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Sirt3 attenuates post-infarction cardiac injury via inhibiting mitochondrial fission and normalization of AMPK-Drp1 pathways

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ABSTRACT

Mitochondrial damage is involved in the pathogenesis of post-infarction cardiac injury. However, the upstream regulators of mitochondrial damage have not yet been identified. The aim of our study is to explore the role of Sirt3 in post-infarction cardiac injury with a particular focus on mitochondrial fission and AMPK-Drp1 pathways. Our results indicated that Sirt3 was downregulated in the progression of post-infarction cardiac injury. Overexpression of Sirt3 attenuated cardiac fibrosis, sustained myocardial function, inhibited the inflammatory response, and reduced cardiomyocyte death. Functional studies illustrated that chronic post-infarction cardiac injury was characterized by increased mitochondrial fission, which triggered mitochondrial oxidative stress, metabolic disorders, mitochondrial potential reduction and caspase-9 apoptosis in cardiomyocytes. However, Sirt3 overexpression attenuated mitochondrial fission and thus preserved mitochondrial homeostasis and cardiomyocyte viability. Furthermore, our results confirmed that Sirt3 repressed mitochondrial fission via normalizing AMPK-Drp1 pathways. Inhibition of AMPK activity re-activated Drp1 and thus abrogated the inhibitory effect of Sirt3 on mitochondrial fission. Altogether, our results indicate that Sirt3 enhancement could be an effective approach to retard the development of post-infarction cardiac injury via disrupting mitochondrial fission and normalizing the AMPK-Drp1 axis.

1. Introduction

Acute myocardial infarction presenting as ST-segment elevation (STEMI) is the result of abrupt occlusion of an epicardial coronary artery, which is associated with significant morbidity and mortality worldwide. Timely restoration of blood flow to the ischemic myocardium to limit infarct size, improve long-term myocardial function, and reduce mortality has become the standard treatment for patients. Unfortunately, the limited regenerative ability of cardiomyocytes to replenish infarcted tissues precedes the cardiac dysfunction, which is defined as the post-infarction cardiac injury. At the molecular levels, post-infarction cardiac injury is associated with excessive cardiomyocyte death and microvascular malfunction [1,2]. Additionally, the excessive inflammation response, elevated oxidative stress, increased cardiac preload and/or afterload also works together to exacerbate the extent of post-infarction cardiac injury [3] which is manifested by collagen deposition, functional cell loss, and myocardial contraction reduction [4,5]. Accordingly, developing approaches to increase the viability of cardiomyocytes is vital to attenuate post-infarction cardiac injury.

Notably, several studies have found that deregulation of cardiomyocyte viability is connected to mitochondrial abnormality [6]. Hearts contain abundant mitochondria, which convert food to energy, ensuring the myocardial function [7]. However, more robust data concerning the causal relationship of mitochondrial damage and post-infarction cardiac injury have been provided by genetic loss- and gain-of-function studies [6,8,9]. Recently, mitochondrial fission, a type of mitochondria dynamic, has been acknowledged to have a decisive effect on mitochondrial damage in various cardiovascular illness [10,11]. For example, in acute cardiac ischemia-reperfusion injury, activated

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mitochondrial fission promotes cardiomyocyte death via multiple pathways [12,13]. In chronic metabolic cardiac injury, such as myocardial hypertrophy [14], cardiac fibrosis [15], and doxorubicin-induced cardiotoxicity [16], inhibition of mitochondrial fission sustains heart structure and function. Although several careful studies have been attempted to explain the detailed role of mitochondrial fission in a model of post-infarction cardiac injury [6], the upstream regulators for mitochondrial fission have not been adequately explored.

Sirtuin 3 (Sirt3) is an NAD-dependent deacetylase and is primarily expressed in the mitochondria. Several mitochondrial biological processes are affected by Sirt3, including mitochondrial oxidative stress, calcium overload, mitochondrial apoptosis, mitochondrial permeability transition pore (mPTP) opening, and mitochondrial biological synthesis [17,18]. Notably, in neuron ischemia reperfusion [19], cervical cancer stress [20], and white fat metabolism [21], mitochondrial fission is preferentially regulated by Sirt3. Based on these facts, we wanted to determine whether Sirt3 could attenuate post-infarction cardiac injury via modulating mitochondrial fission.

According to recent studies, mitochondrial fission can be regulated by either the JNK-Mff [12,13] axis or the AMPK-Drp1 cascade [22,23] in the heart. Notably, different disease models seem to have various impacts on these two pathways. For example, in acute stress, such as cardiac ischemia-reperfusion injury, activated JNK pathways promote mitochondrial fission factor (Mff) phosphorylation and the latter mediates cardiomyocyte and endothelium death [12,13,24]. However, in chronic cardiac injury, such as diabetic cardiomyopathy [22], mitochondrial fission can be inhibited by AMPK pathways via repressing dynamin-1-like protein (Drp1) phosphorylation. Additionally, in diabetic tubulopathy [23], Parkinson's disease [25], and age-associated vascular dysfunction [26], the AMPK pathway is a more relevant mediator of mitochondrial fission. Accordingly, we ask whether AMPK-Drp1 pathways are involved in regulating mitochondrial fission in a model of post-infarction cardiac injury. Accordingly, the aim of our study is to explore the role of Sirt3 in ameliorating post-infarction cardiac injury via inhibiting mitochondrial fission in a manner dependent on AMPK-Drp1 pathways.

2. Methods and material

2.1. Animals and intervention

The animal studies were performed according to the NIH Guidelines on the Care and Use of Laboratory Animals. WT mice and Sirt3 transgenic (Sirt3-TG) mice (C57BL/6 background) were constructed by K&D Gene Technology (Wuhan, China). These mice at 12 weeks old were used to establish the myocardial infarction (MI) model. In brief, ligation of left anterior descending (LAD) coronary artery was performed to achieve the cardiac MI model according to a previous study [27]. After 28 days, the hearts were isolated to analyze the chronic cardiac post-infarction injury (n = 6/group).

2.2. Primary cardiomyocyte culture and treatment

The primary cardiomyocytes were isolated from the ventricles of the neonatal hearts in WT mice and Sirt3-TG mice. Cardiomyocytes were cultured with H-DMEM supplemented with 10% FBS. To induce the chronic cardiac post-infarction injury in vitro, cardiomyocytes were treated with hypoxia for 48 h according to a previous study [6]. The hypoxic condition was performed via a hypoxic incubator (95% N_2 and 5% CO_2). To inhibit the activity of AMPK pathways, Compound C (CC, Selleck Chemicals, Houston, TX, USA) was added into the medium of cardiomyocytes for 2 h.

2.3. Cardiac function detection

Twenty-eight days after MI, cardiac function was assessed using echocardiography according to a previous study. An M-mode system with a 15-MHz linear transducer (VisualSonics Vevo 2100, Toronto, Canada) was used to analyze the left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), E/A ratio and left ventricular volume in systole (LV vol-s).

2.4. Sample preparation and histological analysis

Four percent buffered formalin fixed heart tissues were embedded in paraffin based on a previous study. Tissue sections with 5 μm thickness were prepared and stained with Masson trichrome stain according to the previous study. The changes in tissue morphology were observed through light microscope and captured by a camera. Blood samples were collected to analyze the concentration of lactate dehydrogenase (LDH), troponin T, creatine kinase-MB (CK-MB), laminin and pre-collagen III using ELISA assays as previously described [13].

2.5. Cell shortening/relengthening assay

Cardiomyocyte mechanical properties were evaluated using a SoftEdge MyoCam system (IonOptix, Milton, MA) according to a previous study [27]. Cell shortening and relengthening parameters include PS (peak shortening), TPS (time to peak shortening), TR90 (time to 90% relengthening), and ± dL/dt (the maximal velocity of shortening and relengthening).

