

Androgen Receptor Promotes Abdominal Aortic Aneurysm Development via Modulating Inflammatory Interleukin-1 α and Transforming Growth Factor- β 1 Expression

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Abstract—Sex difference is a risk factor for abdominal aortic aneurysm (AAA) formation yet the reason for male predominance remains unclear. Androgen and the androgen receptor (AR) influence the male sex difference, indicating that AR signaling may affect AAA development. Using angiotensin II–induced AAA in apolipoprotein E null mouse models (82.4% AAA incidence), we found that mice lacking AR failed to develop AAA and aorta had dramatically reduced macrophages infiltration and intact elastic fibers. These findings suggested that AR expression in endothelial cells, macrophages, or smooth muscle cells might play a role in AAA development. Selective knockout of AR in each of these cell types further demonstrated that mice lacking AR in macrophages (20% AAA incidence) or smooth muscle cells (12.5% AAA incidence) but not in endothelial cells (71.4% AAA incidence) had suppressed AAA development. Mechanism dissection showed that AR functioned through modulation of interleukin-1 α (IL-1 α) and transforming growth factor- β 1 signals and by targeting AR with the AR degradation enhancer ASC-J9 led to significant suppression of AAA development. These results demonstrate the underlying mechanism by which AR influences AAA development is through IL-1 α and transforming growth factor- β 1, and provides a potential new therapy to suppress/prevent AAA by targeting AR with ASC-J9. (*Hypertension*. 2015;66:881-891. DOI: 10.1161/HYPERTENSIONAHA.115.05654.)

• **Online Data Supplement**

Key Words: androgen receptors ■ angiotensin II ■ apolipoproteins ■ interleukin-1 α ■ transforming growth factor- β 1

Abdominal aortic aneurysm (AAA) is a disease of the cardiovascular system that is characterized by aortic dilation that exceeds the normal aortic diameter by >50%. Age, atherosclerosis, hypertension, male sex, and smoking are major AAA risk factors.^{1,2} In epidemiological studies, the sex difference shows a male dominance with a ratio of 4:1. In inflammatory AAA, the male sex is even a stronger risk factor resulting in male dominance of AAA ranging from 6:1 to 30:1.² Androgen and its receptor, the androgen receptor (AR), play important roles in male sexual characteristic maturation³ and may be involved in the increased risk of AAA development associated with the male sex.

Previous studies of the effect of castration on the AAA formation demonstrated that castration in male mice reduced AAA incidence to 18% to 30%.^{4,5} Surgical castration results in no circulating androgen in the animals and could account for loss of androgen-induced AR activity in AAA development

rather than a direct effect of AR. AR has been shown to be activated by several different kinds of growth factors, including insulin-like growth factor 1, epidermal growth factor, and keratinocyte growth factor,⁶ implying that growth factors activated AR signaling might account for the remaining 18% to 30% incidence of AAA in castrated male mice.

Endothelial cells, monocytes/macrophages, and smooth muscle cells (SMCs) are critical cell types involved in AAA development.⁷ Early studies have investigated that the potent androgen, dihydrotestosterone, could promote monocytes/macrophages adhesion on endothelial cells through vascular cell adhesion molecule 1 and this regulation might be through nuclear factor κ B signaling.^{8,9} In addition, male macrophages have been shown to express higher AR mRNA than female macrophages.¹⁰ However, there have been controversial observations about the effects of androgen on tumor necrosis factor- α (TNF- α)–induced inflammatory cytokines.

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Another group suggested that dihydrotestosterone treatment could inhibit TNF- α -induced inflammatory cytokines and this effect was reversed by antiandrogen treatment.¹¹ Androgen effects on SMCs have been well characterized about their behaviors, apoptosis, and migration in AAA development.¹² Testosterone treatment could induce vascular SMCs migration through NADPH oxidase and c-Src-dependent pathways.¹³ Testosterone also induces apoptosis in vascular SMCs.¹⁴ Together, all evidence indicates that AR exists in those cell types and is involved in the cellular behaviors of those cells.

In this study, we investigated the role of AR in angiotensin II (Ang II)-induced AAA formation as the reason for the male sex predominance, in whole body general AR knockout (GARKO) mice. Furthermore, we determined the contribution of specific cell types using macrophage-specific, SMC-specific, and endothelial cell-specific AR knockout mice. The influence of interleukin-1 α (IL-1 α), transforming growth factor- β 1 (TGF- β 1), infiltrating macrophages, matrix metalloproteinases (MMPs) production, and fibrosis were also determined. Together, these results indicate the importance of cell type-specific AR expression in the sex-dependent risk for AAA development.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

AR Deficiency Blocks Ang II-Induced AAA Formation In Vivo

To investigate whether AR influences AAA initiation and progression, we established GARKO mice in the apolipoprotein E null (*apoE*^{-/-}) background (GARKO-*apoE*^{-/-}). Mating strategy and genotyping results are shown in Figure 1A and 1B. There were marked decreases of testosterone and dihydrotestosterone levels in GARKO-*apoE*^{-/-} mice compared with wild-type *apoE*^{-/-} (WT-*apoE*^{-/-}) mice (Figure S1A and S1B in the online-only Data Supplement). Ang II was used to induce AAA formation for 4 weeks in GARKO-*apoE*^{-/-} and WT-*apoE*^{-/-} mice. Consistent with previous reports, we found that 4 weeks of Ang II infusion promoted AAA formation in *apoE*^{-/-} mice.⁴ Ang II infusion significantly increased blood pressure in *apoE*^{-/-} mice (Figure S1C and S1D), but no gross differences in aortic morphology were observed between saline-infused WT-*apoE*^{-/-} and GARKO-*apoE*^{-/-} mice (Figure 1C). Surprisingly, we found that none of the GARKO-*apoE*^{-/-} mice developed AAAs after Ang II induction (Figure 1D). In contrast, 82% of WT-*apoE*^{-/-} mice developed AAA (Figure 1C and 1D). The maximal aortic diameter outcome also exhibited significant reduction in Ang II-treated GARKO-*apoE*^{-/-} mice compared with Ang II-treated WT-*apoE*^{-/-} mice (Figure 1E). These results suggest that AR plays an essential role in Ang II-induced AAA formation.

Macrophage infiltration is one of the critical events involved in AAA development.² We next investigated whether differences could be observed in macrophage infiltration. We found that Ang II infusion promoted macrophage infiltration and elastic fiber fragmentation/degeneration as indicated with

arrowheads (the left side of arrowheads with pink color is the aneurysmal tissue) in WT-*apoE*^{-/-} mice, but not in GARKO-*apoE*^{-/-} mice (Figure 1F and 1G) suggesting that AR may promote AAA formation via modulating infiltrating macrophages and aortic wall degeneration, which are 2 important factors promoting AAA development.^{15,16} Vascular endothelial cells and vascular SMCs are the major types of cells contributing to the maintenance of integrity in the aortic wall¹⁷ and our observations showing the importance of AR in these processes is of great significance.

