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NRIP1 is a downstream target of YY1 in promoting OGD/R-induced H9c2 cardiomyocyte injury and mitochondrial dysfunction

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Summary. Background and objective. From a clinical standpoint, myocardial ischemia/reperfusion injury (MIRI) has always been an enormous challenge for the treatment of acute myocardial infarction (AMI). Molecular targeting therapy may help overcome this challenge. The present work aimed to elucidate the possible involvement of Yin-Yang 1 (YY1)/nuclear receptor-interacting protein 1 (NRIP1) and discover the molecular mechanism of MIRI.

Methods. Herein, a cardiomyocyte ischemia/reperfusion (I/R) model was established via oxygen-glucose deprivation/re-oxygenation (OGD/R) damage in H9c2 cardiomyocytes. Reverse transcription-quantitative PCR and western blotting were conducted to measure the levels of YY1 and NRIP1 at the RNA and protein levels, respectively. H9c2 cell viability and apoptosis were assayed using the Cell Counting Kit-8, flow cytometry, and western blotting. In addition, superoxide dismutase, glutathione peroxidase, and malondialdehyde levels were analyzed as markers of oxidative stress. Additionally, mitochondrial membrane potential, which was measured via JC-1 staining, ATP content, Complex I activity, mitochondrial DNA copy number, and mitochondrial permeability transition pore (mPTP) opening rate were analyzed to evaluate mitochondrial activity. Moreover, luciferase reporter and chromatin immunoprecipitation assays experimentally validated the predicted affinity of YY1 with the NRIP1 promoter according to the HumanTFDB online tool.

Results. YY1/NRIP1 were both highly expressed in OGD/R-injured H9c2 cardiomyocytes. Downregulation of NRIP1 improved cell viability, whereas it inhibited cell apoptosis and oxidative stress, and suppressed mitochondrial dysfunction in OGD/R-injured H9c2 cardiomyocytes. Importantly, it was verified that YY1 could bind to the NRIP1 promoter to positively regulate NRIP1 expression. The protective effects of NRIP1 knockdown against cardiomyocyte damage and mitochondrial dysfunction in OGD/R-injured H9c2 cardiomyocytes were partly abolished through overexpression of YY1.

Conclusion. NRIP1 emerged as a downstream target of YY1 in promoting OGD/R-induced H9c2 cardiomyocyte injury and mitochondrial dysfunction, providing novel ideas for targeted treatments to alleviate MIRI.

Key words: Cardiomyocyte ischemia/reperfusion injury, Mitochondrial dysfunction, NRIP1, YY1

Introduction

Acute myocardial infarction (AMI) is a dominant contributor to death and disability worldwide (Bahit et al., 2018). It is estimated that over 7,000,000 new cases of AMI occur annually and ~30% of deaths are attributed to untreated AMI (Beom et al., 2021). To reduce the extent of myocardial infarction and improve clinical prognosis, thrombolytic agents and primary percutaneous coronary angioplasty remain the most effective treatment regimens (Ding et al., 2023). Nonetheless, the reperfusion process can trigger ischemia/reperfusion (I/R) injury, which increases the likelihood of an unfavorable prognosis and even death (Lahnwong et al., 2020). Effective treatment modalities are unavailable to reduce myocardial ischemia/reperfusion injury (MIRI) following AMI, although great efforts have been made (Hu et al., 2023). Therefore, the development of novel targets is imperative to improve the prognosis of patients with AMI.

Previous studies highlighted the implication of mitochondrial dysfunction in MIRI (Sun et al., 2023; Xu et al., 2023). Mitochondria, responsible for intracellular

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ATP production, drive the contraction of the myocardium, hence supplying energy for cardiac blood pumping (Huang and Zhou, 2023). During MIRI, mitochondria play a role in cell death, which involves a series of detrimental events, such as oxidative damage activated by the generation of reactive oxygen species (ROS), reduced mitochondrial membrane potential (MMP), and opening of the mitochondrial permeability transition pore (mPTP), which in turn triggers the discharge of apoptotic factors that eventually lead to cell death (Crola Da Silva et al., 2023). Consequently, mitochondrial homeostasis might be a potential target for MIRI therapy.

Nuclear receptor-interacting protein 1 (NRIP1), also named receptor-interacting protein 140 (RIP140), is a co-regulator containing four inhibitory domains (Flindris et al., 2021). Intriguingly, elevated expression of NRIP1 contributes to hypertrophy and dysfunction of the myocardium (Fritah et al., 2010). In Down's syndrome, NRIP1 functions as a pivotal regulator of mitochondrial signaling and a possible treatment target (Izzo et al., 2014). Furthermore, NRIP1 was shown to modulate the biogenesis of mitochondria and to participate in the inflammatory response as well as metabolism in the myocardium (Zhang et al., 2014). Nevertheless, whether NRIP1 is involved in the physiology of MIRI etiology is vague and demands deeper exploration.

The NRIP1 promoter was predicted to interact with Yin-Yang 1 (YY1) based on the HumanTFDB. Belonging to the polycomb group protein family, YY1 is a ubiquitous zinc-finger transcription factor possessing multiple functions (Huang et al., 2021). For instance, YY1 plays complex regulatory roles in cardiovascular diseases. Elevated YY1 expression in patients with heart failure was demonstrated to downregulate Myosin heavy chain α isoform promoter 5, which is harmful to cardiac function (Sucharov et al., 2003). In addition, YY1 was shown to facilitate mitochondrial dysfunction during tubulointerstitial fibrosis related to early diabetic nephropathy (Yang et al., 2023). Therefore, YY1 is speculated to be involved in MIRI etiology.

On this basis, the current study assumed that YY1 might bind the NRIP1 promoter, corroborating the hypothesis that NRIP1 exacerbates cardiomyocyte injury upon OGD/R induction via intensification of mitochondrial dysfunction through interaction with YY1.

Materials and methods

Cell culture and treatments

H9c2 cells (American Type Culture Collection; ATCC, VA, USA) were cultured in DMEM complete medium supplemented with 10% FBS and 100 μg/ml antibiotics at 37°C in a 5% CO_2 saturated moist environment. In detail, after being subjected to 4h of cultivation in DMEM deprived of glucose under hypoxic conditions (1% O_2, 5% CO_2, and 94% N_2), H9c2 cells were reoxygenated (95% room air-5% CO_2, 37°C) for 24h in normal DMEM to mimic OGD/R damage.

Plasmid construction

Cells were transfected with 15 nM YY1 overexpression plasmid (Oe-YY1), 500 ng/μl short hairpin (sh)RNA against NRIP1 (sh-NRIP1#1 and sh-NRIP1#2) or their corresponding negative controls (sh-NC and Oe-NC; Shanghai GenePharma Co., Ltd.) using Lipofectamine 3000™ (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Cell viability assay

Briefly, $5x10^3$ cells were seeded into a 96-well culture plate. After 24h of incubation, each well received 10 μl of Cell Counting Kit-8 (CCK-8) reaction agent (Vazyme, Nanjing, China), and the plate was incubated at 37°C for 4h. The optical density at 450 nm was measured for each well using a microplate reader.

Apoptosis detection

Following centrifugation, the trypsinized cells were harvested and then doubly stained with Annexin V-FITC (10 μl) and propidium iodide (PI; 5 μl) shielded from light for 15 min at room temperature. A flow cytometry device (BD Biosciences, CA, USA) equipped with Cell Quest software was adopted for the analysis of cell apoptotic rate.

Measurement of lactate dehydrogenase (LDH) release

Intracellular LDH generation was assessed with the aid of the LDH Cytotoxicity Assay Kit (cat. No. C0016; Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions.

Determination of oxidative stress markers

Superoxide dismutase (SOD; cat. No. A001-3-2), glutathione peroxidase (GSH-Px; cat. No. A005-1-2), as well as malondialdehyde (MDA; cat. No. A003-1-2) levels were measured using commercial kits from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions.

