Supplemental Material

Brown Adipocyte ADRB3 Mediates Cardioprotection via Suppressing Exosomal iNOS

Running Title: ADRB3 Activation in BAT mediates Cardioprotection

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Materials and methods

Animals

Ucp1-Cre (C57BL/6J background) and *Adrb3*^{flox} (C57BL/6J background) mice were obtained from the GemPharmatech Co. Ltd. *Adrb3*^{flox} mice were bred with *Ucp1*-Cre mice to produce *Ucp1*-Cre+; *Adrb3*^{flox} heterozygote mice. Then the heterozygote mice were bred with *Adrb3*$^{flox/flox}$ mice to produce *Ucp1*-Cre+; *Adrb3*$^{flox/flox}$ (BKO) homozygote and littermates *Ucp1*-Cre-; *Adrb3*$^{flox/flox}$ (Ctrl) mice. Male mice aged 8-10 weeks were in used in all experiments. BKO and Ctrl were infused with 1000 ng/kg per minute of Ang II or saline for 4 weeks via subcutaneously implanted Alzet miniosmotic pumps. Tail-cuff measurements of systolic blood pressure (SBP) was obtained following Ang II or saline surgery using Visitech BP-2000 tail cuff system. All animal experiments were conducted in accordance with guidelines approved by the Institutional Guidelines established by the Committee of Ethics on Animal Experiments at Shanghai Jiao Tong University School of Medicine.

Echocardiography

Anesthetized mice (1-2% isofluorane) were subjected to transthoracic echocardiography by using a Vevo 2100 instrument (Fuji Film Visual Sonics) with a MS-400 transducer (18-38 MHz) as previously described.[23] M-mode recording was performed at the midventricular level. All images were analyzed using dedicated software (Vevo 2100 version 1.4). Left ventricular (LV) interventricular septal thickness (IVS), LV internal dimension (LVID) at diastole, and systole (IVSd, LVIDd, and IVSs, LVIDs) were measured. Ejection fraction (EF) and fractional shortening (FS) were determined from M-mode measurements.

Cell Culture

Brown adipose tissue was obtained from 5-6 weeks-old mice and digested as described previously.[24] The preadipocytes were treated with differentiation medium (10% FBS DMEM/F12 culture medium, supplemented with 0.02 mM insulin, 0.5 mM IBMX, 1 mM rosiglitazone, 1 nM triiodothyronine [T3], and 100 nM dexamethasone) for 4 days and then maintained in 0.02 mM insulin and 1 nM T3 and harvested at days 6-8 days. Primary cardiac fibroblasts (CFs) were isolated from 1-2 weeks-old male mice as previously described.[25] CFs were grown in DMEM supplemented with 15% FBS and 1% penicillin and streptomycin. Neonatal mouse cardiomyocytes (NMCMs) were isolated from 1-3 days-old male mice as previously described.[26] NMCMs were cultured in DMEM supplemented with 15% FBS, 1% penicillin and streptomycin and 100 mM 5-bromodeoxyuridine.

Isolation and characterization of exosomes

The exosomes were isolated from cultured brown adipocyte conditioned medium using two different methods as previously described.[27] Culture supernatant was collected on ice and cleared by serial centrifugation at 300 × g for 10 min to remove live cells, 2,000 × g for 10 min to remove dead cells and 10,000 × g for 30 min to sediment debris and microvesicles. Then, the supernatant was filtered through a 0.8 μm syringe filter, followed by ultracentrifugation with two rounds of centrifugation at 100,000 ×g for 80 min (method 1) or using an affinity column (exoEasy Maxi Kit from QIAGEN) (method 2) following the manufacturer's introductions. Isolated exosomes were confirmed to be present by transmission electron microscopy and Delta Nano C particle analyzer and analyzed for the presence of the specific marker protein CD63 and TSG101 by Western blot.

Exosome detection

For in vitro exosome uptake experiments, exosomes were stained with PKH26 red fluorescent dye at 100 μM for 10 minutes according to the manufacturer's instructions. Labeled exosomes were then added to cardiac fibroblasts for 24h. Then, uptake assays were performed under fluorescence microscopy. For in vivo detection, exosomes were labeled with the lipophilic near-infrared red dye DiR (Invitrogen). Exosomes were incubated with 2 μM DiR for 30 min and injected intravenously into WT mice (4mg/kg body weight). One hour later, mouse hearts were collected and placed in Tissue OCT-Freeze Medium. These hearts were subsequently snap-frozen in liquid nitrogen. Frozen sections of 8 μm were cut and counterstained with Alexa Fluor 488-conjugated anti-vimentin antibody (CST).

Co-culture experiments

Co-culture experiment was performed using 6.5 mm diameter and 8.0 μm pore size Transwells. The membrane permits the free exchange of media between insert and well. Approximately 1×10^5 brown adipocytes were seeded on the upper chamber, and 2×10^5 cardiac fibroblasts/well were co-cultured in the insert.

Western blot analysis

Western blot was performed as previously reported.[28] Total protein was extracted from brown adipocytes, mouse hearts, exosomes, or exosome-treated cells. Proteins were separated by SDS-PAGE and blotted onto 0.45-mm PVDF membranes. The dilutions of antibodies used were as follows: rabbit anti-αSMA (1:1000), anti-collagen I (1:1000), anti-TGFβ (1:1000), anti-iNOS (1:1000), anti-CD63 (1:1000), anti-TSG101 (1:1000), anti-ERK (1:2000), anti-p-ERK (1:2000).

Quantitative real-time PCR

Total RNA was isolated by using a RNeasy Plus Mini Kit. cDNA was generated by reverse-transcribed total RNA (1 μg) using oligo (dT) primer and ReverTra Ace reverse transcriptase (EZBioscience). Q-PCR was performed and analyzed by kinetic real-time PCR using the ABI Prism 7900HT sequence detection system (Applied Biosystems) with TB Green Realtime PCR Master Mix kit (TaKaRa) for relative quantification of the indicated genes. The sequence of primers used in this study was shown in Supplemental Material. GAPDH transcript was used for internal normalization.

