

Sirt1 attenuates calcific aortic valve disease by inhibiting ferroptosis and enhancing mitochondrial function via the activation of the Nrf2/HO-1/FTH1 axis

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Abstract. Calcific aortic valve disease (CAVD) is a progressive disorder characterized by valvular calcification and currently lacks effective pharmacological therapies. Sirtuin 1 (Sirt1) has emerged as a cardioprotective factor; however, its role in CAVD remains unclear. The present study aimed to investigate whether Sirt1 attenuates CAVD by regulating iron homeostasis, ferroptosis and mitochondrial function through the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1)/ferritin heavy chain 1 (FTH1) signaling axis. Sirt1 expression was evaluated in human calcified and non-calcified aortic valves. Primary human aortic valve interstitial cells (hAVICs) were transfected with pcDNA3.1-Sirt1 and subjected to osteogenic induction with or without the Nrf2 inhibitor, ML385, or the ferroptosis inducer, erastin. Osteogenic differentiation, mitochondrial function, ferroptosis and Nrf2/HO-1/FTH1 signaling were analyzed. *In vivo*, apolipoprotein E-deficient (ApoE^{-/-}) mice fed a Western diet containing 21% fat and 0.15% cholesterol (w/w) were treated with the Sirt1 agonist, SRT2104, with or without AAV-mediated HO-1 or FTH1 silencing, to evaluate aortic valve calcification. The results revealed that Sirt1 expression was significantly reduced in calcified human valves and osteogenically induced hAVICs. Sirt1 overexpression inhibited osteogenic differentiation, reduced RUNX2 nuclear translocation, restored mitochondrial function and suppressed ferroptosis. Mechanistically, Sirt1 activated Nrf2/HO-1 signaling, upregulated the downstream effector, FTH1, reduced intracellular Fe²⁺ accumulation and

maintained iron homeostasis. These protective effects were abolished by treatment with ML385 or erastin. In ApoE^{-/-} mice, SRT2104 attenuated aortic valve calcification and pathological remodeling, whereas HO-1 or FTH1 silencing partially reversed these benefits. Collectively, the present study demonstrates that Sirt1 protects against CAVD by activating the Nrf2/HO-1 pathway, leading to the FTH1-dependent maintenance of iron homeostasis, the suppression of ferroptosis, the preservation of mitochondrial function and the inhibition of osteogenic differentiation. These findings identify the Sirt1/Nrf2/HO-1/FTH1 axis as a potential therapeutic target for CAVD.

Introduction

Calcific aortic valve disease (CAVD) is the most common heart valve disease affecting adults. It is characterized by fibrosis, inflammation and calcific deposition in the aortic valve, resulting in aortic valve sclerosis, stenosis, heart failure and, ultimately, in premature mortality (1). The epidemiological characteristics of CAVD profoundly reflect the global trends of population aging and the increasing prevalence of metabolic disorders. According to estimates from the Global Burden of Disease study, ~12.6 million individuals were affected by CAVD worldwide in 2017, representing an increase of ~124% compared with 1990, and the disease accounted for ~102,700 deaths globally (2). Of particular concern, the progression of CAVD is irreversible. Although CAVD progresses at a slow rate, once angina, syncope or heart failure occur, the 2-year mortality rate escalates to 50%, and the 5-year survival rate declines to <20% (3). Despite advancements in medical research, existing treatment strategies for CAVD have critical limitations, particularly in early-stage intervention and the regulation of pathological mechanisms. Pharmacological therapy has proven entirely ineffective, leaving surgical aortic valve replacement as the only viable therapeutic option (1). However, the incomplete elucidation of the pathogenesis of CAVD has markedly hindered the development of novel therapeutic strategies.

Aortic valve interstitial cells (AVICs), the predominant cellular component of the aortic valve, primarily exhibit a fibroblast-like phenotype and are crucial for matrix

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remodeling, injury repair and the pathogenesis of CAVD (2). During the progression of CAVD, AVICs undergo phenotypic transformation in response to oxidative stress and mechanical stimuli. These AVICs differentiate into osteoblast-like cells and secrete bone matrix proteins, promoting valvular calcification (4). In recent years, ferroptosis, a novel form of programmed cell death, has garnered increasing attention for its role in the pathogenesis of CAVD. Research has demonstrated that an imbalance in iron metabolism and lipid peroxidation stress are significantly elevated in tissues affected by CAVD (5), suggesting that ferroptosis plays a crucial role in the occurrence and progression of CAVD. Notably, ferroptosis is an iron-dependent form of cell death characterized by the accumulation of lipid peroxides, with its primary mechanism involving elevated intracellular free iron levels. Nesfatin-1 can attenuate the calcification of AVICs by inhibiting ferroptosis (6). However, the specific mechanisms by which CAVD regulates ferroptosis in AVICs remain unclear. Further investigations into the molecular mechanisms of AVIC ferroptosis may provide novel insight into the pathogenesis of CAVD.

In studies on various cardiovascular diseases, Sirtuin 1 (Sirt1) has been identified as a crucial protective molecule with multiple biological functions, including anti-inflammatory, antioxidant and anti-apoptotic properties (7). As a member of the NAD⁺-dependent deacetylase family, Sirt1 exerts broad cellular regulatory functions by modulating multiple target proteins, such as peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC1 α). The involvement of Sirt1 in the regulation of ferroptosis has garnered increasing attention in recent years. It has been demonstrated that Sirt1 can reduce ferroptosis by enhancing intracellular antioxidant stress responses (8). Furthermore, Sirt1 plays a role in maintaining cellular iron homeostasis and preventing the Fenton reaction, thereby mitigating ferroptosis (9). Research has revealed that the expression of Sirt1 is markedly down-regulated in the valvular tissue of patients with CAVD (10). The exogenous activation of Sirt1 has been demonstrated to inhibit cellular osteogenic differentiation and vascular calcification (11,12). These findings suggest that Sirt1 may serve as a key regulator of ferroptosis in AVICs and may play a crucial role in the onset and progression of CAVD. However, the precise mechanisms by which Sirt1 regulates ferroptosis and mitochondrial function in AVICs remain largely unknown. Moreover, the potential crosstalk between Sirt1 and the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway in the context of CAVD has not yet been clearly elucidated, at least to the best of our knowledge. Addressing this gap is essential, as uncovering upstream modulators of ferroptosis may provide novel therapeutic strategies beyond symptomatic surgical interventions.

Therefore, the present study aimed to systematically investigate whether Sirt1 inhibits the osteogenic differentiation of and ferroptosis in AVICs via the activation of the Nrf2/HO-1 pathway, and to validate its protective role both *in vitro* and in a mouse model of CAVD. The findings presented herein may provide further insight into the mechanisms of the pathogenesis of CAVD and identify Sirt1 as a promising molecular target for early pharmacological intervention.

Materials and methods

Collection of clinical samples. The present retrospective observational study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Hainan Medical University on January 24, 2025 (Approval no. 2025-KYL-016). Sample collection and processing were conducted in strict accordance with the Declaration of Helsinki and the Regulations on the Management of Human Genetic Resources. Written informed consent was obtained from all participants prior to tissue collection.

Calcified aortic valves were collected from patients who underwent aortic valve replacement at the First Affiliated Hospital of Hainan Medical University between January and December, 2024 (CAV group, n=30), and non-calcified aortic valves were obtained from patients with aortic valve regurgitation due to aortic annular dilatation (normal group, n=30). The sex and age characteristics of the patients were as follows: CAV group: 18 males and 12 females; median age, 68 years (range, 56-68 years); Normal group: 17 males and 13 females; median age, 64 years (range, 48-76 years). Following ethics approval on January 24, 2025, archived specimens and corresponding clinical data were retrieved from February 1, 2025, and laboratory analyses commenced on February 17, 2025.

As regards patients in the CAV group, the diagnosis of severe aortic valve calcification (aortic valve area <1.0 cm², transvalvular flow velocity, >4 msec) was confirmed using echocardiography (transthoracic echocardiogram or transesophageal echocardiogram) and cardiac computed tomography, with the exclusion of secondary calcifications, such as those caused by rheumatic heart disease, infective endocarditis, or metastatic calcification associated with malignancies. For the patients in the normal group, the criteria for inclusion were as follows: Echocardiography confirming an aortic valve annulus diameter >27 mm (in males) or >25 mm (in females), absence of calcific lesions on imaging examinations, and preserved leaflet structure and flexibility, as documented in surgical records. Exclusion criteria included bicuspid aortic valve, congenital valve abnormalities, prior valve interventions and advanced chronic kidney disease.

Isolation and culture of primary human AVICs. Primary human AVICs (hAVICs) were isolated according to methods described in previous studies (13,14). In brief, the non-calcified aortic valve was collected and washed in sterile phosphate-buffered saline (PBS, Gibco; Thermo Fisher Scientific, Inc.). Subsequently, excess connective tissue and blood vessels were carefully removed using ophthalmic scissors and forceps, leaving only the valve tissue. The processed valve tissue was then cut into small sections of ~1 mm³ and transferred to a new sterile centrifuge tube. Subsequently, 0.25% trypsin was added, and the mixture was incubated at 37°C in a water bath or on a shaker for 15 min. The sample was then centrifuged at 300 x g for 5 min at room temperature (25°C), and the supernatant was discarded. Subsequently, 2 mg/ml collagenase type I solution was added, and the mixture was incubated at 37°C on a shaking incubator for 4 h. Following incubation, an equal volume of DMEM/F-12 medium containing 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.) was added. The cell suspension was filtered through a 70- μ m

cell strainer and centrifuged at 300 x g for 5 min at room temperature (25°C). The supernatant was discarded, and the cells were resuspended in DMEM/F-12 medium (containing 10% FBS). The cell suspension was then seeded into culture dishes and incubated at 37°C with 5% CO₂. When the cells reached 70% confluency, subculture was performed. Cells from passages 3-5 were used in subsequent experiments.

Subsequently, hAVICs at 60-70% confluency were assigned to the following six groups: Control, OM, OM + vector, OM + oe-Sirt1, OM + oe-Sirt1 + ML385, and OM + oe-Sirt1 + Erastin. Specifically, cells in the control group were cultured in standard complete medium containing 10% FBS without osteogenic induction or other interventions. Cells in the OM group were cultured in osteogenic induction medium containing 10 mM β -glycerolphosphate, 50 μ g/ml ascorbic acid, 100 nM dexamethasone (MilliporeSigma), and 10% FBS until osteogenic differentiation was achieved. Cells in the OM + vector and OM + oe-Sirt1 groups were transfected with the negative control plasmid, pcDNA3.1, and the pcDNA3.1-Sirt1 plasmid (Shanghai GenePharma Co., Ltd.), respectively, using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following incubation at 37°C for 6-8 h, the medium was replaced with fresh osteogenic induction medium, and the cells were cultured until osteogenic differentiation was complete. Sirt1 overexpression efficiency was verified by RT-qPCR, and the corresponding validation results are provided in Fig. S1A. As for the cells in the OM + oe-Sirt1 + ML385 and OM + oe-Sirt1 + erastin groups, they were transfected with the pcDNA3.1-Sirt1 plasmid as described above; following 6-8 h of incubation at 37°C, the culture medium was replaced with osteogenic induction medium, supplemented with either 1 μ M of the Nrf2 inhibitor, ML385 (MedChemExpress; OM + oe-Sirt1 + ML385 group), or 10 μ M of the ferroptosis inducer erastin (MedChemExpress, OM + oe-Sirt1 + erastin group); the cells were then cultured until osteogenic differentiation was achieved.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Aortic valve tissues (50 mg), either fresh or stored in liquid nitrogen, were added to 1 ml TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and thoroughly homogenized. Total RNA was extracted using the chloroform-isopropanol method as described in the instructions provided with the TRIzol reagent kit, and the extracted RNA was purified with anhydrous ethanol (MilliporeSigma). As for the cell samples, they were washed with PBS and then lysed directly in 1 ml TRIzol reagent. Total RNA was subsequently extracted. The RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Subsequently, 1,000 ng total RNA were reverse transcribed into cDNA using the PrimeScript RT Reagent kit (RR047Q, Takara Bio, Inc.). Equal amounts of cDNA were quantified using TB Green® Premix Ex Taq™ II FAST quantitative polymerase chain reaction (CN830S, Takara Bio, Inc.). The PCR thermocycling conditions were initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing/extension at 60°C for 30 sec. GAPDH was used as the internal reference gene. Data were analyzed using the 2^{- $\Delta\Delta$ C_q} method (15). The primer sequences used are listed in Table I.

Enzyme-linked immunosorbent assay (ELISA). Following treatment, the culture supernatants of hAVICs were collected and analyzed for PGC1 α (cat. no. KTE61195, Abbkine Scientific Co. Ltd.), mitofusin 2 (MFN2; at. no. KTE61636, Abbkine Scientific Co. Ltd.), optic atrophy 1 (OPA1; at. no. USEE291, Wuhan USCN Business Co., Ltd.), fission 1 (FIS1; at. no. USEJ105, Wuhan USCN Business Co., Ltd.), dynamin-related protein 1 (DRP1; at. no. ab315308, Abcam) levels using commercially available human ELISA kits. All procedures were performed in accordance with the manufacturer's instructions. Briefly, standards and samples were added to pre-coated 96-well plates, followed by incubation at 37°C for 60 min, washing, enzyme conjugation and substrate reaction. The absorbance was measured at 450 nm using a Synergy H1 microplate reader (BioTek Instruments, Inc.), and protein concentrations were calculated based on the standard curve. All samples were measured in duplicate, and the average value was used for statistical analysis.

