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Article

Estrogen Inhibits the Phenotypic Switching of Vascular Smooth Muscle Cells through ER-α/CREB in Aortic Dissection

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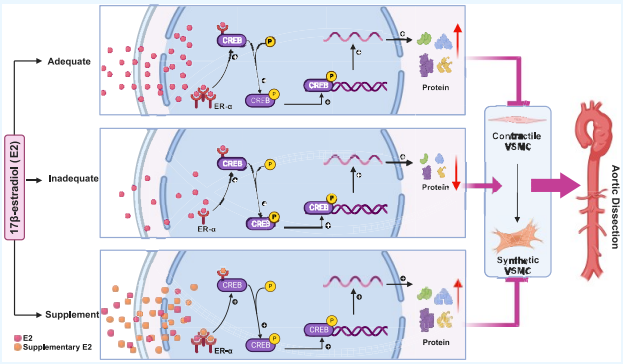
ABSTRACT: **Objective:** To examine the alterations in estrogen levels in patients with aortic dissection (AD) and its protective effect on AD patients through the inhibition of vascular smooth muscle cells (VSMCs) phenotypic switching via the ER-α/CREB pathway. **Methods:** Demographic data were collected to assess sex disparity in AD patients, and serum 17β-estradiol (E2) levels were measured using ELISA. Phenotypic switching markers were analyzed in aortic tissues from AD patients and controls. Bioinformatics analysis identified estrogen-related pathways, focusing on the ER-α/CREB axis, with expression levels confirmed via immunohistochemistry and Western blot. AD mouse models were developed in male and ovariectomized female mice, with the effects of E2 supplementation on AD progression and VSMCs phenotypic switching evaluated. An AD cellular model was also employed to verify these findings through targeted pathway inhibition. **Results:** AD prevalence was higher in males, with reduced serum E2 levels observed in both male and postmenopausal female patients. Ovariectomized female mice showed increased AD incidence, while E2 supplementation reduced AD progression by inhibiting the phenotypic switching of VSMCs. Downregulation of ER-α and p-CREB/CREB expression was observed in AD patients, and E2 enhanced ER-α expression and CREB phosphorylation, preventing VSMC phenotypic switching. E2 also promoted ER-α/CREB interaction, and silencing ER-α inhibited CREB phosphorylation, leading to increased VSMC phenotypic switching. **Conclusions:** Estrogen (E2) plays a crucial role in preventing AD by maintaining VSMCs synthetic phenotype through the ER-α/CREB signaling pathway, providing a protective effect against the development of AD.



INTRODUCTION

Aortic dissection (AD) is a severe vascular disease that poses a serious risk to patients' lives due to its sudden onset and high mortality rate.[1] While advancements in medical technology have led to a decrease in the mortality rate of AD patients, the latest data from the International Registry of Acute Aortic Dissection (IRAD) shows that the initial mortality rate of AD remains at 27.4% globally. Specifically, the Stanford type A AD hospital mortality rate is 22%, while the Stanford type B AD hospital mortality rate is 13%.[2] Currently, the treatment of AD primarily focuses on surgical or interventional treatment after the disease has already occurred. However, there is a pressing need for in-depth research into the mechanisms underlying the onset of the disease and the development of early intervention methods.

Structural abnormalities in the inner layer of the aorta are the pathological basis of AD,[1,3] specifically related to cystic necrosis[4] and phenotypic switching of vascular smooth muscle cells (VSMCs).[5] The transition of VSMCs from a contractile to a synthetic phenotype is a critical step in aortic wall remodeling and rupture. This transition is marked by an increase in the synthetic marker protein osteopontin (OPN) and a decrease in the contractile marker α-smooth muscle actin (α-SMA) and smooth muscle 22α (SM22α).[5] Therefore, identifying factors that can inhibit the phenotypic transformation of VSMCs may have a protective effect on the occurrence and development of AD. The IRAD data shows that men account for 65% of all AD cases, with significantly fewer cases in women. Female AD patients also tend to be older than men, with over 50% of female AD cases occurring in individuals aged 70 and above.[2] Our preliminary research also indicates a significant male predominance among AD patients, with age-related patterns observed particularly among female patients.[6,7] This sex disparity in AD patients suggests that sex hormones may play a crucial role in the pathogenesis of AD.

Estrogen, one of the primary sex hormones in women, is primarily synthesized in the granulosa cells of the ovaries, with small amounts also produced by Sertoli cells in men.[8] 17β-estradiol (E2), the main active form of estrogen, has been shown

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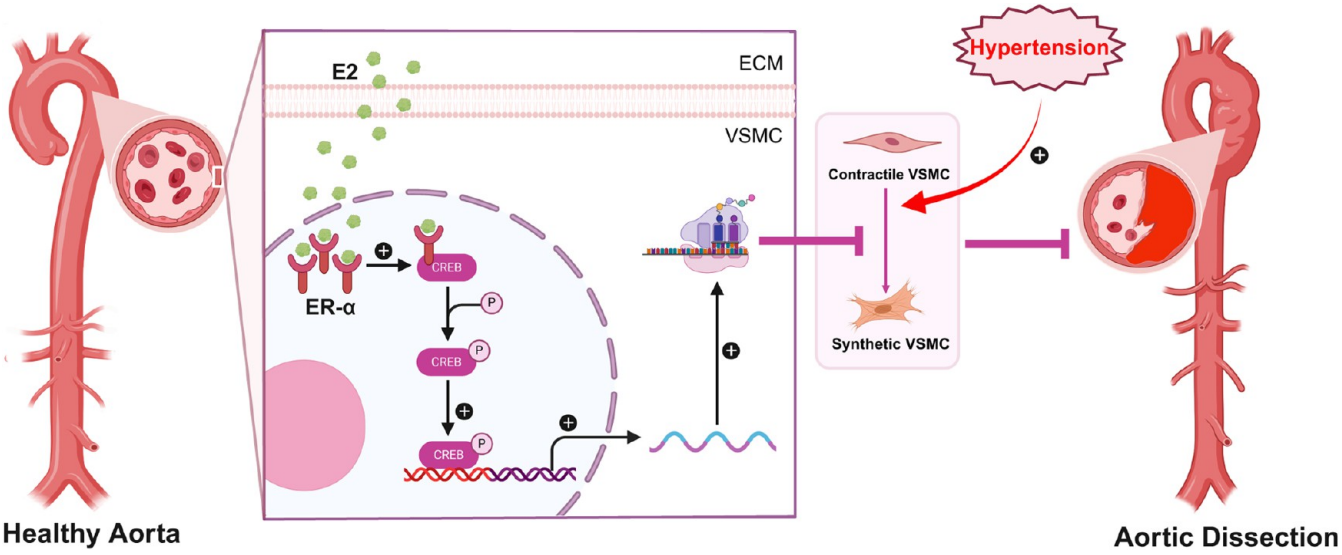

Figure 1. Proposed E2 inhibition mechanism of VSMCs phenotypic switching in AD mediated by ER-α/CREB. Estrogen (E2, 17β-estradiol) binds to ER-α and promotes the interaction of ER-α with CREB, thereby activating downstream CREB phosphorylation (p-CREB) and regulating the expression of genes involved in the maintenance of VSMC phenotype. This process inhibits the transition of VSMC from a contractile to a synthetic phenotype, potentially preventing AD progression. Schematic diagram of the molecular mechanism created in https://BioRender.com. E2: 17β-estradiol; VSMCs: vascular smooth muscle cells; AD: aortic dissection; ER-α: estrogen receptor α; CREB: cAMP-response element binding protein.

to play important roles in the physiological and pathological processes of the cardiovascular system.[9,10] It regulates vascular wall function and has anti-inflammatory and antioxidant effects. Consistent with this, premenopausal women demonstrate lower incidences of hypertension and coronary artery disease compared to age-matched men.[11] This protective effect of estrogen diminishes after menopause, although early estrogen replacement therapy in postmenopausal women has been shown to somewhat reduce the risk of hypertension and coronary artery disease.[12,13]

Estrogen primarily acts through three major receptors: estrogen receptor α (ER-α), G protein-coupled estrogen receptor, and estrogen receptor β (ER-β). ER-α is the main effector receptor.[14] Notably, studies have shown that estrogen treatment does not confer its protective effects against vascular injury in mice with ER-α gene knockout, indicating the essential role of ER-α in mediating vascular injury responses.[15,16] Additionally, estrogen induces G1 cell cycle arrest in VSMCs, inhibiting their proliferation, and upregulates the expression of ER-related genes *ESR1* and *ESR2* in a concentration-dependent manner.[17]

The cAMP-response element binding protein (CREB) is a protein that regulates gene transcription. Recent studies have suggested that CREB plays a crucial role in maintaining vascular integrity and preventing pathological vascular remodeling.[18] Importantly, CREB signaling is involved in the regulation of VSMC differentiation and survival, which are key factors in AD progression. C-terminus of CREB serves as the DNA-binding domain, and its N-terminus serves as the transcription activation domain. CREB contains a phosphorylation motif with multiple phosphorylation sites that can be phosphorylated by various protein kinases, including protein kinase C and protein kinase A. Phosphorylation of CREB at Ser133 is the primary mechanism through which it induces transcription and exerts its biological activity. In this context, evidence from ischemic stroke models and myocardial cells has shown that estrogen can exert protective effects on neurons and cardiomyocytes through the CREB pathway.[18,19] However, its specific role in AD remains poorly understood.