Histological and immunostaining analysis

Hearts and intrascapular brown adipose tissue (iBAT) were obtained, fixed in 10% buffered formalin, and embedded in paraffin. Sections (5 μm in thickness) were stained

with hematoxylin eosin and Masson's Trichrome according to the manufacturer's instructions. For IHC staining, heart sections were incubated with primary antibody for α-SMA, Col1a1, and iBAT sections were incubated with primary antibody for UCP1 (abcam). Wheat germ agglutinin (WGA) staining was conducted using an FITC-conjugated probe (Sigma-Aldrich). All images were obtained using Zeiss Microscopy or confocal laser-scanning microscopy (Zeiss). Imaging analysis was performed using ImageJ and Image-Pro Plus software. Cross-sectional area was measured in samples from six hearts in each group, and about 100 myocytes with centrally located nuclei were analyzed per section.

Depletion of macrophages

In vivo macrophage depletion was conducted by neutralizing antibody CSF-1R (Bioxcell). Mice were injected intraperitoneally with anti-CSF-1R (1 mg) every 2 days during Ang II infusion.

Catecholamine secretion measurements

The concentrations of dopamine, adrenaline and norepinephrine in iBAT was measured using 3-CAT Research ELISA kit (LDN) as described previously.[29] Analyses were performed according to the manufacturer's instructions.

Adeno-associated virus injection

Male mice (6-8 weeks) were anaesthetized with isoflurane and the interscapular area was exposed. About 1×10^{11} adeno-associated virus (AAV) particles were injected into each BAT lobe. Open wounds were sutured after viral injection. Mice were allowed to recover for 1-2 weeks for further in vivo experiments. AAV-DIO-iNOS and AAV-DIO-shRNA(iNOS) were packaged at OBiO Technology (Shanghai).

Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM). All data sets were subjected to the Shapiro-Wilk normality test for the normality distribution analysis. Statistical analysis was performed using unpaired, two-tailed Student's t test (for two groups), one-way ANOVA (one independent variable), and two-way ANOVA (two independent variables) followed by the Tukey multiple comparison test (for multiple groups). If datasets did not follow a normal distribution, or $n<6$, statistical analysis was performed using a non-parametric test: 2-tailed Mann-Whitney U test (for two groups) and Kruskal-Wallis with Dunn's multiple comparisons test (for multiple groups) (details in Figure legends). A P value <0.05 was considered statistically significant. No

corrections for multiple testing were made across tests. The representative image was chosen from one of the repeats that best matched the average data in each experiment. Please see the Major Resources Table in the Supplemental Materials.

Primers

Gene	**Forward (5'-3')**	**Reverse (5'-3')**
Adrb3	CCGTGAAGATCCAGCAAGGA	GGTTCTGGAGCGTTGGAGAGT
Gapdh	GTATGACTCCACTCACGGCAA	CTTCCCATTCTCGGCCTTG
Col1a1	GAGCGGAGAGTACTGGATCG	TACTCGAACGGGAATCCATC
Tgfβ	CAACAATTCCTGGCGTTACCTTGG	GAAAGCCCTGTATTCCGTCTCCTT
αSma	CTGACAGAGGCACCATGAA	AGAGGCATAGAGGGACAGCA
Anp	TCTTCCTCGTCTTGGCCTTT	CCAGGTGGTCTAGCAGGTTC
Bnp	TGGGAGGTCACTCCTATCCT	GGCCATTTCCTCCGACTTT
β-Mhc	CGGACCTTGGAAGACCAGAT	GACAGCTCCCCATTCTCTGT
Ucp1	AGGCTTCCAGTACCATTAGGT	CTGAGTGAGGCAAAGCTGATTT
Pparγ	TTAGATGACAGTGACTTGGC	TCTTCTGGAGCACCTTGG
Prdm16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
Cidea	TGCTCTTCTGTATCGCCCAGT	GCCGTGTTAAGGAATCTGCTG