Western blot analysis. Aortic valve tissues (50 mg) that were previously ground in liquid nitrogen, or treated hAVICs, were lysed with 500 μ l radioimmunoprecipitation assay buffer containing 1 mM phenylmethylsulfonyl fluoride (Beyotime Biotechnology). Additionally, cytoplasmic and nuclear proteins were isolated using a nuclear protein extraction kit (cat. no. P0027, Beyotime Biotechnology). The samples were sonicated on ice (10 sec x 3 cycles, with 30-sec intervals). The lysates were centrifuged at 12,000 x g for 15 min at 4°C, and the supernatant was collected. Protein concentrations were measured using the bicinchoninic acid protein assay kit (cat. no. P0012, Beyotime Biotechnology) according to the standard curve. The protein concentration of all samples was adjusted to 1-2 μ g/ μ l, after which 5X sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (Beyotime Biotechnology) was added, and the samples were denatured in a 95°C metal bath for 5 min. The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (stacking gel: 80 V for 30 min; separating gel: 120 V for 90 min). The proteins were then transferred onto methanol-activated polyvinylidene fluoride (PVDF) membranes by wet transfer at 300 mA for 90 min. The membranes were blocked with 5% skim milk for 1 h at room temperature and subsequently incubated with the corresponding primary antibodies overnight at 4°C. The primary antibodies used were as follows: Sirt1 (1:1,000, cat. no. 9475, Cell Signaling Technology, Inc.), glutathione peroxidase 4 (GPX4; 1:1,000, cat. no. ab125066, Abcam), acyl-CoA synthetase long-chain family member 4 (ACSL4; 1:1,000, cat. no. 9189, Cell Signaling Technology, Inc.), Runt-related transcription factor 2 (RUNX2; 1:1,000, cat. no. ab192256, Abcam), solute carrier family 7 member 11 (SLC7A11; 1:1,000, cat. no. 26864-1-AP, Proteintech Group, Inc.), ferritin heavy chain 1 (FTH1; 1:1,000, cat. no. ab75973, Abcam), Kelch-like ECH-associated protein 1 (KEAP1; 1:1,000, cat. no. 10503-2-AP, Proteintech Group, Inc.), HO-1 (1:1,000, cat. no. 5853, Cell Signaling Technology, Inc.), NAD(P)H quinone dehydrogenase 1 (NQO1; 1:1,000, cat. no. ab80588, Abcam), Nrf2 (1:3,000, cat. no. PA5-27882, Thermo Fisher Scientific, Inc.), β -actin (1:5,000, cat. no. A5441, MilliporeSigma) and histone H3 (1:1,000, cat. no. 4499, Cell Signaling Technology, Inc.). After washing, the membrane was incubated with

Table I. Primer sequences used for RT-qPCR analysis.

Gene	Species	Primer sequences (5'-3')
Sirt1	Human	F: CTGGAACAGTGAGAAAATGCTG, R: TTCATCCTCCATGGGTTCTTC
RUNX2	Human	F: CCATAACCGTCTTCACAAATCC, R: GGCGGGGTGTAAGTAAAGG
OPN	Human	F: TCTCACCAGTCTGATGAGTC, R: CTTGTATGCACCAATCAACTCC
Osterix	Human	F: AGTTCATATGGCTCCAGTC, R: TGGGAAAAGGGAGGGTAATC
ALP	Human	F: AGTCCTTCAAAGCTGGAGTC, R: CATTGGCCATAAGTCACTGG
HO-1	Human	F: ATGCTGAGTTCATGAGGAACCTT, R: GGTGCAGCTCTTCTGGGAA
FTH1	Human	F: GTGGCTTTGAAGAACTTTGCC, R: GGGGTCATTTTGTCTAGTGG
GAPDH	Human	F: AAGATCATCAGCAATGCCTCC, R: AGGTTTTTCTAGACGGCAGG
RUNX2	Mouse	F: CATCCCAGTATGAGAGTAGG, R: CTCTTCTTACTGAGAGAGGAAGG
OPN	Mouse	F: GACTGAGGTCAAAGTCTAGG, R: AACTTGTGGCTCTGATGTTCC
Osterix	Mouse	F: TTCTCAAGCACCAATGGACTC, R: AGCCATAGGGATGAGTCATATC
ALP	Mouse	F: CGAAGAACAGAACTGATGTGG, R: TTGAGGTTTTTGGTCAGGATCC
GAPDH	Mouse	F: AGGTCGGTGTGAACGGATTG, R: TGTAGACCATGTAGTTGAGGTCA

F, forward; R, reverse; Sirt1, sirtuin 1; HO-1, heme oxygenase 1; RUNX2, RUNX family transcription factor 2; OPN, osteopontin; ALP, alkaline phosphatase; HO-1, heme oxygenase 1; FTH1, ferritin heavy chain 1.

HRP-conjugated secondary antibodies [anti-rabbit IgG (1:5,000, cat. no. 7074, Cell Signaling Technology, Inc.) and anti-mouse IgG (1:5,000, cat. no. 7076, Cell Signaling Technology, Inc.)] at room temperature for 1 h. Subsequently, enhanced chemiluminescence substrate (cat. no. P0018S, Beyotime Biotechnology) was added to the membrane for 30 sec of incubation. The band grayscale values were quantified using ImageJ software (version 1.53, National Institutes of Health), and the relative expression of the target protein was calculated using β -actin and histone H3 as internal controls.

ALP staining and Alizarin Red S staining. A total of 1×10^4 hAVICs were seeded in each well of a 24-well plate. Upon different treatments and osteogenic induction, the cells were washed twice with PBS and then fixed in 4% paraformaldehyde (Gibco; Thermo Fisher Scientific, Inc.) at room temperature for 15 min. Following three additional PBS washes, the cells were subjected to ALP staining and Alizarin Red S staining.

The ALP staining working solution was prepared according to the instructions provided with the ALP Staining kit (C3206, Beyotime Biotechnology). BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitro blue tetrazolium), supplied with

the kit, were mixed at a 1:1 ratio. Subsequently, 500 μ l ALP working solution were added to each well to cover the cells. The cells were then incubated at 37°C for 30 min in the dark until the blue-violet precipitate appeared. Subsequently, the staining solution was discarded, and the cells were washed three times with PBS. Finally, the blue-violet ALP-positive cells were observed and photographed under an optical microscope (Olympus Corporation), and the staining intensity and area were quantified using ImageJ software (version 1.53, National Institutes of Health).

Alizarin Red S staining solution was prepared according to the instructions provided with the Alizarin Red S Staining kit (C0148S, Beyotime Biotechnology). Cells in each well was supplemented with 500 μ l Alizarin Red S staining solution and incubated at room temperature for 30 min. The staining solution was then removed, and the cells were washed three times with PBS. The stained cells were then observed and photographed under an optical microscope (Olympus Corporation). Immediately following image acquisition, a 10% citric acid-ammonia buffer (pH 4.1, supplied with the kit) was added to the wells, followed by a 30-min incubation period at room temperature to completely dissolve the staining substance.

Lastly, the absorbance at 415 nm was measured using a microplate reader (FLx800, BioTek; Agilent Technologies, Inc.) to quantify the mineralization level.

Measurement of cell-related parameters. The cells were seeded in 96- or 6-well plates and treated when they reached a confluency of 60-80%. Following the indicated osteogenic induction and pharmacological/genetic treatments described above, the culture medium was removed, and the cells were gently washed twice with PBS. Subsequently, the total reactive oxygen species (ROS) levels, mitochondrial ROS levels, mitochondrial membrane potential (JC-1), mitochondrial DNA (mtDNA), adenosine triphosphate (ATP) levels and cellular malondialdehyde (MDA), glutathione (GSH), ferrous ion (Fe^{2+}) and total iron levels were determined as described below.

Measurement of intracellular and mitochondrial ROS levels. Total ROS levels in the cells were measured using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, S0034S, Beyotime Biotechnology) and mitochondrial ROS levels were assessed using MitoSOX™ Red (S0061S, Beyotime Biotechnology). The cells were incubated with 10 μM DCFH-DA or 5 μM MitoSOX™ Red at 37°C for 30 min, followed by three washes with PBS. Fluorescence was then detected using a microplate reader (FLx800, BioTek; Agilent Technologies, Inc.) with DCFH-DA [excitation/emission (Ex/Em)=488/525 nm] or MitoSOX™ Red (Ex/Em=510/580 nm).

Quantification of mtDNA. Relative changes in mtDNA were evaluated by qPCR. Mitochondrial DNA was first extracted using a mitochondrial DNA extraction kit. Mitochondrial COX1 was selected as the target gene, and quantification was performed using SYBR-Green Polymerase Chain Reaction Master Mix (cat. no. A25742; Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. GAPDH was used as the internal control, and data were analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method (15). The primer sequences used were as follows: COX1 forward, 5'-GAATGCCACCTTCATCCGAGAAG-3' and reverse, 5'-GCTCACATTGGAGAAGGACTCC-3'; and GAPDH forward, 5'-AAGATCATCAGCAATGCCTCC-3' and reverse, 5'-AGGTTTTTCTAGACGGCAGG-3'.

Measurement of ATP content. The ATP content in the cells was measured using the ATP assay kit (cat. no. S0026, Beyotime Biotechnology). Specifically, ATP lysis buffer was added to a 6-well plate and the cells were lysed on ice for 5 min. The lysate was then centrifuged at 10,000 g for 10 min at 4°C. Immediately following centrifugation, 50 μl of the supernatant were mixed with 50 μl of the fluorescent substrate and incubated at room temperature for 5 min. The ATP levels were then measured using a microplate reader (FLx800, BioTek; Agilent Technologies, Inc.) at Ex/Em=485/520 nm, and the ATP concentration was calculated based on the standard curve.

Measurement of lipid peroxidation and iron metabolism parameters. The levels of MDA, GSH, Fe^{2+} and total iron were measured according to the instructions provided with the

MDA assay kit (cat. no. S0131M, Beyotime Biotechnology), GSH assay kit (cat. no. S0053, Beyotime Biotechnology), Fe^{2+} assay kit (cat. no. BC5415, Beijing Solarbio Science & Technology Co., Ltd.) and total iron assay kit (cat. no. BC5315, Beijing Solarbio Science & Technology Co., Ltd.). Briefly, cells from a 6-well plate were harvested and lysed, after which reaction solutions were added. The absorbance was then measured using a microplate reader (FLx800, BioTek; Agilent Technologies, Inc.), and the MDA, GSH, Fe^{2+} and total iron levels were calculated based on the standard curve.

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential was measured using the JC-1 mitochondrial membrane potential assay kit (cat. no. C2003S, Beyotime Biotechnology). The cells were incubated with JC-1 working solution at 37°C for 20-30 min, followed by three washes with PBS. The ratio of red fluorescence (Ex/Em=550/600 nm) to green fluorescence (Ex/Em=488/525 nm) was detected using a microplate reader (FLx800, BioTek; Agilent Technologies, Inc.).

Establishment and maintenance of mouse models. All animal experiments in the present study were conducted in accordance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, 2011 revision) (16) and approved by the Ethics Committee of Hainan Medical University (Approval no. HYALL-2025-028). Male apolipoprotein E-deficient ($\text{ApoE}^{-/-}$) mice (8 weeks old, weighing 20-25 g; $n=30$) were purchased from the Guangdong Provincial Laboratory Animal Center. Mice were housed under specific pathogen-free conditions at $22\pm 2^\circ\text{C}$ and 50-60% relative humidity, with a 12/12-h light/dark cycle and *ad libitum* access to food and water.

Following acclimatization, mice were randomly assigned to the following three groups ($n=10$ per group): Normal, calcified and calcified + SRT2104. The mice in the normal group received standard laboratory chow (Beijing Keao Xieli Feed Co., Ltd. China), consisting primarily of corn, soybean meal, fish meal, wheat flour, yeast powder, vegetable oil, vitamins and minerals, with $\geq 18\%$ crude protein and $\geq 4\%$ crude fat. The mice in the calcified group received a Western diet (Beijing Keao Xieli Feed Co., Ltd.) containing 21% fat and 0.15% cholesterol. The mice in the calcified + SRT2104 group received the same Western diet supplemented with SRT2104, a Sirt1 agonist (MedChemExpress), at 1.33 g/kg diet, corresponding to ~ 100 mg/kg/day based on the average daily food intake per mouse under standard housing conditions.

For mechanistic validation, recombinant AAV9 vectors carrying shRNAs targeting mouse HO-1 or FTH1, together with the corresponding negative-control vector (AAV-NC), were commercially constructed, packaged, purified and titrated by GeneChem Co., Ltd. The shRNA sequences used for HO-1 and FTH1 silencing are provided in Table S1. The purified viral preparations were supplied at a titer of $\sim 1 \times 10^{12}$ vg/ml. Each mouse received 1×10^{11} vg in a final volume of 100 μl via tail-vein injection. Successful knockdown was subsequently verified in mouse aortic valve tissues by measuring HO-1 and FTH1 mRNA expression (Fig. S1).

All mice were housed in a Specific Pathogen-Free (SPF) facility under a 12/12-h light/dark cycle. The experimental period

lasted for 24 weeks. For terminal tissue collection, anesthesia was induced with 3–4% isoflurane and maintained with 1.5–2.0% isoflurane throughout the procedure. At the experimental endpoint, mice were euthanized by inhalation of 5% isoflurane until complete loss of the pedal withdrawal reflex and spontaneous respiration. Death was confirmed by the absence of both heartbeat and respiration before tissue collection, in accordance with institutional animal care and ethical guidelines.

Histopathological staining of mouse aortic valve tissues. After completing the experimental protocol, mice were anesthetized with 2.5% isoflurane, followed by left ventricular perfusion with PBS and 4% paraformaldehyde for tissue fixation. The heart and aorta were then excised and further fixed in 4% paraformaldehyde at 4°C for 24 h. Following washing with PBS, the tissues were immersed in 15% sucrose at 4°C for 12 h, and then transferred to 30% sucrose at 4°C for a further 12 h for gradient dehydration. Subsequently, the tissues were embedded in optimal cutting temperature compounds, frozen at -20°C, and sectioned at a thickness of 5 μ m. For H&E staining, the frozen sections were brought to room temperature, washed with PBS, and stained with hematoxylin and eosin staining solutions (Wuhan Servicebio Technology Co., Ltd.). Sections were stained with hematoxylin for 5 min at room temperature, rinsed with running water, differentiated and blued, and subsequently counterstained with eosin for 2 min at room temperature. After washing, the sections were dehydrated, cleared and mounted. Histopathological changes in the aortic valve leaflets were examined and photographed using a light microscope (Olympus Corporation).