2.6. TUNEL assay

The terminal deoxynucleotidyl transferase UTP nick end-labeling (TUNEL) assay was performed on frozen tissue sections using an In Situ Cell Death Detection Kit (Roche Diagnostics, 12156792910, Branford, CT, USA) according to the manufacturer's instructions [28]. After washing 3 times for 5 min each in PBS, the sections were mounted in fluorescence mounting medium with DAPI (Invitrogen) to identify the nuclei. All the paired sections were examined under a confocal laser scanning microscope.

2.7. MTT assay and caspase activity detection

An MTT assay was performed to detect the cellular viability according to a previous study [29]. Cells were treated with 50 μl MTT at 37 °C for approximately 4 h. Subsequently, the cells were incubated with 200 μl dimethyl sulfoxide for approximately 10 min at 37 °C. The optical density at a wavelength of 570 nm was then determined. To analyze changes in caspase-3/9, caspase-3/9 activity kits (Beyotime Institute of Biotechnology, China; Catalog No.C1158) were used according to the manufacturer's protocols. To analyze caspase-3 activity, 5 μL of DEVD-p-NA substrate (4 mM, 200 μM final concentration) was added to the samples for 2 h at 37 °C. In brief, to measure caspase-9 activity, 5 μl of LEHD-p-NA substrate (4 mM, 200 μM final concentration) was added to the samples for 1 h at 37 °C. Then, the wavelength at 400 nm was recorded via a microplate reader to reflect the caspase-3 and caspase-9 activities.

2.8. Measurement of mitochondrial reactive oxygen species (mROS) and the mitochondrial membrane potential (ΔΨm)

JC-1 staining was performed to observe mitochondrial potential. Cells at a density of 1×10^6 were treated with a MitoProbe™ JC-1 assay kit (Thermo Fisher Scientific Inc.) at 37 °C in the dark for 15–20 min. After washing with PBS, mitochondrial potential was observed using a fluorescence microscope. Techniques to measure mitochondrial ROS were performed using MitoSOX red mitochondrial superoxide indicator (Molecular Probes, USA) as previously described [30,31]. Flow cytometry was performed to analyze the levels of mitochondrial ROS.

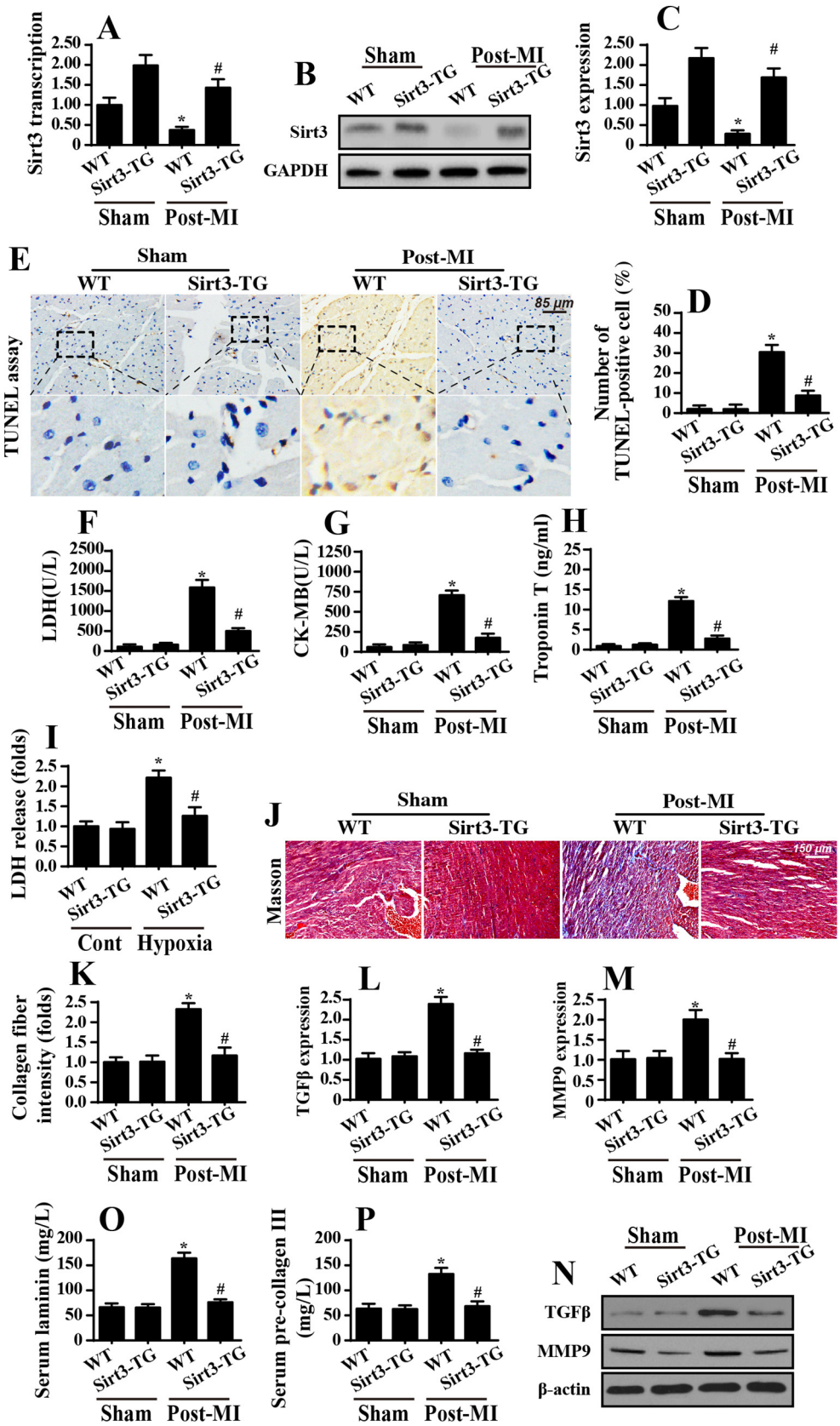


Fig. 1. Sirt3 attenuates cardiomyocyte death and cardiac injury in post-infarction hearts. A. qPCR assay for Sirt3. Wild-type mice underwent myocardial infarction (MI), and then the hearts were isolated for qPCR analysis 28 days after MI. B–C. Proteins were isolated from hearts and then western blotting for Sirt3 expression was performed. D–E. TUNEL staining for cell death. WT mice and Sirt3-TG mice were treated with MI. Then, hearts were stained with TUNEL 28 days after MI. The number of TUNEL positive cells was counted. F–H. Cardiac damage markers were detected using ELISA in WT mice and Sirt3-TG mice. I. Cardiomyocytes were isolated from WT mice and Sirt3-TG mice. Then, 48 h of hypoxia stimulation was performed in vitro to mimic the post-infarction cardiac injury. Cell viability was determined using an LDH release assay. J–K. Cardiac fibrosis was observed using Masson staining. L–N. The expression of fibrosis-related proteins was analyzed using western blotting. O–P. The concentration of serum laminin and pre-collagen III was measured in blood using ELISA. *p < .05 vs. sham group or control (cont) group; #p < .05 vs. WT + post-MI group or WT + hypoxia group.

2.9. Western blot analysis

Protein expression was analyzed via western blotting [32]. Primary antibodies against the following proteins were used in the present study: Bcl2 (1:1000, Cell Signaling Technology, #3498), Bax (1:1000, Cell Signaling Technology, #2772), caspase 9 (1:1000, Cell Signaling Technology, #9504), x-IAP (1:1000; Abcam; #ab28151), cyt-c (1:1000; Abcam; #ab90529), Drp1 (1:1000, Abcam, #ab56788), complex III

subunit core (CIII-core2, 1:1000, Invitrogen, #459220), complex II (CII-30, 1:1000, Abcam, #ab110410), Opa1 (1:1000, Abcam, #ab42364), Mfn2 (1:1000, Abcam, #ab56889), Fis1 (1:1000, Abcam, #ab71498), Mff (1:1000, Cell Signaling Technology, #86668), Sirt3 (1:1000, Abcam, no. ab86671), AMPK (1:1000, Abcam, #ab131512), p-AMPK (1:1000, Abcam, #ab23875), Tom20 (1:1000, Abcam, #ab186735), and p-Drp1^{Ser616} (1:1000, Cell Signaling Technology, #4494). The second antibodies used in the present study were as follows: Horseradish peroxidase-conjugated secondary antibodies (1:2000; cat. nos. 7076 and 7074; Cell Signaling Technology, Inc.) for 1 h at room temperature. Band intensities were normalized to the respective internal standard signal intensity (GAPDH (1:1000, Cell Signaling Technology, #5174) and/or β-actin (1:1000, Cell Signaling Technology, #4970) using Quantity One Software (version 4.6.2; Bio-Rad Laboratories, Inc.).