Supplemental Figures

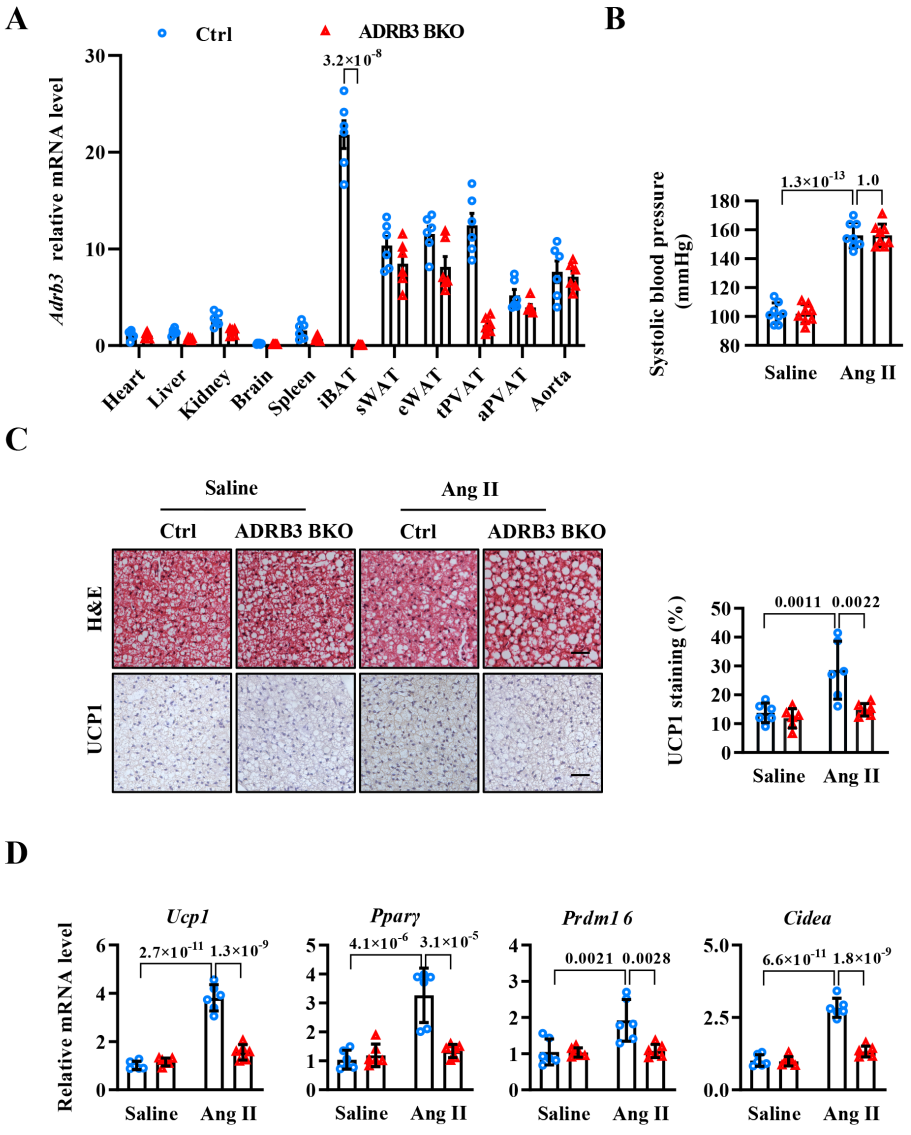


Figure S1. A, Quantification of *Adrb3* mRNA level in different tissues of brown adipocyte-specific ADRB3 knockout (BKO) and littermate control mice (Ctrl) mice. **B,** Systolic blood pressure of BKO and Ctrl mice treated with saline or Angiotensin II (Ang II). **C,** Hematoxylin-eosin (H&E) and uncoupling protein 1 (UCP1) immunohistochemical staining and quantification of UCP1 in intrascapular brown adipose tissue (iBAT) of BKO and Ctrl treated with saline or Ang II. **D,** Quantification of *Ucp1*, peroxisome proliferator-activated receptor γ (*Pparγ*), PRD1-BF1-RIZ1 homologous domain containing 16 (*Prdm16*), and cell death-inducing DNA fragmentation factor-like effector A (*Cidea*) mRNA levels in iBAT of BKO and Ctrl mice treated with saline or Ang II. Scale bar: 100 μm. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by Tukey's multiple comparisons test (n=6).

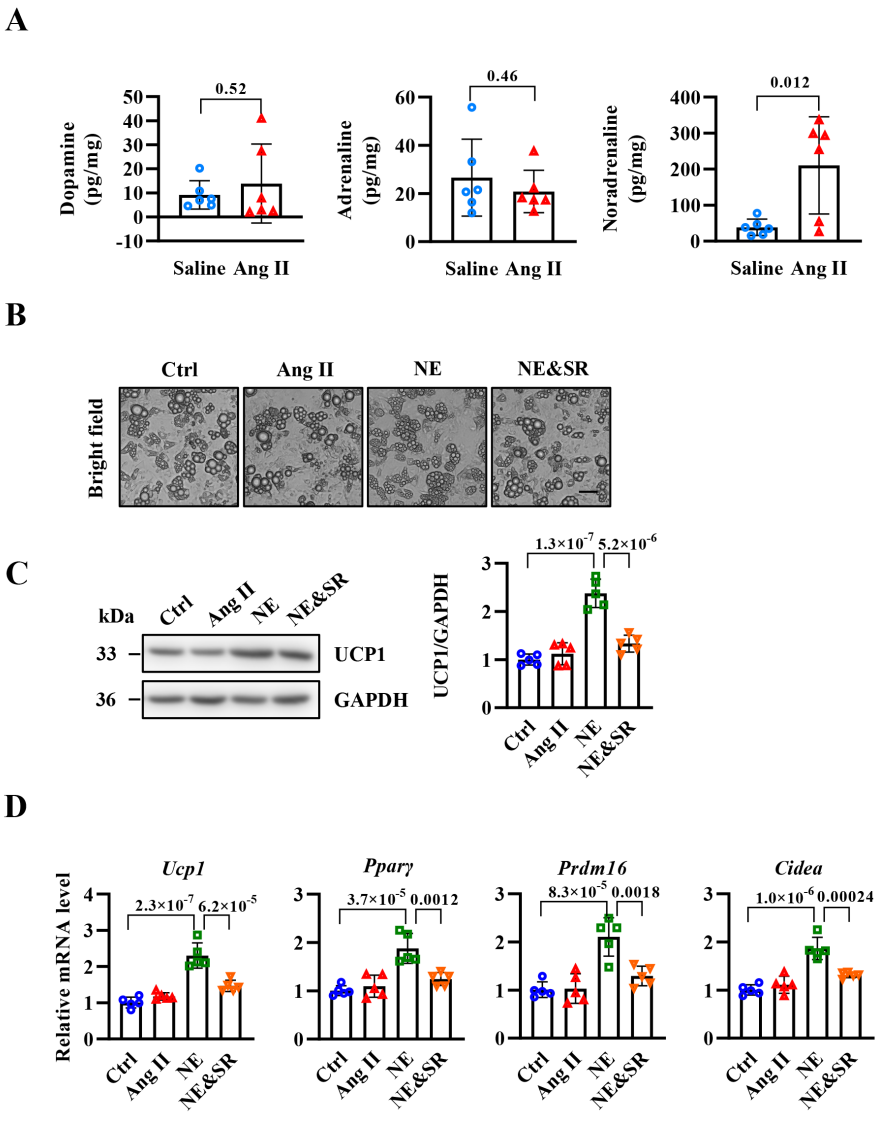


Figure S2. A, Quantification of dopamine, adrenaline and noradrenaline levels in iBAT of mice treated with saline or Ang II. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by Tukey's multiple comparisons test (n=6). **B,** Bright field images of adipocytes treated with Ang II or noradrenaline (NE) or SR59230A(SR). **C,** Western blot of UCP1 in adipocytes treated with Ang II or NE or SR. **D,** Quantification of *Ucp1*, *Pparγ*, *Prdm16* and *Cidea* mRNA levels in adipocytes treated with Ang II or NE or SR. Scale bar: 100 μm. Significant differences were examined by Kruskal-Wallis with Dunn's multiple comparisons test (n=5).

