

Dopamine beta-hydroxylase mediates atherosclerosis progression by regulating copper metabolism *via* PHOX2A

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ABSTRACT

Atherosclerosis (AS) is a major cardiovascular disease whose underlying molecular mechanisms remain incompletely understood. This study investigates the role of dopamine beta-hydroxylase (DBH) in AS pathogenesis and its regulation of copper metabolism via the transcription factor PHOX2A. Through transcriptomic profiling of aortic tissues from ApoE^{-/-} mice fed a high-fat diet, we identified 2891 differentially expressed genes (DEGs) between AS and control mice, with DBH being significantly upregulated. DBH expression was also elevated in the serum of AS patients and in ox-LDL-treated human vascular smooth muscle cells (HVSMDs). Mechanistically, we demonstrate that PHOX2A directly activates the DBH promoter, and that DBH knockdown attenuates ox-LDL-induced apoptosis and lipid accumulation in HVSMDs while PHOX2A overexpression exacerbates these effects. Importantly, DBH knockdown in ApoE^{-/-} mice not only reduced atherosclerotic plaque burden and improved serum lipid profiles but also restored serum copper concentrations and altered the expression of cuproptosis-related proteins (FDX1, LIAS, DLAT). These findings identify DBH as a key mediator of AS progression linked to PHOX2A-dependent regulation of copper metabolism and cuproptosis, suggesting that targeting the DBH/PHOX2A axis may represent a novel therapeutic strategy for AS.

Key words: atherosclerosis; dopamine beta-hydroxylase; PHOX2A; copper metabolism; cuproptosis.

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Ethics approval: this study involving human participants was conducted in accordance with the ethical standards of the Institutional Review Board (IRB) of The First Affiliated Hospital of Wannan Medical University and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all participants prior to their inclusion in the study. The ethical approval for this study was granted by the Ethics Committee of The First Affiliated Hospital of Wannan Medical University (Approval no. (2023)-lunshen-(147)). All animal experiments were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Wannan Medical College (Approval no. WNMC-AWE- 2023287). All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

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Introduction

Atherosclerosis (AS) is a chronic, progressive inflammatory disease of the arterial wall characterized by the accumulation of lipids, immune cells, and extracellular matrix components, leading to the formation of atherosclerotic plaques.^{1,2} It is a leading cause of morbidity and mortality worldwide, with its prevalence rising due to aging populations, unhealthy diets, and lifestyle factors.³ Despite advances in medical treatments, including statins and other lipid-lowering therapies, the clinical management of AS remains suboptimal.⁴ Current therapies primarily target lipid levels, but they do not address the underlying inflammatory and metabolic dysregulations that drive plaque formation and progression. Therefore, there is an urgent need to identify novel therapeutic targets and molecular mechanisms involved in AS pathogenesis.

One such emerging factor is copper metabolism, which plays a pivotal role in maintaining cellular homeostasis and oxidative stress balance.⁵ Copper ions are essential cofactors for enzymes involved in antioxidant defense, collagen synthesis, and lipid metabolism, all of which are crucial processes in vascular health.⁶ Recent studies have suggested that dysregulation of copper homeostasis can promote endothelial dysfunction, oxidative damage, and foam cell formation, all of which contribute to the development of AS.⁶⁻⁸ Furthermore, an emerging concept, known as «copper-induced cell death» or cuproptosis, has been implicated in vascular cell apoptosis and plaque instability, adding another layer of complexity to the pathophysiology of AS.⁹ However, the exact mechanisms by which copper metabolism and cuproptosis contribute to AS remain poorly understood.

Dopamine beta-hydroxylase (DBH), an enzyme responsible for the conversion of dopamine to norepinephrine, has been shown to influence vascular tone and smooth muscle cell function.^{10,11} Increasing evidence suggests that DBH also plays a role in copper metabolism. Specifically, DBH is involved in regulating copper homeostasis, potentially affecting cuproptosis in vascular cells.^{12,13} The transcription factor PHOX2A has been identified as a regulator of DBH expression, linking it to cellular stress responses and copper regulation.^{14,15} However, the interaction between DBH, PHOX2A, and copper metabolism in AS remains poorly understood.

In this study, we investigate the role of DBH in AS, focusing on its regulation of copper metabolism through PHOX2A. Using ApoE^{-/-} mice and ox-LDL-treated human vascular smooth muscle cells (HVSMCs), we explore how DBH influences lipid metabolism, cellular apoptosis, and copper homeostasis. Our findings provide new insights into the molecular mechanisms underlying AS and suggest potential therapeutic targets for this prevalent cardiovascular disease.

Materials and Methods

Animals and AS model

Male ApoE^{-/-} mice (8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were randomly divided into two groups: the AS group and the control group. The AS group was fed a high-fat diet (HFD, containing 21% fat and 0.15% cholesterol; Research Diets, New Brunswick, NJ, USA) for 4-8 weeks, while the control group was fed a standard chow diet (LabDiet, St. Louis, MO, USA). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of The First Affiliated Hospital of Wannan Medical

College and conducted in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Histological analysis

After euthanasia, the aortas were dissected and fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA). Tissues were embedded in paraffin and sectioned at 5 µm thickness. Hematoxylin and eosin (H&E) staining and Masson's trichrome staining were performed using standard protocols. For lipid deposition analysis, frozen aortic sections were stained with Oil Red O (Sigma-Aldrich). Images were captured using a Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan) and analyzed with ImageJ software (NIH, Bethesda, MD, USA).

Study population and clinical sample collection

This study enrolled 36 patients diagnosed with carotid atherosclerosis (AS group) and 53 age- and sex-matched healthy controls (NC group) who underwent routine health examinations at The First Affiliated Hospital of Wannan Medical College between November 2023 and March 2025. The diagnosis of carotid atherosclerosis was established by carotid duplex ultrasonography, defined as the presence of carotid intima-media thickness (IMT) ≥1.0 mm and/or the presence of atherosclerotic plaque.

Inclusion criteria for the AS group were: i) age between 18 and 75 years; ii) ultrasonographic confirmation of carotid atherosclerotic plaque and/or IMT thickening; iii) willingness to provide written informed consent. Exclusion criteria included: i) acute cardiovascular or cerebrovascular events within the preceding month (e.g., acute myocardial infarction, acute ischemic stroke); ii) autoimmune disease, active infection, or malignancy; iii) severe hepatic or renal insufficiency; iv) pregnancy or lactation; v) current use of medications known to affect copper or catecholamine metabolism; vi) incomplete clinical data.

Healthy controls were recruited from individuals undergoing routine physical examination during the same period, with no evidence of carotid atherosclerosis on ultrasonography and no history of cardiovascular, cerebrovascular, or systemic metabolic disease. The same exclusion criteria applied to the control group. Baseline demographic and clinical characteristics of the AS and NC groups are summarized in Table 1.

