

# Melatonin prevents Drp1-mediated mitochondrial fission in diabetic hearts through SIRT1-PGC1 $\alpha$ pathway

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## Abstract

Myocardial contractile dysfunction is associated with an increase in mitochondrial fission in patients with diabetes. However, whether mitochondrial fission directly promotes diabetes-induced cardiac dysfunction is still unknown. Melatonin exerts a substantial influence on the regulation of mitochondrial fission/fusion. This study investigated whether melatonin protects against diabetes-induced cardiac dysfunction via regulation of mitochondrial fission/fusion and explored its underlying mechanisms. Here, we show that melatonin prevented diabetes-induced cardiac dysfunction by inhibiting dynamin-related protein 1 (Drp1)-mediated mitochondrial fission. Melatonin treatment decreased Drp1 expression, inhibited mitochondrial fragmentation, suppressed oxidative stress, reduced cardiomyocyte apoptosis, improved mitochondrial function and cardiac function in streptozotocin (STZ)-induced diabetic mice, but not in SIRT1<sup>-/-</sup> diabetic mice. In high glucose-exposed H9c2 cells, melatonin treatment increased the expression of SIRT1 and PGC-1 $\alpha$  and inhibited Drp1-mediated mitochondrial fission and mitochondria-derived superoxide production. In contrast, SIRT1 or PGC-1 $\alpha$  siRNA knockdown blunted the inhibitory effects of melatonin on Drp1 expression and mitochondrial fission. These data indicated that melatonin exerted its cardioprotective effects by reducing Drp1-mediated mitochondrial fission in a SIRT1/PGC-1 $\alpha$ -dependent manner. Moreover, chromatin immunoprecipitation analysis revealed that PGC-1 $\alpha$  directly regulated the expression of Drp1 by binding to its promoter. Inhibition of mitochondrial fission with Drp1 inhibitor mdivi-1 suppressed oxidative stress, alleviated mitochondrial dysfunction and cardiac dysfunction in diabetic mice. These findings show that melatonin attenuates the development of diabetes-induced cardiac dysfunction by preventing mitochondrial fission through SIRT1-PGC1 $\alpha$  pathway, which negatively regulates the expression of Drp1 directly. Inhibition of mitochondrial fission may be a potential target for delaying cardiac complications in patients with diabetes.

## KEYWORDS

diabetes, Drp1, melatonin, mitochondrial fission, PGC-1 $\alpha$ , silent information regulator 1

Mingge Ding, Na Feng, Daishi Tang contributed equally to this study.

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## 1 | INTRODUCTION

The prevalence of diabetes mellitus appears to increase rapidly during recent years in the world, currently estimated at 11.6% in China<sup>1</sup> and 8.3% in America.<sup>2</sup> Diabetic cardiomyopathy (DCM) is one major complication in diabetics, which impairs myocardial performance even without coronary artery disease or hypertension.<sup>3</sup> Multiple factors are responsible for the pathogenesis of DCM. Mounting evidence from current studies has indicated that the generation of reactive oxygen species (ROS) contributes to the progression of diabetes-induced cardiac dysfunction.<sup>4,5</sup> Mitochondria are critical organelles for ROS generation and energy production in cardiomyocytes. Recent work has highlighted the important role of mitochondrial fusion/fission dynamics in mitochondrial homeostasis.<sup>6</sup> Mitochondrial fusion is considered to be beneficial because it is associated with an increase in the mitochondrial function and ATP production. In contrast, excessive mitochondrial fission seems to be detrimental because it is associated with decreased mitochondrial function and increased ROS.<sup>7,8</sup> Increased mitochondrial fission was observed in several types of cultured cells from the cardiovascular system under prolonged hyperglycemic conditions.<sup>9</sup> Moreover, myocardial contractile dysfunction is associated with an increase in mitochondrial fission among diabetic patients.<sup>10</sup> However, whether inhibition of mitochondrial fission is efficient to protect against diabetes-induced cardiac dysfunction is still largely unknown.

Melatonin, chemically N-acetyl-5-methoxytryptamine, has been proved to exert its protective properties in a variety of cardiovascular diseases.<sup>11-15</sup> Recent studies have reported that melatonin has the ability to protect against diabetes-induced cardiac dysfunction.<sup>16,17</sup> Our previous study demonstrated that melatonin alleviated posttraumatic myocardial injury and cardiac dysfunction. The mechanisms were associated with improved performance of mitochondrial dynamics and function.<sup>18</sup> Several recent studies from others also demonstrated that melatonin-inhibited mitochondrial fission under some pathological conditions,<sup>19-22</sup> indicating that melatonin exerts a substantial influence on the regulation of mitochondrial dynamics. Given the potentially important role of mitochondrial dynamics in diabetic hearts, we speculated that melatonin may alleviate diabetes-induced cardiac dysfunction via inhibition of mitochondrial fission. Moreover, the exact underlying mechanisms that how melatonin protects against mitochondrial fission remain poorly understood.

Silent information regulator 1 (SIRT1) is an NAD<sup>+</sup>-dependent protein deacetylase involved in the cardioprotective effects of melatonin. Melatonin reduced myocardial ischemia-reperfusion injury in both nondiabetic and diabetic animals via activation of SIRT1.<sup>23,24</sup> SIRT1 exerts its beneficial effects via the reduction of oxidative stress and endoplasmic reticulum stress.<sup>25,26</sup> Decreased SIRT1 expression

was found in diabetic hearts while normalizing or activating SIRT1 signaling alleviated diabetes-induced cardiac dysfunction.<sup>27,28</sup> It has been reported that activation of SIRT1 improves mitochondrial function and attenuates cardiac dysfunction in diabetic animals.<sup>29,30</sup> However, whether SIRT1 signaling is involved in the regulatory effect of melatonin on mitochondrial fission is unknown.

Therefore, the aims of this study were (i) to determine whether melatonin alleviates diabetes-induced cardiac dysfunction via inhibition of mitochondrial fission; (ii) if so, to investigate whether melatonin prevents mitochondrial fission via activation of SIRT1 signaling. Using *in vivo* diabetic mice and *in vitro* hyperglycemia-treated cells, our results demonstrate for the first time that melatonin prevents Drp1-mediated mitochondrial fission in diabetes in a SIRT1/PGC-1 $\alpha$ -dependent manner.

## 2 | MATERIALS AND METHODS

### 2.1 | Reagents

Melatonin (Mel) and streptozocin (STZ) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Mdivi-1 was obtained from MedChem Express (MCE, NJ, USA). Dihydroethidium (DHE) probe, mitochondria isolation kits, malondialdehyde (MDA), and manganese superoxide dismutase (MnSOD) activity assay kits were purchased from Beyotime Biotechnology (Jiangsu, China). Mitochondrial complex activity assay kits were purchased from GENMED Scientific Inc (Arlington, MA, USA). PGC-1 $\alpha$  siRNA, Drp1 siRNA, and control siRNA were obtained from Santa Cruz Biotechnology (Catalog Number: sc-72151, sc-270298, and sc-37007, Dallas, TX, USA). MitoTracker Red CMXRos probe, MitoSOX probe, and Lipofectamine RNAiMAX reagent were obtained from Invitrogen (Carlsbad, CA, USA). Caspase-3 and ATP assay kits were obtained from Biovision (CA, USA). Terminal deoxynucleotidyl nick-end labeling (TUNEL) assay kits were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Adenoviruses expressing SIRT1, PGC-1 $\alpha$  or GFP were purchased from Hanbio Technology Ltd (Shanghai, China). The primary antibodies against Drp1, Opa-1, Mfn2, Nox4, and COX IV were obtained from Abcam Biotechnology (Cambridge, MA, USA). PGC-1 $\alpha$  antibody was obtained from Novus Biologicals (Littleton, CO, USA). The primary antibody against Fis1 was purchased from GeneTex (Irvine, CA, USA). The goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Beyotime (Jiangsu, China). SIRT1 siRNA (Product Number: 12241S), SIRT1 antibody,  $\beta$ -actin antibody, and SimpleChIP Plus Enzymatic Chromatin IP kits were obtained from Cell Signaling Technology (Beverly, MA, USA).