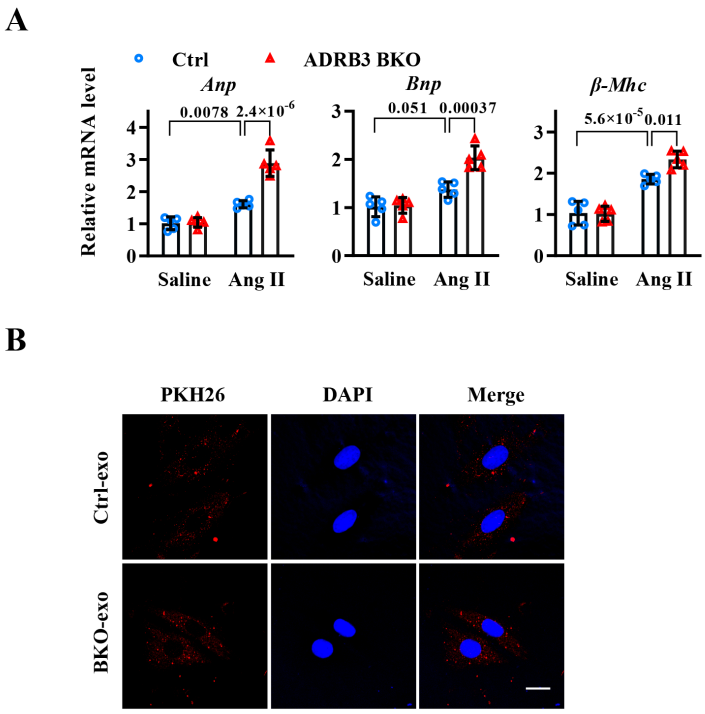
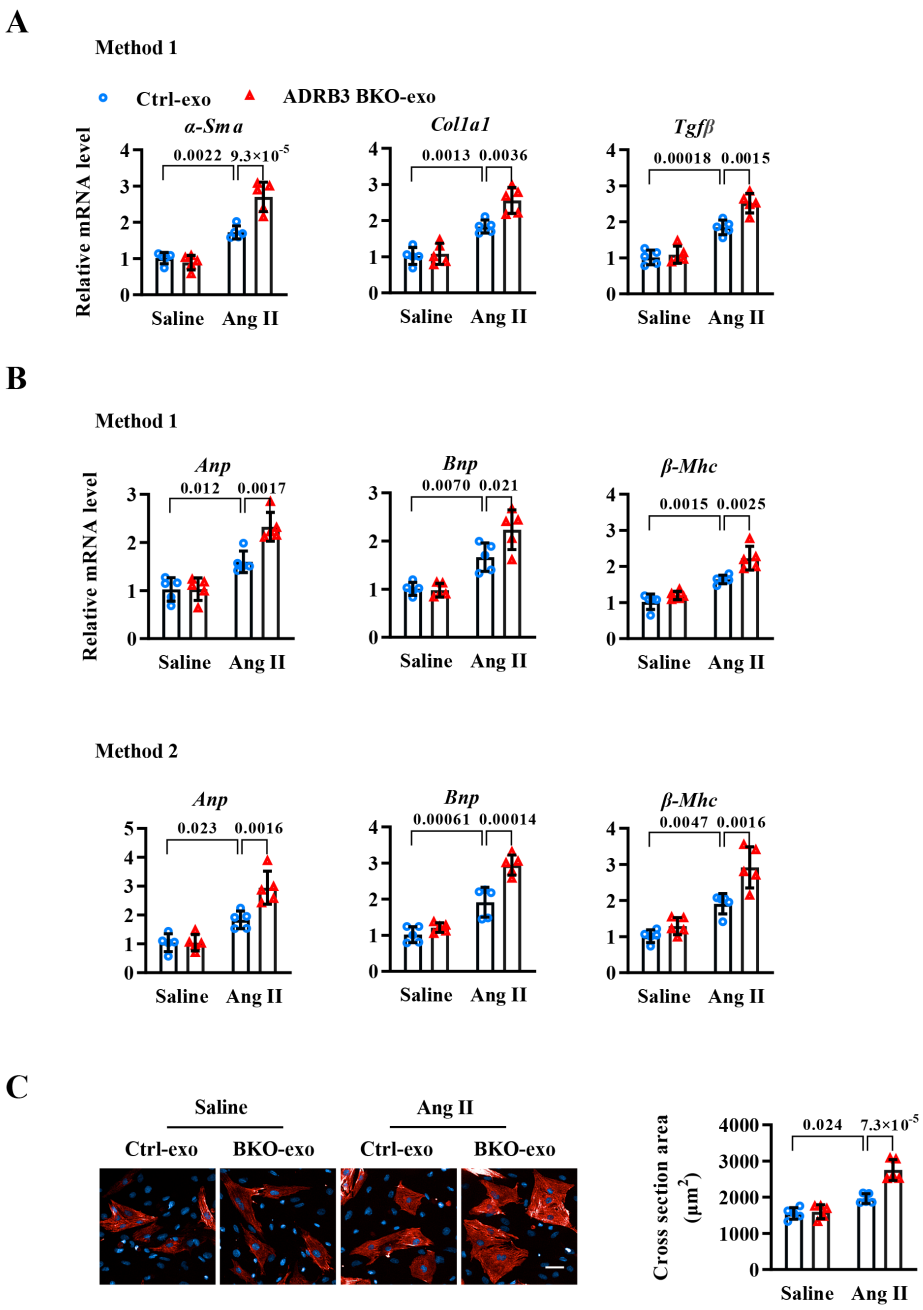
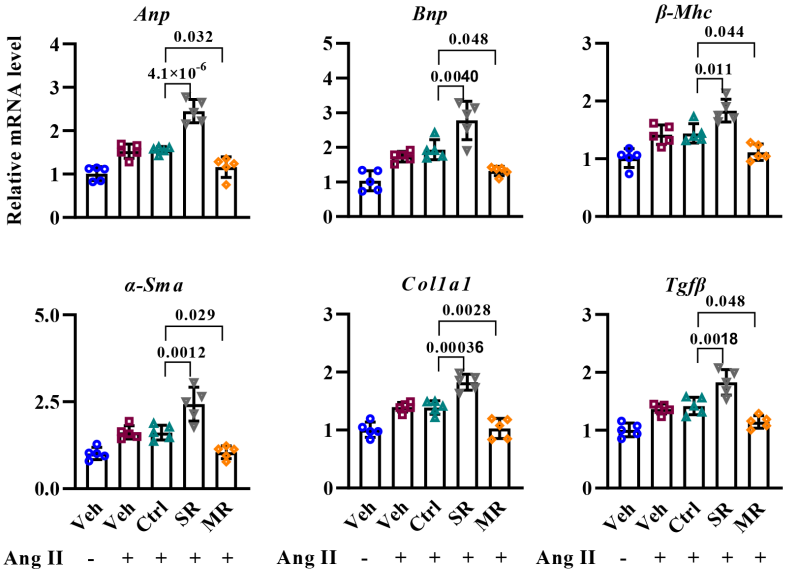


Figure S3. A, Quantification of atrial natriuretic peptide (*Anp*), brain natriuretic peptide (*Bnp*) and myosin heavy chain β (*β-Mhc*) mRNA levels in neonatal cardiac myocytes (NMCMs) cocultured with brown adipocytes from BKO and Ctrl mice treated with saline or Ang II. **B,** Representative images of brown adipocyte-derived exosomes staining of BKO and Ctrl mice in NMCMs. Nuclei stained by DAPI in blue and exosomes stained by PKH26 in red. Scale bar: 30μm. Significant differences were examined by Kruskal-Wallis with Dunn's multiple comparisons test (n=5).


