SUPPLEMENTAL MATERIAL

PTP1B Inhibition Improves Mitochondrial Dynamics to Alleviate Calcific Aortic Valve Disease via Regulating OPA1 Homeostasis

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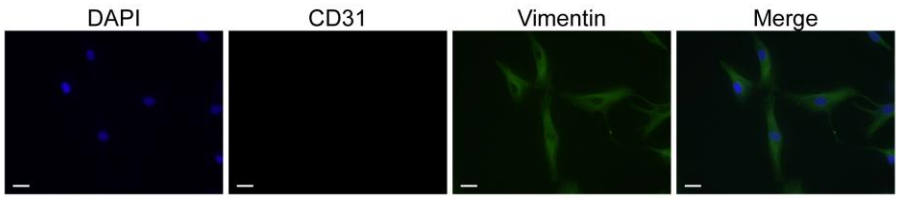
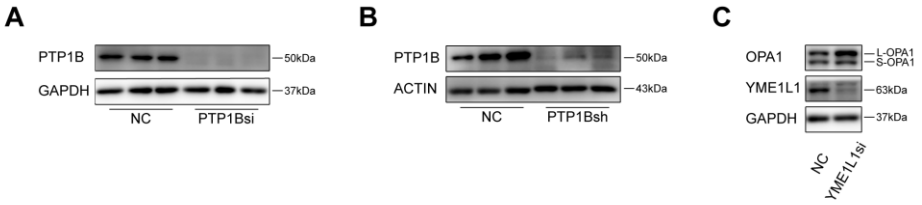
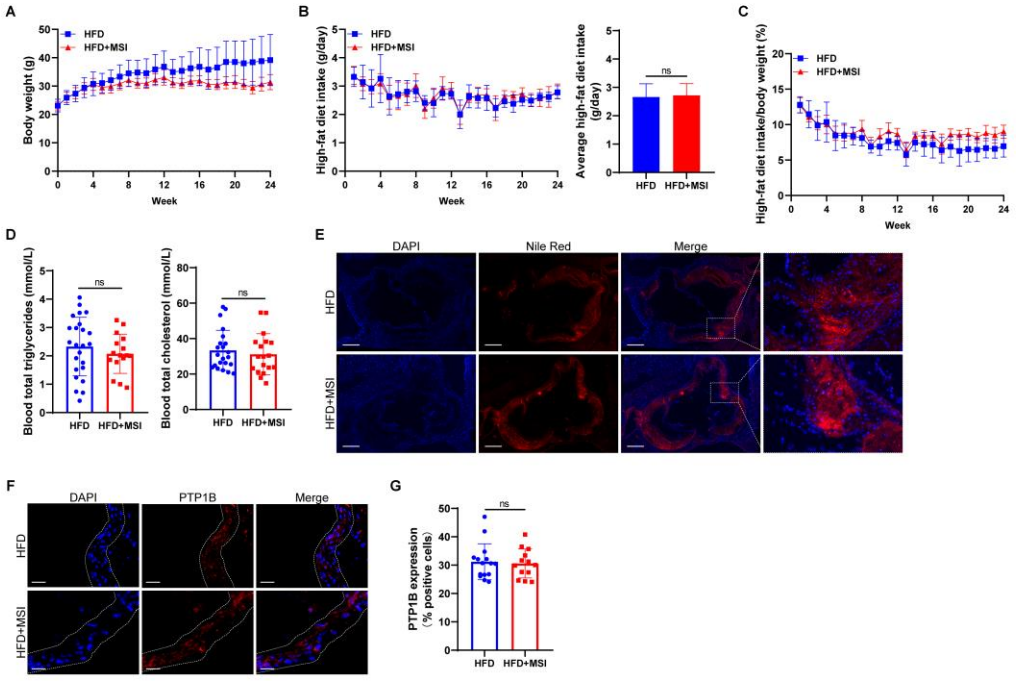
Supplemental Figures and Figure Legends