Statistical analysis. Statistical analyses were performed using SPSS version 26.0 (IBM Corp.) and graphs were generated with GraphPad Prism 9.2.0 (GraphPad Software). Data distribution was assessed using the Shapiro-Wilk test for normality and Levene's test for homogeneity of variance. All data are presented as the mean $\pm$ standard deviation (SD). For comparisons between two groups, an unpaired two-tailed Student's t-test was applied. For comparisons among multiple groups, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Sirt1 expression is downregulated in calcified aortic valve tissues and osteogenically-induced hAVICs. To explore the role of Sirt1 in CAVD, the present study first examined its expression in human aortic valve tissues and *in vitro* osteogenic models. The results of RT-qPCR and western blot analysis disclosed that, compared with the normal group, Sirt1 expression was significantly lower in the aortic valve tissue of the CAV group (Fig. 1A and B). Similarly, osteogenic stimulation significantly reduced the expression of Sirt1 in cultured hAVICs relative to the control group (Fig. 1C and D). These data suggested the consistent downregulation of Sirt1 in both clinical and experimental calcification models.

Overexpression of Sirt1 attenuates the osteogenic differentiation of hAVICs. Given the reduced expression of Sirt1 in

calcified settings, the present study then investigated whether restoring its expression could influence osteogenic transition. To further investigate the association between Sirt1 and CAVD, Sirt1 expression was increased in hAVICs *in vitro* using an overexpression vector (pcDNA3.1-Sirt1) (Fig. 2A). The cells were subjected to osteogenic induction, and ALP and Alizarin Red S staining were carried out when the cells reached full maturation. The experimental results revealed that in comparison with the control group, the cells in the OM group exhibited a significantly higher ALP activity, enhanced mineralization and the increased formation of calcified nodules. By contrast, the OM + oe-Sirt1 group displayed a significantly reduced ALP activity, lower mineralization and decreased calcified nodule formation compared with the OM + vector group (Fig. 2B and C). Consistently, the calcifying conditions markedly increased nuclear RUNX2 accumulation, whereas Sirt1 overexpression significantly reduced nuclear RUNX2 levels, indicating the inhibition of RUNX2 nuclear translocation (Fig. 2D). RT-qPCR revealed elevated mRNA levels of the osteogenic markers, RUNX2, OPN, osterix and ALP in the OM group, which were all significantly downregulated in the OM + oe-Sirt1 group (Fig. 2E). These data demonstrated that the overexpression of Sirt1 inhibited the calcification of osteogenically-induced hAVICs.

Overexpression of Sirt1 enhances mitochondrial function in osteogenically-induced hAVICs. Recent research has proposed that alterations in mitochondrial metabolism are a key determinant in the development of CAVD (17). Based on this, herein, it was hypothesized that Sirt1 may serve as a critical link between CAVD and mitochondrial function. As shown in Fig. 3A and B, the overexpression of Sirt1 significantly decreased both total and mitochondrial ROS levels in hAVICs compared to the OM + vector group. Correspondingly, JC-1 assay revealed that mitochondrial membrane potential, which was reduced in the OM-treated cells, was markedly restored by the overexpression of Sirt1 (Fig. 3C). In line with this, the mtDNA content and intracellular ATP levels were also significantly elevated in the OM + oe-Sirt1 group (Fig. 3D and E). ELISA revealed that the overexpression of Sirt1 reversed the OM-induced downregulation of PGC1 α , MFN2 and OPA1, and suppressed the upregulation of FIS1 and DRP1, indicating improved mitochondrial dynamics (Fig. 3F–J). These results suggested that the overexpression of Sirt1 improved mitochondrial function under conditions of osteogenic stress.

Overexpression of Sirt1 inhibits ferroptosis in osteogenically-induced hAVICs. Additionally, it has previously been demonstrated that ferroptosis plays a critical role in CADV (5). Notably, Sirt1, mitochondria and ferroptosis are often interrelated (18). Therefore, in the present study, it was hypothesized that Sirt1 may induce ferroptosis in cells. To validate this hypothesis, the levels of MDA, GSH, Fe²⁺ and total iron in hAVICs were measured following the overexpression of Sirt1. The results revealed that the overexpression of Sirt1 significantly reduced the MDA, Fe²⁺ and total iron levels, while it significantly restored the GSH levels in the OM-treated hAVICs (Fig. 4A–D). In parallel, the results of western blot analysis revealed that the overexpression of Sirt1 increased the expression of the anti-ferroptotic proteins, GPX4, SLC7A11

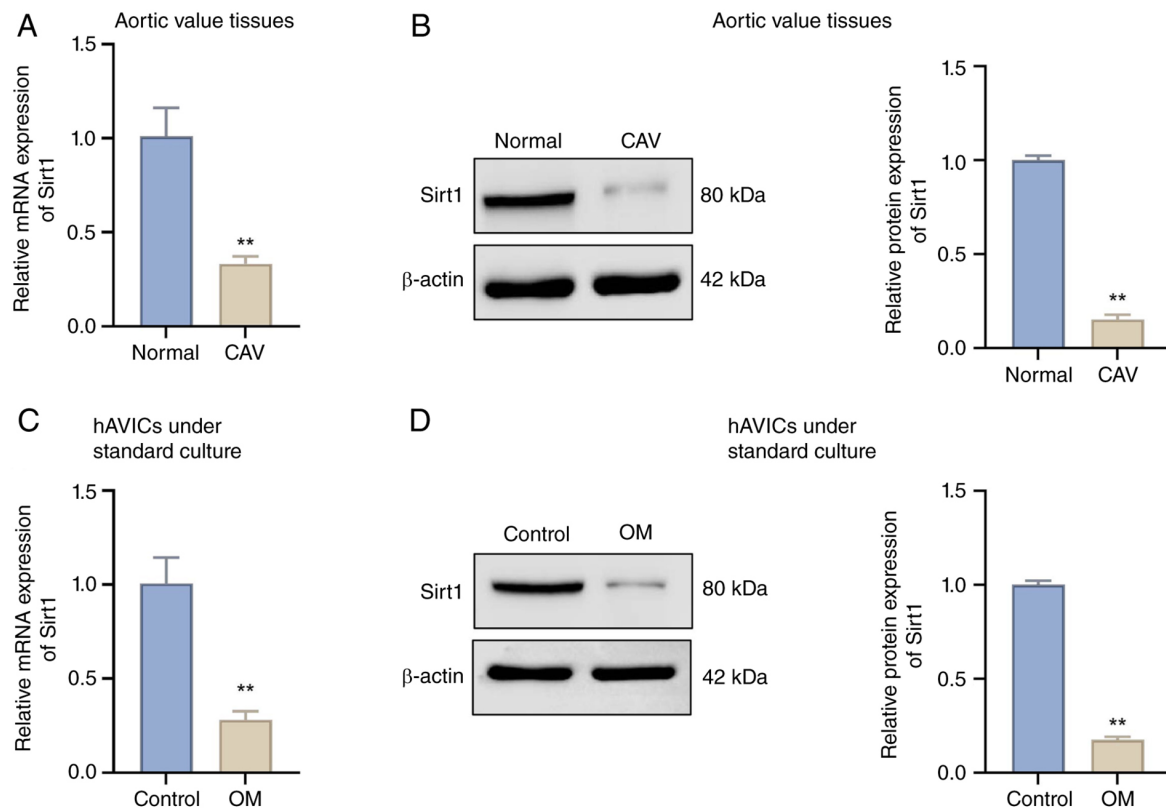


Figure 1. Downregulation of Sirt1 in calcified aortic valve tissues and osteogenic-induced aortic valve interstitial cells. (A) RT-qPCR analysis of Sirt1 expression levels in aortic valve tissues from the CAV and normal groups. (B) Western blot analysis of Sirt1 expression levels in aortic valve tissues from the CAV and Normal groups. (C and D) RT-qPCR and western blot analysis of Sirt1 expression levels in hAVICs under standard culture (control) and osteogenic medium (OM) stimulation. Data represent the mean $\pm$ SD from three independent experiments. Statistical significance was determined using an unpaired two-tailed Student's t-test. ** $P < 0.01$ vs. normal/control. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CAV, calcific aortic valve; Sirt1, sirtuin 1; hAVICs, human aortic valve interstitial cells.

and FTH1, and suppressed the pro-ferroptotic marker, ACSL4, compared to the OM + vector group (Fig. 4E and F). These findings suggest that Sirt1 inhibits ferroptosis in hAVICs under conditions of osteogenic stress.

Overexpression of Sirt1 activates the Nrf2/HO-1 pathway in osteogenically-induced hAVICs. Previous studies have demonstrated that Sirt1 activates the Nrf2/HO-1 pathway through its deacetylase activity, thereby inhibiting ferroptosis (19,20). Therefore, it was hypothesized that the Nrf2/HO-1 pathway may be the key signaling pathway responsible for the induction of ferroptosis following the reduced expression of Sirt1 in CAVD. Western blot analysis revealed that, relative to the control group, the OM group exhibited a significant upregulation in KEAP1 protein expression, a marked downregulation in the levels of HO-1 and NQO1 proteins, and a significant increase in the Nrf2 protein levels in the cytoplasm, while the nuclear Nrf2 protein levels were significantly decreased. Conversely, compared to the OM + vector group, the OM + oe-Sirt1 group had significantly lower KEAP1 levels, higher HO-1 and NQO1 levels, as well as reduced Nrf2 levels in the cytoplasm, and elevated Nrf2 levels in the nucleus (Fig. 5). These findings suggest that the overexpression of Sirt1 activates the Nrf2/HO-1 pathway in osteogenically-induced hAVICs.

Inhibition of Nrf2/HO-1 or the induction of ferroptosis reverse the protective effects of Sirt1 overexpression in

hAVICs. To verify whether the anti-calcification effects of Sirt1 are dependent on Nrf2/HO-1 activation and the suppression of ferroptosis, hAVICs overexpressing Sirt1 were treated with Nrf2 inhibitor (ML385) or ferroptosis inducer (erastin). Initially, ALP staining and Alizarin Red S staining revealed that, compared to the OM + oe-Sirt1 group, the OM + oe-Sirt1 + ML385 and OM + oe-Sirt1 + erastin groups exhibited a significantly increased ALP activity, mineralization and calcified nodule formation (Fig. 6A and B). Consistently, the expression levels of the osteogenic markers, RUNX2, OPN, osterix and ALP, were upregulated (Fig. 7A). Moreover, the levels of the mitochondrial function markers, PGC1 α , MFN2 and OPA1, were downregulated, whereas the levels of FIS1 and DRP1 were upregulated (Fig. 7B). Additionally, in comparison with the OM + oe-Sirt1 group, the OM + oe-Sirt1 + ML385 and OM + oe-Sirt1 + erastin groups exhibited significantly reduced levels of GPX4, SLC7A11 and FTH1, and a significant increase in ACSL4 protein expression (Fig. 7C). These data indicated that the inhibition of the Nrf2/HO-1 pathway or the promotion of ferroptosis enhanced the calcification of osteogenically-induced hAVICs.

Sirt1-mediated anti-calcification effects involve both HO-1 and FTH1 in hAVICs. To further investigate the roles of HO-1 and FTH1 in the Sirt1-mediated anti-calcification effects, hAVICs overexpressing Sirt1 were subjected to HO-1 or FTH1 knockdown under osteogenic conditions.