To explore potential regulatory mechanisms, we performed a bioinformatics analysis and identified a protein−protein interaction between ER-α and CREB, suggesting that these two factors may work together in regulating VSMC phenotype. This finding, combined with prior studies on estrogen's vascular protective effects and CREB's role in VSMC regulation, forms the basis of our hypothesis that estrogen (E2) may regulate the conversion of VSMCs phenotype through the ER-α/CREB pathway, potentially offering protection against the occurrence and development of AD (Figure 1).To elucidate the protective role of estrogen in AD, we will analyze clinical samples, perform bioinformatics analysis, utilize AD mouse models, conduct human aortic vascular smooth muscle cells (HASMCs) cultures, and carry out in vivo and in vitro experiments. Our aim is to demonstrate how E2 inhibits the phenotypic conversion of HASMCs through the ER-α/CREB pathway, thus providing new insights for AD prevention.

MATERIALS AND METHODS

Study Population Data and Samples. AD patients who were confirmed by computer tomography angiography (CTA)[20] of the aorta and treated at the Second Xiangya Hospital of Central South University from January 2015 to December 2018 were collected. Details of these patients' demographic information and clinical characteristics can be found in our previous publication.[21] Patients with traumatic AD, Marfan syndrome, and pregnancy were excluded. Blood samples (Table S1): Patients diagnosed with AD by aortic CTA at the Second Xiangya Hospital of Central South University from May 2023 to December 2023 were collected as the case group, and patients with traumatic AD, Marfan syndrome, and pregnancy were excluded. A total of 33 cases were included, consisting of 21 men and 12 postmenopausal women, with samples obtained from the first blood draw upon arrival at the emergency department of the Second Xiangya Hospital of Central South University. For comparison, serum samples from 27 age-matched healthy

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individuals during the same period were collected as the healthy control group, including 18 men and 9 postmenopausal women. All participants signed informed consent before sample collection. All blood samples were collected using procoagulant-containing blood collection tubes and stored at 4 °C overnight. After serum precipitation, the supernatant was centrifuged and stored at −80 °C until analysis. Aortic samples: Human aortic samples involved in the study were obtained with signed consent from either the patient or their family in accordance with the Declaration of Helsinki. AD tissue specimens were collected from AD patients ($n = 3$), and normal control aortic tissue specimens were collected from organ donors ($n = 3$). All aortic tissues were frozen in liquid nitrogen or fixed with 4% paraformaldehyde until the next experiment. These three studies were reviewed and approved by the Ethics Committee of the Second Xiangya Hospital of Central South University (protocol code 2022−621).

Animal Experiments. All animal experiments were carried out in accordance with the regulations approved by the Institutional Animal Care and Use Committee of the Second Xiangya Hospital of Central South University. All experimental mice were C57BL/6J (Silaikejingda, Hunan, China).

Ovariectomy (OVX) or sham surgery in female mice: Two-week-old female mice were anesthetized by subcutaneous injection of tribromoethanol. The abdominal cavity was opened along the paravertebral side, the bilateral ovaries were lifted, and the ovaries were ligated and removed. The sham group had part of the adipose tissue around the ovary removed. Samples from the vaginal cells and tail vein blood of female mice were collected before OVX or sham surgery and on the seventh day after surgery. Vaginal cells were fixed on slides for further testing. Blood samples were separated and stored at −80 °C for subsequent experiments. Mice that died within 7 days of surgery were automatically excluded from the next experiment.

AD mouse model: The AD mouse model was established by administering fumarate 3-aminopropionitrile (BAPN) (Sigma-Aldrich). Briefly, C57BL/6J mice were fed water containing 0.25% BAPN for 4 weeks.[22] A corn oil solution dissolved with 17β-estradiol (Sigma) was injected subcutaneously at 0.1 mg/kg for 4 weeks according to the body weight of the mice. Throughout the experiment, any animals that died were harvested immediately, and their aortas were fixed. At the end of the experimental cycle, the diameter of the aorta was measured by Doppler ultrasound in all surviving animals, and then euthanized, and their aortas were collected and fixed for further analysis.

Animal groups: After adaptive feeding to 3-weeks-old, 40 wild-type males were randomly selected and randomly divided into normal Saline group, E2 group, BAPN group and BAPN + E2 intervention group. Similarly, 20 mice were randomly selected from each of the 3-week-old female mice operated on OVX and sham, and divided into Sham group, Sham + BAPN group, OVX + BAPN group and OVX + BAPN + E2 group were intervened.

Analysis of Differentially Expressed Genes in the Transcriptome. The high-throughput RNA sequencing data set GSE153434 for human AD was obtained from the Gene Expression Omnibus (GEO) database by searching for the keyword "aortic dissection".This data set consisted of 20 samples, including 10 cases in the AD group and 10 cases in the donor group. To identify genes related to the estrogen signaling pathway hsa04915, a search was conducted on the Kyoto Encyclopedia of Genes and Genomes (KEGG) Web site, resulting in a total of 137 genes.

The "limma" package in R was then used to filter, background correct, logarithmic transform (with a cardinality of 2), normalize, and perform differential gene analysis. The differentially expressed genes were determined with an adjusted P-value <0.05 and an absolute log 2 fold change ≥ 1. The "ggplot2" package was used to create volcano plots, histograms, and heat maps of the differential genes. The resulting differential genes from the GSE153434 data set and the estrogen signaling pathway gene data set were merged to obtain the differential genes associated with the estrogen signaling pathway. The protein interaction network diagram was obtained from the STRING Web site.

Enzyme-Linked Immunosorbent Assay (ELISA). The levels of E2 in human and mouse blood samples were measured using an ELISA kit from Jonln, China.

Histology. Mouse aortic tissues were fixed in 4% paraformaldehyde for more than 24 h. Dehydration was performed using a gradient of alcohol in a dehydrator, and the aortic tissue was then embedded in an optimal cutting temperature compound and sectioned with a 4-μm microtome. Staining was carried out using hematoxylin-eosin (HE) and Elastica van Gieson (EVG).

Immunohistochemistry (IHC). Paraffin sections of human aortic tissue were deparaffinized and subjected to antigen retrieval using citric acid. A solution of 3% H_2O_2 was applied at room temperature for 30 min, and the tissue sections were circled. Subsequently, 10% goat serum was incubated at 37 °C for 30 min. The primary antibodies (ER-α at a 1:200 dilution, 21244−1-AP, Proteintech; CREB at a 1:100 dilution, 12208−1-AP, Proteintech; p-CREB at a 1:100 dilution, 28792−1-AP, Proteintech) were diluted in 10% serum and incubated overnight at 4 °C. The sections were washed with TBST and then incubated with the secondary antibody at 37 °C for 30 min. Finally, DAB solution was used for color development.

Cell Culture. HASMC lines (ATCC) were cultured with DMEM (Gibco, C11995500BT) with 10% FBS (NEWZENUM, FBS-PA500) and 1% penicillin-streptomycin (Abiowell, AWH0529). Cell models mimicking AD were constructed using HASMCs treated with angiotensin II (Ang II) at 0.10 μM (Sigma-Aldrich). HASMCs AD cell models were cultured with E2 (saline, 10^{-4} μM, 10^{-3} μM, 10^{-2} μM, Sigma-Aldrich), ER-α receptor antagonist (saline, 10^{-3} μM, 10^{-2} μM, 0.10 μM, Selleck, AZD9496), and CREB phosphorylation inhibitor (saline, 10^{-3} μM, 10^{-2} μM, 0.10 μM, Target MOI, 666−15) to obtain the optimal intervention concentration.

Co-Immunofluorescence (Co-IF). Human and mouse aortic tissue paraffin sections were deparaffinized into water, and antigen retrieval was performed using citric acid. Next, the sections were incubated with 10% goat serum at 37 °C for 30 min. They were subsequently incubated overnight at 4 °C with primary antibodies diluted in 10% serum. After washing with TBST, the sections were incubated with two secondary antibodies at 37 °C for 30 min. Nuclei were stained using DAPI working solution. The fluorescent colors used were DAPI (blue), ER-α (488, green), and α-SMA (555, orange-red). The primary antibodies used were ER-α (1:200 dilution, 21244−1-AP, Proteintech) and α-SMA (1:1000 dilution, 12208−1-AP, Abcam).

