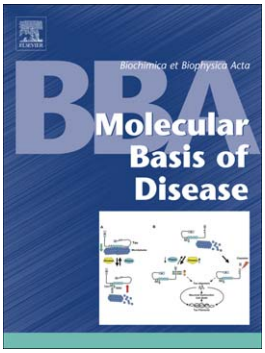

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Androgen inhibits key atherosclerotic processes by directly activating *ADTRP* transcription

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ABSTRACT

Low androgen levels are associated with an increased risk of coronary artery disease (CAD), thrombosis and myocardial infarction (MI), suggesting that androgen has a protective role. However, little is known about the underlying molecular mechanism. Our genome-wide association study identified the *ADTRP* gene encoding the androgen-dependent TFPI regulating protein as a susceptibility gene for CAD and MI. The expression level of *ADTRP* was regulated by androgen, but the molecular mechanism is unknown. In this study, we identified the molecular mechanism by which androgen regulates *ADTRP* expression and tested the hypothesis that androgen plays a protective role in cardiovascular disease by activating *ADTRP* expression. Luciferase assays with an *ADTRP* promoter luciferase reporter revealed that androgen regulated *ADTRP* transcription in a dose-and time-dependent manner, and the effect was abolished by three different androgen inhibitors, including pyrvinium pamoate, bicalutamide, and cyproterone acetate. Chromatin-immunoprecipitation showed that the androgen receptor bound to a half androgen response element (ARE, TGTTCT) located at +324bp from the *ADTRP* transcription start site. The ARE is required for concentration-dependent transcriptional activation of *ADTRP*. HL-60 monocyte adhesion to EAhy926 endothelial cells (ECs) and transmigration across the EC layer, the two processes critical to development of CAD and MI, were inhibited by androgen, but the effect was rescued by *ADTRP* siRNA and exacerbated by overexpression of *ADTRP* and its downstream genes *PIK3R3* and *MIA3*. These data suggest that one molecular mechanism by which androgen confers protection against CAD is

stimulation of *ADTRP* expression.

Keywords

Androgen

Androgen receptor (AR)

ADTRP

Atherosclerosis

Coronary artery disease (CAD)

Myocardial infarction (MI)

Monocyte adhesion to endothelial cells

Monocyte transmigration across endothelial cells

Transendothelial migration of monocytes

1. Introduction

Coronary artery disease (CAD) and its main complication myocardial infarction (MI) are the leading cause of death worldwide [1-5]. CAD and MI are caused by genetics factors, environmental factors and interactions among these factors. Many risk factors have been identified for CAD and MI, including male gender, smoking, hyperlipidemia, diabetes, hypertension and obesity. There has been a long history of interest in the relationship between androgen and cardiovascular disease. Lower serum androgen/testosterone levels were associated with insulin resistance, diabetes and metabolic syndrome, which all increase risk of cardiovascular disease [6]. Moreover, many studies showed that lower serum androgen/testosterone levels were associated with atherosclerosis, cardiovascular risk, stroke and cardiovascular mortality in men [6]. Androgen can bind to its receptor, the androgen receptor (AR), in the cytoplasm, which activates the AR and translocates it to the nucleus. The AR is a DNA-binding transcriptional factor that regulates gene expression by directly binding to the androgen response element (ARE) at the promoter/regulatory region of target genes [7-10]. Knockout of the *AR* gene increased atherosclerosis in mice under the *ApoE*$^{-/-}$ knockout background [11, 12]. Thus, the atheroprotective effect of androgen was also inferred in animal models. However, the molecular mechanism by which androgen reduces risk of atherosclerotic CAD is unknown.

Genome-wide association studies (GWAS) have been effective in identifying genomic variants that confer risk or protection of CAD and MI [13, 14]. Our first GWAS for CAD and MI in a non-European ancestry population identified a

susceptibility single nucleotide polymorphism (SNP) for CAD and MI, rs6903956 in the *ADTRP* gene encoding the androgen-dependent TFPI regulating protein [15]. The ADTRP protein showed homology to AIG1 (androgen-induced protein 1). ADTRP was found to be expressed on the cell surface of endothelial cells (ECs) and regulate the expression of TFPI (Tissue Factor Pathway Inhibitor) [16], which is a key natural inhibitor of coagulation and thrombosis [16]. Recently, we reported that *ADTRP* regulates cell cycle progression, proliferation, and apoptosis by global regulation gene expression [17]. Moreover, we also reported that *ADTRP* regulates the levels of *MIA3/TANGO1* (another CAD and MI-associated gene identified by GWAS), collagen VII and ApoB by positively regulating the expression of *PIK3R3* and activation of AKT [18]. The ADTRP-MIA3-collagen VII/ApoB network was associated with monocyte adhesion to endothelial cells and transmigration of monocytes across the endothelial cell layer, the two cellular processes directly relevant to atherosclerosis [18]. Chinetti-Gbaguidi et al recently showed that ADTRP is expressed in human macrophages and atherosclerotic lesions, and its expression is regulated by the peroxisome proliferator-activated receptor (PPAR)γ in human primary macrophages [19].

Similar to AIG1[20], the expression levels of both *ADTRP* mRNA and ADTRP protein on the endothelial cell surface were up-regulated by androgen (dihydrotestosterone) [16], but the molecular mechanism remains to be identified. In the present study, we characterized the molecular mechanism for the regulation of *ADTRP* by androgen. We found that the transcriptional activation of *ADTRP* is

induced by androgen and by the direct binding of the AR to the *ADTRP* promoter/regulatory region. Importantly, we demonstrated that androgen exerts the atheroprotective effect by decreasing monocyte adhesion to endothelial cells and monocyte transmigration across endothelial cells via up-regulation of *ADTRP*.

2. Materials and methods

2.1. Cell culture

The EAhy926 endothelial cell line was purchased from Shanghai Institute of Biochemistry and Cell Biology of SIBCB (Shanghai, China) and maintained in the human endothelial basal growth medium supplemented with 15% fetal bovine serum (FBS) from Gibco, Life Technologies, CA, USA. The HeLa cell line was purchased from ATCC (American Type Culture Collection, VA, USA) and maintained in the Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. The HL-60 cells line was purchased from Pricells (Wuhan, Hubei, China) and maintained in the 1640 medium supplemented with 20% FBS from Gibco, Life Technologies, California, USA. All cells were cultured at 37°C in a humidified incubator with 5% CO_2.

2.2. Plasmids and siRNA

The promoter/regulatory region of the *ADTRP* gene starting from -789bp to +724bp from the transcriptional start site (TSS) was amplified by PCR analysis using human genomic DNA as the template, the Prime STAR HS DNA polymerase

(TaKaRa, Dalian, China) and PCR primers *ADTRP*-F-*KpnI* 5'-CGGGGTACCCCTCC TTGTCCAGCCTACAG-3' and *ADTRP*-R-*XhoI* 5'-CCGCTCGAGCCCCTCTTTGA GCTCATCTG-3'. The PCR product was digested with restriction enzymes *Kpn I* and *Xho I* (TaKaRa, Dalian, China), and sub-cloned into the multiple cloning site of the pGL3-Basic luciferase vector. This generates a luciferase reporter for the *ADTRP* promoter, pGL3-Basic-ADTRPp-Luc, in which the *ADTRP* promoter/ regulatory region is inserted upstream of the firefly *luciferase* coding region.

The half ARE sequence at the position of +324bp from the TSS was mutated from TGTTCT to AAAAAT and TAAAAA using site-directed mutagenesis as described elsewhere [21, 22], resulting in two mutant reporters pGL3-Basic-ADTRP p-Luc-Mut1 and pGL3-Basic-ADTRPp-Luc-Mut2. The primers for mutagenesis from TGTTCT to AAAAAT included a forward primer 5'-TGCATATACCACTTCCT AAAAATGAGCTGGTATACTTTCC-3' and a reverse primer 5'-GGAAAGTATACC AGCTCATTT TTAGGAAGTGGTATATGCA-3' (for pGL3-Basic-ADTRPp-Luc-Mut1). The primers for mutagenesis from TGTTCT to TAAAAA included a forward primer 5'-TGCATATACCACTTCCTTAAAAAGAGCTGGTATACTTTCC-3' and a reverse primer 5'-GGAAAGTATACCAGTTCTTTTTAAGGAAGTGGTATATGCA-3' (for pGL3-Basic-ADTRPp-Luc-Mut 2).

A plasmid with the full-length cDNA for the human *AR* gene, pEZ-M02 AR, was purchased from Gene Copoeia (Guangzhou, China) (human genome build hg19 mRNA: NC_000023.11, Entrez GeneID 367, Ensembl: ENSG00000169083). The full length cDNA for *AR* was amplified by PCR analysis using pEZ-M02 AR as the

template and primers *AR* (920aa) 2763bp F-*BglII*: 5'-GGAAGATCTGGATGGAAGT GCAGTTAGGGCTGGG-3'and R-*XhoI*:5'-CCGCTCGAGTCACTGGGTGTGGAA ATAGATGGGCT-3'. The PCR product was digested using restriction enzymes *Bgl II* and *Xho I* (TAKARA, Dalian, China) and sub-cloned into the multiple cloning site of eukaryotic tag expression vector pCMV-Myc, generating a mammalian expression plasmid for *AR*, pCMV-Myc-*AR*.

The full-length cDNA for *PIK3R3* (NC_000001.11, EntrezGeneID8503, Ensembl: ENSG00000117461) was obtained by RT-PCR analysis using RNA samples isolated from HeLa cells. The PCR product was digested with *EcoR I* and *Kpn I* (TAKARA, Japan) and sub-cloned into the multiple cloning site of pCDNA3.1(-), resulting in an expression plasmid for *PIK3R3*, pCDNA3.1(-)-*PIK3R3*.

