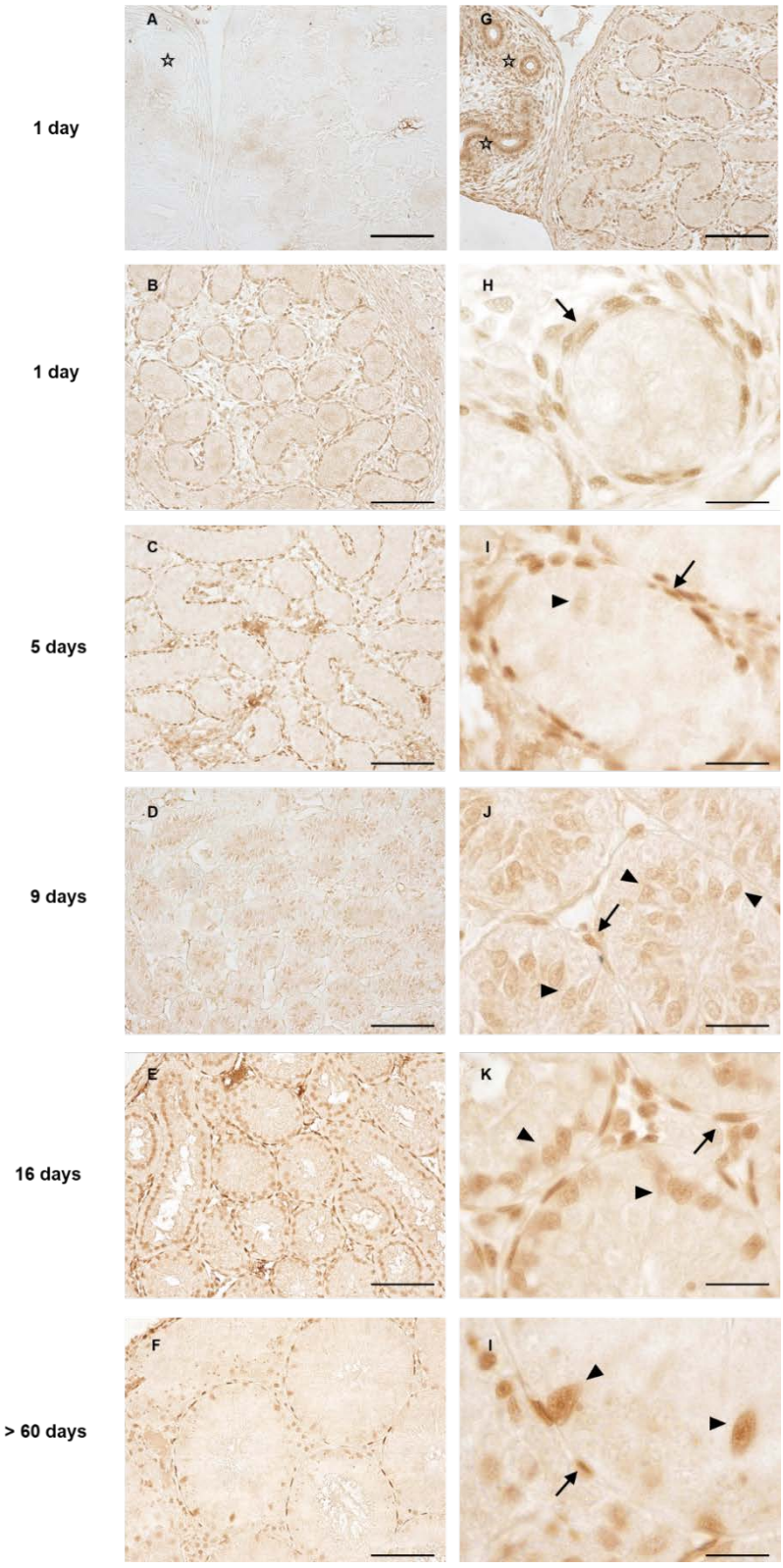
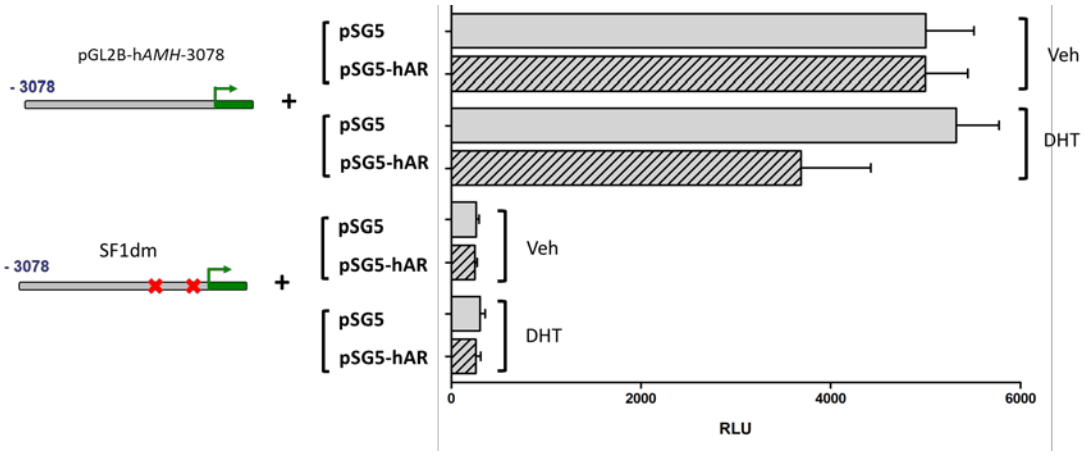
Supplementary Figure 1