ShRNA Transfection. The shRNA lentivirus against ER-α (ESR1-shRNA) was designed by General Biol (Anhui, China). Blank-shRNA lentivirus was used as a negative control. The

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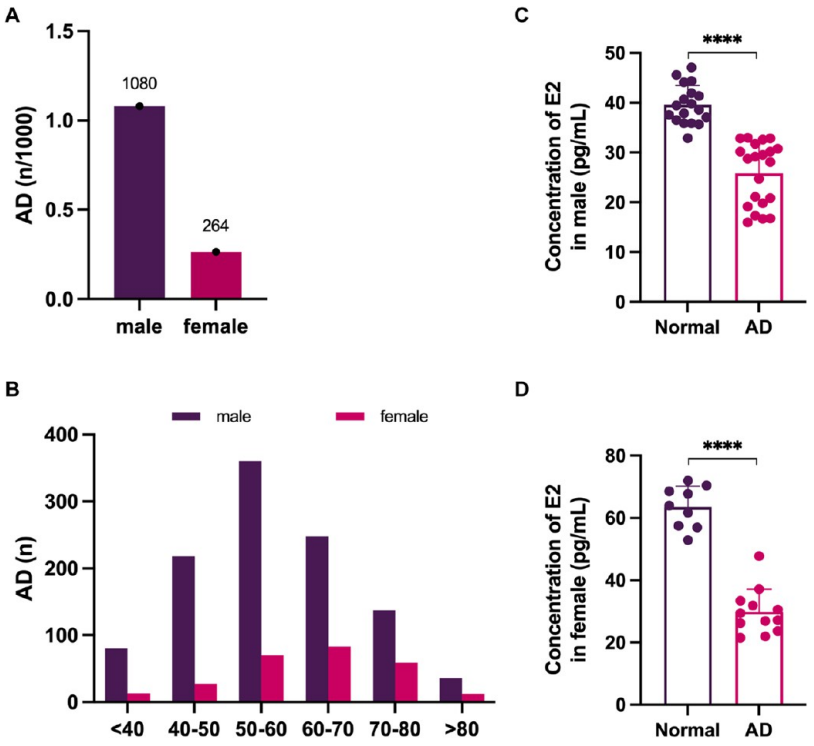

Figure 2. Sex differences and E2 levels in AD patients. (A) Sex distribution of AD patients diagnosed by CTA from January 2015 to December 2018, in the Second Xiangya Hospital of Central South University. (B) Age trends in male and female AD patients. (C) Serum estrogen levels in male AD patients (n = 21) and age-matched healthy controls (n = 18). Data are expressed as mean ± SEM ****P < 0.0001. (D) Serum estrogen levels in postmenopausal women with AD (n = 12) and in postmenopausal healthy women (n = 9). 2-way analysis of variance followed by the Bonferroni posthoc test was used for data analysis. ****P < 0.0001. E2: 17β-estradiol; AD: aortic dissection; CTA: computer tomography angiography.

sequence of the ESR1-shRNA is shown in Table S2. The lentivirus was added to HASMCs cultured in 24-well plates with multiplicities of infection (MOIs) of 10, 50, and 100, and the optimal MOI range for initial screening was 50−100. Next, the optimum MOI was determined by filtering with MOIs of 50, 65, 80, and 95. To select for stable transfection, 0.5, 1, 2, and 4 μg/mL of puromycin were added to the HASMCs cultured in 6-well plates, with 2 μg/mL determined as the optimal concentration. After 72 h of optimal lentiviral MOI transfection of HASMCs, 2 μg/mL puromycin was added to screen for stably transfected cells.

Western Blotting. The human aortic tissues or HASMCs were washed with ice-cold PBS. Lysates containing RIPA, PMSF, and phosphatase inhibitors were prepared at a ratio of 100:1:1. Human aortic tissue was added to the lysate and fully ground on a tissue grinder to obtain whole tissue protein. HASMCs were lysed on ice for 20 min, and the whole-cell proteins were obtained by fully lysing them with an ultrasonic apparatus. Equal amounts of total protein from human aortic tissue or HASMCs were separated using a 4−12% gradient SDS-PAGE (ACE, China), and the proteins were transferred to poly(vinylidene difluoride) membranes (MilliporeSigma). The membrane was incubated with the following primary antibodies: Tubulin (1:5000 dilution, AF7011, Affinity), OPN (1:1000 dilution, 22952−1-AP, Proteintech), α-SMA (1:1000 dilution, 14395−1-AP, Proteintech), SM22α (1:1000 dilution, 15502−1-AP, Proteintech), ER-α (1:1000 dilution, 21244−1-AP, Proteintech), CREB (1:1000 dilution, 12208−1-AP, Proteintech), p-CREB (1:1000 dilution, 28792−1-AP, Proteintech), and HRP-conjugated secondary antibodies. The protein signals were visualized using an enhanced chemiluminescence system (ECL, Tanon, 5200).

Co-Immunoprecipitation (Co-IP). HASMCs were washed with ice-cold PBS. Lysates containing RIPA, PMSF, and phosphatase inhibitors were prepared at a ratio of 100:1:1. HASMCs were lysed on ice for 20 min, and the whole cell proteins were obtained by fully lysing them. Target proteins (ER-α at a 1:100 dilution, CREB at a 1:100 dilution) were then precipitated using immunomagnetic beads, following the instructions provided by the Co-IP kit (Bioss, C0951).

RNA Extraction and Quantitative Real-Time PCR. According to the instructions of the RNA extraction kit (AG21024, AG), total RNA was extracted from cultured HASMCs using Trizol (AG221101, AG). RNA concentration was measured using a NanoDrop device (ThermoFisher). Reverse transcription was performed using 1 μg RNA according to the instructions of the reverse transcription kit (AG11728, AG). qRT-PCR of mRNA was performed using the SYBR Green qPCR Super Mix UDG Kit (AG11702, AG), and experiments were conducted on a Roche real-time PCR system. The primer sequences are shown in Table S3.

Statistical Analysis. R language (version 4.3.1) was used for statistical analysis. A t-test was used for normally distributed data and variance homogeneity analysis, while one-way analysis of variance was used for comparative analysis between multiple groups. GraphPad Prism 9 software was used for statistical plotting, and P < 0.05 was considered statistically significant.

RESULTS

Sex Disparities and Estrogen Levels in AD. In our previous studies, pronounced gender disparities in aortic

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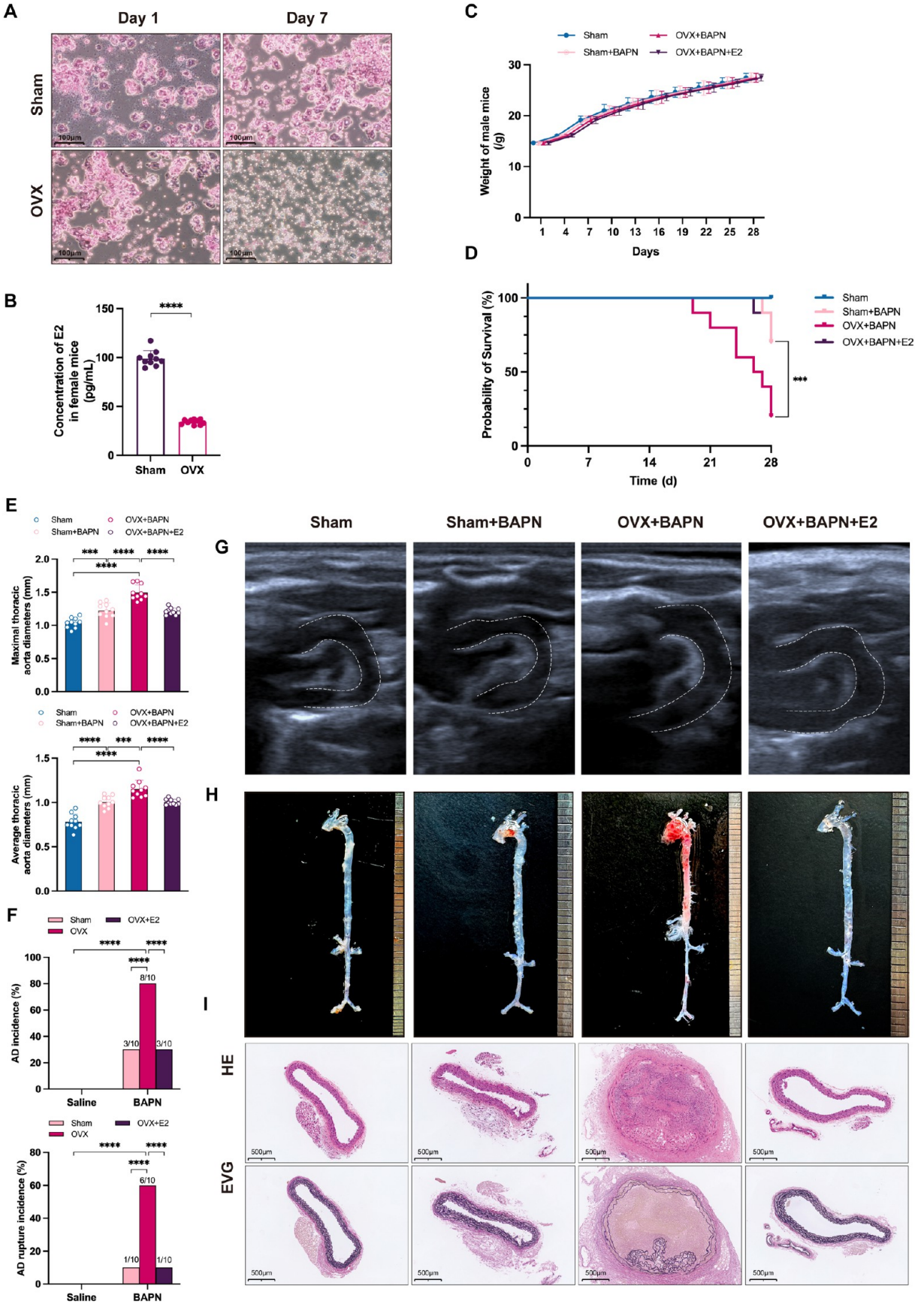