JC-1 staining

Alterations in the MMP were investigated using the JC-1 Mitochondrial Membrane Potential Assay Kit (Beyotime Institute of Biotechnology). After washing with PBS, the treated H9c2 cells were incubated with JC-1 working solution (500 μl) for 20 min at 37°C. After incubation, the cells were washed with JC-1 staining buffer (1X). The stained cells were imaged using a fluorescence microscope (Olympus, Corporation).

Analysis of ATP levels

To obtain the supernatants, samples were homogenized in lysis buffer in an ice bath and then

subjected to 10 min centrifugation at 12,000 rpm at 4°C. The ATP content of the supernatants was then measured using an ATP Assay Kit (cat. No. S0026; Beyotime Institute of Biotechnology).

Complex I activity

The supernatants were obtained following homogenization and centrifugation. Complex I activity in the supernatants was analyzed using the Complex I Enzyme Activity Dipstick Assay (Abcam, Cambridge, UK), according to the manufacturer's instructions. The absorbance of the samples was measured at 450 nm using a VICTOR X3 Multilabel Plate Reader (PerkinElmer, Inc.). Results are expressed as enzyme activity with respect to the control.

Quantification of mitochondrial (mt)DNA copy number

Using the QIAmp DNA mini kit (Qiagen), total DNA was isolated and purified according to the manufacturer's instructions. PCR was performed with 20 ng of DNA sample, mtDNA target-specific (MT-CO2) and nuclear DNA (nucDNA)-specific (β2-microglobulin) primers. The mtDNA content was normalized to the total level of nucDNA.

Analysis of the mitochondrial permeability transition pore (mPTP)

mPTP was assessed according to the instructions of the mPTP kit (cat. No. C2009S; Beyotime Institute of Biotechnology). Briefly, after rinsing with PBS, the cells were labeled using an appropriate volume of calcein AM staining solution for 30 min at 37°C away from the light. Subsequently, fresh preheated complete medium for normal cell culture was used for 30 min at 37°C. A fluorescence microscope (Olympus Corporation) was used for imaging using an excitation wavelength of 494 nm and measuring the light emission at 515 nm.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted using TRIzol™ Reagent (Invitrogen; Thermo Fisher Scientific) and reverse transcribed into cDNA using the PrimeScript RT Kit (Takara Bio, Inc.), according to the manufacturer's instructions. Subsequently, the SYBR Premix Ex Taq kit was used for PCR amplification in an ABI Detection System. Relative NRIP1 and YY1 RNA expression were quantified using the $2^{-\Delta\Delta Cq}$ method and GAPDH as the internal standard for normalization.

Western blot assay

Total protein was extracted from cells using the RIPA lysis buffer (Beyotime Institute of Biotechnology) according to the manufacturer's instructions and then quantified using the BCA method. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked in 5% skim milk powder for 1h at room temperature. Subsequently, the membranes were incubated with antibodies against NRIP1, YY1, Bax, Bcl-2, and GAPDH at 4°C overnight. Following primary antibody incubation, the membranes were incubated with horse-radish peroxidase secondary antibody for 1h at room temperature. The protein bands were detected using an enhanced chemiluminescence solution and then analyzed with a Bio-Rad imaging system (Bio-Rad Laboratories, Inc.) and normalized to GAPDH.

Dual-luciferase report assay

The affinity of YY1 with the NRIP1 promoter was demonstrated using a dual-luciferase reporter assay. Cells were co-transfected with NRIP1 wild-type (WT)/mutant (MUT) reporter plasmids and Oe-YY1/Oe-NC using the Lipofectamine™ 3000 transfection reagent according to the manufacturer's instructions. After transfection, the luciferase activity was assessed using the Luciferase Reporter System (Promega Corporation) that employs *Renilla luciferase* for normalization.

Chromatin immunoprecipitation (ChIP) assay

Cells were cross-linked with 1% formaldehyde, which was halted with 0.125 M glycine. Then, SDS lysis buffer was utilized for the lysis of cells and 100-500 bp fragments were acquired from chromatin through sonication. Cell lysates were labeled using protein A/G agarose beads for 2h and then subjected to overnight incubation with anti-YY1 and anti-IgG antibodies. The ChIP-enriched DNA was quantitated through RT-qPCR.

Statistical analyses

All data are shown as mean ± standard deviation. One-way analysis of variance followed by Tukey's *post hoc* test or unpaired Student's t-test was applied for group comparisons. $p<0.05$ was considered to indicate a statistically significant difference.

Results

NRIP1 expression is increased in OGD/R-injured H9c2 cardiomyocytes

To establish the cardiomyocyte I/R model, after being subject to 4h culture under hypoxic conditions, H9c2 cells were reoxygenated in a standard environment for 24h. H9c2 cells that were subject to OGD/R injury displayed increased NRIP1 expression, suggesting a potential role of NRIP1 in MIRI (Fig. 1).

Knockdown of NRIP1 enhances H9c2 cell viability whilst inhibiting cell apoptosis upon OGD/R cell injury

To elucidate the biological role of NRIP1 during MIRI, sh-NRIP1#1 or sh-NRIP1#2 was transfected into H9c2 cells to downregulate NRIP1 expression for

functional experiments. Sh-NRIP1#2 showed the greatest interference efficacy and, therefore, was used in the subsequent experiments (Fig. 2A). OGD/R induction damaged H9c2 cell activity whereas it promoted intracellular LDH generation. NRIP1 knockdown improved the cell activity of OGD/R-injured H9c2 cells and decreased LDH generation, thereby relieving OGD/R-elicited cardiomyocyte damage (Fig. 2B,C). The flow cytometry data underlined that OGD/R induction promoted H9c2 cell apoptosis and NRIP1 knockdown hampered OGD/R-injured H9c2 cell apoptosis (Fig. 2D). In addition, the increased Bax and reduced Bcl-2 levels observed in H9c2 cells due to OGD/R were partially counterbalanced by NRIP1 knockdown, also suggesting that under OGD/R conditions, NRIP1 knockdown could reduce H9c2 cell apoptosis (Fig. 2E).

Knockdown of NRIP1 alleviates oxidative damage in H9c2 cardiomyocytes after OGD/R

Oxidative stress was triggered in H9c2 cells after OGD/R, manifested as decreased SOD and GSH-Px activities, and increased MDA levels. Conversely, the increased SOD and GSH-Px activity, and decreased MDA level after NRIP1 knockdown highlighted that NRIP1 deletion could mitigate oxidative stress in H9c2 cells with OGD/R injury (Fig. 3).

Knockdown of NRIP1 decreases the mitochondrial dysfunction of OGD/R-injured H9c2 cardiomyocytes

As shown through JC-1 staining, red fluorescence aggregates in normal mitochondria and green fluorescence monomers were noticeable in injured mitochondria. Enhanced green fluorescence staining indicated impaired mitochondria in H9c2 cells after OGD/R. Intensified red fluorescence and reduced green fluorescence upon NRIP1 silencing suggested that NRIP1 knockdown could restore damaged mitochondria (Fig. 4A). Meanwhile, OGD/R induction reduced intracellular ATP levels, inhibited complex I activity, and decreased mtDNA copy number in H9c2 cells, which was partially reversed by NRIP1 knockdown (Fig. 4B-D). In addition, OGD/R induction increased the mPTP opening rate of H9c2 cells; NRIP1 knockdown partially reversed the influence of OGD/R on the mPTP opening rate (Fig. 4E). These findings collectively proved the protective effects of NRIP1 knockdown against mitochondrial dysfunction.