2.10. Immunofluorescence confocal microscopy

Samples were observed using a Leica DM IL LED inverted fluorescence microscope (magnification, ×400; Leica Microsystems, Inc.). Primary antibodies against the following proteins were used in the present study: cyt-c (1:500; Abcam; #ab90529), troponin T (1:1000, Abcam, #ab8295), p-AMPK (1:1000, Abcam, #ab23875), F4/80 (1:1000, Abcam, #ab111101), p-Drp1^{Ser616} (1:1000, Cell Signaling Technology, #4494), and Tom-20 (1:1000, Abcam, #ab186735). Mitochondrial fission was evaluated via measuring mitochondrial length [12]. Fluorescence intensity was calculated using Image-Pro Plus 6.0 software. Firstly, fluorescence pictures were converted to the grayscale pictures with the help of Image-Pro Plus 6.0 software. Then, fluorescence intensities were separately recorded as the grayscale intensity. Subsequently, relative grayscale intensity was expressed as a ratio to that of control group.

2.11. ELISA

Cellular glutathione (GSH), glutathione peroxidase (GPX) and SOD were measured via ELISA assay according to the manufacturer's instructions. Cellular lactate production in the medium was measured via a lactate assay kit (#K607-100; BioVision, Milpitas, CA, USA) according to a previous study [33]. The glucose uptake rate was detected via a glucose absorption assay kit (#K606-100; BioVision).

2.12. RNA isolation and qPCR

Total RNA was isolated using Trizol reagent (Invitrogen). Then, a Reverse Transcription kit (Kaneka Eurogentec S.A., Seraing, Belgium) was applied to transcribe RNA (1 μg in each group) into cDNA. Then, qPCR was performed with primers and matched probes from the Universal Fluorescence-labeled Probe Library (Roche Applied Science) using SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, Inc.) [34]. The primers used in the present study were as follows: Sirt3 forward, 5′-GACATCTCTTGGAGTGCTTC-3′ and reverse, 5'-GCTGCTGGATTCGGAAGCAA-3′; IL6 Forward, 5′-CAGACTCGCGCCTCTAAGGAGT3′; Reverse, 5′-GATAGCCGATCCGTCGAA-3′; MCP1 Forward, 5′-GGATGGATTGCACAGCCATT-3′; Reverse, 5′-GCGCCGACTCAGAGGTGT-3′; TNFα Forward, 5′- AGATGGAGCAACCTAAGGTC-3′; Reverse, 5′-GCAGACCTCGCTGTTCTAGC-3′; and GAPDH forward, 5'-GCTACAGCACCACCACA-3′ and reverse, 5′-GCCTGTCTCGAGCAGTC-3′. The cycling conditions were as follows: 95 °C for 8 min, followed by 35 cycles of 95 °C for 10 s and 72 °C for 12 s, for telomere PCR. Fold changes of PTEN mRNA expression were normalized by GAPDH as an internal control.

2.13. Statistics

Data are presented as the mean ± S.E.M. and were representative of at least three independent experiments. Statistical analyses were performed by one-way analysis of variance (ANOVA). P values < .05 were considered statistically significant.

3. Results

3.1. Overexpression of Sirt3 reduces cardiomyocyte death and cardiac fibrosis in post-infarcted hearts

To verify the role of Sirt3 in post-infarcted hearts, qPCR was performed to analyze the transcription of Sirt3 after myocardial infarction (MI). Compared to the sham group, mRNA transcription of Sirt3 was statically downregulated 28 days after MI (Fig. 1A). This finding was further supported via analyzing protein expression. Compared to the sham group, Sirt3 expression was significantly reduced in post-infarcted hearts (Fig. 1B-C). The above results indicate that Sirt3 is absent in the progression of post-infarction cardiac injury. Subsequently, Sirt3 transgenic (Sirt3-TG) mice were used to determine the contribution of Sirt3 in regulating post-infarction cardiac injury. Then, cardiomyocyte death was measured using a TUNEL assay in vivo. Compared to the control group, the number of TUNEL-positive cells was significantly increased in the hearts after MI, and this phenotypic alteration was repressed by Sirt3 overexpression (Fig. 1D–E). Similarly, cardiac damage markers, including LDH, CK-MB and troponin-T, were higher in WT mice that underwent MI (Fig. 1F–H), and this alteration could be attenuated by Sirt3 overexpression. In vitro, cardiomyocytes were isolated from WT mice and Sirt3-TG mice. Then, chronic hypoxia stimulation was used to mimic the post-infarction cardiac injury according to a previous study [6]. Consistent with the results of the in vivo study, cell viability measured via LDH assay also demonstrated that cardiomyocytes isolated from Sirt3-TG mice released less LDH into the medium compared to the cardiomyocytes isolated from WT mice (Fig. 1I).

Subsequently, we observed cardiac fibrosis via Masson staining. Compared to the control group, Sirt3 overexpression significantly reduced the levels of cardiac fibrosis (Fig. 1J–K). Moreover, the critical factors related to cardiac fibrosis, including TGFβ and MMP9 (Fig. 1L–N), were also increased in post-infarcted hearts and were reduced to near-normal levels with Sirt3 overexpression. Additionally, fibrosis markers, such as serum laminin (Fig. 1O) and pre-collagen III (Fig. 1P), were notably elevated in WT mice that underwent MI. However, Sirt3 overexpression reduced the concentration of serum laminin and pre-collagen III (Fig. 1O–P). Altogether, our results indicate that post-infarction cardiac injury may be associated with a deficiency in Sirt3, which regulates cardiomyocyte viability and myocardial fibrosis.

3.2. Sirt3 overexpression sustains myocardial function and attenuates the inflammatory response

Next, cardiac function was measured using echocardiography. As shown in Fig. 2A-B, cardiac contractile parameters, including LVEF and LVFS, were significantly reduced in WT mice after MI, and this phenotypic alteration could be reversed by Sirt3 overexpression. Additionally, the cardiac diastolic index, assessed via E/A ratio and LV volume (Fig. 2C-D), was better in Sirt3-TG mice than in the WT mice. These data suggest that Sirt3 prevents the decrease in myocardial function during chronic cardiac injury.

Furthermore, cardiomyocytes were isolated from mice and were used to observe mechanical parameters in the setting of post-infarction cardiac injury. Compared to the sham group, neither post-infarction injury nor Sirt3 overexpression had effects on the resting cell length of cardiomyocytes (Fig. 2E). However, chronic post-infarction injury obviously reduced PS and ± dL/dt (Fig. 2F–J), and these phenotypic alterations were rescued by Sirt3 overexpression. Similarly, TPS and TR90 were also augmented in cardiomyocyte isolated from the post-infarcted hearts and were reduced to normal levels with Sirt3