Figure 3. Estrogen supplementation prevents the formation of AD in female mice. (A) Vaginal cells were collected on days 1 and 7 from female mice undergoing OVX surgery and from the sham group. Cell smears and HE staining were performed. The cells that are large and irregular in shape are keratinocytes, and the cells that are small and round are leukocytes. (B) Serum estrogen levels were measured on day 7 after surgery in female mice

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Figure 3. continued

undergoing OVX surgery and female mice undergoing sham surgery. 2-way analysis of variance followed by the Bonferroni posthoc test was used for data analysis. ****$P < 0.0001$. (C) Curves showing changes in body weight in the indicated groups of female mice ($n = 10$ per group). mean ± SEM was used for data expression. (D) Survival curves for the indicated groups of female mice ($n = 10$ per group). The Kaplan−Meier method and compared with log-rank tests were used for survival data analysis. ***$P < 0.001$. (E) Quantification of the maximum (upper) and average (down) diameters of the thoracic aorta, measured by ultrasound in the indicated groups of female mice ($n = 6$−10 per group). two-way ANOVA was used for data analysis, followed by the Bonferroni posthoc test. ***$P < 0.001$; ****$P < 0.0001$. (F) The rate of AD rupture, instigated by β-aminopropionitrile (BAPN), was examined across various groups of female mice ($n = 10$ per group). The statistical evaluation of these data was conducted employing Fisher's exact test, which yielded an exceedingly significant result, indicated by ****$P < 0.0001$. (G) Typical echocardiographic images capturing the anatomical details of the thoracic aorta were obtained from the designated cohorts of female mice. (H) Characteristic macroscopic photographs of AD were taken in the specified groups of female mice. (I) Representative images of HE and EVG staining in the indicated groups of female mice. AD: aortic dissection; OVX: ovariectomy; BAPN: fumarate 3-aminopropionitrile; HE: hematoxylin and eosin; EVG: elastin-van Gieson staining.

dissection were identified within the clinical data of 1344 patients, comprising 1080 men and 264 women, revealing an alarming 4:1 ratio (Figure 2A). Additionally, the number of AD patients increases with age, with a peak age of 50−60 years for men and 60−70 years for women (Figure 2B). To explore the underlying causes of these sex differences, we measured E2 levels in AD patients compared to healthy controls. We observed that serum E2 levels decreased in male AD patients compared to healthy men of the same age group (Figure 2C). Similarly, in postmenopausal women, E2 levels decreased in AD patients compared to healthy individuals (Figure 2D).

E2 Represses AD Formation In Vivo. To determine the role of estrogen in the development of AD, we administered E2 in male and female AD mouse models. In female mice, we first reduced their estrogen levels through oophorectomy. The physiological cycle of female mice lasts about 4−6 days; therefore, we assessed estrogen levels by staining and measuring vaginal cells on day 1 and day 7 to evaluate the success of the OVX surgery. Compared to the sham group, the OVX group showed vaginal cells dominated by leukocytes and a significant reduction in keratinocytes (Figure 3A), with corresponding significant decreases in E2 levels (Figure 3B).

In the BAPN-induced AD model, BAPN was administered for 28 days, followed by E2 infusion (Figure 4A). The body weights of the different groups of female and male mice were not statistically significant (Figures 3C and 4B). In other words, theoretically, there was no statistically significant difference in the amount of BAPN consumed by the mice in different groups. In female mice, ovarian castration mice (OVX + BAPN group) exhibited shorter survival, more severe vasodilation, and a higher occurrence of AD compared to sham mice (Sham + BAPN group). However, E2 supplementation in the ovarian castration mice (OVX + BAPN + E2 group) improved these outcomes (Figure 3D−G). Similarly, in male mice, E2 supplementation (BAPN + E2 group) led to improved survival, reduced aortic dilation, and decreased the occurrence and rupture of AD in mice (Figure 4C−F). Morphologically, histological analysis showed that E2 intervention reduced aortic lumen enlargement and elastic fiber rupture (Figures 3H,I and 4G,H). These results suggest that estrogen can inhibit the onset and progression of AD.

E2 Regulates the Phenotypic Switching of Aortic VSMCs through ER-α. Phenotypic switching is an important pathophysiological factor in the development of AD. We found that patients with AD had significantly reduced expression of α-SMA and SM22α (contraction markers) and increased expression of OPN (a synthetic marker) in the aortic tissue compared to the control group (Figure 5A). To investigate the role of phenotypic switching of VSMCs in the protective effect of estrogen on AD, we examined the expression of OPN, α-SMA, and SM22α in the aortic wall of male and female mice. In male mice, BAPN-induced AD resulted in decreased α-SMA and SM22α expression and increased OPN expression, indicating a transition of VSMCs from a contractile to a synthetic phenotype. However, supplementation with E2 increased α-SMA and SM22α expression and inhibited the increased expression of OPN (Figure 5B). Similarly, in female mice, BAPN-induced AD led to increased OPN expression and decreased α-SMA and SM22α expression. However, sham surgery or E2 supplementation restored α-SMA and SM22α expression and reduced OPN expression (Figure 5C). Furthermore, in an AD cell model using HASMCs exposed to Ang II, estrogen intervention decreased OPN expression and increased α-SMA and SM22α expression (Figure 5D).

To better understand the connection between estrogen and the onset of AD, as well as to identify potential downstream candidates, we analyzed the high-throughput RNA sequencing GSE153434 data set of the human aorta. Specifically, we examined differentially expressed genes with adjusted $P < 0.05$ and $|\log 2[\text{fold change}]| \geq 1$. Our bioinformatics analysis of gene expression differences revealed that among the three main estrogen receptors, only ER-α exhibited reduced expression levels in AD, while no statistically significant difference was observed between ER-β and GPR30 (Figure 6A). Subsequently, we investigated the expression levels of ER-α in aortic tissues from AD patients and organ donors using IHC, Western blotting, and coimmunofluorescence. Our findings confirmed a decrease in ER-α expression levels in the aortic tissues of AD patients (Figure 6B−E). Additionally, in Ang II-mediated HASMCs, we observed a gradual increase in ER-α expression levels with higher estrogen-stimulated concentrations, as indicated by coimmunofluorescence following E2 intervention (Figure 7A,B).

To investigate how ER-α mediates estrogen's effect on the transition from contractile to synthetic HASMCs, we utilized AZD9496, a specific ER-α receptor inhibitor. By inhibiting the binding of E2 to ER-α in the Ang II-treated cell model, we found that the use of AZD9496 resulted in decreased expression levels of α-SMA and SM22α, while the expression of OPN increased in HASMCs (Figure 7C).

CREB as a Target of E2 Regulating Aortic SMC Phenotypic Switching via ER-α. We further analyzed the possible downstream role of estrogen-regulated genes in the phenotypic transformation of aortic SMCs through ER-α. By integrating the estrogen signaling pathway-related gene set Hsa04915 obtained from the KEGG with differentially expressed genes in the transcriptome of AD patients from GSE153434, we identified 21 possible downstream targets.

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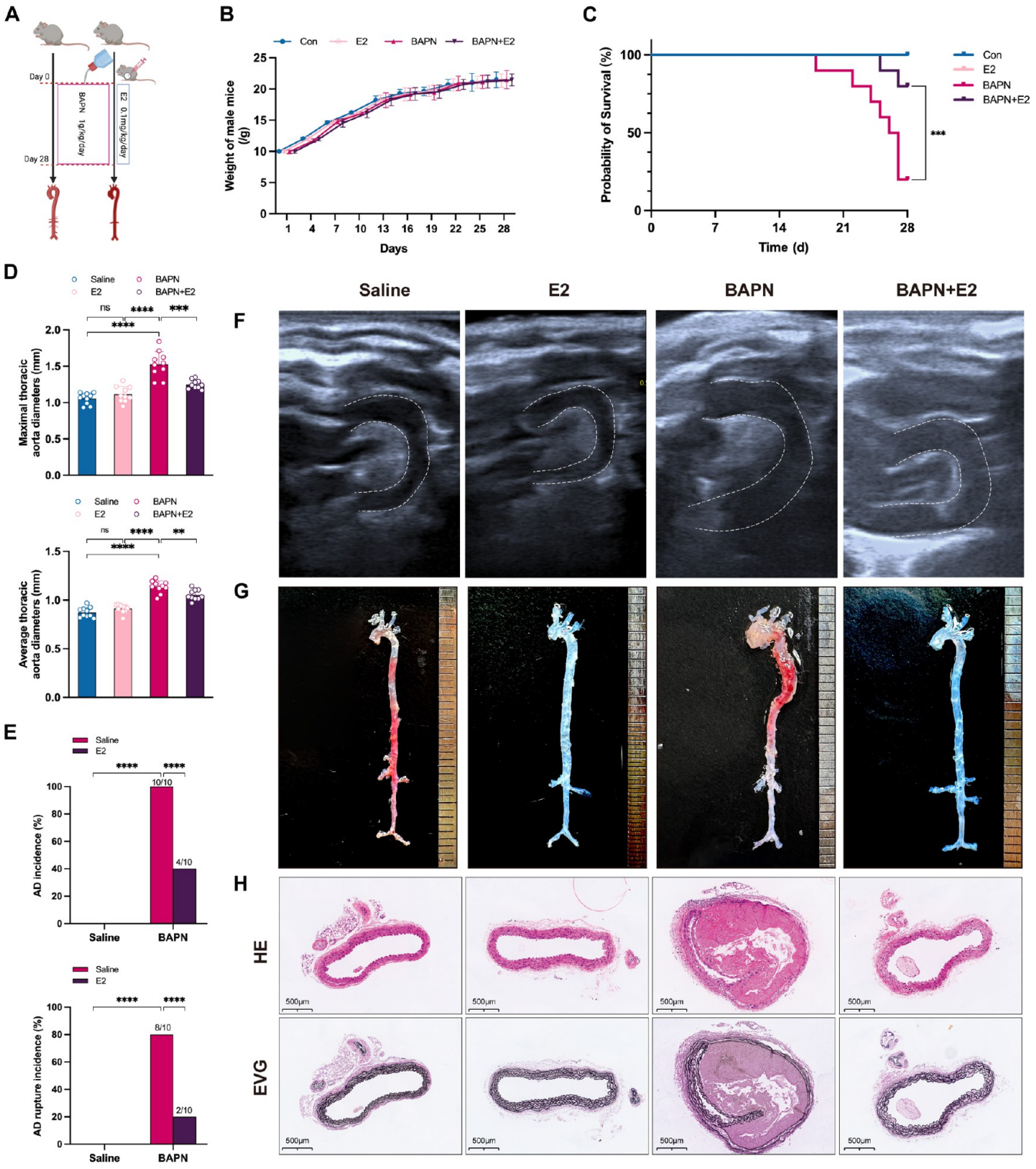