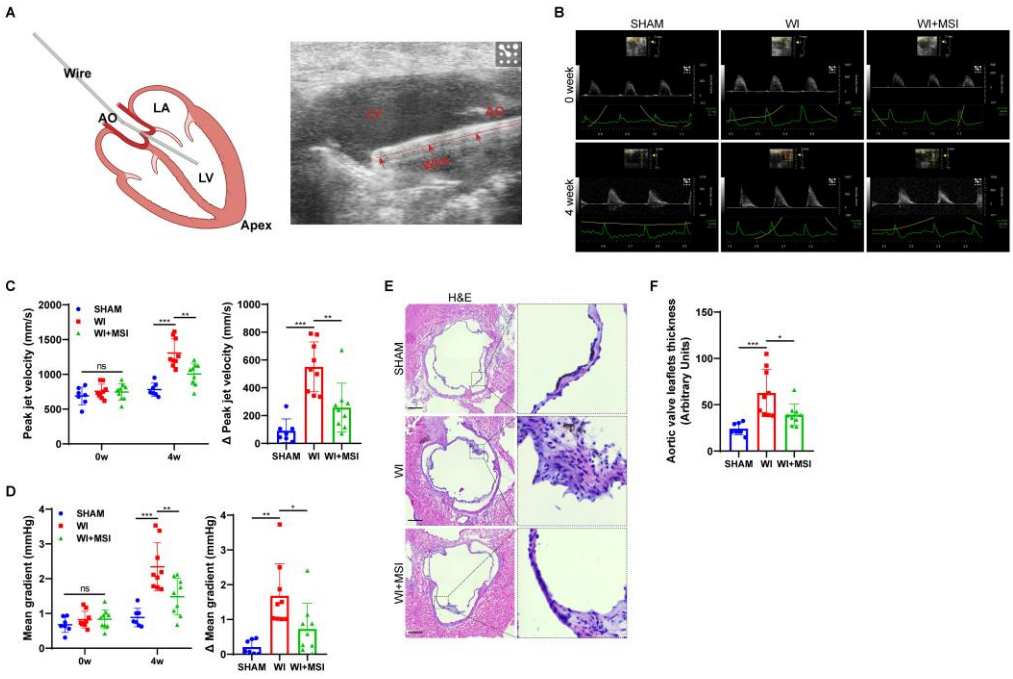
Supplemental Figure 1 Characterization of Isolated Valvular Interstitial Cells Immunofluorescence staining images of interstitial cell marker (Vimentin) and endothelial cell marker (CD31) in valvular interstitial cells isolated from human aortic valve leaflet. Scale bar: 20 μm.



Supplemental Figure 2 Identification of PTP1B siRNA, PTP1B shRNA and YME1L siRNA (A and B) The protein level of PTP1B in VICs transfected with PTP1B siRNA (PTP1Bsi; A; n = 3) and PTP1B shRNA (PTP1Bsh; B; n = 3) treatment. (C) The protein level of OPA1 and YME1L1 in VICs transfected with YME1L1 siRNA (YME1L1si; n = 1).