The expression plasmid for *MIA3/TANGO1*, pcDNA3.1(+)-TANGO1-HA, was as described previously [23-28] and kindly provided by Dr. Vivek Malhotra and colleagues.

We purchased siRNAs from Genepharma (Suzhou, China). The sequences for siRNAs are 5'-GGAUCCUCUUUCUCUACAATT-3' (sense) and 5'-UUGUAGAGA AAGAGGAUCCTT-3' (antisense) (*ADTRP*), 5'-GGACUUGCUUUAUGGGAAAdT dT-3' (sense) and 5'-UUUCCCAUAAAGCAAGUCC dTdT-3'(antisense) (*PIK3R3*), and 5'-GGUGAAGUCUGAAUGCCAUTT-3' (sense) and 5'-AUGGCAUUCAGAC UUCACCTT-3'(antisense) (*MIA3/TANGO1*).

2.3. Androgen receptor inhibitors

Three different inhibitors for the AR were analyzed for their effects on androgen-

induced transcriptional activation of the *ADTRP* promoter. The first potent AR inhibitor is pyrvinium pamoate salt hydrate from Sigma-Aldrich, MO, USA. Pyrvinium pamoate inhibits the AR activity by inhibiting androgen receptor-dependent gene expression via a distinct signaling mechanism [29]. It does not block DNA occupancy by the AR or binding to the ligand-binding domain of the androgen receptor [30]. Pyrvinium pamoate binds to the DNA binding domain of AR at the interface to the minor groove [31].

The second AR inhibitor is bicalutamide from Sigma-Aldrich, MO, the USA. It is a selective antagonist for the AR and blocks androgen binding to the AR and subsequent AR activation [32].

The third AR inhibitor is cyproterone acetate (Selleck Chemicals, Texas, USA). Cyproterone acetate is a potent competitive AR antagonist, which directly blocks binding of androgen to the AR and subsequent activation of the AR [33].

2.4. Cell transfection

For plasmid DNA, HeLa cells were transfected with 500 ng of wild type (WT) pGL3-Basic-ADTRPp-Luc or mutant reporters together with 25 ng of Renilla *luciferase* reporter plasmid using Lipofectamine®2000 (Invitrogen, Thermo Fisher Scientific, the Massachusetts, USA) according to the manufacturer's instruction. Transfection of EAhy926 cells was carried out using the Fu GENE® HD Transfection Reagent from Promega (Madison, Wisconsin, USA).

Transfection of siRNA was performed for HeLa and EAhy926 cells using

Lipofectamine® RNAi MAX (Invitrogen, Thermo Fisher Scientific, USA) and Fu GENE®HD Transfection Reagent (Promega, Madison, Wisconsin, the USA), respectively.

2.5. Dual luciferase assays for the ADTRP promoter

Cells were cultured and transfected as describe above. Cells were harvested 48h after transfection and used for dual luciferase assays using the Dual-luciferase reporter assay system (Promega, Madison, Wisconsin, the USA) according to the manufacturer's instructions and as described [34-36]. The firefly luciferase activity was normalized to the corresponding renilla luciferase activity. Each luciferase assay was performed in triplicate and repeated at least three times.

2.6. Cell proliferation and toxicity assay

HeLa and EAhy926 cells (5×10^3 per well) were plated in 96-well plates, and transfected as described above. The transfected cells were then treated for 12 hours with androgen (testosterone, 5nM) and an AR-antagonist (pyrvinium pamoate, bicalutamide or cyproterone acetate) at the concentration of 0nM, 0.02nM, 0.05nM, 0.1nM, 0.25nM and 0.5nM, respectively. Cell proliferation assays were carried out using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instruction [17]. Each assay was performed in triplicate and repeated at least three times.

2.7. Chromatin immunoprecipitation (ChIP) assays

ChIP was performed using the chromatin immunoprecipitation linked PCR assay with the EZ-ChIP kit (Merck Millipore, Germany). Cross-linked chromatin was extracted from HeLa cells and EAhy926 endothelial cells transfected with the Myc-tagged AR expression plasmid pCMV-Myc-AR and immunoprecipitated using an anti-Myc antibody (Merck Millipore, Germany). Rabbit IgG was used as a negative control, whereas an anti-RNA polymerase II antibody (Merck Millipore, Germany) was used as a positive control. The precipitated DNA was used for PCR detection of the ARE bound by the AR and the promoter bound the RNA polymerase II. The PCR primers for the ARE include forward primer 5'-GCATATACCACTTCCTTGTT CTGAGCTG-3' and reverse primer 5'-TCATATATTTCCACCTTGCACCATTTG-3'. The negative control for PCR was performed for a distal, non-ARE site using forward primer 5'-CTTCCCAGTTTACAGTATGTTGTTATAGCA-3' and reverse primer 5'-GAGTTCTGCATTGTAACCATGTTTTTTT-3'. The PCR primers for the promoter (RNA polymerase II) included the forward primer 5'-GCAACTTCCTTGTT ATATGCAAATGAAAAG-3' and reverse primer 5'-CTGTTTCACTAGGGCCTGGG GCA-3'. Each ChIP assay was replicated at least three times.

2.8. Monocyte adhesion to endothelial cells assay

An assay for monocyte adhesion to ECs was carried out as described [18, 37]. In brief, EAhy926 endothelial cells were transfected with siRNA or plasmid DNA as described above. The cells were seeded in 6-well plates at a density of 8×10^4 cells/

well, grown to fully confluent and stimulated with oxidized LDL (0.8mg/ml ox-LDL, Yesen, Shanghai, China) for 6hours. Human HL-60 promyelocytic leukemia cells were cultured and fluorescently labeled with Calcein AM (Fanbo, Beijing, China) for 60 min at 37°C, and seeded into the wells coated with EAhy926 endothelial cells. After 1 hour, the media were removed and fluorescence intensity was measured with a standard fluorometer with an inverse microscope (Nikon, The Tokyo metropolitan, Japan). The fluorescent units reflecting the number of adhered HL-60 cells to EAhy926 cells (exit: 490 nm, emission: 515 nm) was determined and analyzed with Image Pro Plus6.0software (Media Cybernetics, Bethesda, MD, the USA). Each experiment was repeated at least three times.

2.9. Transmigration of monocytes across endothelial cells

Transmigration of monocytes across endothelial cells was analyzed by a Transwell assay using the Boyden chamber method as described [17, 18, 37]. In brief, HL-60cells (3×10^5) were loaded into 0.4 μm pore size Transwell membranes within a Boyden chamber (Corning), which were plated with confluent EAhy926 endothelial cells transfected with siRNA or plasmid DNA. Cells were stimulated for 6h with 0.25 μg of ox-LDL 24 hours after the transfection. The HL-60 cells migrated to the bottom chamber 24 h after their administration were counted under an Olympus 81 FV1000 microscope. Each experiment was repeated at least three times.

2.10. Expression analyses

Real-time RT-PCR analysis was performed as described previously by us [17, 18, 38]. The primer sequences used are as follows:

ADTRP Forward: 5'-GCCGCATCCTATGGCTCTACTTTG-3'

ADTRP Reverse: 5'-CAAGTAGGTAGATGCTGGCGATGA-3'

MIA3 Forward: 5'-TACAAGCGGAGAATTGAAGAAATGG-3'

MIA3 Reverse: 5'-GCCAGTTTTCATGAGCTTTCTTCT-3'

GAPDH Forward: 5'-ATGGGGAAGGTGAAGGTCG-3'

GAPDH Reverse: 5'-GGGGTCATTGATGGCAACAATA-3'

PIK3R3 Forward: 5'-ATGTACAATACGGTGTGGAGTATG-3'

PIK3R3 Reverse: 5'-GCTGGAGGATCCATTTCAAT-3'

Western blot analysis was carried out as described previously by us [17, 18]. The primary antibodies used include a rabbit polyclonal antibody against ADTRP (*C6orf105*) (1:800 dilution; Sigma, MO,USA), a rabbit polyclonal antibody against MIA3/TANGO1 (1:500; Santa Cruz Biotechnology, Dallas, TX,USA), a mouse monoclonal antibody against α-tubulin (1:5000; Merck Millipore, a part of Merck KGaA, Darmstadt, Germany), a polyclonal anti-goat antibody against ApoB (1:1000, Abcam, MA, USA), and a rabbit polyclonal antibody against collagen VII (1:500, Abcam, MA, USA). The secondary antibodies were HRP-labeled rabbit anti-goat antibody, goat anti-rabbit antibody and goat anti-mouse antibody (1:20,000 dilution) (Thermo Fisher Scientific).

2.11. Statistical analysis

The data were presented as means ± standard error of means (SEM) unless specifically noted and were from at least three independent experiments. Statistical analysis was carried out using a Student's t-test or ANOVA with SPSS version17.0 software (SPSS, Chicago, IL, USA). Statistical significance was considered at a *P* value of 0.05 or less.