A
Method 1
Ctrl-exo
ADRB3 BKO-exo
α-Sma
Col1a1
Tgfβ
Relative mRNA level
0.0022
9.3×10-5
0.0013
0.0036
0.00018
0.0015
Saline
Ang II
B
Method 1
Anp
Bnp
β-Mhc
0.012
0.0017
0.0070
0.021
0.0015
0.0025
Method 2
0.023
0.0016
0.00061
0.00014
0.0047
0.0016
C
Saline
Ang II
Ctrl-exo
BKO-exo
Cross section area (μm2)
0.024
7.3×10-5

D



E

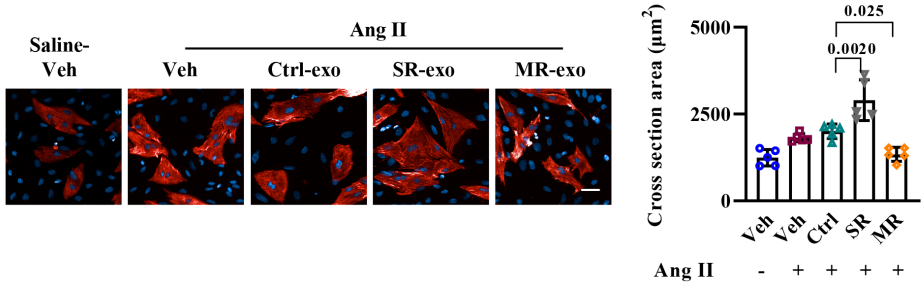


Figure S4. A, Quantification of alpha smooth muscle actin (*α-Sma*), alpha-1 type I collagen (*Col1a1*) and transforming growth factor beta 1 (*Tgfβ*) mRNA levels in cardiac fibroblast (CFs) cultured with brown adipocyte-derived exosomes from BKO (BKO-exo) and Ctrl (Ctrl-exo) mice treated with saline or Ang II using method 1. **B,** Quantification of *Anp*, *Bnp, β-Mhc* mRNA levels in NMCMs cultured with BKO-exo and Ctrl-exo treated with saline or Ang II using method 1&2. **C,** Immunofluorescence for α-actinin (red) and cross-section area of NMCMs cultured with BKO-exo and Ctrl-exo with saline or Ang II. **D,** Quantification of *Anp*, *Bnp, β-Mhc, α-Sma*, *Col1a1* and *Tgf β* mRNA levels in NMCMs or CFs cultured with exosomes isolated from brown adipocytes treated with control (Ctrl-exo), SR59230A (SR-exo) or mirabegron (MR-exo) with saline or Ang II. **E,** Immunofluorescence for α-actinin (red) and cross-section area of NMCMs cultured with Ctrl-exo, SR-exo or MR-exo with saline or Ang II. Scale bar: 100 μm. Significant differences were examined by Kruskal-Wallis with Dunn's multiple comparisons test (n=5).

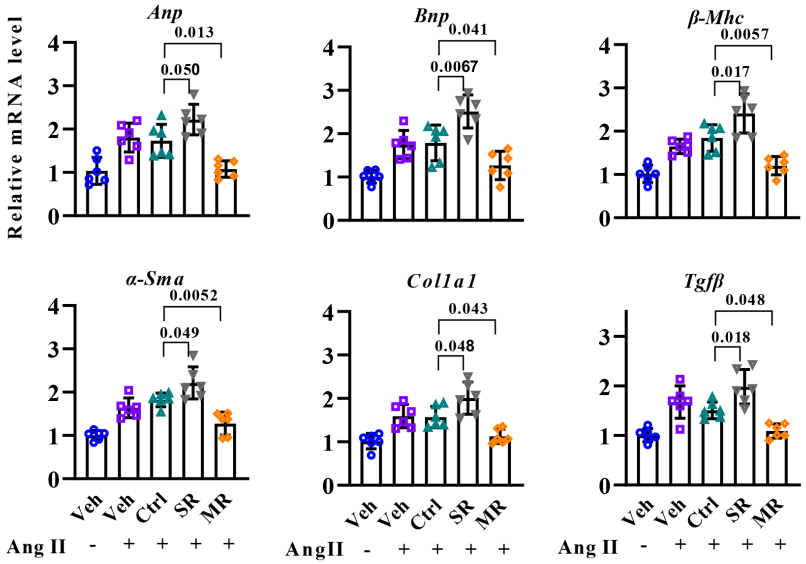
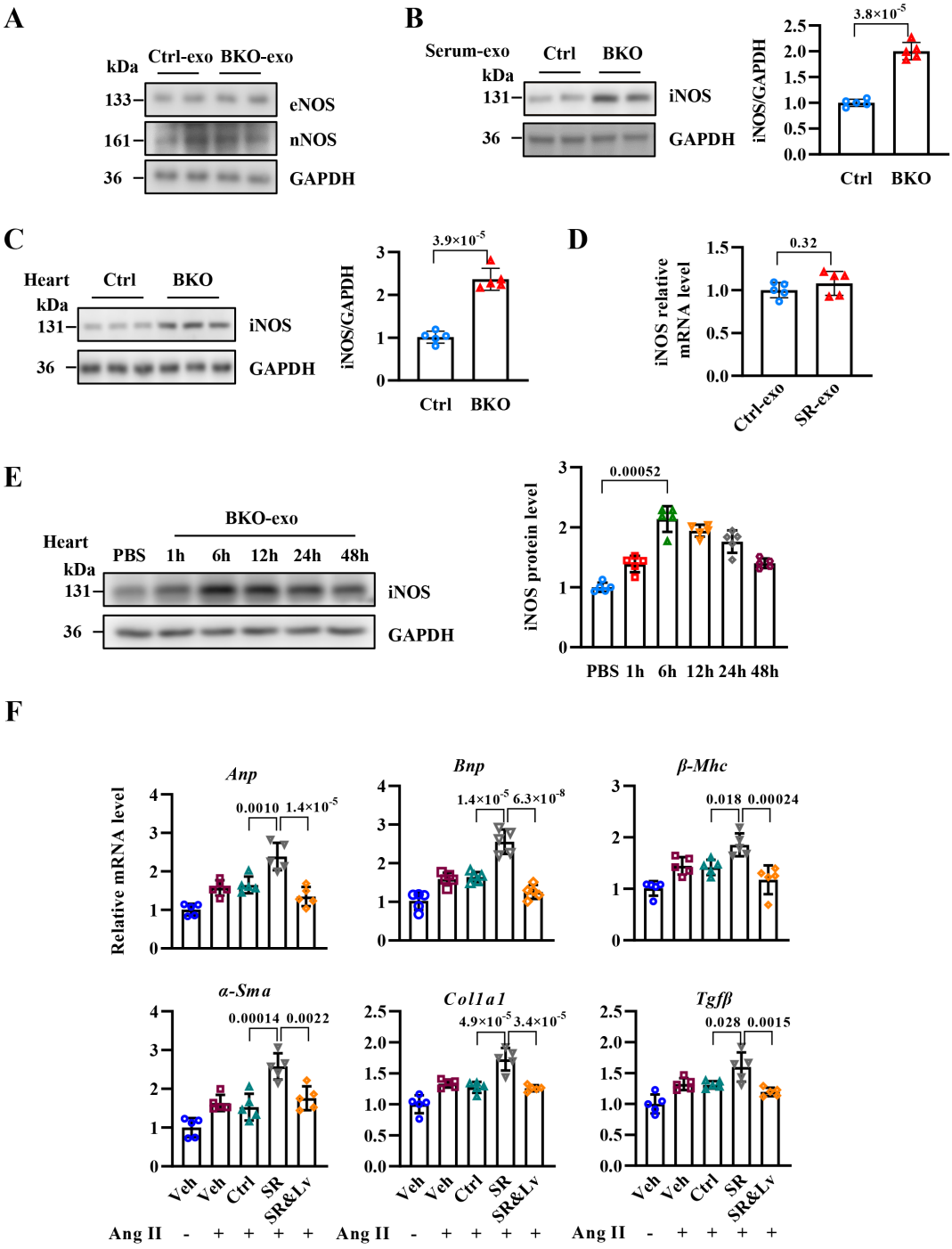