Figure 4. E2 supplementation prevents the formation of AD in male mice. (A) Schematic diagram of the protocol: WT male and female mice were orally administered saline or BAPN for 28 days while simultaneously being subcutaneously injected with estrogen for 28 days (n = 10 per group). (B) Curves showing changes in body weight in the indicated groups of male mice (n = 10 per group). mean ± SEM was used for data expression. (C) Kaplan−Meier survival analysis was employed to delineate the survival trajectories for distinct cohorts of male mice (n = 10 per group). The survival outcomes were subsequently juxtaposed utilizing the log-rank test to discern intergroup disparities. A statistically significant threshold was observed, denoted by ***P < 0.001, thereby underscoring the robustness of the observed distinctions in survival patterns. (D) Quantification of the maximum (upper) and average (down) diameters of the thoracic aorta, measured by ultrasound in the indicated groups of male mice (n = 6−10 per group). Data were analyzed by two-way ANOVA, followed by the Bonferroni posthoc test. **P < 0.01; ***P < 0.001; ****P < 0.0001. (E) In the indicated groups of male mice, the incidence of BAPN-induced AD rupture (n = 10 per group). Data were analyzed using Fisher's exact test. ****P < 0.0001. (F) Typical sonographic depictions of the thoracic aorta were captured from the specified groups of male mice. (G) Typical macroscopic illustrations of AD development were documented in the respective groups of male mice. (H) Representative images of HE and EVG staining in the indicated groups of male mice. AD: aortic dissection; BAPN: fumarate 3-aminopropionitrile; HE: hematoxylin and eosin; EVG: elastin-van Gieson staining.

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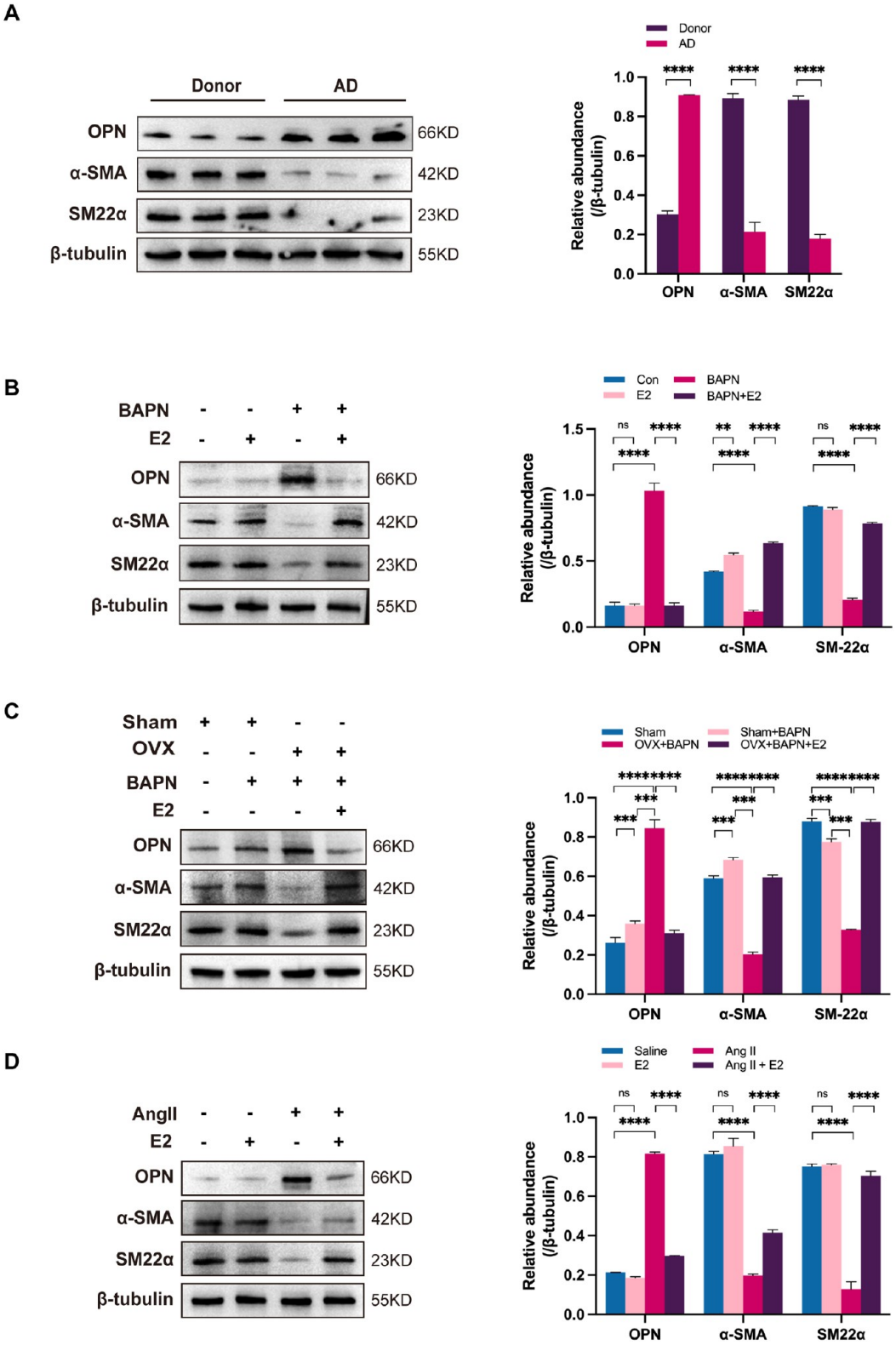

Figure 5. Estrogen inhibits the transition of VSMCs from contractile to synthetic phenotypes in AD. (A) Protein expression of OPN, α-SMA, and SM22α measured by Western blotting in aortic tissue from human organ donors and AD patients (n = 3 per group). Student's t-test was used for data analysis. ****P < 0.0001. (B) Protein expression of OPN, α-SMA, and SM22α measured by Western blotting in aortas of male mice. Student's t-test was used for data analysis. P < 0.01; **P < 0.0001. (C) Protein expression of OPN, α-SMA, and SM22α measured by Western blotting in aortas of female mice. Student's t-test was used for data analysis. ***P < 0.001; ****P < 0.0001. (D) Protein expression of OPN, α-SMA, and SM22α measured by Western blotting in HASMCs supplemented with Ang II and estrogen. Student's t-test was used for data analysis. ****P < 0.0001. VSMCs: vascular smooth muscle cells; OPN: osteopontin; α-SMA: α-smooth muscle actin; SM22α: smooth muscle 22α; HASMC: human aortic smooth muscle cell; Ang II: angiotensin II.