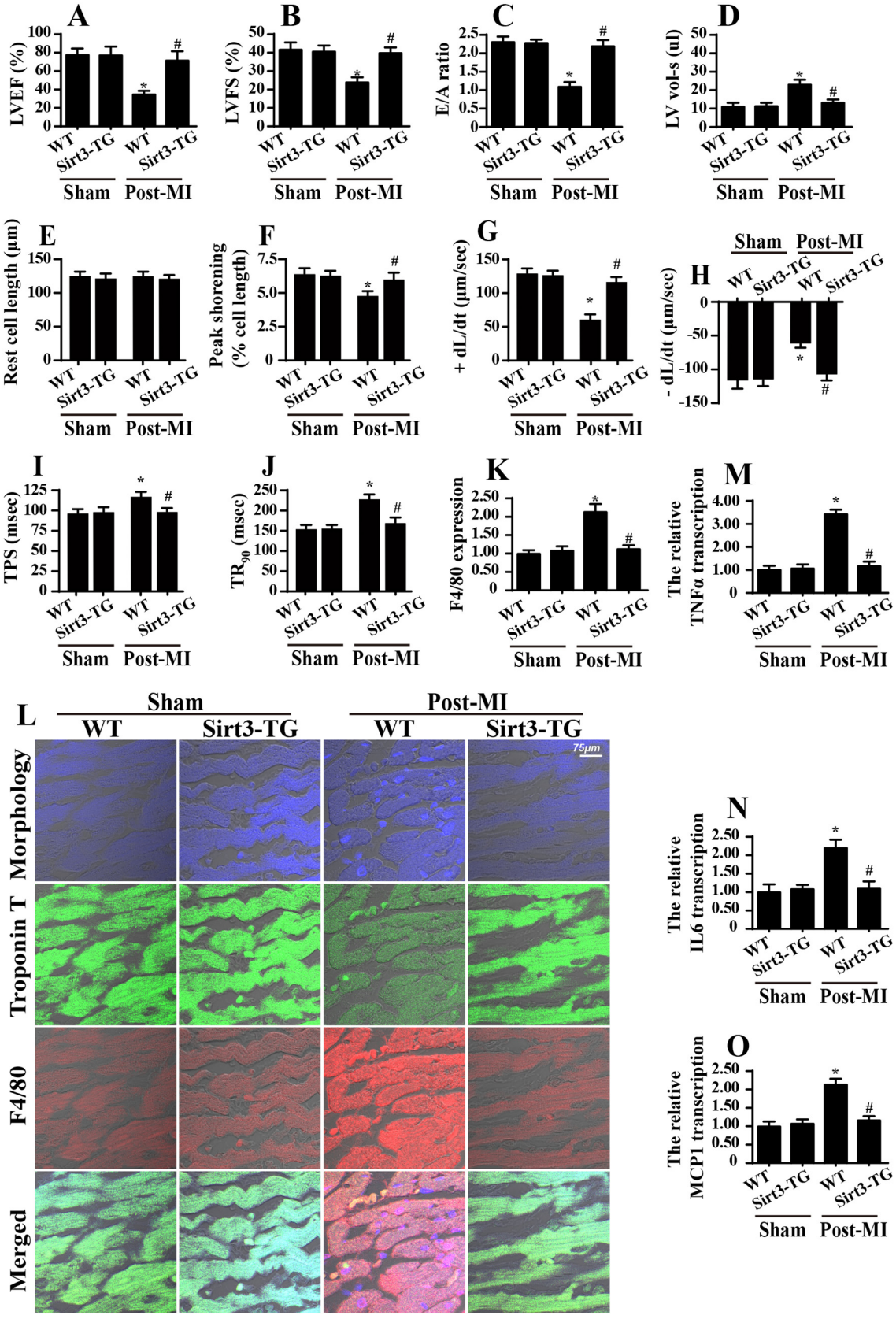


Fig. 2. Sirt3 maintains heart function and inhibits an excessive inflammatory response. A–D. The cardiac function was detected as LVEF, LVFS, E/A ratio and LV vol-s via echocardiography in WT and Sirt3-TG mice. E–J. The contractile properties of cardiomyocytes in WT mice and Sirt3-TG mice in the context of post-MI injury. +dL/dt is the maximal velocity of shortening. −dL/dt is the maximal velocity of relengthening. TPS is the time to peak shortening. TR90 is the time to 90% relengthening. K–L. F4/80-positive macrophage migration into myocardial tissue was measured using an immunofluorescence assay in WT mice and Sirt3-TG mice. M-O. The transcriptional alterations of inflammation factors (TNFα, IL6 and MCP1) were detected via qPCR in the post-infarcted hearts. *p < .05 vs. sham group or control (cont) group; #p < .05 vs. WT + post-MI group or WT + hypoxia group.

overexpression (Fig. 2F–J).

Further, the cardiac inflammatory response is a primary factor mediating post-infarction cardiac injury. Accordingly, immunofluorescence was used to observe inflammatory cell accumulation in post-infarcted hearts. As shown in Fig. 2K–L, the expression of F4/80, a specific surface marker of macrophages, was significantly upregulated in post-infarcted myocardium and was reduced to near-normal levels with Sirt3 overexpression. As a consequence of inflammatory cell accumulation, the mRNA transcription of inflammatory factors, such as TNFα, IL-6 and MCP1, were obviously increased in the post-MI heart, and these effects were negated by Sirt3 overexpression (Fig. 2M–O). Altogether, these findings confirm the protective role played by Sirt3 in alleviating post-infarction cardiac injury via maintaining myocardial function and inhibiting the inflammatory response.

4. Sirt3 inhibits mitochondrial fission in post-infarcted hearts

Experiments were then performed to analyze the role of mitochondrial fission in post-infarction cardiac injury. First, western blotting was conducted to detect the alterations of mitochondrial fission-related proteins. Compared to the sham group, the expression of pro-fission factors, such as Mff and Fis1, were elevated in the post-MI hearts (Fig. 3A–F). Moreover, chronic heart injury also caused an increase in the phosphorylation of Drp1 at Ser616, indicative of Drp1 activation in post-MI hearts (Fig. 3A–F). Interestingly, Sirt3 overexpression prevented the upregulation of pro-fission proteins (Fig. 3A–F). Additionally, we also observed a change in mitochondrial fusion, a compensatory system to correct the aberrant mitochondrial fission. Compared to the sham group, the expression of Mfn2 and Opa1 were significantly downregulated in post-MI hearts and were reversed to near-normal levels with Sirt3 overexpression (Fig. 3A–F). These data indicate that Sirt3 also influences mitochondrial fission via reversing mitochondrial fusion.

In vitro, cardiomyocytes were isolated from WT mice and Sirt3 mice. Then, chronic hypoxia stimulation was used to mimic post-infarction cardiac injury according to a previous study [6]. Subsequently, an immunofluorescence assay was used to analyze the mitochondrial morphology using a mitochondrial-specific Tom-20 antibody. As shown in Fig. 3G–H, the mitochondria in the control group were highly interconnective and formed the network dispersed throughout the cytoplasm. Interestingly, in response to hypoxia stress, mitochondria were small and round, and this alteration could be abrogated by Sirt3 overexpression (Fig. 3G–H). To further quantify mitochondrial fission, the average mitochondrial length was measured. Compared to the control group, the mitochondria length was reduced to approximately 2.6 μm with hypoxia treatment, and increased to approximately 8.8 μm (Fig. 3G–H). Additionally, co-immunofluorescence of p-Drp1^{Ser616} was also performed. As shown in Fig. 3H–I, a small amount of p-Drp1 was observed in the control group. However, hypoxia augmented the expression of p-Drp1^{Ser616}; the effect was accompanied by mitochondrial debris (Fig. 3H–I). Interestingly, Sirt3 overexpression repressed Drp1 phosphorylation and thus sustained the mitochondrial network (Fig. 3H–I). Collectively, our results in vivo and in vitro support the functional importance of Sirt3 in repressing mitochondrial fission in post-infarcted hearts.

5. Inhibition of mitochondrial fission by Sirt3 maintains mitochondrial function

Mitochondrial dysfunction is associated with cardiomyocyte viability and the inflammatory response. Subsequently, we aimed at determining the role of Sirt3-modified mitochondrial fission in mitochondrial dysfunction. To address this question, loss- and gain-of-function assays of mitochondrial fission were performed using Mdivi-1 and FCCP, respectively. Mdivi-1, an antagonist of mitochondrial fission, was added to the control group, which was used as a negative control group for Sirt3 overexpression. In contrast, FCCP, an agonist of mitochondrial fission, was administered to the Sirt3-overexpressed cells, which was used to re-activate mitochondrial fission. Then, mitochondrial ROS was determined via flow cytometry. The levels of mitochondrial ROS were significantly increased in response to hypoxia stress, and this effect was nullified by Mdivi-1 treatment or Sirt3 overexpression (Fig. 4A–B). Interestingly, activation of mitochondrial fission via FCCP abolished the antioxidant property of Sirt3 (Fig. 4A–B). As a consequence of mitochondrial ROS overproduction, the concentrations of cellular antioxidant factors (GSH, GPX and SOD) were reduced with hypoxia stress (Fig. 4C–E). However, Mdivi-1 treatment or Sirt3 overexpression reversed the levels of cellular antioxidant factors; these effects could be invalidated by FCCP supplementation (Fig. 4C–E).