AR in Macrophages and SMCs Promotes AAA Formation but AR in Endothelial Cells Has No Impact on AAA Formation

Because macrophages, vascular endothelial cells, and vascular SMCs could contribute to AR-dependent AAA formation, we used cell type-specific knockout strategies via the Cre-loxP recombination system to develop cell-specific AR knockout in endothelial cells, macrophages, and SMCs to determine how AR in these specific cell types contributes to AAA formation. As shown in Figure S2, we developed macrophage, SMC, and endothelial cell AR-specific knockout mice by breeding specific cell driven Cre mice (lysozyme Cre mice for monocyte/macrophage, transgelin Cre mice for SMCs, and vascular endothelial cadherin Cre mice for endothelial cells) with the *apoE* null mice. We then used these resultant mice to cross with floxed AR/AR-*apoE*^{-/-} mice to generate macrophage AR-specific knockout mice in *apoE* null background (named MARKO-*apoE*^{-/-}; Figure S2A and S2D), SMC-specific AR knockout mice with *apoE* knockout background (named SARKO-*apoE*^{-/-}; Figure S2B and S2D) and endothelial cell-specific AR knockout mice on *apoE*^{-/-} background (named EARKO-*apoE*^{-/-}; Figure S2C and S2D). The cell-specific ARKO effects have been determined in our previous study¹⁸ and the genotyping data indicate that these cell-specific ARKO mice used in this study have ARKO in aortic tissue (Figure S2D). EARKO-*apoE*^{-/-}, MARKO-*apoE*^{-/-}, SARKO-*apoE*^{-/-}, and WT-*apoE*^{-/-} mice were then treated with Ang II to induce AAA formation. Consistent with previous results, we found that WT-*apoE*^{-/-} mice infused with Ang II developed a significant increase in the size of the aorta, although there were significant reductions of AAA incidence and maximal aortic diameter in MARKO-*apoE*^{-/-} mice (20% AAA incidence; Figure 2A) and SARKO-*apoE*^{-/-} mice (12.5% AAA incidence; Figure 2B). However, endothelial cell-specific AR knockout did not affect AAA development (Figure 2C). Furthermore, macrophage infiltration was attenuated in MARKO-*apoE*^{-/-} and SARKO-*apoE*^{-/-} mice but not in EARKO-*apoE*^{-/-} mice (Figure S2E).

In summary, using a cell type-specific ARKO strategy to dissect AR functions of specific cell types in AAA formation, we found that AR in macrophages and SMCs, but not endothelial cells, mediates AAA formation.

AR Promotes AAA Formation Through Modulating IL-1 α -Mediated Inflammatory Response

Early studies indicated that androgens might promote Ang II receptor type 1 (AT1R) expression in SMCs that might

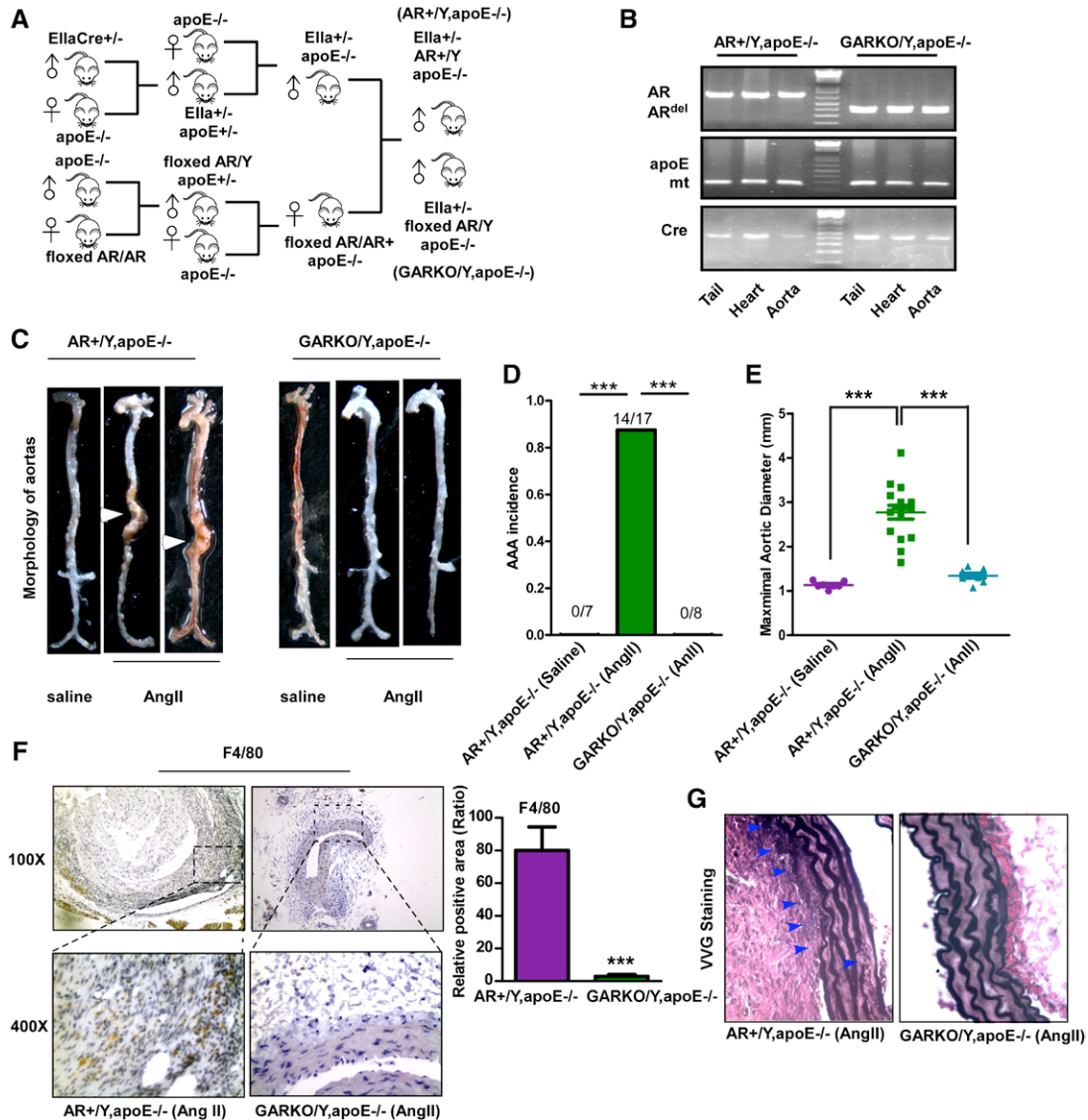


Figure 1. Knockout of androgen receptor (AR) inhibits angiotensin II-induced abdominal aortic aneurysms (AAAs). **A**, Mating strategy of general AR knockout (GARKO) and apolipoprotein E null (*apoE*^{-/-}) mouse generation. **B**, Genotyping results of wild-type (WT)-*apoE*^{-/-} and GARKO-*apoE*^{-/-} mice in tissues from tail, heart, and aorta. **C**, Representative images of whole aortas from saline-infused WT-*apoE*^{-/-}, angiotensin II (Ang II)-infused WT-*apoE*^{-/-}, saline-infused GARKO-*apoE*^{-/-}, and Ang II-infused GARKO-*apoE*^{-/-} mice. **D**, AAA incidence in saline-infused WT-*apoE*^{-/-}, Ang II-infused WT-*apoE*^{-/-}, and GARKO-*apoE*^{-/-} mice. **E**, Maximal aortic diameter in saline-infused WT-*apoE*^{-/-}, Ang II-infused WT-*apoE*^{-/-}, and GARKO-*apoE*^{-/-} mice, n=7–14. **F**, Infiltrating macrophages were determined using F4/80 staining. **Top**, ×100 magnification; **bottom**, ×400 magnification. Quantification results of F4/80 staining are on right, n=5–6. **G**, Verhoeff-Van Gieson (VVG) staining of Ang II-infused WT-*apoE*^{-/-} and GARKO-*apoE*^{-/-} mice. Arrowheads indicate elastin degeneration area.