3. Results

3.1. Androgen induces ADTRP transcriptional activation in a dose-dependent and time-dependent manner

Lupu et al. used immunostaining analysis to show that androgen increased the cell surface expression level of the ADTRP protein by about 2-fold [16]. However, it is unknown whether the regulation of *ADTRP* expression by androgen is on the transcriptional level. We constructed an *ADTRP* promoter *luciferase* reporter to demonstrate that androgen activates *ADTRP* expression on the transcriptional level. HeLa cells were transfected with 500ng of the pGL3Basic-ADTRP-Luc reporter plasmid and 25ng of Renilla luciferase reporter plasmid, treated with different concentrations of testosterone, and collected at different time points for luciferase assays. Luciferase assays revealed that testosterone induced transcriptional activation of the *ADTRP* promoter in a dose-dependent manner in HeLa cells (Fig. 1A). Testosterone also induced activation of the *ADTRP* promoter in a time-dependent manner in HeLa cells, especially at lower concentrations of androgen

(Fig. 1A). Similar observations were made in EAhy926 endothelial cells (Fig. 1B).

3.2. Androgen receptor inhibitors and antagonists block androgen-induced transcriptional activation of the ADTRP promoter

To further verify that androgen regulates *ADTRP* transcriptional activation, we studied three androgen receptor inhibitors and antagonists for their effects on transcriptional activation of the *ADTRP* promoter using luciferase assays. Testosterone induced strong transcriptional activation of the *ADTRP* promoter, but AR inhibitor pyrvinium pamoate abolished androgen-induced transactivation of the *ADTRP* promoter in HeLa cells (Fig. 2A) and EAhy926 cells (Fig. 2B). Similar results were obtained for AR antagonist bicalutamide (Fig. 2C-2D) and cyproterone acetate (Fig. 2E-2F). Further luciferase assays showed that bicalutamide and cyproterone acetate blocked androgen-induced *ADTRP* transcriptional activation in a dose-dependent manner in both HeLa cells (Fig. 3A-3D) and EAhy926 cells (Fig. 3E-3H). The doses of bicalutamide and cyproterone acetate used in the study were not toxic to cells (Fig. S1), suggesting that the effects of androgen antagonists on activation of *ADTRP* promoter were not due to toxic effects on cell growth.

3.3. Androgen activates ADTRP transcription through the androgen responsive element (ARE) at the ADTRP promoter/regulatory region

To identify the molecular mechanism by which androgen regulates transcriptional activation of the *ADTRP* promoter, we searched for potential binding sites for the AR

at the *ADTRP* promoter/regulatory region and found one half androgen-responsive element (ARE) (TGTTCT) at the position of +324bp from the transcriptional start site (Fig. 4A). We mutated the half ARE site from TGTTCT to either AAAAAT (mutation 1) or TAAAAA (mutation 2). Compared to the WT *ADTRP* promoter, both mutant promoters lost responses to testosterone induction in both HeLa cells (Fig. 4B) and EAhy926 endothelial cells (Fig. 4C). These data suggest that the half ARE is responsible for the androgen-induced transcriptional activation of the *ADTRP* promoter.

3.4. Androgen receptor interacts with the half ARE at the ADTRP promoter/regulatory region

We performed chromatin immunoprecipitation (ChIP) analysis to determine whether the AR binds to the half ARE at the *ADTRP* promoter/regulatory region *in vivo*. HeLa cells were transfected with an expression plasmid for a Myc-tagged AR. The protein-DNA complex formed between the half ARE and Myc-tagged AR was immunoprecipitated using an anti-Myc antibody. The DNA associated with AR was pulled down by ChIP and detected by PCR analysis using primers spanning the ARE (Fig. 5A). As shown in Fig. 5B, the anti-Myc (AR) antibody successfully pulled the ARE down, but the control IgG failed to pull the ARE down. As negative controls, neither anti-Myc nor IgG pulled DNA at a distal non-specific control region down (Fig. 5A-5B). We used an anti-RNA Polymerase II antibody as a positive control for ChIP analysis (Fig. 5C). The anti-RNA Polymerase II antibody, but not the negative

control rabbit IgG, was able to pull DNA at the TSS of the *ADTRP* promoter down (Fig. 5C). Similar data were obtained in EAhy926 endothelial cells (Fig. 5D-5E). These data suggest that the AR binds to the half ARE at the *ADTRP* promoter/ regulatory region.

3.5. *Androgen has similar effects on endothelial cell proliferation, cell cycle progression, migration and apoptosis as ADTRP*

We previously reported that *ADTRP* regulates the proliferation, cell cycle progression and apoptosis of endothelial cells [17]. Because androgen regulates the expression of *ADTRP*, we hypothesized that androgen may have similar effects on endothelial cell functions as *ADTRP*. As shown in Fig. 6A-6B, similar to overexpression of *ADTRP*, EAhy926 cells treated with androgen showed significantly increased proliferation at three time points of 24, 48 and 72 hours compared to cells treated with vehicle. For the number of cells at the S phase, similar to overexpression of *ADTRP*, EAhy926 cells treated with androgen showed a significantly increased number of cells compared to cells treated with vehicle (Fig. 6C-6D). Similarly, androgen had similar effect on promoting migration of EAhy926 cells as overexpression of ADTRP (Fig. 6E-6F). For apoptosis, similar to overexpression of *ADTRP*, EAhy926 cells treated with androgen showed a significantly decreased rate of apoptosis compared to cells treated with vehicle (Fig. 6G-6H).

3.6. Androgen regulates the levels of ADTRP-downstream genes/proteins PIK3R3, MIA3/TANGO1, collagen VII and ApoB

We previously reported that *ADTRP* regulates the expression of *PIK3R3,*which activates AKT and increases the expression level of *MIA3/TANGO1*, resulting in increased levels of collagen VII and ApoB [18]. As androgen regulates the expression of *ADTRP*, we examined the effect of androgen on the levels of *PIK3R3, MIA3/TANGO1,* collagen VII and ApoB. Western blot analysis showed that similar to the effect of expression of *ADTRP* (Fig. 7A-7B) androgen treatment of EAhy926 cells significantly increased the levels of MIA3/TANGO1 (Fig. 7C-7D), collagen VII (Fig. 7E-7F), and ApoB (Fig. 7G-7H), whereas the effects were almost abolished by androgen inhibitors bicalutamide and cyproterone acetate. Similar effects of androgen were observed on the mRNA levels for *PIK3R3* and *MIA3* by real-time RT-PCR analysis (Fig. 8). The real-time RT-PCR analysis was not performed for COL7A1 encoding collagen VII and ApoB because their regulation is on the level of protein trafficking [18].

3.7. Androgen inhibits monocyte adhesion to endothelial cells, a functional process directly relevant to atherosclerosis, via activating ADTRP expression

Reduced androgen levels were shown to be associated with risk of cardiovascular disease, therefore, androgen is protective of cardiovascular disease [18, 39]. The molecular and cellular mechanisms by which androgen reduces risk of cardiovascular disease are unknown. Because androgen induces transcriptional activation of

ADTRP, a susceptibility gene for CAD and MI, we hypothesized that androgen reduces risk of cardiovascular disease by activating expression of *ADTRP*. To test the hypothesis, we characterized the effects of androgen in combination with knockdown or overexpression of *ADTRP* on monocyte adhesion to the endothelium (a layer of ECs), the first event involved in the initiation of atherosclerosis [37]. Upon stimulation with oxidized-LDL (ox-LDL), significantly less HL-60 cells showed adhesion to EAhy926 cells plated on the bottom of culture wells when treated with androgen than treatment with vehicle ($P<0.001$) (Fig. 9A and 9N). The data are consistent with the protective effect of androgen on atherosclerosis. The effect of androgen was partially rescued by knockdown of *ADTRP* expression with siRNA (Fig. 9B, 9N) and by siRNAs for *PIK3R3* and *MIA3/TANGO1* (Fig. 9L-9M and 9N). The data suggest that androgen reduces monocyte adhesion to endothelial cells by activating *ADTRP* expression and expression of *ADTRP*-downstream genes *PIK3R3* and *MIA3/TANGO1* (9C, 9F, 9I and 9N). On the contrary, overexpression of *ADTRP* by transient transfection of EAhy926 cells with an *ADTRP* expression further reduced HL-60 cell adhesion to endothelial cells by androgen (Fig. 9C and 9N). As controls, androgen receptor antagonists bicalutamide and cyproterone acetate blocked the androgen-induced reduction of adhesion of HL-60 to endothelial cells (Fig. 9D-9E and 9N). Similar to *ADTRP*, overexpression of *ADTRP*-downstream genes *PIK3R3* and *MIA3* also further reduced HL-60 cell adhesion to endothelial cells by androgen (Fig. 9F, 9I and 9N), but the effects were abolished by bicalutamide or cyproterone acetate (Fig. 9G-9H, 9J-9K,and 9N).

The effects of androgen and *ADTRP* as well as *ADTRP*-downstream genes *PIK3R3* and *MIA3* on monocyte adhesion to endothelial cells occurred only when cells were treated with ox-LDL, but not with native LDL (Fig. 9A-9E, 9F-9M and 9O).