The primary role of mitochondria is to generate sufficient ATP to ensure cell metabolism. Moreover, the ATP production was inhibited by chronic hypoxia treatment and was reversed to near-normal levels with Sirt3 overexpression (Fig. 4F). Mitochondrial ATP production is primarily handled by mitochondrial respiratory complex. However, the expression of a mitochondrial respiratory complex was significantly downregulated in response to hypoxia stress (Fig. 4G–I), and this effect was negated by Mdivi-1 treatment or Sirt3 overexpression. Notably, activation of mitochondrial via FCCP re-induced a decrease in the levels of the mitochondrial respiratory complex (Fig. 4G–I), indicating that mitochondrial fission impairs mitochondrial respiratory function. This finding was further supported via analyzing the glucose uptake of cardiomyocytes. Hypoxia-treated cardiomyocytes utilized less glucose in the medium (Fig. 4J), and this effect could be rescued by Sirt3 via repressing mitochondrial fission. In response to a decrease in glucose intake, the production of lactic acid in the medium was also reduced in hypoxia-treated cardiomyocytes and was reversed to near-normal levels in Sirt3-overexpressed cells via regulating mitochondrial fission (Fig. 4K). This finding indicates that inhibition of mitochondrial fission by Sirt3 sustains mitochondrial function and metabolism.

6. Sirt3 blocks mitochondrial apoptosis via repressing mitochondrial fission

Excessive mitochondrial damage is associated with the activation of mitochondrial apoptosis. Next, we performed experiments to analyze whether Sirt3-modified mitochondrial fission participated in mitochondrial apoptosis in the setting of post-infarction cardiac injury. The early event of mitochondrial apoptosis is a decline in mitochondrial membrane potential. Using JC-1 staining, we found that mitochondrial membrane potential was reduced in response to hypoxia stress, and this effect was reversed by Mdivi-1 treatment or Sirt3 overexpression (Fig. 5A–B). Notably, Sirt3-stabilized mitochondrial membrane potential could be reversed via activation of mitochondrial fission (Fig. 5A-B). Further, the characterizations of mitochondrial apoptosis are the translocation of mitochondrial pro-apoptotic factors into the nucleus and activation of the caspase-9-related apoptotic pathway. As shown in Fig. 5C-D, hypoxia promoted mitochondrial cyt-c liberation into the cytoplasm/nucleus, and this effect was negated by Sirt3 overexpression via inhibiting mitochondrial fission. In response to cyt-c translocation, the expression of mitochondrial proapoptotic proteins, such as Bax and caspase-9, were significantly upregulated (Fig. 5E–I); the effect was accompanied by a decline in the expression of antiapoptotic factors, such as Bcl-2 and x-IAP (Fig. 5E–I). Interestingly, Sirt3 overexpression obviously reversed the balance between antiapoptotic proteins and proapoptotic factors via regulating mitochondrial fission (Fig. 5E–I). Altogether, our results underscore that Sirt3 sustains mitochondrial homeostasis via repressing mitochondrial fission.

7. Sirt3 regulates mitochondrial fission via modulating AMPK-Drp1 pathways

Finally, we explored the molecular mechanism by which Sirt3

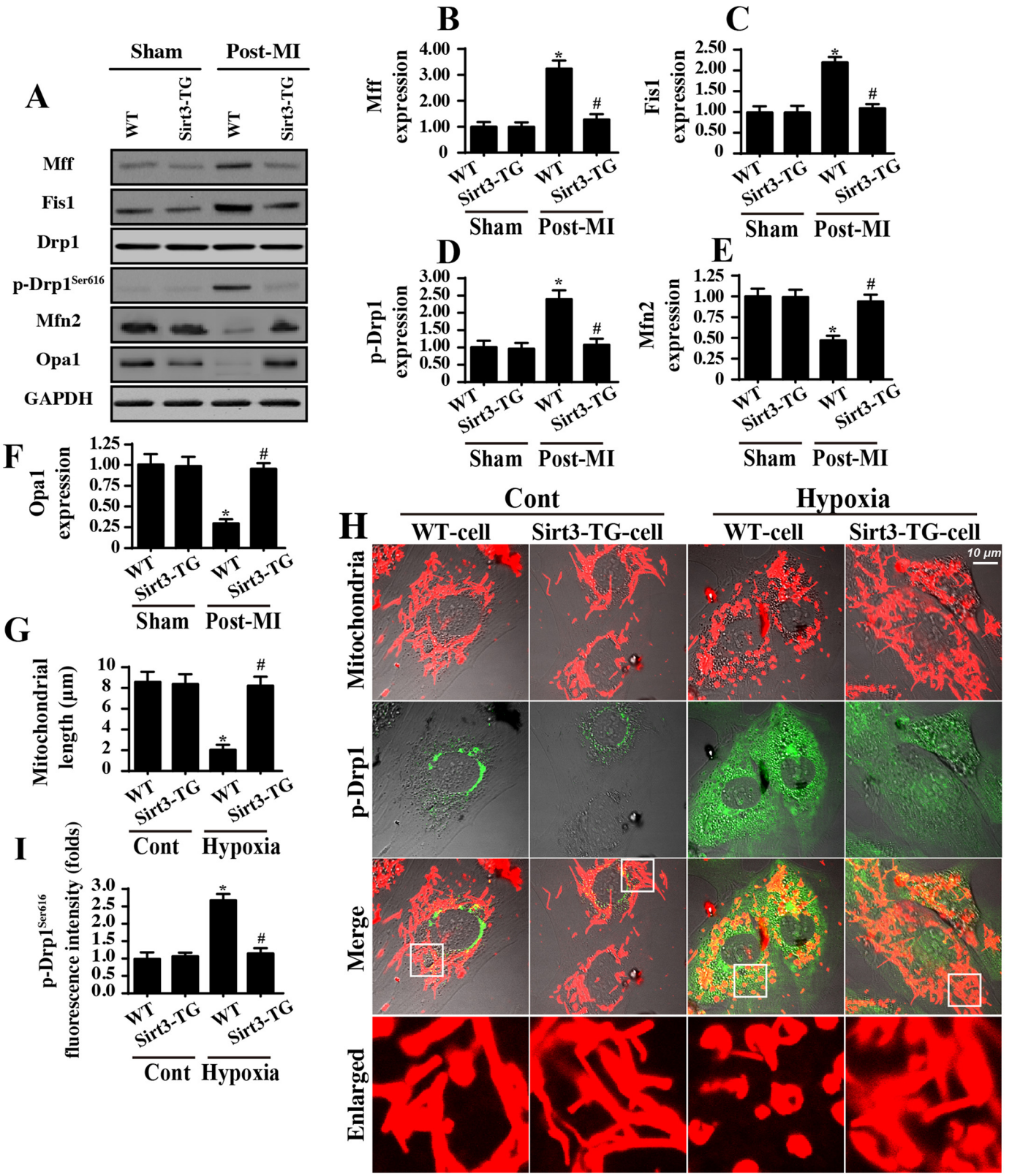
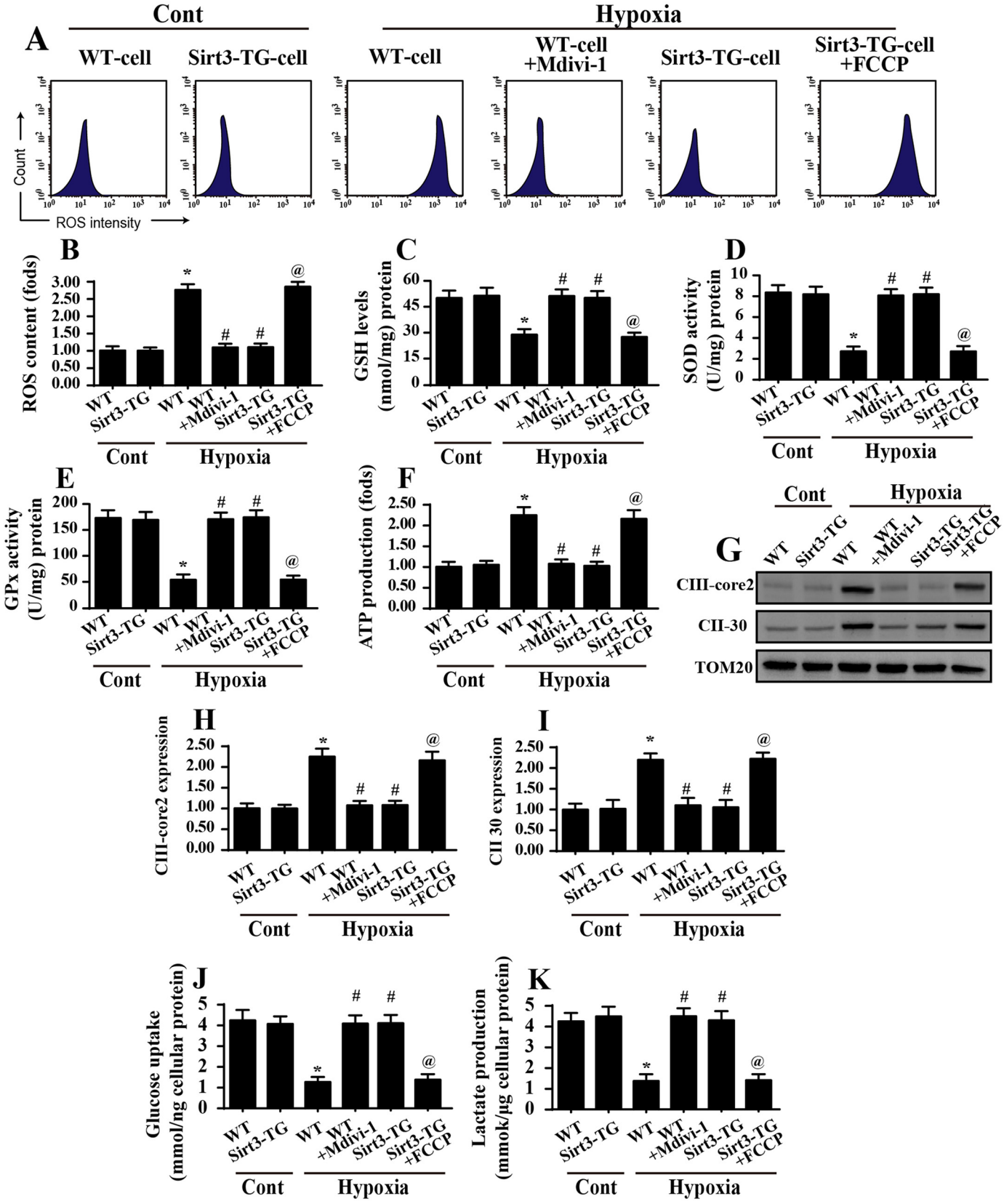