## 2.2 | Animal experiments

All animal experiments were performed in compliance with the NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University. Mice homozygous for the floxed *sirt1* alleles (>99% C57BL/6 genetic background, *SIRT1<sup>fl/fl</sup>*) were obtained from National Resource Center for Mutant Mice (NRCMM), initially from The Jackson Laboratory (stock number: 008041). Mutated estrogen receptor (Mer)-Cre-Mer Tg mice under the  $\alpha$ -myosin heavy chain promoter (*Myh6-MerCreMer*) were obtained from NRCMM (>99% C57BL/6 genetic background). Cardiac-specific *SIRT1*-knockout (*SIRT1<sup>-/-</sup>*) mice were generated by crossing *SIRT1<sup>fl/fl</sup>* mice with *Myh6-MerCreMer* Tg mice, followed by 2-week oral administration of tamoxifen (30 mg/kg/d in chow) as described previously.<sup>31</sup> Male cardiac-specific *SIRT1*-knockout (*SIRT1<sup>-/-</sup>*) aged 8-10 weeks were applied to the study at 2 weeks after the last administration of tamoxifen. Age-matched male littermates (*SIRT1<sup>fl/fl</sup>* with tamoxifen) were served as controls.

The mice were injected with STZ (50 mg/kg/d) in citrate buffer (pH 4.3) intraperitoneally for 5 consecutive days. Hyperglycemic mice with fasting blood glucose >11.1 mmol/L from three samplings 2 weeks after the first injection of STZ were considered to have diabetes. Nondiabetic animals were administrated with an equivalent volume of citrate buffer. Diabetic mice were treated with the vehicle, melatonin (10 mg/kg, once daily, intraperitoneally) or mdivi-1 (10 mg/kg, twice per week, intraperitoneally), respectively for another 10 weeks. Melatonin was injected at 9:00 AM every morning when serum melatonin level was relatively low. The dosage of melatonin or mdivi-1 treatment was chosen based on previous studies about the effect of melatonin or mdivi-1 on diabetic complications.<sup>16,32,33</sup> At the end of the experiments (12 weeks after the first injection of STZ), hearts were collected for mitochondrial dynamics, apoptosis, ROS generation, and Western blot analyses.

## 2.3 | Echocardiography measurements

Echocardiography was performed in M-mode with a VEVO 2100 echocardiography system (Visual Sonics, Toronto, ON, Canada) as described previously.<sup>34</sup> Left ventricular end-systolic volume (LVESV), Left ventricular fractional shortening (LVFS), and ejection fraction (LVEF) were measured in M-mode images using computer algorithms.

## 2.4 | Transmission electron microscopy

Heart samples from the ventricular anterior wall were obtained and fixed in 2.5% glutaraldehyde (pH = 7.2) and 1%

osmium tetroxide, and processed as described previously.<sup>35</sup> The slices were viewed with one transmission electron microscope (JEM-1230, JEOL Ltd., Tokyo, Japan). Images were obtained by a technician blinded to the treatment. Mitochondrial size and the number of mitochondria were analyzed with Image-Pro Plus software. The percentage of mitochondria that classified into three size categories in a given field (<0.6  $\mu\text{m}^2$ , within 0.6-1.0  $\mu\text{m}^2$ , >1.0  $\mu\text{m}^2$ ) was counted as described previously.<sup>34,36</sup>

## 2.5 | Mitochondrial electron transport chain (ETC) complex activities and ATP content

Cardiac mitochondria were isolated from fresh cardiac tissues using the Mitochondria Isolation Kit (Beyotime, Jiangsu, China). Mitochondrial ETC complex (I-V) activities and ATP content were determined according to manufacturer's instructions (GENMED, MA, USA).

## 2.6 | Determination of myocardial apoptosis and ROS production

Myocardial apoptosis was determined by caspase-3 activity measurement and TUNEL staining as described previously.<sup>34</sup> Dihydroethidium (DHE) staining was utilized for detection of intracellular superoxide anion levels in fresh heart tissues. MitoSOX staining was utilized for detection of mitochondrial ROS levels. Measurements of malondialdehyde (MDA) level and manganese superoxide dismutase (MnSOD) activity were performed with commercial assay kits according to manufacturer's protocols.

## 2.7 | Cell culture

H9c2 cells (obtained from Tiancheng Biotechnology, Shanghai, China) were grown in Dulbecco's modified Eagle's medium containing normal glucose (5.5 mmol/L, NG) with 10% fetal bovine serum supplement. Cells were incubated in humidified air (5%  $\text{CO}_2$ ) at 37°C. The media were changed every two days, and the cells were used in the experiments once they reached 70-80% confluence. The cells were incubated in the vehicle or melatonin (100  $\mu\text{mol/L}$ ) for 4 hours<sup>17,18</sup> and subjected to high glucose culture (33 mmol/L, HG) for 48 hours.

## 2.8 | siRNA transfection

H9c2 cells were transfected with *SIRT1* siRNA, *PGC-1 $\alpha$*  siRNA, *Drp1* siRNA, or control siRNA by employing Lipofectamine RNAiMAX reagent (Invitrogen). After siRNA transfection, the cells were treated with vehicle or melatonin (100  $\mu\text{mol/L}$ ) for 4 hours and subjected to HG for 48 hours.

## 2.9 | Adenoviral transfection

H9C2 cells were transfected with adenoviruses harboring SIRT1, PGC-1 $\alpha$ , or GFP as described previously.<sup>37</sup> The titers of adenoviruses employed in this study were  $1.2 \times 10^{10}$  PFU/mL, and the multiplicity of infection (MOI) was 100:1. After adenoviral transfection, the cells were subjected to NG or HG for 48 hours.

## 2.10 | Assessment of mitochondrial morphology in the cells

Mitochondrial morphology was evaluated in H9c2 cells that were stained with MitoTracker Red CMXRos probe (100 nmol/L, 30 minutes at 37°C). Images were acquired by means of a confocal laser scanning microscope (Olympus FV 1000, Japan) as described previously.<sup>18</sup> Mitochondrial volume and the number of mitochondria were analyzed and quantified. Percentage of cells with fragmented mitochondria (small and round) was determined.

## 2.11 | Western blotting analysis

Mice hearts and H9c2 cells were lysed with lysis buffer containing protease inhibitor cocktail. Western blotting was carried out using the standard method as described previously.<sup>26</sup> The primary antibodies used were as follows: SIRT1, Drp1, Fis1, Opa1, Mfn2, Nox4, COX IV, PGC-1 $\alpha$ , and  $\beta$ -actin.

## 2.12 | Real-time quantitative PCR

RNA extraction and Real-time quantitative PCR were performed as described previously.<sup>26</sup> The primer sequences were as follows: Drp1 forward GGTGGAATTGGAGATGGTGGTCGA, reverse TTCGTGCAACTGGAAGTGG CACA; Actin forward GTCCCTCACCTCCCAAAG, reverse GCTGCCTCAACACCTCAACCC. Data were normalized relative to actin and expressed as a relative ratio.

## 2.13 | Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was carried out using the SimpleChIP Plus Enzymatic Chromatin IP kit (Cell Signaling) as described previously.<sup>38</sup> In brief, H9c2 cells were fixed with formaldehyde and then quenched with glycine. The sheared chromatin was incubated with PGC-1 $\alpha$  antibody and protein G magnetic beads. DNA released from the precipitation was detected by PCR analysis. The primers specific to the PGC-1 $\alpha$  binding region within Drp1 promoter region were as follows: Drp1 promoter forward: 5'-CAGATTCACGGACCCAGCTT-3', reverse:

5'-CAAAGGCTGTCGGGAGATGT-3'. IgG was used as the negative control.

## 2.14 | Statistical analysis

All data were presented as mean  $\pm$  standard error (SEM). All measured data were subjected to One-way ANOVA followed by Bonferroni post hoc test with the utilization of GraphPad Prism software version 5.0.  $P < .05$  was taken as statistically significant.

## 3 | RESULTS

### 3.1 | Melatonin reduced cardiomyocyte apoptosis and improved cardiac function in diabetic mice but not in SIRT1<sup>-/-</sup> diabetic mice

