Supplemental Material

Small Extracellular Vesicles from Cardiomyocytes Activate Microglia Aggravating HFpEF

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Short Title: Cardiomyocyte sEVs Activate Microglia in HFpEF

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Expanded Materials and Methods

Data Availability

The data in this study are available in the main text and supplementary materials. Detailed descriptions of materials and methods are provided in the supplementary methods section. The other data supporting the findings are available from the corresponding author upon reasonable request.

Animals

All animal experiments were approved by the Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). C57BL/6J mice (RRID: IMSR_JAX:000664) were obtained from GemPharmatech (RRID: SCR_017239, Wuhan, China) for male cohorts and from Wuhan Vital River Laboratory Animal Technology Co., Ltd. for female cohorts.

HFpEF Mouse Model

To establish the HFpEF model, 8-9 weeks old male C57BL/6J mice were randomly assigned to a two-hit protocol combining a high-fat diet (60% fat, Research Diets, Cat#D12492) with the nitric oxide synthase inhibitor L-NAME (0.5 g/L, Merck, Cat#51298-62-5) in drinking water, and maintained on these diets and drinking water for 12 weeks. Control male mice received standard chow diet. For the female cohort, 9-10 weeks old female mice were fed either a control diet or the same two-hit diet (high-fat diet plus L-NAME) for 8 weeks. Sample sizes were determined empirically based on effect sizes observed in prior studies using comparable experimental systems.

Microglia Depletion with PLX3397

The selective colony-stimulating factor-1 receptor (CSF1R) kinase antagonist PLX3397 was administered via the diet to deplete microglia. In male mice, after 10 weeks of HFpEF induction, animals were switched to a 60% kcal fat rodent diet supplemented with 290 ppm PLX3397 (Research Diets, Cat#M23052401) or a standard rodent diet containing 290 ppm PLX3397 (Research Diets, Cat#M18072603) for an additional 2 weeks. In female mice, after 6 weeks of control or HFpEF diet feeding, both groups were switched to the corresponding PLX3397-containing diets for 2 weeks.

Isolation and In Vivo Administration of Myocardial sEVs

Small extracellular vesicles (sEVs) were isolated from myocardial tissues of control and HFpEF model mice as described the sEVs isolation and characterization section. The protein concentration of the isolated sEVs was determined using a BCA assay (Wuhan Promoter Biological CO., LTD, Cat#P5026). Healthy male recipient mice were randomly divided into two groups ($Ctrl^{sEV}$ and $HFpEF^{sEV}$) and received 100 μg of the corresponding sEVs (derived from control or HFpEF mouse myocardium, respectively) diluted in sterile PBS (Servicebio, Cat#4202) to a final volume of 100 μL per injection. Injections were administered via the tail vein every 5 days for a total of 15 days (three injections per mouse). This sEVs transfer experiment was performed

exclusively in male mice and was designed to evaluate the effects of myocardial sEVs on microglial activation and hypothalamic inflammation.

Inhibition of sEV Biogenesis with GW4869

GW4869 (MedChemExpress, Cat#HY-19363), an inhibitor of sEV biogenesis, was administered intraperitoneally at 2.5 mg/kg every three days. In male mice, GW4869 or its vehicle (5% DMSO in PBS, Merck, Cat#276855) was administered concurrently with the two-hit HFpEF protocol for 12 weeks, at an injection volume of 150 μL per mouse, adjusted weekly based on body weight. In female mice, the same dosing regimen was administered for 8 weeks.

Cardiomyocyte-Specific miR-200c-3p Sponge via AAV9

The miR-200c-3p sponge and the negative control (NC) sponge were purchased from WZBio. The sponge was packaged into adeno-associated virus serotype 9 (AAV9) under the control of the cardiac troponin T (cTnT) promoter to achieve cardiomyocyte-specific expression. For male mice, at 8 weeks of age, a single tail vein injection of 2.5×10^{12} viral genome copies of either miR-200c-3p sponge or NC sponge were administered in sterile PBS (100 μL total volume) using a tail vein injector (YLS-Q9G, Yiyan Technology Development Co., Ltd., Jinan, China) while mice were briefly restrained without anesthesia. Four weeks after AAV9 injection, the two-hit model of HFpEF was initiated and continued for 12 weeks. Thus, the total experimental period from AAV9 injection to the end of the study was 16 weeks.

For female C57BL/6J mice, the same AAV9 constructs (the miR-200c-3p sponge or the NC sponge) were diluted in sterile PBS and administered via tail vein injection at 9 weeks of age at an identical dose of 9.8×10^{11} viral genome copies per mouse in a volume of 100 μL. Injections were performed using a tail vein injector (YLS-Q9G, Yiyan Technology Development Co., Ltd., Jinan, China) while mice were briefly restrained without anesthesia. The two-hit HFpEF protocol was initiated 2 weeks after AAV9 injection and maintained for 6 weeks.

Randomization and Blinding

Both male and female mice were included in this study. Group allocation was performed using simple randomization, and baseline body weights were compared across groups to rule out any systematic imbalance at the start of the experiment. Inclusion criteria: All animals that survived to the predetermined endpoint were included in the final analysis. Exclusion criteria: Mice that died prematurely before reaching the terminal time point were excluded. These criteria were established a priori. There was no attrition or dropout of animals for any reason other than the premature mortality already noted; all animals that survived to the terminal time point completed the study. Group sizes were determined based on empirically observed effect sizes from previous studies using comparable experimental systems; no formal a priori power calculation was performed. To minimize observer bias, a double-blind approach was used throughout the in vivo work. Specifically, animal caretakers responsible for daily husbandry and body weight monitoring were blinded to group identity, and the

investigator who performed terminal assessments and tissue collection was also unaware of treatment allocation.

Body Weight and Cardiac Morphometry

Body weight was recorded weekly for all mice throughout the experimental period. For the specific cohort involving female mice that received viral injection, body weight measurements were initiated two weeks post-injection (immediately prior to the commencement of the high-fat diet combined with L-NAME treatment) and were continued weekly thereafter until the end of the experiment. At the end of the experiment, the heart was dissected and weighed, and the tibia length was uniformly measured from the left hindlimb in all mice.

Echocardiography

Echocardiography was performed using the Color Doppler ultrasound diagnostic instrument V6 LAB (VINNO Technology, Suzhou, China). Anaesthesia was induced with 5% isoflurane (RWD, Cat#R510-22), at a gas flow rate of 500 mL/min in an induction chamber for approximately 180 s, confirmed by loss of the righting reflex. The mouse was then maintained on 1.5% isoflurane via a face mask, with the depth adjusted according to heart rate during the measurement. With the animal in the supine position and the probe oriented at the 1 o'clock direction, a parasternal short-axis view was obtained to measure left ventricular ejection fraction, fractional shortening, and heart rate. At the 3 o'clock direction, an apical four-chamber view was used to obtain pulsed-wave Doppler images of the mitral inflow (E and A waves) and tissue Doppler images of the mitral annular early diastolic velocity (e').

Hemodynamic Measurements

The hemodynamic parameters were measured using the Millar catheter system, as described previously[41]. Briefly, mice were anesthetized via intraperitoneal injection of pentobarbital sodium (1%, 150 μL, with dose adjusted by body weight, Merck, Cat#P3761). The right common carotid artery was isolated through a midline neck incision and cannulated with a microtip catheter. The catheter was then advanced retrograde into the left ventricle under continuous pressure waveform monitoring via LabChart software. Upon transition to a characteristic ventricular pressure trace, the catheter position was fixed, and hemodynamic parameters were recorded for 15 min. Data analysis included the maximum rate of rise in left ventricular pressure (dP/dt_{max}), peak rate of left ventricular pressure fall (dP/dt_{min}).

Assessment of Glucose Homeostasis

The oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were performed after 8 or 9 weeks of modelling. Mice were fasted for 16 h (starting at 4:00 pm until 8:00 am the next day) prior to OGTT and for 5 h (starting at 9:00 am until 2:00 pm) prior to ITT with free access to water during fasting. For OGTT mice were given 2 g/kg glucose (Solarbio, Cat#G8150) by oral gavage, with the aid of a glucometer (Safe AQ Smart, Sinocare, Changsha, China), blood glucose was measured from mouse tail veins

at 0, 15, 30, 60, 90, and 120 min. For ITT mice were administered via intraperitoneal injection with a bolus of 1.0 unit/kg insulin (M&C Gene Technology, Cat#CC101) and blood glucose was measured from mouse tail veins at 0, 15, 30, 45, and 60 min. In addition, at the end of the experiment, random (non-fasted) blood glucose levels were measured in the $Ctrl^{sEV}$ and $HFpEF^{sEV}$ groups of mice.

Noninvasive Blood Pressure Measurement

At the end of each experiment, systolic and diastolic blood pressures were measured via tail-cuff plethysmography using a noninvasive blood pressure system (CODA, Kent Scientific Corporation). Briefly, each mouse was placed in a purpose-built holder that prevented free movement while leaving the tail exposed. An occlusion cuff (OCUFF) was fitted around the base of the tail with moderate tightness, and a volume pressure recording (VPR) sensor was positioned approximately 1 cm distal to the OCUFF. The holder was then covered with a black cloth to minimize external interference. The measurement protocol consisted of three preliminary cycles followed by five experimental cycles, with a deflation time of 20 seconds per mouse. During the recording, the room lights were turned off and ambient noise was kept to a minimum.

Treadmill Exercise Tolerance Test

The treadmill endurance test was performed as described previously, with minor modifications[42]. The treadmill (ZS-PT-III, Zhongshi Technology, Beijing, China) was set to a 20° incline and operated in constant-current electrical stimulation mode (0.06 mA, with a tolerance time of 10 s). For acclimation, mice underwent three consecutive daily sessions. On day 1, the belt speed was set at 5 m/min for 4 min, then gradually increased to 8 m/min over 6 min and maintained for 10 min, and finally increased to 10 m/min for 7 min. On day 2, the speed was set at 10 m/min for 4 min, rapidly increased to 11 m/min within 10 s and held for 5 min, and then raised to 12 m/min for 7 min. On day 3, mice ran at 5 m/min for 4 min, followed by an increase to 14 m/min within 10 s for 2 min, and finally a progressive ramp to 50 m/min until exhaustion. After a subsequent 24-hour rest period, mice were subjected to an exhaustive running test. The test began with a warm-up at 5 m/min for 4 min, followed by increases to 14 m/min for 2 min and finally to 50 m/min until exhaustion. The total running time and distance to exhaustion were measured.

Measurement of W/D ratio

The lung tissue was gathered and rinsed with PBS. Subsequently, filter paper was used to soak up the liquid and the wet weight of the lung was measured. The samples were dried at 70°C for 48 hours to determine the dry weight. The lung W/D ratio was computed to evaluate pulmonary edema.

Small extracellular vesicles (sEVs) isolation and characterization

Mouse cardiac tissue was minced to 1×1×1 mm pieces and digested in DMEM (Wuhan Promoter Biological CO., LTD, Cat#KGL1211-500) containing Type II collagenase (Biosharp, Cat#9001-12-1) at 37°C in a constant temperature water bath for 1 hour. The

digested tissue suspension was centrifuged at 300 × g for 10 min. The supernatants centrifugation at 2000 × g for 20 min, and then collected and filtered through a 0.22 μm filter (Merck, Cat#SLGP033R), followed by ultracentrifugation (Optima XPN-100, Beckman Coulter, Brea, CA, USA) at 10000 × g for 30 min. Next, the collected supernatants ultracentrifuged at 100000 × g for 70 min. The sEVs were washed in 56 mL of sterile PBS and re-centrifuged at 100000 × g for another 70 min. The final pellet was resuspended in 100 μL of sterile PBS. Similarly, the supernatants from HL-1 cells with or without palmitic acid (PA, 100 μM, Kunchuang Biotechnology, Cat#KC002) were collected and sEVs were isolated following the above centrifugation steps. sEVs were lysed in 10× cell lysis buffer (Antgene, Cat#ANT071) and the amount of sEVs proteins recovered was quantified by a BCA assay (Wuhan Promoter Biological CO., LTD, Cat#P5026). The morphology and size of the isolated sEVs were determined using transmission electron microscopy (TEM) according to the standard protocols. A total of 20 μg sEVs were used for Western blot experiments. The sEVs were verified by using primary antibodies such as HSP70 (HUABIO, Cat#ET1601-11, RRID: AB_3069593, 1:1000 dilution), TSG101 (HUABIO, Cat#T1701-59, RRID: AB_3068599, 1:1000 dilution) and CD9 (HUABIO, Cat#ET1601-9, RRID: AB_3069620, 1:1000 dilution). Flow NanoAnalyzer (N30E, NANOFCM, Medical sub-center of Analytical and Testing Center, Huazhong University of Science and Technology) was used to measure the concentration and particle size distribution of sEVs derived from mouse myocardial tissue.

Small extracellular vesicles (sEVs) uptake assay

For the in vitro sEVs uptake assay, 20 μg of sEVs were stained with PKH26 (MKBio, Cat#MX4021) according to the manufacturer's protocol. Specifically, 250 μL of diluent C-loaded sEVs were mixed with 250 μL of diluent C-loaded PKH26 Dye prepared in Diluent C and incubated at 37°C in the dark for 20 min. Then, 500 μL of 5% BSA (Sigma-Aldrich, Cat#A9418) was added to stop the staining, and the mixture was transferred to a 2 mL benchtop ultracentrifuge tube (Beckman Coulter, Cat#344625). The labeled sEVs were washed with PBS and collected by ultracentrifugation (Optima MAX-XP, Beckman Coulter, Brea, CA, USA) at 100000 × g for 70 min. The PKH26-labeled sEVs were resuspended in DMEM (Wuhan Promoter Biological CO., LTD, Cat#KGL1211-500) and incubated with BV2 cells for 24 hours. Fluorescence confocal laser scanning microscopy (Nikon, Shanghai, China) was used for imaging. DiR (MKBio, Cat#MX4005) was prepared by dissolving in DMSO at a concentration of 1 mM as a stock solution for the in vivo sEVs uptake assay. 100 μg of sEVs isolated from mouse myocardial tissue mixed with DiR to a final concentration of 5 μM, and incubate at 37°C in the dark for 20 min according to the manufacturer's protocol. The sEVs were washed with PBS and ultracentrifuged at 100000 × g for 70 min to remove free DiR. Next, DiR-labeled sEVs were resuspended in PBS and injected into mice through the tail vein. After 12 hours, all images were acquired using the SPECTRAL Lago X imaging system (SI Imaging, Medical sub-center of Analytical and Testing Center, Huazhong University of Science and Technology).

To quantitatively determine the brain biodistribution and cellular uptake of

cardiomyocyte-derived sEVs, DiR-labeled sEVs were administered intravenously to healthy mice. Animals were euthanized at serial time points post-injection (30 min, 6 h, 12 h, and 24 h), and then the whole brain was rapidly removed on ice and briefly rinsed with ice-cold PBS to eliminate blood residues. The hypothalamus, amygdala, and cerebral cortex were quickly dissected and processed for cryosectioning at a thickness of 10 μm. Hypothalamic sections were first rewarmed to room temperature, washed in PBS, subjected to antigen retrieval, and then blocked and permeabilized. Thereafter, the sections were incubated with primary antibody anti-IBA1 (ABclonal, Cat#A19776, RRID: AB_3711469, 1:400 dilution) overnight at 4°C on a shaker. To validate antibody specificity and distinguish genuine target staining from background, parallel negative control sections were processed either by omitting the primary antibody or by using an isotype-matched rabbit IgG at the same concentration as the primary antibody. After three washes with PBS, the sections were incubated with an Alexa Fluor 488-conjugated secondary antibody (Goat anti-Rabbit IgG (H+L), ABclonal, Cat#AS053, RRID: AB_2768320, 1:500 dilution) for 1 hour at room temperature, followed by three additional PBS washes. Finally, the sections were mounted onto glass slides using an anti-fade mounting medium containing DAPI (MKBio, Cat#MM1402). All procedures involving DiR-labeled sEVs, from injection to tissue dissection, were performed under minimal light exposure to prevent photobleaching and quenching of the fluorescent signal.

Cell culture and transfection

HL-1 (National Collection of Authenticated Cell Cultures, Cat#GNM46, CSTR: 19375.09.3101MOUGNM46) and BV2 (China Center for Type Culture Collection CCTCC, Cat#GDC0311, RRID: CVCL_0182) cells were cultured in DMEM (Wuhan Promoter Biological CO., LTD, Cat#KGL1211-500) with 10% FBS (NEWZERUM, Cat#FBS-S500) and 1% penicillin-streptomycin (Sigma-Aldrich, Cat#A5955) in a humidified incubator with 5% CO_2 at 37°C. Cells were subcultured every 2-3 days, and all experiments were performed using cells between passages 5 and 15. 5×10^3 cells per cm^2 for passage maintenance. For experimental treatments, cells were seeded at a density of 1×10^5 cells per well in 6-well plates, and allowed to adhere for 8 h prior to any treatment. The passage number and seeding density were consistent across all experimental replicates. HL-1 and BV-2 cell lines were authenticated by the source vendors using short tandem repeat (STR) profiling. Cells were routinely tested for mycoplasma contamination every 3 months using a PCR-based detection kit (Beyotime, Cat#C0301S), and all test results were negative. No additional authentication was performed by the authors.