This study was approved by the Ethics Committee of The First Affiliated Hospital of Wannan Medical College (Approval No.: (2023)-lunshen-(147)) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants prior to enrollment.

Blood sample collection and processing

Peripheral venous blood samples (approximately 5 mL) were collected from all participants after an overnight fast of at least 8 h. Samples were allowed to clot at room temperature for 30 min and then centrifuged at 3,000 rpm for 10 min at 4°C. The resulting serum was aliquoted and stored at -80°C until further analysis, avoiding repeated freeze-thaw cycles.

ELISA

Serum concentrations of dopamine beta-hydroxylase (DBH) were measured using a commercially available human DBH ELISA kit (ab277076; Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, 50 µL of serum from each sample was added to pre-coated microplate wells and incubated with the detection antibody, followed by incubation with

horseradish peroxidase (HRP)-conjugated secondary antibody and substrate solution. The optical density (OD) was measured at 450 nm using a microplate reader (Infinite 200 Pro; Tecan Group Ltd., Männedorf, Switzerland), and DBH concentrations were calculated based on a standard curve generated from serial dilutions of the provided standards. All samples were assayed in duplicate, and the intra- and inter-assay coefficients of variation were <10% and <15%, respectively.

mRNA transcriptome sequencing

Total RNA was extracted from aortic tissues using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). mRNA sequencing libraries were prepared using the NEBNext Ultra RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA) and sequenced on the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA). Differential gene expression analysis was performed using the DESeq2 package in R, with a threshold of |log2 fold change| > 1 and adjusted *p*-value <0.05.

Screening of cuproptosis-related differentially expressed genes

Based on the mRNA sequencing data, differential expression analysis was conducted using the “limma” R package to identify DEGs. The screening criteria were set as |log2 fold change (log2FC)| >2 and an adjusted *p*-value (*p*.adj) <0.01. To focus on cuproptosis-related genes (CRGs), the identified DEGs were intersected with a curated list of 46 CRGs obtained from published literature.^{13,16}

The differential expression patterns of the target genes were visualized using the “ggplot2” R package, and statistical significance was assessed using the Wilcoxon rank-sum test. Additionally, heatmaps were generated to illustrate the expression profiles of the CRGs using the “pheatmap” R package.

For functional enrichment analysis, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed on the DEGs using the “clusterProfiler” R package. The *p*-values were adjusted using the Benjamini-Hochberg (BH) method to control for false discovery rates, while all other parameters were maintained at their default settings. This comprehensive approach enabled the identification and functional characterization of cuproptosis-related genes potentially involved in the pathogenesis of AS.

Cell culture and treatment

Human vascular smooth muscle cells (HVSMCs) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco). Cells were treated with 50 µg/mL oxidized low-density lipoprotein (ox-LDL, Yiyuan Biotechnologies, Guangzhou, China) for 24 h to induce AS-like conditions.

Transfection of siDBH, shDBH, and OE-PHOX2A in HVSMCs

The transfection experiments for siDBH (small interfering RNA targeting DBH), shDBH (short hairpin RNA targeting DBH), and OE-PHOX2A (PHOX2A overexpression) were performed using Lipofectamine 3000 Transfection Reagent (Invitrogen) according to the manufacturer’s instructions. HVSMCs (ATCC) were seeded in 6-well plates at a density of 2 × 10⁵ cells per well and cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C in a 5% CO₂ incubator until reaching 70-80% confluence. For transfection, the culture medium was replaced with Opti-MEM Reduced Serum Medium (Gibco). For siDBH transfection, 50 nM of DBH-targeting siRNA (Sigma-Aldrich) or scramble

Table 1. Baseline demographic and clinical characteristics of the AS and NC groups.

Variable	AS group (n=36)	NC group (n=53)	<i>p</i>
Age, years	60.61±8.04	60.34±7.68	0.874
Male sex, n (%)	21 (58.3)	27 (50.9)	0.492
BMI, kg/m ²	26.05±2.67	24.14±2.98	0.002
Smoking, n (%)	16 (44.4)	14 (26.4)	0.077
Drinking, n (%)	8 (22.2)	11 (20.8)	0.868
Hypertension, n (%)	20 (55.6)	12 (22.6)	0.002
Diabetes, n (%)	13 (36.1)	6 (11.3)	0.005
SBP, mmHg	139.36±12.70	123.57±10.99	<0.001
DBP, mmHg	84.53±7.95	77.70±7.14	<0.001
TC, mmol/L	5.38±0.97	4.76±0.64	0.002
TG, mmol/L	2.04±0.86	1.49±0.64	0.002
LDL-C, mmol/L	3.59±0.88	2.75±0.63	<0.001
HDL-C, mmol/L	1.10±0.23	1.35±0.28	<0.001
FBG, mmol/L	6.09±0.98	5.14±0.47	<0.001
hs-CRP, mg/L*	4.01 (2.81–6.03)	1.43 (0.91–2.19)	<0.001

AS, atherosclerosis; NC, normal control; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; FBG, fasting blood glucose; hs-CRP, high-sensitivity C-reactive protein. Continuous variables were compared using the independent-samples *t*-test; categorical variables were compared using the chi-square test. Data are presented as mean ± SD, median (interquartile range), or n (%); *hs-CRP was not normally distributed and was compared using the Mann-Whitney U test; values are presented as median (interquartile range).

siRNA (negative control, Sigma-Aldrich) was diluted in 125 μ L Opti-MEM and mixed with 5 μ L Lipofectamine 3000 diluted in 125 μ L Opti-MEM. The mixture was incubated at room temperature for 15 min to form transfection complexes, which were then added dropwise to the cells. For shDBH transfection, 2 μ g of pLKO.1-shDBH plasmid (Addgene, Watertown, MA, USA) or empty pLKO.1 vector (negative control, Addgene) was diluted in 125 μ L Opti-MEM and mixed with 5 μ L Lipofectamine 3000 in 125 μ L Opti-MEM. For OE-PHOX2A transfection, 2 μ g of pcDNA3.1-PHOX2A plasmid (Addgene) or empty pcDNA3.1 vector (negative control, Addgene) was diluted in 125 μ L Opti-MEM and mixed with 5 μ L Lipofectamine 3000 in 125 μ L Opti-MEM. After 15 min of incubation at room temperature, the transfection complexes were added to the cells. The cells were incubated at 37°C for 6 h, after which the transfection medium was replaced with fresh complete DMEM. The cells were further cultured for 48 h before harvesting for downstream analyses, including qPCR, Western blot, and functional assays. Transfection efficiency was confirmed by qPCR and Western blot analysis of DBH and PHOX2A expression levels. All experiments were performed in triplicate to ensure reproducibility.

qPCR

Total RNA was extracted from cells or tissues using TRIzol Reagent and reverse-transcribed into cDNA using the PrimeScript

RT Reagent Kit (Takara, Shiga, Japan). qPCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). Gene expression levels were normalized to *GAPDH* and calculated using the $2^{-\Delta\Delta C_t}$ method.