YY1 modulates NRIP1 by binding with the NRIP1 promoter

The NRIP1 promoter was predicted to interact with YY1 based on the HumanTFDB (Fig. 5A). H9c2 cells showed increased YY1 expression after OGD/R (Fig. 5B). Subsequently, the Oe-YY1 plasmid was transfected into H9c2 cells to overexpress YY1 for subsequent research (Fig. 5C). The potent affinity between YY1 and NRIP1 was corroborated through a dual-luciferase reporter assay (Fig. 5D). The binding site of the NRIP1 promoter region to YY1 was identified via ChIP assay (Fig. 5E). Furthermore, it was demonstrated that YY1 overexpression enhanced NRIP1 expression in H9c2 cells after OGD/R (Fig. 5F), indicating a positive moderating effect of YY1 on NRIP1 expression.

NRIP1 is a downstream target of YY1, reducing H9c2 cardiomyocyte activity whilst promoting apoptosis after OGD/R

Decreased H9c2 cell activity and accelerated intracellular LDH generation following co-transfection with sh-NRIP1 and Oe-YY1 indicated that the enhancing impact of NRIP1 depletion on H9c2 cell viability after OGD/R was partly abolished by YY1 overexpression (Fig. 6A,B). Additionally, the results of flow cytometry and the variation in the expression of apoptosis-related proteins following co-transfection with sh-NRIP1 and Oe-YY1 indicated that the inhibiting impact of NRIP1 knockdown on H9c2 cell apoptosis after OGD/R was partly abolished by YY1 over-expression (Fig. 6C,D).

NRIP1 is a downstream target of YY1, exacerbating oxidative damage in H9c2 cardiomyocytes after OGD/R

Compared with the OGD/R + sh-NRIP1 + Ov-NC group, reduced SOD and GSH-Px activities, and elevated MDA levels in the OGD/R + sh-NRIP1 + Ov-YY1 group illustrated that the alleviating impact of NRIP1 knockdown on oxidative stress in H9c2 cells

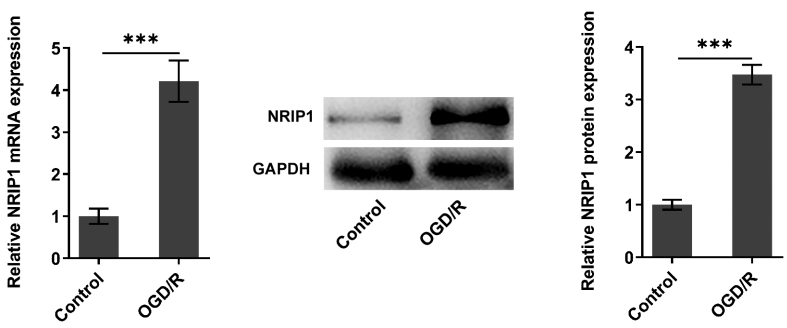

Fig. 1. NRIP1 expression increases in H9c2 cardiomyocytes after OGD/R. After being subjected to 4h of cultivation under hypoxic conditions, H9c2 cells were reoxygenated for 24h in normal DMEM to mimic OGD/R damage. Reverse transcription-quantitative PCR and western blot analysis of NRIP1 expression in H9c2 cells. ***$p<0.001$. NRIP1, nuclear receptor-interacting protein 1; OGD/R, oxygen-glucose deprivation/re-oxygenation.

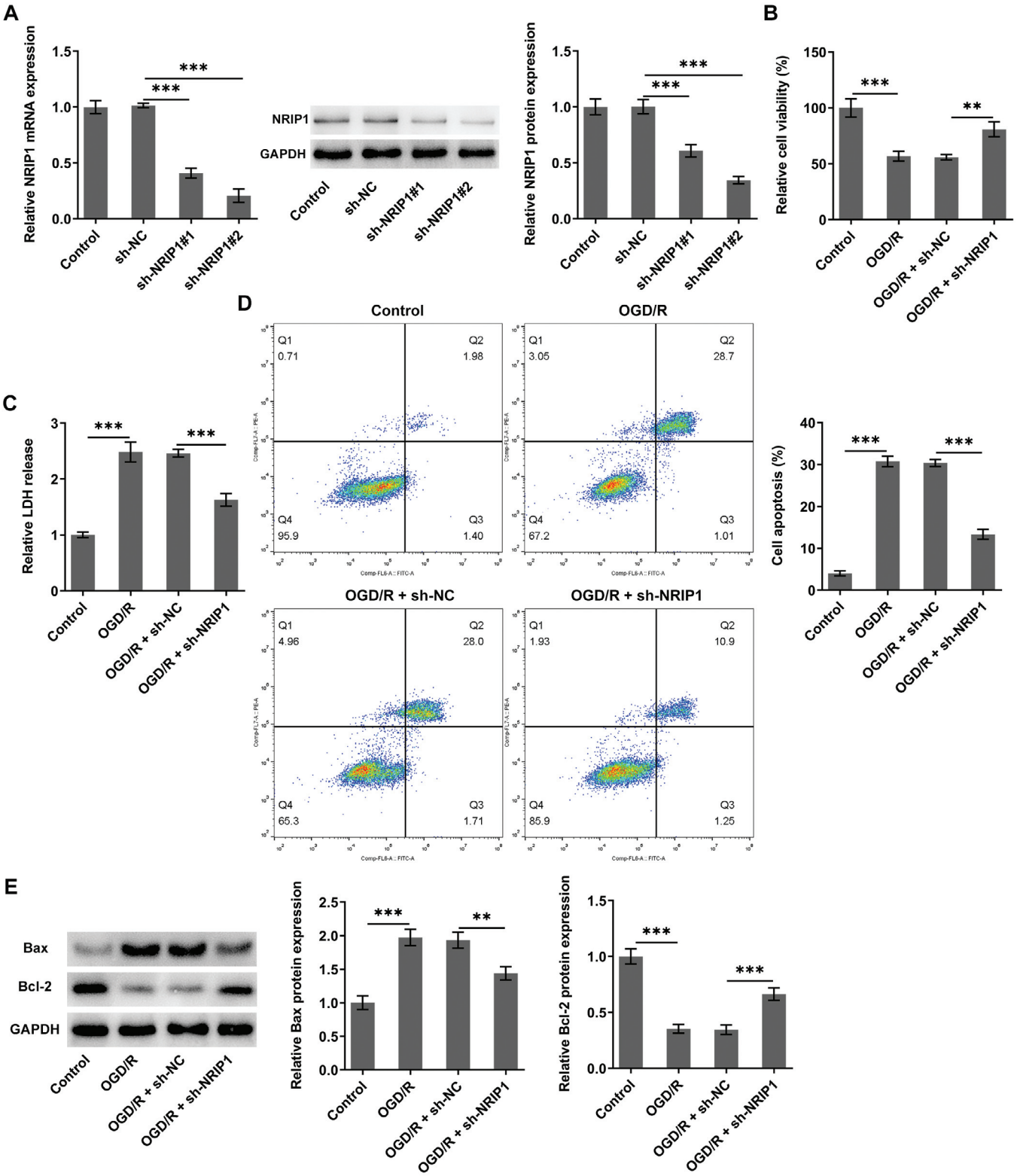

Fig. 2. NRIP1 knockdown enhances H9c2 cell viability whilst it inhibits cell apoptosis after OGD/R. **A.** sh-NRIP1#1 or sh-NRIP1#2 was transfected into H9c2 cells. Transfection efficiency was evaluated through reverse transcription-quantitative PCR and western blot analysis. **B.** sh-NRIP1 was transfected into H9c2 cells after OGD/R. The cell Counting Kit-8 method was used to measure cell viability. **C.** sh-NRIP1 was transfected into H9c2 cells after OGD/R. The production of intracellular LDH in H9c2 cells was determined with an LDH assay kit. **D.** sh-NRIP1 was delivered into H9c2 cells after OGD/R. Flow cytometry analysis of H9c2 cell apoptosis. **E.** sh-NRIP1 was transfected into H9c2 cells after OGD/R. Western blot analysis of Bax and Bcl-2 expression. $^{**}p<0.01$, $^{***}p<0.001$. LDH, lactase dehydrogenase; sh-, short hairpin; NRIP1, nuclear receptor-interacting protein 1; OGD/R, oxygen-glucose deprivation/re-oxygenation.