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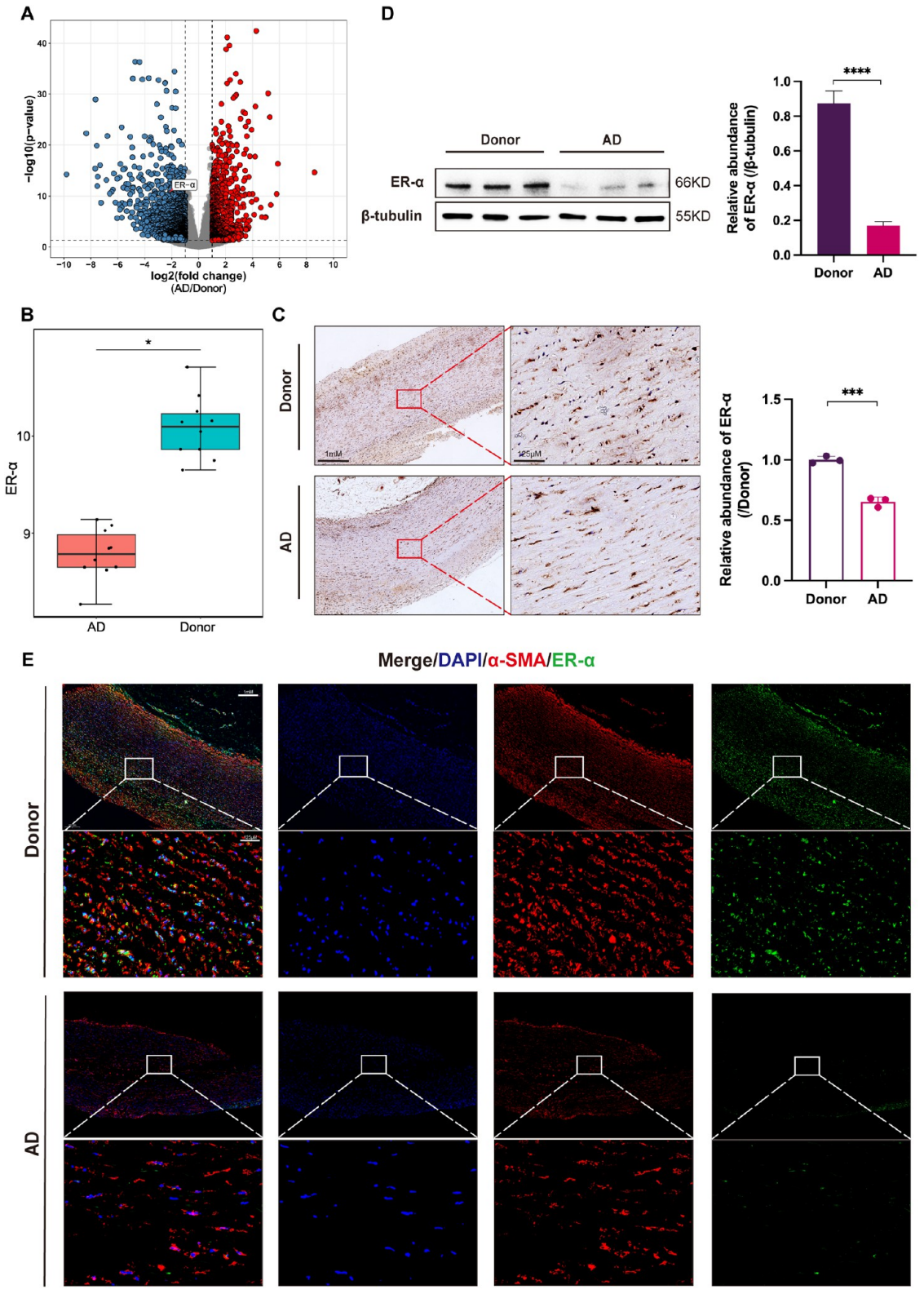

Figure 6. ER-α is downregulated in AD aortas. (A) Volcano plot showing differential genes in the GSE153434 (n = 10 per group) sequencing data set of the AD transcriptome. Genes identified as differentially expressed were those exhibiting Benjamini−Hochberg-adjusted P-values below the threshold of 0.05, coupled with a magnitude of change denoted by an absolute log 2 fold change value of 1 or greater. (B) Box plot showing ER-α mRNA expression in the indicated groups from the GSE153434 data set. (C) Representative images of IHC staining showing ER-α expression in aortic tissue from human organ donors and AD patients. Student's t-test was used for data analysis. ***$P < 0.001$. (D) ER-α protein expression measured by Western blotting in aortic tissue from human organ donors and AD patients (n = 3 per group). Student's t-test was used for data analysis. ****$P <$ 0.0001. (E) Representative images of ER-α expression by coimmunofluorescence staining in aortic tissue from human organ donors and AD patients, including costaining with α-SMA and DAPI. ER-α: estrogen receptor α; AD: aortic dissection; IHC: immunohistochemistry; α-SMA: α-smooth muscle actin; DAPI: 4′6-diamidino-2-phenylindole.

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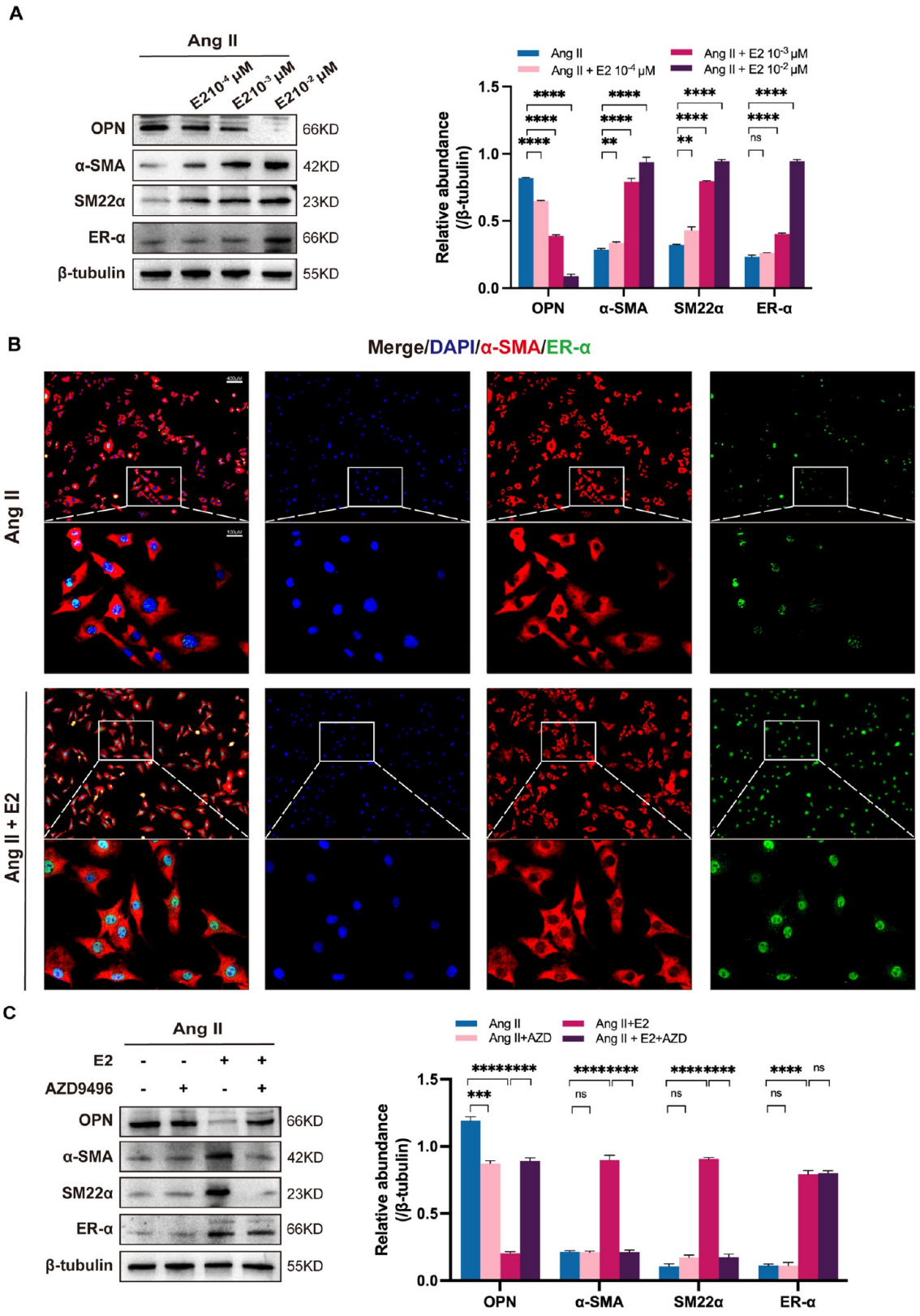

Figure 7. ER-α is the direct target of estrogen in inhibiting the transition of HASMCs from contractile to synthetic phenotypes in AD. (A) ER-α is the direct target of estrogen in inhibiting the transition of HASMCs from contractile to synthetic phenotypes in AD. (A) Protein expression of OPN, α-SMA, SM22α, and ER-α measured by Western blotting in HASMCs supplemented with Ang II and estrogen. Student's t-test was used for data analysis. ****$P < 0.0001$. (B) Representative images of ER-α expression by coimmunofluorescence staining in Ang II-supplemented HASMCs with or without estrogen, including costaining with α-SMA and DAPI. (C) Protein expression of OPN, α-SMA, SM22α, and ER-α measured by Western blotting in HASMCs supplemented with Ang II, estrogen, and the ER-α inhibitor AZD9496. Student's t-test was used for data analysis. ***$P < 0.001$; ****$P < 0.0001$. ER-α: estrogen receptor α; HASMCs: human aortic smooth muscle cells; AD: aortic dissection; OPN: osteopontin; α-SMA: α-smooth muscle actin; SM22α: smooth muscle 22α; ER-α: estrogen receptor α; Ang II: angiotensin II.