contribute to androgen/AR signaling-mediated AAA progression.¹⁹ We also found that dihydrotestosterone could promote AT1R expression but knockout of AR could not suppress AT1R expression (Figure S3). Therefore, we decided to further dissect these complicated mechanisms via investigating the inflammatory pathways because the inflammatory response is one of the underlying mechanisms contributing to AAA development.² To understand the risk posed by the male sex, we used aortic tissues of GARKO-*apoE*^{-/-} versus WT-*apoE*^{-/-} mice and performed a focused quantitative polymerase chain reaction array to screen for inflammatory cytokines/chemokines that are modulated by AR in AAA formation. Aortic morphology, blood pressure, and MMP9 expression were measured to confirm that Ang II-induced AAAs were

as previously described.¹⁵ We found aortic morphology was consistent with previous observations and Ang II treatments increased blood pressures in WT-*apoE*^{-/-} and GARKO-*apoE*^{-/-} mice compared with saline treatments (Figure S1C and S1D; Figure 4A). Upregulation of MMP9 expression occurred in WT-*apoE*^{-/-} mice, but not in GARKO-*apoE*^{-/-} mice after Ang II induction (Figure S4B). Array analysis revealed that several inflammation-associated genes, including *CCL4*, *CCL9*, *IL-1α*, *TNF-α*, and *TGF-β1* were significantly suppressed in aortic tissues of Ang II-treated GARKO-*apoE*^{-/-} mice compared with Ang II-treated WT-*apoE*^{-/-} mice (Table S1). To validate the array analysis, we measured *IL-1α* expression in aortic tissues of these mice as evidence indicates that *IL-1* is a major inflammatory cytokine elevated in patients with AAA.²⁰

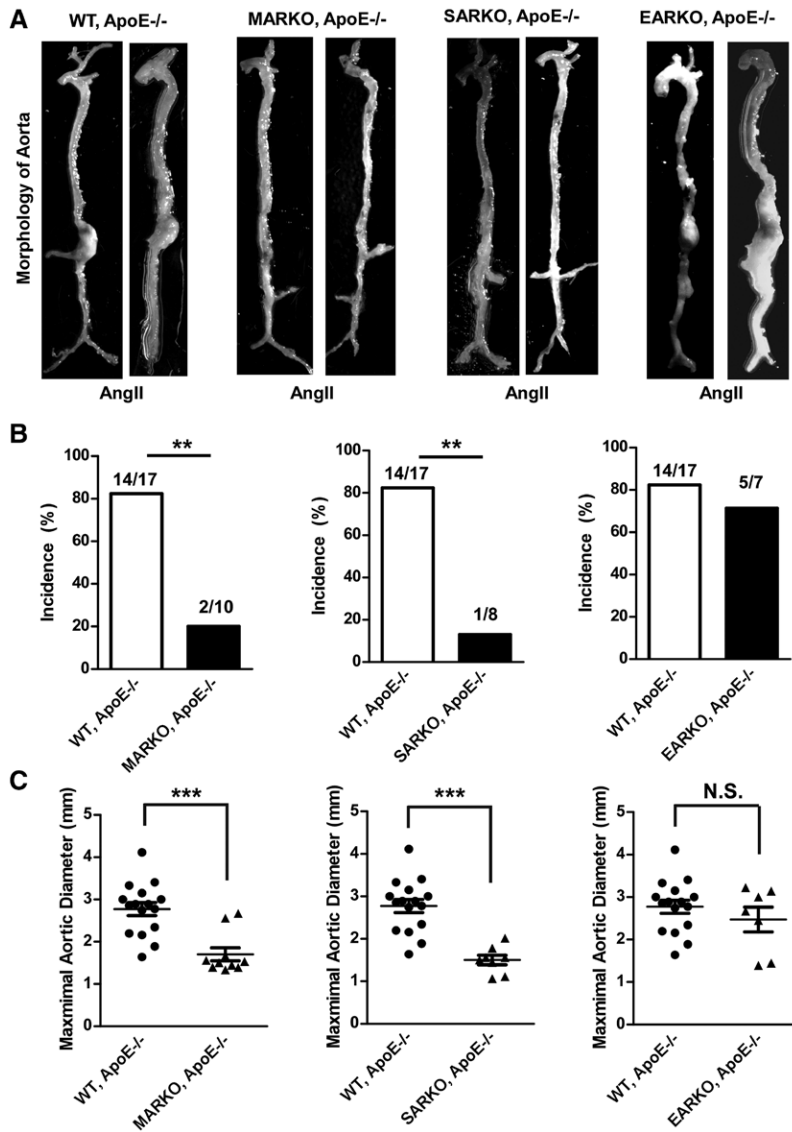


Figure 2. Androgen receptor in macrophages and smooth muscle cells, but not in endothelial cells, promotes abdominal aortic aneurysm (AAA) development. **A**, Representative images of whole aortas from angiotensin II (Ang II)-infused mice with the genotypes of wild-type (WT)-apoE^{-/-}, specific ARKO in macrophages, and apoE null background (MARKO-apoE^{-/-}), specific ARKO in smooth muscle cells (SMCs) and apoE null background (SARKO-apoE^{-/-}), and specific ARKO in endothelial cells and apoE null background (EARKO-apoE^{-/-}). **B**, AAA incidences were measured in Ang II-infused MARKO-apoE^{-/-}, SARKO-apoE^{-/-}, EARKO-apoE^{-/-} mice, and their related control WT-apoE^{-/-} mice, n=7–17. **C**, Maximal aortic diameters were determined in Ang II-infused MARKO-apoE^{-/-}, SARKO-apoE^{-/-}, EARKO-apoE^{-/-} mice, and their related control WT-apoE^{-/-} mice. N.S. indicates not significant.

Ang II treatment promoted IL-1 α expression in WT-apoE^{-/-} mice compared with vehicle control (Figure 3A); however, this enhancement was reduced when AR was knocked out (Figure 3B and 3C).

IL-1 has been shown to enhance macrophage infiltration and stimulate MMPs in macrophages and other cell types.^{21,22} Macrophage chemo-attractant protein 1 (MCP-1) is a crucial downstream target of IL-1 α . We therefore determined how AR modulates this signaling cascade. Loss of AR significantly inhibited MCP-1 expression in Ang II-infused apoE^{-/-} mice (Figure 3D). Similarly, MMP2 and MMP9 expression levels were dramatically increased in Ang II-induced apoE^{-/-} mice, whereas knockout of AR inhibited MMP2 and MMP9 expression at the mRNA and protein levels (Figure 3E–3H). Consistently, knockdown of AR in THP-1 cells, human monocytes/macrophages, also inhibited those inflammatory cytokines and expression levels of MMPs (Figure 3I). These results suggest that AR modulates expression of IL-1 α and key downstream target proteins.