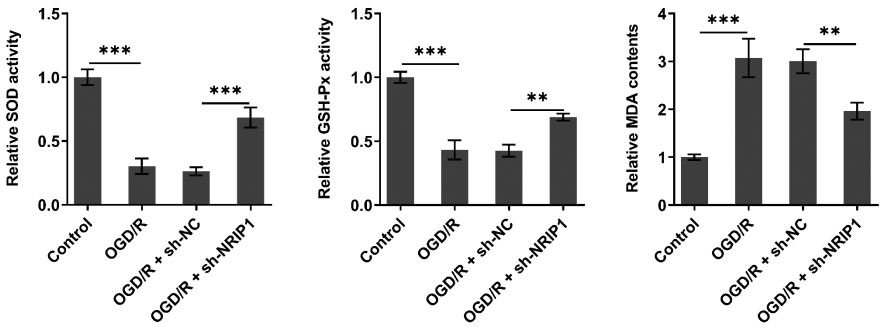

Fig. 3. Knockdown of NRIP1 alleviates oxidative damage in H9c2 cardiomyocytes after OGD/R. sh-NRIP1 was transfected into H9c2 cells after OGD/R. Commercial kits were used to examine superoxide dismutase and glutathione peroxidase activity, and malonaldehyde levels. **p<0.01, ***p<0.001. sh-, short hairpin; NRIP1, nuclear receptor-interacting protein 1; OGD/R, oxygen-glucose deprivation/re-oxygenation.

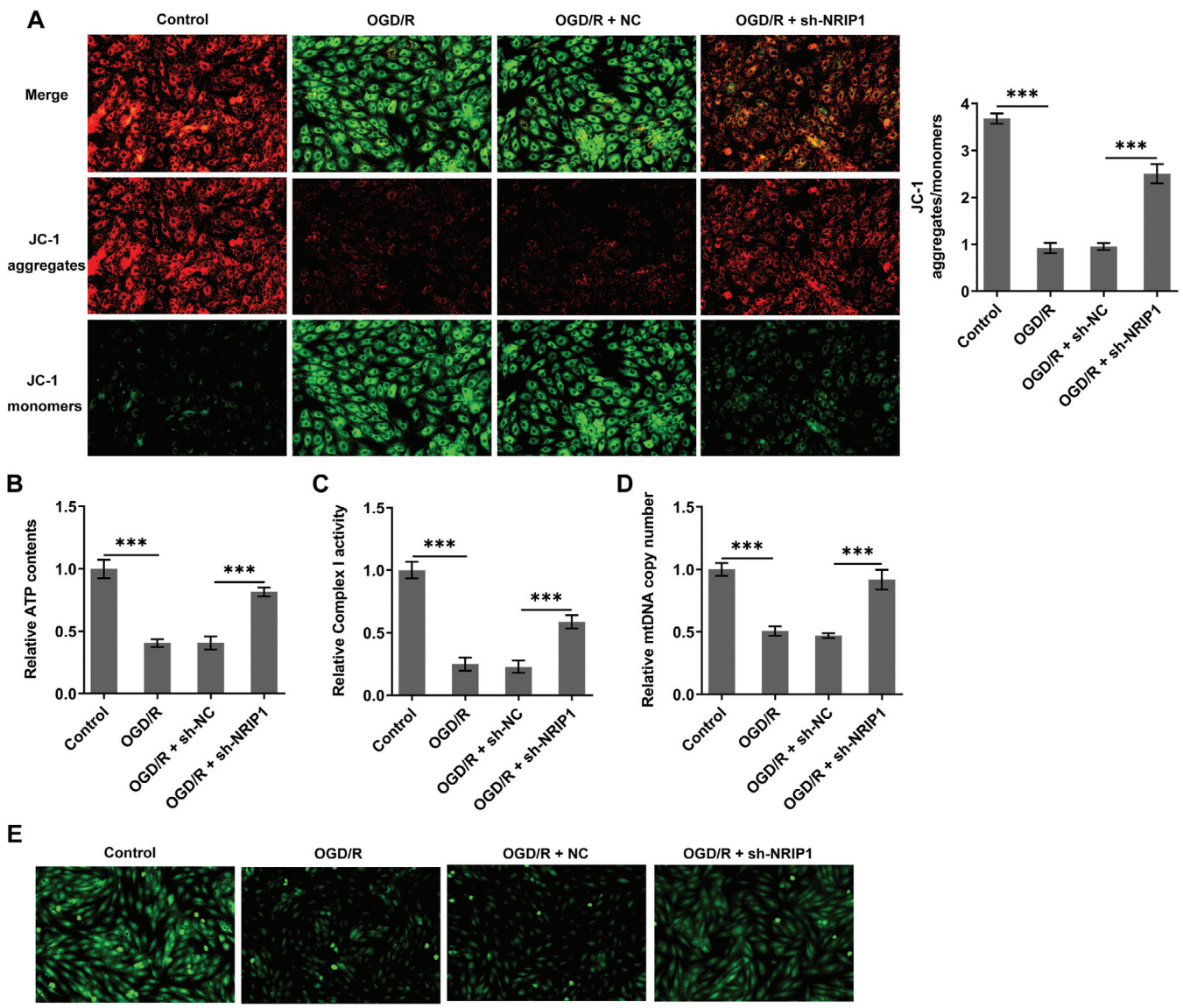

Fig. 4. Knockdown of NRIP1 diminishes mitochondrial dysfunction in OGD/R-injured H9c2 cardiomyocytes. **A.** Mitochondrial membrane potential was monitored by staining with the fluorescent dye JC-1. **B.** ATP contents were determined with an ATP assay kit. **C.** Complex I activity was assessed with a relevant assay kit. **D.** Reverse transcription-quantitative PCR analysis of mitochondrial DNA copy number. **E.** The opening rate of the mitochondrial permeability transition pore was appraised using the Calcein AM fluorescence probe. ***p<0.001. NRIP1, nuclear-receptor-interacting protein 1; OGD/R, oxygen-glucose deprivation/re-oxygenation.

after OGD/R was partly abrogated by YY1 overexpression (Fig. 7).

NRIP1 is a downstream target of YY1, aggravating the mitochondrial dysfunction of OGD/R-injured H9c2 cardiomyocytes.