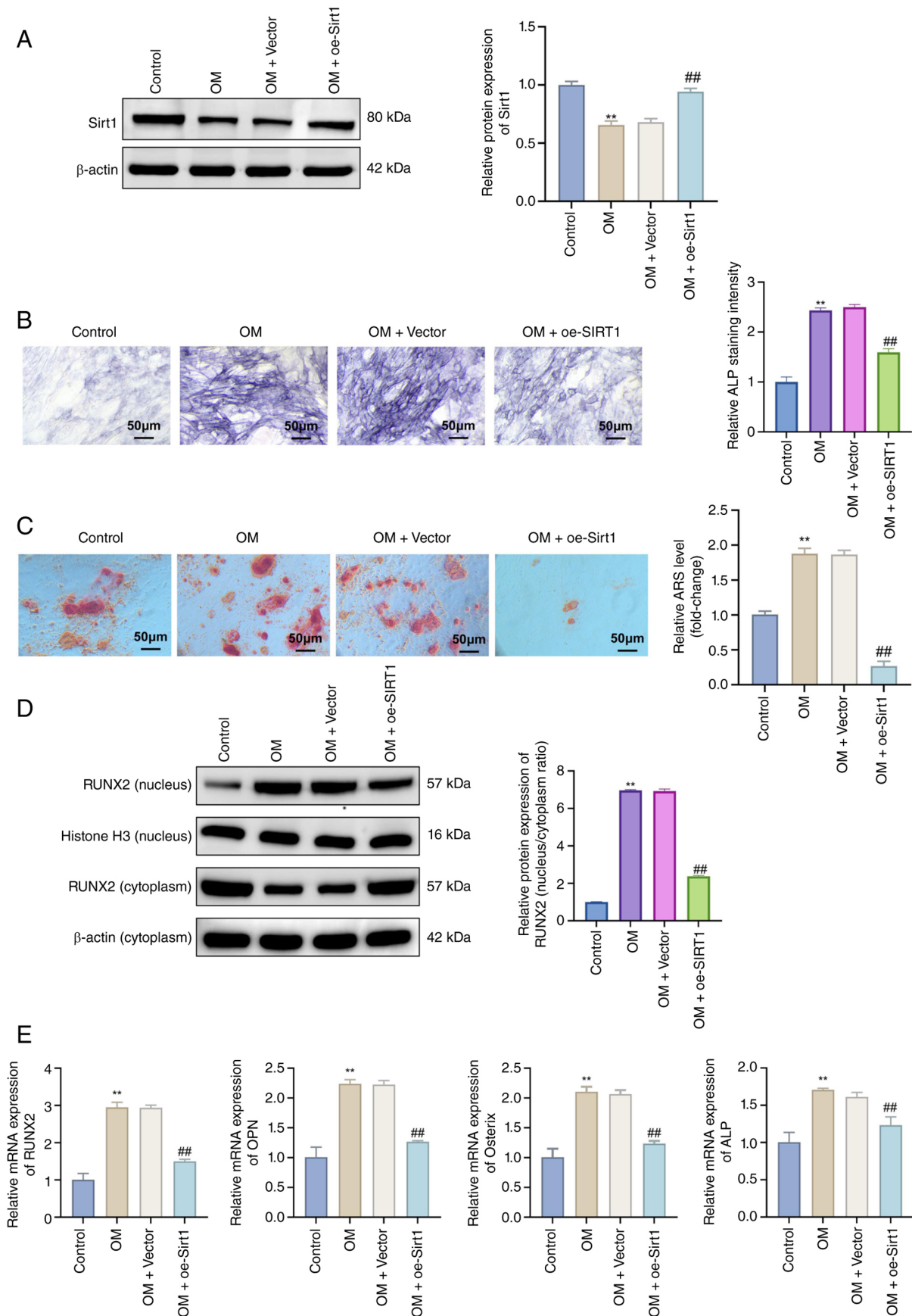


Figure 2. Overexpression of Sirt1 *in vitro* suppresses the calcification of osteogenically-induced aortic valve interstitial cells. (A) Western blot analysis of Sirt1 protein expression in each treatment group; (B) ALP staining illustrating ALP activity in each group; (C) Alizarin Red S staining reflecting calcified nodule formation in all groups; (D) western blot analysis of RUNX2 protein expression in nuclear and cytoplasmic fractions; (E) reverse transcription-quantitative polymerase chain reaction analysis of osteogenic markers RUNX2, OPN, osterix and ALP expression levels in all groups. Data represent the mean $\pm$ SD from three independent experiments. Comparisons were performed using one-way ANOVA. ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. OM + vector. Sirt1, sirtuin 1; ALP, alkaline phosphatase; RUNX2, RUNX family transcription factor 2; OPN, osteopontin; OM, osteogenic medium; oe, overexpression.

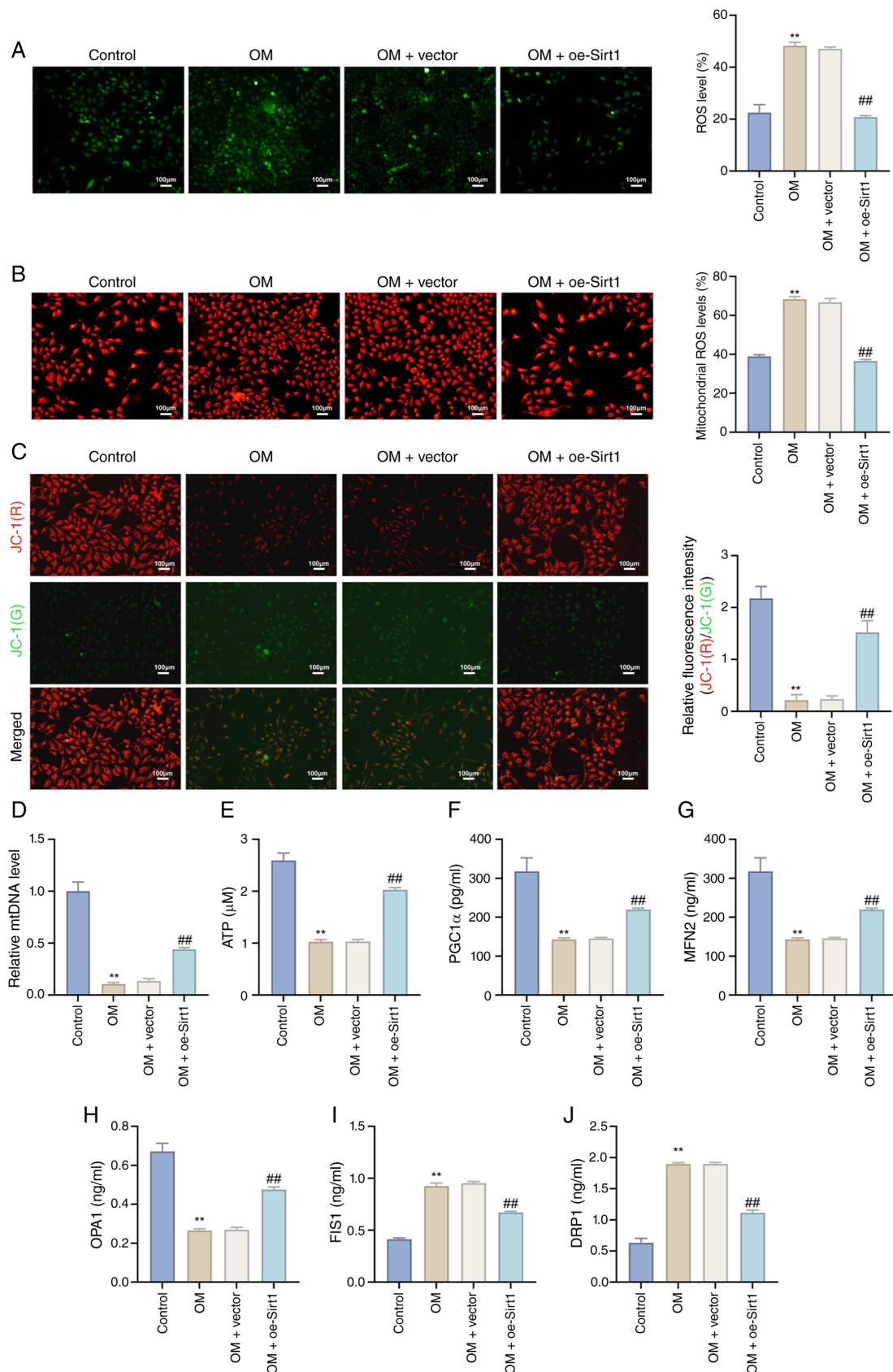


Figure 3. Overexpression of Sirt1 enhances mitochondrial function in osteogenically-induced aortic valve interstitial cells. (A and B) Fluorescent probes were used to detect (A) intracellular ROS and (B) mitochondrial ROS levels. (C) JC-1 staining was applied to assess mitochondrial membrane potential (JC-1). (D) Reverse transcription-quantitative polymerase chain reaction was used to measure the mtDNA content. (E) Intracellular ATP levels were detected using a luminescence-based ATP assay; (F-J) ELISA was performed to evaluate the expression of mitochondrial function-related proteins (PGC1 α , MFN2, OPA1, FIS1 and DRP1). Data represent the mean $\pm$ SD from three independent experiments. Comparisons were performed using one-way ANOVA. ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. OM + vector. ROS, reactive oxygen species; mtDNA, mitochondrial DNA; ATP, adenosine triphosphate; PGC1 α , peroxisome proliferator-activated receptor gamma coactivator 1-alpha; MFN2, mitofusin 2; OPA1, optic atrophy 1; FIS1, fission 1; DRP1, dynamin-related protein 1; Sirt1, siirtuin 1; OM, osteogenic medium; oe, overexpression.

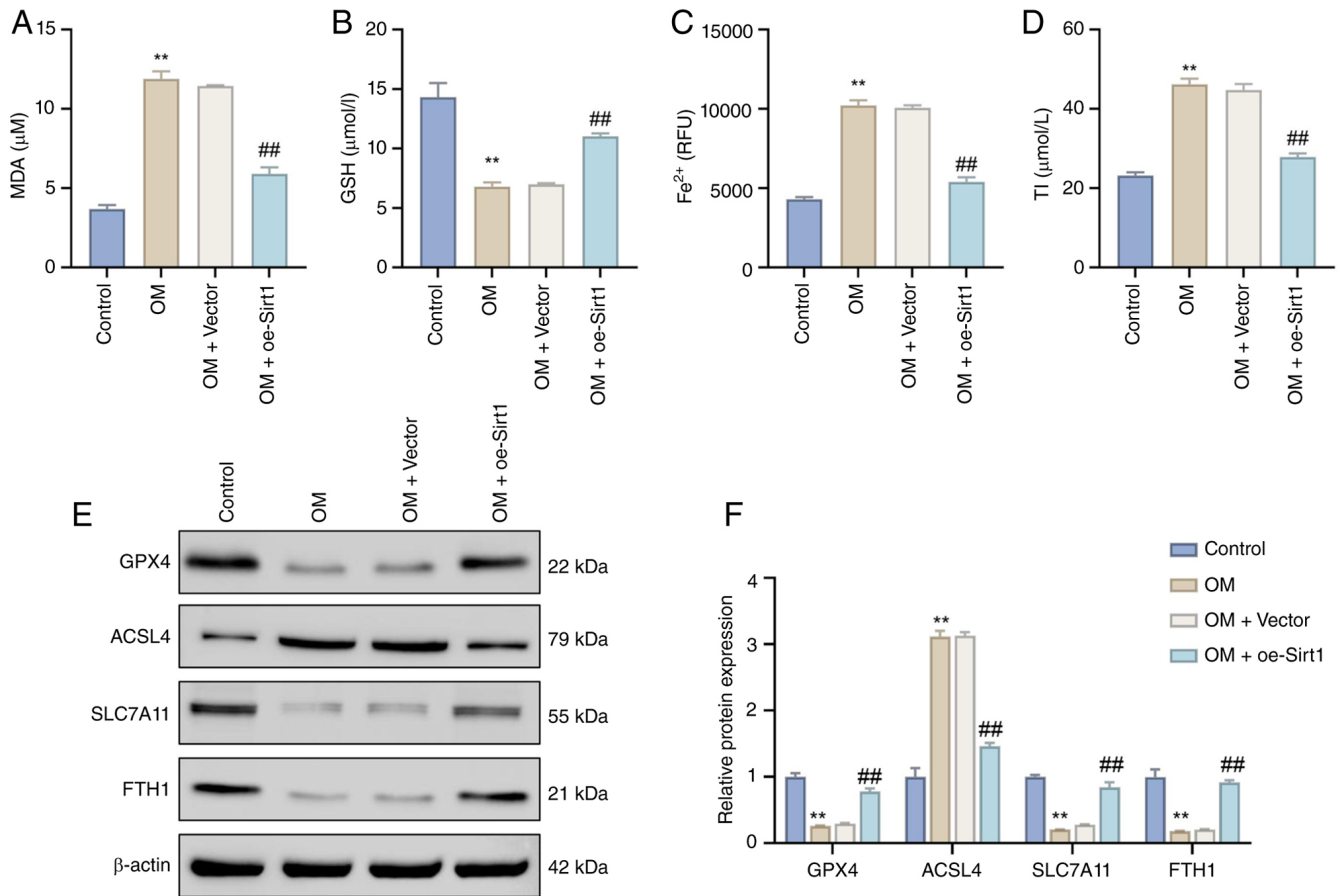


Figure 4. Overexpression of Sirt1 inhibits ferroptosis in osteogenically-induced aortic valve interstitial cells. (A-D) Biochemical assays were used to quantify intracellular (A) MDA, (B), (C) Fe²⁺, and (D) total iron levels. (E and F) Western blot analysis was performed to evaluate the expression of ferroptosis-related proteins, including GPX4, ACSL4, SLC7A11 and FTH1. Data represent the mean ± SD from three independent experiments. Comparisons were performed using one-way ANOVA. **P<0.01 vs. control; ##P<0.01 vs. OM + vector. MDA, malondialdehyde; GSH, glutathione; Fe²⁺, ferrous ion; GPX4, glutathione peroxidase 4; ACSL4, acyl-CoA synthetase long-chain family member 4; SLC7A11, solute carrier family 7 member 11; FTH1, ferritin heavy chain 1; Sirt1, sirtuin 1; OM, osteogenic medium; oe, overexpression.

Alizarin Red S and ALP staining demonstrated that the overexpression of Sirt1 markedly reduced mineralization and osteogenic activity, whereas silencing either HO-1 or FTH1 partially reversed these inhibitory effects (Fig. 8A and B). Both interventions increased calcified nodule formation and ALP activity compared with the Sirt1 overexpression group.

At the transcriptional level, the overexpression of Sirt1 significantly increased Sirt1 and HO-1 mRNA expression, whereas HO-1 knockdown effectively reduced the HO-1 levels. By contrast, FTH1 expression was decreased following FTH1 silencing, confirming the efficiency of gene-specific knockdown (Fig. 8C). Consistently, the expression of osteogenic markers, including RUNX2, OPN, osterix and ALP, was significantly suppressed by Sirt1 overexpression, but was restored upon the knockdown of either HO-1 or FTH1 (Fig. 8D).

These findings were further supported by western blot analysis, which revealed an increased nuclear RUNX2 accumulation following HO-1 or FTH1 silencing compared with the Sirt1 overexpression group (Fig. S2A). To further investigate the underlying signaling pathway, key components of the Nrf2/HO-1 axis were analyzed. Compared with the OM and OM + vector + shNC groups, Sirt1 overexpression markedly reduced KEAP1 protein expression and increased the nuclear

Nrf2 levels, accompanied by a decrease in cytoplasmic Nrf2, and the upregulation of HO-1 and NQO1 expression. Upon HO-1 knockdown, HO-1 protein expression was significantly reduced, and NQO1 levels were correspondingly decreased. In addition, KEAP1 expression and both nuclear and cytoplasmic Nrf2 levels exhibited no obvious changes compared with the Sirt1 overexpression group. By contrast, FTH1 silencing did not significantly affect the expression of HO-1, NQO1, KEAP1, or the nuclear and cytoplasmic distribution of Nrf2 compared with the Sirt1 overexpression group (Fig. S2B).