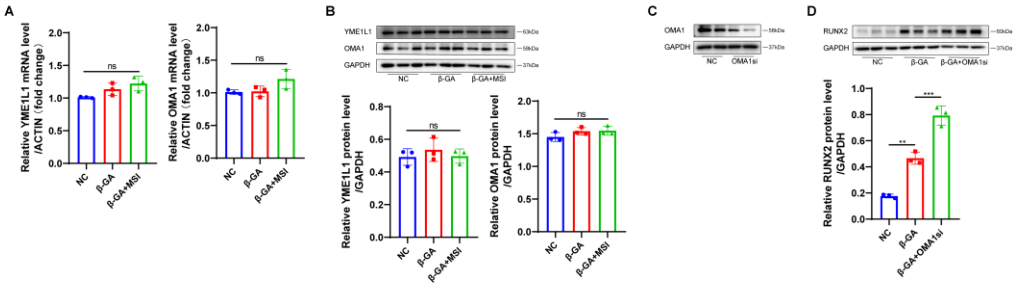


Supplemental Figure 3 Metabolic indices of LDLR$^{-/-}$ mice fed a high-fat diet (A) Body weight of HFD-fed mice treated with saline with MSI-1436 (MSI, 10 mg/kg, intraperitoneally, monthly) or DMSO during the course of the experiment. (B) Daily high-fat diet intake was not significantly different in HFD-fed mice after MSI-1436 treatment. (C) Ratio between high-fat diet intake and body weight in HFD-fed mice treated with MSI-1436 or DMSO during the course of the experiment. (D) Blood total triglycerides and total cholesterol were not significantly different between HFD-fed mice treated with MSI-1436 and DMSO at week 24. (E) Representative immunofluorescence staining images of Nile Red in aortic valves from HFD-fed mice treated with MSI-1436 or DMSO. Scale bar: 30 μm. (F) Representative immunofluorescence staining images of PTP1B in aortic valves from HFD-fed mice treated with MSI-1436 or DMSO. Scale bar: 15 μm. (G) Quantitative data showed no significant difference in the HFD group (n = 15) versus the HFD+MSI group (n = 13). Values are mean ± SD. The unpaired t-test was performed. ns: not significant.



Supplemental Figure 4 PTP1B inhibition alleviated the aortic valve stenosis in wire injury mice model (A) Schematic representation and exemplary parasternal long-axis views of wire injury induced aortic valve stenosis mice model. AO: aortic; LA: left atrium; LV: left ventricle. (B) Representative echocardiographic images of wire injury mice at week 0 and week 4. Male C57BL/6 mice were divided into: 1) SHAM, saline with DMSO; 2) wire injury (WI), saline with DMSO; 3) wire injury, saline with MSI-1436 (MSI, 10 mg/kg, intraperitoneally). (C and D) Continuous-wave Doppler assessment of the aortic valve function indicated that mice in the WI group (n = 9) showed a significant increase in transvalvular peak jet velocity (C) and mean gradient (D) compared with that of the SHAM group (n = 7) at week 4, whereas MSI-1436 treatment (n = 9) reversed these changes. (E) Representative H&E staining images of aortic valves in wire injury mice from different groups. Scale bar: 150 μm. (F) Quantitative analysis of aortic valve leaflet thickness was assessed by ImageJ (n= 7, 9, 9). Values are mean ± SD. The ANOVA with Tukey's post-hoc test was performed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$,

ns: not significant.



Supplemental Figure 5 Knockdown of OMA1 promoted RUNX2 expression in β-GA-induced osteogenic differentiation of VICs (A and B) Expression of YME1L1 and OMA1 at the mRNA (A) and protein (B) levels was assessed in β-GA-treated VICs under MSI-1436 or DMSO treatment (n = 3). (C) The protein level of OMA1 in VICs transfected with OMA1 siRNA (OMA1si; n = 2). (D) The relative RUNX2 protein level was increased in VICs transfected with OMA1si under β-GA medium treatment (n = 3). Values are mean ± SD. The ANOVA with Tukey's post-hoc test was performed. $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant.

Supplemental Tables

Supplemental Table 1 Sequence for siRNA and shRNA

	Sequence (5' to 3')
PTP1B siRNA	Sense: GACCCUUCUUCCGUUGAUATT
	Anti-sense: UAUCAACGGAAGAAGGGUCTT
YME1L1 siRNA	Sense: CAUGCAAAGGAGCAUAAGATT
	Anti-sense: UCUUAUGCUCCUUUGCAUGTT
OMA1 siRNA	Sense: GGAAGUAAGUCCAAUCACATT
	Anti-sense: UGUGAUUGGACUUACUUCCTT

PTP1B shRNA	Sense: CCGGGCTGCTCTGCTATATGCCTTACT
	CGAGTAAGGCATATAGCAGAGCAGCTTTTTG
	Anti-sense: AATTCAAAAAGCTGCTCTGCTATATG
	CCTTACTCGAGTAAGGCATATAGCAGAGCAGC