Compared with the OGD/R + sh-NRIP1 + Ov-NC group, the weakened red fluorescence and intensified green fluorescence observed through JC-1 staining, as well as reduced intracellular ATP levels, inhibited complex I activity, decreased mtDNA copy number, and elevated mPTP opening in the OGD/R + sh-NRIP1 + Ov-YY1 group delineated that the suppressing impact of NRIP1 knockdown on mitochondrial dysfunction in H9c2 cells upon OGD/R was partly abolished by YY1

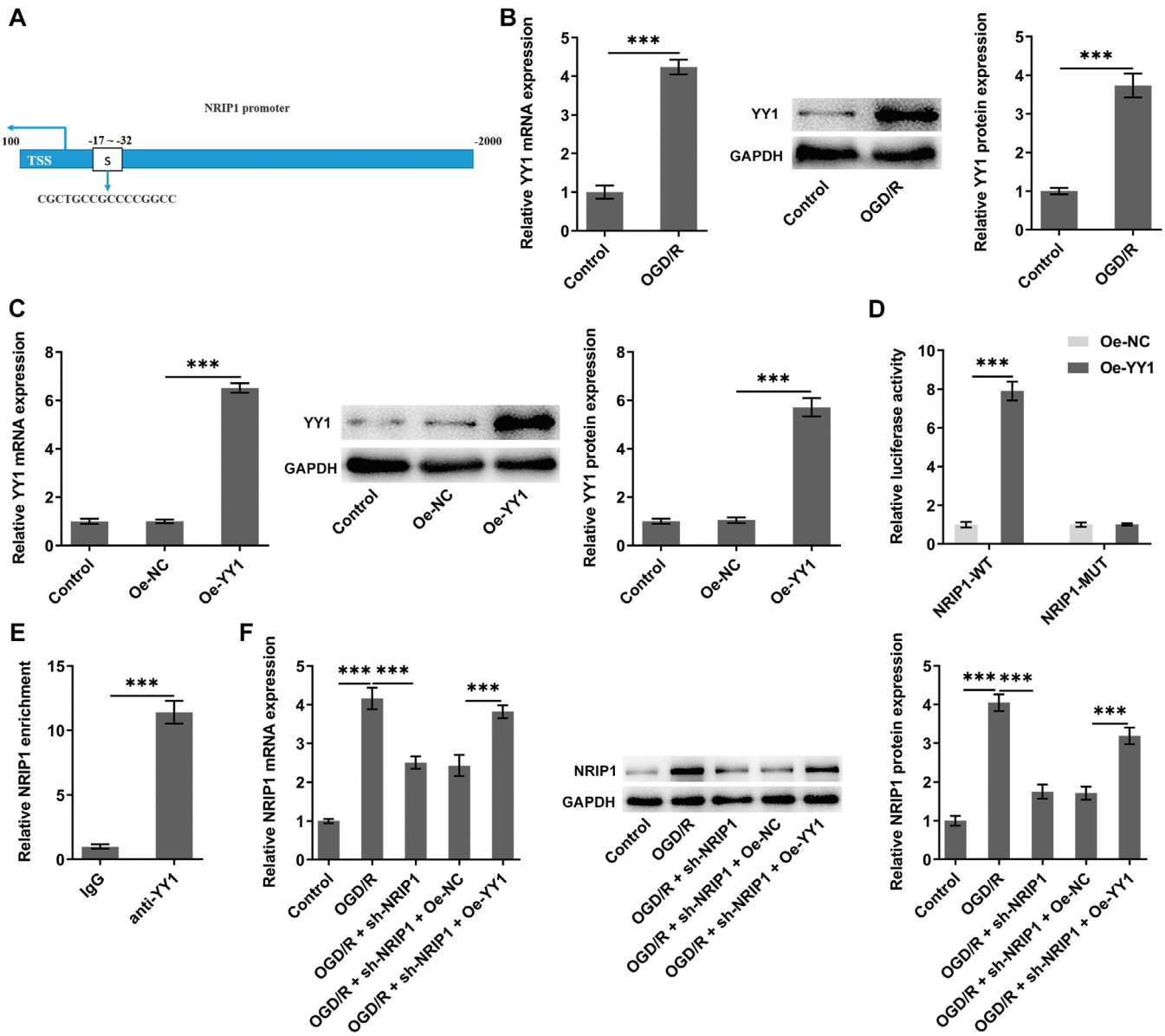

Fig. 5. YY1 modulates NRIP1 by binding to the NRIP1 promoter. **A.** The NRIP1 promoter was predicted to interact with YY1 based on the HumanTFDB. **B.** After being subjected to hypoxic conditions for 4h, H9c2 cells were reoxygenated for 24h in normal DMEM to mimic OGD/R damage. RT-qPCR and western blot analysis of YY1 expression in H9c2 cells. **C.** Oe-YY1 was transfected into H9c2 cells and the overexpression efficacy was evaluated through RT-qPCR and western blot analysis. **D.** The luciferase reporter assay corroborated the affinity of YY1 with NRIP1. **E.** The YY1 binding site in the NRIP1 promoter region was identified via the chromatin immunoprecipitation assay. **F.** sh-NRIP1 or combined sh-NRIP1 and Oe-YY1 was delivered into H9c2 cells after OGD/R. RT-qPCR and western blot analysis of NRIP1 expression in H9c2 cells. ***p<0.001. sh-, short hairpin; NRIP1, nuclear receptor-interacting protein 1; YY1, Yin Yang 1; OGD/R, oxygen-glucose deprivation/re-oxygenation; RT-qPCR, reverse transcription-quantitative PCR.