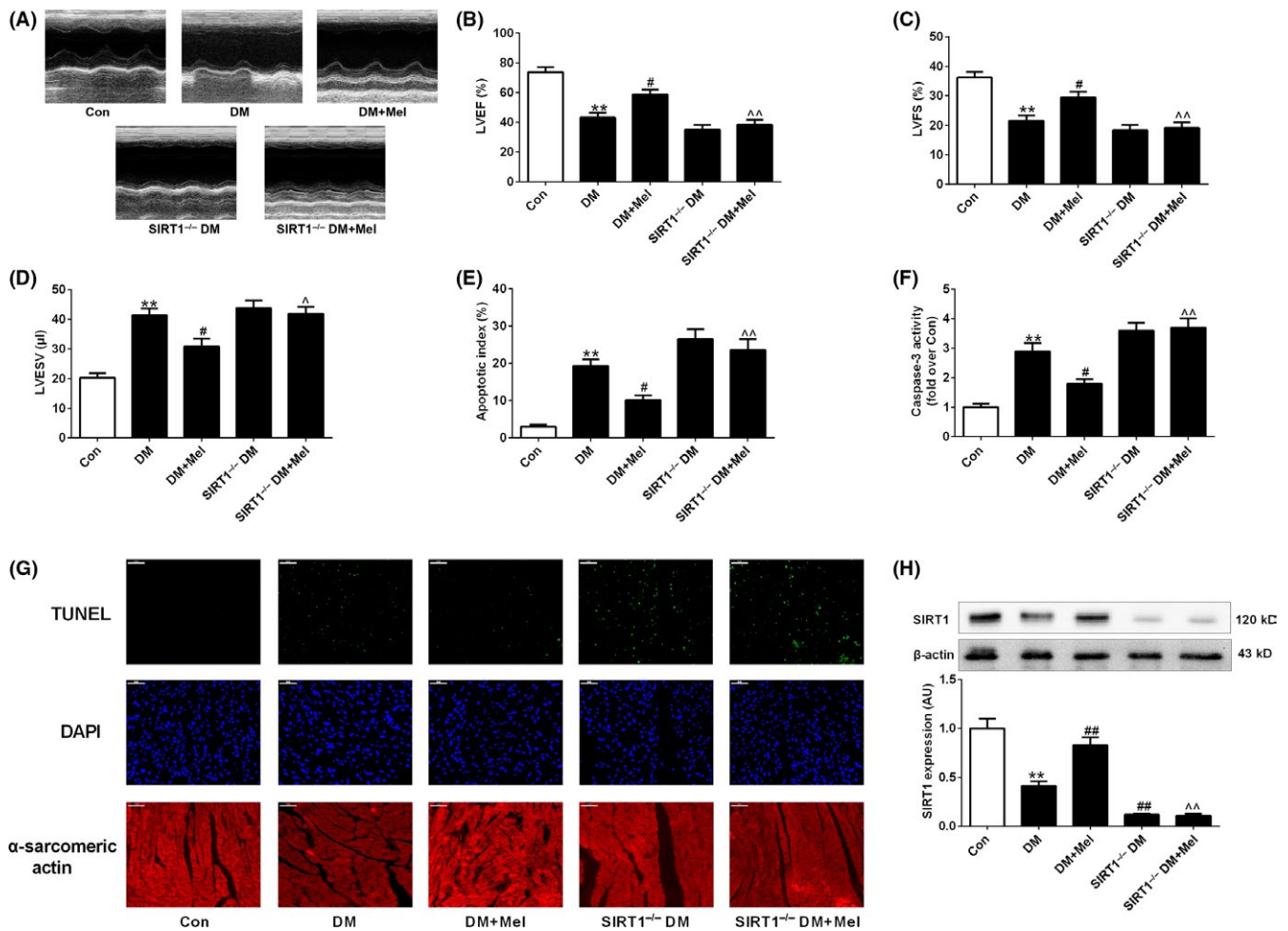
Cardiac-specific SIRT1-knockout (SIRT1<sup>-/-</sup>) mice were generated by crossing SIRT1<sup>fl/fl</sup> mice with Myh6-MerCreMer Tg mice (Figure S1A,B), followed by 2-week tamoxifen administration. There was almost no SIRT1 protein expression in cardiac tissues of SIRT1<sup>-/-</sup> mice after tamoxifen treatment. In other tissues such as the brain, liver, and muscle, the protein levels of SIRT1 were comparable between wild-type (WT), SIRT1<sup>fl/fl</sup> and SIRT1<sup>-/-</sup> mice (Figure S1C). Cardiac function assessed at 2 weeks after tamoxifen treatment showed that there were no significant changes in LVEF and LVESV between SIRT1<sup>fl/fl</sup> and SIRT1<sup>-/-</sup> mice (Figure S1D-F).

Compared with nondiabetic control mice, diabetic mice developed significantly cardiac dysfunction as evidenced by decreased LVEF and LVFS and increased LVESV at 12 weeks after STZ injection (Figure 1A-D). Concomitantly, myocardial caspase-3 activity and apoptosis index were significantly increased in diabetic hearts (Figure 1E-G). Although melatonin treatment did not significantly affect blood glucose and body weight in diabetic animals (Table S1), administration of melatonin alleviated cardiac dysfunction and reduced cardiomyocyte apoptosis in diabetic hearts (Figure 1A-G). Moreover, melatonin administration restored the decreased expression of SIRT1 in diabetic hearts (Figure 1H). Nevertheless, when cardiac SIRT1 was knocked out, melatonin treatment failed to improve cardiac dysfunction and inhibit myocardial apoptosis in diabetic mice (Figure 1A-H).

### 3.2 | Melatonin prevented Drp1-mediated mitochondrial fission and improved mitochondrial function in diabetic mice but not in SIRT1<sup>-/-</sup> diabetic mice

There was no significant difference in the number of mitochondria per  $\mu\text{m}^2$  among all the groups (Figure 2A,B). Mean mitochondrial size was smaller in diabetic hearts compared to control hearts (Figure 2A,C). Moreover, the percentage



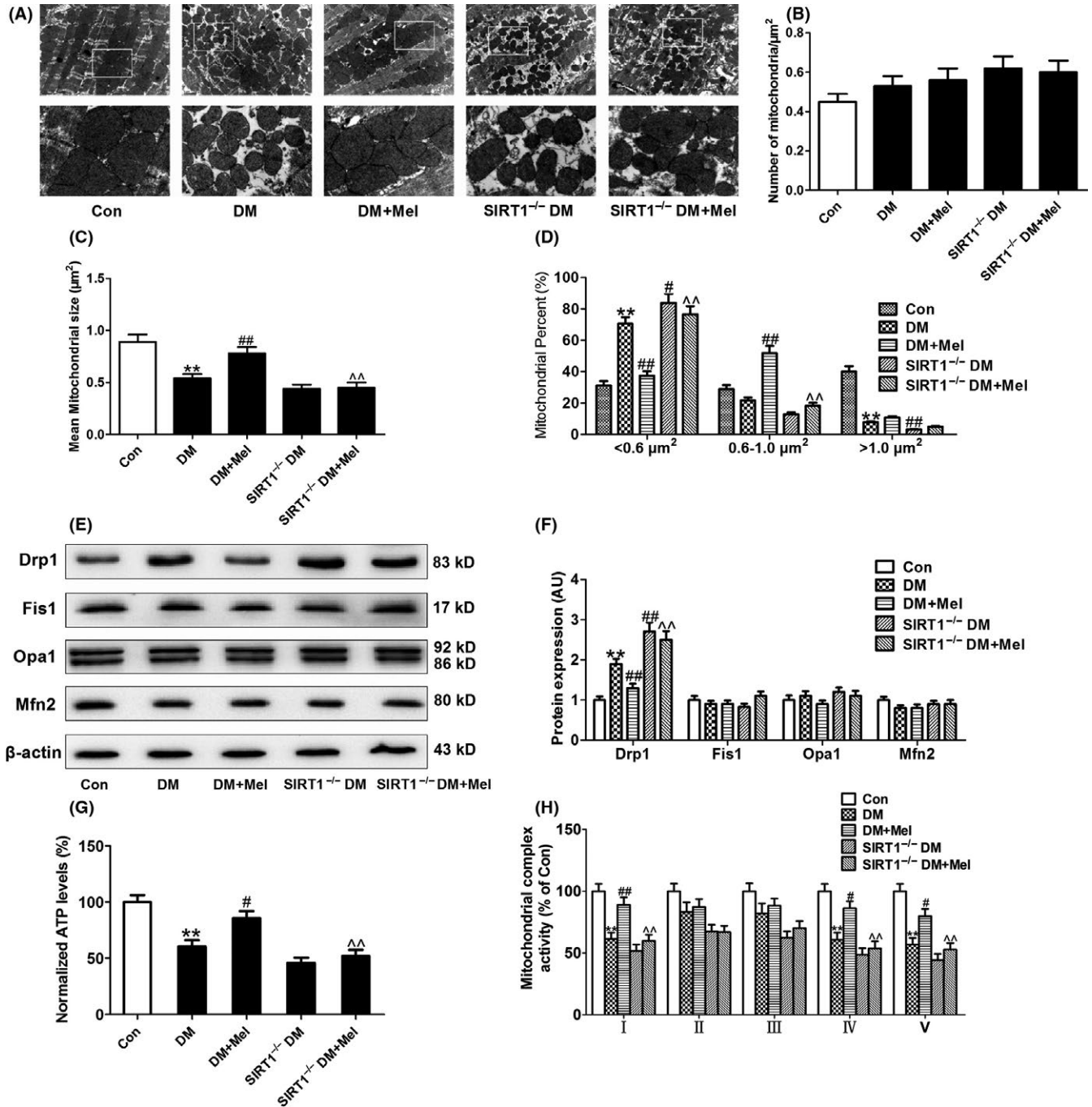


**FIGURE 1** Melatonin reduced cardiomyocyte apoptosis and improved cardiac function in diabetic mice but not in SIRT1<sup>-/-</sup> diabetic mice. (A) Representative echocardiography images. (B) Left ventricular ejection fraction (LVEF). (C) Left ventricular fractional shortening (LVFS). (D) Left ventricular end-systolic volume (LVESV). (E) Apoptosis index. (F) Myocardial caspase-3 activity (fold over Con). (G) Representative photomicrographs of TUNEL-stained and DAPI-stained heart sections. Original magnification  $\times 400$ . (H) Protein expression of SIRT1. Presented values are means  $\pm$  SEM. DM, diabetes mellitus; Mel, melatonin.  $n = 8$  in each group. \*\* $P < .01$  vs Con. #  $P < .05$ , ##  $P < .01$  vs DM.  $\Delta$   $P < .05$ , ^^  $P < .01$  vs DM+Mel