3.7. Androgen inhibits monocyte transmigration across a layer of endothelial cells, a functional process directly relevant to atherosclerosis, via activating ADTRP expression

Trans-endothelial migration of monocytes is one of the most important processes in the initiation of atherosclerosis [18, 37]. We coated the Transwell membrane with confluent EAhy926 endothelial cells transfected with siRNA or plasmid DNA and inserted it into the Boyden chamber. Cells were treated with ox-LDL or other agents. HL-60 cells were then loaded on the top of the endothelial cell layer. The HL-60 cells migrated across the endothelial layer were counted and analyzed. Androgen treatment significantly inhibited ox-LDL-inducedHL-60 migration across the layer of EAhy926 cells (Fig. 10), which is consistent with the protective effect of androgen on atherosclerosis. Knockdown of *ADTRP* expression by siRNA attenuated the effect of androgen (Fig. 10), suggesting that androgen reduces trans-endothelial migration of monocytes by activating *ADTRP* expression. Similar to *ADTRP* siRNA, PIK3R3 and MIA3/TANGO1 siRNAs also attenuated the effect of androgen on monocyte transmigration of endothelial cells (Fig. 10). Consistent with this, overexpression of *ADTRP* by transient transfection of EAhy926 cells with an *ADTRP* expression further

reduced HL-60 cell migration across a layer of EAhy926endothelial cells by androgen (Fig. 10). As controls, androgen receptor antagonists bicalutamide and cyproterone acetate blocked the androgen-induced reduction of HL-60 migration across to a layer of EAhy926endothelialcells (Fig. 10). Similar to *ADTRP*, overexpression of *ADTRP*- downstream gene *PIK3R3* and *MIA3*in EAhy926 cells further reduced HL-60 cell migration across a layer of EAhy926endothelial cells by androgen, and the effects were abolished by androgen receptor antagonists bicalutamide and cyproterone acetate (Fig. 10).

4. Discussion

Previously, Lupu et al. used qRT-PCR to show that *ADTRP* mRNA expression was up-regulated in endothelial cells by 30nMdihydrotestosteronetreatment for 24 hours [16]. In addition, immunostaining showed that the cell surface expression of the ADTRP protein was also increased by dihydrotestosterone treatment [16]. However, it is unknown whether androgen regulates *ADTRP* expression at the transcriptional level. In this study, we further assessed the regulation of *ADTRP* expression by androgen using luciferase assays of an *ADTRP* promoter luciferase reporter gene. Testosterone treatment increased the *ADTRP* promoter luciferase reporter activity in a dose-and time-dependent manner and three different androgen inhibitors/antagonists, including pyrvinium pamoate, bicalutamide and cyproterone acetate, abrogated the effects of testosterone (Figs. 1-3). These data suggest that androgen regulation of *ADTRP* expression is on the transcriptional level. ChIP

analysis showed that the androgen receptor binds to the half ARE (TGTTCT) at the promoter/regulatory region of *ADTRP* (Fig. 5). Mutational analysis showed that the half ARE is critical to the transcriptional activation of ADTRP and mutations of the ARE abolished the transcriptional activation of *ADTRP* (Fig. 4). Our data suggest that androgen induces transcriptional activation of *ADTRP* through direct binding of the androgen receptor to the ARE at the *ADTRP* promoter/regulatory region.

Atherosclerosis in coronary arteries is the cause of CAD and MI [40, 41]. Atherosclerosis is initiated with the activation of endothelial cells (endothelium) by ox-LDL or other inflammatory molecules, which attracts adhesion of monocytes to the endothelium [40, 41]. The monocytes then transmigrate across the endothelium and become macrophages. Macrophages ingest ox-LDL and other lipids, attract other cells, and induce apoptosis, which leads to formation of foam cells and plaques, the hallmark of atherosclerosis [40, 41]. The molecular mechanism by which low levels of androgen increase the risk of atherosclerosis is unknown. Our data in this study identified one potential mechanism (the first molecular mechanism to the best of knowledge): androgen regulates expression of CAD/MI susceptibility gene *ADTRP* and reduces risk of cardiovascular disease by activating *ADTRP* expression (Fig. 6 and Fig. 7). Androgen inhibited monocyte adhesion to EAhy926 endothelial cells and blocked monocyte transmigration across the endothelial cells (Fig. 6 and Fig. 7). These effects of androgen were reversed by knockdown of *ADTRP* expression (Fig. 6 and Fig. 7). The data suggest that *ADTRP* expression is important for androgen-mediated inhibition of monocyte adhesion to endothelial cells and monocyte

transmigration across the endothelial cells, the two key processes in atherosclerosis. Together, our data suggest that the molecular mechanism by which androgen confers protection against CAD and MI is stimulation of *ADTRP* expression.

Although knockdown of *ADTRP* expression by siRNA reversed the effects of androgen on monocyte adhesion to endothelial cells and migration of monocytes across endothelial cells, the reversal was not 100% (Fig. 6 and Fig. 7). The data suggest that in addition to *ADTRP*, other downstream target genes regulated by androgen may also be involved in the action of androgen on atherosclerosis. As the androgen receptor is a transcriptional factor, it regulates expression of numerous genes. Future studies are needed to identify other downstream target genes of the androgen and AR and to distinguish which other AR-regulated genes are also involved in reducing the risk of atherosclerosis, in addition to *ADTRP*.

In conclusion, the data in this study showed that androgen induced up-regulation of *ADTRP* expression on the transcriptional level and the underlying molecular mechanism is the direct binding of the androgen receptor to the half ARE at the promoter/regulatory region, which activate transcription of *ADTRP*. Most importantly, we showed that androgen blocked two key endothelial functions/ processes associated with atherosclerotic CAD: monocyte adhesion to the endothelium and transmigration of monocytes across the endothelium, and the effects were mediated by activating the transcription of the CAD susceptibility gene *ADTRP*, at least partly.

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Conflict of interest

K.L., W.Z., E.P., S.H.C., C.P., M.B., J.H., and V.L. are current or formal employees of Bayer. Other authors are the members of Wang Laboratory, which received funding from Bayer AG.

Disclosures

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Supplementary material

Supplementary Figure 1.Supplementary material is available at BBA Molecular Basis of Disease.

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Figure legends:

Fig. 1 *ADTRP* expression is induced by testosterone in HeLa and EAhy926 endothelial cells in a dose-and time-dependent manner. An *ADTRP* promoter luciferase reporter pGl3-Basic-ADTRPp-luc reporter (500 ng) and a renilla luciferase plasmid (25 ng) were co-transfected into HeLa cells (A) or EAhy926 cells (B), cultured for 48 hours, and used for luciferase assays. Treatments with testosterone were made at the time points of 36 hours (12 hour treatment), 39 hours (9 hour treatment) and 42 hours (6 hour treatment). Cells were collected and lysed and used for dual luciferase assays. ***, $P<0.001$ (n=4).

Fig.2 AR inhibitors and antagonists block transcriptional activation of the *ADTRP* promoter stimulated by testosterone. HeLa cells and EAhy926 cells were treated and cultured as described in the Fig. 1 legend except for addition of a treatment group with both testosterone and an androgen inhibitor or antagonist. Cells were collected and lysed and used for dual luciferase assays. (A) Effect of AR inhibitor pyrvinium pamoate on androgen-induced *ADTRP* transactivation in HeLa cells. (B) Effect of AR inhibitor pyrvinium pamoate on androgen-induced *ADTRP* transactivation in EAhy926 cells. (C) Effect of AR antagonist bicalutamide on androgen-induced *ADTRP* transactivation in HeLa cells. (D) Effect of AR antagonist bicalutamide on androgen-induced *ADTRP* transactivation in EAhy926 endothelial cells. (E) Effect of AR antagonist cyproterone acetate on androgen-induced *ADTRP* transactivation in HeLa cells. (F) Effect of AR antagonist cyproterone acetate on androgen- induced *ADTRP* transactivation in EAhy926 endothelial cells. ***, $P<0.001$ (n=4).

Fig. 3 AR antagonists block testosterone-induced *ADTRP* transactivation in a dose-

dependent manner. HeLa cells and EAhy926 cells were treated and cultured as described in the Fig. 2 legend except that the concentration of testosterone was fixed at 5 nM and that different concentrations of AR antagonists were used. Cells were collected and lysed and used for dual luciferase assays. (A) Effect of AR antagonist bicalutamide (0-5 nM) on androgen-induced *ADTRP* transactivation in HeLa cells. (B) Effect of AR antagonist bicalutamide (0-0.5 nM) on androgen-induced *ADTRP* transactivation in HeLa cells. (C) Effect of AR antagonist cyproterone acetate (0-5 nM) on androgen-induced *ADTRP* transactivation in HeLa cells. (D) Effect of AR antagonist cyproterone acetate (0-0.5 nM) on androgen- induced *ADTRP* transactivation in HeLa cells. (E) Effect of AR antagonist bicalutamide (0-5 nM) on androgen-induced *ADTRP* transactivation in EAhy926 endothelial cells. (F) Effect of AR antagonist bicalutamide (0-0.5 nM) on androgen- induced *ADTRP* transactivation in EAhy926 endothelial cells. (G) Effect of AR antagonist cyproterone acetate (0-5 nM) on androgen-induced *ADTRP* transactivation in EAhy926 endothelial cells. (H) Effect of AR antagonist cyproterone acetate (0-0.5nM) on androgen-induced *ADTRP* transactivation in EAhy926 endothelial cells. ***, $P<0.001$ (n=4).

Fig.4 Androgen-induced *ADTRP* transactivation is mediated by a half androgen response element in the *ADTRP* promoter/regulatory region. (A) Schematic diagram showing the structure of the *ADTRP* promoter/regulatory region. ARE, a half androgen-responsive element (TGTTCT); TSS, transcriptional start site (marked as position +1). (B) Transcriptional activation activity (luciferase activity) from wild

type (WT) and mutant *ADTRP* promoters with the ARE mutated in HeLa cells. Mutation 1, ARETGTTCT mutated to AAAAAT; Mutation 2, ARETGTTCT mutated to TAAAAA. (C) Transcriptional activation activity (luciferase activity) from wild type (WT) and mutant *ADTRP* promoters with the ARE mutated in EAhy926 endothelial cells. Cells were treated with different concentrations of testosterone varying from 0 to 5 nM for 12 hours. **, $P<0.01$ (n=4); ***, $P<0.001$ (n=4).