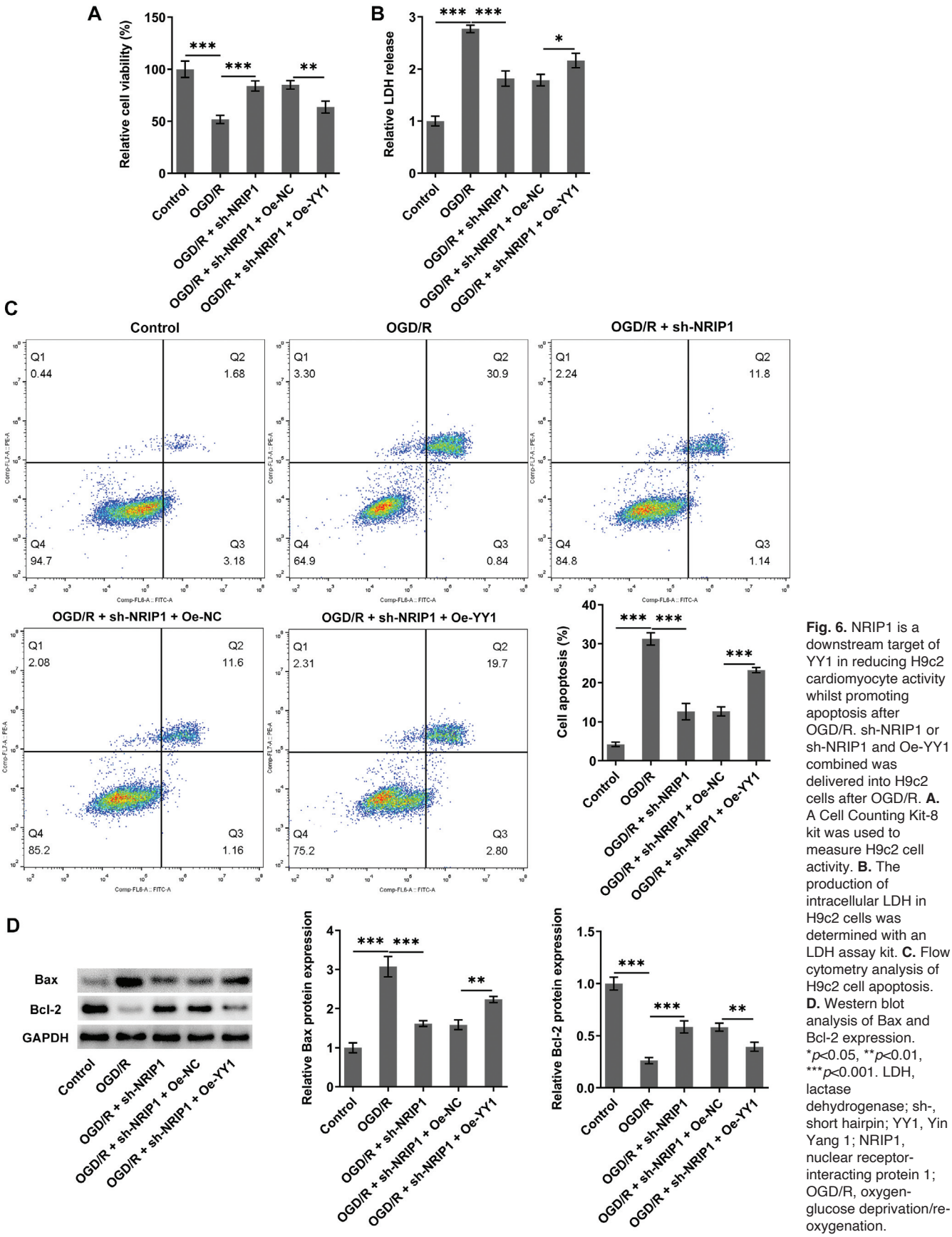

Fig. 6. NRIP1 is a downstream target of YY1 in reducing H9c2 cardiomyocyte activity whilst promoting apoptosis after OGD/R. sh-NRIP1 or sh-NRIP1 and Oe-YY1 combined was delivered into H9c2 cells after OGD/R. **A.** A Cell Counting Kit-8 kit was used to measure H9c2 cell activity. **B.** The production of intracellular LDH in H9c2 cells was determined with an LDH assay kit. **C.** Flow cytometry analysis of H9c2 cell apoptosis. **D.** Western blot analysis of Bax and Bcl-2 expression. *p<0.05, **p<0.01, ***p<0.001. LDH, lactase dehydrogenase; sh-, short hairpin; YY1, Yin Yang 1; NRIP1, nuclear receptor-interacting protein 1; OGD/R, oxygen-glucose deprivation/re-oxygenation.

overexpression (Fig. 8A-E).

Discussion

Coronary blood flow restoration remains the most effective intervention to improve survival by reducing myocardial ischemic damage. On the other hand, extra myocardial injury can occur during the reperfusion process (Ding et al., 2023). Given that, the development of modalities for the diagnosis and therapy of MIRI is imperative. The present study demonstrated a novel role of NRIP1 knockdown in reducing myocardial injury and mitochondrial dysfunction mediated by I/R. The levels of NRIP1 were regulated by the level of YY1, which directly binds to the NRIP1 promoter. Additionally, YY1 was discovered to promote the mitochondrial dysfunction elicited by MIRI by upregulating NRIP1, thereby aggravating MIRI.

The pathology of myocardial I/R injury is characterized by several pathophysiological features. The harmful impacts of MIRI related to mitochondrial dysfunction were identified in a previous study (Xu et al., 2023). Aberrant electron transport chain activity, decreased ATP release, dysregulated metabolic substrate transport, increased ROS, facilitated mtDNA injury, cristae disruption, as well as metabolic defects, are responsible for mitochondrial dysfunction (Nguyen et al., 2019). Cardiomyocytes have a high density of mitochondria. MIRI results in superabundant mitochondrial fission and ATP generation is impeded in the resulting fragmented mitochondria (Zhu et al., 2021). Persistent mPTP opening imposed by ATP deficiency results in intercellular calcium deposition, and eventually mitochondrial depolarization and MMP decline (Kim et al., 2012; Rizzuto et al., 2012). MMP decline as well as augmented ROS release from mitochondria are drivers of oxidative damage in cardiomyocytes (Lahnwong et al., 2020). Also, the release of cytochrome C from mitochondria to the cytosol attributed to persistent mPTP opening successively activated caspases-3-related apoptotic signaling (Zhang et al., 2020). In the present study, it was observed that OGD/R injury causes mitochondrial dysfunction and cell apoptosis in myocardial cells.

To the best of our knowledge, the present work is the first to evaluate the role of NRIP1 in MIRI. Another study showed that NRIP1 deficiency enhanced cardiac fuel metabolism and protected against heart failure in mice (Yamamoto et al., 2023). In addition, the rapid and progressive postnatal cardiomyopathy in RIP140 transgenic mice results in premature death mainly on account of damaged energy generation (Fritah et al., 2010). In the present study, NRIP1 expression was increased in H9c2 cells after OGD/R, which promoted cell apoptosis and aggravated cardiomyocyte injury, highlighting the harmful impacts of NRIP1 in MIRI. Notably, NRIP1 was shown to repress oxidative metabolism and mitochondrial biogenesis (Nautiyal et al., 2013). Moreover, NRIP1 was associated with mitochondrial dysfunction (Zhang et al., 2014). It is noteworthy that, in the present study, mitochondrial dysfunction, cell apoptosis, and oxidative stress were suppressed after NRIP1 was silenced, compared with the OGD/R-induced group. Furthermore, it is interesting to find a close association between NRIP1 and Protein kinase C epsilon (PKCε), which acts as a nuclear export trigger of NRIP1 (Gupta et al., 2008; Ho and Wei, 2012). PKCε is a key regulator of mitochondrial homeostasis and also plays important roles during I/R injury in cardiovascular diseases by regulating oxidative stress,

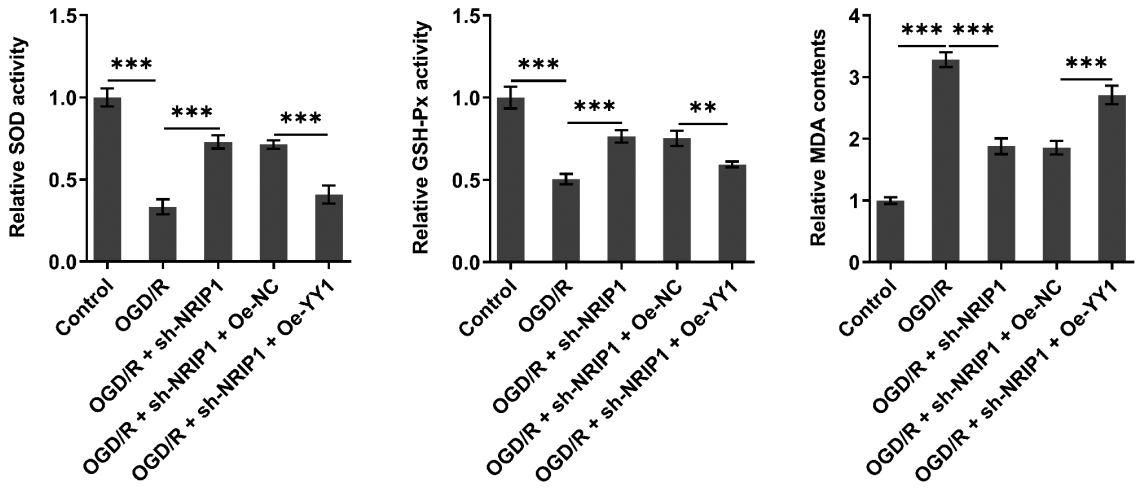

Fig. 7. Nuclear receptor-interacting protein 1 is a downstream target of YY1 in exacerbating oxidative damage in H9c2 cardiomyocytes upon OGD/R circumstances. sh-NRIP1 or sh-NRIP1 and Oe-YY1 combined was delivered into H9c2 cells after OGD/R. Relevant kits examined superoxide dismutase, glutathione peroxidase, and malondialdehyde levels. **p<0.01, ***p<0.001. sh-, short hairpin; YY1, Yin Yang 1; NRIP1, nuclear receptor-interacting protein 1; OGD/R, oxygen-glucose deprivation/re-oxygenation.

cell apoptosis, and mitochondrial dysfunction (Benoist et al., 2019; Carubbi et al., 2019; Chen et al., 2021). Therefore, the protective role of NRIP1 against OGD/R-induced cell apoptosis, oxidative stress, and mitochondrial dysfunction was partly associated with PKCε, which may be an interesting topic that needs to be explored in our future work.