To further dissect the molecular mechanism by which AR modulates IL-1 α expression, we examined whether AR directly

binds to the IL-1 α promoter region and regulates its transcription. Most identified androgen responsive elements are located within the promoter region of AR target genes with >9 bases matching with consensus androgen responsive element-binding motif.²³ By scanning, we identified many potential androgen responsive elements in the IL-1 α promoter region and confirmed that AR could bind to one of these regions (Figure 3J, bottom) and promoted IL-1 α transactivation (Figure 3J, top). These results suggest that AR modulates AAA formation by elevating IL-1 α expression, which in turn increases expression levels of MCP-1 and MMPs.

Restoration of IL-1 α Partially Reverses the AR Knockout Effects on Promoting AAA Formation

To test whether MMPs activation is a consequence of increased IL-1 α expression by AR, we used an in vitro culture system using THP-1 cells to determine the consequent molecular mechanism events. Knockdown of AR resulted in suppressed MMP2 and MMP9 expression (Figure 4A and 4B). However, addition of recombinant IL-1 α reversed the effects of AR knockdown on MMP9 expression (Figure 4A

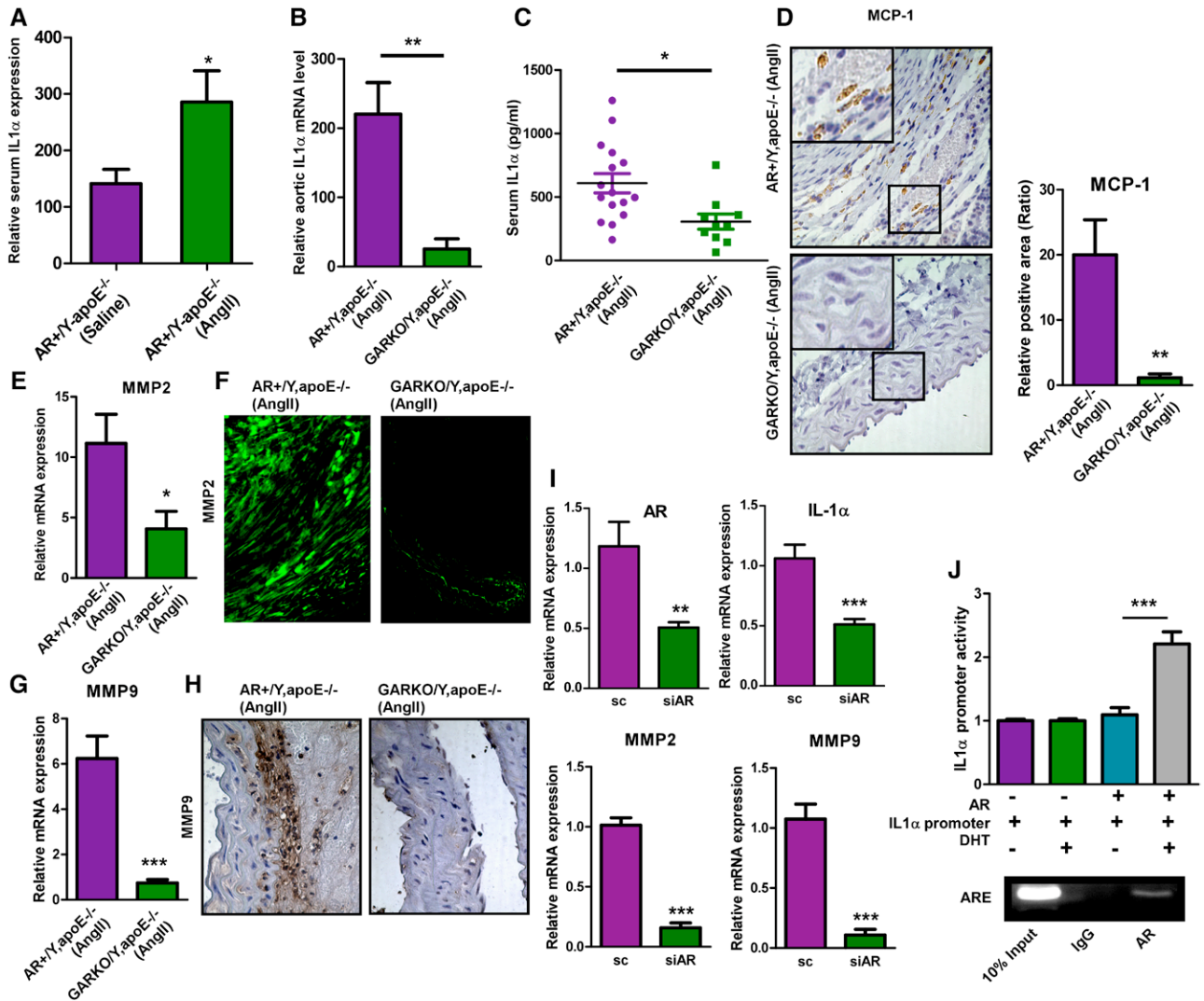


Figure 3. Androgen receptor (AR) mediates abdominal aortic aneurysms development via stimulating inflammatory cytokines including interleukin-1α (IL-1α) to enhance matrix metalloproteinase (MMP)2 and MMP9 production in macrophages. **A**, Relative serum IL-1α protein expression levels were measured in saline and angiotensin II (Ang II)-infused wild-type (WT)-*apoE*^{-/-} mice, n=11. **B**, Aortic IL-1α mRNA and **C** serum IL-1α protein expression levels were determined in Ang II-infused WT-*apoE*^{-/-} and general AR knockout (GARKO)-*apoE*^{-/-} mice, n=10–17. **D**, Macrophage chemo-attractant protein 1 (MCP-1) expressions were determined in WT-*apoE*^{-/-} and GARKO-*apoE*^{-/-} mice. Enlarged images in the upper left corners were from the small squares inside images, quantification at right, n=3–6. **E**, MMP2 mRNA and **F** protein levels were examined in Ang II-infused WT-*apoE*^{-/-} and GARKO-*apoE*^{-/-} mice. **G**, MMP9 mRNA and **H** protein expressions were determined in Ang II-infused WT-*apoE*^{-/-} and GARKO-*apoE*^{-/-} mice, n=3–6. **I**, AR, IL-1α, MMP2, and MMP9 were measured in THP-1 cells infected with lentivirus packing with scramble (sc) or siRNA-AR (siAR), n=3–6. **J**, Luciferase assay (top) was performed with HEK-293T cells to measure IL-1α (–1335 to –65) promoter activities in the absence or presence of AR with or without 10 nmol/L dihydrotestosterone (DHT), n=3. Chromatin immunoprecipitation (CHIP) assay (bottom) was used to verify putative androgen responsive elements (AREs) located within 3 kb promoter region of IL-1α. Gel image showed CHIP results that were amplified using primers specific to IL-1α ARE in the chromatin pulled down either by IgG control or by AR antibody; 10% input used as positive control.

and 4B), but not on MMP2 expression. To further validate whether IL-1α is critical for AR-dependent AAA formation in vivo, we locally delivered IL-1α to the surrounding area of the suprarenal aorta in the AAA mouse models infused with Ang II. Elevated IL-1α in GARKO-*apoE*^{-/-} mice was shown by green fluorescence protein staining to monitor the exogenously delivered IL-1α (Figure S5A). Restoration of IL-1α reduced AR knockout effects on AAA formation as judged by morphological changes, AAA incidence, and maximal abdominal aortic diameter (Figure 4C–4E). Restoration of IL-1α also reversed the AR knockout effect on decreasing MMPs and MCP-1 expression as shown in Figure 4F–4I. Both

in vitro and in vivo IL-1α restoration studies provide strong evidence that IL-1α is a molecular target mediating AR function in AAA formation.