Primer sequences: DBH Forward primer (F): 5'-GCTGGAG-GAGGAGTTCGAAG-3'; Reverse primer (R): 5'-TCCTGGT-GATGGTGATGAGG-3'. PHOX2A Forward primer (F): 5'-CAGCAGCAGCAGCAGCAGTA-3'; Reverse primer (R): 5'-GCTGGTGGTGGTGGTGGTATAG-3'. AOC3 Forward primer (F): 5'-AGGTGAAGCTGCAGTTCAGG-3'; Reverse primer (R): 5'-TCCTTGAGCTCCTGGTTGAA-3'. ATP7A Forward primer (F): 5'-GCTGTGCTGTGGTTCATCATC-3'; Reverse primer (R): 5'-CCACAATGTCCAGGATCACC-3'. LOXL2 Forward primer (F): 5'-TGCTGTGCAGAAAGATCCAGA-3'; Reverse primer (R): 5'-GGATGTTGTGGTTGCTGTCC-3'. CDKN2A Forward primer (F): 5'-GCTCAACTCACGCCTAACCA-3'; Reverse primer (R): 5'-TCATCAGGAATGCCACGTC-3'. GAPDH Forward primer (F): 5'-GGAGCGAGATCCCTCCAAAAT-3'; Reverse primer (R): 5'-GGCTGTTGTCATACTTCTCATGG-3'.

Western blot

Protein extracts were prepared using RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with protease inhibitors (Roche, Basel, Switzerland). Proteins were separated by

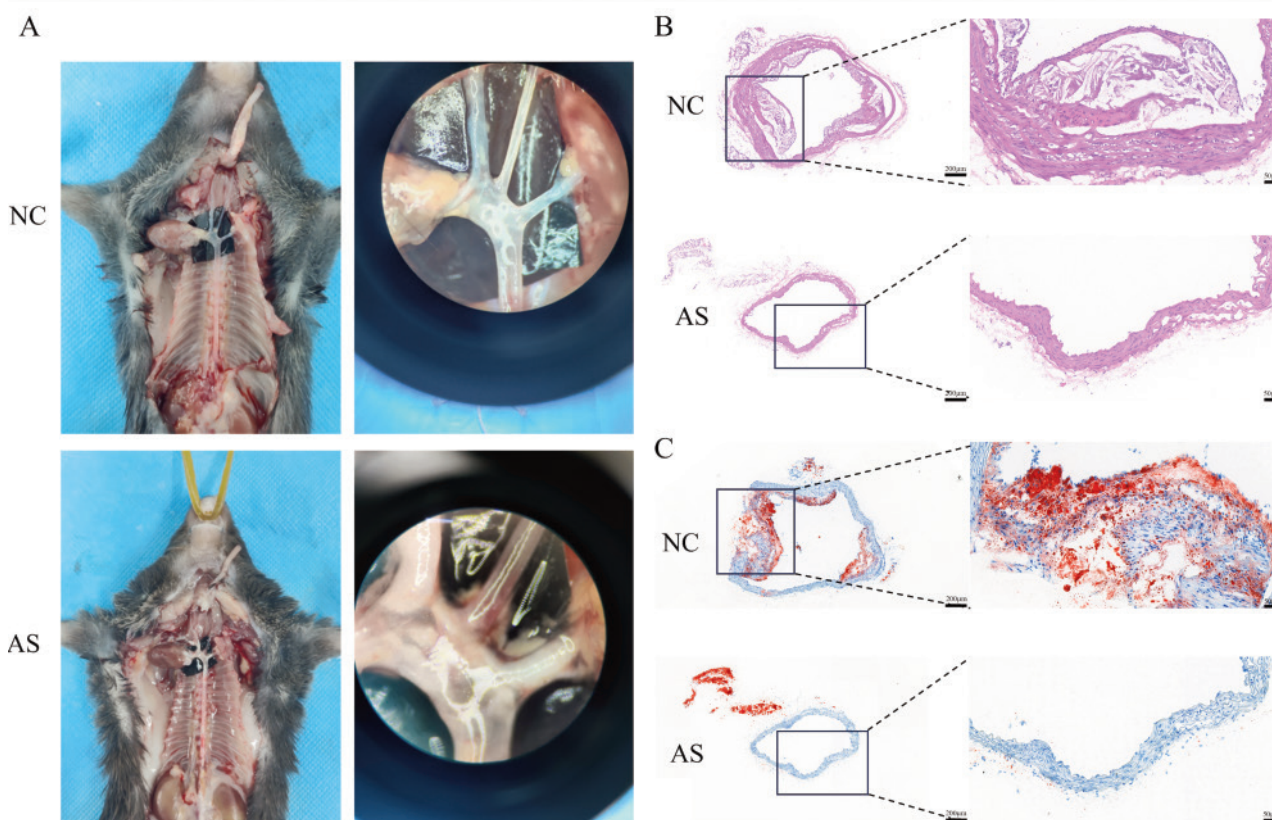


Figure 1. Establishment and histochemical characterization of the AS model in Apo^{E-/-} mice. **A)** Representative images of aortic isolation from normal control (NC) and AS mice; the AS group exhibits visible vascular alterations compared with the NC group. **B)** H&E staining of aortic cross-sections showing vascular morphology and plaque formation; the AS group displays marked intimal thickening and lesion development compared with the NC group; boxed regions indicate areas shown at higher magnification. **C)** Oil Red O staining of aortic sections illustrating lipid deposition within the vascular wall; extensive lipid accumulation is observed in the AS group, whereas minimal staining is detected in the NC group; boxed regions indicate areas shown at higher magnification.

SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat milk and incubated with primary antibodies against DBH, PHOX2A, FDX1, LIAS, DLAT and GAPDH overnight at 4°C. After incubation with HRP-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA), protein bands were visualized using the ECL Plus Detection Kit (Thermo Fisher Scientific, Waltham, MA, USA). Primary antibody information: DBH (10777-1-AP, 1:4000; Proteintech, Rosemont, IL, USA), PHOX2A (ab155084, 1:1000; Abcam), GAPDH (60004-1-Ig, 1:10000; Proteintech), LIAS (11577-1-AP, 1:2500; Proteintech), DLAT (DF14046, 1:1000; Affinity), FDX1 (12592-1-AP, 1:10000; Proteintech).

Dual-luciferase reporter assay

The DBH promoter region was cloned into the pGL3-Basic vector (Promega, Madison, WI, USA). HVMSCs were co-transfected with the DBH promoter reporter plasmid and a PHOX2A overexpression plasmid using Lipofectamine 3000 (Invitrogen). After 48 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and normalized to Renilla luciferase activity.