Fig. 3. Sirt3 inhibits mitochondrial fission in post-infarcted hearts. A–F. Proteins were isolated from WT mice and Sirt3-TG mice. Then, the expression of mitochondrial fission-related proteins was determined. G–H. In vitro, cardiomyocytes were isolated from WT mice and Sirt3-TG mice. Then, 48 h of hypoxia stimulation was used to mimic the post-infarction cardiac injury. Subsequently, immunofluorescence was performed to observe mitochondrial fission using a mitochondrial-specific Tom-20 antibody. The average length of mitochondria was recorded to quantify mitochondrial fission. I. Co-immunofluorescence assay for p-Drp1. The relative fluorescence intensity of p-Drp1 was determined. *$p < .05$ vs. sham group or control (cont) group; #$p < .05$ vs. WT + post-MI group or WT + hypoxia group.



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regulated mitochondrial fission. Because AMPK-Drp1 pathways are associated with chronic metabolic injury, such as diabetes, Parkinson's disease, and age-associated vascular dysfunction, we determined whether AMPK-Drp1 pathways were involved in Sirt3-mediated mitochondrial fission in post-infarcted hearts. As shown in Fig. 6A–C, compared to the control group, AMPK phosphorylation was

Fig. 4. Inhibition of mitochondrial fission sustains mitochondrial function in hypoxia-treated cardiomyocytes. A-B. Mitochondrial ROS was detected using flow cytometry. Loss- and gain-of-function assays of mitochondrial fission were performed using Mdivi-1 and FCCP, respectively. Mdivi-1, an antagonist of mitochondrial fission, was added to the control group, which was used as a negative control group for Sirt3 overexpression. In contrast, FCCP, an agonist of mitochondrial fission, was administered to the Sirt3-overexpressed cells, which was used to reactivate mitochondrial fission. C-E. ELISA for cellular antioxidant factors. F. Cellular ATP production was measured via ELISA. Mdivi-1 was added to the control group, which was used as a negative control group for Sirt3 overexpression. FCCP was administered to the Sirt3-overexpressed cells, which was used to reactivate mitochondrial fission. G-I. Proteins were isolated from cardiomyocytes in the presence of hypoxia stress. Then, the expression of the mitochondrial respiratory complex was evaluated via western blotting. J-K. The glucose intake and lactic acid production were measured in cardiomyocytes. *p < .05 vs. sham group or control (cont) group; #p < .05 vs. WT + post-MI group or WT + hypoxia group; @p < .05 vs. Sirt3-TG + hypoxia group.

significantly downregulated in response to chronic hypoxia stress; the effect was accompanied with an increase in p-Drp1^{Ser616} (Fig. 6A–C). Interestingly, Sirt3 overexpression reversed the levels of p-AMPK and thus reduced the expression of p-Drp1^{Ser616}. This finding identifies Sirt3 as the upstream regulator of AMPK-Drp1 pathways. To demonstrate whether AMPK-Drp1 was involved in Sirt3-mediated mitochondrial fission, Compound C (CC), a pathway blocker of AMPK, was added to Sirt3-overexpressed cells to inhibit AMPK activation. Then, mitochondrial fission was evaluated again using immunofluorescence. Compared to the control group, hypoxia forced mitochondria to divide into several fragmentations (Fig. 6D–F). However, Sirt3 overexpression maintained the mitochondrial network, and this effect was abolished by CC (Fig. 6D–F). Additionally, mitochondrial length was shorter in hypoxia-treated cells and was reversed to near-normal levels with Sirt3 overexpression (Fig. 6D–F). However, CC treatment negated the inhibitory effects of Sirt3 on mitochondrial fission. Further, the expression of p-Drp1^{Ser616} was further estimated via co-immunofluorescence. Compared to the control group, p-Drp1^{Ser616} expression was upregulated in response to hypoxia and was downregulated by Sirt3 overexpression (Fig. 6D–F), and this effect of Sirt3 was dependent on AMPK activity because inhibition of AMPK blocked Sirt3-mediated p-Drp1^{Ser616} downregulation. Lastly, we wanted to determine whether the AMPK-Drp1 cascade was involved in cardiomyocyte death. Therefore, a TUNEL assay was used in the presence of Sirt3 overexpression and/or CC treatment. Compared to the control group, hypoxia stress significantly increased the ratio of TUNEL-positive cells, and this effect was negated by Sirt3 overexpression (Fig. 6G–H). Interestingly, inhibition of AMPK via CC could abrogate the anti-apoptotic actions of Sirt3 on cardiomyocytes, indicating that AMPK activation is required for Sirt3-mediated cardiomyocyte survival (Fig. 6G–I). Collectively, our results suggest that the AMPK-Drp1 axis is required for Sirt3-modulated mitochondrial fission.