After PA stimulation for 24 hours, HL-1 cell supernatants were collected, centrifuged at 1000 rpm for 5 min and filtered through 0.22 μm filters (Merck, Cat#SLGP033R) as HL-1 conditioned medium (CM). Supernatant from untreated HL-1 cells served as the control (CON). The BV2 cells were treated with CM or CON supernatants for 24 hours. To test the effect of sEVs on inflammatory phenotype of BV2 cells, BV2 cells were stimulated with sEVs isolated from the supernatant of HL-1 cells cultured with or without PA. To investigate the relationship between miR-200c-3p and DUSP1 and its

impact on the inflammatory phenotype of BV2 cells, we transfected BV2 cells with miR-200c-3p mimic NC (RIBOBIO, Cat#miR1N0000001-1-5) or mimic (RIBOBIO, Cat#miR1CM001) for 24 hours using Lipofectamine 2000 (Thermo Fisher Scientific, Cat#11668019). While stimulating BV2 cells with sEVs extracted from HL-1 cells treated with PA, we simultaneously transfected them with miR-200c-3p inhibitor NC (RIBOBIO, Cat#miR2N0000001-1-5) or inhibitor (RIBOBIO, Cat #miR2CM001) for 24 hours using Lipofectamine 2000 (Thermo Fisher Scientific, Cat #11668019). In addition, DUSP1 was overexpressed (WZBIO, China) on the basis of mimic transfection. To investigate whether miR-200c-3p is packaged into sEVs for intercellular communication, a transwell co-culture system was established. HL-1 cardiomyocytes were seeded in the upper chamber, while BV2 microglia were cultured in the lower chamber. The cells were co-cultured in serum-free DMEM (Wuhan Promoter Biological CO., LTD, Cat#KGL1211-500) medium containing PA, in the presence or absence of GW4869. Subsequently, the expression levels of miR-200c-3p were quantified by qRT-PCR. U6 small nuclear RNA was used as the endogenous control for normalization of miRNA expression, detected using the Bulge-Loop U6 qPCR Primer Set (RIBOBIO, Cat#MQPS0000002-1-500) according to the manufacturer's instructions.

For in vitro experiments, three independent biological replicates were performed, each consisting of three technical replicates. Biological replicates refer to independently cultured cell passages on different days. Technical replicates refer to repeated measurements (e.g., multiple wells or repeated qRT-PCR runs) from the same biological sample. The passage number and seeding density were kept consistent across all replicates.

Fluorescence in situ hybridization (FISH) combined with immunofluorescence

Paraffin-embedded heart and hypothalamus tissues were sectioned (5 μm), mounted on slides, and processed for FISH using custom-designed digoxigenin-labeled locked nucleic acid miR-200c-3p probes (Genomeditech, #GM-SI-145528). The miR-200c-3p probe was provided as a 2OD liquid stock and used at a 1:100 dilution for hybridization. Following deparaffinization and rehydration, sections underwent antigen retrieval (10 mM sodium citrate buffer, pH 6.0, boiling for 10 min, Servicebio, Cat#G1202), permeabilization with proteinase K (20 μg/mL, 37°C, 15 min, Servicebio, Cat#G1234), and post-fixation. Hybridization with the miR-200c-3p probe was performed overnight at 37°C, followed by stringent washes. Probe detection was achieved using anti-digoxigenin HRP and tyramide signal amplification (TSA) with fluorophore conjugated tyramide (TSA Plus Fluorescence Kit, PerkinElmer, Cat#NEL741001KT). Subsequently, sections were co-stained with primary antibodies, anti-cardiac troponin T (cTnT, HUABIO, Cat#ET1610-51, RRID: AB_3069936, 1:200 dilution) for heart, and anti-IBA1 (ABclonal, Cat#A19776, RRID: AB_3711469, 1:400 dilution) for hypothalamus, incubated overnight at 4°C, followed by Alexa Fluor-conjugated secondary antibodies (Goat anti-Rabbit IgG (H+L), ABclonal, Cat#AS053, RRID: AB_2768320, 1:500 dilution, 1 h at room temperature). Nuclei were counterstained with DAPI. Slides were then mounted with anti-fade mounting medium (Servicebio,

Cat#G1401) and coverslipped. Negative controls included omission of the miRNA probe (for FISH) and omission of primary antibody or use of isotype-matched IgG (for immunofluorescence). All sections were mounted on standard glass slides with a thickness of 1.0-1.2 mm (Citotest Labware, Cat#80312-2101) and covered with #1.5 coverslips (0.17 mm thick). Representative images were selected as those most representative of the typical staining pattern observed across at least three independent experiments. All images were acquired under identical confocal settings (Nikon, Shanghai, China).

Mutation of the DUSP1 3'-UTR

To disrupt the specific interaction between miR-200c-3p and its target, we introduced a precise point mutation into the seed-region binding site within the DUSP1 3'-UTR using CRISPR/Cas9-mediated homology-directed repair (HDR). The donor plasmid and the CRISPR/Cas9 plasmid (Genomeditech, #GM-MC-151677) were co-transfected into BV2 cells. Stable polyclonal populations were selected with 5 μg/mL blasticidin (MedChemExpress, #HY-103401) for 7-10 days. For subsequent transfection with miR-200c-3p mimic, the stable BV2 cells were seeded at a density of 1×10^5 cells per well in 6-well plates and transfected using Lipofectamine 3000 (Thermo Fisher Scientific, Cat#L3000015) according to the manufacturer's instructions. These cells were then transfected with miR-200c-3p mimic (RIBOBIO, Cat#miR1CM001) to assess changes in the expression of inflammatory cytokines.

Western blot

Frozen myocardial tissue, hypothalamic tissue and cells were lysed using RIPA buffer (Solarbio, #R0020) combined with protease inhibitors (MedChemExpress, #HY-K0010) and phosphatase inhibitors (MedChemExpress, #HY-0022). After protein quantification by BCA assay, added loading buffer (YEASEN, #20315ES05) was added and heated at 100°C for 5 min. 20-45 μg of total protein samples and 2-5 μL ColorMixed Protein Marker (Thermo Scientific, Cat#26616 or ABclonal, Cat#RM19001P) were separated by 10% SDS-PAGE (Vazyme, #E303-01) and transferred to PVDF membranes (0.45 μm, Merck, Cat#IPVH00010). Following blocking with 5% non-fat milk, the membranes were incubated overnight at 4℃ with the following primary antibodies, BNP (ABclonal, #A23996, RRID: AB_3712732, 1:1000 dilution), ANP (ABclonal, #A1609, RRID: AB_2763531, 1:1000 dilution), Collagen I (Proteintech, #14695-1-AP, RRID: AB_2082037, 1:1000 dilution), Collagen III (HUABIO, #HA720050, RRID: AB_3071990, 1:1000 dilution), Nos2 (Proteintech, #22226-1-AP, RRID: AB_2879038, 1:1000 dilution), p-IRE1α (HUABIO, #HA721980, RRID: AB_3096844, 1:1000 dilution), IRE1α (HUABIO, #HA723225, RRID: AB_3683560, 1:1000 dilution), IL-6 (ABclonal, #A27935, 1:1000 dilution, RRID: AB_3751225), IL-1β (HUABIO, #ET1701-39, RRID: AB_3070207, 1:1000 dilution) and TNF-α (HUABIO, #ER65189, RRID: AB_3720451, 1:1000 dilution), CD86 (HUABIO, #ET1606-50, RRID: AB_3069745, 1:5000 dilution), CD206 (HUABIO, #ET1702-04, RRID: AB_2893392, 1:1000 dilution), DUSP1 (HUABIO, #ET1701-82, RRID: AB_3070247, 1:1000 dilution), βTubulin (ABclonal, #A12289, RRID:

AB_2861647, 1:10000 dilution), and αTubulin (ABclonal, #A6830, RRID: AB_2863541, 1:5000 dilution). Membranes were then incubated with goat anti-rabbit IgG secondary antibody (ABclonal, #AS014, RRID: AB_2769854) at 1:5000 dilution, or with goat anti-mouse IgG secondary antibody (ABclonal, #AS003, RRID: AB_2769851) at 1:5000 dilution, for 1 hour at room temperature. The signal intensity was detected using a chemiluminescence kit (Abbkine, #BMU101-CN) and analyzed with ImageJ (RRID: SCR_003070). If the molecular weight of the target protein is close to that of the loading control, after target protein exposure, the PVDF membrane is stripped with stripping buffer (Theorell, Cat#PS107) for 15 min, blocked with non-fat milk for 1 hour, and then re-probed with the primary antibody against the loading control. For western blots, representative blots were chosen as those showing band intensities closest to the group mean based on densitometric analysis using ImageJ (RRID: SCR_003070), with subsequent statistical analysis of the quantified data. Molecular weight markers were run on each gel, and their corresponding sizes (in kDa) are annotated next to the respective bands on all blot images. Blots are representative of independent biological replicates.

Antibody Specificity Controls

To validate antibody specificity and distinguish genuine target staining from background, the following negative controls were included in parallel for each staining run: isotype control, sections were incubated with an isotype-matched control antibody (e.g., rabbit or mouse IgG) at the same concentration as the primary antibody, replacing the specific primary antibody, to assess non-specific binding via Fc receptors or other interactions. Secondary antibody only control, sections were processed without primary antibody incubation, followed by the same secondary antibody and detection steps, to evaluate non-specific staining contributed by the secondary antibody itself. All antibody concentrations and staining conditions were optimized to minimize background, and serial sections from the same tissue block were used for all control and experimental stainings to ensure comparability.

Immunofluorescent staining

Immunofluorescence staining was performed as described below. Detailed information on antibody specificity controls is provided in the "Antibody Specificity Controls" section. The paraffin-embedded myocardial tissue sections were cut at a thickness of 4 μm, then deparaffinized, rehydrated, and subjected to antigen retrieval; after blocking 5% goat serum (Boster, #AR0009), the sections were incubated overnight at 4°C with primary antibodies, such as tyrosine hydroxylase (HUABIO, #ET1611-12, RRID: AB_3069989, 1:200 dilution), synaptophysin (HUABIO, #ET1606-56, RRID: AB_3069749, 1:200 dilution). Then, sections were incubated with fluorophore-conjugated secondary antibodies. Nuclei were counterstained with DAPI, and images were acquired using a confocal microscope. For the hypothalamic tissue (4 μm thick), multiple or single immunofluorescence staining was carried out using anti-IBA1 (ABclonal, Cat#A19776, RRID: AB_3711469, 1:1000 dilution), CD86 (HUABIO, #ET1606-50, RRID: AB_3069745, 1:200 dilution), FosB (HUABIO, #ET1611-75,

RRID: AB_3070055, 1:400 dilution), IL-6 (ABclonal, #A27935, 1:200 dilution, RRID: AB_3751225), IL-1β (HUABIO, #ET1701-39, RRID: AB_3070207, 1:100 dilution), TNF-α (HUABIO, #ER65189, RRID: AB_3720451, 1:600 dilution), and DUSP1 (HUABIO, #ET1701-82, RRID: AB_3070247, 1:2000 dilution). Images were acquired using a confocal microscope (Nikon, Shanghai, China) or an upright fluorescence microscope. All sections were mounted on standard glass slides with a thickness of 1.0-1.2 mm (Citotest Labware, Cat#80312-2101) and covered with #1.5 coverslips (0.17 mm thick). Representative images were selected as those showing staining intensities or cell counts closest to the group mean, as determined by quantitative analysis using ImageJ (RRID: SCR_003070) across at least three independent experiments.

Immunohistochemistry

Immunohistochemistry staining was performed as described below. Detailed information on antibody specificity controls is provided in the "Antibody Specificity Controls" section. The cardiac tissue was fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 4 μm thickness. The myocardial tissue was stained with hematoxylin-eosin (H&E), masson's trichrome or sirius red according to standard protocols. For immunohistochemical staining, sections were incubated with primary antibodies against tyrosine hydroxylase (HUABIO, #ET1611-12, RRID: AB_3069989, 1:2000 dilution), synaptophysin (HUABIO, #ET1606-56, RRID: AB_3069749, 1:4000 dilution), and F4/80 (Cell Signaling Technology, #70076, RRID: AB_2799771, 1:500 dilution) respectively in accordance with the manufacturer's instructions. Representative images were selected as those showing staining intensities or cell counts closest to the group mean, as determined by quantitative analysis using ImageJ (RRID: SCR_003070) across at least three independent experiments. All sections were mounted on standard glass slides with a thickness of 1.0-1.2 mm (Citotest Labware, Cat#80312-2101) and covered with #1.5 coverslips (0.17 mm thick).

ELISA

After anesthetizing the mice with 0.5% pentobarbital, blood was collected via orbital puncture and centrifuged at 3000 rpm for 15 min. The supernatant was stored at -80°C for later use. Equal amounts of heart tissue were cut, homogenized and centrifuged at 12,000 rpm for 20 min at 4°C to obtain the supernatant. All assays of norepinephrine (Elabscience, #E-EL-0047), and corticosterone (Elabscience, #E-OSEL-M0001) were performed in duplicate strictly according to the manufacturers' protocols. Briefly, 50 μL of serially diluted standards, standard diluent (blank), or test samples were added to designated wells, followed immediately by 50 μL of biotinylated antibody working solution. The plate was incubated at 37°C for 45 min, washed three times, and then 100 μL of HRP-enzyme conjugate working solution was added to each well. After sealing with a membrane, the plate was incubated at 37°C for 30 min and washed five times. Then, 90 μL of tetramethylbenzidine (TMB) substrate solution was added to each well, and the plate was sealed and incubated at 37°C for approximately 15 min in the dark. The reaction was terminated by adding 50 μL of stop solution, and the optical density was immediately measured at 450 nm using a microplate reader.

Serum total cholesterol measurement

Serum total cholesterol levels were measured using a total cholesterol colorimetric assay kit (Elabscience, #E-BC-K109-M) according to the manufacturer's instructions. Serum samples were thawed to room temperature before use. The blank well received 2.5 μL of double-distilled water, the standard well received 2.5 μL of the provided standard solution (Reagent 2), and each sample well received 2.5 μL of the test serum. Subsequently, 250 μL of enzyme working solution was added to each well. The plate was gently mixed, then incubated at 37°C for 10 min. The optical density (OD) of each well was measured at a wavelength of 510 nm using a microplate reader. Total cholesterol concentrations were calculated from a standard curve and expressed as mmol/L.

sEVs miRNA analysis

sEVs isolated from myocardial tissues of mice in control group and the HFpEF group (n=5) were detected using the DNBSEQ platform. The differences in the expression levels of small RNA sequencing were compared to screen out miRNAs with significantly different expressions. The conditions for screening differentially expressed miRNAs by DEGseq and PossionDis are $|\log2FC| \geq 1$ and Qvalue ≤ 0.05. The results were visualized using cluster heatmaps and volcano plots to illustrate the expression profiles of the significantly altered miRNAs. The microRNA sequencing data generated during this study will be available at the public repository GEO through accession number GSE334160.

Dual-Luciferase reporter assay

The DUSP1 3'-UTR mut plasmid was purchased from WZBio (Shandong, China). HEK293T cells (ATCC, Cat#CRL-3216, RRID: CVCL_0063) were seeded in 24-well plates at a density of 8×10^4 cells per well and cultured overnight to reach 70-80% confluence at the time of transfection. Then, cells were transfected with 1 μg of plasmid and 50nM miR-200c-3p mimic using Lipofectamine 3000 (Thermo Fisher Scientific, #L3000015). After 24 hours, a dual-luciferase assay was conducted to measure the luciferase activity coupled with the 3'-UTR according to the manufacturer's instructions, which was normalized to Renilla luciferase activity.

qRT-PCR

Total RNA was isolated from frozen hypothalamic tissue or cells using the RNA extraction kit (ABclonal, #RK30120), and cDNA was synthesized using a reverse transcription reagent (ABclonal, #RK20432) following the manufacturer's instructions. qRT-PCR was performed with SYBR Green (ABclonal, #RK21203) under the following cycling conditions: 40 cycles, denaturation at 95°C for 10 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 15 seconds. Primers are listed in Online Table S1. For microRNA reverse transcription, we employed a stem-loop reverse transcription method using specifically designed stem-loop primers for mmu-miR-200c-3p (RIBOBIO, #MQPSCM001-2), pri-miR-200c (RIBOBIO, #MQP-0102),

and U6 (RIBOBIO, #MQPS0000002-1-100). Reverse transcription was performed according to the manufacturer's protocol (Vazyme, #MR101-01) to generate cDNA. Additionally, for reverse transcription of multiple microRNAs, a poly(A)-tailing method was employed using poly(A) polymerase, reverse transcriptase (Vazyme, #R211-01), and the corresponding primers (Sangon, Table S2). Relative RNA expression was calculated using the $2^{-}\Delta\Delta Ct$ method. Rn18s was used as the endogenous control for normalization of mRNA expression. For microRNA expression level quantification, U6 small nuclear RNA was used as the endogenous control. The expression levels were then scaled so that the mean value of the control group was set to 1, while retaining the full biological variation within the control samples.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0 software (RRID: SCR_002798). No custom code was generated or used for data analysis in this study. All data were obtained from a minimum of three independent experiments and are reported as mean±standard error of the mean (SEM). For comparisons involving a small sample size ($n<6$ per group), non-parametric tests were applied exclusively. Specifically, the Mann-Whitney U test was used for two-group comparisons, and Dunn's multiple comparisons test (following a Kruskal-Wallis test) for comparisons among three or more groups. For datasets with $n\geq6$ per group, normality was assessed using the Shapiro-Wilk test, and homoscedasticity was verified where applicable. When normality and variance homogeneity were satisfied, parametric tests were employed, an unpaired two-tailed Student's t-test for two groups, and one-way ANOVA with Tukey's multiple comparisons test for multiple groups. Heatmaps, z-scores, and volcano plots depicting differentially expressed miRNAs were generated with GraphPad Prism 9.0. Exact *P* values and sample sizes (n) are provided in the figures or their legends. Statistical significance was defined as *P* values<0.05. ImageJ (RRID: SCR_003070, Version 1.53e) was utilized for the quantitative assessment of Western blot bands, immunohistochemistry images and fluorescence intensity.