Copper ion concentration measurement

Copper ion content in cells was determined using a Copper Ion Detection Kit (Solarbio, Beijing, China). According to the kit's

instructions, an appropriate amount of cell suspension (1×10^6 cells/mL) was added to a centrifuge tube, and copper ions were extracted following the protocol provided by the kit. After preparation, the cell lysate was centrifuged at 12,000 rpm for 10-15 min at 4°C using a centrifuge (Eppendorf SE, Hamburg, Germany). The supernatant was collected and incubated with the copper ion colorimetric reagent at 37°C for 5 min. Absorbance at 570 nm was measured using a microplate reader (Tecan Group Ltd.).

IHC

Paraffin-embedded aortic sections were deparaffinized, rehydrated, and subjected to antigen retrieval using citrate buffer (pH 6.0; Sigma-Aldrich). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (Sigma-Aldrich). Sections were then incubated with primary antibodies against DBH (10777-1-AP, 1:4000; Proteintech), FDX1 (12592-1-AP, 1:10000; Proteintech), LIAS (11577-1-AP, 1:2500; Proteintech), and DLAT (DF14046, 1:1000; Affinity) overnight at 4°C. After washing, sections were incubated with HRP-conjugated secondary antibodies (Jackson ImmunoResearch) and visualized using the DAB Substrate Kit (Vector Laboratories, Burlingame, CA, USA). Slides were counterstained with hematoxylin, dehydrated, and mounted. Images were acquired using a Nikon Eclipse Ti microscope and analyzed with ImageJ software.

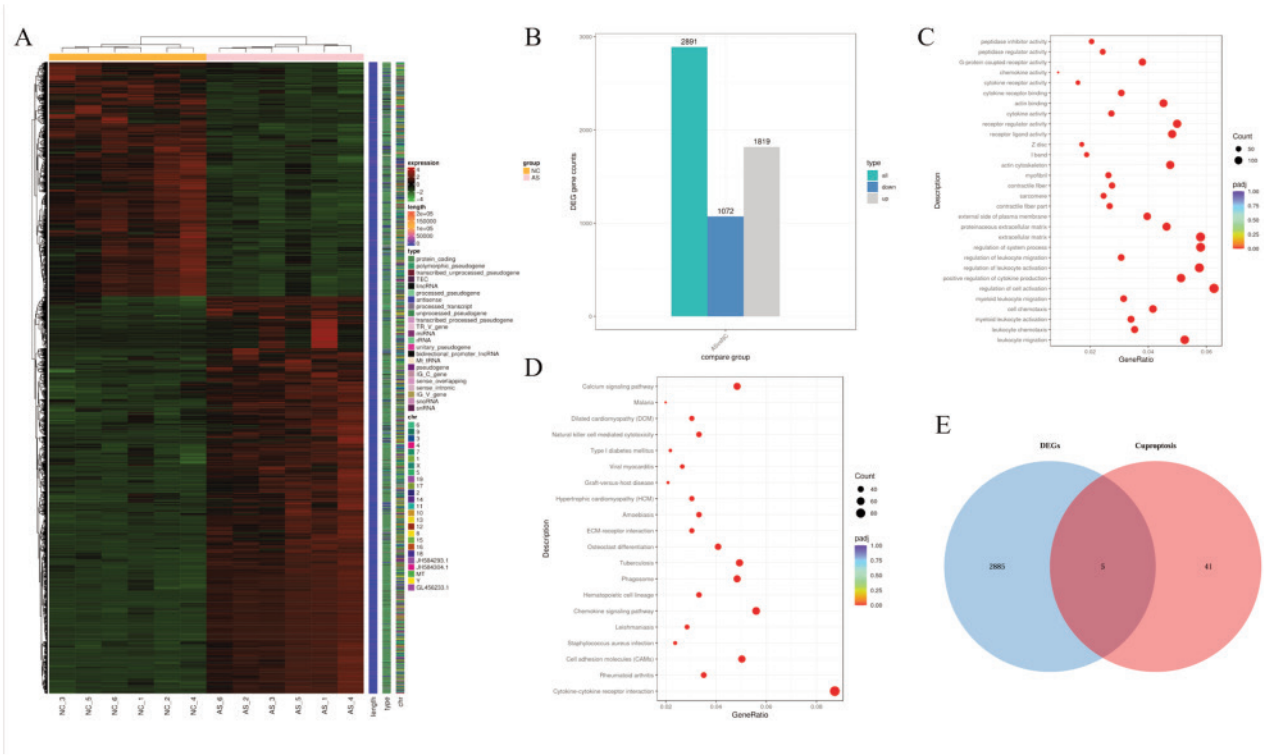


Figure 2. mRNA transcriptome analysis reveals atherosclerosis-related genes. **A)** Heatmap of DEGs between AS and NC groups. **B)** Volcano plot showing 2,891 DEGs (1,072 downregulated and 1,819 upregulated) with $|\log_2FC| > 1$ and $p_{adj} < 0.05$. **C)** GO enrichment analysis of DEGs, highlighting biological processes related to immune response and muscle contraction. **D)** KEGG pathway analysis showing enriched pathways, including calcium signaling and immune dysregulation. **E)** Venn diagram illustrating the overlap between DEGs and CRG.

Statistical analysis

Data are presented as mean $\pm$ SD. Statistical analyses were performed using GraphPad Prism 9.0. Differences between groups were analyzed using Student's *t*-test or one-way ANOVA followed by Tukey's *post-hoc* test. A *p*-value <0.05 was considered statistically significant.

Results

Establishment of the AS animal model and aortic pathological analysis

In this study, we successfully established an AS animal model. AS was induced in ApoE^{-/-} mice by high-fat feeding for 4 to 8 weeks. In contrast, the control group mice were fed a standard diet for the same period. Upon dissection of the aorta and imaging (Figure 1A), prominent atherosclerotic plaques were observed in the aortas of the AS group mice, while no abnormalities were seen in the control group. Histological examination using H&E staining

(Figure 1B) revealed large and numerous atherosclerotic plaques in the aorta of the AS group, which were absent in the control group. Oil Red O staining, which detects lipid content, showed a significant increase in lipid deposition in the AS group (Figure 1C), while almost no lipid accumulation was detected in the control group, further confirming the successful establishment of the AS model.

mRNA transcriptomic analysis reveals AS-related genes

To explore the molecular mechanisms underlying AS, we performed mRNA transcriptomic sequencing on aortic tissues from both the AS and control groups. The heatmap of DEGs demonstrated significant differences in gene expression between the AS and control groups (Figure 2A). A total of 2891 DEGs were identified, with 1072 genes downregulated and 1819 genes upregulated (Figure 2B). GO enrichment analysis indicated that these DEGs were significantly enriched in immune response pathways (such as cell migration and cytokine activity) and muscle contraction pathways (such as myofibril and sarcomere function) (Figure 2C). KEGG pathway analysis further highlighted the critical roles of calcium signaling, immune dysregulation, and infection pathways