Fig. 5 Chromatin immunoprecipitation (ChIP) for detection of the interaction between the AR and a half ARE at the *ADTRP* promoter/regulatory region. (A) Schematic diagram of the promoter/regulatory region of the human *ADTRP* gene. TSS, the transcription start site (+1 position); ARE, androgen-responsive element. (B) ChIP analysis showing that a Myc-tagged AR interacts with the ARE at the proximal *ADTRP* promoter/regulatory region in HeLa cell. An anti-Myc antibody pulled down chromatin containing the *ADTRP* ARE, but not a non-specific, control distal genomic DNA fragment. (C) Positive control for ChIP in HeLa cells. An anti-RNA Polymerase II antibody pulled down the chromatin containing the *ADTRP* TSS, but not a non-specific, control distal genomic DNA fragment. (D) Similar ChIP analysis as in (B), but in EAhy926 endothelial cells. (E) Similar control ChIP analysis as in (C), but in EAhy926 endothelial cells. ***, $P<0.001$ (n=4); NS, not significant.

Fig. 6 Androgen has similar effects on EC proliferation, cell cycle progression, migration and apoptosis as overexpression of *ADTRP*. (A) Cell proliferation analysis for EAhy926 cells treated with androgen vs. vehicle (no androgen) control.

***, *P*<0.001 (n=3). (B) Cell proliferation analysis for EAhy926 cells with overexpression of *ADTRP* (transfection with an expression plasmid for *ADTRP*) or without *ADTRP* overexpression (transfection with the empty vector). **, *P*<0.01 (n=3); ***, *P*<0.001 (n=3). (C) Representative flow cytometry data showing the cell number at the S phase of cell cycle for EAhy926 cells with or without overexpression of *ADTRP* vs. vector, and treated with androgen vs. vehicle. (D) Summary data from (C). *, *P*<0.05 (n=3); **, *P*<0.01 (n=3). (E) Representative images for scratch-based cell migration analysis for EAhy926 cells with or without overexpression of *ADTRP* vs. vector, and treated with androgen vs. vehicle. (F) Summary data from (E). ***, *P*<0.001 (n=3). (G) Representative flow cytometry images for analysis of apoptosis for EAhy926 cells with or without overexpression of *ADTRP* vs. vector, and treated with androgen vs. vehicle. (H) Summary data from experiments as shown in (G), ***, *P*<0.001 (n=3).

Fig. 7 Androgen regulates the levels of *ADTRP*-downstream targets PIK3R3 and MIA3/TANGO1 as well as the levels of collagen VII and ApoB downstream of MIA3/TANGO1. (A) Western blot analysis showing that the expression level of ADTRP in EAhy926 cells was induced by androgen and inhibited by androgen receptor antagonists bicalutamide and cyproterone acetate. (B) Summary data from (A). (C) Western blot analysis showing that the expression level of MIA3/TANGO1 in EAhy926 cells was induced by androgen and inhibited by androgen receptor antagonists bicalutamide and cyproterone acetate. (D) Summary data from (C). (E) Western blot analysis showing that the expression level of collagen VII in EAhy926

cells was induced by androgen and inhibited by androgen receptor antagonists bicalutamide and cyproterone acetate. (F) Summary data from (E). (G) Western blot analysis showing that the expression level of ApoB in EAhy926 cells was induced by androgen and inhibited by androgen receptor antagonists bicalutamide and cyproterone acetate. (H) Summary data from (G). ***, $P<0.001$ (n=3).

Fig. 8 Androgen regulates the levels of *ADTRP*-downstream targets *PIK3R3* and *MIA3/TANGO1*. (A) Real-time RT-PCR analysis showing that the expression level of *ADTRP* in EAhy926 cells was induced by androgen and inhibited by androgen receptor antagonists bicalutamide and cyproterone acetate. (B) Real-time RT-PCR analysis showing that the expression level of *ADTRP* in HeLa cells was induced by androgen and inhibited by androgen receptor antagonists bicalutamide and cyproterone acetate. (C) Real-time RT-PCR analysis showing that the expression level of *PIK3R3* in EAhy926 cells was induced by androgen and inhibited by androgen receptor antagonists bicalutamide and cyproterone acetate. (D) Real-time RT-PCR analysis showing that the expression level of *PIK3R3* in HeLa cells was induced by androgen and inhibited by androgen receptor antagonists bicalutamide and cyproterone acetate. (E) Real-time RT-PCR analysis showing that the expression level of *MIA3* in EAhy926 cells was induced by androgen and inhibited by androgen receptor antagonists bicalutamide and cyproterone acetate. (F) Real-time RT-PCR analysis showing that the expression level of *MIA3* in HeLa cells was induced by androgen and inhibited by androgen receptor antagonists bicalutamide and cyproterone acetate. *, $P<0.05$ (n=4); **, $P<0.01$ (n=4); ***, $P<0.001$ (n=4).

Fig. 9 Androgen inhibits ox-LDL-mediated monocyte adhesion to endothelial cells by inducing expression *of ADTRP* and its downstream genes *PIK3R3* and *MIA3/TANGO1*. (A) Representative images for analysis of monocyte adhesion to EAhy926 endothelial cells stimulated by ox-LDL (native LDL as control) in the presence and absence of testosterone treatment (5 nM). (B) Effect of *ADTRP* siRNA on androgen-blocked HL-60 monocyte adhesion to EAhy926 endothelial cells. (C) Effect of *ADTRP* overexpression on androgen-blocked monocyte adhesion to EAhy926 endothelial cells. (D) Effect of bicalutamide on androgen-blocked monocyte adhesion to EAhy926 endothelial cells. (E) Effect of cyproterone acetate on androgen-blocked monocyte adhesion to EAhy926 endothelial cells. (F) Effect of *MIA3/TANGO1* overexpression on androgen-blocked monocyte adhesion to EAhy926 endothelial cells. (G) Effect of both *MIA3/TANGO1* overexpression and bicalutamide on androgen-blocked monocyte adhesion to EAhy926 endothelial cells. (H) Effect of both *MIA3/TANGO1* overexpression and cyproterone acetate on androgen-blocked monocyte adhesion to EAhy926 endothelial cells. (I) Effect of *PIK3R3* overexpression on androgen-blocked monocyte adhesion to EAhy926 endothelial cells. (J) Effect of both *PIK3R3* overexpression and bicalutamide on androgen-blocked monocyte adhesion to EAhy926 endothelial cells. (K) Effect of both *PIK3R3* overexpression and cyproterone acetate on androgen-blocked monocyte adhesion to EAhy926 endothelial cells. (L) Effect of *MIA3/TANGO1* siRNA on androgen-blocked monocyte adhesion to EAhy926 endothelial cells. (M) Effect of

PIK3R3 siRNA on androgen-blocked monocyte adhesion to EAhy926 endothelial cells. (N) Summary data from experiments in (A-M) with ox-LDL treatment. (O) Summary data from experiments in (A-M) with native LDL treatment. **, $P<0.01$ (n=4); ***, $P<0.001$; NS, not significant.

Fig. 10 Androgen inhibits ox-LDL-mediated transmigration of monocytes across a layer of EAhy926 endothelial cells by inducing expression of *ADTRP* and its downstream genes *PIK3R3* and *MIA3*. HL-60monocytes (3×10^5) were loaded on the top of confluent EAhy926 endothelial cells transfected with siRNA or plasmid DNA and stimulated with ox-LDL (native LDL as control) in the presence and absence of testosterone treatment (5 nM) cultured on 0.4 μm pore size Transwell membranes within a Boyden chamber. Twenty-four hours later, the HL-60 cells that have migrated to the bottom of the Transwell and into the chamber were manually counted under a microscope. Each experiment was repeated at least three times. Each experiment was repeated at least three times. ***, $P<0.001$ (n=4).