In addition, biochemical analysis revealed that Sirt1 overexpression reduced MDA levels and increased the GSH content, while these effects were reversed by the knockdown of either HO-1 or FTH1 (Fig. S2C), indicating a restoration of oxidative stress and ferroptosis-related imbalance. Collectively, these results indicate that both HO-1 and FTH1 are involved in mediating the inhibitory effects of Sirt1 on osteogenic differentiation and ferroptosis in hAVICs.

Activation of Sirt1 attenuates aortic valve calcification in Apoe^{-/-} mice. To validate the *in vitro* findings *in vivo*, a mouse model of CAVD was established using Apoe^{-/-} mice fed a Western diet, with or without the administration of SRT2104,

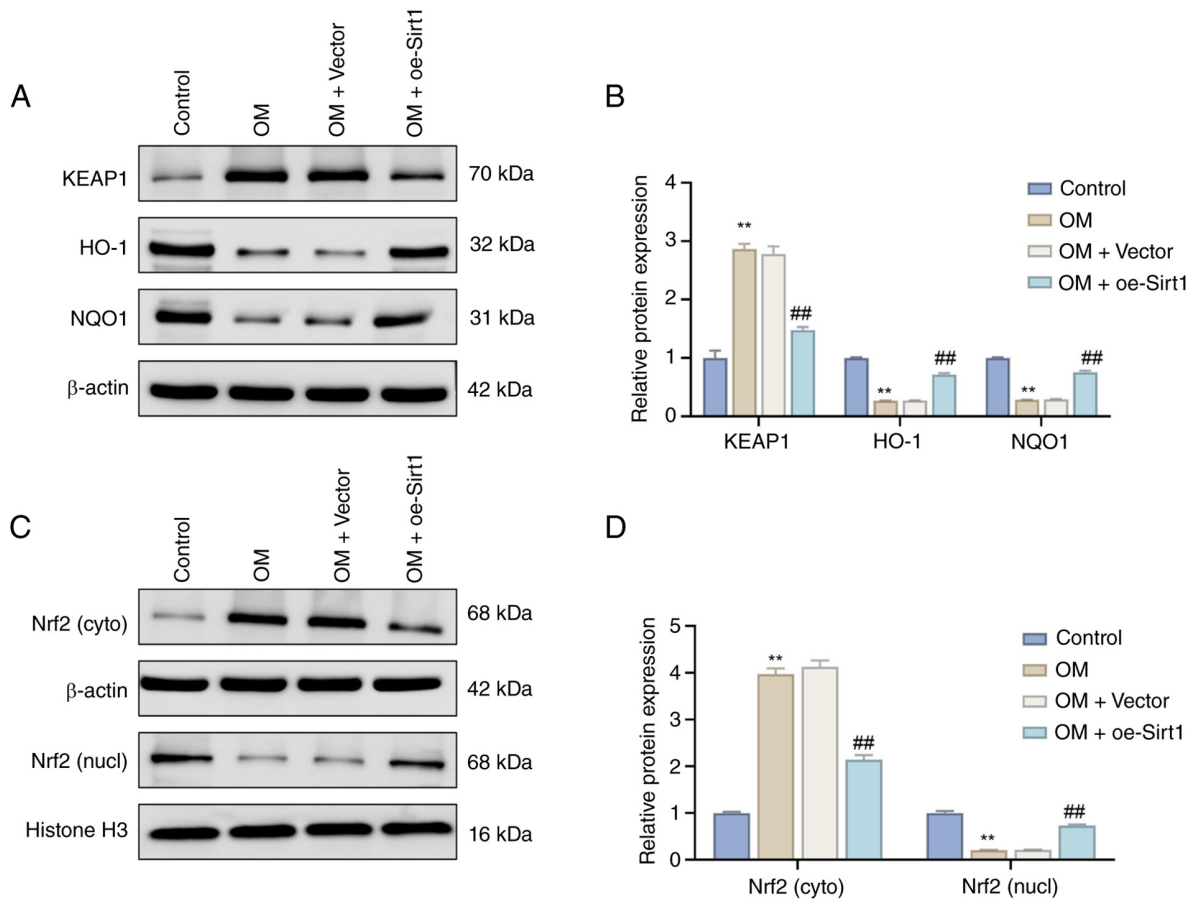


Figure 5. Overexpression of Sirt1 activates the Nrf2/HO-1 pathway in osteogenically-induced aortic valve interstitial cells. (A and B) Western blot analysis of KEAP1, HO-1 and NQO1 protein levels in cells from each group. (C and D) Cytoplasmic and nuclear fractions were isolated to evaluate Nrf2 protein localization using western blot analysis. Data represent the mean $\pm$ SD from three independent experiments. Comparisons were performed using one-way ANOVA. **P<0.01 vs. control; ##P<0.01 vs. OM + vector. KEAP1, Kelch-like ECH-associated protein 1; HO-1, heme oxygenase 1; NQO1, NAD(P)H quinone dehydrogenase 1; Sirt1, sirtuin 1; OM, osteogenic medium; oe, overexpression.

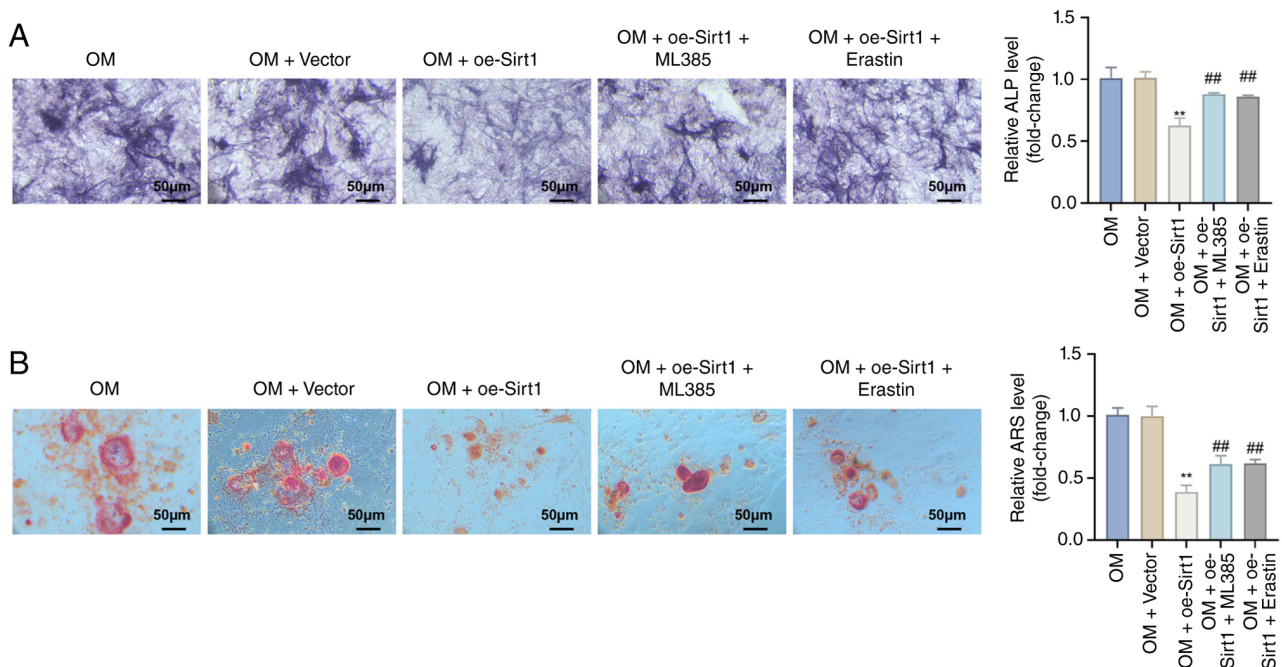


Figure 6. Inhibition of Nrf2 signaling or induction of ferroptosis reverses the inhibitory effect of Sirt1 overexpression on osteogenic differentiation and mineralization in hAVICs. (A) ALP and (B) Alizarin Red S staining were used to assess ALP activity and bone mineralization. Data represent the mean $\pm$ SD from three independent experiments. Comparisons were performed using one-way ANOVA. **P<0.01 vs. OM + vector; ##P<0.01 vs. OM + oe-Sirt1. ALP, alkaline phosphatase.

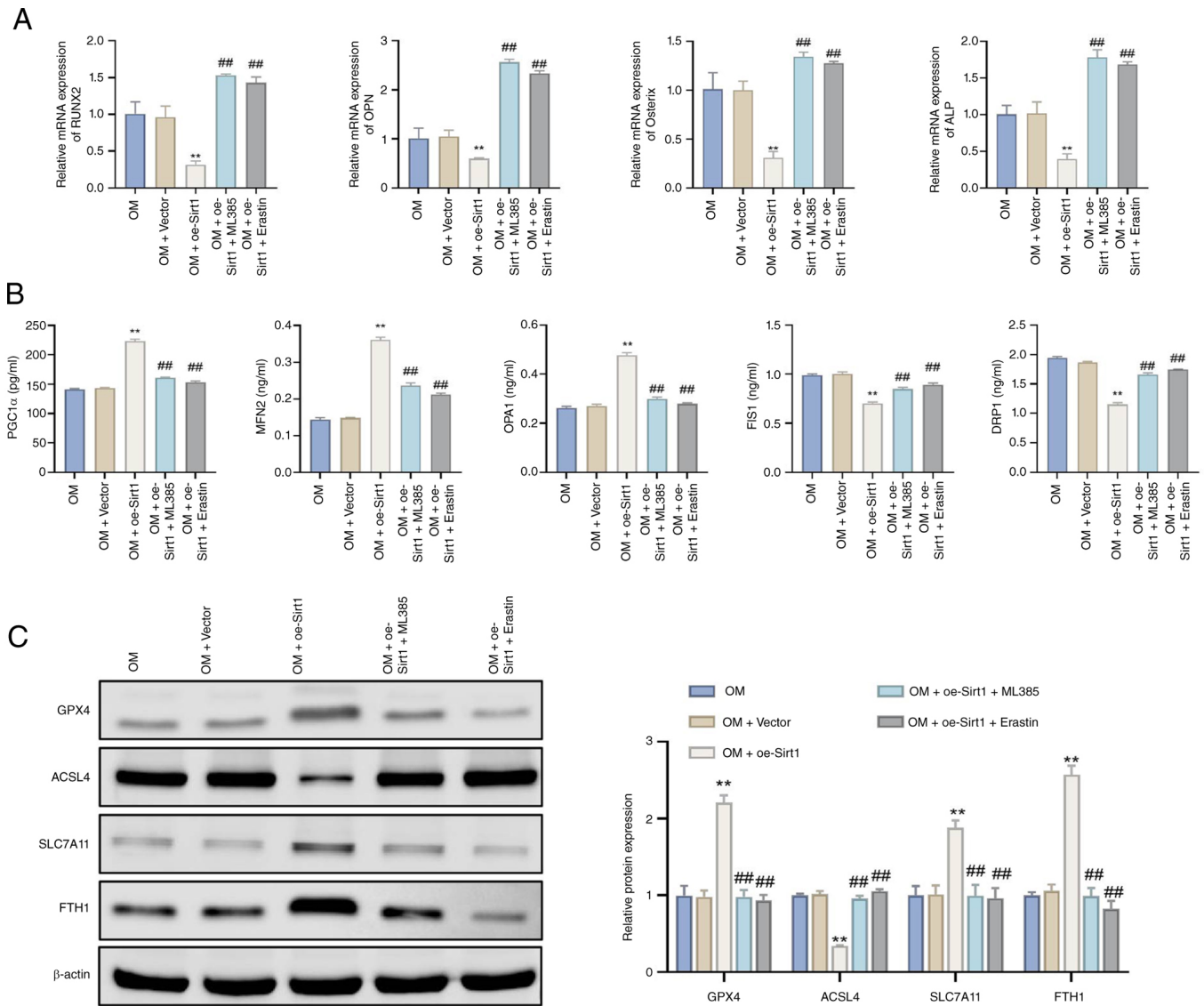


Figure 7. Inhibition of Nrf2 signaling or induction of ferroptosis reverses the molecular protective effects of Sirt1 overexpression in hAIVCs. (A) Reverse transcription-quantitative polymerase chain reaction was used to evaluate the expression of the osteogenic markers, RUNX2, OPN, osterix and ALP; (B) Mitochondrial function-related proteins PGC1 α , MFN2, OPA1, FIS1 and DRP1 were measured using ELISA; (C) Western blot analysis of ferroptosis-related proteins (GPX4, ACSL4, SLC7A11 and FTH1). Data represent the mean $\pm$ SD from three independent experiments. Comparisons were performed using one-way ANOVA. ** $P < 0.01$ vs. OM + vector; ## $P < 0.01$ vs. OM + oe-Sirt1. ALP, alkaline phosphatase; RUNX2, RUNX family transcription factor 2; OPN, osteopontin; PGC1 α , peroxisome proliferator-activated receptor gamma coactivator 1-alpha; MFN2, mitofusin 2; OPA1, optic atrophy 1; FIS1, fission 1; DRP1, dynamin-related protein 1; GPX4, glutathione peroxidase 4; ACSL4, acyl-CoA synthetase long-chain family member 4; SLC7A11, solute carrier family 7 member 11; FTH1, ferritin heavy chain 1. ML385, Nrf2 inhibitor; erastin, ferroptosis inducer; Sirt1, sirtuin 1; OM, osteogenic medium; oe, overexpression.

a pharmacological Sirt1 activator. Following 24 weeks of feeding, the tissue sample was collected, and H&E staining was performed for histological evaluation.

The histological evaluation of aortic valve tissues via H&E staining revealed that the calcified tissues exhibited marked valve thickening, structural disorganization and inflammatory cell infiltration. By contrast, the SRT2104-treated group demonstrated preserved trilaminar architecture, reduced leaflet thickness and diminished inflammatory features, suggesting alleviated pathological remodeling (Fig. 9A). These findings indicate that SRT2104 ameliorates calcification-associated structural changes *in vivo*.