Supplemental Tables 1-3

Table S1. Gene primers used for qRT-PCR and sequences of mRNA

Gene	Forward	Reverse
IL-1β	CACTACAGGCTCCGAGATGAACAAC	TGTCGTTGCTTGGTTCTCCTTGTAC
TNF-α	CGCTCTTCTGTCTACTGAACTTCGG	GTGGTTTGTGAGTGTGAGGGTCTG
IL-6	CTTCTTGGGACTGATGCTGGTGAC	TCTGTTGGGAGTGGTATCCTCTGTG
CD206	GGCTTCCGTCACCCTGTATGC	GACCTTCCATCTGCTCCACAATCC
CD86	TCTGCCGTGCCCATTTACAAAGG	AAGTTGGCGATCACTGACAGTTCTG
DUSP1	GCCACCATCTGCCTTGCTTACC	CTCCGCCTCTGCTTCACAAACTC
rn18s	GGCCGTTCTTAGTTGGTGGA	TCAATCTCGGGTGGCTGAAC

Table S2. Gene-specific primers for poly(A)-tailed miRNA qRT-PCR

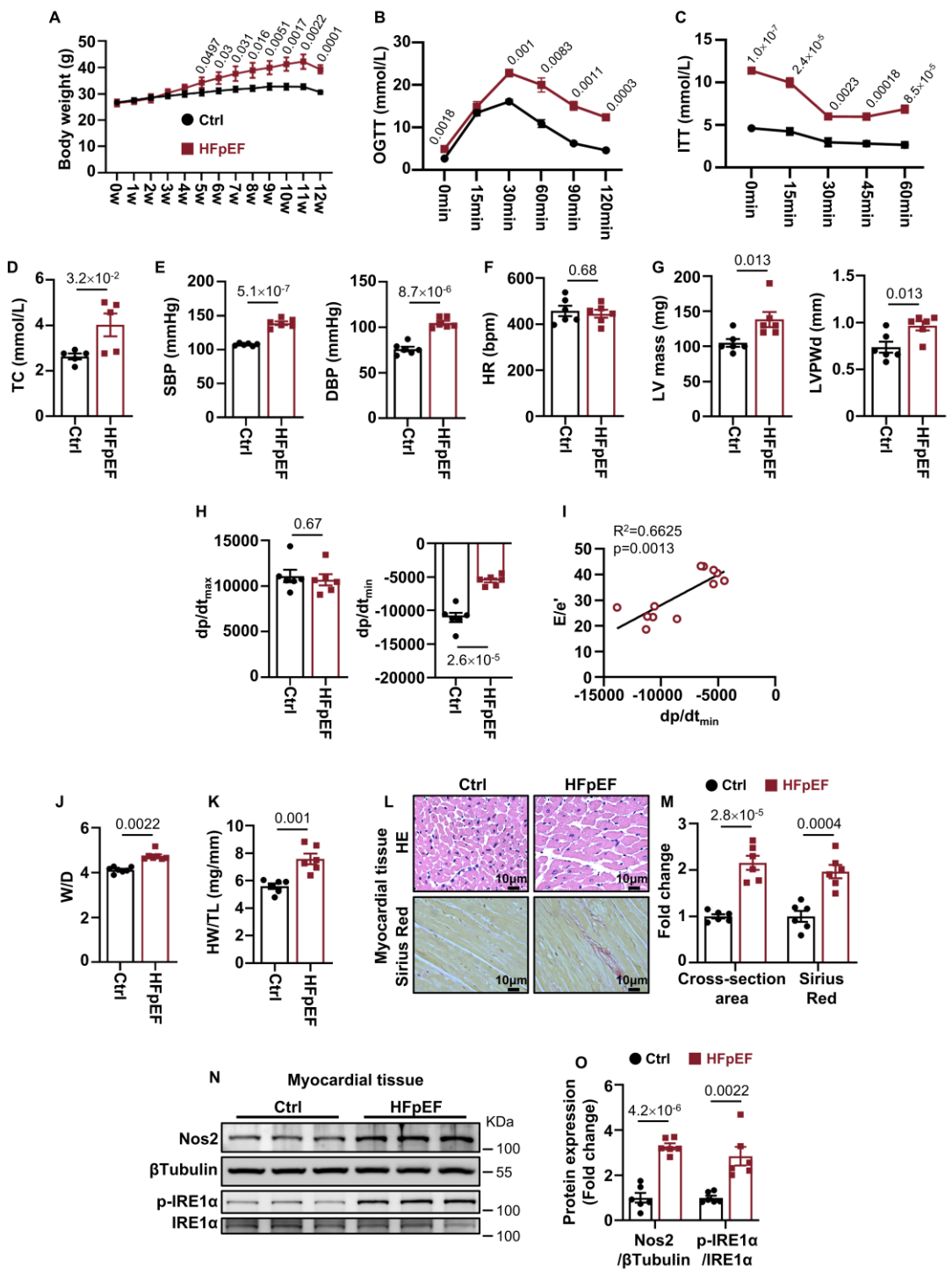
Gene	Oligo sequence
mmu-mir-421-3p	GCATCAACAGACATTAATTGGGCGC
mmu-mir-376b-5P	CGCCGGTGGATATTCCTTCTATGGTTA
mmu-mir-3473b	TAGGGCTGGAGAGATGGCTCA
mmu-mir-214-3p	GCATTAACAGCAGGCACAGACAGG
mmu-mir-210-3p	ATAATCTGTGCGTGTGACAGCGG
mmu-mir-200c-3p	CCGCTAATACTGCCGGGTAATGATG
mmu-mir-199a-3p	GGGCACAGTAGTCTGCACATTGGTTA
mmu-mir-141-3p	GCGCTAACACTGTCTGGTAAAGATGG
mmu-mir-135b-5p	CCCGCTATGGCTTTTCATTCCTATGTGA
mmu-mir-124-3p	ATTAAGGCACGCGGTGAATGC
mmu-mir-72-3p	TTATATAACGGTGGGGGGCGGG
mmu-mir-325-3p	TAATATAAGTGGTGGCGGTGGTGGC
mmu-mir-495-3p	GCGCAAACAAACATGGTGCACTTCTT
mmu-mir-205-5p	ATAGGAGGGAACGCAGTCTGAGTG
mmu-mir-335-5p	GGCGCTCAAGAGCAATAACGAAAAATGT

582 **Table S3. Echocardiography in the different groups of mice**

Condition	HR (bpm)	MV E/A ratio	MV E/e' ratio	EF (%)	FS (%)	LV Mass (mg)	LVPWd (mm)
male							
Ctrl	457.0±22.47	1.22±0.06	23.9±1.33	76.8±2.91	40.2±2.51	105.0±5.63	0.74±0.06
HFpEF	445.2±16.98	1.95±0.07	40.4±1.19	76.2±2.70	39.3±2.50	138.3±10.78	0.97±0.05
male							
Ctrl	415.3±5.69	1.22±0.03	22.5±0.61	75.6±1.74	36.7±0.96	83.3±2.11	0.61±0.02
PLX3397	457.8±19.33	1.19±0.08	22.8±1.24	76.4±1.56	39.7±1.39	78.3±3.07	0.55±0.02
HFpEF	421.8±13.05	2.02±0.08	41.1±0.97	76.5±1.39	39.7±0.78	111.7±7.49	0.76±0.03
HFpEF+ PLX3397	437.5±10.44	1.33±0.06	21.4±0.90	76.3±1.68	39.4±1.50	86.7±2.11	0.66±0.02
male							
Ctrl	439.2±13.51	1.44±0.04	20.8±0.66	75.6±1.10	39.8±0.78	83.3±3.33	0.60±0.02
GW4869	446.8±22.08	1.42±0.06	19.0±0.92	75.3±1.13	39.3±1.38	85.0±5.00	0.60±0.04
HFpEF	442.2±14.49	1.97±0.05	27.8±1.71	78.0±1.15	39.9±1.09	133.3±10.9	0.78±0.02
HFpEF+ GW4869	435.2±13.03	1.61±0.08	21.0±1.05	75.4±1.07	39.8±1.46	100.0±6.83	0.68±0.04
male							
$Ctrl^{sEV}$	471.2±23.05	1.54±0.02	22.9±1.12	74.0±1.89	37.6±1.54	90.0±10.0	0.60±0.04
$HFpEF^{sEV}$	473.0±26.74	1.48±0.04	23.9±1.34	75.2±1.04	37.1±1.06	96.7±3.33	0.61±0.02
male							
NC	453.5±25.09	1.47±0.02	22.4±0.94	77.3±2.13	40.2±1.85	60.0±3.65	0.55±0.03
NC+ HFpEF	472.7±21.30	1.93±0.04	32.8±2.75	77.3±2.55	39.4±2.11	98.3±7.49	0.81±0.05
Sponge	458.3±29.22	1.49±0.02	22.2±1.54	76.1±3.15	39.8±2.83	63.3±4.22	0.50±0.04
Sponge+ HFpEF	478.0±24.90	1.60±0.02	25.8±1.01	76.8±2.13	40.0±2.06	75.0±6.19	0.63±0.02
female							
Control	466.0±15.34	1.47±0.03	17.5±1.22	73.9±1.40	37.5±1.26	78.3±5.43	0.52±0.04
GW4869	467.9±20.51	1.41±0.05	20.8±0.46	73.3±2.88	37.3±2.49	70.0±3.65	0.58±0.04
HFpEF	463.7±16.48	2.18±0.16	27.6±0.78	73.8±1.67	37.1±1.36	101.7±9.80	0.78±0.03
HFpEF+ GW4869	464.0±10.26	1.46±0.07	20.2±0.93	73.4±2.46	36.8±1.91	75.0±4.28	0.65±0.03
female							
Control	474.9±7.97	1.46±0.03	19.0±1.12	73.6±0.93	36.9±0.79	75.0±2.24	0.54±0.02
PLX3397	474.7±5.70	1.39±0.05	20.1±0.98	72.6±2.09	36.1±1.97	75.0±5.00	0.65±0.03
HFpEF	472.1±8.54	1.98±0.13	28.4±1.62	75.5±2.07	36.4±2.18	95.0±4.28	0.75±0.04
HFpEF+	474.0±14.99	1.52±0.06	17.3±1.43	74.6±1.0	37.3±0.90	76.7±4.22	0.60±0.04

PLX3397							
female							
NC	487.4±9.20	1.35±0.05	20.0±1.03	70.8±2.12	36.6±1.42	80.0±5.16	0.67±0.02
NC+ HFpEF	465.8±11.01	2.16±0.09	28.8±1.96	69.8±1.60	35.5±2.19	103.3±4.22	0.79±0.02
Sponge	483.6±14.58	1.44±0.03	19.7±1.13	70.8±2.21	35.0±1.77	80.0±5.16	0.58±0.02
Sponge+ HFpEF	488.7±12.39	1.57±0.05	20.1±1.74	71.0±1.72	35.9±1.11	88.3±6.01	0.65±0.02

Supplementary Figures



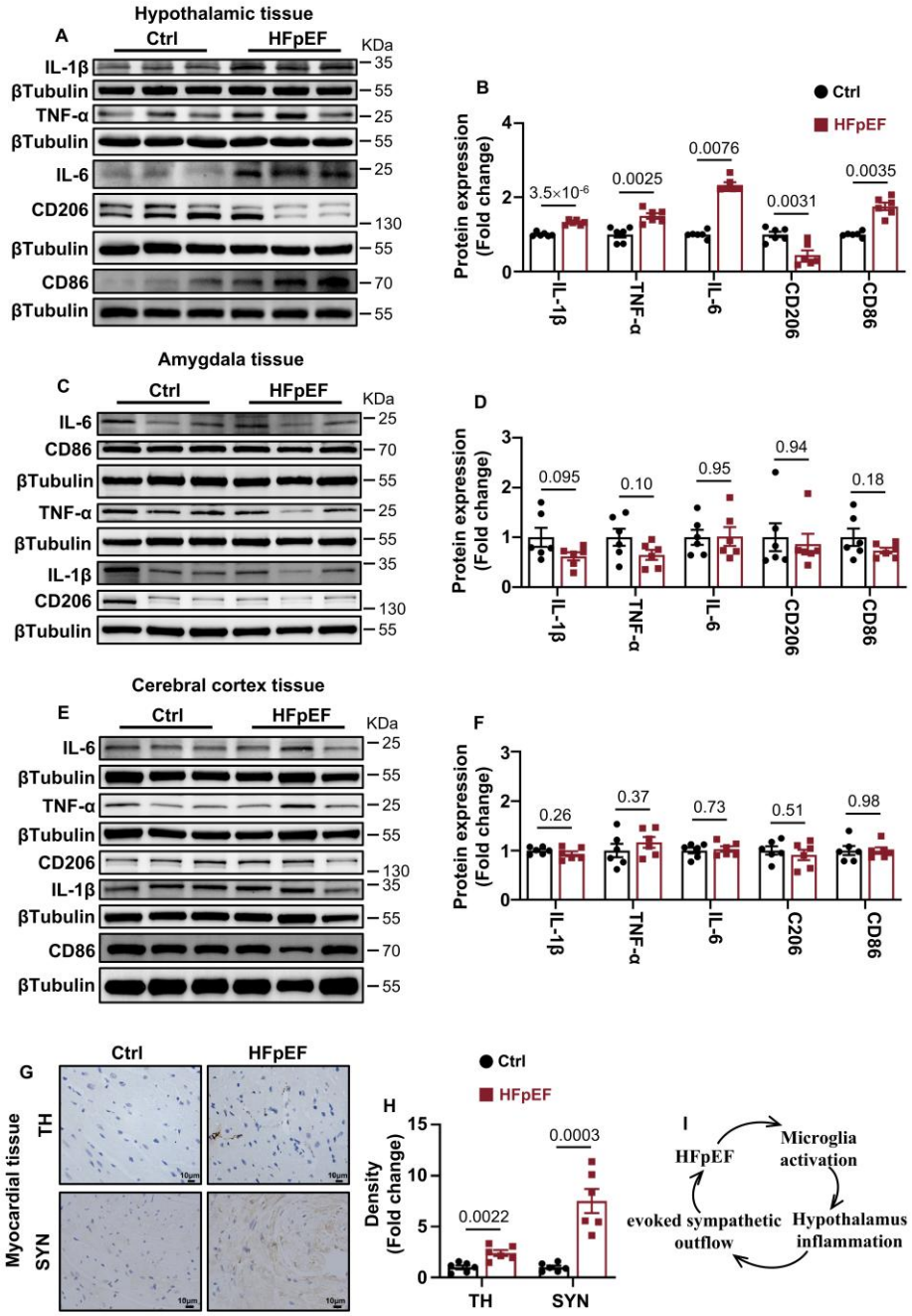
Supplementary Figure 1. HFpEF Model Generated by Two-hit Strategy in Male Mice.

A, Body weight of mice after two-hit treatment (n=6 per group).

B, Oral glucose tolerance test (OGTT) after 12 weeks of two-hit treatment (n=6 per group).

C, Insulin tolerance test (ITT, n=6 per group).