of mitochondria smaller than  $0.6 \mu\text{m}^2$  was significantly increased in diabetic hearts compared to control hearts. The percentage of mitochondria bigger than  $1 \mu\text{m}^2$  was decreased in diabetic hearts compared to control hearts (Figure 2D). Meanwhile, the expression pattern of the main mitochondrial fission-related proteins (Drp1 and Fis1) and fusion-related proteins (Opa1 and Mfn2) were assessed. Compared with nondiabetic control hearts, diabetic hearts exhibited higher levels of Drp1, while there were no significant changes in the protein expressions of Fis1, Opa1, and Mfn2 between diabetic and control hearts (Figure 2E,F). These results suggested that Drp1-mediated mitochondrial fission was enhanced in diabetic hearts. In addition, mitochondrial complex I, IV, V activities, and ATP levels were lower in diabetic hearts compared with control hearts (Figure 2G,H).

Diabetic mice treated with melatonin displayed reduced levels of mitochondrial fission compared to vehicle-treated

diabetic mice as evidenced by increased mean size of mitochondria, decreased percentage of mitochondria smaller than  $0.6 \mu\text{m}^2$  and increased percentage of mitochondria between  $0.6 \mu\text{m}^2$  and  $1 \mu\text{m}^2$  (Figure 2A,C,D). Melatonin treatment reduced diabetes-enhanced Drp1 expression but had no significant effect on the expression of Fis1, Opa1, and Mfn2 in mouse hearts (Figure 2E,F). Mitochondrial complex I, IV, V activities, and ATP levels were increased in melatonin-treated diabetic hearts (Figure 2G,H). Compared with diabetic hearts, SIRT1<sup>-/-</sup> diabetic hearts exhibited further enhanced Drp1-mediated mitochondrial fission evidenced by increased Drp1 expression, elevated percentage of mitochondria smaller than  $0.6 \mu\text{m}^2$  and lowered percentage of mitochondria bigger than  $1 \mu\text{m}^2$  (Figure 2A,D,E,F). Treatment with melatonin did not affect mitochondrial dysfunction and Drp1-mediated mitochondrial fission in SIRT1<sup>-/-</sup> diabetic mice (Figure 2A-H).

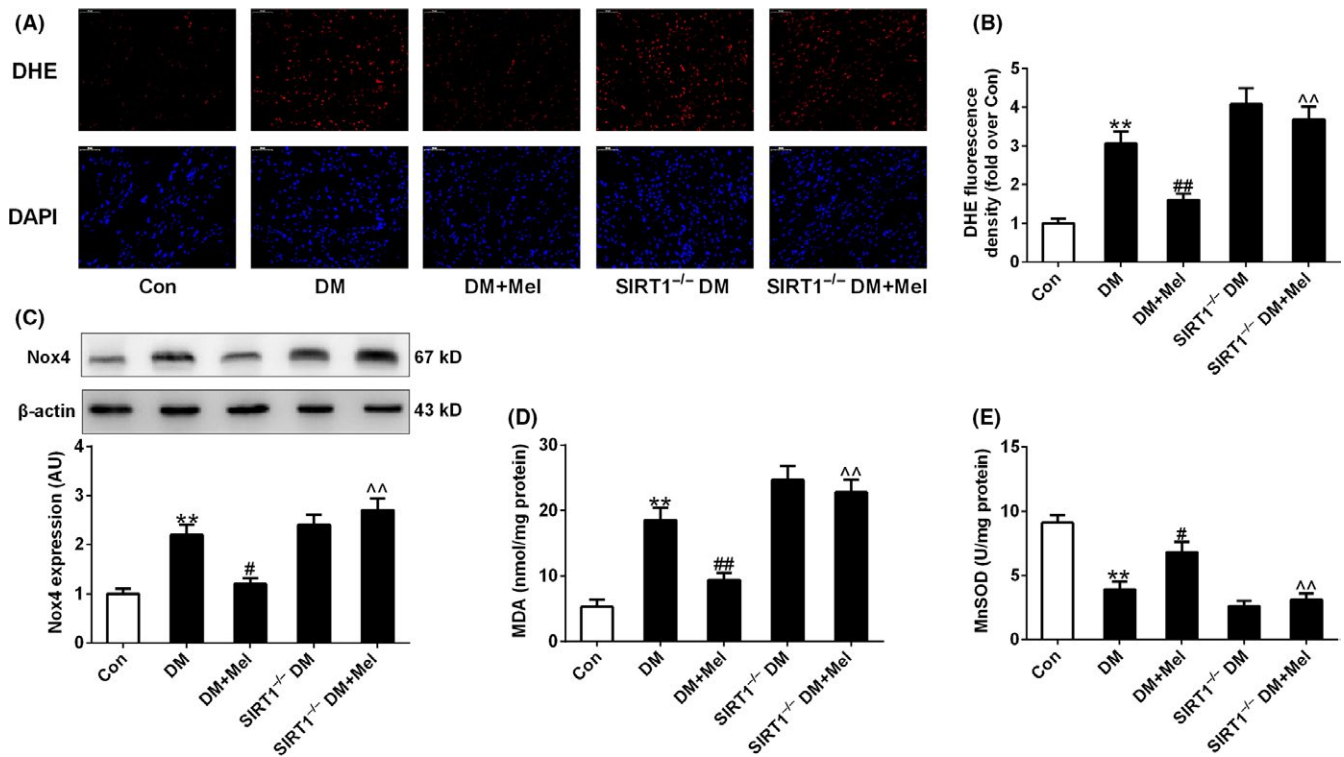


**FIGURE 2** Melatonin prevented Drp1-mediated mitochondrial fission and improved mitochondrial function in diabetic mice but not in  $SIRT1^{-/-}$  diabetic mice. (A) Representative transmission electron microscopic images of the myocardium (major finding is B-D). (B) The number of mitochondria per  $\mu\text{m}^2$ . (C) Mean size of mitochondria. (D) Percentage of mitochondria that sorted into three size categories based on size. (E,F) Protein expressions of mitochondrial fission-related proteins (Drp1 and Fis1) and fusion-related proteins (Opa1 and Mfn2). (G) Normalized ATP levels. (H) Mitochondrial complex activity (percentage of Con). Presented values are means  $\pm$  SEM. DM, diabetes mellitus; Mel, melatonin.  $n = 4-8$  in each group.  $**P < .01$  vs Con.  $\#P < .05$ ,  $\#\#P < .01$  vs DM.  $^{\wedge}P < .05$ ,  $^{\wedge\wedge}P < .01$  vs DM+Mel

### 3.3 | Melatonin inhibited oxidative stress in diabetic hearts but not in $SIRT1^{-/-}$ diabetic hearts

Diabetic hearts has been reported to exhibit enhanced ROS generation and oxidative stress. Nox4-derived ROS is the

major source of oxidative stress in diabetic hearts and contributes to myocardial cell injury at early stages.<sup>39</sup> As expected, myocardial superoxide anion production (stained by DHE) and Nox4 expression and MDA levels were significantly higher in diabetic mice compared with control animals (Figure 3A-D), while MnSOD activity of diabetic hearts



**FIGURE 3** Melatonin inhibited oxidative stress in diabetic hearts but not in SIRT1<sup>-/-</sup> diabetic hearts. (A) Representative microphotographs of DHE staining in heart sections. Original magnification  $\times 400$ . (B) Quantitative analysis of DHE fluorescence density (fold over Con). (C) Protein expression of Nox4. (D) Myocardial malondialdehyde (MDA) content. (E) Mitochondrial manganese superoxide dismutase (MnSOD) activity. Presented values are means  $\pm$  SEM. DM, diabetes mellitus; Mel, melatonin.  $n = 8$  in each group. \*\* $P < .01$  vs Con. #  $P < .05$ , ##  $P < .01$  vs DM. <sup>^^</sup>  $P < .01$  vs DM+Mel

was lowered (Figure 3E). There was a small trend toward enhanced myocardial oxidative stress (elevated superoxide anion production, increased MDA levels, and decreased MnSOD activity) in SIRT1<sup>-/-</sup> diabetic hearts compared to diabetic hearts while the difference did not reach a significant level. Melatonin treatment inhibited the increase in myocardial oxidative stress in diabetic mice, but not in SIRT1<sup>-/-</sup> diabetic mice (Figure 3A-E).