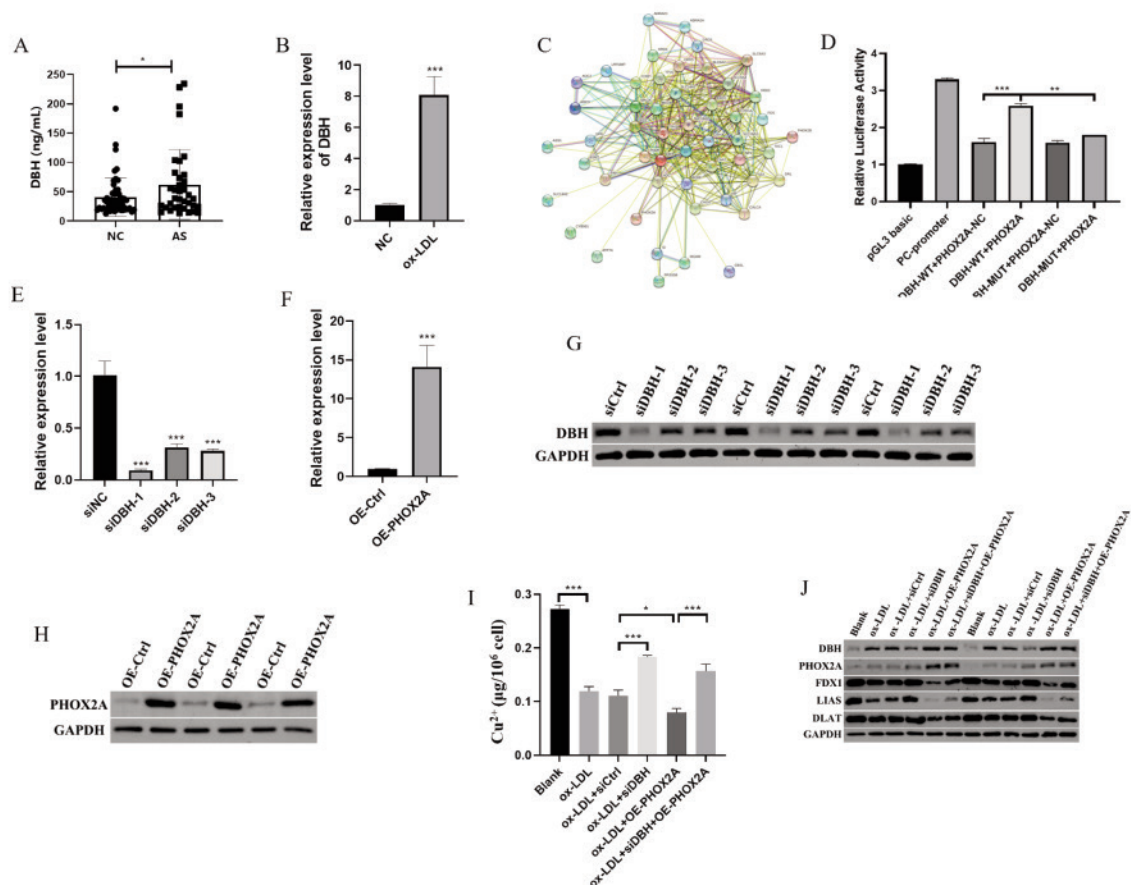


Figure 3. DBH regulates copper metabolism via PHOX2A transcription factor. **A)** ELISA analysis of DBH expression in serum samples from AS patients and healthy controls. **B)** qPCR analysis of DBH expression in HVSMCs treated with ox-LDL. **C)** PPI network analysis suggesting PHOX2A as a potential regulator of DBH. **D)** Dual-luciferase reporter assay demonstrating PHOX2A-mediated activation of the DBH promoter; after DBH knockdown or PHOX2A overexpression, qPCR (**E-F**) and Western blot (**G-H**) verified the expression of DBH and PHOX2A in HVSMC. **I)** Copper ion concentration in mouse serum was measured. **J)** Western blot was used to detect the expression of cuproptosis-related proteins (FDX1, LIAS, and DLAT) in HVSMCs. Data are expressed as mean $\pm$ SD; *n*=3; ***p*<0.01, ****p*<0.001.

in AS (Figure 2D). Venn diagram analysis (Figure 2E) revealed five genes -*AOC3*, *Atp7a*, *Loxl2*, *DBH*, and *Cdkn2a*- that intersected between DEGs and copper-induced cell death-related proteins, suggesting that these genes may play key roles in both AS and copper-mediated cell death.

To further prioritize among the five candidate genes identified by the Venn analysis, we performed qPCR validation in aortic tissues from NC and AS mice. As shown in Supplementary Figure S1, *DBH* exhibited the most pronounced upregulation in AS tissues compared with NC controls, while the other four candidate genes showed relatively minor changes. Based on these results, *DBH* was selected for subsequent mechanistic studies.

DBH regulates copper metabolism via the PHOX2A transcription factor

Having identified *DBH* as a candidate of interest, we further investigated the role of *DBH* in AS. Elevated expression of *DBH* was observed both in the serum of AS patients and in ox-LDL-

treated HVSMC cells (Figure 3 A,B). The use of ox-LDL, a well-established model for inducing atherosclerosis-like conditions *in vitro*,¹⁷ was crucial for mimicking the oxidative stress and lipid accumulation characteristic of AS, thereby providing a relevant cellular context to study *DBH*'s role in this disease. Protein-protein interaction (PPI) network analysis suggested that *DBH* might be regulated by the transcription factor PHOX2A (Figure 3C). Dual-luciferase reporter assays (Figure 3D) demonstrated a significant increase in the activity of the *DBH* gene promoter under the influence of PHOX2A (***p*<0.001), whereas mutations in the *DBH* promoter region led to a reduced activity (***p*<0.01), confirming PHOX2A's regulatory effect on *DBH* transcription. We then validated the role of *DBH* and PHOX2A in copper metabolism by knocking down *DBH* and overexpressing *PHOX2A*. qPCR and Western blotting confirmed the changes in *DBH* and PHOX2A expression at the mRNA and protein levels, respectively (Figure 3 E-H). Copper ion concentration assays (Figure 3 I,J) revealed a significant reduction in copper levels in the ox-LDL-treated group,