As a multifunctional zinc-finger transcription factor, YY1 is a modulator of the cell cycle, apoptosis, and mitochondrion-associated genes (Kawamura et al., 2021; Guan et al., 2023). Numerous studies underlined the involvement of YY1 in cardiovascular disorders. For instance, YY1 expression and activity were increased in heart failure patients (Sucharov et al., 2003). Administration of isoproterenol increased YY1 while it decreased CuZn-SOD, thus triggering H9c2 cardiomyocyte oxidative damage and apoptosis (Srivastava et al., 2007). In addition, YY1 was also shown to aggravate

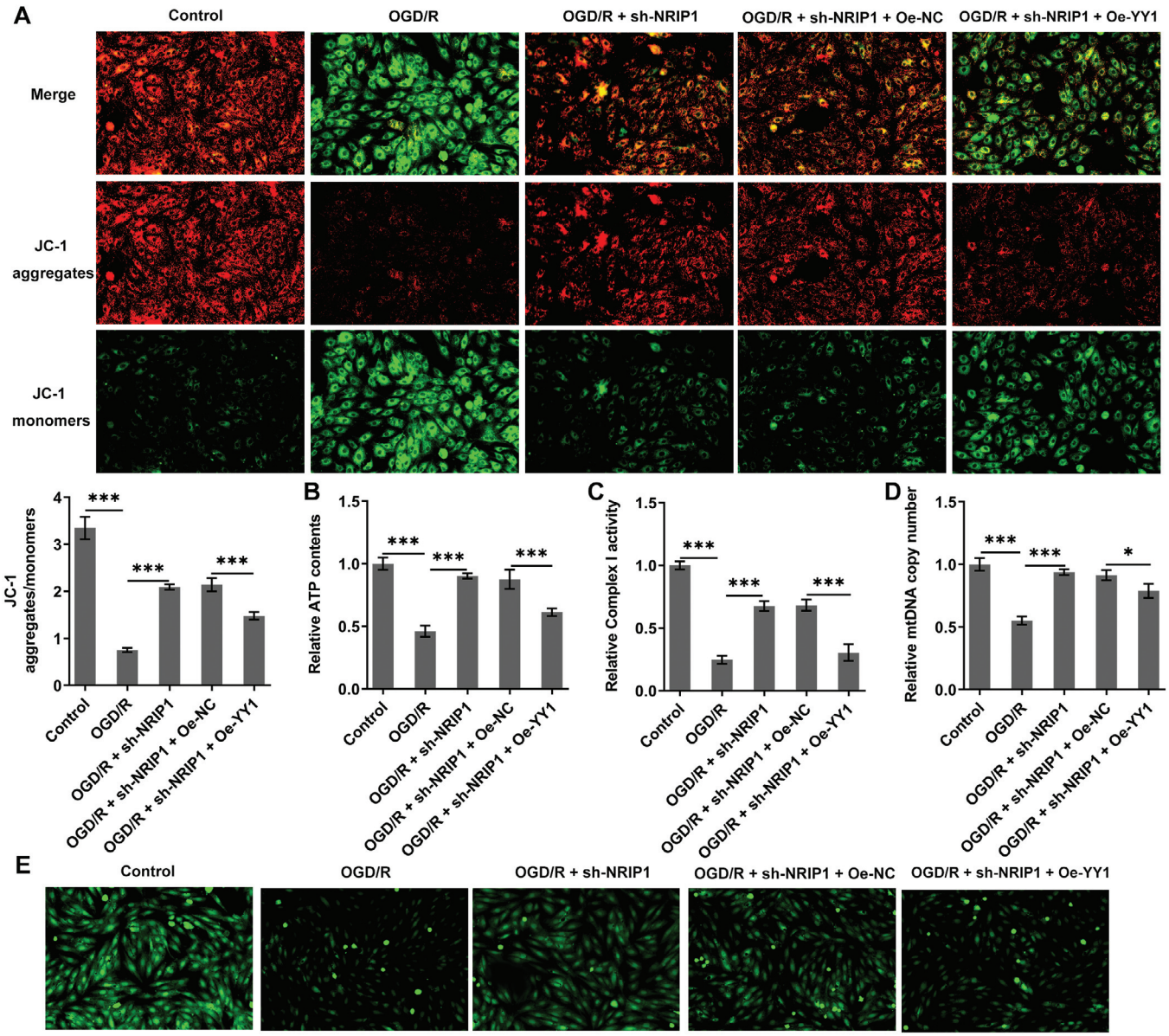

Fig. 8. NRIP1 is a downstream target of YY1 in aggravating mitochondrial dysfunction of OGD/R-injured H9c2 cardiomyocytes. sh-NRIP1 or sh-NRIP1 and Oe-YY1 combined was transfected into H9c2 cells after OGD/R. **A.** Mitochondrial membrane potential was monitored by staining with the fluorescent dye JC-1. **B.** ATP levels were measured using an ATP assay kit. **C.** Complex I activity was assessed with a relevant assay kit. **D.** Reverse transcription-quantitative PCR analysis of mitochondrial DNA copy number. **E.** The opening rate of the mitochondrial permeability transition pore was appraised with a Calcein AM fluorescence probe. *p<0.05, ***p<0.001. sh-, short hairpin; YY1, Yin Yang 1; NRIP1, nuclear receptor-interacting protein 1; OGD/R, oxygen-glucose deprivation/re-oxygenation.

myocardial and cerebral injuries elicited by I/R (Wan et al., 2019), implying its stimulatory role in the course of I/R-induced injury. Moreover, overexpression of YY1 stimulated mitochondrial dysfunction in HK-2 cells under normal glucose conditions; however, under high glucose, YY1 deletion could improve mitochondrial dysfunction, highlighting the critical role of YY1 in mediating mitochondrial functions (Yang et al., 2023). In the present work, the relationship between YY1 and NRIP1 was corroborated via a mechanistic study. YY1 was abundant in H9c2 cells after OGD/R. YY1 overexpression reversed the effects of NRIP1 knockdown on cell viability, apoptosis, oxidative damage, and mitochondrial dysfunction in OGD/R-challenged H9c2 cardiomyocytes.

Conclusion

In conclusion, the present data demonstrated that NRIP1 and YY1 were upregulated in OGD/R-treated H9c2 cells. Elevation of NRIP1 by YY1 was noted to promote MIRI progression by inducing cell apoptosis, oxidative stress, and mitochondrial dysfunction, unraveling a novel action mechanism behind MIRI and highlighting a novel treatment target.

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References

Bahit M.C., Kochar A. and Granger C.B. (2018). Post-myocardial infarction heart failure. JACC Heart Fail. 6, 179-186.

Benoist L., Chadet S., Genet T., Lefort C., Heraud A., Danila M.D., Muntean D.M., Baron C., Angoulvant D., Babuty D., Bourguignon T. and Ivanes F. (2019). Stimulation of P2Y11 receptor protects human cardiomyocytes against Hypoxia/Reoxygenation injury and involves PKCε signaling pathway. Sci. Rep. 9, 11613.

Beom J.H., Kim J.H., Seo J., Lee J.H., Chung Y.E., Chung H.S., Chung S.P., Kim C.H. and You J.S. (2021). Targeted temperature management at 33 degrees C or 36°C induces equivalent myocardial protection by inhibiting HMGB1 release in myocardial ischemia/reperfusion injury. PLoS One 16, e0246066.

Carubbi C., Masselli E., Pozzi G., Mattioli M., Martini S., Goldoni M., Aloe R., Cervellin G., Vitale M. and Gobbi G. (2019). Combination of platelet expression of PKCepsilon and cardiac troponin-I for early diagnosis of chest pain patients in the emergency department. Sci. Rep. 9, 2125.

Chen L., Shi D. and Guo M. (2021). The roles of PKC-δ and PKC-ε in myocardial ischemia/reperfusion injury. Pharmacol. Res. 170, 105716.

Crola Da Silva C., Baetz D., Vedere M., Lo-Grasso M., Wehbi M., Chouabe C., Bidaux G. and Ferrera R. (2023). Isolated mitochondria state after myocardial ischemia-reperfusion injury and cardioprotection: Analysis by flow cytometry. Life 13, 707.