### 3.4 | Melatonin inhibited Drp1-mediated mitochondrial fission and mitochondria-derived superoxide production in hyperglycemia-treated cells

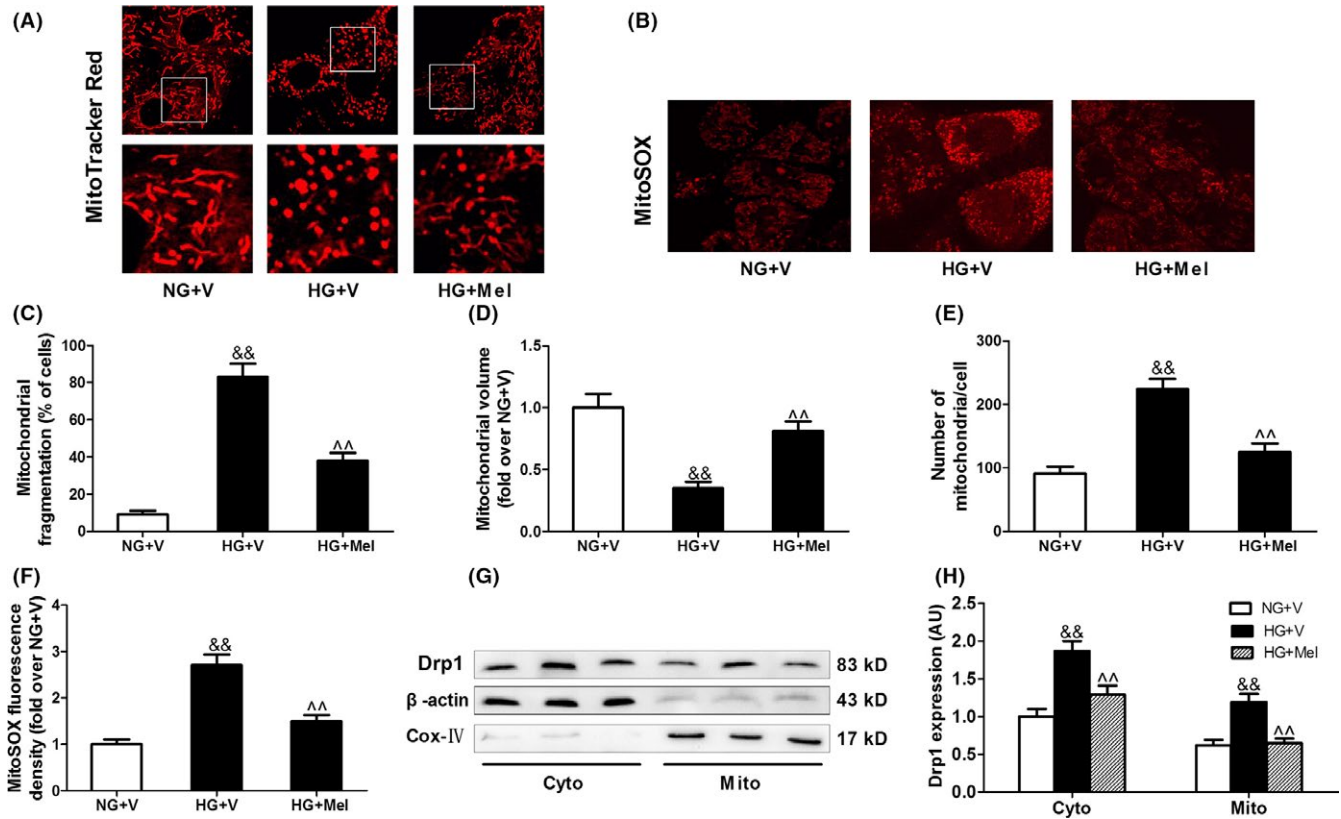
Very few reports have studied mitochondrial dynamics in cultured adult ventricular cardiomyocytes in vitro, as acute manipulation of fission and fusion proteins resulted in significant morphological changes in animal hearts and H9c2 cells but only mild changes in adult cardiomyocytes.<sup>35,40</sup> We then investigated whether melatonin inhibits high glucose-induced mitochondrial fragmentation in H9c2 cells by visualizing mitochondrial morphology with MitoTracker Red probe. As shown in Figure S2, in H9c2 cells cultured in normal glucose medium (NG, 5.5 mmol/L) for 48 hours, mitochondrial morphology mainly appeared as elongated tubules with

highly interconnecting networks. There were no significant changes in mitochondrial morphology in cells cultured in osmotic control (OC, 27.5 mmol/L mannitol plus 5.5 mmol/L glucose) medium for 48 hours compared with those cultured in NG. After stimulation with high glucose (HG 33 mmol/L) for 48 hours, mitochondria became spherical and shorter. The volume of mitochondria was decreased and the number of mitochondria was increased, indicating mitochondrial fragmentation. Compared with NG and OC treatments, HG increased Drp1 protein expression without affecting Fis1, Opa1, and Mfn2 expression (Figure S2E-F). Notably, melatonin treatment attenuated HG-induced mitochondrial fragmentation (Figure 4A,C-E) and reduced the expression of Drp1 in both cytoplasmic fractions and mitochondrial fractions (Figure 4G,H). Moreover, melatonin treatment reduced mitochondria-derived superoxide production (stained by MitoSOX) in the cells cultured in HG medium (Figure 4B,F).

### 3.5 | Melatonin reduces Drp1 expression and mitochondrial fission through SIRT1-PGC1 $\alpha$ signaling pathway

Whether SIRT1 is essential for the melatonin-induced inhibition of mitochondrial fission was also tested in the cells. As shown in Figure 5, silencing SIRT1 expression with small