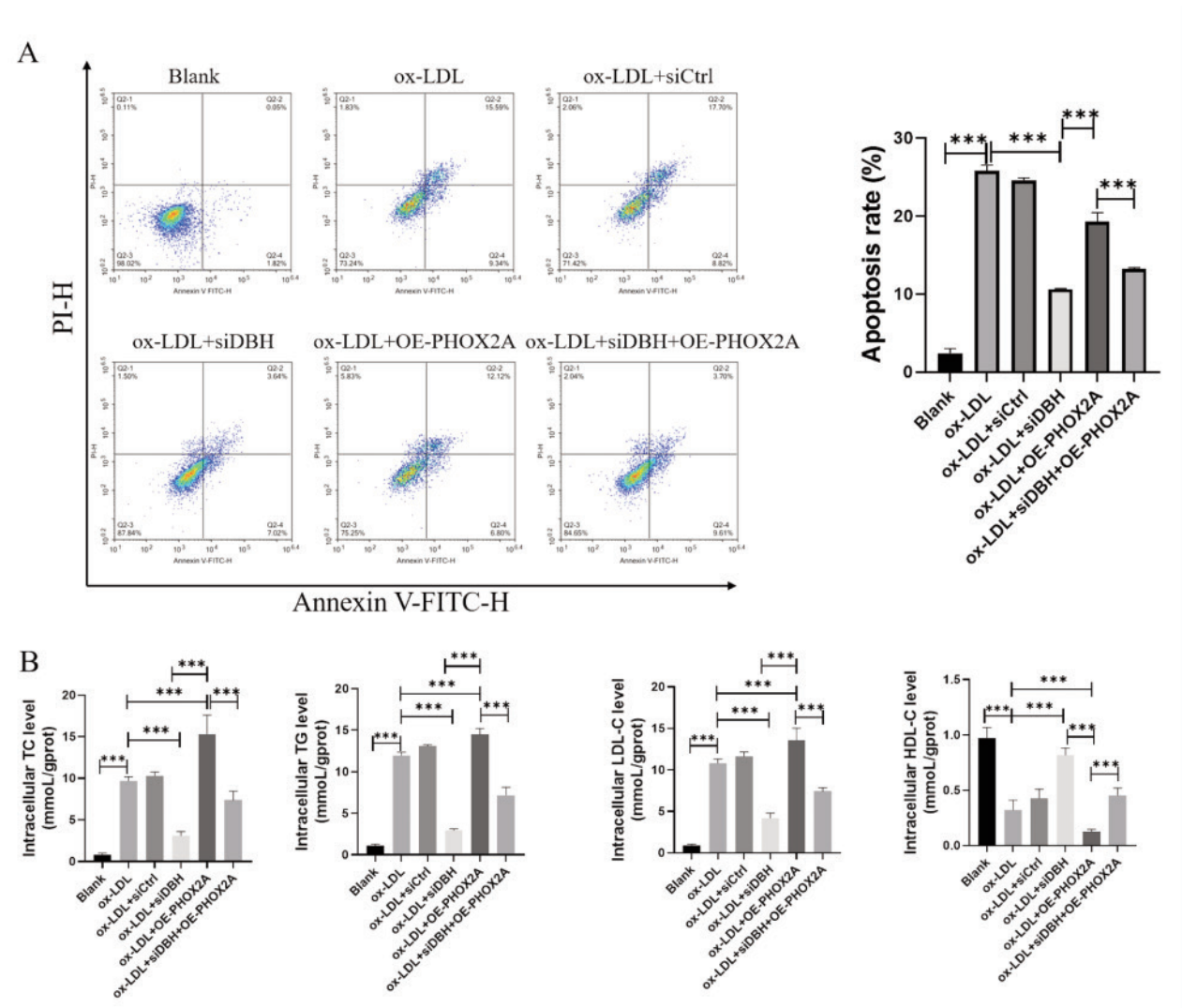


Figure 4. DBH knockdown attenuates ox-LDL-induced HVSMC injury. **A**) Flow cytometry analysis of apoptosis in HVSMCs treated with ox-LDL (50 µg/mL, 24 h) after DBH knockdown or PHOX2A overexpression. **B**) ELISA analysis of lipid profiles (TC, TG, LDL-C, and HDL-C) in HVSMCs under the same conditions. Data are presented as mean ±SD; n=3 ****p*<0.001.

which was restored upon *DBH* knockdown, while *PHOX2A* overexpression led to a further decrease in copper levels, corroborating the interactive role of *DBH* and *PHOX2A* in copper metabolism.

DBH knockdown reduces ox-LDL-induced injury in HVSMC cells

To explore the functional role of *DBH* in AS, we assessed the effect of *DBH* knockdown on ox-LDL-treated HVSMC cells. Flow cytometry analysis (Figure 4A) showed a significant increase in apoptosis in ox-LDL-treated HVSMCs, which was alleviated by *DBH* knockdown. Overexpression of *PHOX2A*, on the other hand, promoted apoptosis. ELISA results (Figure 4B) revealed that ox-LDL treatment elevated the levels of TC, TG, and LDL-C in cells,

while *DBH* knockdown significantly reduced these lipid levels, and *PHOX2A* overexpression further exacerbated lipid accumulation. HDL-C levels showed an inverse correlation with these lipid indicators. These findings suggest that *DBH* knockdown may protect HVSMC cells from ox-LDL-induced damage by modulating lipid metabolism and apoptosis.

DBH knockdown ameliorates AS in mice

In vivo experiments showed that *DBH* knockdown significantly alleviated the severity of AS. Aortic imaging from the shCtrl and shDBH groups (Figure 5A) revealed a marked reduction in plaque area in the shDBH group. Oil Red O staining and plaque area quantification (Figure 5B) further confirmed that the shDBH group