8. Discussion

In the present study, our in vivo and in vitro studies demonstrated that post-infarction cardiac injury was closely associated with Sirt3 downregulation. Sirt3 deficiency mediated heart dysfunction, induced myocardial oxidation, promoted cardiac fibrosis, and evoked cardiomyocyte death. At the molecular levels, loss of Sirt3 induced mitochondrial damage including mitochondrial ROS production, cell oxidative stress, ATP depletion, mitochondrial potential reduction, mitochondrial cyt-c liberation, and caspase-9 apoptotic pathway activation. Further, we confirmed that Sirt3-mediated mitochondrial damage and cardiomyocyte death were attributable to mitochondrial fission. However, overexpression of Sirt3 repressed mitochondrial fission via attenuating Drp1 phosphorylation. Molecular investigations illustrated that Sirt3 regulated mitochondrial fission and Drp1 phosphorylation via the AMPK pathway; inhibition of the AMPK pathway reactivated Drp1 phosphorylation and mitochondrial fission in Sirt3-overexpressed cells. Altogether, our results identify Sirt3 as a novel mediator for post-infarction cardiac injury via influencing mitochondrial fission and normalization of the AMPK-Drp1 pathway. To our knowledge, this is the first study to verify the protective role played by Sirt3 in post-infarction cardiac injury via modifying mitochondrial fission.

Several mechanisms have been introduced to explain the pathogenesis of post-infarction cardiac injury. Oxidative stress [35], an excessive inflammatory response [36], cellular calcium overload [37], and cardiomyocyte death [38] have been noted in the progression of post-infarction cardiac injury. Interestingly, the above pathogenic processes are closely associated with mitochondrial integrity. Damaged mitochondria cannot capture electrons to complete oxidative phosphorylation, leading to ROS overproduction and ATP undersupply [39]. The excessive ROS generation consumes a large amount of antioxidant factors, leading to cell oxidative stress [40,41]. Chronic oxidative stress disrupts protein synthesis, promotes phospholipid membrane oxidation, and impairs cardiomyocyte viability [42,43]. Moreover, ATP shortage due to mitochondria damage inhibits the cell's ability to recycle calcium, leading to calcium overload [12,44]. Additionally, uncontrolled mitochondrial ROS can activate cardiomyocyte apoptosis, and this effect is followed by an increased inflammatory response [45]. Accordingly, some researchers propose that mitochondrial homeostasis is a potential target to ameliorate post-infarction cardiac injury. This conclusion is supported by our results. We demonstrated that chronic hypoxia stress induced mitochondrial metabolism disorder and excessive mitochondrial injury. These results reconfirm that mitochondrial damage is a primary pathogenic pathway for the initiation of post-infarction cardiac injury.

Further, our results demonstrated that post-infarcted mitochondrial damage was associated with Sirt3 downregulation. Overexpression of Sirt3 protected mitochondrial structure and function against chronic hypoxia stress. The beneficial effects of Sirt3 on mitochondrial homeostasis have been extensively reported. For example, Sirt3 attenuates mitochondrial oxidative stress in neuronal injury [18], sustains mitochondrial metabolism in skeletal muscle [46], and promotes mitochondrial biogenesis in age-associated vascular dysfunction [26]. Similarly, the mitochondrial protective action of Sirt3 are also observed in Alzheimer's disease [47], cervical cancer [20], and diabetic cardiomyopathy [48]. These findings highlight that Sirt3 is an endogenous defender, sustaining mitochondrial function. At the molecular levels, Sirt3 upregulates MnSOD gene transcription via FoxO3a [49], reverses the NAD^+/NADH ratio [50], activates mitochondrial autophagy [2,51], and increases mitochondrial cardiolipin content [46]. Interestingly, in this study, we found that Sirt3 overexpression repressed mitochondrial ROS overproduction, reversed ATP generation, disrupted mitochondrial cyt-c translocation and blocked caspase-9 mitochondrial apoptosis. This result further illustrates the protective effects of Sirt3 on mitochondrial stress. Accordingly, strategies to enhance Sirt3 expression would lead to more clinical benefits for patients suffering from post-infarction cardiac injury.

Our results demonstrated that Sirt3 protected mitochondrial function via inhibiting mitochondrial fission. The activation of mitochondrial fission led to mitochondrial damage and cardiomyocyte death despite overexpression of Sirt3. Notably, mitochondrial fission has been defined as the initial signal for mitochondrial damage [30,45]. Excessive mitochondrial fission promotes the formation of mitochondrial debris, which contains intact DNA. These mitochondrial fragmentations cannot effectively produce ATP. Moreover, several researchers have reported that fragmented mitochondria are the source of ROS [27,52]. More importantly, mitochondrial fission largely initiates mitochondrial apoptosis in several disease models [15,53,54]. These findings are