D, Serum total cholesterol (TC) levels (n=5 per group).
E, Systolic blood pressure (SBP) and diastolic blood pressure (DBP) of mice (n=6 per group).
F, To minimize the influence of heart rate variability on cardiac functional parameters, the changes of heart rate were statistically analyzed after inhaling isoflurane anesthesia in mice (n=6 per group).
G, The calculated left ventricular mass and left ventricular posterior wall thickness at end-diastole (LVPWd, n=6 per group).
H, Hemodynamic assessment of left ventricular contractility dp/dt_{max} and relaxation dp/dt_{min} via cardiac catheterization (n=6 per group).
I, Correlation analysis between the diastolic function index E/e' and the hemodynamic relaxation parameter dP/dt_{min}.
J, Lung wet/dry weight ratio (W/D, n=6 per group).
K, Heart weight (HW) normalized to tibia length (TL) (n=6 per group).
L-M, Representative images of hematoxylin-eosin (H&E) staining, Sirius Red staining, along with quantification of cardiomyocyte cross-sectional area (scale bar=10 μm, n=6 per group, 3 random areas per heart), fibrotic area (scale bar=10 μm, n=6 per group, 3 random areas per heart).
N-O, Western blot analysis was employed to quantify the expression of Nos2, p-IRE1α, and IRE1α in the myocardial tissue of control and two-hit-treated mice (n=6 per group). The relative protein levels were determined by comparing them to the first sample in the control group. Data points represent individual mice for in vivo. Data are shown as mean±SEM and analyzed using unpaired t-test (E, F, H, K, and M) or Mann-Whitney U test (D, G, J, and O) or repeated measures two-way ANOVA with Bonferroni's multiple comparisons test (A-C). Data were analyzed by Spearman's rank correlation (I). Exact P values are indicated in corresponding figures.



Supplementary Figure 2. Neuroinflammation in Different Brain Regions and Sympathetic Activation in Male HFpEF Mice.

A-B, Western blot analysis was employed to quantify the expression of IL-1β, IL-6, TNF-α, CD86, and CD206 in the hypothalamic tissue of control and two-hit-treated mice (n=6 per group).

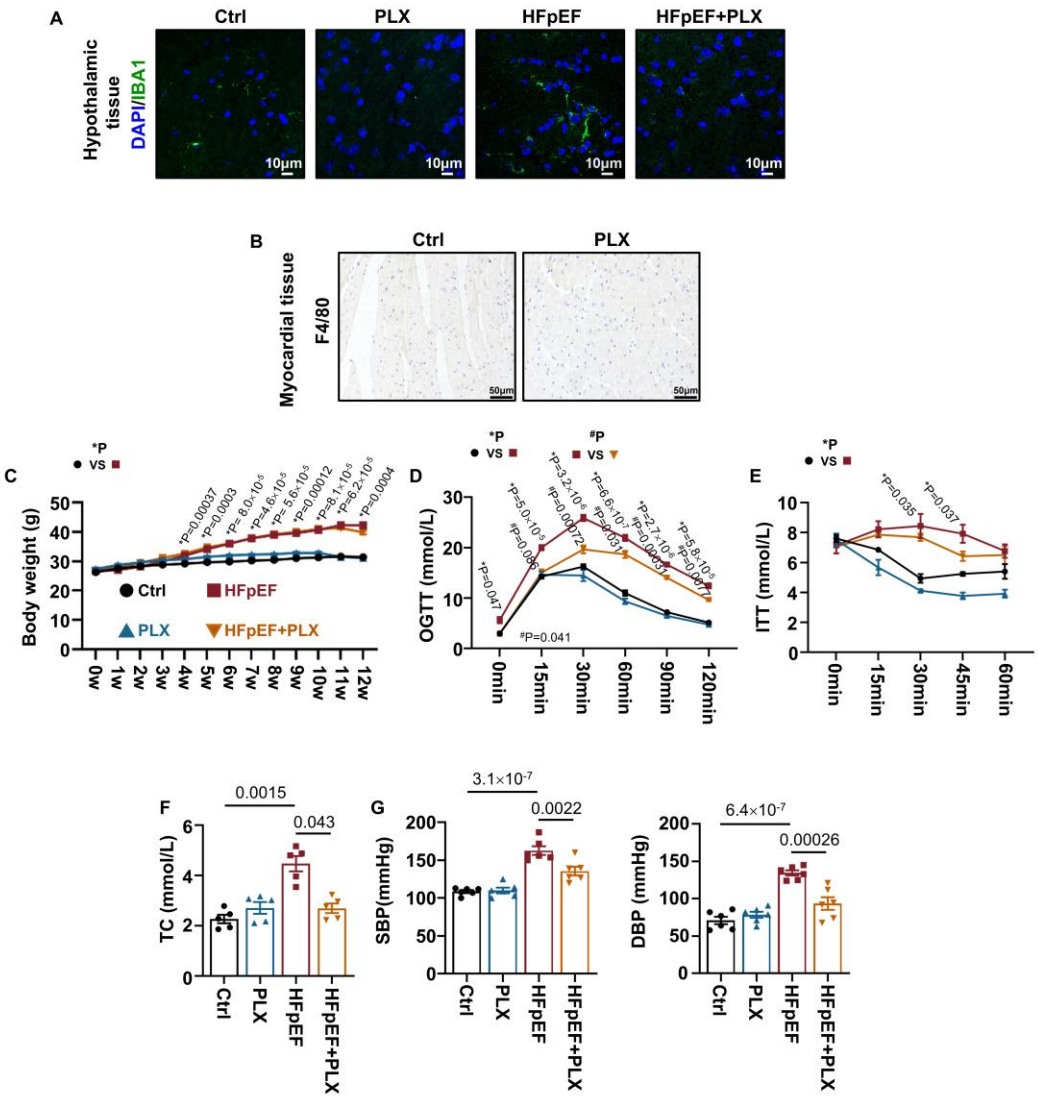
C-D, Western blot analysis was employed to quantify the expression of IL-1β, IL-6, TNF-α, CD86, and CD206 in the amygdala tissue of control and two-hit-treated mice (n=6 per group).

E-F, Western blot analysis was employed to quantify the expression of IL-1β, IL-6, TNF-α, CD86, and CD206 in the cerebral cortex tissue of control and two-hit-treated mice (n=6 per group).

G-H, Representative immunohistochemistry images of tyrosine hydroxylase (TH) and synaptophysin (SYN) in myocardial tissue (scale bar=10 μm, n=6 per group, 3 random areas per heart).

I, Schematic illustration.

The relative protein levels were determined by comparing them to the first sample in the control group. Blots are representative of independent biological replicates. Data points represent individual mice for in vivo. Data are shown as mean±SEM and analyzed using unpaired t-test (for B, F, and H, and for IL-1β, TNF-α, IL-6, and CD86 in E) or Mann-Whitney U test (for CD206 in E). Exact P values are indicated in corresponding figures.



Supplementary Figure 3. Microglia Depletion with PLX3397 Ameliorates Metabolic Phenotypes in Male HFpEF Mice.

A, Representative immunofluorescence images of IBA1 in hypothalamic tissue (scale bar=10 μm).

B, Representative immunohistochemistry images of F4/80 in myocardial tissue (scale bar=50 μm).

C, Body weight (n=6 per group).

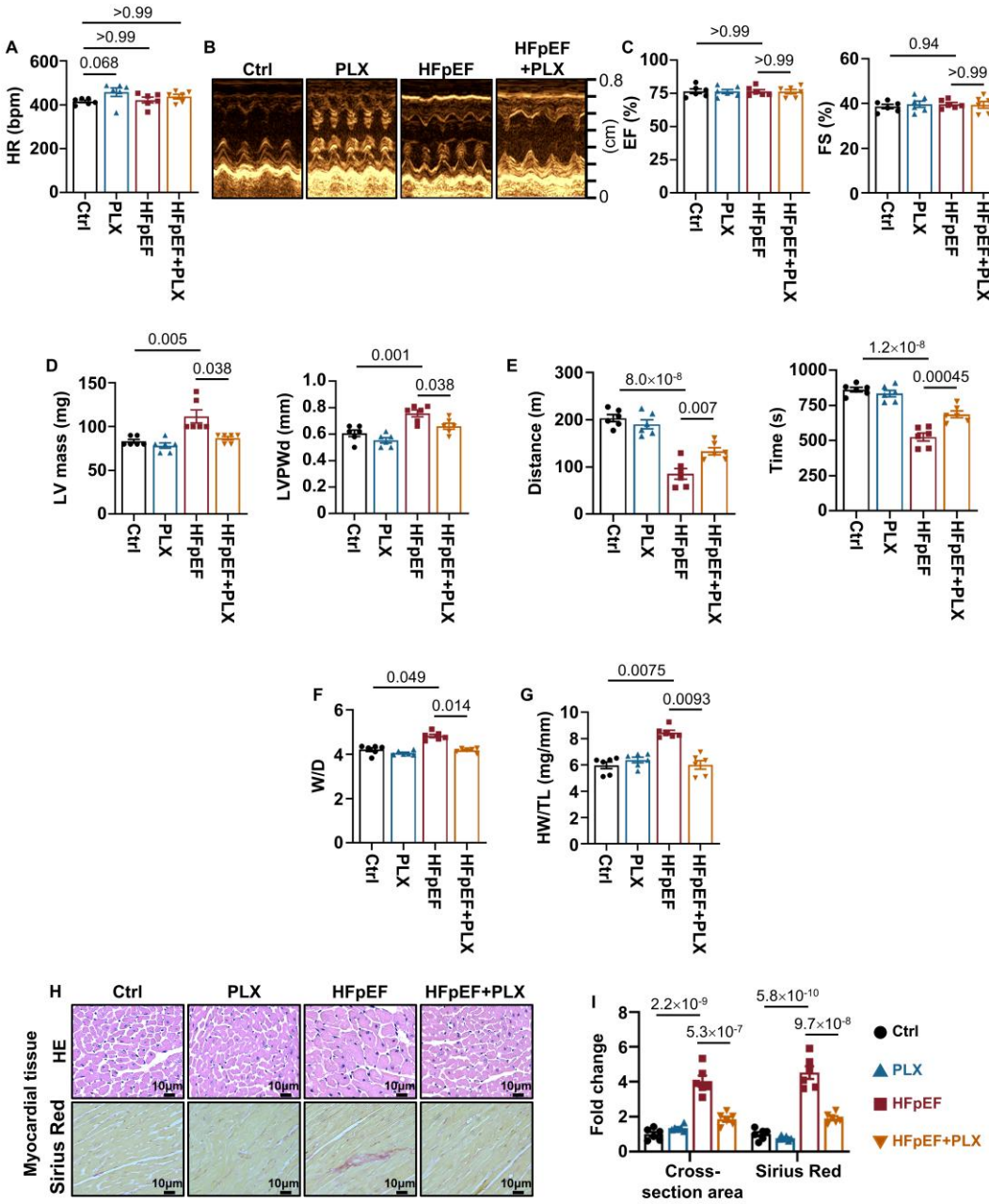
D, Oral glucose tolerance test (OGTT, n=6 per group).

E, Insulin tolerance test (ITT, n=6 per group).

F, Serum total cholesterol (TC) levels (n=5 per group).

G, Systolic blood pressure (SBP) and diastolic blood pressure (DBP) (n=6 per group).

Data points represent individual mice for in vivo. Data are shown as mean±SEM and analyzed using one-way ANOVA with Tukey's multiple comparisons test (G), Kruskal-Wallis test (F), or two-way ANOVA with Bonferroni's multiple comparisons test (C, D, and E). Exact P values are indicated in corresponding figures.



Supplementary Figure 4. Microglia Depletion with PLX3397 Attenuates Cardiac Dysfunction in Male HFpEF Mice.

A, To minimize the influence of heart rate variability on cardiac functional parameters, heart rate was recorded and analyzed under isoflurane anesthesia (n=6 per group).

B-C, Representative echocardiographic images and statistical analysis of left ventricular ejection fraction (EF) and fractional shortening (FS, n=6 per group).

D, The calculated left ventricular mass and left ventricular posterior wall thickness at end-diastole (LVPWd, n=6 per group).

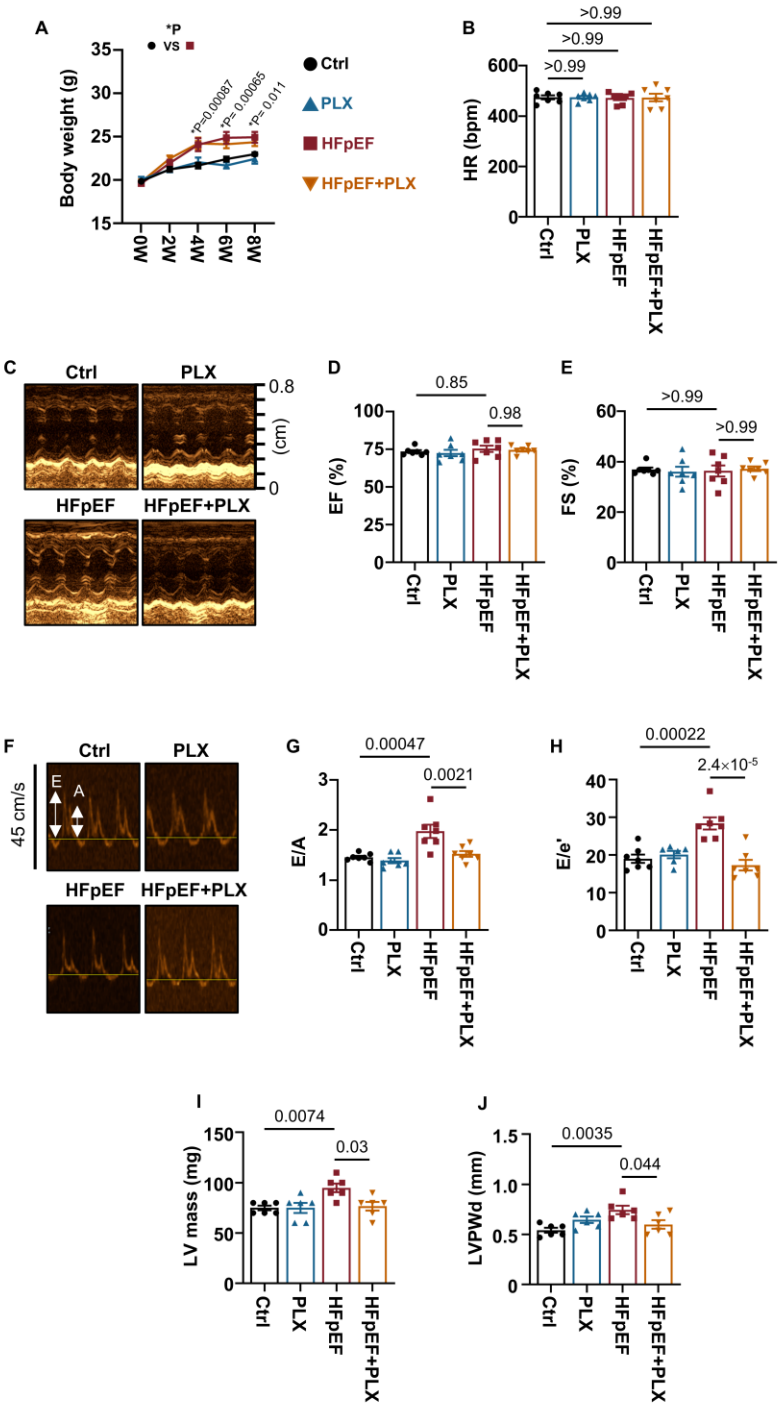
E, Exercise time and distance in the mouse treadmill exhaustion test (n=6 per group).

F, Lung wet/dry weight ratio (W/D, n=6 per group).

G, Heart weight (HW) normalized to tibia length (TL, n=6 per group).

H-I, Representative images of hematoxylin-eosin (H&E) staining, Sirius Red staining,

along with quantification of cardiomyocyte cross-sectional area (scale bar=10 μm, n=6 per group, 3 random areas per heart), fibrotic area (scale bar=10 μm, n=6 per group, 3 random areas per heart). Each point represents a mouse. Data are shown as mean±SEM and analyzed using one-way ANOVA with Tukey's multiple comparisons test (C, LVPWd in D, E, and I) or Kruskal-Wallis test (A, LV mass in D, F, and G). Exact P values are indicated in corresponding figures.