AR Modulates AAA Formation Through Stimulating Fibrosis via TGF-β1 Signaling

Fibrosis of the adjacent retroperitoneum is a major characteristic in inflammatory aneurysms,²⁴ and TGF-β1 is known to play a key role in fibrosis development.²⁵ Therefore, we next addressed whether AR might modulate fibrosis to exacerbate AAA. Consistent with previous reports, Ang II-induced AAA has elevated TGF-β1 expression in WT-*apoE*^{-/-} mice

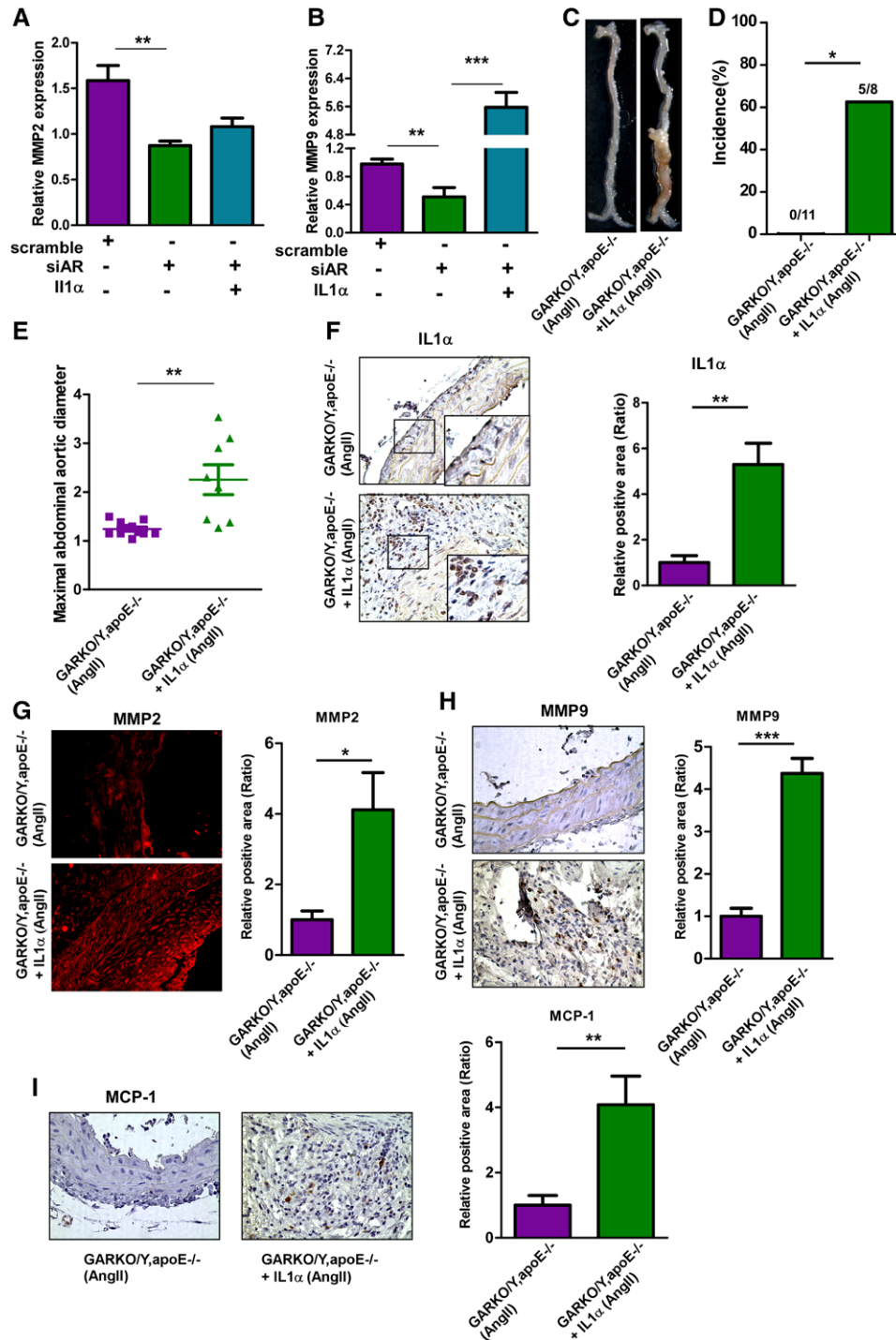


Figure 4. Androgen receptor (AR) promotes abdominal aortic aneurysm (AAA) progression through modulating interleukin-1 α (IL-1 α) and resulting in elevated matrix metalloproteinase (MMP)2 and MMP9 generations in AAA. **A**, MMP2 and **B** MMP9 expression levels were measured in THP-1 cells manipulated with scramble, siAR, and siAR plus treatments with 10 ng/mL recombinant IL-1 α protein, $n=3-6$. **C-E**, Representative image of whole aortas, AAA incidence, and maximal abdominal aortic diameter were shown in angiotensin II (Ang II)-infused general AR knockout (GARKO)- $apoE^{-/-}$ mice and GARKO- $apoE^{-/-}$ mice treated with IL-1 α , $n=8-11$. Expression levels of IL-1 α (**F**), MMP2 (**G**), MMP9 (**H**), and macrophage chemo-attractant protein 1 (MCP-1) (**I**) were determined in Ang II-infused GARKO- $apoE^{-/-}$ mice and GARKO- $apoE^{-/-}$ mice treated with IL-1 α . Graphics beside the immunohistochemical images are quantitation results of indicated genes. $n=3-8$.

compared with saline-infused WT- $apoE^{-/-}$ mice (data not shown).²⁶ Interestingly, deletion of AR suppressed TGF- β 1 expression in Ang II-induced $apoE^{-/-}$ mice compared with WT- $apoE^{-/-}$ mice (Figure 5A and 5B, arrows). To demonstrate the involvement of TGF- β 1, we examined mothers against

decapentaplegic homolog 3 (SMAD3), which initiates intracellular signaling by TGF- β 1-dependent phosphorylation and the result showed that AR deletion inhibited pSMAD3 in Ang II-induced AAA mouse model (Figure 5C). Collagen levels were also examined to demarcate the fibrotic areas of