At the molecular level, RT-qPCR analysis revealed that the levels of osteogenic markers (RUNX2, OPN, osterix and ALP)

were significantly upregulated in the calcified group and were markedly reduced following SRT2104 treatment (Fig. 9B), indicating the suppression of osteogenic transformation.

To determine whether the protective effects were mediated via the Nrf2/HO-1 pathway, the levels of related proteins were assessed. Compared to the calcified group, the SRT2104-treated mice exhibited reduced KEAP1 levels, an elevated expression of HO-1 and NQO1, and an enhanced nuclear accumulation of Nrf2, along with decreased cytoplasmic retention (Fig. 9C and D). Collectively, these findings suggest that the pharmacological activation of Sirt1 attenuates calcification-associated pathological remodeling *in vivo*, at least in part, through the reactivation of Nrf2/HO-1 signaling and the suppression of osteogenic transformation.

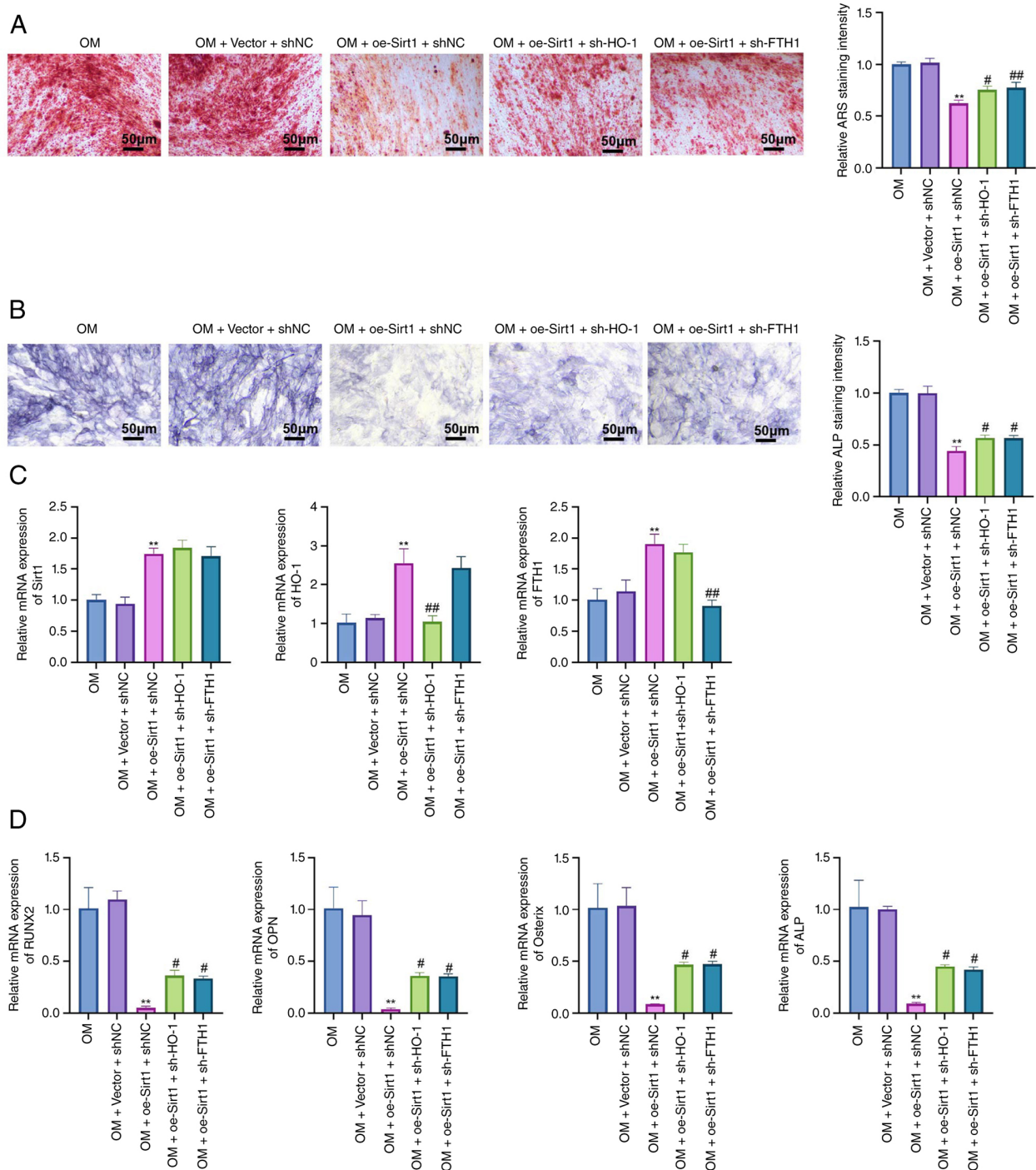


Figure 8. HO-1 and FTH1 contribute to the Sirt1-mediated inhibition of osteogenic differentiation and calcification in hAVICs. (A) Alizarin Red S staining and (B) ALP staining were performed to assess mineralization and osteogenic activity in each group; (C) RT-qPCR analysis of Sirt1, HO-1 and FTH1 mRNA expression levels in the different groups; (D) RT-qPCR analysis of osteogenic markers RUNX2, OPN, osterix and ALP expression levels. Data represent the mean $\pm$ SD from three independent experiments. Comparisons were performed using one-way ANOVA. ** $P < 0.01$ vs. OM + vector + shNC; # $P < 0.05$ and ## $P < 0.01$ vs. OM + oe-Sirt1+ shNC. RT-qPCR, reverse transcription-quantitative PCR; OM, osteogenic medium; Sirt1, sirtuin 1; HO-1, heme oxygenase 1; FTH1, ferritin heavy chain 1; RUNX2, RUNX family transcription factor 2; OPN, osteopontin; ALP, alkaline phosphatase.

FTH1 mediates the protective effects of Sirt1 against aortic valve calcification in vivo. To validate the relative contributions of HO-1 and FTH1 *in vivo*, Apoe^{-/-} mice treated with SRT2104 were further subjected to AAV-mediated silencing of HO-1 or FTH1. Histological analyses, including H&E and Alizarin Red S staining, revealed that SRT2104 markedly attenuated aortic valve pathological remodeling and calcium deposition compared with the calcified group. The silencing of either

HO-1 or FTH1 partially reversed these protective effects, as evidenced by increased leaflet thickening, structural disorganization, inflammatory infiltration and Alizarin Red-positive staining. The AAV-shHO-1 and AAV-shFTH1 groups exhibited comparable pathological changes and calcium deposition (Fig. 10A and B).

At the molecular level, RT-qPCR demonstrated that SRT2104 significantly reduced the expression of osteogenic

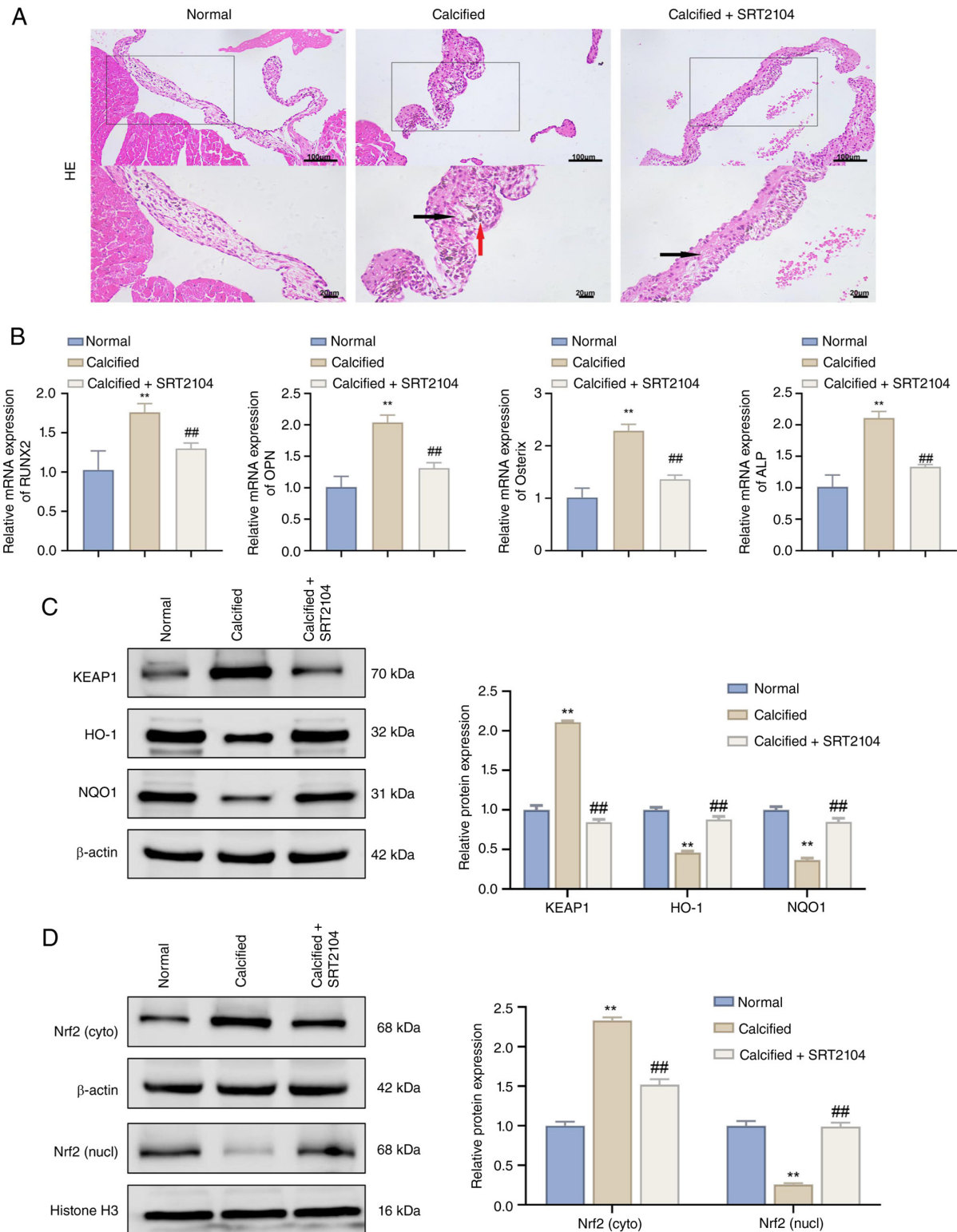


Figure 9. Activation of Sirt1 *in vivo* attenuates calcification-associated pathological remodeling in *Apoe*^{-/-} mice. (A) H&E staining for examining the pathological damage of aortic valve tissues from all groups; (B) reverse transcription-quantitative PCR analysis of osteogenic marker (RUNX2, OPN, osterix and ALP) expression levels in aortic valve tissues; (C) western blot analysis of KEAP1, HO-1 and NQO1 protein levels in aortic valve tissues; (D) western blot analysis of Nrf2 protein levels in cytoplasmic and nuclear fractions. For animal experiments, each individual mouse was considered as one biological replicate (n=10 per group). Comparisons were performed using one-way ANOVA. **P<0.01 vs. normal; ##P<0.01 vs. calcified + SRT2104. H&E, hematoxylin and eosin; RUNX2, RUNX family transcription factor 2; OPN, osteopontin; ALP, alkaline phosphatase; KEAP1, Kelch-like ECH-associated protein 1; HO-1, heme oxygenase 1; NQO1, NAD (P) H quinone dehydrogenase 1. SRT2104, a pharmacological sirtuin 1 activator.

markers (RUNX2, OPN, osterix and ALP), whereas the knock-down of HO-1 or FTH1 partially restored their expression levels (Fig. 10C). Consistently, western blot analysis revealed

that SRT2104 decreased nuclear RUNX2 protein expression, while this effect was reversed following the silencing of HO-1 or FTH1 (Fig. S3A).