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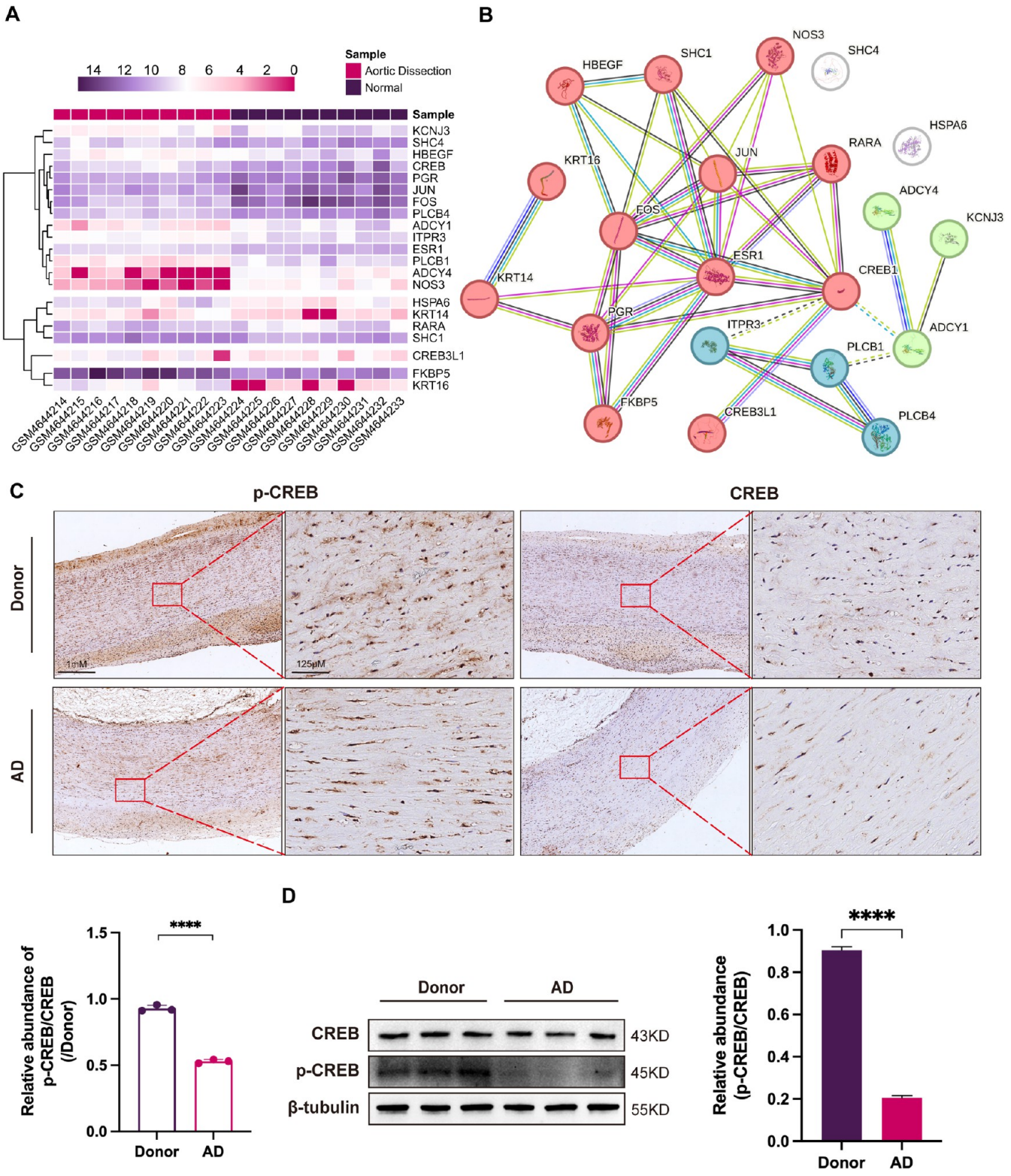

Figure 8. CREB phosphorylation is downregulated in AD aortas. (A) Common differentially expressed genes in the AD transcriptome sequencing data set GSE153434 (n = 10 per group) and estrogen signaling pathway data set hsa04915. Differentially expressed genes are defined as genes with Benjamini−Hochberg-adjusted P-values < 0.05 and |log 2 (fold change)| ≥ 1. (B) PPI network of the indicated differentially expressed genes according to the STRING Web site. (C) Representative images of IHC staining in aortic tissue from human organ donors and AD patients. Student's t-test was used to analyze the data. ****P < 0.0001. (D) CREB protein phosphorylation measured by Western blotting in aortic tissue from human organ donors and AD patients (n = 3 per group). Student's t-test was used to analyze the data. ****P < 0.0001. CREB: cAMP-response element binding protein; AD: aortic dissection; PPI: protein−protein interaction; IHC: immunohistochemistry.

Protein interaction network analysis using STRING revealed direct interactions between CREB and ER-α (Figure 8A,B). To confirm this finding, we analyzed the expression of p-CREB/CREB in the aortic tissue of AD patients using IHC and Western

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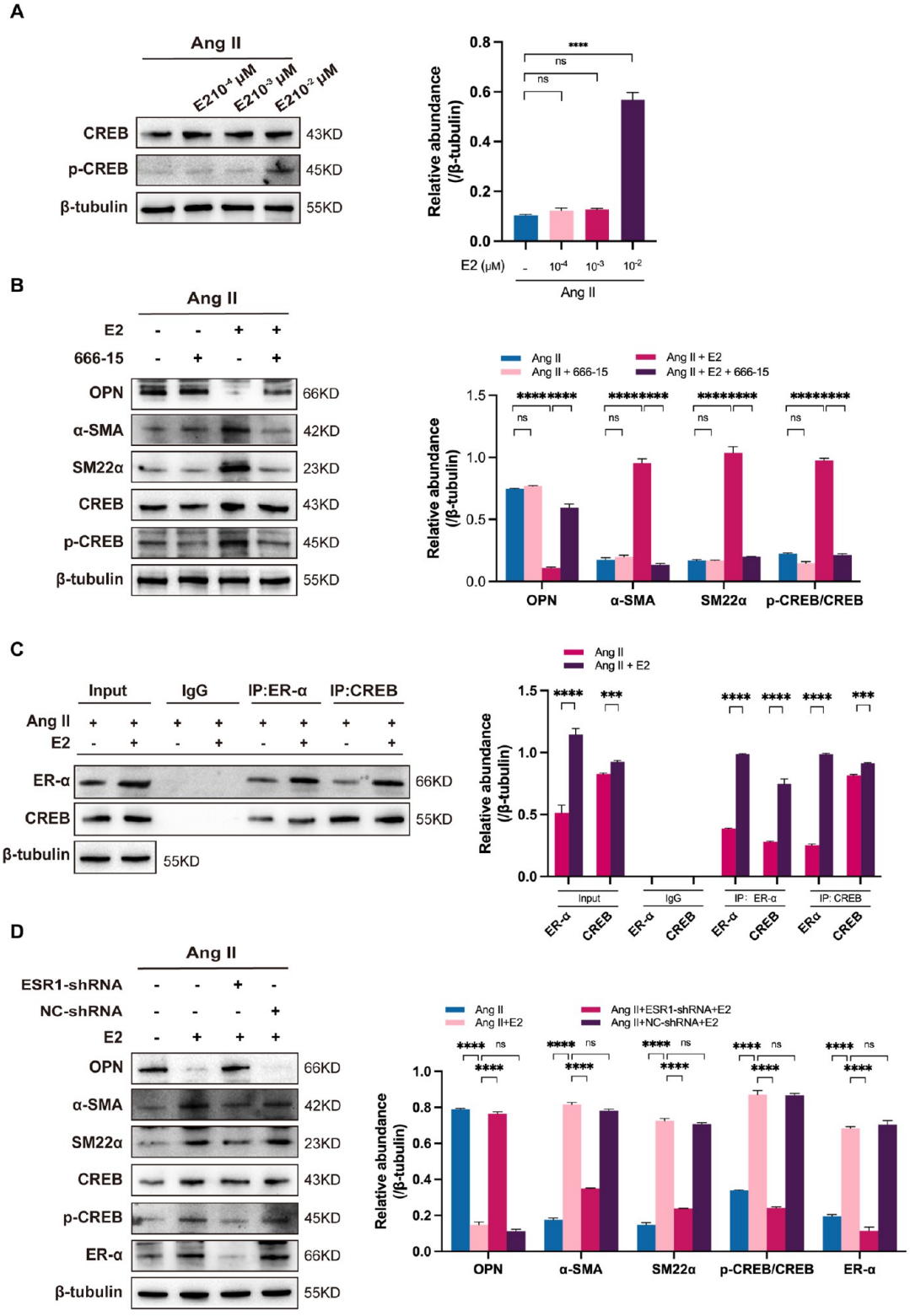

Figure 9. ER-α regulates the phosphorylation of CREB in HASMCs. (A) CREB and p-CREB protein expression in HASMCs supplemented with Ang II and estrogen was measured by Western blotting. Student's *t* test was used to analyze the data. ****$P < 0.0001$. (B) OPN, α-SMA, SM22α, CREB, and p-CREB protein expression measured by Western blotting in HASMCs supplemented with Ang II, estrogen, and the CREB phosphorylation inhibitor 666−15. Student's *t* test was used for data analysis. ****$P < 0.0001$. (C) ER-α protein expression measured by Western blotting following CO-IP in HASMCs supplemented with Ang II and estrogen. Student's *t* test was used for data analysis. ***$P < 0.001$; ****$P < 0.0001$. (D) OPN, α-SMA, SM22α, ER-α, CREB, and p-CREB protein expression measured by Western blotting in HASMCs with and without ER-α silencing and supplemented with Ang II and estrogen. Student's *t* test was used to analyze the data. ****$P < 0.0001$. ER-α: estrogen receptor α; CREB: cAMP-response element binding protein; AD; HASMCs: human aortic smooth muscle cells; Ang II: angiotensin II; OPN: osteopontin; α-SMA: α-smooth muscle actin; SM22α: smooth muscle 22α; CO-IP: coimmunoprecipitation.