Supplementary Figure 2



Supplementary Table 1: Plasmids used in this study

Abbreviated name	Construct	Description	Ref.
pGL2B	pGL2-Basic	Empty control vector carrying luciferase gene	Promega
pGL2B-*AMH*-3078	pGL2B-5'*AMH*[-3078 to -1]-luc	3078 bp 5' upstream of the TSS for the human *AMH* gene	[1]
pGL2B-*AMH*[-3078 to -1926]	pGL2B-5'*AMH*[-3078 to -1926]-luc	Comprises the [-3078 to -1926] 5' region of the human *AMH* promoter	[2]
pGL2B-*AMH*-1926	pGL2B-5'*AMH*[-1926 to -1]-luc	1926 bp 5' upstream of the TSS for the human *AMH* gene	[2]
pGL2B-*AMH*-433	pGL2B-5'*AMH*[-433 to -1]-luc	433 bp 5' upstream of the TSS for the human AMH gene	[2]
pGL2B-*AMH*-GATA4tm	pGL2B-5'*AMH*[-3078 to -1]GATA4tm-luc	3078 bp 5' upstream of the TSS for the human *AMH* gene with GATA4 sites mutated	[3]
pGL2B-*AMH*-AP1m	pGL2B-5'*AMH*[-3078 to -1]AP1m-luc	3048 bp 5' upstream of the TSS for the human *AMH* gene with AP1 site mutated	[3]
pGL2B-*AMH*-SF1dm	pGL2B-5'*AMH*[-3078 to -1]SF1dm-luc	3078 bp 5' upstream of the TSS for the human *AMH* gene with SF1 sites mutated	[3]
pGL2B-*AMH*-SF1m-102	pGL2B-5'*AMH*[-3078 to -1]SF1m-102-luc	3078 bp 5' upstream of the TSS for the human *AMH* gene with SF1 site at -102 mutated	[3]
pGL2B-*AMH*-SF1m-228	pGL2B-5'*AMH*[-3078 to -1]SF1m-228-luc	3078 bp 5' upstream of the TSS for the human *AMH* gene with SF1 site at -228 mutated	[3]
pSG5	pSG5	Empty control vector for AR constructs	Agilent
pSG5-hAR	pSG5-hAR	Human androgen receptor	[4]
pMIR-*lacZ*	pMIR-*lacZ*	Plasmid for β-galactosidase expression. Transfection control.	Thermo Fisher Scientific

Note: For *AMH* promoter constructs site notation according to Picard *et al.* [5] was used, reflecting a 10bp to the 5' displacement when compared with the references [2, 3, 6].

Abbreviations: TSS, transcription start site.

Supplementary Table 2: Antibodies used in this study

Antibody	Description	Information	Research Resource Identifier
Anti-androgen receptor	Rabbit polyclonal antibody for the AR	Santa Cruz Biotechnology Cat# sc-816	RRID:AB_1563391
Control IgG	Normal rabbit IgG	Santa Cruz Biotechnology Cat# sc-2027	RRID:AB_737197

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