734

735 **Supplementary Figure 5. Microglia Depletion with PLX3397 Attenuates Cardiac**
736 **Dysfunction in Female HFpEF Mice.**

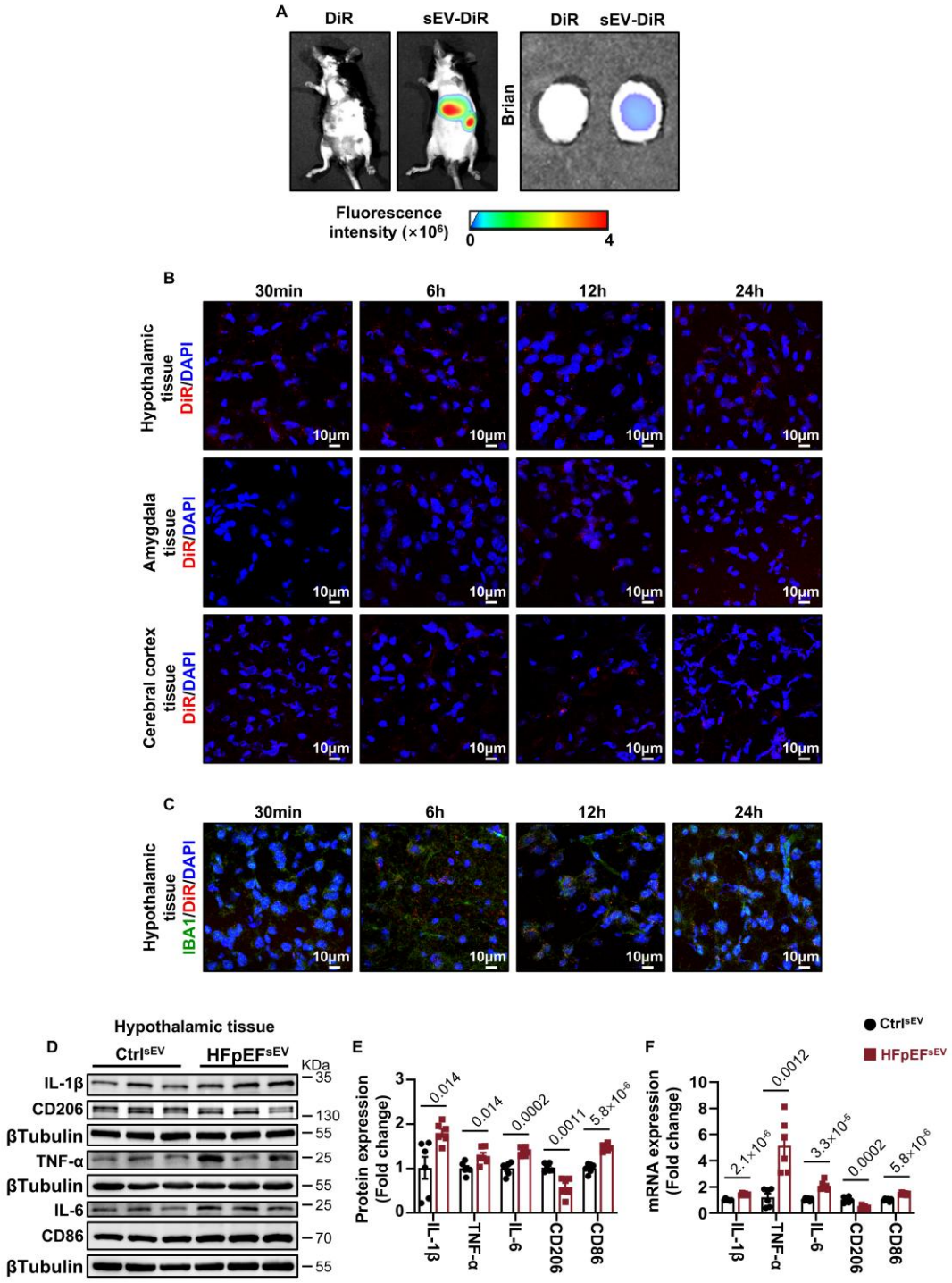
737 In the female cohort, 9-week-old mice received either a control diet or the HFpEF-
738 inducing diet for 8 weeks.

739 A, Body weight (n=7 per group).

740 B, To minimize the influence of heart rate variability on cardiac functional parameters,
741 heart rate was recorded and analyzed under isoflurane anesthesia (n=7 per group).

742 C-E, Representative echocardiographic images and statistical analysis of left

ventricular ejection fraction (EF) and fractional shortening (FS) in female mice (n=7 per group).
F-H, Representative echocardiographic images and statistical data of diastolic function E/A and E/e' in female mice (n=6-7 per group).
I, The calculated left ventricular mass at end-diastole (n=6 per group).
J, The calculated left ventricular mass and left ventricular posterior wall thickness at end-diastole (LVPWd) in female mice (n=6 per group).
Data points represent individual mice for in vivo. Data are shown as mean±SEM and analyzed using one-way ANOVA with Tukey's multiple comparisons test (B, D, G, H, and J), Kruskal-Wallis test (E and I) or two-way ANOVA (A). Exact P values are indicated in corresponding figures.



Supplementary Figure 6. Small Extracellular Vesicles (sEVs) Derived from HFpEF Myocardium Induce Hypothalamic Inflammation in Male Mice.

A, Myocardial sEVs were isolated and labeled with DiR and then injected into normal mice via the tail vein. To control for non-specific signal, a separate group of mice was injected with an equivalent dose of DiR dye alone to account for dye distribution and clearance. Representative in vivo fluorescence images of whole mice and isolated brains.

B, Representative fluorescence microscopy images of brain sections showing

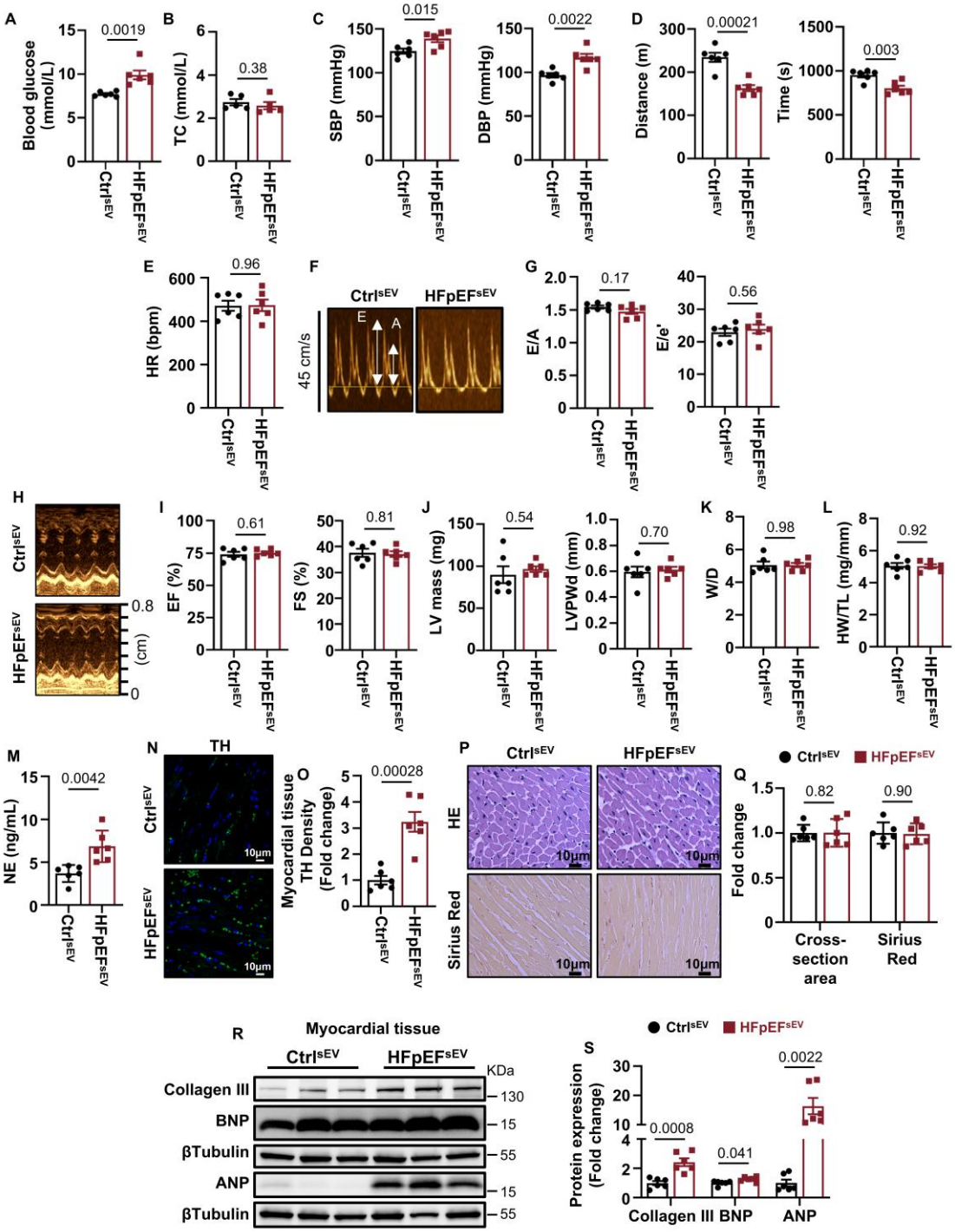
DiR-labeled sEVs (red) and DAPI-stained nuclei (blue) in the hypothalamus, amygdala, and cerebral cortex at 30 min, 6 h, 12 h, and 24 h after injection.

C, Hypothalamic sections collected at 30 min, 6 h, 12 h, and 24 h after injection were immunostained for the microglial marker IBA1 (green) and imaged for co-localization with DiR-labeled sEVs (red). Nuclei were counterstained with DAPI (blue).

D-E, Western blot analysis and quantification of IL-1β, IL-6, TNF-α, CD86, and CD206 in hypothalamic tissue of $Ctrl^{sEV}$ and $HFpEF^{sEV}$ group (n=6 per group).

F, The mRNA expression levels of IL-1β, IL-6, TNF-α, CD86, and CD206 in hypothalamic tissue of $Ctrl^{sEV}$ and $HFpEF^{sEV}$ group (n=6 per group).

The relative protein levels were determined by comparing them to the first sample in the control group. The relative mRNA levels were determined by comparing them to the average expression in the control group. Data points represent individual mice for in vivo. Data are shown as mean±SEM and analyzed using unpaired t-test (E and F). Exact P values are indicated in corresponding figures.



Supplementary Figure 7. HFpEF-derived sEVs Induce Metabolic Abnormalities, Sympathetic Activation, and Cardiac Dysfunction in Male Mice.

A, Random blood glucose levels (n=6 per group).

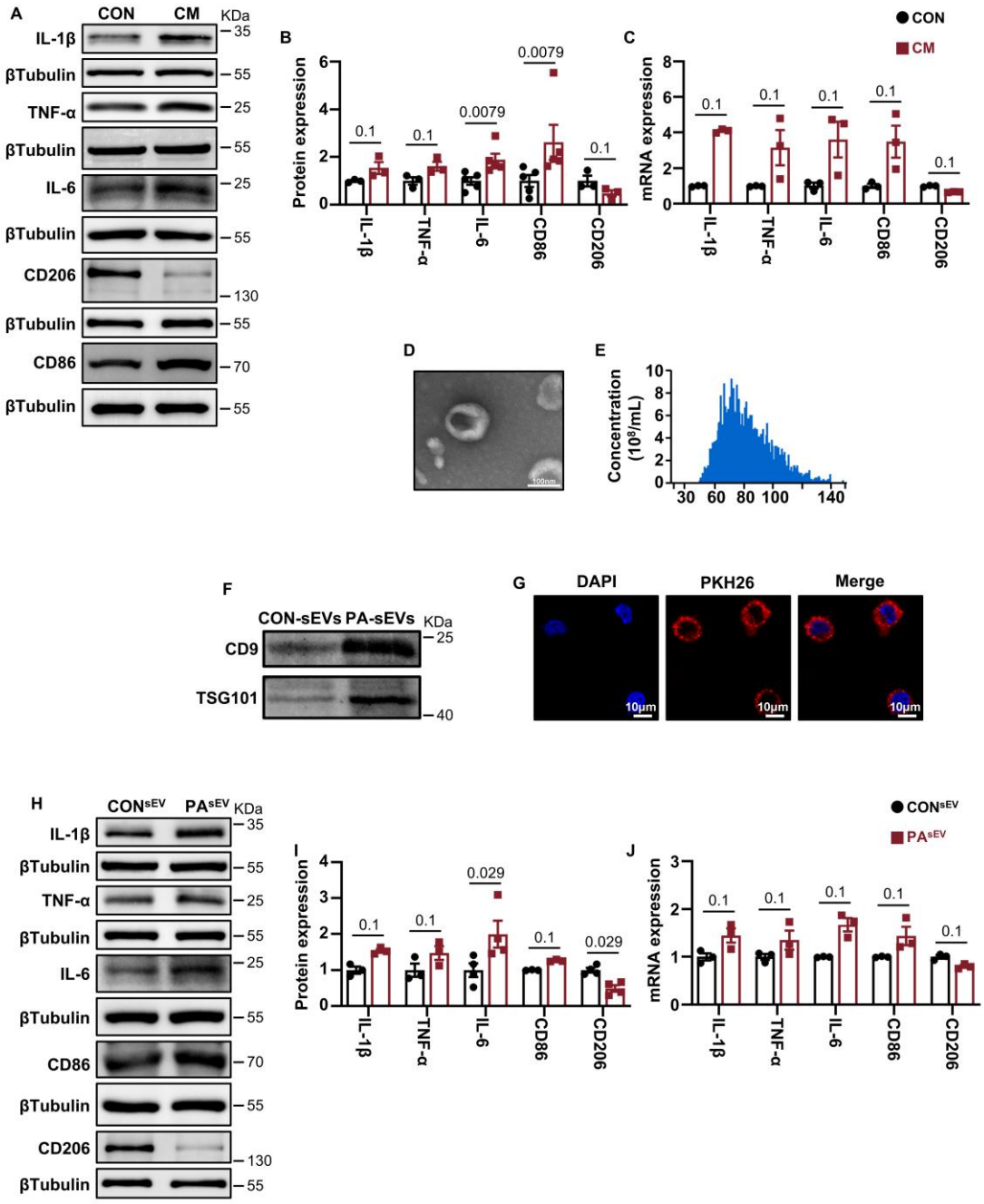
B, Serum total cholesterol (TC) levels (n=5 per group).

C, Systolic blood pressure (SBP) and diastolic blood pressure (DBP) (n=6 per group).

D, Exercise time and distance in the mouse treadmill exhaustion test (n=6 per group).

E, To minimize the influence of heart rate variability on cardiac functional parameters, heart rate was recorded and analyzed under isoflurane anesthesia in male mice (n=6 per group).

F-G, Representative echocardiographic images and statistical data of diastolic function E/A and E/e' (n=6 per group).
H-I, Representative echocardiographic images and statistical analysis of left ventricular ejection fraction (EF) and fractional shortening (FS, n=6 per group).
J, The calculated left ventricular mass and left ventricular posterior wall thickness at end-diastole (LVPWd, n=6 per group).
K, Lung wet/dry weight ratio (W/D, n=6 per group).
L, Heart weight (HW) normalized to tibia length (TL, n=6 per group).
M, Quantitative data of serum norepinephrine (NE) level (n=6 per group).
N-O, Representative immunofluorescence images and quantitative analysis of tyrosine hydroxylase (TH) in myocardial tissue (scale bar=10 μm, n=6 per group, 3 random areas per heart).
P-Q, Representative images of hematoxylin-eosin (H&E) staining, Sirius Red staining, along with quantification of cardiomyocyte cross-sectional area (scale bar=10 μm, n=6 per group, 3 random areas per heart), fibrotic area (scale bar=10 μm, n=6 per group, 3 random areas per heart).
R-S, Western blot analysis and quantification of Collagen III, ANP, and BNP in myocardial tissue of $Ctrl^{sEV}$ and $HFpEF^{sEV}$ group (n=6 per group). The relative protein levels were determined by comparing them to the first sample in the control group.
Data points represent individual mice for in vivo. Data are shown as mean±SEM and analyzed using unpaired t-test ((for A, C, D, E, G, I, J, K, L, M, O, fibrotic area in Q, and Collagen III band intensity in S). Non-normally distributed parameters were compared using the Mann-Whitney U test (for B, cardiomyocyte cross-sectional area in Q, and ANP and BNP band intensity in S). Exact P values are indicated in corresponding figures.