Figure S5. Quantification of *Anp*, *Bnp, β-Mhc, α-Sma*, *Col1a1* and *Tgfβ* mRNA levels in heart tissue of WT mice injected with Ctrl-exo, SR-exo or MR-exo treated with saline or Ang II. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by Tukey's multiple comparisons test (n=6).


A
kDa
Ctrl-exo
BKO-exo
133
eNOS
161
nNOS
36
GAPDH
B
Serum-exo
Ctrl
BKO
kDa
131
iNOS
36
GAPDH
iNOS/GAPDH
3.8×10-5
Ctrl
BKO
C
Heart
Ctrl
BKO
kDa
131
iNOS
36
GAPDH
iNOS/GAPDH
3.9×10-5
Ctrl
BKO
D
iNOS relative mRNA level
0.32
Ctrl-exo
SR-exo
E
BKO-exo
Heart
PBS
1h
6h
12h
24h
48h
kDa
131
iNOS
36
GAPDH
iNOS protein level
0.00052
PBS
1h
6h
12h
24h
48h
F
Relative mRNA level
Anp
0.0010
1.4×10-5
Bnp
1.4×10-5
6.3×10-8
β-Mhc
0.018
0.00024
α-Sma
0.00014
0.0022
Col1a1
4.9×10-5
3.4×10-5
Tgfβ
0.028
0.0015
Veh
Veh
Ctrl
SR
SR&Lv
Ang II
-
+
+
+
+