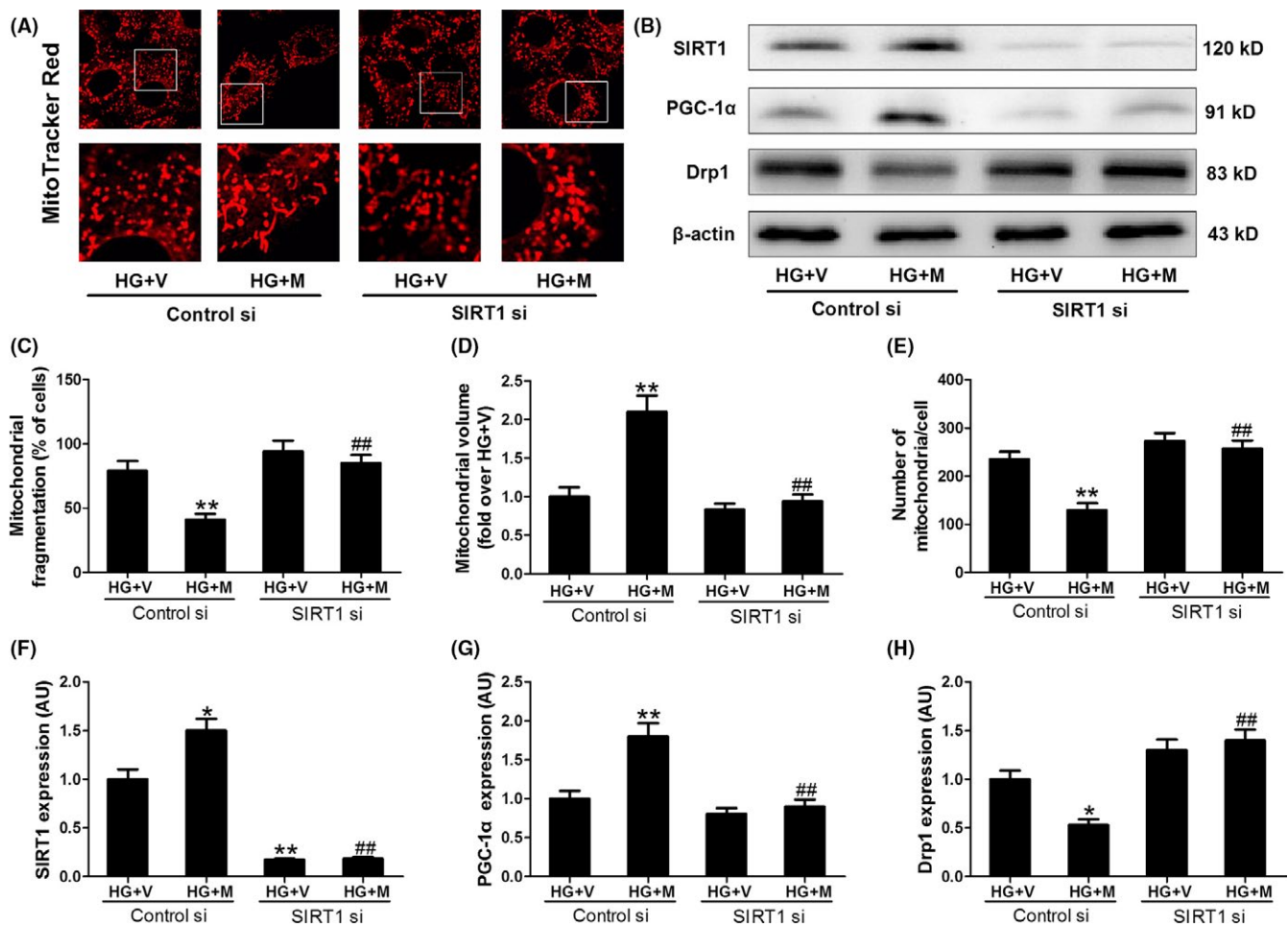
**FIGURE 4** Melatonin inhibited Drp1-mediated mitochondrial fission and mitochondrial-derived superoxide production in hyperglycemia-treated H9c2 cells. (A) Representative confocal microscope images showing mitochondrial morphology stained by MitoTracker Red (major finding is C-E). Original magnification  $\times 600$ . (B) Representative confocal microscope images showing mitochondria-derived superoxide production stained by MitoSOX (major finding is F). Original magnification  $\times 600$ . (C) The percentage of cells with fragmented mitochondria. (D) Mean volume of mitochondria (fold over NG+V). (E) The number of mitochondria per cell. (F) Quantitative analysis of MitoSOX fluorescence density (fold over NG+V). (G,H) Western blot analysis of Drp1 protein expression in cytoplasmic (Cyto) and mitochondrial (Mito) fractions. Presented values are means  $\pm$  SEM. NG, normal glucose (5.5 mmol/L); HG, high glucose (33 mmol/L glucose); V, vehicle; Mel, melatonin.  $n = 6$  in each group. &&  $P < .01$  vs NG+V. ^^  $P < .01$  vs HG+V

interfering (si)RNA blocked the inhibitory effect of melatonin on Drp1 expression and mitochondrial fission in HG-treated H9c2 cells. On the other hand, transfection with the adenovirus encoding SIRT1 (Ad SIRT1) increased the expression of SIRT1 and reduced Drp1 protein expression and mitochondrial fission in HG-treated cells (Figure 6). These data suggest that melatonin prevents HG-induced mitochondrial fission via the SIRT1-dependent down-regulation of Drp1 protein expression.

We further explored how the protein expression of Drp1 was down-regulated by SIRT1. As shown in Figure S3, Drp1 mRNA expression was also increased in HG-treated cells, while over-expression of SIRT1 (Ad SIRT1) reduced Drp1 mRNA expression. It seemed that the expression of Drp1 was regulated at the level of transcription. PGC-1 $\alpha$  is one key transcription factor, which has been considered as a target of SIRT1. As expected, SIRT1 siRNA blocked the up-regulation effect of melatonin on PGC-1 $\alpha$  expression in HG-treated H9c2 cells (Figure 5B,G), and SIRT1 knockout

blunted up-regulation effect of melatonin on PGC-1 $\alpha$  expression in diabetic mice (Figure S4). Over-expression of SIRT1 increased PGC-1 $\alpha$  expression in the cells cultured in NG or HG medium (Figure 6B,G). Subsequently, we determined whether PGC-1 $\alpha$  was responsible for the regulation of Drp1 expression and mitochondrial morphology. It was shown that PGC-1 $\alpha$  siRNA blocked the inhibitory effect of melatonin on Drp1-mediated mitochondrial fission in HG-treated H9c2 cells (Figure 7 and Figure S3C). Over-expression of PGC-1 $\alpha$  (Ad PGC-1 $\alpha$ ) reduced Drp1 expression in both protein and mRNA levels and prevented mitochondrial fission in the cells cultured in HG medium (Figure 8 and Figure S3D). Moreover, ChIP and PCR analysis revealed that PGC-1 $\alpha$  directly regulated the expression of Drp1 by binding to its promoter (Figure 8C). These data suggest that SIRT1-PGC1 $\alpha$  signaling is directly responsible for the protective effects of melatonin against Drp1-mediated mitochondrial dynamics under hyperglycemia condition.





**FIGURE 5** SIRT1 siRNA blunted the effect of melatonin on PGC-1 $\alpha$ , Drp1 and mitochondrial fission in hyperglycemia-treated H9c2 cells. (A) Representative confocal microscope images showing mitochondrial morphology stained by MitoTracker Red (major finding is C-E). Original magnification  $\times 600$ . (B,F-H) Protein expressions of SIRT1, PGC-1 $\alpha$ , and Drp1 were determined by Western blotting. (C) The percentage of cells with fragmented mitochondria. (D) Mean volume of mitochondria (fold over HG+V). (E) The number of mitochondria per cell. Presented values are means  $\pm$  SEM. HG, high glucose (33 mmol/L glucose); V, vehicle; Mel, melatonin.  $n = 6$  in each group. \* $P < .05$ , \*\* $P < .01$  vs HG+V with Control si. ##  $P < .01$  vs HG+M with Control si

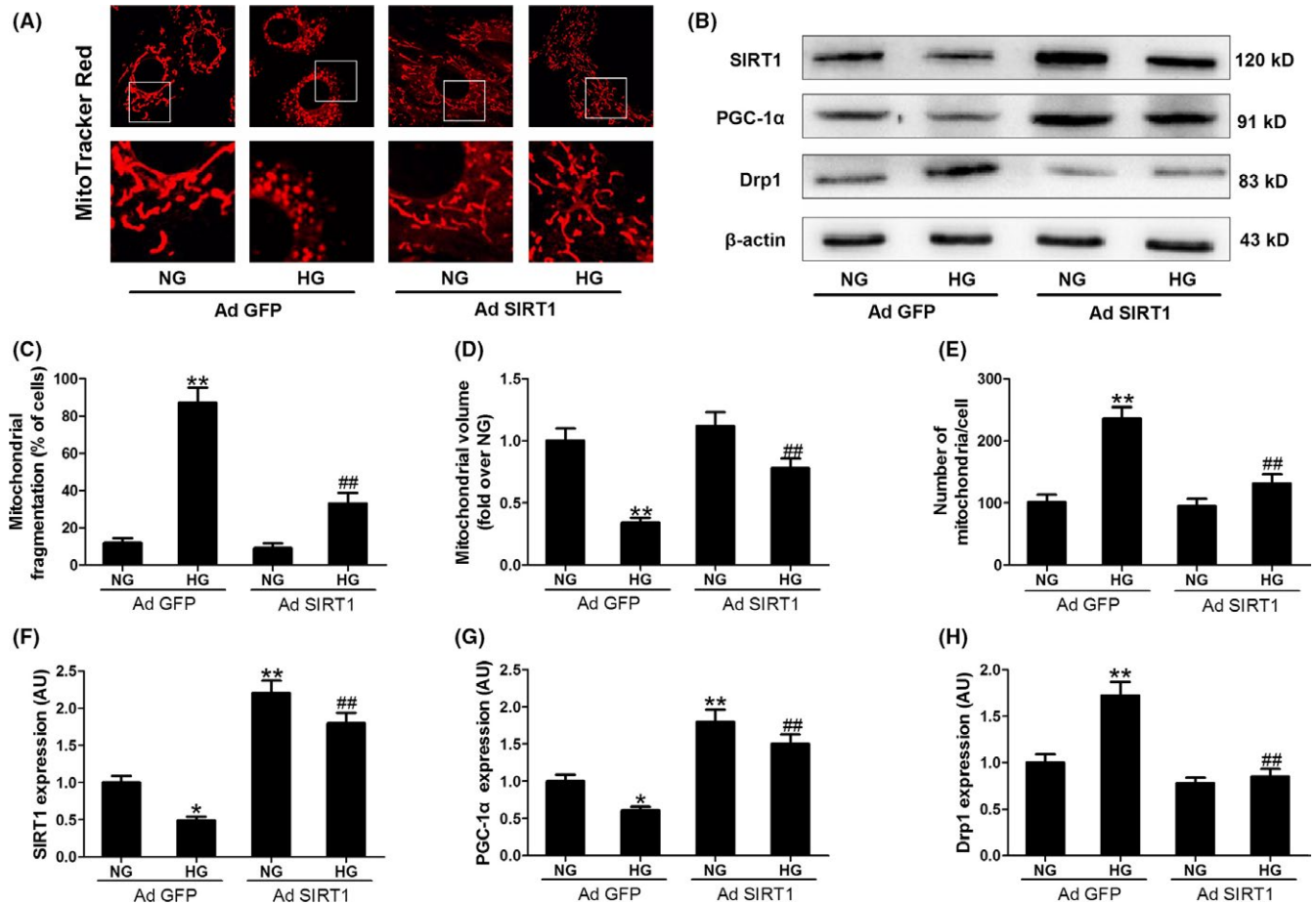
### 3.6 | Inhibition of mitochondrial fission suppressed oxidative stress and alleviated mitochondrial dysfunction in diabetes