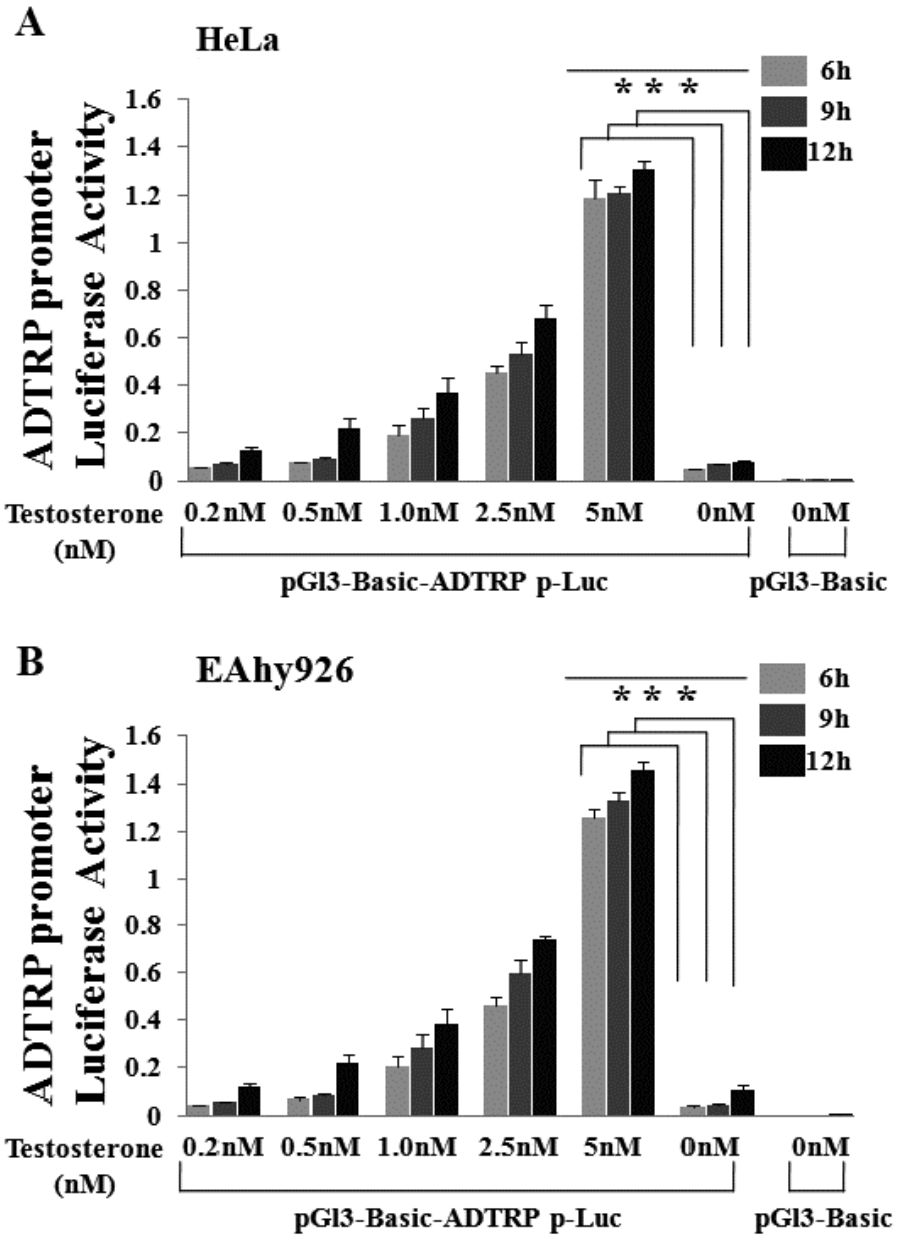


Figure 1

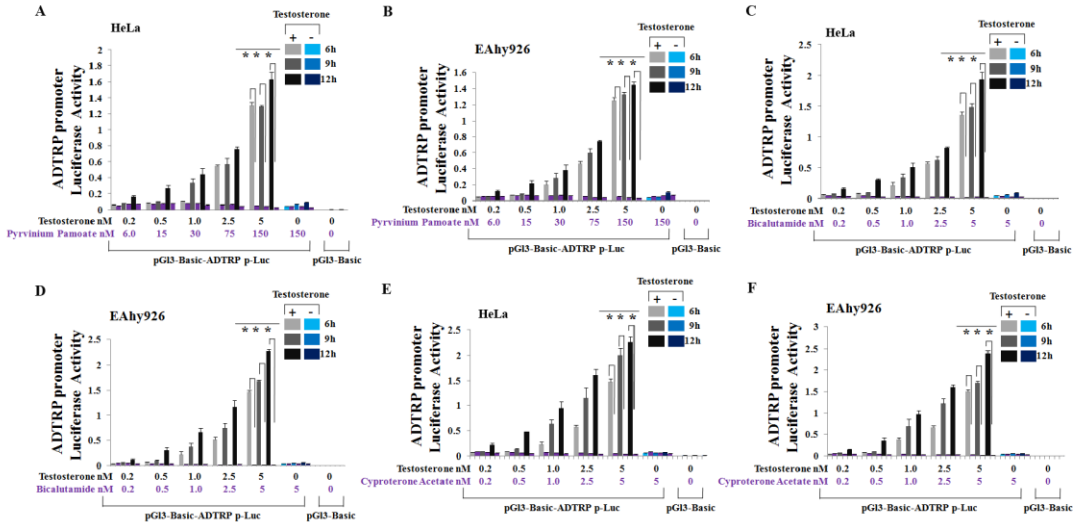
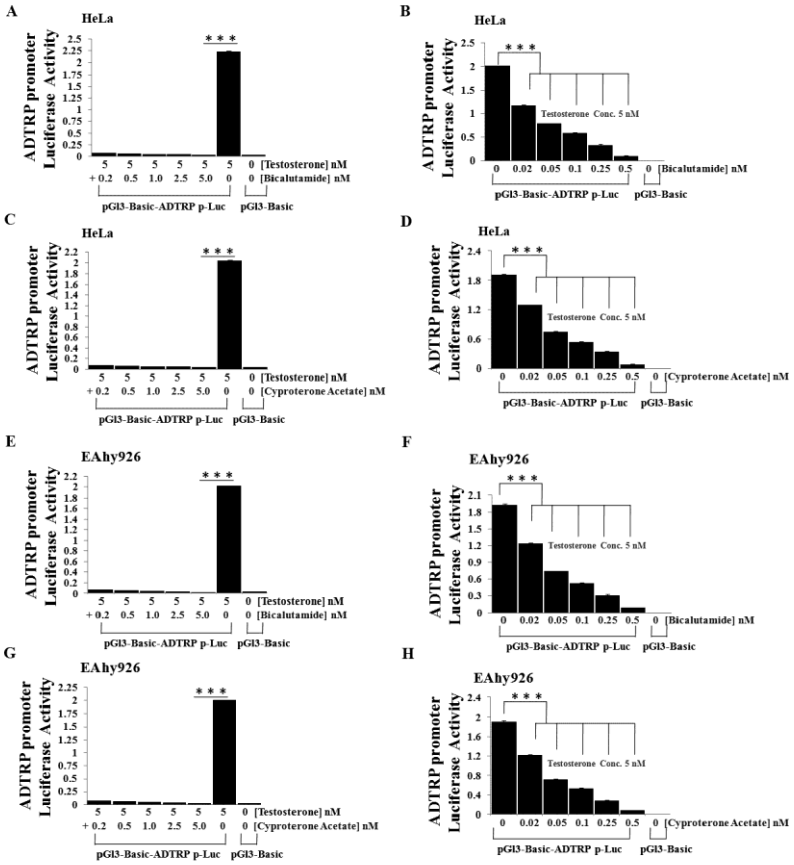


Figure 2



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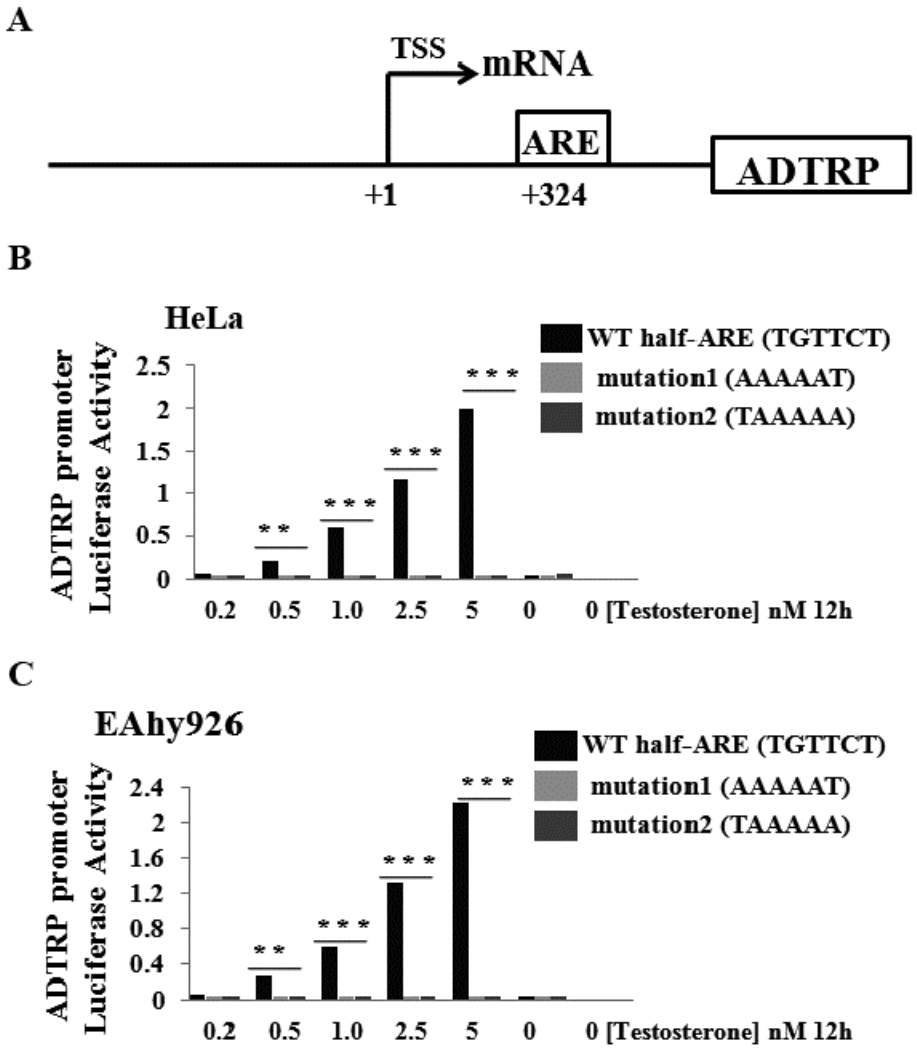