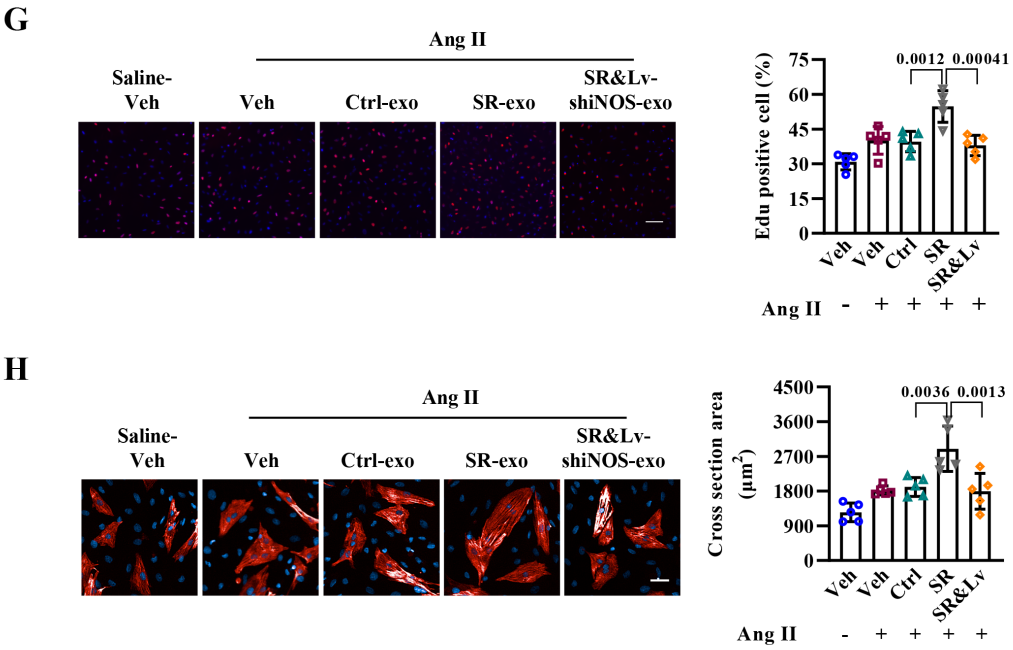


Figure S6. A, Western blot of eNOS and nNOS in BKO-exo and Ctrl-exo mice treated with Ang II. **B,** Western blot and quantitative analysis of iNOS in serum-derived exosomes of BKO and Ctrl mice treated with Ang II. **C,** Western blot and quantitative analysis of iNOS in the heart tissue of BKO and Ctrl mice treated with Ang II. **D,** Quantification of iNOS mRNA level in heart tissue of WT mice injected with Ctrl-exo or SR-exo under Ang II treatment. Significant differences were examined by unpaired 2-tailed Mann-Whitney U test (n=5). **E,** Western blot and quantitative analysis of iNOS in the heart tissue of WT mice injected with BKO-exo in different times. **F,** Quantification of *Anp*, *Bnp, β-Mhc, α-Sma*, *Col1a1* and *Tgfβ* mRNA levels in NMCMs or CFs cultured with Ctrl-exo, SR-exo or SR&Lv-exo (exosomes isolated from primary brown adipocytes treated with iNOS-silencing Lentivirus virus construct and SR) with saline or Ang II. **G,** Representative image and quantitative analysis of BrdU incorporation in CFs cultured with Ctrl-exo, SR-exo or SR&Lv-exo with saline or Ang II. **H,** , Immunofluorescence for α-actinin (red) and cross-section area of NMCMs cultured with Ctrl-exo, SR-exo or SR&Lv-exo with saline or Ang II. Scale bar: 100 μm. Significant differences were examined by Kruskal-Wallis with Dunn's multiple comparisons test (n=5).

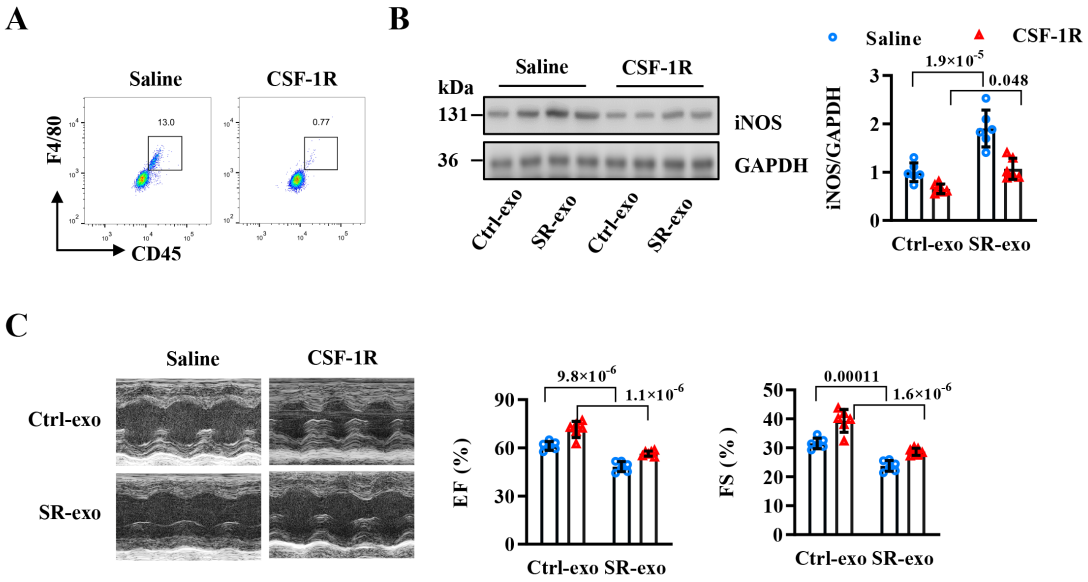


Figure S7. A, Flow cytometric analysis of positive macrophage F4/80 in the serum of mice injected with saline or anti-CSF-1R antibody. **B,** Western blot and quantitative analysis of iNOS in the heart of mice injected with saline or anti-CSF-1R antibody every 2 days during Ang II infusion. **C,** Representative M-mode echocardiography, Ejection fraction (EF) and fractional shortening (FS) of mice injected with saline or anti-CSF-1R antibody during Ang II infusion. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by Tukey's multiple comparisons test (n=6).