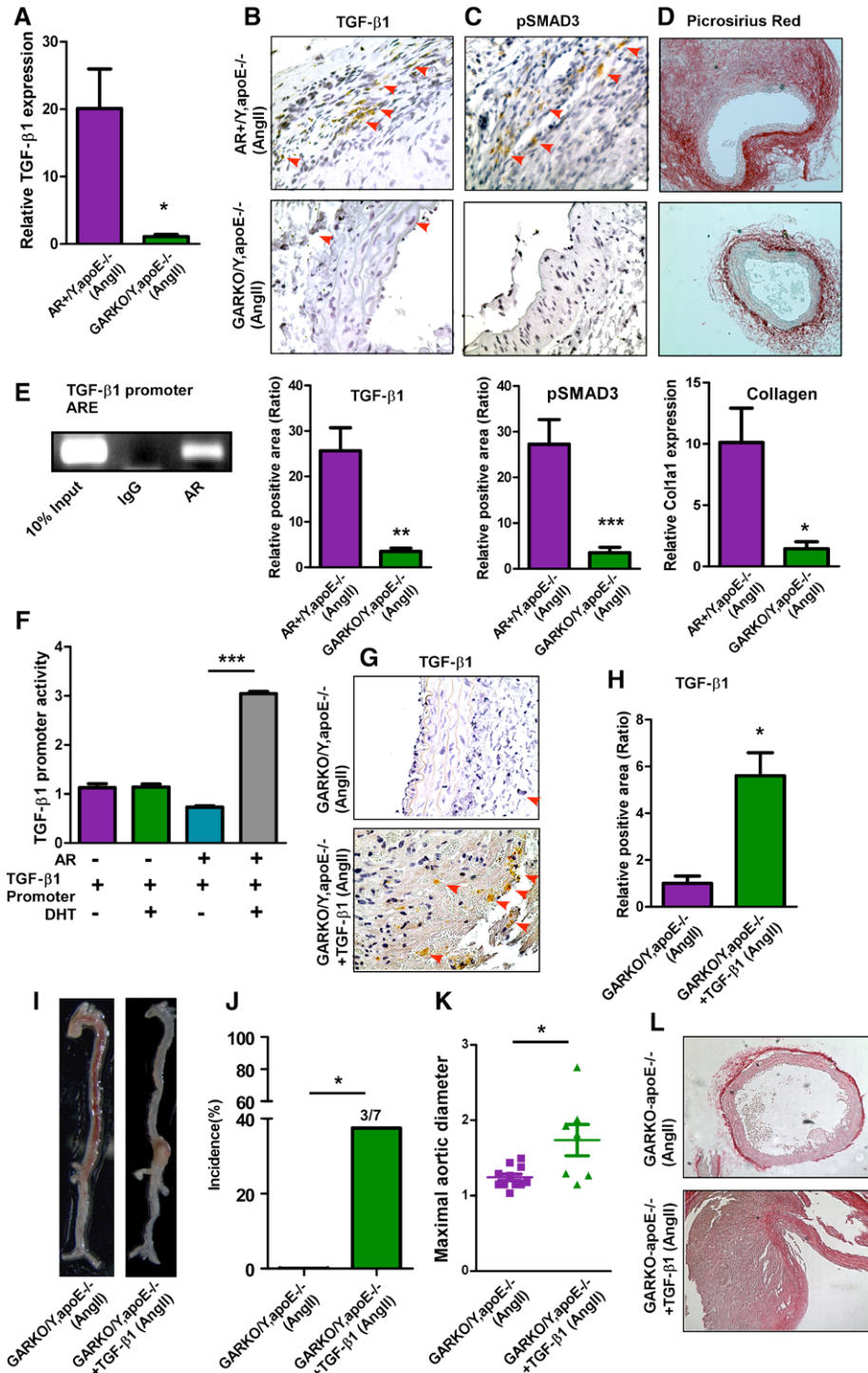


Figure 5. Loss of Androgen receptor (AR) blocks transforming growth factor-β1 (TGF-β1) inhibition of abdominal aortic aneurysm (AAA) formation and restoration of TGF-β1 partially reverses AR deficiency effects on AAA development. **A** and **B**, TGF-β1 mRNA and protein expressions were determined in angiotensin II (Ang II)-infused wild-type (WT)-apoE^{-/-} and general AR knockout (GARKO)-apoE^{-/-} mice, n=3–6. **C**, TGF-β1 downstream, pSMAD3 (phosphorylated mothers against decapentaplegic homolog 3) was determined in Ang II-infused WT-apoE^{-/-} and GARKO-apoE^{-/-} mice. **D**, Gel image for chromatin immunoprecipitation (CHIP) assay, which was used to verify putative AR responsive element (ARE) located within 3 kb promoter region of TGF-β1. **E**, Cross-linked chromatin was immunoprecipitated with IgG control and AR antibody; 10% input was used as positive control. Eluted chromatin was amplified with specific primers for putative AREs of TGF-β1. **F**, Results of luciferase assay to measure TGF-β1 promoter transcriptional activity in HEK-293 cells with or without AR in the presence or absence of 10 nmol/L dihydrotestosterone (DHT), n=3. **G**, TGF-β1 levels were determined in Ang II-infused GARKO-apoE^{-/-} and GARKO-apoE^{-/-} mice treated with TGF-β1. **H**, Quantitation results of TGF-β1, n=5–8. **I**, Representative images of whole aortas from Ang II-infused GARKO-apoE^{-/-} and GARKO-apoE^{-/-} mice treated with TGF-β1. **J**, AAA incidence and (**K**) maximal abdominal aortic diameter were shown in Ang II-infused GARKO-apoE^{-/-} and GARKO-apoE^{-/-} mice treated with TGF-β1, n=7–11. **L**, Fibrosis areas were determined in Ang II-infused GARKO-apoE^{-/-} and GARKO-apoE^{-/-} mice treated with TGF-β1 using picrosirius red staining.

WT-*apoE*^{-/-} and GARKO-*apoE*^{-/-} mice infused with Ang II. The results demonstrated that knockout of AR significantly reduced collagen deposition and fibrosis in GARKO-*apoE*^{-/-} mice (Figure 5D). Mechanism dissection revealed that AR could bind to the putative androgen responsive element sequence located from -3132 to -3118 in the TGF- β 1 promoter region and AR expression is a positive regulator for TGF- β 1 transcriptional regulation (Figure 5E and 5F).

To determine whether TGF- β 1 is critical in AR-mediated AAA formation, we investigated whether in vivo restoration of TGF- β 1 could reverse the protective effect of AR deletion on AAA formation. Green fluorescence protein-labeled TGF- β 1 was delivered into Ang II-infused GARKO-*apoE*^{-/-} mice and AAA formation was monitored. TGF- β 1 levels and green fluorescence protein expression confirmed successful delivery

of TGF- β 1 into the aortic area (Figure 5G and 5H; S5B). Restoration of TGF- β 1 partially reversed the suppressive effect of AR knockout on AAA formation in GARKO-*apoE*^{-/-} mice as judged by morphology, disease incidence, and maximal abdominal aortic diameter (Figure 5I–5K). Increased collagen staining indicates increased fibrotic area in TGF- β 1-delivered GARKO-*apoE*^{-/-} mice (Figure 5L). Taken together, we conclude that AR promotes AAA formation partially through increasing fibrosis via upregulation of TGF- β 1 signaling.