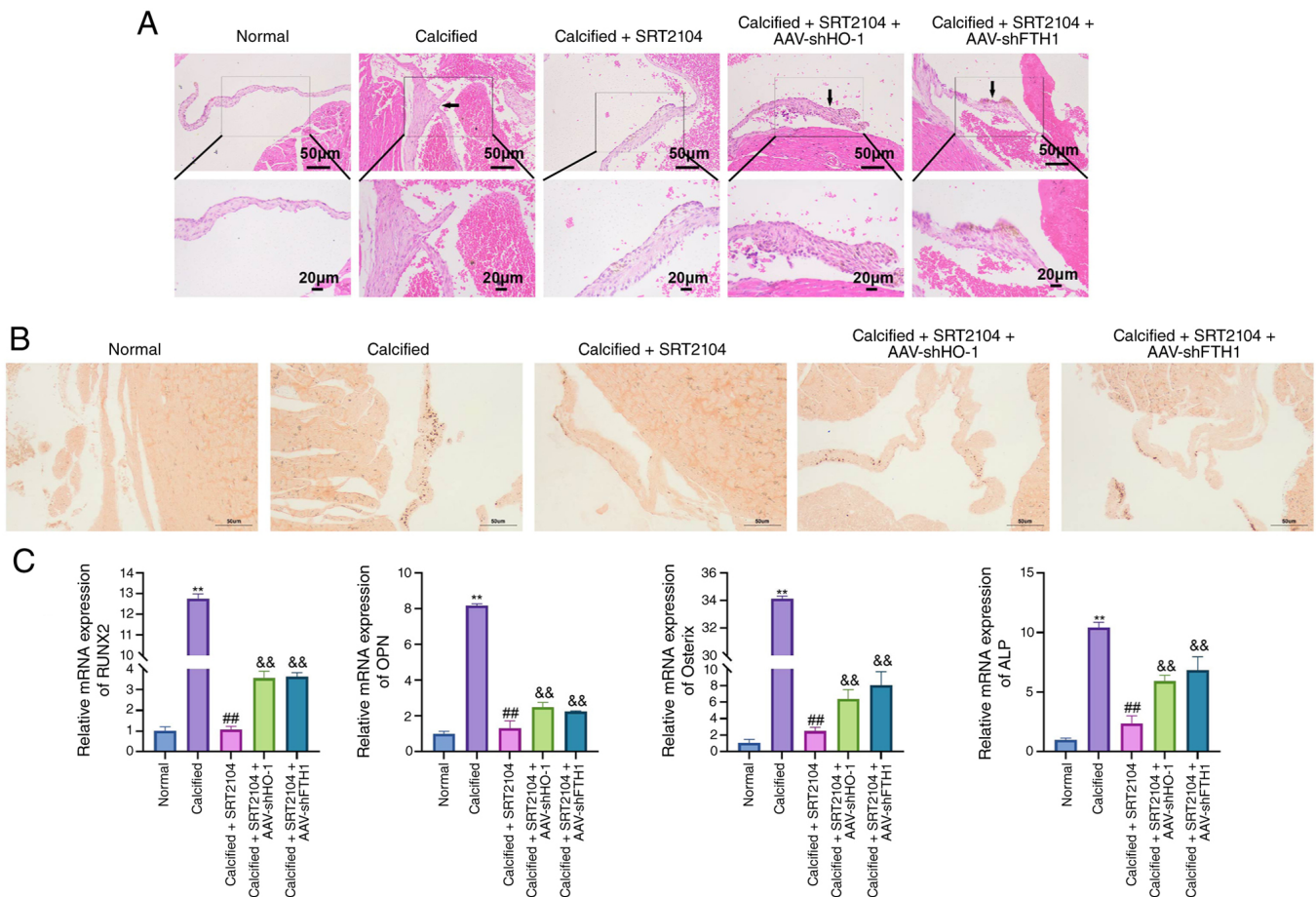


Figure 10. HO-1 and FTH1 contribute to the protective effects of Sirt1 activation against aortic valve calcification *in vivo*. Representative (A) H&E staining and (B) Alizarin Red S staining of mouse aortic valve sections illustrating leaflet morphology and pathological remodeling in different experimental groups. (C) Reverse transcription-quantitative PCR analysis of the expression levels of the osteogenic markers, RUNX2, OPN, osterix and ALP. Data represent the mean $\pm$ SD. For animal experiments, each individual mouse was considered as one biological replicate (n=10 per group). Comparisons were performed using one-way ANOVA. **P<0.01 vs. normal; ##P<0.01 vs. calcified; &&P<0.01 vs. calcified + SRT2104. Normal, Apoe^{-/-} mice on a standard diet; calcified, Apoe^{-/-} mice fed a Western diet; Calcified + SRT2104, Apoe^{-/-} mice treated with SRT2104; AAV-shHO-1, adeno-associated virus-mediated HO-1 knockdown; AAV-shFTH1, adeno-associated virus-mediated FTH1 knockdown; RUNX2, RUNX family transcription factor 2; OPN, osteopontin; ALP, alkaline phosphatase; KEAP1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase 1; NQO1, NAD(P)H quinone dehydrogenase 1; FTH1, ferritin heavy chain 1.

Further analysis of the Nrf2/HO-1 signaling pathway revealed that SRT2104 treatment decreased KEAP1 expression, increased nuclear Nrf2 levels, reduced cytoplasmic Nrf2 levels, and upregulated the expression of HO-1, NQO1 and FTH1. Notably, FTH1 expression was reduced in calcified mice compared with the normal controls and was restored following SRT2104 treatment. Upon HO-1 knockdown, HO-1 protein expression was markedly reduced, accompanied by decreases in the NQO1 and FTH1 levels, whereas KEAP1 expression and Nrf2 nuclear and cytoplasmic distribution exhibited no obvious changes compared with the SRT2104-treated group. By contrast, FTH1 silencing markedly reduced FTH1 protein expression, while the KEAP1, Nrf2 (nuclear and cytoplasmic), HO-1 and NQO1 levels remained largely unaltered compared with the SRT2104-treated group (Fig. S3B). Collectively, these results indicate that both HO-1 and FTH1 participate in the protective effects of Sirt1 activation against aortic valve calcification *in vivo*.

Discussion

CAVD is a progressive degenerative cardiovascular disorder characterized by a markedly increased prevalence with

advancing age; in severe cases, it may progress to calcific aortic stenosis, ultimately leading to heart failure (1). Given the absence of effective drug therapies capable of preventing or reversing CAVD progression, there is an urgent need to unravel the underlying molecular mechanisms of CAVD pathogenesis and to identify potential therapeutic targets. It is important to acknowledge that the pathogenesis of CAVD is multifactorial, involving a complex interplay of chronic inflammation, lipid dysregulation, oxidative stress, aberrant cell differentiation, and extracellular matrix remodeling. The present study focused on investigating a specific regulatory axis within this network. The present provides evidence that Sirt1 may play a pivotal role in the progression of CAVD by regulating ferroptosis, providing important insights into potential strategies for delaying disease progression.

As a deacetylase, Sirt1 regulates the activity of multiple signaling pathways and has long been demonstrated to be closely associated with vascular calcification. For instance, Bartoli-Leonard *et al* (21) reported that the loss of Sirt1 expression in patients with diabetes accelerated DNA damage and promoted vascular calcification. Liu *et al* (11) demonstrated that the upregulation of Sirt1 expression by spermidine effectively

inhibited mineral deposition in rat and human vascular smooth muscle cells. Moreover, Yu *et al* (22) revealed that luteolin could directly bind to and activates Sirt1, thereby inhibiting oxidative stress and enhancing autophagy to protect against vascular calcification. These findings suggest that Sirt1 is a key participant in the process of vascular calcification. Consistently, the present study revealed that Sirt1 expression was markedly downregulated in aortic valve tissues from both patients with CAVD and cellular models. Notably, Sirt1 overexpression effectively attenuated AVIC calcification, consistent with the previously reported effects of Sirt1 on calcification in other cardiovascular diseases (23,24). These observations indicate that Sirt1 plays a decisive role in the progression of CAVD.

In recent years, mitochondrial dysfunction has been recognized as a key driving factor in the onset and progression of CAVD (25). In contrast to nuclear DNA, mtDNA is not bound to histones, rendering mtDNA repair mechanisms relatively inefficient. As a result, mtDNA is inherently unstable and susceptible to mutations and deletions (26). Rothwell *et al* (27) observed that DNA polymerase γ -deficient mice displayed substantial mtDNA damage and aggravated vascular calcification. Additionally, extensive mitochondrial fragmentation has been observed in calcified cardiovascular diseases, and this fragmentation has been shown to induce oxidative stress and promote mitochondrial depolarization by regulating the expression of mitochondrial fission-fusion proteins (28). Notably, research has revealed that DRP1 is enriched in calcified areas of the human carotid artery, and inhibiting DRP1 can alleviate oxidative stress, thereby mitigating cardiovascular calcification (29). In the present study, the expression of mitochondrial fusion proteins (MFN2 and OPA1) was decreased, while the expression of mitochondrial fission proteins (DRP1 and FIS1) was increased in CAVD cells, suggesting a shift in mitochondrial dynamics towards fission (30). Furthermore, mitochondrial membrane potential was significantly reduced, indicating mitochondrial dysfunction (31). Additionally, mtDNA replication capacity decreased, ATP production was reduced, and both ROS and mitochondrial-specific ROS levels were significantly elevated, all indicating mitochondrial damage in CAVD cells. However, it was also observed that the overexpression of Sirt1 effectively alleviated mitochondrial damage in CAVD cells.

Similarly, ferroptosis has been linked to various cardiovascular calcification-related diseases (32). The core mechanism of ferroptosis is the accumulation of lipid peroxides, primarily mediated by iron-dependent Fenton reactions, resulting in elevated ROS levels and damage to the cell membrane (33). In the process of cardiovascular calcification, vascular endothelial cells and smooth muscle cells are exposed to high phosphate environments, inflammatory cytokines and oxidative stress, resulting in mitochondrial dysfunction. This, in turn, promotes the accumulation of iron ions and lipid peroxidation, ultimately inducing ferroptosis (34). It has been demonstrated that ferroptosis not only exacerbates AVICs, but also induces the mineralization of the extracellular matrix, accelerating calcific deposition in the valve (6). The present study revealed that under osteogenic medium induction, MDA, Fe^{2+} and total iron levels accumulated in AVICs, while GSH was depleted. Additionally, a significant decrease in the expression of key negative regulators of ferroptosis, including GPX4, SLC7A11 and FTH1 was observed, while the expression of the ferroptosis-promoting

protein, ACSL4, was upregulated, all of which are consistent with the results of previous research (35,36). These findings indicate an elevated level of ferroptosis in CAVD cells.

More notably, the present study revealed that the overexpression of Sirt1 effectively inhibited mitochondrial dysfunction and ferroptosis in cells. As a deacetylase, Sirt1 regulates the activity of multiple signaling pathways. Among these, the activation of the Nrf2/HO-1 pathway is considered to reduce cardiovascular calcification (37). Notably, the protective role of the Nrf2/HO-1 axis against calcification may extend beyond HO-1 itself, involving its downstream effector, H-ferritin (FTH1). A well-established regulatory circuit exists where HO-1 catalyzes heme degradation, releasing iron which in turn transcriptionally upregulates ferritin, particularly the heavy chain (FTH1), via iron-responsive elements (38). Herein, the authors did consider this important scenario. The data presented herein demonstrated that Sirt1 activation concurrently upregulated both HO-1 and FTH1 in cells, and reduced labile Fe^{2+} levels. This pattern is consistent with the described feedback mechanism where HO-1 induction leads to increased cellular iron, which post-transcriptionally induces FTH1 synthesis to manage the iron load and exert its ferroxidase activity. The observed reduction in labile Fe^{2+} upon Sirt1 overexpression is plausible given the concurrent upregulation of FTH1. The ferroxidase activity of H-ferritin is crucial for sequestering labile iron and mitigating iron-catalyzed oxidative damage (39), thereby exerting anti-calcification effects in vascular smooth muscle cells and valvular interstitial cells (40-42). While HO-1 and ferritin expression levels are often connected in various pathologies (43), their functions can be distinct, and the benefit against mineralization has been specifically attributed to the ferroxidase activity of H-ferritin rather than to HO-1-derived products such as carbon monoxide or bilirubin (42). Lu *et al* (44) discovered that the activation of the Nrf2/HO-1 pathway activity reduced vascular calcification in mice with chronic kidney disease; it also inhibits osteoblast maturation and mineralization (45). Sirt1-mediated deacetylation can suppress Keap1-mediated ubiquitination degradation, activating Nrf2 and facilitating its nuclear translocation to induce the expression of HO-1 and other antioxidant and iron homeostasis genes (46). Of note, in the present study, loss-of-function experiments further refined the hierarchical association between HO-1 and FTH1 within this axis. *In vitro*, the silencing of HO-1 led to a reduction in NQO1 expression without significantly affecting KEAP1 or Nrf2 distribution, whereas FTH1 knockdown had a minimal impact on upstream Nrf2/HO-1 signaling components. Similarly, *in vivo* experiments demonstrated that HO-1 silencing reduced both HO-1 and NQO1 expression, while FTH1 knockdown selectively decreased FTH1 levels without altering KEAP1 or Nrf2 localization. These findings suggest that HO-1 primarily functions as an upstream regulator within the Nrf2 signaling pathway, whereas FTH1 functions as a downstream effector involved in iron sequestration and ferroptosis modulation. Therefore, the anti-calcification effects of Sirt1 are likely mediated through coordinated regulation of both signaling activation (via HO-1) and iron homeostasis control (via FTH1). HO-1 contributes to cellular iron metabolism by catalyzing heme degradation, whereas the sequestration of labile iron is primarily mediated by ferritin, particularly FTH1. The reductive capacity of HO-1 can effectively reduce mitochondrial ROS levels, thereby protecting mitochondrial function (47). The findings

of the present study confirm that Sirt1 effectively activates the Nrf2/HO-1 pathway, and *in vivo* experiments in mice provided evidence that Sirt1 activation may alleviate aortic calcification via the Nrf2/HO-1 pathway.

However, the present study has several limitations which should be acknowledged. First, the temporal changes in Sirt1 expression during different stages of CAVD progression were not explored, which may provide deeper insight into its role in disease development. Second, although the involvement of the Nrf2/HO-1 pathway was supported by pharmacological inhibition, the absence of genetic validation (e.g., siRNA or knockout models) limits the conclusiveness of pathway specificity. Third, the present study utilized a mixed osteogenic cocktail (containing β -glycerophosphate) for induction. Future studies are required to examine whether Sirt1 exerts similar protective effects against calcification induced specifically by elevated inorganic phosphate, a key pathological driver of calcification in chronic kidney disease-related CAVD. Finally, *in vivo* experiments relied on the pharmacological activation of Sirt1, and direct histological assessment of calcium deposition (e.g., Alizarin Red or OsteoSense staining) was not performed; therefore, stronger evidence could be obtained by incorporating genetic models (e.g., Sirt1 knockout or conditional knockout) and more specific mineralization assays in future studies. Further studies are thus warranted to enhance mechanistic clarity and provide translational relevance.