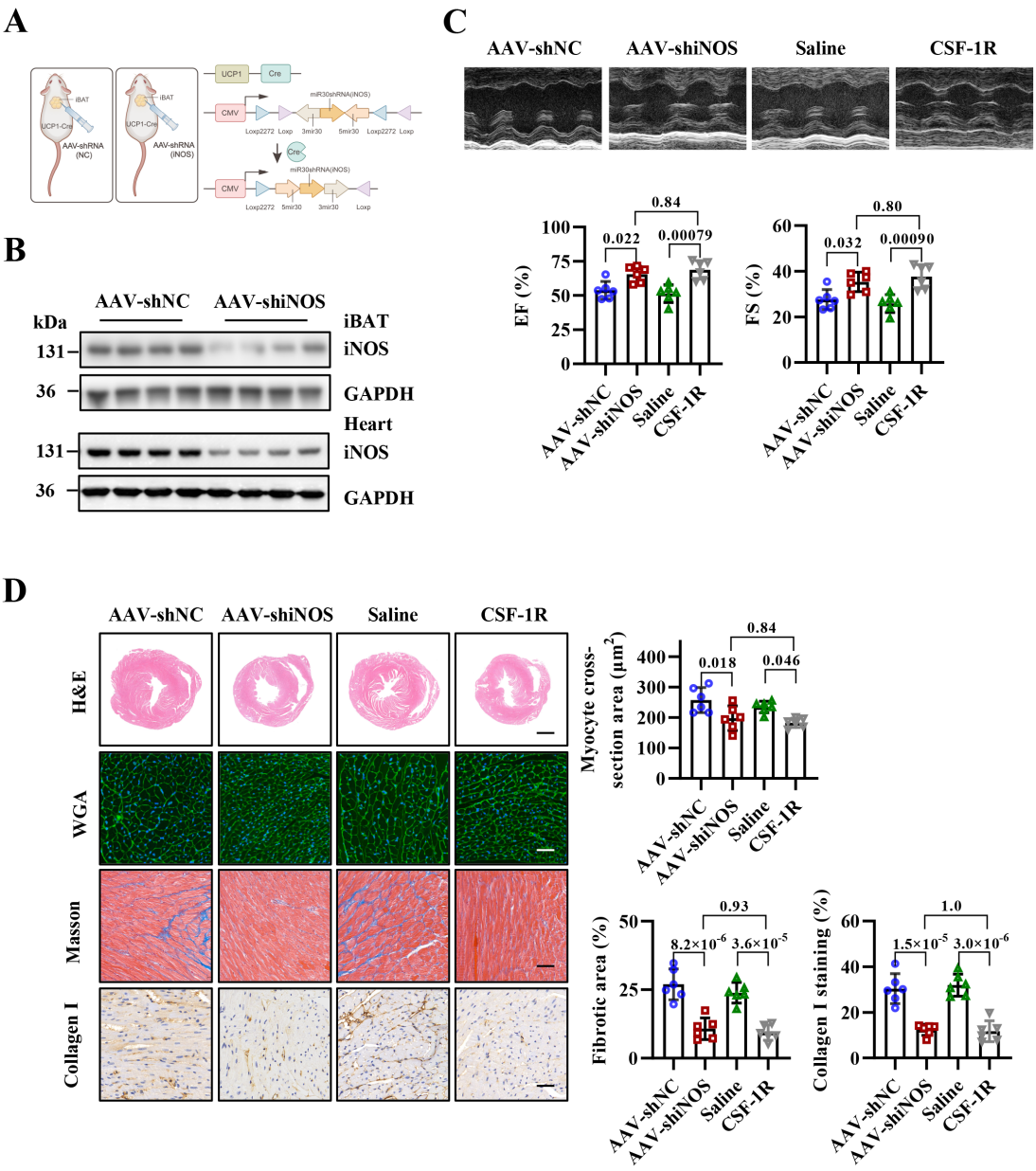


Figure S8. A, Overview for AAV-mediated specific knockdown of iNOS in iBAT of adult mice. **B,** Western blot of iNOS in iBAT and heart after AAV injection. **C,** Representative M-mode echocardiography, EF and FS of UCP1-cre mice injected with AAV-shRNA (NC), AAV-shRNA (iNOS), saline or anti-CSF-1R antibody during Ang II infusion. **D,** H&E and WGA, Masson trichrome and collagen I immunohistochemical staining in heart tissue of UCP1-cre mice mice injected with AAV-shRNA (NC), AAV-shRNA-iNOS, saline or anti-CSF-1R antibody during Ang II infusion. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by Tukey's multiple comparisons test (n=6).

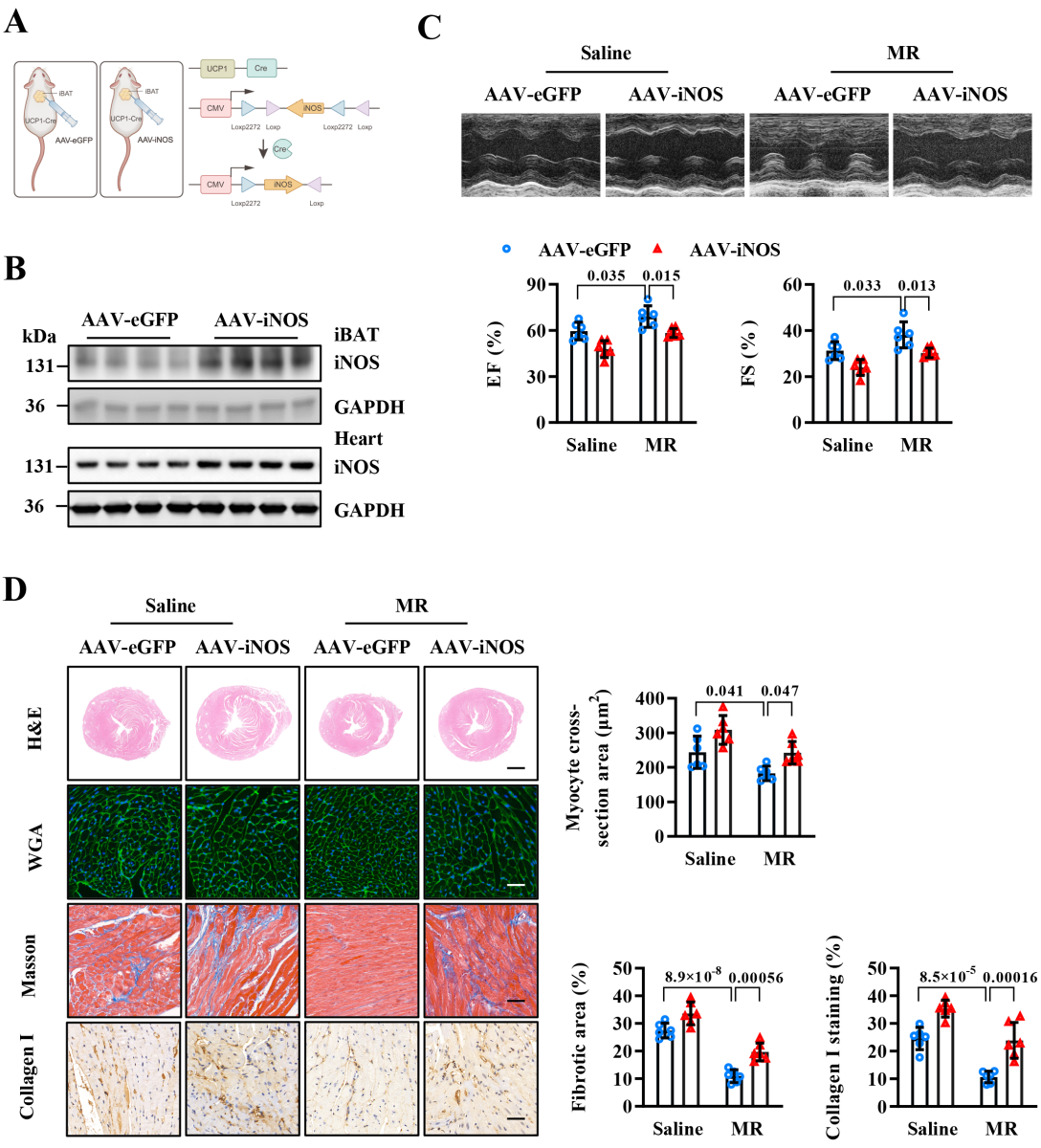


Figure S9. A, Overview for AAV-mediated specific overexpression of iNOS in iBAT of adult mice. **B,** Western blot of iNOS in iBAT and heart after AAV injection. **C,** Representative M-mode echocardiography, EF and FS of UCP1-cre mice injected with AAV-eGFP, AAV-iNOS, with or without MR treatment during Ang II infusion. **D,** H&E and WGA, Masson trichrome and collagen I immunohistochemical staining in heart tissue of UCP1-cre mice injected with AAV-eGFP, AAV-iNOS, with or without MR treatment during Ang II infusion. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by Tukey's multiple comparisons test (n=6).