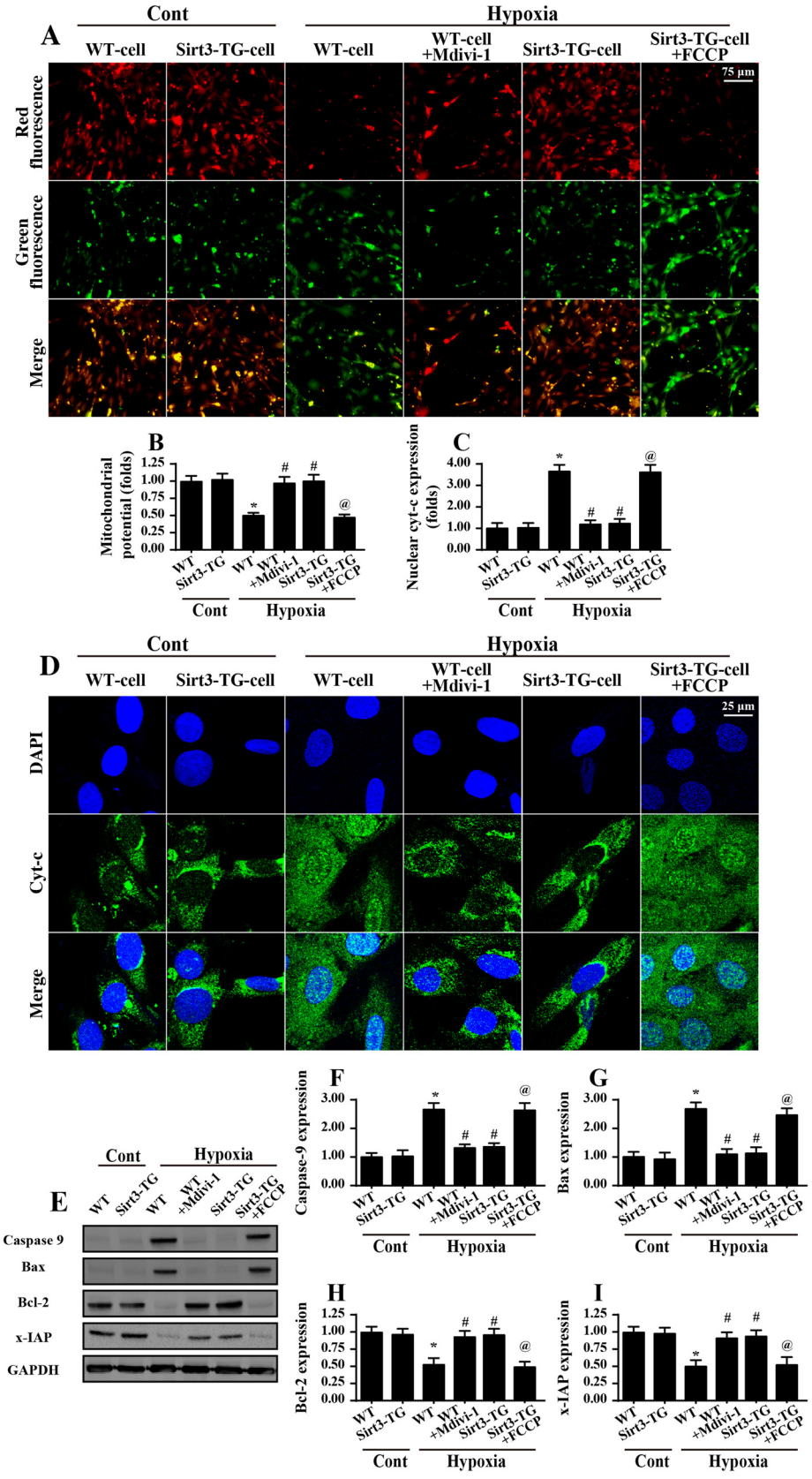

Fig. 5. Mitochondrial fission regulates mitochondrial apoptosis in cardiomyocytes. A-B. Mitochondrial potential was observed using JC-1 staining. Loss- and gain-of-function assays of mitochondrial fission were performed using Mdivi-1 and FCCP, respectively. Mdivi-1, an antagonist of mitochondrial fission, was added to the control group, which was used as a negative control group for Sirt3 overexpression. In contrast, FCCP, an agonist of mitochondrial fission, was administered to the Sirt3-overexpressed cells, which was used to reactivate mitochondrial fission. C-D. Immunofluorescence assay for cyt-c translocation. DAPI was used to tag the nucleus. The relative expression of nuclear cyt-c was quantified. E-I. Proteins were isolated from cardiomyocytes in the presence of hypoxia stress. Then, the expression of mitochondrial apoptosis proteins was evaluated via western blotting. *p < .05 vs. sham group or control (cont) group; #p < .05 vs. WT + post-MI group or WT + hypoxia group; @p < .05 vs. Sirt3-TG + hypoxia group.

similar to our results. Further, we demonstrated that Sirt3 regulated mitochondrial fission via AMPK-Drp1 pathways. AMPK is the upstream regulator of mitochondrial fission [22,23], and activated AMPK represses Drp1 phosphorylation or Mff expression, attenuating mitochondrial fission in diabetic cardiomyopathy [22], oxidative-mediated endothelial damage [55,56], and iron-loaded mesenchymal stromal cells [57,58]. Accordingly, our results, combined with previous findings, lay the foundation for detailed study of molecular mechanisms

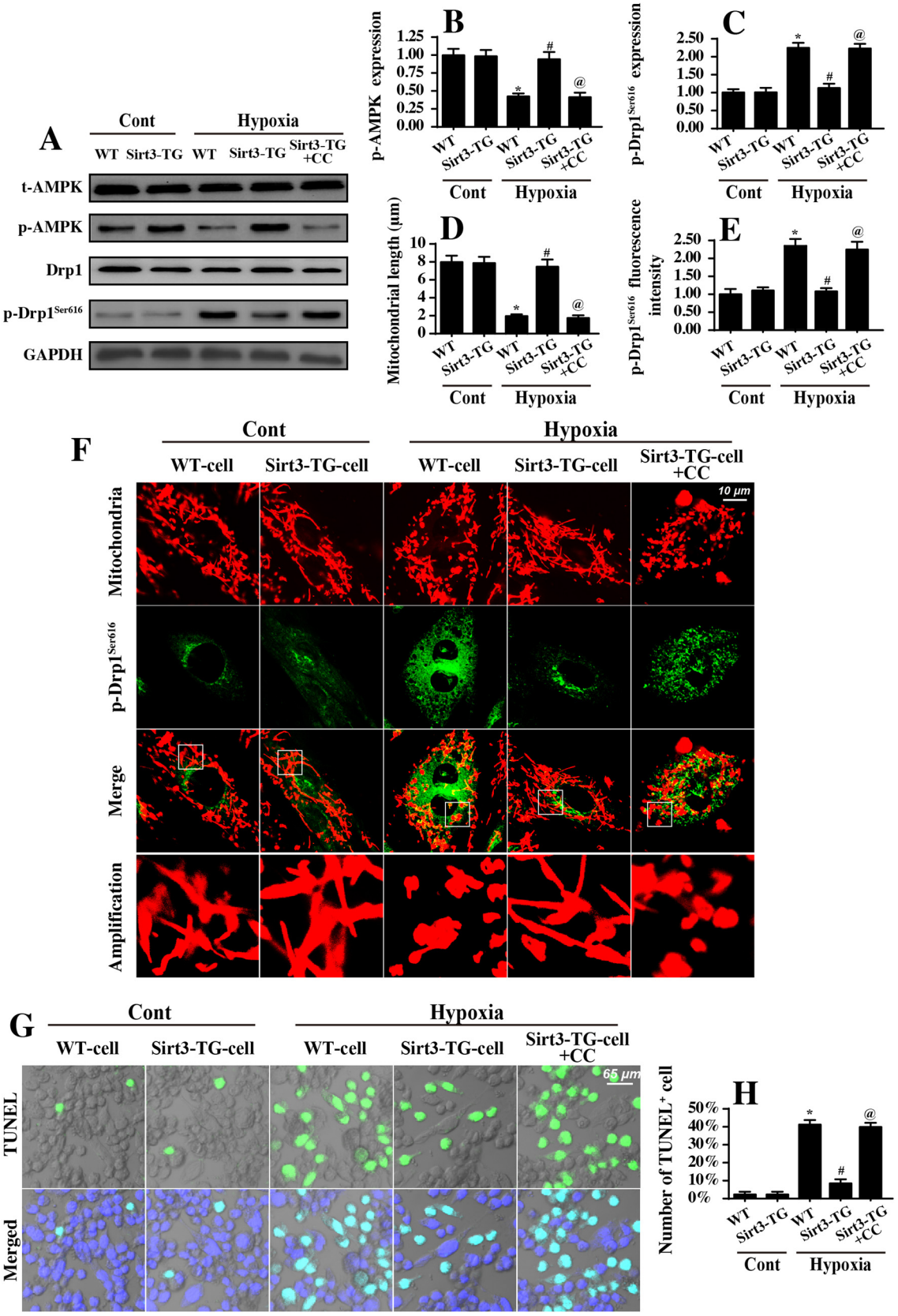

Fig. 6. Sirt3 modulates mitochondrial fission via AMPK-Drp1 pathways. A-C. Proteins were isolated from cardiomyocytes in the presence of hypoxia stress. Then, the expression of p-AMPK and p-Drp1 was measured via western blotting. D-F. Mitochondrial fission was measured using immunofluorescence. The average length of mitochondria was estimated. Compound C (CC), a pathway blocker of AMPK, was added to Sirt3-overexpressed cells to inhibit AMPK activation. The relative fluorescence intensity of p-Drp1 was determined. G-H. TUNEL assay for cell death quantification. Compound C (CC), a pathway blocker of AMPK, was added to Sirt3-overexpressed cells to inhibit AMPK activation. The number of TUNEL positive cells was evaluated. *p < .05 vs. sham group or control (cont) group; #p < .05 vs. WT + post-MI group or WT + hypoxia group; @p < .05 vs. Sirt3-TG + hypoxia group.

of mitochondrial fission initiation and regulation in chronic post-infarction cardiac injury.

Collectively, our study reported that Sirt3 downregulation promoted post-infarcted cardiac injury via mitochondrial malfunction. *Re*-activation of Sirt3 enhanced the resistance of post-infarcted hearts to chronic damage via inhibiting mitochondrial fission and normalizing AMPK-Drp1 pathways. Based on this finding, the preservation of mitochondrial integrity is of the utmost importance when designing cardioprotective therapies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JXL, WY, KLH and XJZ made substantial contributions to the concept and design of the present study, QJ, JDW, HAZ, CLL and ZJS contributed to the performance of experiments, data analysis and interpretation, and manuscript writing.

Ethics approval and consent to participate

The animal study was performed in accordance with the Declaration of Helsinki. All experimental protocols were approved by the Ethics Committee of the Department of Cardiology, Chinese PLA General Hospital, Beijing, China, 100853. The ethics reference number is PLA-33SSP1.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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