In conclusion, the present study demonstrates that Sirt1 protects against CAVD by activating the Nrf2/HO-1 pathway, leading to FTH1-dependent maintenance of iron homeostasis, the suppression of ferroptosis, the preservation of mitochondrial function and the inhibition of osteogenic differentiation in AVICs, thereby attenuating calcification-associated pathological remodeling. These findings identify the Sirt1/Nrf2/HO-1/FTH1 signaling axis as a key regulatory pathway linking iron homeostasis, ferroptosis, mitochondrial dysfunction and CAVD progression, and highlight Sirt1 as a therapeutic target for CAVD.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

FY was involved in the conceptualization of the study, in data curation, formal analysis, data investigation, study supervision, data validation and visualization, as well as in the writing of the original draft, and in the writing, reviewing and editing of the manuscript. LS was involved in data curation, formal analysis,

investigation, in the study methodology, and in the writing, reviewing and editing of the manuscript. YWa was involved in data curation, data investigation, in the study methodology, and writing, reviewing and editing of the manuscript. YZ was involved in data curation and in the formal analysis, as well as in the writing, reviewing and editing of the manuscript. YWu was involved in the formal analysis, data investigation, in the study methodology, and in the writing, reviewing and editing of the manuscript. DX, YL, NW, HC and MZ were involved in data curation and in the formal analysis, as well as in the writing, reviewing and editing of the manuscript. FY and MZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Prior to initiation, the present study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Hainan Medical University (Approval no. 2025-KYL-016). Sample collection and processing were conducted in strict accordance with the Declaration of Helsinki and the Regulations on the Management of Human Genetic Resources. Written informed consent was obtained from all participants prior to tissue collection. All animal experiments were approved by Ethics Committee of Hainan Medical University (Approval no. HYALL-2025-028) and were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Kraler S, Blaser MC, Aikawa E, Camici GG and Lüscher TF: Calcific aortic valve disease: From molecular and cellular mechanisms to medical therapy. *Eur Heart J* 43: 683-697, 2022.
2. Yu J, Wang Z, Bao Q, Lei S, You Y, Yin Z and Xie X: Global burden of calcific aortic valve disease and attributable risk factors from 1990 to 2019. *Front Cardiovasc Med* 9: 1003233, 2022.
3. Tanase DM, Valasciuc E, Gosav EM, Floria M, Costea CF, Dima N, Tudorancea I, Maranduca MA and Serban IL: Contribution of oxidative stress (OS) in calcific aortic valve disease (CAVD): From pathophysiology to therapeutic targets. *Cells* 11: 2663, 2022.
4. Liu Z, Dong N, Hui H, Wang Y, Liu F, Xu L, Liu M, Rao Z, Yuan Z, Shang Y, *et al*: Endothelial cell-derived tetrahydrobiopterin prevents aortic valve calcification. *Eur Heart J* 43: 1652-1664, 2022.
5. Li XZ, Xiong ZC, Zhang SL, Hao QY, Gao M, Wang JF, Gao JW and Liu PM: Potential ferroptosis key genes in calcific aortic valve disease. *Front Cardiovasc Med* 9: 916841, 2022.
6. Wang S, Gu J, Bian J, He Y, Xu X, Wang C, Li G, Zhang H, Ni B, Chen S, *et al*: Nesfatin-1 mitigates calcific aortic valve disease via suppressing ferroptosis mediated by GSH/GPX4 and ZIP8/SOD2 axes. *Free Radic Biol Med* 222: 149-164, 2024.
7. Budbazar E, Rodriguez F, Sanchez JM and Seta F: The role of sirtuin-1 in the vasculature: Focus on aortic aneurysm. *Front Physiol* 11: 1047, 2020.
8. Chen H, Lin X, Yi X, Liu X, Yu R, Fan W, Ling Y, Liu Y and Xie W: SIRT1-mediated p53 deacetylation inhibits ferroptosis and alleviates heat stress-induced lung epithelial cells injury. *Int J Hyperthermia* 39: 977-986, 2022.

9. Chen Z, Li J, Peng H, Zhang M, Wu X, Gui F, Li W, Ai F, Yu B and Liu Y: Meteorin-like/Meteorin- β protects LPS-induced acute lung injury by activating SIRT1-P53-SLC7A11 mediated ferroptosis pathway. *Mol Med* 29: 144, 2023.
10. Li SJ, Cheng TY, Kao YH, Chen WY, Chung CC, Trang NN, Chen PH and Chen YJ: Uremic toxin p-cresyl sulfate enhances the calcification of aortic valvular interstitial cells via klotho/sirtuin-1 signaling. *Mol Med Rep* 33: 162, 2026.
11. Liu X, Chen A, Liang Q, Yang X, Dong Q, Fu M, Wang S, Li Y, Ye Y, Lan Z, *et al*: Spermidine inhibits vascular calcification in chronic kidney disease through modulation of SIRT1 signaling pathway. *Aging Cell* 20: e13377, 2021.
12. Ouyang X, Ding Y, Yu L, Xin F and Yang X: LncRNA TUG regulates osteogenic differentiation of bone marrow mesenchymal stem cells via miRNA-204/SIRT 1. *J Musculoskelet Neuronal Interact* 22: 401-410, 2022.
13. Li Y, Zeng X, Lu D, Yin M, Shan M and Gao Y: Erastin induces ferroptosis via ferroportin-mediated iron accumulation in endometriosis. *Hum Reprod* 36: 951-964, 2021.
14. Song J, Wang H, Sheng J, Zhang W, Lei J, Gan W, Cai F and Yang Y: Vitexin attenuates chronic kidney disease by inhibiting renal tubular epithelial cell ferroptosis via NRF2 activation. *Mol Med* 29: 147, 2023.
15. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
16. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals: Guide for the Care and Use of Laboratory Animals. 8th edition. National Academies Press (US), Washington, DC, 2011.
17. Combi Z, Potor L, Nagy P, Sikura KÉ, Ditrói T, Jurányi EP, Galambos K, Szerafin T, Gergely P, Whiteman M, *et al*: Hydrogen sulfide as an anti-calcification stratagem in human aortic valve: Altered biogenesis and mitochondrial metabolism of H₂S lead to H₂S deficiency in calcific aortic valve disease. *Redox Biol* 60: 102629, 2023.
18. Zeng Y, He Y, Wang L, Xu H, Zhang Q, Wang Y, Zhang J and Wang L: Dihydroquercetin improves experimental acute liver failure by targeting ferroptosis and mitochondria-mediated apoptosis through the SIRT1/p53 axis. *Phytomedicine* 128: 155533, 2024.
19. Dang R, Wang M, Li X, Wang H, Liu L, Wu Q, Zhao J, Ji P, Zhong L, Licinio J and Xie P: Edaravone ameliorates depressive and anxiety-like behaviors via Sirt1/Nrf2/HO-1/Gpx4 pathway. *J Neuroinflammation* 19: 41, 2022.
20. Lv QK, Tao KX, Yao XY, Pang MZ, Cao BE, Liu CF and Wang F: Melatonin MT1 receptors regulate the Sirt1/Nrf2/Ho-1/Gpx4 pathway to prevent α -synuclein-induced ferroptosis in Parkinson's disease. *J Pineal Res* 76: e12948, 2024.
21. Bartoli-Leonard F, Wilkinson FL, Schiro A, Serracino Inglott F, Alexander MY and Weston R: Loss of SIRT1 in diabetes accelerates DNA damage-induced vascular calcification. *Cardiovasc Res* 117: 836-849, 2021.
22. Yu X, Xu L, Su C, Wang C, Wang Z, Wang Y, Lu X and Sun H: Luteolin protects against vascular calcification by modulating SIRT1/CXCR4 signaling pathway and promoting autophagy. *AAPS J* 26: 111, 2024.
23. Lu CL, Liao MT, Hou YC, Fang YW, Zheng CM, Liu WC, Chao CT, Lu KC and Ng YY: Sirtuin-1 and Its relevance in vascular calcification. *Int J Mol Sci* 21: 1593, 2020.
24. Ren Z, Song B, Peng Y, Bian C, Shen Q, Zhou M, Li B and Jin X: Mechanism and treatment of Sirtuin family in vascular calcification. *Front Cardiovasc Med* 12: 1732281, 2025.
25. Li M, Zhu Y, Jaiswal SK and Liu NF: Mitochondria homeostasis and vascular medial calcification. *Calcif Tissue Int* 109: 113-120, 2021.
26. de Souza-Pinto NC, Mason PA, Hashiguchi K, Weissman L, Tian J, Guay D, Lebel M, Stevnsner TV, Rasmussen LJ and Bohr VA: Novel DNA mismatch-repair activity involving YB-1 in human mitochondria. *DNA Repair (Amst)* 8: 704-719, 2009.
27. Rothwell S, Ng I, Shalchy-Tabrizi S, Kalinowski P, Taha OM, Paris I, Baniqued A, Lin L, Mezei MM, Lehman A, *et al*: Loss-of-function mitochondrial DNA polymerase gamma variants cause vascular smooth muscle cells to secrete a diffusible mitogenic factor. *Front Physiol* 15: 1488248, 2025.
28. Wu S, Zhou F, Zhang Z and Xing D: Mitochondrial oxidative stress causes mitochondrial fragmentation via differential modulation of mitochondrial fission-fusion proteins. *FEBS J* 278: 941-954, 2011.
29. Rogers MA, Maldonado N, Hutcheson JD, Goettsch C, Goto S, Yamada I, Faits T, Sesaki H, Aikawa M and Aikawa E: Dynamin-related protein 1 inhibition attenuates cardiovascular calcification in the presence of oxidative stress. *Circ Res* 121: 220-233, 2017.
30. Liu YZ, Li ZX, Zhang LL, Wang D and Liu YP: Phenotypic plasticity of vascular smooth muscle cells in vascular calcification: Role of mitochondria. *Front Cardiovasc Med* 9: 972836, 2022.
31. Wang PW, Pang Q, Zhou T, Song XY, Pan YJ, Jia LP and Zhang AH: Irisin alleviates vascular calcification by inhibiting VSMC osteoblastic transformation and mitochondria dysfunction via AMPK/Drp1 signaling pathway in chronic kidney disease. *Atherosclerosis* 346: 36-45, 2022.
32. Chen Z, Sun X, Li X and Liu N: Oleoylethanolamide alleviates hyperlipidaemia-mediated vascular calcification via attenuating mitochondrial DNA stress triggered autophagy-dependent ferroptosis by activating PPAR α . *Biochem Pharmacol* 208: 115379, 2023.
33. Ma WQ, Sun XJ, Zhu Y and Liu NF: Metformin attenuates hyperlipidaemia-associated vascular calcification through anti-ferroptotic effects. *Free Radic Biol Med* 165: 229-242, 2021.
34. Wang Y, Zhang Y, Gao M, Chen Z, Lu J, Li Y, Di Y, Zhao Y, Liu B and Tang R: Lipocalin-2 promotes CKD vascular calcification by aggravating VSMCs ferroptosis through NCOA4/FTH1-mediated ferritinophagy. *Cell Death Dis* 15: 865, 2024.
35. Ye Y, Chen A, Li L, Liang Q, Wang S, Dong Q, Fu M, Lan Z, Li Y, Liu X, *et al*: Repression of the antiporter SLC7A11/glutathione/glutathione peroxidase 4 axis drives ferroptosis of vascular smooth muscle cells to facilitate vascular calcification. *Kidney Int* 102: 1259-1275, 2022.
36. Li M, Wang ZW, Fang LJ, Cheng SQ, Wang X and Liu NF: Programmed cell death in atherosclerosis and vascular calcification. *Cell Death Dis* 13: 467, 2022.
37. Chowdhury A, Balogh E, Ababneh H, Tóth A and Jeney V: Activation of Nrf2/HO-1 antioxidant pathway by heme attenuates calcification of human lens epithelial cells. *Pharmaceuticals (Basel)* 15: 493, 2022.
38. Balla J, Jacob HS, Balla G, Nath K, Eaton JW and Vercellotti GM: Endothelial-cell heme uptake from heme proteins: Induction of sensitization and desensitization to oxidant damage. *Proc Natl Acad Sci USA* 90: 9285-9289, 1993.
39. Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW and Vercellotti GM: Ferritin: A cytoprotective antioxidant stratagem of endothelium. *J Biol Chem* 267: 18148-18153, 1992.
40. Becs G, Zarjou A, Agarwal A, Kovács KÉ, Becs Á, Nyitrai M, Balogh E, Bánya E, Eaton JW, Arosio P, *et al*: Pharmacological induction of ferritin prevents osteoblastic transformation of smooth muscle cells. *J Cell Mol Med* 20: 217-230, 2016.
41. Sikura KÉ, Potor L, Szerafin T, Zarjou A, Agarwal A, Arosio P, Poli M, Hendrik Z, Méhes G, Oros M, *et al*: Potential role of H-ferritin in mitigating valvular mineralization. *Arterioscler Thromb Vasc Biol* 39: 413-431, 2019.
42. Zarjou A, Jeney V, Arosio P, Poli M, Antal-Szalmás P, Agarwal A, Balla G and Balla J: Ferritin prevents calcification and osteoblastic differentiation of vascular smooth muscle cells. *J Am Soc Nephrol* 20: 1254-1263, 2009.
43. Balla J, Vercellotti GM, Jeney V, Yachie A, Varga Z, Jacob HS, Eaton JW and Balla G: Heme, heme oxygenase, and ferritin: How the vascular endothelium survives (and dies) in an iron-rich environment. *Antioxid Redox Signal* 9: 2119-2137, 2007.
44. Lu CW, Lee CJ, Hsieh YJ and Hsu BG: Empagliflozin attenuates vascular calcification in mice with chronic kidney disease by regulating the NFR2/HO-1 anti-inflammatory pathway through AMPK activation. *Int J Mol Sci* 24: 10016, 2023.
45. Kook SH, Kim KA, Ji H, Lee D and Lee JC: Irradiation inhibits the maturation and mineralization of osteoblasts via the activation of Nrf2/HO-1 pathway. *Mol Cell Biochem* 410: 255-266, 2015.
46. Huang WC, Liou CJ, Shen SC, Hu S, Chao JC, Huang CH and Wu SJ: Punicagin from pomegranate ameliorates TNF- α /IFN- γ -induced inflammatory responses in HaCaT cells via regulation of SIRT1/STAT3 axis and Nrf2/HO-1 signaling pathway. *Int Immunopharmacol* 130: 111665, 2024.
47. Jin W, Zhu X, Yao F, Xu X, Chen X, Luo Z, Zhao D, Li X, Leng X and Sun L: Corrigendum to 'Cytoprotective effect of Fufang Lurong JIANGU capsule against hydrogen peroxide-induced oxidative stress in bone marrow stromal cell-derived osteoblasts through the Nrf2/HO-1 signaling pathway' [Biomed. Pharmacother. 121 (2020) 109676]. *Biomed Pharmacother* 122: 109808, 2020.