Targeting AR With the AR Degradation Enhancer ASC-J9 Effectively Blocks AAA Formation

Our studies of ARKO mice indicate that AR is a critical factor in Ang II-induced AAA formation, suggesting the possibility that targeting AR might be therapeutically beneficial for

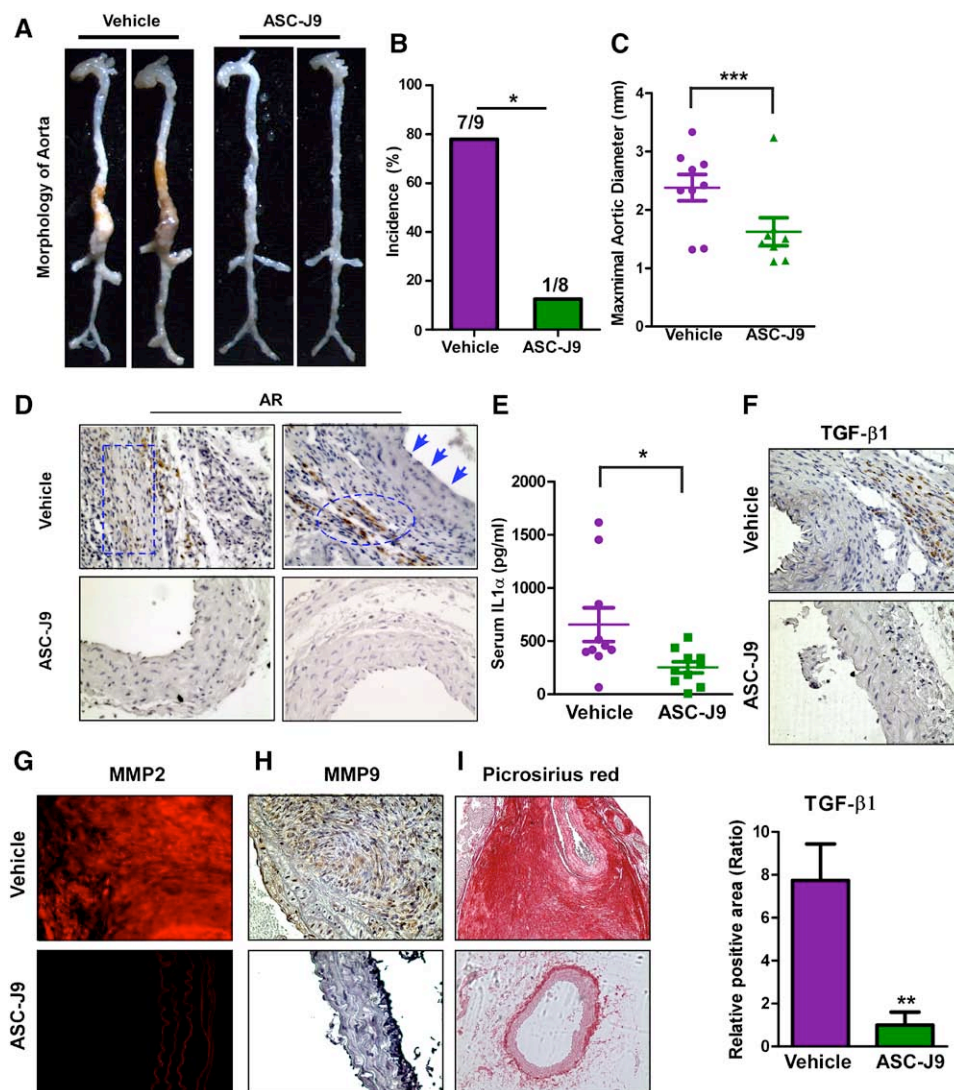


Figure 6. Targeting androgen receptor with ASC-J9 inhibits abdominal aortic aneurysm (AAA) development. **A**, Representative of whole aortas in vehicle or ASC-J9-treated *apoE*^{-/-} mice infused with angiotensin II (Ang II) for 28 days. **B**, AAA incidence and **C** maximal aortic diameter were determined in the vehicle or ASC-J9-treated *apoE*^{-/-} mice infused with Ang II, n=8–9. **D**, Androgen receptor (AR) expression was monitored using immunohistochemistry staining. Rectangle with dotted line shows AR expression in smooth muscle layer. Ellipse with dotted line shows AR expression in infiltrated cells, including monocytes/macrophages. Arrowheads indicate no AR expression in endothelium. **E**, Serum IL-1 α and **F** aortic transforming growth factor- β 1 (TGF- β 1) levels were measured in the vehicle and ASC-J9-treated AAA mice. Graph below **F** is quantitation of TGF- β 1, n=5–10. **G**, matrix metalloproteinase (MMP)2 and **H** MMP9 expressions were detected in the suprarenal aortas of vehicle or ASC-J9-treated *apoE*^{-/-} mice infused with Ang II. **I**, Picrosirius red staining was used to determine the fibrotic areas in vehicle and ASC-J9-treated *apoE*^{-/-} mice infused with Ang II.

patients with AAA. ASC-J9, an AR degradation enhancer, has been well characterized with better efficacy but little side effects on fertility (Figure S6) in treating AR-related diseases, such as spinal and bulbar muscular atrophy.²⁷ To test whether ASC-J9 might have therapeutic effects in treating AAA, we treated Ang II-induced AAA *apoE*^{-/-} mice with this compound and compared results with mice receiving vehicle only. ASC-J9 treatment significantly suppressed AAA incidence and reduced maximal abdominal aortic diameter in Ang II-treated *apoE*^{-/-} mice compared with control (Figure 6A–6C). In addition, ASC-J9 reduced AR, IL-1 α , and TGF- β 1 expression levels (Figure 6D–6F). Expression of MMP2 and MMP9, downstream of IL-1 α , was significantly lower in ASC-J9-treated mice than in control (Figure 6G and 6H). Furthermore, ASC-J9 treatment reduced fibrosis in Ang II-treated *apoE*^{-/-} mice (Figure 6I). These findings suggest that targeting AR with ASC-J9 is a novel therapeutic approach for treatment of AAA.

Discussion

In this study, we demonstrated that AR plays a proinflammatory role in AAA development. We first found that AR is required for Ang II induction of AAA. Using the Cre-loxP recombination strategy, we were able to knockout AR specifically in macrophages, SMCs, and endothelial cells, and showed the importance of AR in macrophages and SMCs in AAA progression. Our inflammatory cytokine analysis found that inflammatory cytokines were significantly suppressed when AR was deleted. One of these inflammatory cytokines, IL-1 α , has been linked to inflammatory AAA formation.^{2,20,28} When we restored IL-1 α in GARKO-*apoE*^{-/-} mice in vivo, knockout of AR effects on AAA development were significantly reversed, suggesting that AR mediates the inflammatory response in AAA partially through IL-1 α . Fibrosis often accompanies inflammatory AAA²⁸ and TGF- β 1 is a critical molecule involved in the fibrosis development in several diseases, including AAA.^{16,29} In this study, loss of AR suppressed TGF- β 1 expression and fibrosis in AAA. Using an in vivo TGF- β 1 delivery strategy in GARKO-*apoE*^{-/-} mice, we found that restoration of TGF- β 1 in AR-deficient mice significantly reversed the AAA phenotype, suggesting that AR mediates AAA formation partially through modulating TGF- β 1.

The male sex is one of the risk factors for AAA development. On the basis of this epidemiological result, previous studies have evaluated androgen/AR signaling in AAA development via removal of androgen by using castration. Indeed, castration showed promising effects on AAA development in different models of AAA formation, including Ang II induction and elastase challenge.^{4,30} However, castration does not completely block AAA development, implying that other pathways might contribute to AAA formation under castration conditions. Moreover, accumulating evidence has indicated that castration does not completely stop androgen production, and both intracellular and adrenal gland synthesis of androgen have been observed in prostate cancer and embryonic stem cells.^{31,32} Androgen-independent activation of AR has also been demonstrated through phosphorylating AR via different kinds of growth factors, such as epidermal growth factor, insulin-like growth factor 1, and keratinocyte growth factor.⁶

A full understanding of AR signaling in AAA formation will be crucial for developing therapeutic approaches especially in males. By using our ARKO mouse models, we explored the importance of AR in AAA progression independently of alternative mechanisms for AR activation, and our results provide important new information as to the significance of targeting AR in patients with AAA.