Supplemental Table 2 Primer Sequence for qRT-PCR

Gene	Primer sequence (5' to 3')
SDHB	Forward: ACAGCTCCCCGTATCAAGAAA
	Reverse: GCATGATCTTCGGAAGGTCAA
ATP5A	Forward: CCCTCCTTGGTCAAGAGCAC
	Reverse: GTATCTCCGGTCGTTTCAGCA
NDUFB1	Forward: GTCCCTATGGGATTTGTCATTGG
	Reverse: CAGTTAGCCGTTCATCACTCTT
MTCO1	Forward: TGTGTTGATGCACTACCCCC
	Reverse: TTGTGCTCACGTAGCCAGAG
UQCR10	Forward: ATCGTGGGCGTCATGTTCTTC
	Reverse: ATGTGGTCGTAGATAGCGTCC
YME1L1	Forward: CCAGCAGTGAGCCTTCACTTA
	Reverse: ACCCCGAGACTGTATGAAAACAT
OMA1	Forward: AAATTGGAGGCCGAAGCTGA
	Reverse: TCGAGGGTCTGGATTAGACAGT
ACTIN	Forward: CATGTACGTTGCTATCCAGGC

Reverse: CTCCTTAATGTCACGCACGAT

Supplemental Methods

Human Aortic Valve Collection

All of samples collected from patients were adhered to the Declaration of Helsinki and were approved by the Ethics Committee of The Second Affiliated Hospital, School of Medicine, Zhejiang University (No. 2014-159). Informed consent was acquired from every patient before surgery. Calcified aortic valve was acquired from patient diagnosed with CAVD who underwent surgical replacement of aortic valve and non-calcified valve, exhibited no thickening or calcium deposition, was acquired from patients with heart transplantation and with aortic valve regurgitation who underwent surgical aortic valve replacement. Exclusion criteria included rheumatic valvular disease, infective endocarditis and congenital valvular disease. After the aortic valve leaflets were extracted, they were placed in phosphate buffered saline (PBS) at 4 ℃. Afterwards, the leaflets were divided into RIPA lysis buffer (50mM Tris (pH 7.4), 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA and leupeptin; P0013B, Beyotime, CHN) for protein extraction, optimum cutting temperature compound (OCT; BDH Laboratory Supplies, Safat, Kuwait) for immunofluorescence and collagenase buffer for primary VICs isolation.

Cell Culture and Treatment

Primary human VICs were isolated from aortic valve leaflets via collagenase II as described previously(1). Briefly, valve leaflets were incubated in medium with 1 mg/ml collagenase II (Sigma-Aldrich, St. Louis, MO, USA) at 37 ℃ for 30 minutes to eliminate valvular endothelial cells. After centrifugation, the left valve tissues were subsequently digested in collagenase II medium for another 4-6 hours. Finally, isolated interstitial cells were seeded and then cultured in Dulbecco's modified Eagle medium (DMEM, Thermo Fisher, Waltham, MA, USA) with 4.5 g/L glucose, 10% fetal bovine serum (FBS, Thermo Fisher), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich). The isolated cells were stained with interstitial cell marker (Vimentin) and endothelial cell marker (CD31) for VIC characterization. VICs of passage 3-7 were used for subsequent experiments. To induce osteogenic differentiation of VICs, 10 mmol/L β-glycerophosphate acid (β-GA), 10 nmol/L dexamethasone and 0.25 mmol/L ascorbic acid (β-GA system) or 2 mmol/L Na_2HPO_4 (all purchased from Sigma-Aldrich) were added to complete medium for 14 days. To induce myofibrogenic differentiation, VICs were incubated in medium with 10 ng/ml recombinant human TGFβ (240-B, R&D Systems, Minneapolis, MN, USA) for 7 days. The PTP1B specific inhibitor, MSI-1436 lactate (MedChemExpress, NJ, USA), was dissolved in DMSO and added to the medium at a concentration of 20 μmol/L according to the results of preliminary experiment.