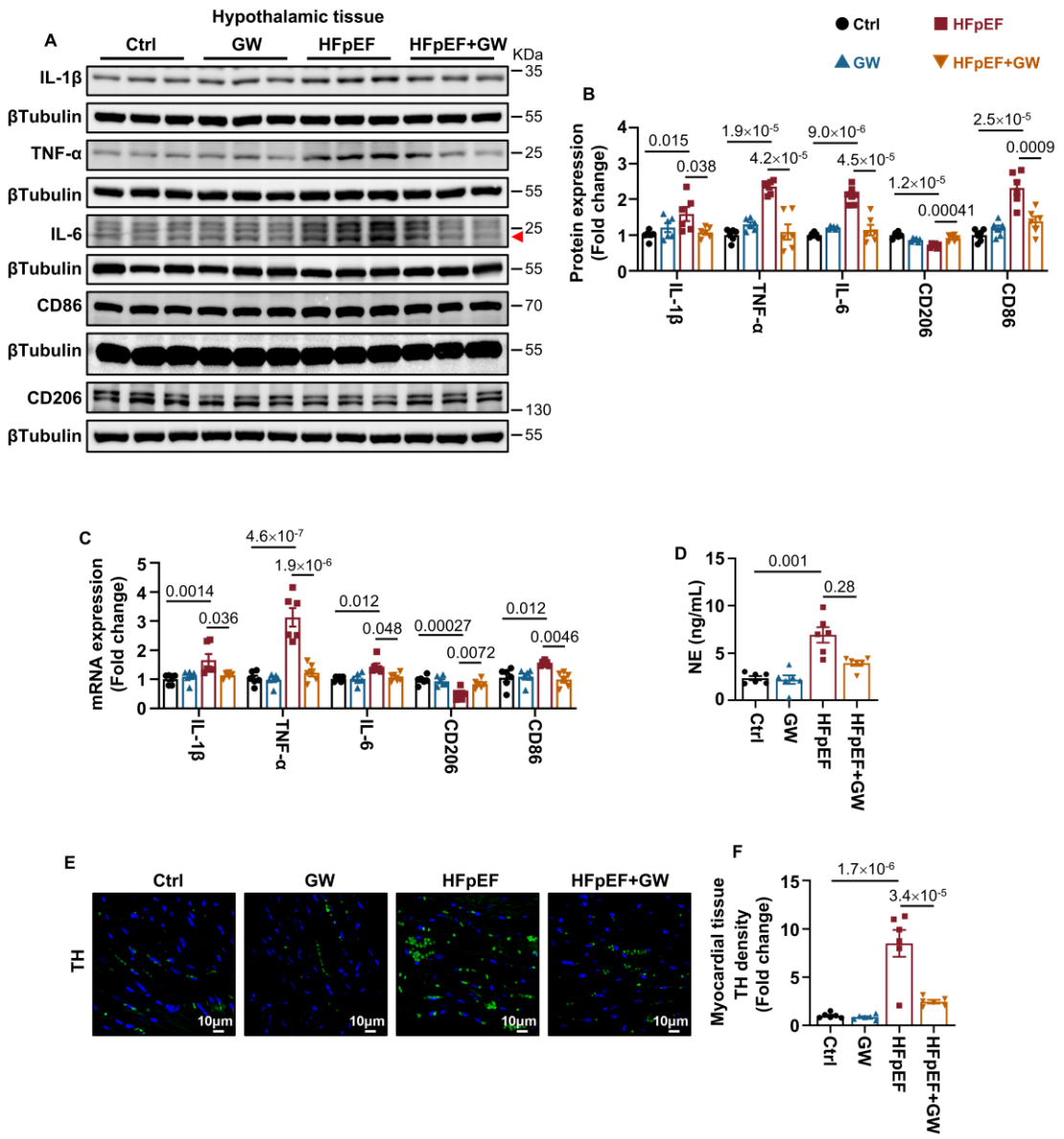
Supplementary Figure 8. The Conditioned Supernatant of HL-1 Cells and the sEVs Secreted from HL-1 Supernatant with PA Stimulation Convert Microglia to a Pro-inflammatory Phenotype.

A-B, BV2 cells were cocultured with conditioned medium (CM) from palmitic acid stimulated HL-1 cells for 24 hours. Western blot analysis and quantification of IL-1β, IL-6, TNF-α, CD86, and CD206 levels in BV2 cells. (n=3-5 per group).

C, The mRNA expression levels of IL-1β, IL-6, TNF-α, CD86, and CD206 in BV2 cells treated with conditioned medium (CM) from palmitic acid stimulated HL-1 cells (n=3 per group).

D, Morphology of sEVs secreted by HL-1 cell supernatant observed by transmission electron microscopy. Scale bar=100 nm.

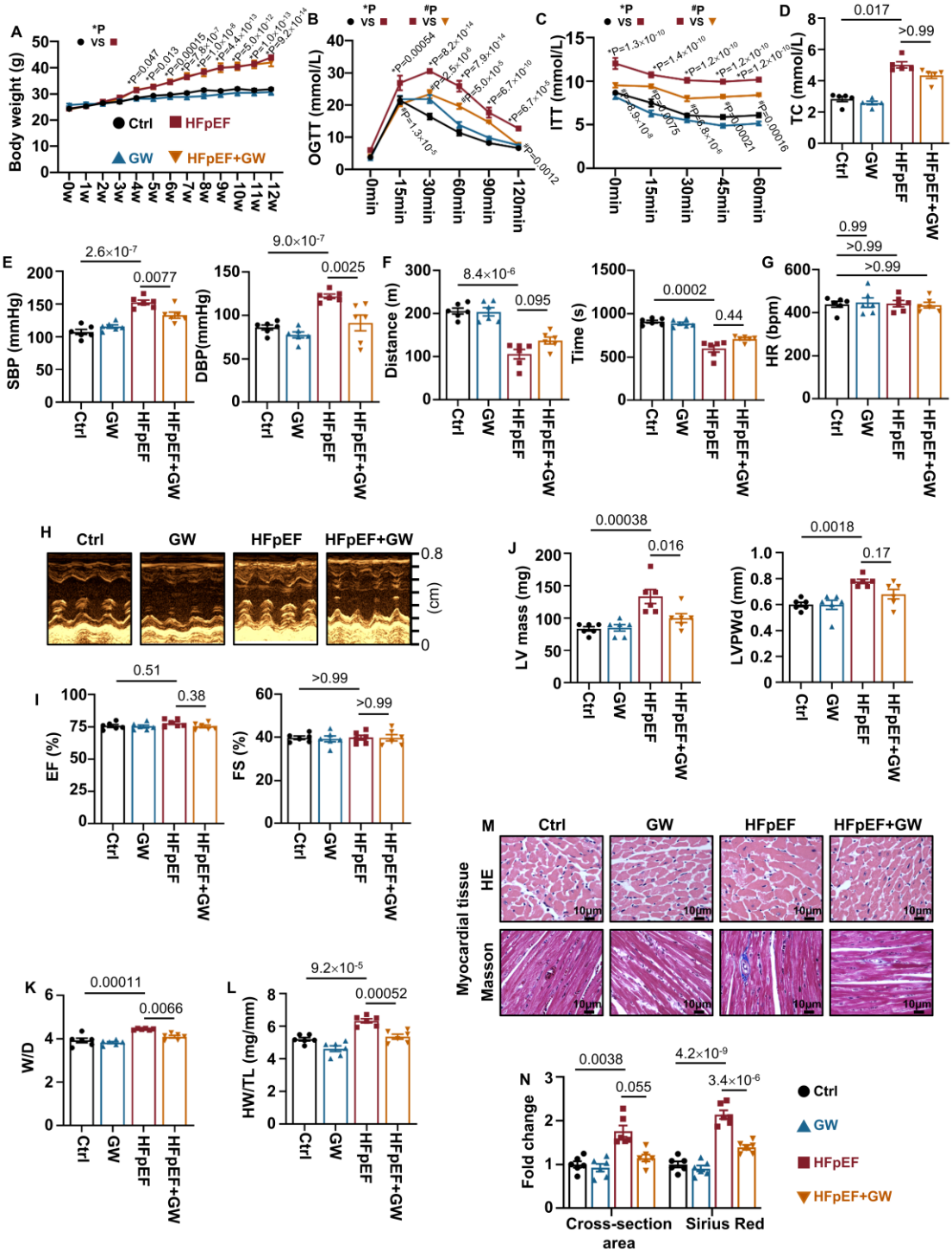
E, Particle size distribution measured by Nanoparticle tracking analysis.
F, Western blot analysis of sEV markers CD9 and TSG101 in sEVs derived from HL-1 cell supernatant. Representative blot from 3 independent experiments.
G, Representative confocal images showing uptake of PKH26-labeled sEVs (red) by BV2 cells after 12 h. Nuclei were stained with DAPI (blue). Scale bar=10 μm.
H-I, Western blot analysis and quantification of IL-1β, IL-6, TNF-α, CD86, and CD206 in BV2 cells stimulated with CON^{sEV} or PA^{sEV} for 24 hours. (n=3-4 per group).
J, The mRNA expression of IL-1β, IL-6, TNF-α, CD86, and CD206 in BV2 cells stimulated with CON^{sEV} or PA^{sEV} for 24 hours. (n=3 per group).
The relative protein levels were determined by comparing them to the first sample in the control group. The relative mRNA levels were determined by comparing them to the average expression in the control group. Each data point represents one independent biological replicate, 2-3 technical replicates were performed per sample and averaged prior to analysis. Data are shown as mean±SEM and analyzed using Mann-Whitney U test (B, C, I, and J). Exact P values are indicated in corresponding figures.



841

842 **Supplementary Figure 9. Inhibition of sEV Biogenesis by GW4869 Alleviates**
843 **Hypothalamic Inflammation and Sympathetic Activation in Male HFpEF Mice.**
844 A-B, Western blot analysis and quantification of IL-1β, IL-6, TNF-α, CD86, and CD206
845 in hypothalamic tissue from mice treated with or without GW4869 (GW, n=6 per group).
846 C, The mRNA expression levels of IL-1β, IL-6, TNF-α, CD86, and CD206 in
847 hypothalamic tissue from mice treated with or without GW4869 (n=6 per group).
848 D, Quantitative data of serum norepinephrine (NE) level (n=6 per group).
849 E-F, Representative immunofluorescence images and quantitative analysis of tyrosine
850 hydroxylase (TH) in myocardial tissue (scale bar=10 μm, n=6 per group, 3 random
851 areas per heart).
852 The relative protein levels were determined by comparing them to the first sample in
853 the control group. The relative mRNA levels were determined by comparing them to
854 the average expression in the control group. Data points represent individual mice for
855 in vivo. For panel D, 2-3 technical replicates were performed per sample and averaged
856 prior to analysis. Data are shown as mean±SEM and analyzed using one-way ANOVA

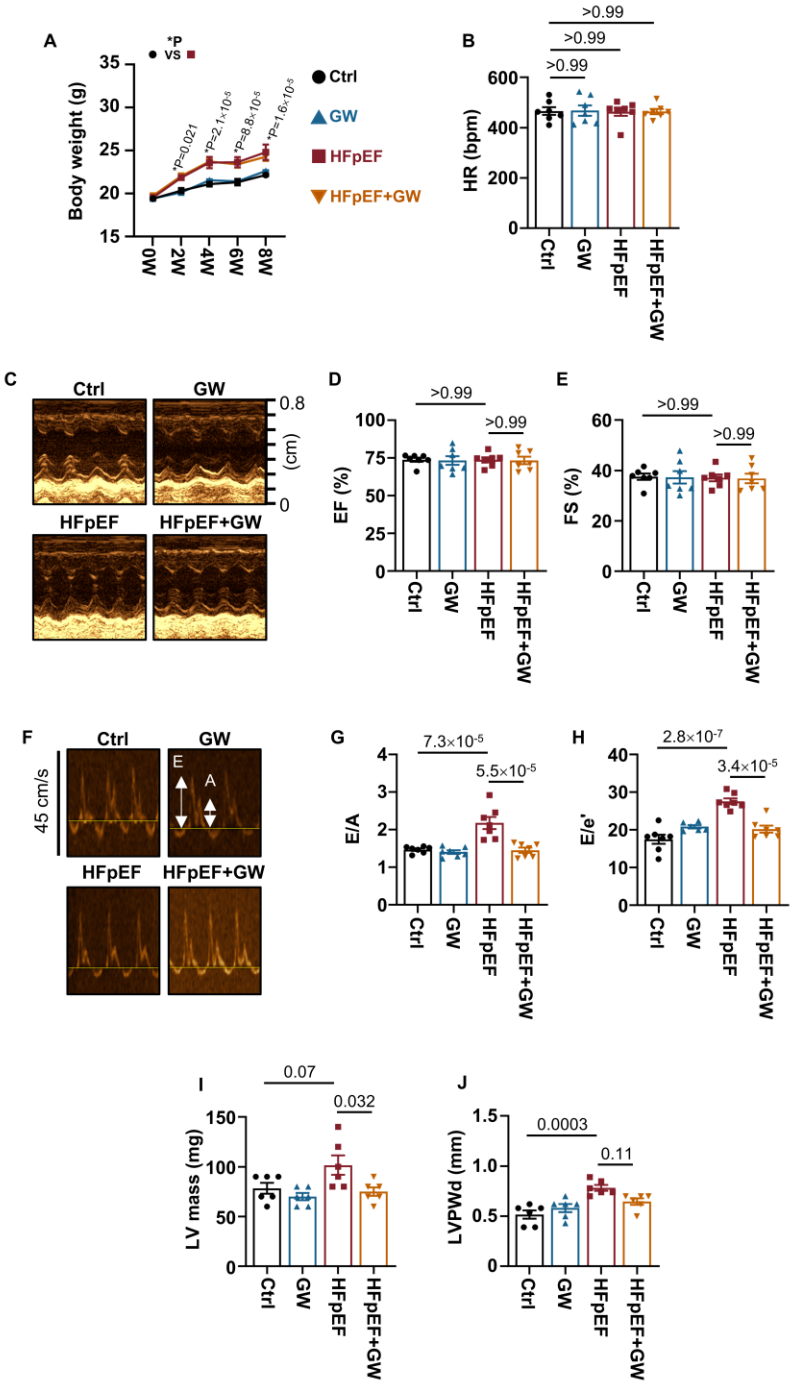
with Tukey's multiple comparisons test (B, TNF-α, CD86, and CD206 in C, and F) or Kruskal-Wallis test (IL-1β and IL-6 in C, and D). Exact P values are indicated in corresponding figures.



Supplementary Figure 10. Inhibition of sEV Biogenesis by GW4869 Alleviates Hypothalamic Inflammation, Sympathetic Activation, and Cardiac Dysfunction in Male HFpEF Mice.

A, Body weight (n=6 per group).

B, Oral glucose tolerance test (OGTT, n=6 per group).
C, Insulin tolerance test (ITT, n=6 per group).
D, Serum total cholesterol (TC) levels (n=5 per group).
E, Systolic blood pressure (SBP) and diastolic blood pressure (DBP) (n=6 per group).
F, Exercise time and distance in the mouse treadmill exhaustion test (n=6 per group).
G, To minimize the influence of heart rate variability on cardiac functional parameters, heart rate was recorded and analyzed under isoflurane anesthesia (n=6 per group).
H-I, Representative echocardiographic images and statistical analysis of left ventricular ejection fraction (EF) and fractional shortening (FS, n=6 per group).
J, The calculated left ventricular mass and left ventricular posterior wall thickness at end-diastole (LVPWd, n=6 per group).
K, Lung wet/dry weight ratio (W/D, n=6 per group).
L, Heart weight (HW) normalized to tibia length (TL, n=6 per group).
M-N, Representative images of hematoxylin-eosin (H&E) staining, Sirius Red staining, along with quantification of cardiomyocyte cross-sectional area (scale bar=10 μm, n=6 per group, 3 random areas per heart), fibrotic area (scale bar=10 μm, n=6 per group, 3 random areas per heart). Data points represent individual mice for in vivo. Data are shown as mean±SEM and analyzed using one-way ANOVA with Tukey's multiple comparisons test (E, exercise distance in F, G, I, LV mass in J, K, L, and fibrotic area in N) or Kruskal-Wallis test (D, exercise time in F, LVPWd in J, and cardiomyocyte cross-sectional area in N) or two-way ANOVA with Sidak's multiple comparisons test (A, B, and C). Exact P values are indicated in corresponding figures.



Supplementary Figure 11. Inhibition of sEV Biogenesis by GW4869 Ameliorates Cardiac Dysfunction in Female HFpEF Mice.

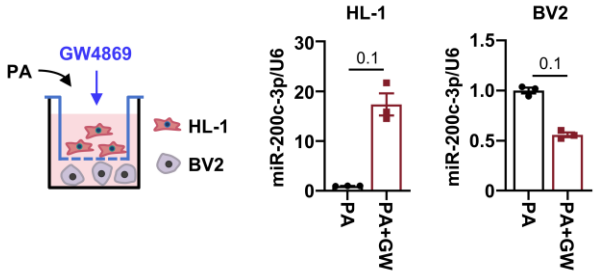
In the female cohort, GW4869 (2.5 mg/kg, intraperitoneally, every three days) or vehicle was administered for 8 weeks concurrently with the two-hit HFpEF protocol.

A, Body weight (n=7 per group).