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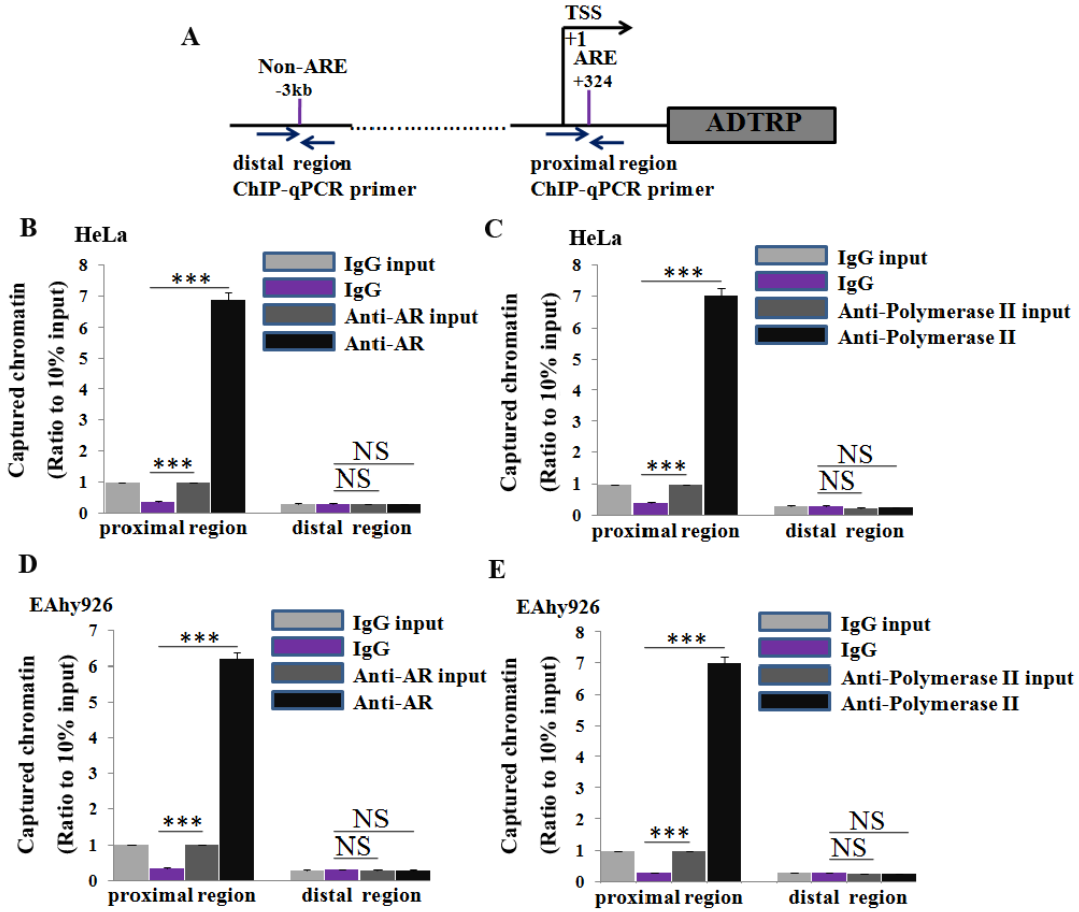

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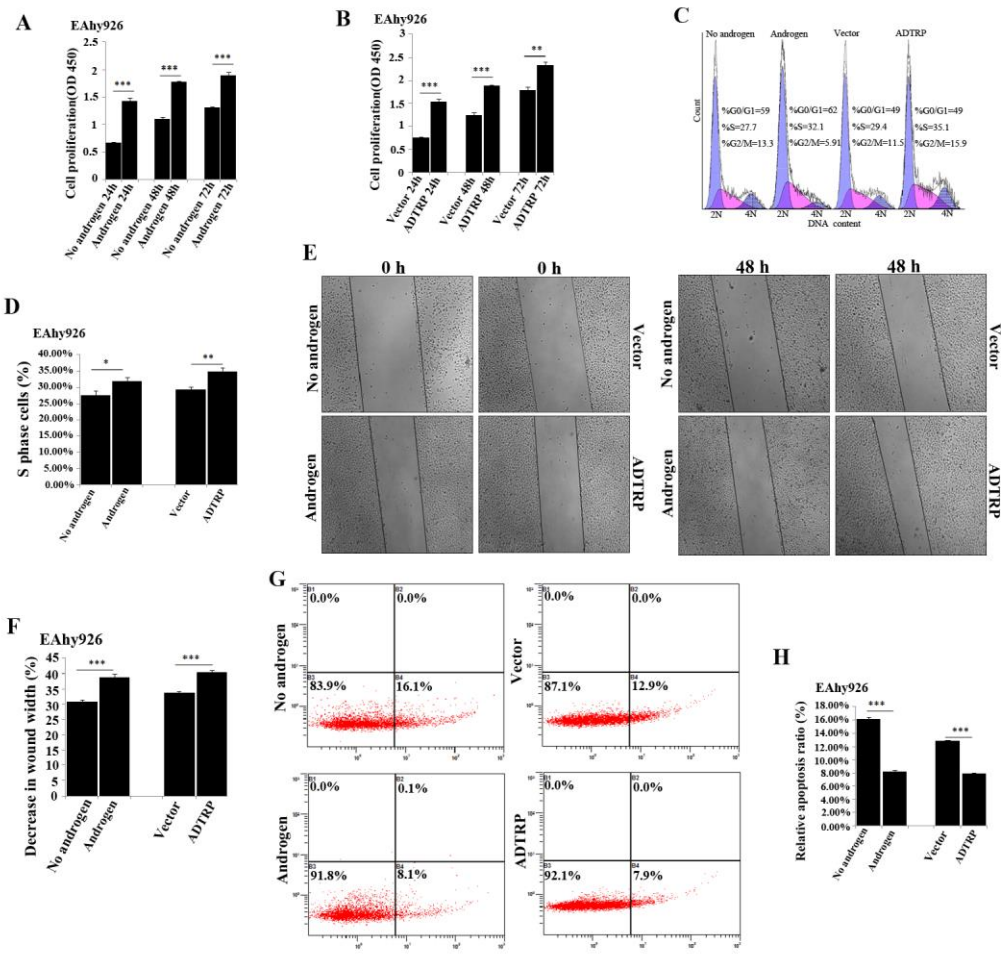
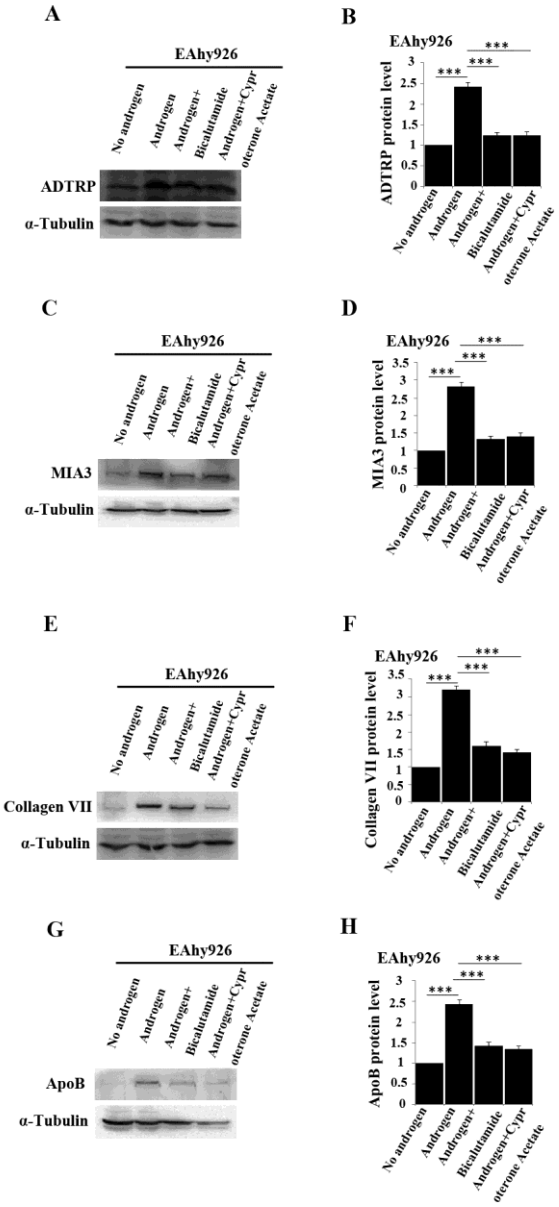