Actually, androgen/AR signaling in cardiovascular diseases is more complicated than we previously thought. To investigate androgen/AR signaling in cardiovascular diseases, we have generated GARKO mice in different backgrounds including double knockout of AR and low density lipoprotein receptor (LDLR), double knockout of AR and apoE as well as only knockout of AR. All GARKO mice generated in different backgrounds displayed the same appearance as we previously observed.³ GARKO-*LDLR*^{-/-} mice had exacerbated atherosclerosis but MARKO-*LDLR*^{-/-} mice had inhibited atherosclerosis progression.¹⁸ Although we found that macrophage infiltration is one of the potential mechanisms accounting for atherosclerosis progression, this might not be able to explain why GARKO in mice results in exacerbated atherosclerosis progression because GARKO-*LDLR*^{-/-} mice had some extent of macrophage infiltration inhibition.^{33,34} One explanation for these differential effects of AR on atherosclerosis is lipid metabolism. GARKO-*LDLR*^{-/-} mice have abnormal lipid profiles because of a dramatic decrease of androgen production. MARKO-*LDLR*^{-/-} mice have relatively normal lipid profiles without the altered androgen production.

In this study, knockout of AR in the whole body, macrophages, and SMCs would consistently suppress AAA formation. However, knockout of AR in endothelial cells had little impact on AAA development. Our unpublished observations indicate that human umbilical vein endothelial cells have low AR mRNA and undetectable AR protein expression. In addition, immunohistochemistry staining data show almost no AR-positive signal in aortic endothelium (Figure 6D), suggesting that AR in endothelial cells might not be critical for AAA development. Although an early study suggested that androgen promotes macrophage adhesion through modulating vascular cell adhesion molecule 1, the study used high androgen concentrations that are not the physiological dose and not seen in mice.⁹ This might, in part, explain why knockout of AR in endothelial cells has no effects on AAA development. Regarding AR-mediated molecular mechanism in AAA development, an early study linked androgen/AR signaling and AT1R to AAA development.⁴ However, we did not observe a dramatic reduction of AT1R in GARKO mice. In addition, castration results in a substantial reduction of AAA formation in elastase-induced AAA model whose AAA might not be initiated through AT1R, suggesting that androgen/AR signaling might simultaneously affect AAA formation through other molecular pathways.³⁰

High blood pressure has been suspected to be involved in AAA development. In this study, GARKO-*apoE*^{-/-} mice actually developed higher blood pressure than WT-*apoE*^{-/-} mice in Ang II infusion condition. This unexpected result provides the first evidence that high blood pressure in some instances might not be the reason for AAA formation as inhibition of androgen/AR signaling consistently suppresses AAA formation in

different AAA animal models. Further studies are needed in order to clarify how AR-mediated blood pressure relates to AAA development.

In clinical observations, males have a 4:1 to 30:1 higher ratio than females for inflammatory AAA incidence, suggesting that androgen/AR signaling might affect AAA development and progression through inflammatory cytokines. Indeed, this study identified that AR would promote IL-1 α expression to facilitate AAA progression. In addition, we did observe that several proinflammatory cytokines, including TNF- α , CCL4, CCL9, CCR1, CCR7, integrin α M, and integrin β 2, were suppressed in ARKO mice. In our rescue experiments, we showed only partially reversed ARKO effects on AAA development by overexpressing IL-1 α . Because ARKO mice have several downregulated inflammatory cytokines, it is possible that other inflammatory cytokines may also be implicated in AR-mediated AAA development as well. Moreover, our previous study has demonstrated that AR transcriptionally regulates TNF- α expression,³⁴ suggesting the possibility that AR might also enhance AAA development through TNF- α signaling. It is uncertain whether other inflammatory cytokines are transcriptionally modulated by AR. To further clarify these possible molecular regulations, future studies will be needed.

ASC-J9, an AR degradation enhancer, has been shown to suppress AR in different kinds of cells²⁵ and is effective in treating AR-related diseases, such as prostate cancer. ASC-J9 enhances cutaneous wound healing by inhibiting the inflammatory response.³⁴ In this study, we demonstrate that ASC-J9 is effective in preventing AAA in a mouse model system. Because the male sex is a strong risk factor for inflammatory AAA, our data support that targeting AR with ASC-J9 has therapeutic potential in treating AAA in humans. In summary, this study clearly demonstrates that AR is required in AAA development, and suggests a novel therapeutic approach to battle AAA via directly targeting the AR.

Perspectives

AAA is a complicated disease and several risk factors have been linked to AAA initiation and progression. The sex difference is one of the risk factors contributing to both atherosclerosis and AAA. Previous studies have demonstrated that knockout of AR in monocytes/macrophages inhibits atherosclerosis in LDLR mutant mouse, but ubiquitous knockout of AR in mouse actually exacerbates atherosclerosis progression. In this study, similar strategies have been used to clarify the role of AR in AAA initiation and progression. Knockout of AR in mice with apoE null background results in complete extinguishment of AAA development. Studies of cell-specific ARKO mice clarified that AR in monocytes/macrophages and SMCs plays a critical role in AAA initiation and progression through IL-1 α and TGF- β 1 signaling pathways. Unexpectedly, we found that ARKO mice actually developed even higher blood pressure than WT mice. Originally, it has been speculated that hypertension might be the potential cause for AAA initiation. In addition, androgen has been thought and demonstrated to promote blood pressure. However, several observations from the studies of ARKO mice are inconsistent with this hypothesis, and further studies will be needed to determine separate effects of AR on AAA development and blood pressure.

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Disclosures

ASC-J9 was patented by the University of Rochester, the University of North Carolina, and AndroScience, and then licensed to AndroScience. Both the University of Rochester and C. Chang own royalties and equity in AndroScience.

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Novelty and Significance

What Is New?

- Previous studies only demonstrated that castration could suppress abdominal aortic aneurysm (AAA) development without clarifying that androgen receptor (AR) is required for AAA development.
- This study clarified that AR in macrophages and smooth muscle cells are important for AAA development but not AR in endothelial cells.
- The molecular mechanism studies suggested that AR mediates AAA progression partially through interleukin-1 α (IL-1 α) and transforming growth factor- β 1 (TGF- β 1), both of which have been shown elevated in patients with AAA.

What Is Relevant?

- Males have higher AAA incidence than females probably because of AR. Studies with genetic knockout of AR demonstrate clearly that AR is re-

quired for AAA development. Unexpectedly, androgen receptor knockout mice developed higher blood pressure than AR wild-type mice, suggesting that AR may modulate blood pressure and AAA formation through different mechanisms.

Summary

AR in smooth muscle cells and macrophages play critical roles in mediating AAA formation through IL-1 α and TGF- β 1 pathways. Targeting AR with ASC-J9 may be a potential therapy for treating AAA disease.