Ding Y., Li W., Peng S., Zhou G., Chen S., Wei Y., Xu J., Gu H., Li J., Liu S. and Liu B. (2023). Puerarin protects against myocardial Ischemia/Reperfusion injury by inhibiting ferroptosis. Biol. Pharm. Bull. 46, 524-532.

Flindris S., Katsoulas N., Goussia A., Lazaris A.C., Navrozoglou I., Paschopoulos M. and Thymara I. (2021). The expression of NRIP1 and LCOR in endometrioid endometrial cancer. *In Vivo* 35, 2631-2640.

Fritah A., Steel J.H., Nichol D., Parker N., Williams S., Price A., Strauss L., Ryder T.A., Mobberley M.A., Poutanen M., Parker M. and White R. (2010). Elevated expression of the metabolic regulator receptor-interacting protein 140 results in cardiac hypertrophy and impaired cardiac function. Cardiovasc. Res. 86, 443-451.

Guan H., Zhang W., Liu H., Jiang Y., Li F., Wu M., Waterhouse G.I.N., Sun-Waterhouse D. and Li D. (2023). Quercetin induces apoptosis in HepG2 cells via directly interacting with YY1 to disrupt YY1-p53 interaction. Metabolites 13, 229.

Gupta P., Ho P.C., Huq M.D., Khan A.A., Tsai N.P. and Wei L.N. (2008). PKCepsilon stimulated arginine methylation of RIP140 for its nuclear-cytoplasmic export in adipocyte differentiation. PLoS One 3, e2658.

Ho P.C. and Wei L.N. (2012). Negative regulation of adiponectin secretion by receptor interacting protein 140 (RIP140). Cell. Signal. 24, 71-76.

Hu C., Liao J., Huang R., Su Q. and He L. (2023). MicroRNA-155-5p in serum derived-exosomes promotes ischaemia-reperfusion injury by reducing CypD ubiquitination by NEDD4. ESC Heart Fail. 10, 1144-1157.

Huang Y. and Zhou B. (2023). Mitochondrial dysfunction in cardiac diseases and therapeutic strategies. Biomedicines 11, 1500.

Huang Y., Li L., Chen H., Liao Q., Yang X., Yang D., Xia X., Wang H., Wang W.E., Chen L. and Zeng C. (2021). The protective role of yin-yang 1 in cardiac injury and remodeling after myocardial infarction. J. Am. Heart Assoc. 10, e021895.

Izzo A., Manco R., Bonfiglio F., Cali G., De Cristofaro T., Patergnani S., Cicatiello R., Scrima R., Zannini M., Pinton P., Conti A. and Nitsch L. (2014). NRIP1/RIP140 siRNA-mediated attenuation counteracts mitochondrial dysfunction in Down syndrome. Hum. Mol. Genet. 23, 4406-4419.

Kawamura K., Higuchi T. and Fujiwara S. (2021). YAF2-mediated YY1-sirtuin6 interactions responsible for mitochondrial downregulation in aging tunicates. Mol. Cell. Biol. 41, e0004721.

Kim J.S., Wang J.H. and Lemasters J.J. (2012). Mitochondrial permeability transition in rat hepatocytes after anoxia/reoxygenation: Role of Ca^{2+}-dependent mitochondrial formation of reactive oxygen species. Am. J. Physiol. Gastrointest. Liver Physiol. 302, G723-731.

Lahnwong S., Palee S., Apaijai N., Sriwichaiin S., Kerdphoo S., Jaiwongkam T., Chattipakorn S.C. and Chattipakorn N. (2020). Acute dapagliflozin administration exerts cardioprotective effects in rats with cardiac ischemia/reperfusion injury. Cardiovasc. Diabetol.

19, 91.

Nautiyal J., Christian M. and Parker M.G. (2013). Distinct functions for RIP140 in development, inflammation, and metabolism. Trends Endocrinol. Metab. 24, 451-459.

Nguyen B.Y., Ruiz-Velasco A., Bui T., Collins L., Wang X. and Liu W. (2019). Mitochondrial function in the heart: The insight into mechanisms and therapeutic potentials. Br. J. Pharmacol. 176, 4302-4318.

Rizzuto R., De Stefani D., Raffaello A. and Mammucari C. (2012). Mitochondria as sensors and regulators of calcium signalling. Nat. Rev. Mol. Cell Biol. 13, 566-578.

Srivastava S., Chandrasekar B., Gu Y., Luo J., Hamid T., Hill B.G. and Prabhu S.D. (2007). Downregulation of CuZn-superoxide dismutase contributes to beta-adrenergic receptor-mediated oxidative stress in the heart. Cardiovasc. Res. 74, 445-455.

Sucharov C.C., Mariner P., Long C., Bristow M. and Leinwand L. (2003). Yin Yang 1 is increased in human heart failure and represses the activity of the human alpha-myosin heavy chain promoter. J. Biol. Chem. 278, 31233-31239.

Sun X., Chen H., Gao R., Qu Y., Huang Y., Zhang N., Hu S., Fan F., Zou Y., Hu K., Chen Z., Ge J. and Sun A. (2023). Intravenous transplantation of an ischemic-specific peptide-TPP-mitochondrial compound alleviates myocardial ischemic reperfusion injury. ACS Nano 17, 896-909.

Wan H., Yang Y., Li M., Liu X., Sun Y., Wang K., Zhang C., Zheng Q. and Wang C. (2019). Activation of AK005401 aggravates acute ischemia/reperfusion mediated hippocampal injury by directly targeting YY1/FGF21. Aging 11, 5108-5123.

Xu L.L., Su H.X., Li P.B. and Li H.H. (2023). Ursolic acid ameliorates myocardial ischaemia/reperfusion injury by improving mitochondrial function via immunoproteasome-PP2A-AMPK signalling. Nutrients 15, 1049.

Yamamoto T., Maurya S.K., Pruzinsky E., Batmanov K., Xiao Y., Sulon S.M., Sakamoto T., Wang Y., Lai L., McDaid K.S., Shewale S.V., Leone T.C., Koves T.R., Muoio D.M., Dierickx P., Lazar M.A., Lewandowski E.D. and Kelly D.P. (2023). RIP140 deficiency enhances cardiac fuel metabolism and protects mice from heart failure. J. Clin. Invest. 133, e162309.

Yang T., Hu Y., Chen S., Li L., Cao X., Yuan J., Shu F., Jiang Z., Qian S., Zhu X., Wei C., Wei R., Yan M., Li C., Yin X. and Lu Q. (2023). YY1 inactivated transcription co-regulator PGC-1α to promote mitochondrial dysfunction of early diabetic nephropathy-associated tubulointerstitial fibrosis. Cell Biol. Toxicol. 39, 391-413.

Zhang L., Chen Y., Yue Z., He Y., Zou J., Chen S., Liu M., Chen X., Liu Z., Liu X., Feng X., Li M. and Liu P. (2014). The p65 subunit of NF-κB involves in RIP140-mediated inflammatory and metabolic dysregulation in cardiomyocytes. Arch. Biochem. Biophys. 554, 22-27.

Zhang L., Wang Y., Li C., Shao C., Zhou H., Yang J., He Y. and Wan H. (2020). Dan hong injection protects against cardiomyocytes apoptosis by maintaining mitochondrial integrity through keap1/nuclear factor erythroid 2-related factor 2/JNK pathway. Front. Pharmacol. 11, 591197.

Zhu H., Tan Y., Du W., Li Y., Toan S., Mui D., Tian F. and Zhou H. (2021). Phosphoglycerate mutase 5 exacerbates cardiac ischemia-reperfusion injury through disrupting mitochondrial quality control. Redox Biol. 38, 101777.

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