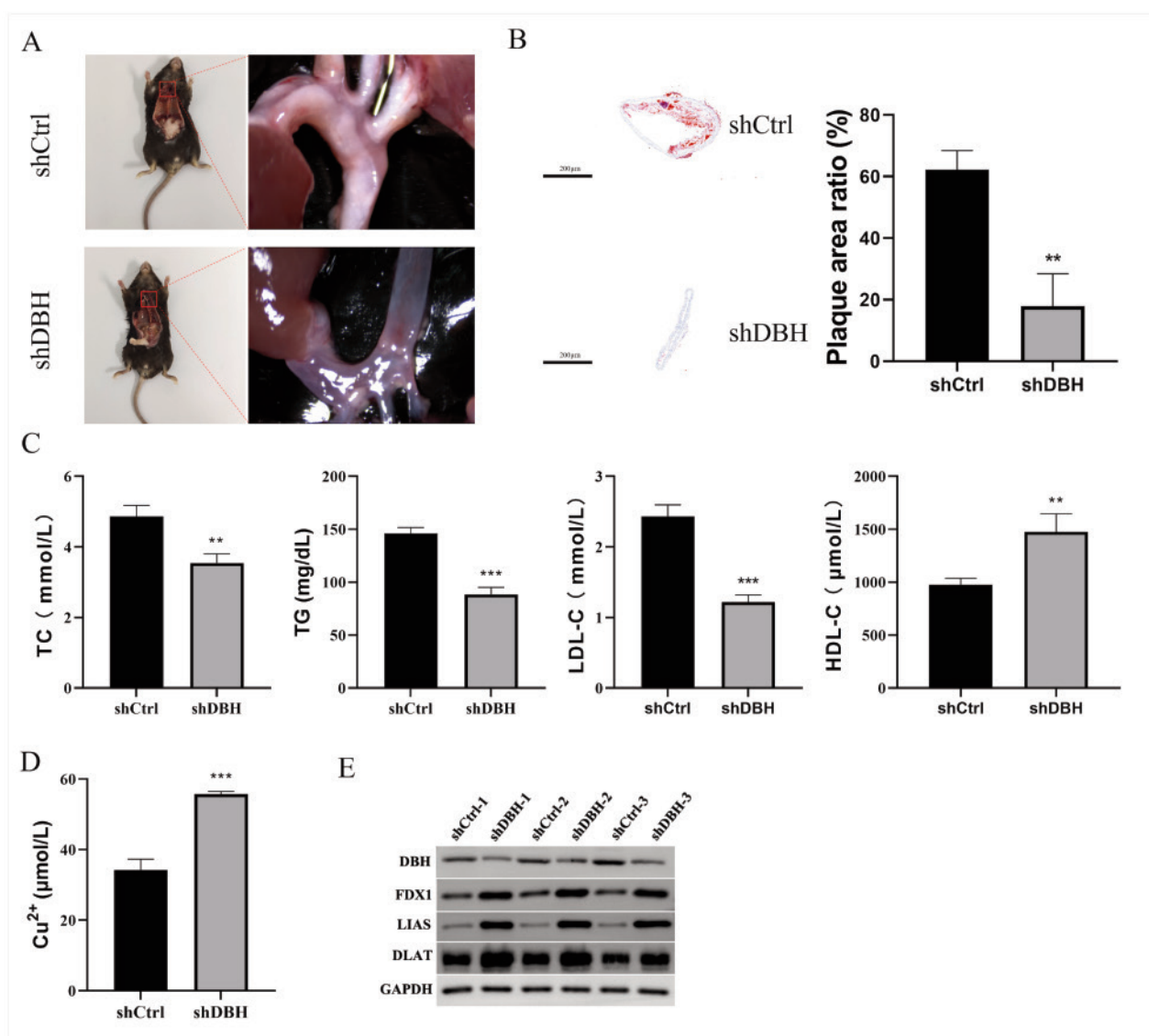


Figure 5. DBH knockdown alleviates AS and modulates lipid metabolism and copper homeostasis *in vivo*. **A)** Representative images of aortic tissues from shCtrl and shDBH mice, showing reduced lesion formation in the shDBH group. The boxed regions indicate areas shown at higher magnification. **B)** Oil Red O staining of aortic sections illustrating lipid deposition within the vascular wall; the shDBH group exhibits markedly reduced lipid accumulation compared with the shCtrl group; right panel: quantitative analysis of plaque area ratio (%). **C)** Serum lipid profiles, including TC, TG, LDL-C, and HDL-C, in shCtrl and shDBH mice. **D)** Copper ion (Cu²⁺) concentration in mouse serum. **E)** Western blot analysis of DBH and cuproptosis-related proteins (FDX1, LIAS, and DLAT) in aortic tissues, with GAPDH as a loading control. Data are presented as mean ±SD; n=5 per group; **p*<0.05, ***p*<0.01, ****p*<0.001 vs shCtrl group.

had a significantly smaller plaque area compared to the shCtrl group. Moreover, serum lipid levels in the shDBH group were significantly reduced, with lower TC, TG, and LDL-C levels, while HDL-C levels were elevated (Figure 5C). Copper ion concentrations were also higher in the shDBH group (Figure 5D), and Western blot and immunohistochemistry staining revealed that DBH knockdown was associated with altered expression of cuproptosis-related proteins (FDX1, LIAS, and DLAT) (Figure 5E and Figure 6). Histopathological analysis by H&E and Masson staining revealed significantly reduced aortic pathological changes in the shDBH group (Figure 6), suggesting that *DBH* knockdown effectively alleviates AS in mice.

Discussion

AS is a complex, multifactorial disease involving lipid accumulation, immune activation, and vascular smooth muscle cell (VSMC) dysfunction, leading to plaque formation and progression.^{1,2} Recent studies have emphasized the role of dysregulated copper metabolism in AS pathogenesis, particularly through mechanisms related to copper overload-induced cell death, termed cuproptosis.^{9,18} In this study, we identify DBH as a key mediator of copper metabolism in AS and provide insights into its regulatory mechanism *via* the transcription factor PHOX2A. Our findings not only expand the understanding of copper-related pathways in AS but also provide new insights into therapeutic strategies targeting the DBH/PHOX2A axis.