Melatonin inhibited Drp1-mediated mitochondrial fission and ROS production and alleviated mitochondrial dysfunction in diabetes; however, whether enhanced Drp1-mediated mitochondrial fission causes oxidative stress and mitochondrial dysfunction in diabetes remains unclear. The Drp1 inhibitor, mdivi-1, and Drp1 siRNA were therefore used to investigate whether the inhibition of Drp1-mediated mitochondrial fission suppressed oxidative stress and alleviated mitochondrial dysfunction in diabetes. Although mdivi-1 treatment did not affect blood glucose and body weight in diabetic animals (Table S2), administration of mdivi-1 significantly attenuated mitochondrial fission (Figure 9A-C) and increased mitochondrial complex I, IV, V activities, and ATP levels in diabetic hearts (Figure 9D,E). Importantly, administration

of mdivi-1 increased LVEF and reduced cardiomyocyte apoptosis in diabetic mice (Figure 9F-I). In addition, mdivi-1 markedly attenuated superoxide anion production (stained by DHE, Figure 10A) and Nox4 expression (Figure 10B) and increased MnSOD activity (Figure 10C) in diabetic hearts. Gene silencing of Drp1 with siRNA (Drp1 si) consistently prevented mitochondrial fission (Figure 10E) and reduced mitochondria-derived superoxide production (stained by MitoSOX, Figure 10F) in HG-treated H9c2 cells.

## 4 | DISCUSSION

In this study, we uncover a novel molecular mechanism that how melatonin prevents against hyperglycemia-induced mitochondrial fission. We provide *in vivo* and *in vitro* evidence that melatonin reduced Drp1 expression and prevented mitochondrial fission in mice with diabetes via activating



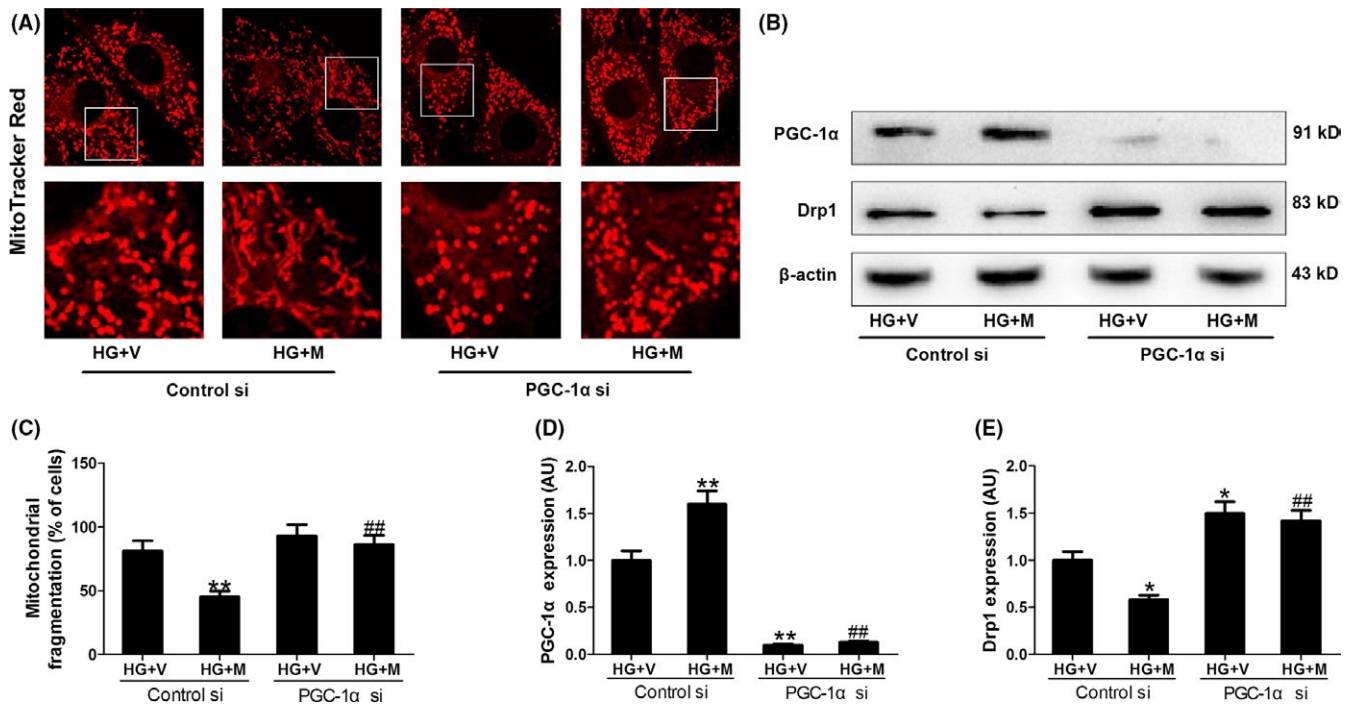
**FIGURE 6** Adenoviral (Ad) over-expression of SIRT1 increased PGC-1 $\alpha$  expression, reduced Drp1 expression and inhibited mitochondrial fission. (A) Representative confocal microscope images showing mitochondrial morphology stained by MitoTracker Red (major finding is C-E). Original magnification  $\times 600$ . (B,F-H) Protein expressions of SIRT1, PGC-1 $\alpha$ , and Drp1 were determined by Western blotting. (C) The percentage of cells with fragmented mitochondria. (D) Mean volume of mitochondria (fold over NG with Ad GFP). (E) The number of mitochondria per cell. Presented values are means  $\pm$  SEM. NG, normal glucose (5.5 mmol/L); HG, high glucose (33 mmol/L glucose).  $n = 6$  in each group. \* $P < .05$ , \*\* $P < .01$  vs NG with Ad GFP. ##  $P < .01$  vs HG with Ad GFP

SIRT1-PGC1 $\alpha$  signaling. Inhibition of mitochondrial fission suppressed mitochondrial ROS production, alleviated mitochondrial dysfunction, reduced cell apoptosis, and consequently improved cardiac function (Figure 11). Our data demonstrate for the first time that melatonin prevents against Drp1-mediated mitochondrial fission in mice with diabetes in a SIRT1/PGC-1 $\alpha$ -dependent manner.