Cell Transfection

Small interfering RNA (siRNA) targeting human PTP1B (PTP1Bsi), YME1 like 1 ATPase (YME1L1si), OMA1 and control vector were purchased from Tsingke (Hangzhou, CHN). VICs were transfected with siRNA using RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The sequences of PTP1Bsi and YMEL1Lsi were listed in Supplemental Table 1. Recombinant lentivirus expressing short hairpin RNA (shRNA) of PTP1B (PTP1Bsh), human OPA1 variant 1 (OPA1v1), OPA1 variant 7 (OPA1v7), OPA1 variant 8 (OPA1v8), YME1L1 and control empty vector were provided by

GeneChem (Shanghai, CHN). The VICs were infected with lentivirus at multiplicity of infection = 30 in complete medium containing 8 μg/mL polybrene (GeneChem) overnight. After replacement of viral suspension to fresh medium, cells were cultured for another 24 hours before subsequent experiments. The verification of transfection was tested by western blot analysis of the target protein.

Immunofluorescence Staining

Frozen aortic valve sections were fixed in 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton X-100 for another 10 minutes and blocked with 5% bovine serum albumin (BSA, AMRESCO, Solon, OH, USA) for 30 minutes. Donkey anti-rabbit IgG H&L (DyLight 550) (1:400 dilution, ab96892, Abcam, Cambridge, UK) and goat anti-rat IgG H&L (Alexa Fluor® 488) (1:400 dilution, ab150165, Abcam) was used after the overnight incubation of the lesions with the following primary antibodies: PTP1B (1:100 dilution, ab244207, Abcam), PTP1B (1:100 dilution, ab75856, Abcam), RUNX2 (1:300 dilution, ab192256, Abcam), BMP2 (1:200 dilution, ab214821, Abcam), CD68 (1:200 dilution, ab53444, Abcam), TOMM20 (1:200 dilution, ab186735, Abcam), CD31 (1:300 dilution, ab76533, Abcam) and Vimentin (1:300 dilution, ab92547, Abcam). DAPI (H-1200-10, Vector Laboratories, USA) was then applied to stain the nuclei. A Leica fluorescence microscope (Leica, Germany) was used for imaging. The images were quantified using ImageJ software (NIH, Bethesda, MD, USA).

Western Blot

Protein lysate concentration was determined using BCA protein assay kit (Thermo Fisher) according to the instructions. In brief, equivalent amounts of proteins from all samples were separated by SDS-PAGE and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk powder (AMRESCO) for 1 hour and subsequently incubated with specific antibodies overnight at 4 °C. The following antibodies were used: HRP-conjugated monoclonal mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000 dilution, KC-5G5, KangChen Biotech, Shanghai, CHN), HRP-conjugated monoclonal mouse anti-beta Actin (ACTIN, 1:10000 dilution, KC-5G5, KangChen Biotech), RUNX2 (1:1000 dilution, 8486s, Cell Signaling Technology, Danvers, MA, USA), ALP (1:1000 dilution, MAB1448, R&D systems), PTP1B (1:1000 dilution, ab75856, Abcam), Fibronectin (1:1000 dilution, ab268020, Abcam), Collagen1a (1:1000 dilution, ab260043, Abcam), αSMA (1:10000 dilution, ab124964, Abcam), p-Smad2 (Ser465/Ser467) (1:1000 dilution, ab18338, Cell Signaling Technology), Smad2 (1:1000 dilution, ab5339, Abcam), DRP1 (1:1000 dilution, ab184247, Abcam), OPA1 (1:1000 dilution, 612607, BD Biosciences, Franklin Lakes, NJ, USA), YME1L1 (1:500 dilution, ab170123, Abcam), OMA1 (1:1000 dilution, 67449-1-Ig, Proteintech, Chicago, IL, USA) and VDAC1 (1:1000 dilution, ab14734, Abcam). After incubation with the corresponding secondary antibodies: HRP-conjugated goat anti-rabbit IgG (1:3000 dilution, 7074s, Cell Signaling Technology), HRP-conjugated goat anti-mouse IgG (1:3000 dilution, 7076s, Cell Signaling Technology) and HRP-conjugated mouse Anti-rabbit IgG (Light-Chain Specific, 1:3000 dilution, 93702, Cell Signaling Technology), the protein blots were visualized using the chemiluminescence (Bio-Rad, Hercules, CA, USA). The quantification of the labeled bands was performed via Image Lab software (Bio-Rad). The relative protein levels detected based on western blot were normalized to GAPDH or ACTIN level.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (TaKaRa, Kyoto, Japan) following the manufacturer's