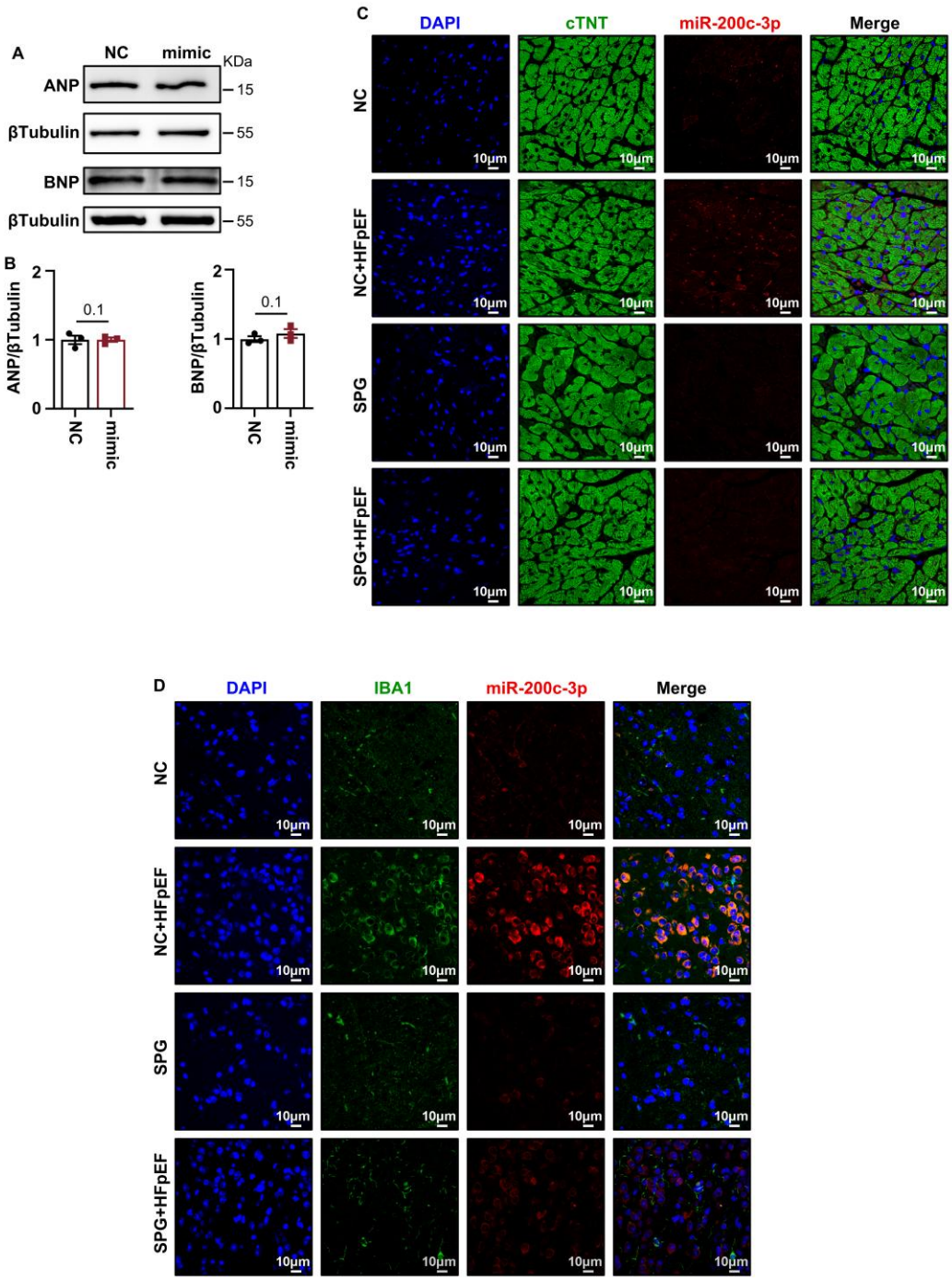
B, To minimize the influence of heart rate variability on cardiac functional parameters, heart rate was recorded and analyzed under isoflurane anesthesia in female mice (n=7 per group).

C-E, Representative echocardiographic images and statistical analysis of left

ventricular ejection fraction (EF) and fractional shortening (FS) in female mice (n=7 per group).
F-H, Representative echocardiographic images and statistical data of diastolic function E/A and E/e' in female mice (n=6-7 per group).
I, The calculated left ventricular mass at end-diastole (n=6 per group).
J, The calculated left ventricular posterior wall thickness at end-diastole (LVPWd) in female mice (n=6 per group).
Data points represent individual mice for in vivo. Data are shown as mean±SEM and analyzed using one-way ANOVA with Tukey's multiple comparisons test (E, G, H, and I), Kruskal-Wallis test (B, D, and J) or two-way ANOVA (A). Exact P values are indicated in corresponding figures.



Supplementary Figure 12. miR-200c-3p Expression Levels in HL-1 Cardiomyocytes and BV2 Microglia. The mRNA expression levels of miR-200c-3p in HL-1 and BV2 cells were measured by qPCR (n=3 per group). The relative mRNA levels were determined by comparing them to the average expression in the control group. Each data point represents one independent biological replicate, 2-3 technical replicates were performed per sample and averaged prior to analysis. Data are shown as mean±SEM and analyzed using Mann-Whitney U test.



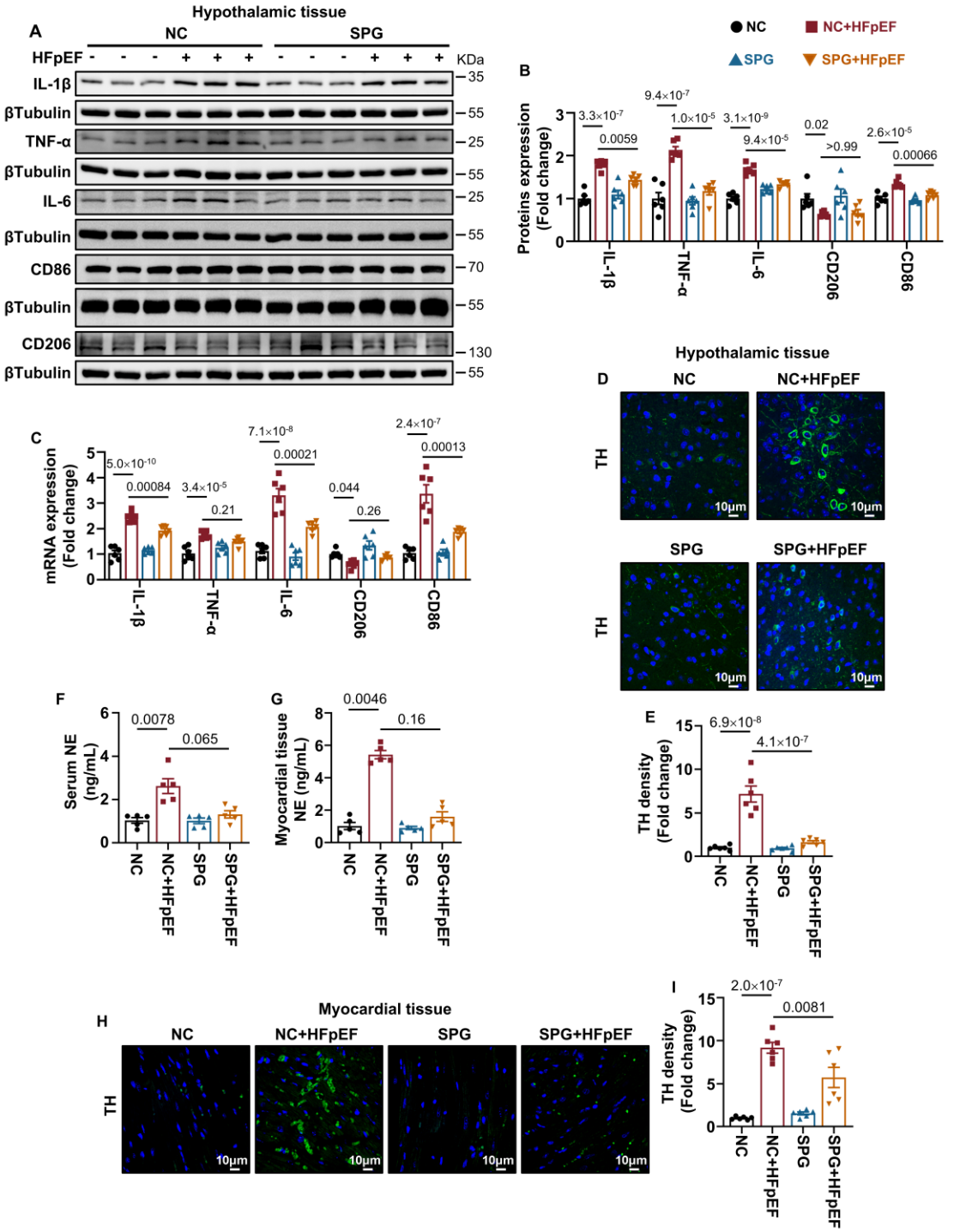
Supplementary Figure 13. miR-200c-3p Expression and Its Effects on Cardiomyocyte Hypertrophy Markers.

A-B, Western blot analysis and quantification of ANP and BNP in HL-1 cells transfected with NC or miR-200c-3p mimic (n=3 per group).

C, Representative fluorescence in situ hybridization (FISH) images of miR-200c-3p (red) combined with immunofluorescence staining for cardiac troponin T (cTnT, green) on paraffin-embedded myocardial tissue sections. Nuclei were counterstained with DAPI (blue). Scale bar=10 μm.

D, Representative FISH images of miR-200c-3p (red) combined with

immunofluorescence staining for IBA1 (green) on paraffin-embedded hypothalamic tissue sections. Nuclei were counterstained with DAPI (blue). Scale bar=10 μm.
The relative protein levels were determined by comparing them to the first sample in the control group. Each data point represents one independent biological replicate, 2-3 technical replicates were performed per sample and averaged prior to analysis. Data are shown as mean±SEM and analyzed using Mann-Whitney U test.



940

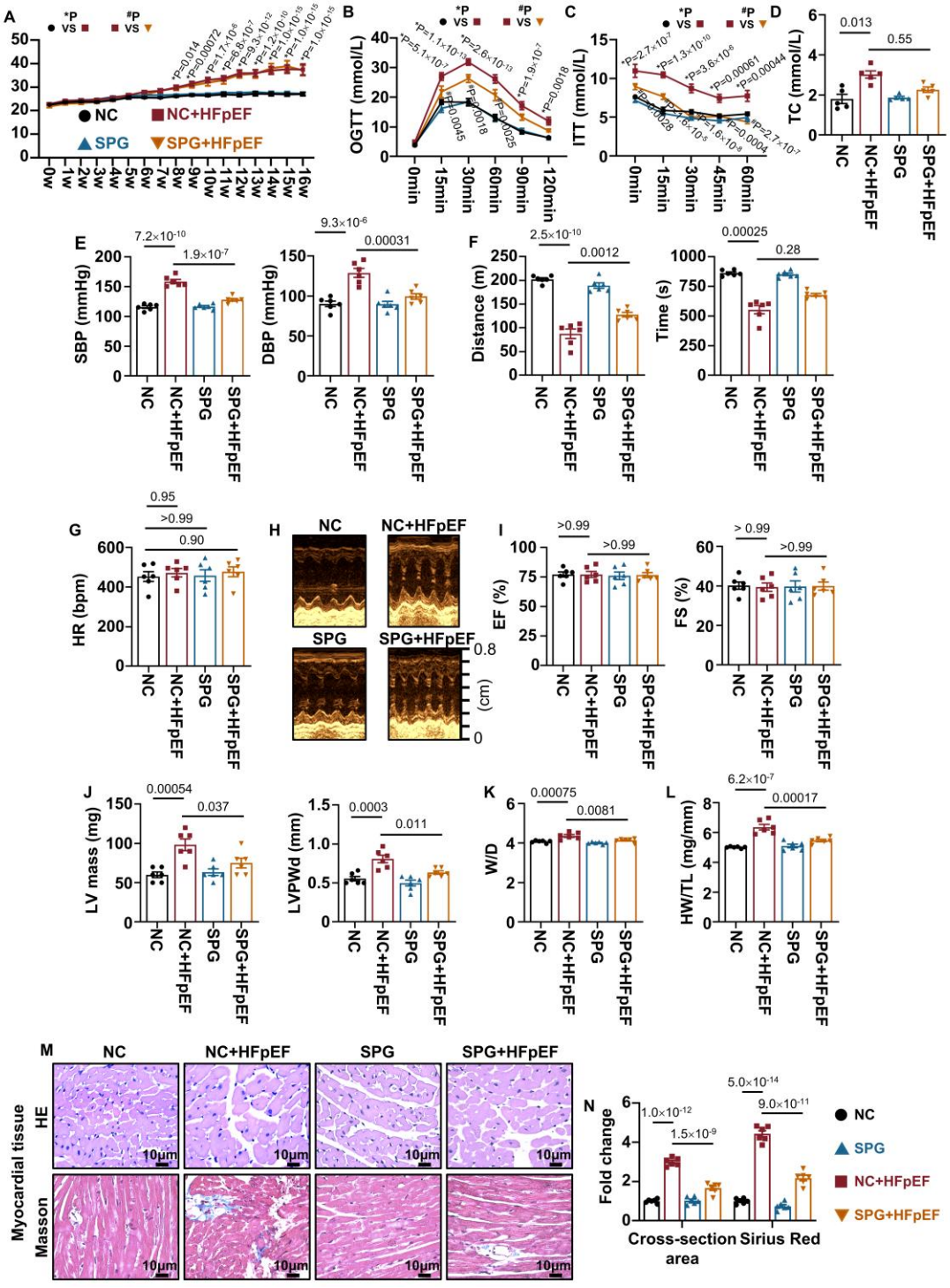
941 **Supplementary Figure 14. Cardiac-specific Inhibition of miR-200c-3p**
942 **Ameliorates Microglial Activation and Hypothalamic Inflammation in Male**
943 **HFpEF Mice.**

944 A-B, Western blot analysis and quantification of IL-1β, IL-6, TNF-α, CD86, and CD206
945 in hypothalamic tissue (n=6 per group).

946 C, The mRNA expression levels of IL-1β, IL-6, TNF-α, CD86, and CD206 in
947 hypothalamic tissue (n=6 per group).

948 D-E, Representative immunofluorescence images and quantitative analysis of tyrosine

hydroxylase (TH) in hypothalamic tissue (scale bar=10 μm, n=6 per group, 3 random areas per hypothalamus).
F, Quantitative data of serum norepinephrine (NE) level (n=5 per group).
G, Quantitative data of norepinephrine (NE) level in myocardial tissue (n=5 per group).
H-I, Representative immunofluorescence images and quantitative analysis of tyrosine hydroxylase (TH) in myocardial tissue (scale bar=10 μm, n=6 per group, 3 random areas per heart).
Data points represent individual mice for in vivo. For panels (F and G), 2-3 technical replicates were performed per sample and averaged prior to analysis. Data are shown as mean±SEM and analyzed using one-way ANOVA with Tukey's multiple comparisons test (for IL-1β, IL-6, TNF-α, CD86 in B, for all five cytokines in C, E, and I) or Kruskal-Wallis test (CD206 in B, F, and G). Exact P values are indicated in corresponding figures.



963

964 **Supplementary Figure 15. Cardiac-specific Inhibition of miR-200c-3p**
965 **Ameliorates Cardiac Dysfunction in Male HFpEF Mice.**

966 A, Body weight (n=6 per group).

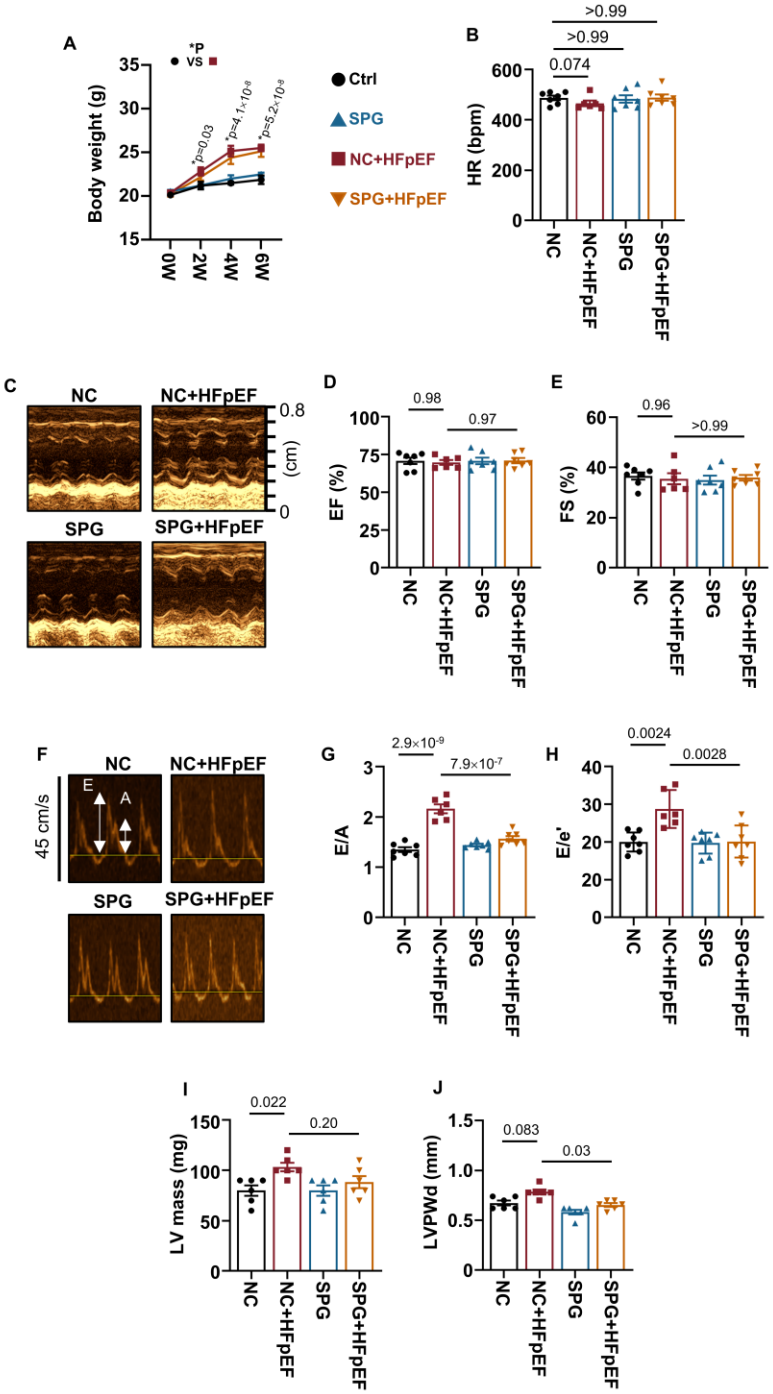
967 B, Oral glucose tolerance test (OGTT, n=6 per group).