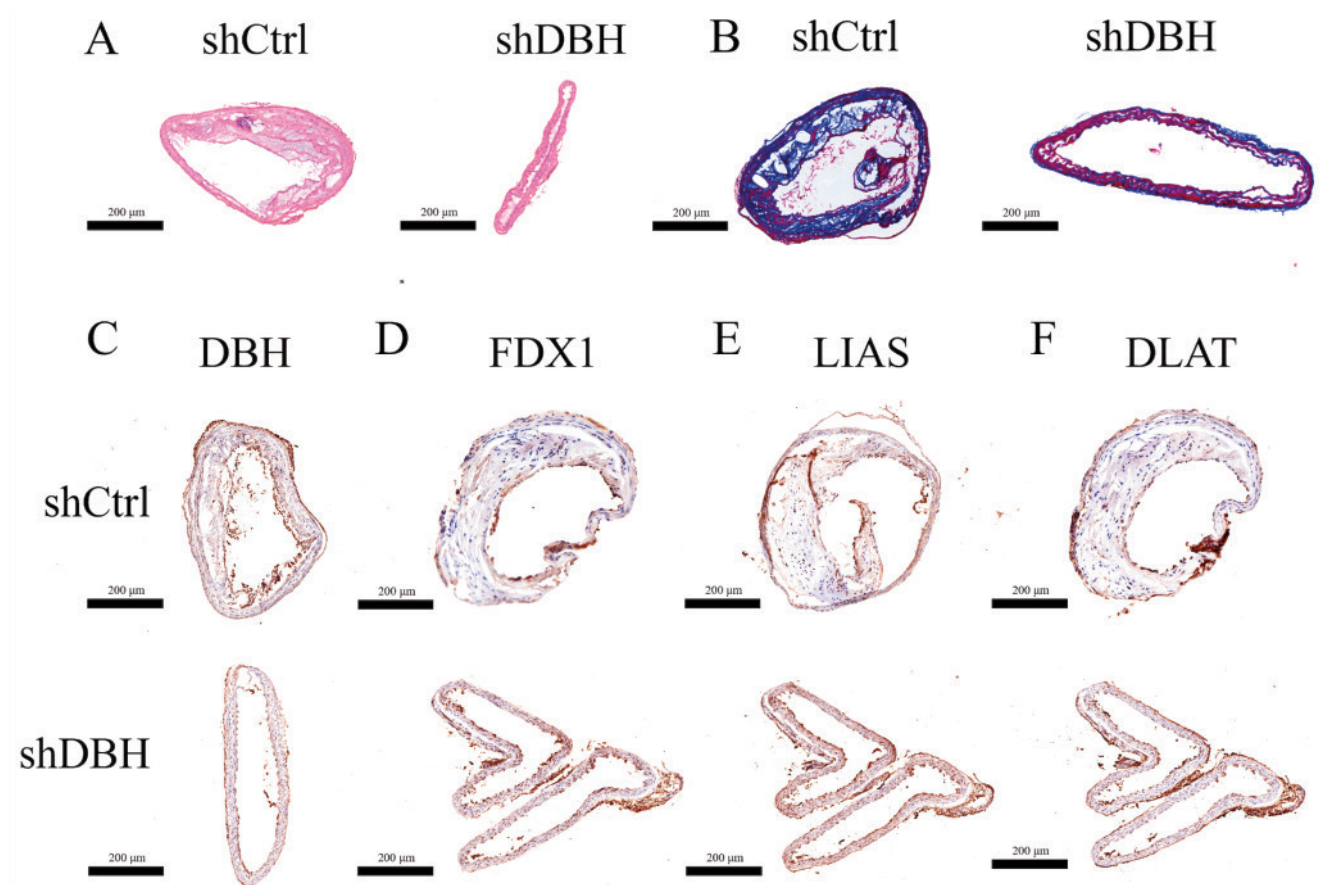


Figure 6. DBH knockdown ameliorates aortic histopathology and modulates cuproptosis-related protein expression in AS. Representative histological and immunohistochemical images of aortic sections from shCtrl and shDBH groups. **A)** H&E staining shows overall vascular morphology and plaque structure. **B)** Masson's trichrome staining highlights collagen deposition and extracellular matrix remodeling within the lesions. Immunohistochemical staining demonstrates expression and spatial distribution of DBH (**C**) and cuproptosis-related proteins, including FDX1 (**D**), LIAS (**E**), and DLAT (**F**), within aortic tissues. Compared with the shCtrl group, DBH knockdown markedly reduced plaque burden, decreased collagen accumulation, and altered the histochemical distribution and intensity of DBH and cuproptosis-associated markers.

We employed mRNA transcriptomic analysis of aortic tissues from ApoE^{-/-} mice to identify genes associated with cuproptosis, identifying five DEGs -*AOC3*, *Atp7a*, *Loxl2*, *DBH*, and *Cdkn2a*- that are linked to both copper metabolism and AS. Given the established role of *DBH* in vascular function and copper regulation,^{10,19,20} together with our qPCR validation showing its most pronounced upregulation among the five candidates, we focused on exploring its role in AS.

Beyond its well-characterized role in neuronal catecholamine synthesis, *DBH* has been detected in vascular tissues, where it may influence vascular tone and remodeling through local norepinephrine production.¹⁰ Importantly, *DBH* is a copper-containing enzyme whose activity is intrinsically linked to copper availability.²¹ This raises the possibility that *DBH* participates in copper homeostasis within the vascular wall through mechanisms beyond its classical enzymatic function, a hypothesis supported by our observation of altered copper levels following *DBH* modulation. Cuproptosis is a newly characterized form of regulated cell death driven by copper overload, distinguished by aggregation of lipoylated mitochondrial proteins and loss of iron-sulfur cluster proteins.¹³ Recent studies have implicated cuproptosis in cardiovascular pathology, with evidence that disturbed blood flow promotes endothelial cell cuproptosis through mitochondrial copper accumulation.²² However, direct evidence of cuproptosis activation within atherosclerotic plaques remains limited, and whether the copper concentrations in lesional tissue reach the threshold for triggering this cell death pathway remains unclear. Our results show that *DBH* is significantly upregulated in both AS mouse models and ox-LDL-treated HVSMSCs, suggesting that *DBH* plays a crucial role in AS pathogenesis. This observation is consistent with previous studies linking *DBH* to cardiovascular diseases, particularly through its regulation of VSMC activity and endothelial function.¹⁰ Moreover, we found that *DBH* interacts with PHOX2A, a transcription factor known to regulate genes in the sympathetic nervous system. Our dual-luciferase assays demonstrated that PHOX2A directly activates the *DBH* promoter, consistent with reports emphasizing PHOX2A's role in neuronal gene regulation.²³ However, this is the first study to link *PHOX2A* to copper metabolism in vascular cells. Overexpression of *PHOX2A* inversely correlated with copper ion concentrations, suggesting a potential link between PHOX2A-driven *DBH* expression and copper homeostasis. Whether this leads to cuproptosis-mediated vascular damage requires further investigation.

Functionally, *DBH* knockdown alleviated ox-LDL-induced apoptosis and lipid accumulation in HVSMSCs, while *PHOX2A* overexpression exacerbated these effects. These results are consistent with studies suggesting that copper chelation mitigates AS progression by reducing oxidative stress.²⁴ Additionally, our *in vivo* data confirmed that *DBH* knockdown significantly reduced aortic plaque area and improved serum lipid profiles, similar to the protective effects of copper supplementation in AS models.^{9,25} Importantly, *DBH* knockdown modulated the expression of cuproptosis-related proteins (FDX1, LIAS, DLAT), supporting the hypothesis that *DBH* is associated with copper-dependent cell death pathways in AS.

Our findings align with and extend previous studies on copper metabolism in AS. While earlier work has largely attributed copper's pro-atherogenic effects to oxidative stress,²⁴ our study suggests *DBH* as a potential regulator of copper availability in the vascular system. Notably, our observation of copper depletion rather than copper overload in the AS model contrasts with some previous reports, a discrepancy that may reflect tissue-specific differences, plaque stage, or the cellular compartment assessed.²⁶ This complexity underscores the need for spatially resolved analyses of

copper distribution in different plaque regions.²⁷

While our findings provide compelling evidence for the role of *DBH* in AS, several limitations must be acknowledged. First, although the ApoE^{-/-} mouse model is widely used, it does not fully recapitulate the complexity of human AS, particularly with regard to chronic inflammatory responses. Second, our focus on HVSMSCs may overlook the contributions of endothelial cells or macrophages, which are key players in plaque instability. Third, the exact molecular interactions between *DBH* and copper ions remain unclear; future studies should employ copper isotope tracing or CRISPR-based screens to further investigate these relationships.

In conclusion, this study identifies *DBH* as a key regulator of copper metabolism in AS, with transcriptional activation driven by PHOX2A. By integrating transcriptomics, biochemistry, and *in vivo* approaches, we provide evidence suggesting that *DBH* knockdown slows AS progression in a manner associated with cuproptosis-related pathways. These findings not only add to our understanding of the role of copper in vascular pathology but also suggest the therapeutic potential of targeting the PHOX2A-*DBH* axis. Future research should explore pharmacological inhibitors of *DBH* or copper-modulating agents as new strategies for AS treatment.

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Online Supplementary Material

Figure S1. qPCR validation of five candidate genes in aortic tissues from NC and AS mice.

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