instructions. Subsequently, cDNA synthesis was performed using the PrimeScript RT Reagent Kit (TaKaRa) following the manufacturer's instructions. The qRT-PCR assays were set up using the SYBR Premix Ex TaqTM Kit (TaKaRa) for quantitative analysis of mRNA on Applied Biosystems 7500 Fast Real-Time PCR System (ABI, Torrance, CA). Analysis of relative gene expression data was carried out using the ΔΔCT method by normalization to ACTIN level. All the primers used were listed in Supplemental Table 2.

Alizarin Red Staining and Von Kossa Staining

For alizarin red staining, after incubation with the osteogenic medium for 14 days, VICs were fixed and then stained with 2% alizarin red solution (Sigma-Aldrich) for 10 minutes. In order to quantify the calcium deposition, staining was resolved in 10% acetic acid and the solution was read at 405 nm via a spectrophotometer.

Von Kossa staining was performed to assess the mineralization in aortic valve leaflets described previously(2). Briefly, the 4% paraformaldehyde fixed aortic valve sections were incubated with 5% silver nitrate solution for 60 minutes with exposure to ultraviolet light, and then treated with 5% sodium thiosulfate solution for 2 minutes. After staining with nuclear fast red solution for another 5 minutes, the calcium deposition in the aortic valve leaflets was observed by a light microscope. Good-quality valve leaflet sections of each group were chosen, and the fractional areas of calcium nodule components (black and brown) in the aortic valve region were measured using ImageJ (NIH). Exclusion criteria included the dense, fusiform dark black mass on the leaflet surface (melanocyte).

Measurement of ALP Activity and ATP Production

The ALP activity assay was conducted in VICs using ALP activity assay kit (P0321, Beyotime). In brief, VICs were harvested and lysed in RIPA lysis buffer. p-Nitrophenyl phosphate (pNPP) was added as the substrate and incubated for 15 minutes at 37 °C, and ALP activity was measured in absorbance at 405 nm. The results were presented as ALP activity normalized to the total protein level.

Total cellular ATP content of VICs was determined by using the luminescence ATP assay kit (S0026, Beyotime) in accordance with the manufacturer's instructions and normalized to the total protein level.

Animal Experiments

All animal experimental protocols complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animal studies were approved by the Institutional Animal Research Committee of Zhejiang University (No. 2019-177). 6-8 weeks old male LDLR$^{-/-}$ mice (C57BL/6 background) were purchased from GemPharmatech Co. Ltd (Nanjing, CHN) and housed in a pathogen-free, temperature-controlled environment (20±2 °C, 12-hours light/dark cycle) with free access to water and diet as described previously(1). The mice were randomly allocated to 3 groups and followed for 24 weeks: 1) mice fed a normal chow (NC group, n = 20); 2) mice fed a high fat-cholesterol diet (HFD group, n = 30, cat. #88137; Harlan Laboratories, Ferndale, WA, USA); 3) mice fed a HFD and treated with MSI-1436 (HFD+MSI group, n = 30). The saline with PTP1B inhibitor MSI-1436 lactate (10 mg/kg) or DMSO was injected intraperitoneally monthly as described previously(3). At the end of the protocol, echocardiographic parameters were determined by transthoracic echocardiography using the Vevo 2100 imaging system (VisualSonics, Toronto, AB, Canada) under 2.5% isoflurane anesthesia. All echocardiographic images and data were collected by an experienced operator blinded to the assignments. After echocardiography,

mice were dissected under 2.5% isoflurane anesthesia and blood was collected for detection of blood glucose, total cholesterol and triglyceride. Mice were then perfused via the left ventricle with PBS prior to tissue collection. Heart with aortic root was carefully dissected and embedded in OCT.