968 C, Insulin tolerance test (ITT, n=6 per group).

969 D, Serum total cholesterol (TC) levels (n=5 per group).

970 E, Systolic blood pressure (SBP) and diastolic blood pressure (DBP) of mice (n=6 per
971 group).

F, Exercise time and distance in the mouse treadmill exhaustion test (n=6 per group).
G, To minimize the influence of heart rate variability on cardiac functional parameters, heart rate was recorded and analyzed under isoflurane anesthesia (n=6 per group).
H-I, Representative echocardiographic images and statistical analysis of left ventricular ejection fraction (EF) and fractional shortening (FS) (n=6 per group).
J, The calculated left ventricular mass and left ventricular posterior wall thickness at end-diastole (LVPWd, n=6 per group).
K, Lung wet/dry weight ratio (W/D, n=6 per group).
L, Heart weight (HW) normalized to tibia length (TL, n=6 per group).
M-N, Representative images of hematoxylin-eosin (H&E) staining, Sirius Red staining, along with quantification of cardiomyocyte cross-sectional area (scale bar=10 μm, n=6 per group, 3 random areas per heart), fibrotic area (scale bar=10 μm, n=6 per group, 3 random areas per heart).
Data points represent individual mice for in vivo. Data are shown as mean±SEM and analyzed using one-way ANOVA with Tukey's multiple comparisons test (E, exercise distance in F, G, I, J, K, L, and N) or as medians with interquartile ranges and analyzed using Kruskal-Wallis test and Dunn multiple comparisons test (D and exercise time in F) or repeated measures 2-way ANOVA and Sidak's multiple comparisons test (A, B, and C). Exact P values are indicated in corresponding figures.



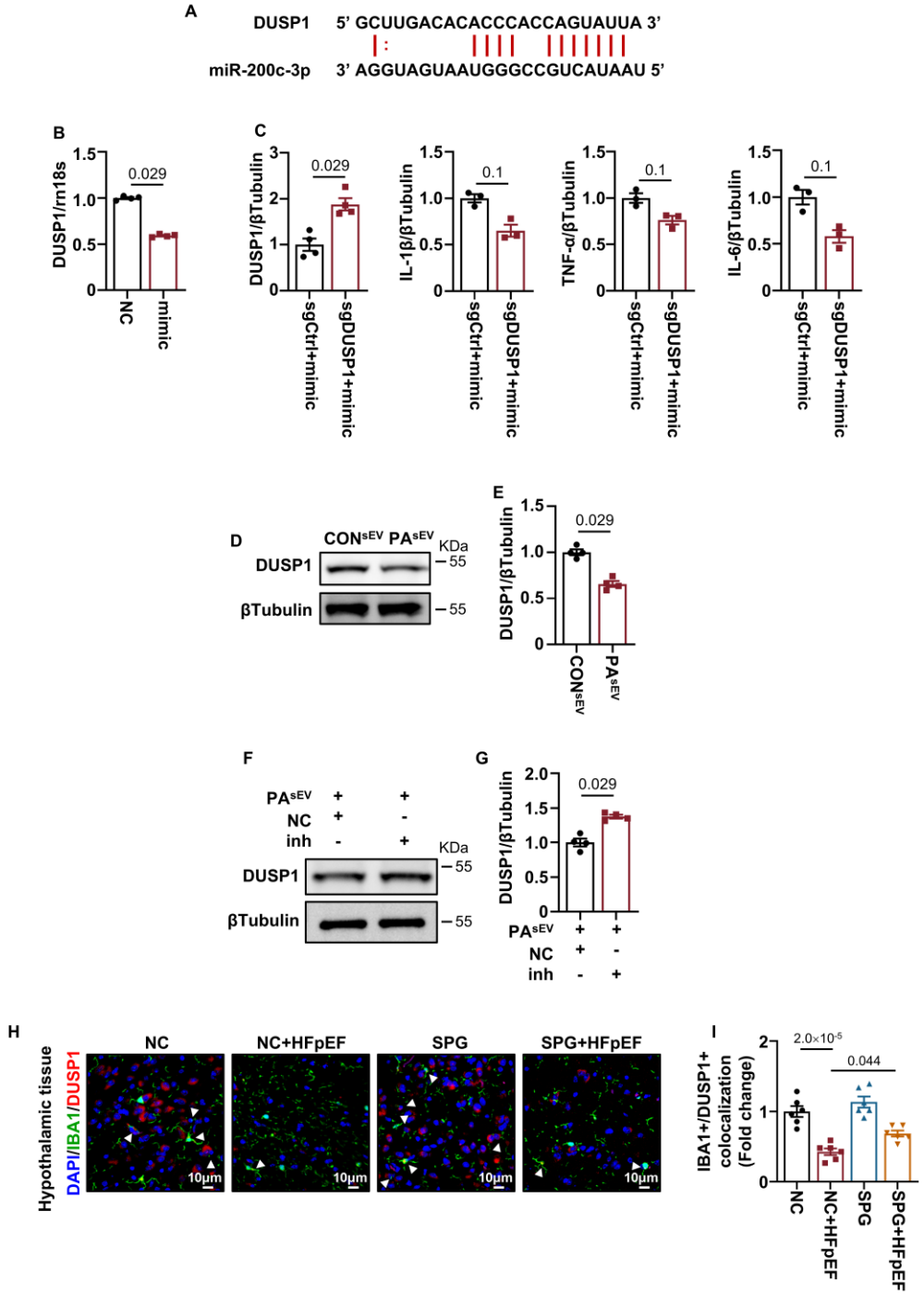
Supplementary Figure 16. Cardiac-specific Inhibition of miR-200c-3p Ameliorates Cardiac Dysfunction in Female HFpEF Mice.

For the female cohort, 9-week-old mice received tail vein injection of AAV9 constructs (miR-200c-3p sponge or NC sponge), followed by a 6-week two-hit HFpEF protocol initiated 2 weeks after injection.

A, Body weight (n=6-7 per group).

B, To minimize the influence of heart rate variability on cardiac functional parameters, heart rate was recorded and analyzed under isoflurane anesthesia in female mice (n=6-7 per group).

C-E, Representative echocardiographic images and statistical analysis of left ventricular ejection fraction (EF) and fractional shortening (FS) in female mice (n=6-7 per group).
F-H, Representative echocardiographic images and statistical data of diastolic function E/A and E/e' in female mice (n=6-7 per group).
I, The calculated left ventricular mass at end-diastole (n=6 per group).
J, The calculated left ventricular posterior wall thickness at end-diastole (LVPWd) in female mice (n=6 per group).
Data points represent individual mice for in vivo. Data are shown as mean±SEM and analyzed using one-way ANOVA with Tukey's multiple comparisons test (D, E, G, H, and I), Kruskal-Wallis test (B and J) or two-way ANOVA (A). Exact P values are indicated in corresponding figures.



Supplementary Figure 17. DUSP1 is a Downstream Target of miR-200c-3p.

A, Predicted binding site of the miR-200c-3p in the DUSP1 3'-UTR.

B, The mRNA level of DUSP1 in BV2 cells transfected with miR-200c-3p mimic or NC (n=4 per group).

C, Quantitative analysis of proteins DUSP1, IL-1β, TNF-α, and IL-6 by Western blot. Mutating the miR-200c-3p binding site in the DUSP1 3'-UTR used the CRISPR/Cas9 gene editing system (sgDUSP1, n=3 per group).

D-E, Western blot analysis and quantification of DUSP1 protein level in BV2 cells after stimulation with CON^{sEV} or PA^{sEV} for 24 hours (n=4 per group).

F-G, BV2 cells were co-transfected with miR-200c-3p inhibitor or NC and then stimulated with PA-sEVs for 24 hours. Western blot analysis and quantification of DUSP1 protein levels (n=4 per group).

H-I, Representative immunofluorescence images and quantitative analysis of IBA1 respectively colocalized with DUSP1 in hypothalamic tissue (scale bar=10 μm, n=6 per group, 3 random areas per hypothalamus).

The relative protein levels were determined by comparing them to the first sample in the control group. The relative mRNA levels were determined by comparing them to the average expression in the control group. Data points represent individual mice for in vivo (I) or independent experiments for in vitro assays (B, C, D, and F). For panels (B, E, and G), 2-3 technical replicates were performed per sample and averaged prior to analysis. Data are shown as mean±SEM and analyzed using Mann-Whitney U test (B, C, E, and G) or one-way ANOVA with Tukey's multiple comparisons test (I). Exact P values are indicated in corresponding figures.

Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Mouse Models Used in This Study

Strain	Vendor or Source	Background Strain	Sex	Other Information (e.g. breeding scheme for F1, F2…if applicable)	Persistent ID / URL
C57BL/6JGpt	GemPharmatech (Jiangsu, China)	C57BL/6JGpt	M	Wild-type control mice used for experiments	N000013
C57BL/6JNifdc	Vital River (Wuhan, China)	C57BL/6JNifdc	F	Wild-type control mice used for experiments	219

(Add additional rows as needed)

Additional Species Used in This Study (if applicable)

Strain	Vendor or Source	Background Strain	Sex	Other Information (e.g. breeding scheme for F1, F2…if applicable)	Persistent ID / URL
N/A					

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)	Persistent ID / URL
BNP	ABclonal	#A23996	1:1000 dilution	-	RRID: AB_3712732
ANP	ABclonal	#A1609	1:1000 dilution	-	RRID: AB_2763531
Collagen I	Proteintech	#14695-1-AP	1:1000 dilution	-	RRID: AB_2082037
Collagen III	HUABIO	#HA720050	1:1000 dilution	-	RRID: AB_3071990
Nos2	Proteintech	#22226-1-AP	1:1000 dilution	-	RRID: AB_2879038
p-IRE1α	HUABIO	#HA721980	1:1000 dilution	-	RRID: AB_3096844
IRE1α	HUABIO	#HA723225	1:1000 dilution	-	RRID: AB_3683560
IL-6	ABclonal	#A27935	1:1000 dilution for WB or 1:200 for IF	-	RRID: AB_3751225
IL-1β	HUABIO	#ET1701-39	1:1000 dilution for WB or 1:100 for IF	-	RRID: AB_3070207
TNF-α	HUABIO	#ER65189	1:1000 dilution for WB or 1:600 for IF	-	RRID: AB_3720451
CD86	HUABIO	#ET1606-50	1:5000 dilution for WB or 1:200 for IF	-	RRID: AB_3069745
CD206	HUABIO	#ET1702-04	1:1000 dilution	-	RRID: AB_2893392
DUSP1	HUABIO	#ET1701-82	1:1000 dilution	-	RRID: AB_3070247

DOI [to be added]

βTubulin	ABclonal	#A12289	1:10000 dilution	-	RRID: AB_2861647
αTubulin	ABclonal	#A6830	1:5000 dilution	-	RRID: AB_2863541
goat anti-rabbit IgG secondary antibody	ABclonal	#AS014	1:5000 dilution	-	RRID: AB_2769854
goat anti-mouse IgG secondary antibody	ABclonal	#AS003	1:5000 dilution	-	RRID: AB_2769851
tyrosine hydroxylase	HUABIO	#ET1611-12	1:200 dilution for IF or 1:2000 for IHC	-	RRID: AB_3069989
synaptophysin	HUABIO	#ET1606-56	1:200 dilution for IF or 1:4000 for IHC	-	RRID: AB_3069749
IBA1	ABclonal	Cat#A19776	1:1000 dilution	-	RRID: AB_3711469
FosB	HUABIO	#ET1611-75	1:400 dilution	-	RRID: AB_3070055
F4/80	Cell Signaling Technology	#70076	1:500 dilution	-	RRID:AB_2799771

DNA/cDNA Clones

Clone Name	Sequence	Source / Repository	Persistent ID / URL
cDNA3.1-3Flag-P2A-GFP-mDusp1	NM_013642.3	WZBio	CM800949

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
HL-1	National Collection of Authenticated Cell Cultures (Cat# GNM46)	F	CSTR:19375.09.3101MOUGNM46
BV2	China Center for Type Culture Collection (CCTCC, Cat# GDC0311)	F	RRID:CVCL_0182
HEK293T	ATCC (Cat# CRL-3216)	F	RRID:CVCL_0063

Data & Code Availability

Description	Source / Repository	Persistent ID / URL
microRNA sequencing data of small extracellular vesicles (sEVs) derived from mouse heart tissue	NCBI Gene Expression Omnibus (GEO)	GSE334160

Other

Description	Source / Repository	Persistent ID / URL

DOI [to be added]

ARRIVE GUIDELINES

The ARRIVE guidelines (https://arriveguidelines.org/) are a checklist of recommendations to improve the reporting of research involving animals. Key elements of the study design should be included below to better enable readers to scrutinize the research adequately, evaluate its methodological rigor, and reproduce the methods or findings.

Study Design

Groups	Sex	Age	Number (prior to experiment)	Number (after termination)	Littermates (Yes/No)	Other description
Group 1 (Control)	Male	8-9 weeks	37	37	Yes	
Group 1 (HFpEF)	Male	8-9 weeks	42	42	Yes	
Group 2 (Control)	Male	8-9 weeks	6	6	Yes	
Group 2 (PLX3397)	Male	8-9 weeks	6	6	Yes	
Group 2 (HFpEF)	Male	8-9 weeks	6	6	Yes	
Group 2 (HFpEF+PLX3397)	Male	8-9 weeks	6	6	Yes	
Group 3 (Control)	Female	9-10 weeks	7	7	Yes	
Group 3 (PLX3397)	Female	9-10 weeks	7	7	Yes	
Group 3 (HFpEF)	Female	9-10 weeks	7	7	Yes	
Group 3 (HFpEF+PLX3397)	Female	9-10 weeks	6	6	Yes	
Group 4 (Control)	Male	8-9 weeks	6	6	Yes	
Group 4 (GW4869)	Male	8-9 weeks	6	6	Yes	
Group 4 (HFpEF)	Male	8-9 weeks	6	6	Yes	
Group 4 (HFpEF+GW4869)	Male	8-9 weeks	6	6	Yes	
Group 5 (Control)	Female	9-10 weeks	7	7	Yes	
Group 5 (GW4869)	Female	9-10 weeks	7	7	Yes	
Group 5 (HFpEF)	Female	9-10 weeks	7	7	Yes	
Group 5 (HFpEF+GW4869)	Female	9-10 weeks	7	7	Yes	
Group 6 $Ctrl^{sEV}$	Male	8-9 weeks	6	6	Yes	
Group 6 $HFpEF^{sEV}$	Male	8-9 weeks	6	6	Yes	
Group 7 NC	Male	8-9 weeks	6	6	Yes	
Group 7 NC+HFpEF	Male	8-9 weeks	6	6	Yes	

DOI [to be added]

Group 7 Sponge	Male	8-9 weeks	6	6	Yes	
Group 7 Sponge+HFpEF	Male	8-9 weeks	6	6	Yes	
Group 8 NC	Female	9-10 weeks	7	7	Yes	
Group 8 NC+HFpEF	Female	9-10 weeks	7	7	Yes	
Group 8 Sponge	Female	9-10 weeks	7	7	Yes	
Group 8 Sponge+HFpEF	Female	9-10 weeks	7	7	Yes	

Sample Size: Please explain how the sample size was decided Please provide details of any a *prior* sample size calculation, if done.
Sample sizes were determined based on preliminary experiments and our experience with similar mouse models, aiming to ensure sufficient statistical power and reproducibility. No statistical methods were used to predetermine sample sizes.

Inclusion Criteria
All animals that survived to the predetermined endpoint were included in the final analysis.

Exclusion Criteria
Mice that died prematurely before reaching the terminal time point were excluded.

Randomization
Simple randomization was used for group allocation. Baseline body weights were compared across groups to rule out any systematic imbalance at the start of the experiment.

Blinding
A double-blind approach was used. Caretakers and the investigator performing tissue collection were both blinded to group allocation until final analyses.

DOI [to be added]