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blotting. We found that the protein expression level of p-CREB/CREB was down-regulated in the AD group compared to the donor group (Figure 8C,D).

The role of CREB in the effect of estrogen on phenotypic conversion in VSMCs is unclear. Therefore, we first measured the level of CREB phosphorylation in a model of AD cells induced by different concentrations of Ang II. We found that the level of CREB phosphorylation in HASMCs increased with increasing estrogen concentrations (Figure 9A). Subsequently, when we added the CREB phosphorylation inhibitor 666−15 to an Ang II-induced AD cell model, we observed that the expression of OPN protein, a marker of synthetic phenotype, increased after estrogen intervention, while the expression of α-SMA and SM22α, markers of contractile phenotype, decreased (Figure 9B). In other words, CREB phosphorylation is involved in estrogen's inhibition of the transition of VSMCs from the contractile to synthetic state.

Our previous protein−protein interaction network analysis suggested a direct interaction between ER-α and CREB. To verify this effect, we performed in vivo Co-IP experiments and found that there was a protein−protein interaction between ER-α and CREB in HASMCs, and this effect was significantly enhanced after the E2 intervention (Figure 9C). Then, we established stable transfection of HASMCs with ER-α silenced and found that after silencing ER-α, CREB phosphorylation was inhibited, the expression of the contractile protein markers α-SMA and SM22α decreased, and the expression of the synthetic protein marker OPN increased (Figure 9D).

DISCUSSION

In this study, we investigated the sex disparity and the role of estrogen in the development of AD. Our findings confirm that there is a significant difference in the number of male and female AD patients, with men outnumbering women by approximately 3.6 times. Additionally, we observed that AD cases increase with age, with different peak incidences for men and women. Estrogen levels were found to be lower in male AD patients compared to healthy individuals and similarly reduced in postmenopausal female AD patients. Furthermore, we identified that estrogen inhibits the phenotypic switching of VSMCs through the ER-α/CREB pathway, suggesting a protective role against AD development. Estrogen is a crucial factor in cardiovascular protection and plays a significant role in cardiovascular disease.[14] It regulates vascular endothelial function through various pathways, including promoting the synthesis and release of nitric oxide,[23] inhibiting the synthesis of endothelin,[24] and reducing platelet activity.[25] This contributes to the stability and elasticity of blood vessels. Additionally, estrogen exerts anti-inflammatory and antioxidant effects, regulates lipid transport, and inhibits the formation and progression of atherosclerotic plaques.[26,27] Moreover, estrogen regulates the remodeling of blood vessel walls by inhibiting the proliferation and migration of VSMCs, thereby reducing the risk of arterial stenosis.[28]

However, it is unclear whether estrogen exerts this protective effect in AD. Epidemiological studies have shown a significant sex disparity in AD, with men having an earlier average age at diagnosis, accompanied by more severe vascular pathological changes and a poor prognosis.[29] In our study, we further collected sex and age data from AD patients and found similar results, with significantly reduced estrogen levels in AD patients. Additionally, we observed that estrogen supplementation in male and female mice alleviated the occurrence and progression of AD. This highlights the potential of estrogen as a protective factor in the pathogenesis of AD. Abnormal phenotypic switching of VSMCs can lead to structural disruption and dysfunction of the vascular wall, thereby promoting the formation and progression of AD.[5,30] This manifests in pathological remodeling,[31,32] involvement in inflammatory responses,[33,34] and impacts on vascular stability.[35] Given the significance of VSMC phenotypic switching in the development of AD, regulating this process may be a potential target for AD treatment. Some studies have demonstrated that phenotypic switching of VSMCs can be modulated by regulating cell signaling pathways and inhibiting inflammatory responses, leading to a reduction in the development and progression of AD. In our study, we found that estrogen could inhibit the conversion of VSMCs from contractile to synthetic phenotypes in both an AD animal model with BAPN intervention and an AD cell model simulated by Ang II. Therefore, we believe that estrogen may play a protective role in the pathogenesis of AD by inhibiting the phenotypic switching of VSMCs.

The role of estrogen depends on its binding to three major receptors and the initiation of downstream signaling pathways, including nuclear receptors (ER-α and ER-β) and membrane receptors.[36] Estrogen nuclear receptors, particularly ER-α and ER-β, are primarily involved in nuclear-initiated steroid signaling and serve as the main receptors for estrogen function. Previous study observed a distinct pattern in aneurysm tissues: male tissues exhibited high expression of androgen receptors, whereas female tissue demonstrated lower expression of ER-β and increased expression of progesterone receptors compared to unaffected aortic tissue.[37] This suggests that sex hormone activity may play a role in the development of aneurysms. To further explore this, we analyzed the transcriptome sequencing data from the AD GSE153434 data set and found that AD patients had reduced levels of ER-α, with no statistically significant difference in the levels of ER-β and GPER1. Protein−protein interaction analysis further revealed a direct interaction relationship between ER-α and CREB.

Estrogen binds to ER-α to dilate blood vessels, reduce vascular resistance, and maintain the stability and elasticity of blood vessels.[38,39] Estrogen can also directly activate the CREB pathway by binding to estrogen receptors on the cell surface, as well as indirectly influence the expression and activity of CREB through other pathways.[40] Furthermore, CREB participates in the regulation of various physiological and pathological processes in the cardiovascular system, such as inhibiting the proliferation of VSMCs,[41,42] inhibiting vascular calcification,[43] improving myocardial hypoxia/reperfusion injury,[44] and limiting ventricular remodeling.[45,46] In this study, immunohistochemistry, immunofluorescence, and Western blot analyses showed reduced levels of ER-α expression and CREB phosphorylation in the aortic tissues of AD patients. Moreover, inhibiting estrogen binding to ER-α or CREB phosphorylation weakened the inhibitory effect of estrogen on the phenotypic transition of HASMCs from contractile to synthetic phenotypes induced by Ang II. Co-IP also demonstrated an increased protein interaction between ER-α and CREB in HSMCs stimulated by estrogen. These findings suggest that estrogen may play a protective role in the pathogenesis of AD by inhibiting the phenotypic switching of VSMCs through the ER-α/CREB pathway. Despite the significant findings, our study has several limitations. First, the observational nature of the clinical data limits our ability to establish a causal relationship between estrogen levels and AD development. Second, the study is based

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on data from a single center, which may not be applicable to other populations. Additionally, while our experimental models provide valuable insights, they may not fully replicate the complex human pathophysiology of AD. Further research involving multicenter studies and more diverse populations is required to validate our findings. Future studies should also aim to explore the therapeutic potential of estrogen or estrogen analogs in preventing or treating AD. Investigating the role of other sex hormones and their interactions with estrogen could provide a more comprehensive understanding of sex differences in AD. Additionally, longitudinal studies tracking hormonal changes over time in AD patients could help clarify the temporal relationship between hormone levels and disease progression. Finally, expanding the research to include genetic and environmental factors that influence estrogen metabolism and VSMC behavior will be crucial in developing targeted interventions for AD.

CONCLUSIONS

Our study identified a higher prevalence of men than women among AD patients, with a significant increase in postmenopausal female patients. We also noted that estrogen levels were lower in AD patients. Importantly, estrogen inhibits the phenotypic transition of VSMCs through the ER/CREB pathway, exerting a protective effect on AD. These findings may offer new insights into the prevention of AD.

ASSOCIATED CONTENT

Data Availability Statement

The data sets used in this study can be accessed from online repositories. The names and accession numbers of these repositories are detailed in this article. If applicable, the data will be shared upon request made to the corresponding author of the manuscript.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c10955.

Information of the population from which the blood sample was collected (Table S1); sequence of ESR1-shRNA (Table S2); primer sequences (Table S3); Western blot raw data (PDF)

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Author Contributions

Y.P.: Formal analysis, investigation, methodology, software, validation, writing—Original draft. Y.Z.: Formal analysis, methodology, writing—review, and editing. T.G.: Formal analysis, software, writing—review, and editing. X.C.: Funding acquisition, project administration, supervision, validation, writing—review, and editing. G.Y.: Funding acquisition, methodology, resources, supervision, validation, writing—review and editing. All authors have read and approved the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

This study was reviewed and approved by the Ethics Committee of the Second Xiangya Hospital of Central South University (protocol code 2022−621).

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ABBREVIATION

AD, aortic dissection; IRAD, acute Aortic Dissection; VSMC, vascular smooth muscle cell; E2, 17β-estradiol; OPN, osteopontin; α-SMA, α-smooth muscle actin; SM22α, smooth muscle 22α; ER-α, estrogen receptor α; ER-β, estrogen receptor β; CREB, cAMP-response element binding protein; HASMCs, human aortic vascular smooth muscle cell; CTA, computer tomography angiography; OVX, ovariectomy; BAPN, fumarate 3-aminopropionitrile; GEO, Gene Expression Omnibus; KEGG, Kyoto Encyclopedia of Genes and Genomes; ELISA, Enzyme-Linked Immunosorbent Assay; HE, hematoxylin-eosin;

EVG, elastica van Gieson; IHC, immunohistochemistry; Co-IP, co-immunofluorescence; MOI, multiplicity of infection

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