Aortic Valve Wire Injury

Male C57BL/6 mice aged 8–10 weeks were operated according to the protocol as described previously(4). The mice were randomly allocated to 3 groups and followed for 4 weeks: 1) Sham (n = 7), Wire Injury (WI, n = 9), Wire Injury with MSI-1436 (WI+MSI, n = 9). The saline with PTP1B inhibitor MSI-1436 lactate (10 mg/kg) or DMSO was injected intraperitoneally at 1 day after operation. In brief, the mice were anesthetized by intraperitoneal injection of 4% chloral hydrate. Right carotid artery was exposed by blunt dissection and blood flow was stopped using ligature loops. A conventional guide wire with a 15° angled tip (ASAHI INTECC MIRACLEbros 3) was used. The wire was introduced into the left ventricle under echocardiographic guidance, passed over the aortic valve and advanced into the left ventricular apex and pulled back into the left ventricular outlet, just below the aortic valve level. Aortic valve leaflet scratching was performed by means of moving the wire in and out of the left ventricle 20 times, and rotating the wire 200 times when it was placed inside left ventricle. After valve injury, the wire was carefully removed and a permanent carotid ligation was performed to avoid hemorrhage. Finally, the skin wound was closed with sutures and mice were monitored until awakening. Sham surgery was performed in the same way without wire insertion into the left ventricle.

Nile Red Staining

Nile Red staining was performed to assess the lipid deposits in aortic valve leaflets. Briefly, the 4% paraformaldehyde fixed aortic valve sections were permeabilized with 0.1% Triton X-100 for another 10 minutes. Then, sections were incubated with 0.1% Nile Red solution for 15 minutes at 37 °C. DAPI was then applied to stain the nuclei. A Leica fluorescence microscope was used for imaging.

Echocardiography and Hemodynamic Measurement

Transthoracic echocardiography was performed in isoflurane anesthetized mice with a heart rate of ~400-550 beats/min using high-resolution in vivo ultrasound imaging system for small animals equipped with a 40-MHz transducer (Vevo 2100 imaging system). Aortic valve function was assessed using continuous-wave Doppler mode that measured aortic valve peak jet velocity and mean gradient. In brief, Doppler flow velocity spectrum of the aortic valve outflow tract of each mouse was recorded. A minimum of three cardiac cycles were traced. Images were taken from the upper right parasternal long axis view and the angle of the transducer was maintained at a proper degree. The cardiac package provided by VisualSonic was used to measure and calculate several parameters including the aortic jet velocity and the mean gradient.

Hematoxylin and Eosin (H&E) Staining and Masson's Trichrome Staining

H&E staining was performed to assess the thickness of aortic valve leaflets as described previously(5). Briefly, sections of aortic valve leaflets were stained with Harris' modified hematoxylin solution for 3 minutes. Then, the sections were washed with running tap water for 5-10 minutes, followed by counterstaining in eosin alcoholic solution for 0.5-1 minutes. Finally, the sections were dehydrated in increasing concentrations of ethyl alcohol and cleared in xylene for 2 minutes. The images were acquired by a light microscope and quantitative analysis of aortic valve leaflet thickness was performed as

described previously(5). Good-quality valve leaflet sections of each group were chosen, and leaflet thickness was measured using Image-J (NIH).

Masson's trichrome staining was performed to assess the fibrosis in aortic valve leaflets as described previously(6). Briefly, after fixing in acetone-methanol for 10 minutes, the sections of mouse aortic valve leaflets were immersed in a Weigert iron hematoxylin solution for 5 minutes. The slides were rinsed again in running tap water for 5 minutes. Then, the sections were incubated in 0.04% acid fuchsin and 0.02% azophloxine solution for 20 minutes and rinsed with 1% acetic acid solution. Finally, they were placed in a 2.5% phosphotungstic acid solution for 10 min and rinsed with 1% acetic acid solution. Good-quality valve leaflet sections of each group were chosen, and the fractional areas of collagen fibrosis components (blue) in the aortic valve region were measured using Image-J (NIH).