Figure 6



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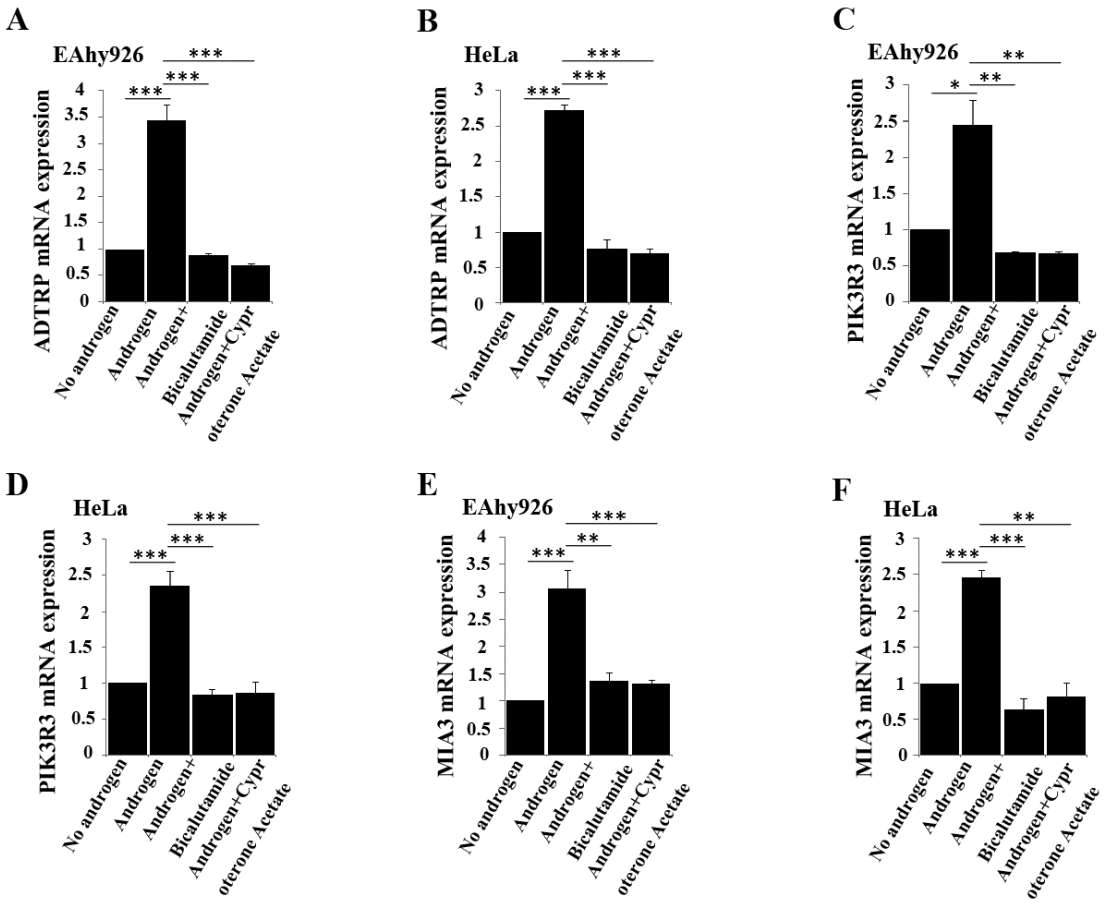

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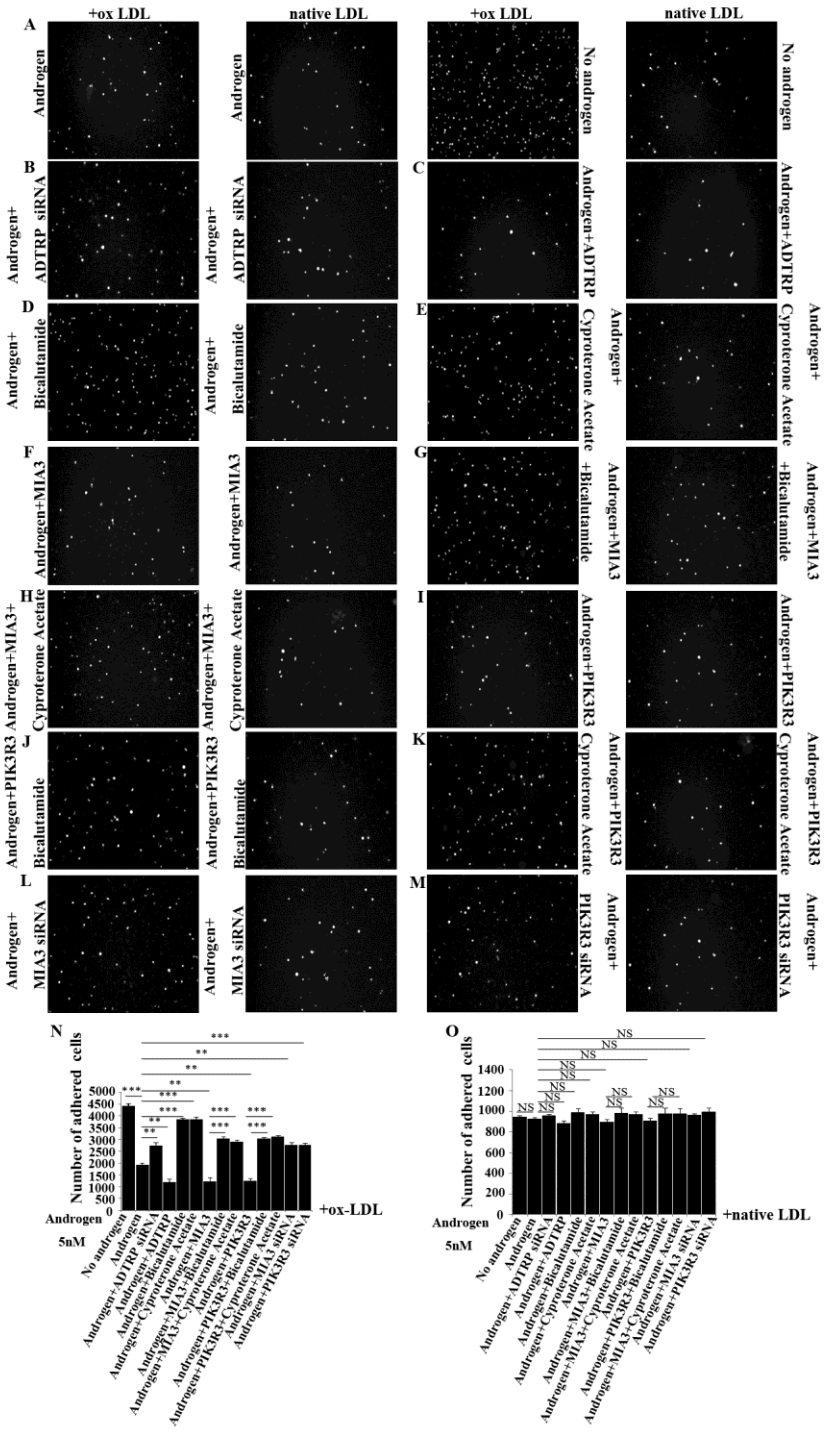

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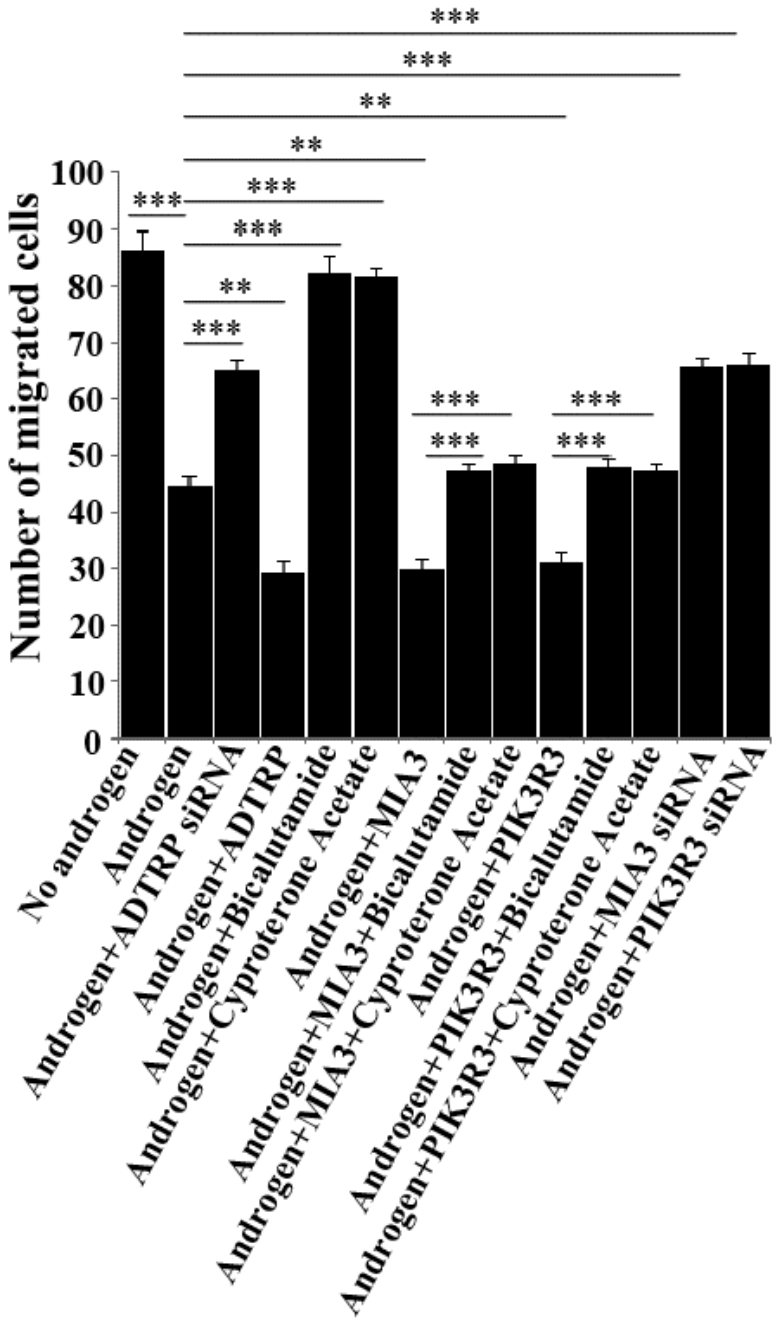

Figure 10

Highlights

1. Androgen induces transcriptional activation of *ADTRP* through the half ARE at +324.

2. Androgen blocks endothelial cell (EC) functions relevant to coronary artery disease.

3. Androgen inhibits monocyte adhesion to ECs by activating *ADTRP*.

4. Androgen inhibits monocyte transmigration across EC layers by activating *ADTRP*.