Reactive Oxygen Species (ROS) Measurement

VICs incubated with culture medium were stained with 10μM DCFH-DA (S0033S, Beyotime) for 20 minutes at 37 ℃. Thereafter, cells were collected after trypsinization and fluorescence was assessed by flow cytometry with a BD FACSCanto II Flow Cytometer (BD Biosciences, San Jose, CA).

Mitochondrial Mass and Mitochondrial Membrane Potential

Mitochondrial mass and quantity were analyzed by flow cytometry. Total mitochondrial content was assessed by using MitoTracker Deep Red (MitoTracker, Invitrogen) staining. VICs were incubated in culture medium with 100 nmol/L MitoTracker for 30 minutes at 37 ℃. For mitochondrial membrane potential measurement, VICs were treated with 100 nmol/L tetramethylrhodamine methyl ester (TMRM, Sigma-Aldrich) or 5 μg/ml JC-1 (C2005, Beyotime) for 30 minutes at 37 ℃. Thereafter, cells were collected after trypsinization and fluorescence was assessed by flow cytometry with a BD FACSCanto II Flow Cytometer (BD Biosciences).

Mitochondrial DNA (mtDNA) Mean Copy Number

Total DNA was extracted from VICs using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. qRT-PCR was used to measure the copy number of mtDNA, which was normalized to the copy number of nuclear DNA. The primers used were purchased from TaKaRa (Cat. #7246).

Transmission Electron Microscope

Transmission electron microscope (TEM) was applied to observe the mitochondrial ultrastructure in VICs as described previously(7). The images were obtained by using Hitachi Model H-7650 TEM to observe the mitochondrial morphology at × 8000 magnification.

Immunoprecipitation

VICs were harvested and lysed in immunoprecipitation (IP) lysis buffer (containing 50 mM Tris-HCl-pH 7.4, 150 mM NaCl, 0.1% NP-40 and protease inhibitors). In total, 10% cell lysates were extracted for input and the remaining supernatant was precipitated in protein A/G immunoprecipitation magnetic beads (Bimake, Houston, TX, USA) buffer containing anti-PTP1B antibody (1:50 dilution, ab244207, Abcam) or normal rabbit IgG (1:100 dilution, 2729, Cell Signaling Technology) at 4°C overnight by gentle agitation. Afterwards, agarose with protein-antibody immunocomplexes was washed, harvested and analyzed via western blot.

Isolation of Cytoplasmic and Mitochondrial Fraction

Isolation of cytoplasmic and mitochondrial fraction was conducted by using cell mitochondria isolation kit (C3601, Beyotime) following the manufacturer's protocol. In brief, VICs were collected by centrifuging cell suspension, and then mitochondria isolation reagent with phenylmethylsulfonyl fluoride was added into the cell pellets. The cell resuspension was homogenized by a glass homogenizer, centrifuged at 1000g for 10 minutes at 4 ℃. The supernatant was transferred to a new tube and centrifuged at 3500g for 10 minutes at 4 ℃. The supernatant was transferred to a new tube, and the pellet contained the isolated mitochondria. Finally, cytoplasmic fraction was derived from the supernatant centrifuged at 12000g for 10 minutes at 4 ℃. Purity of mitochondria was ensured by detecting the expression of ACTIN that was the loading control of cytoplasmic protein.

Statistical Analysis

Quantitative values were denoted as the mean ± standard deviation (SD) and represent data were obtained from at least three independent experimental replicates. The normality of distribution was determined using Shapiro-Wilk test or Kolmogorov-Smirnov test. Statistical differences were determined by two-tailed unpaired Student's t-test for two groups and one-way analysis of variance (ANOVA) for three or more groups followed by Tukey's post-hoc test for multiple pairwise comparisons. For skewed distributions, Kruskal-Wallis test was used to determine significance for multiple pairwise comparisons. Qualitative values were presented as counts with percentages and compared using the chi-square test. Statistical analyses were performed via Prism v8.0.1 and SPSS v26. A value of $p < 0.05$ was considered statistically significant.

References

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