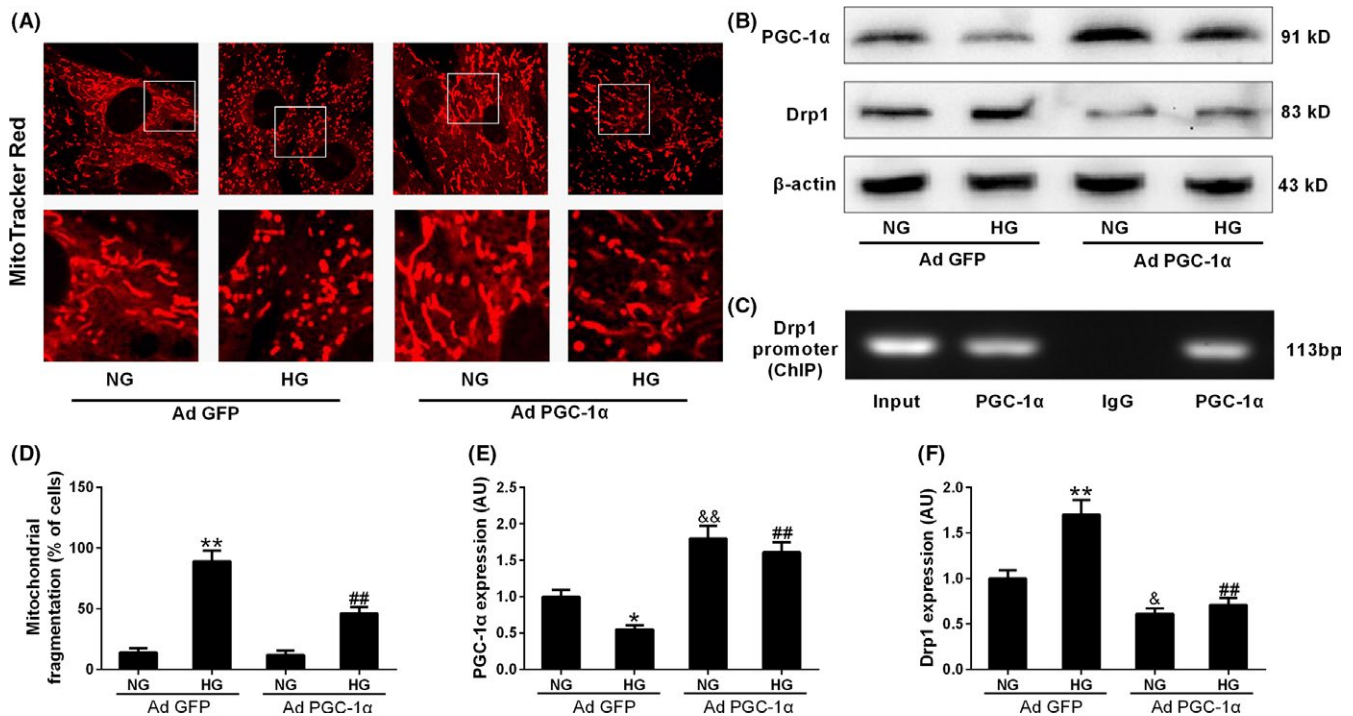
Mitochondria are dynamic organelles that are intimately involved in the regulation of various cellular functions, including energy production, ROS generation, and cell death.<sup>41,42</sup> Accumulating evidence suggests that both mitochondrial oxidative damage and mitochondrial dysfunction contribute to the pathogenesis of diabetes-induced cardiac dysfunction.<sup>43,44</sup> Previous studies have indicated that enhanced mitochondrial fragmentation is associated with increased mitochondrial ROS production in hyperglycemic conditions in vitro.<sup>9,45</sup> However, it is still unclear whether mitochondrial fission is involved in the pathogenesis of diabetes-induced cardiac dysfunction, especially in vivo.

Our study provided compelling evidence showing that diabetes (high glucose) increased Drp1 expression and caused mitochondrial fission both in vivo and in vitro. Inhibition of Drp1 with mdivi-1 alleviated mitochondrial dysfunction and cardiac dysfunction in STZ-induced diabetic mice. Meanwhile, mdivi-1 inhibited mitochondrial ROS production and reduced cell apoptosis. These results suggest that increased mitochondrial fission may accelerate mitochondrial dysfunction and cardiac dysfunction in mice with diabetes. Overall, our study develops a new concept that hyperglycemia-induced mitochondrial fission contributes to cardiac dysfunction in mice with diabetes, partly by enhancing mitochondrial oxidative stress.

Similar to the cardioprotective effects of mdivi-1, we found that melatonin prevented Drp1-mediated mitochondrial fission and alleviated cardiac dysfunction in diabetic mice. Previous studies have reported that melatonin alleviated cardiac dysfunction via activation of retinoic acid-related orphan receptor- $\alpha$  and regulation of autophagy

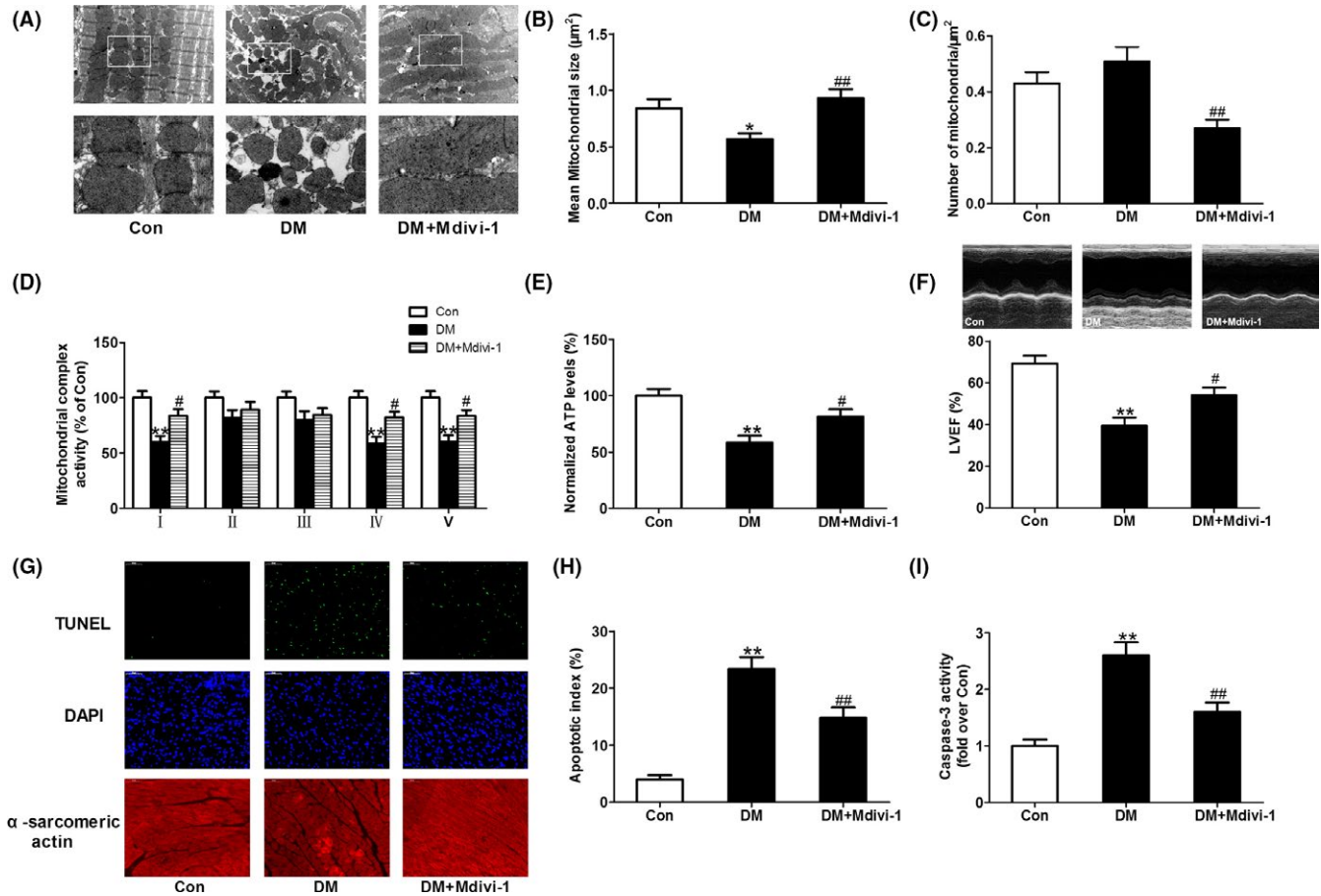


**FIGURE 7** PGC-1 $\alpha$  siRNA blunted the inhibitory effect of melatonin on Drp1 expression and mitochondrial fission in hyperglycemia-treated H9c2 cells. (A) Representative confocal microscope images showing mitochondrial morphology stained by MitoTracker Red (major finding is C). Original magnification  $\times 600$ . (B,D,E) Protein expressions of PGC-1 $\alpha$  and Drp1 were determined by Western blotting. (C) The percentage of cells with fragmented mitochondria. Presented values are means  $\pm$  SEM. HG, high glucose (33 mmol/L glucose); V, vehicle; Mel, melatonin.  $n = 6$  in each group. \* $P < .05$ , \*\* $P < .01$  vs HG+V with Control si. ##  $P < .01$  vs HG+M with Control si



**FIGURE 8** Adenoviral over-expression of PGC-1 $\alpha$  reduced Drp1 expression and inhibited mitochondrial fission. (A) Representative confocal microscope images showing mitochondrial morphology stained by MitoTracker Red (major finding is D). Original magnification  $\times 600$ . (B,E,F) Protein expressions of PGC-1 $\alpha$  and Drp1 were determined by Western blotting. (C) ChIP analysis for PGC-1 $\alpha$  binding to the Drp1 promoter in H9c2 cells. (D) The percentage of cells with fragmented mitochondria. Presented values are means  $\pm$  SEM. NG, normal glucose (5.5 mmol/L); HG, high glucose (33 mmol/L glucose).  $n = 6$  in each group. \* $P < .05$ , \*\* $P < .01$  vs NG with Ad GFP. ##  $P < .01$  vs HG with Ad GFP





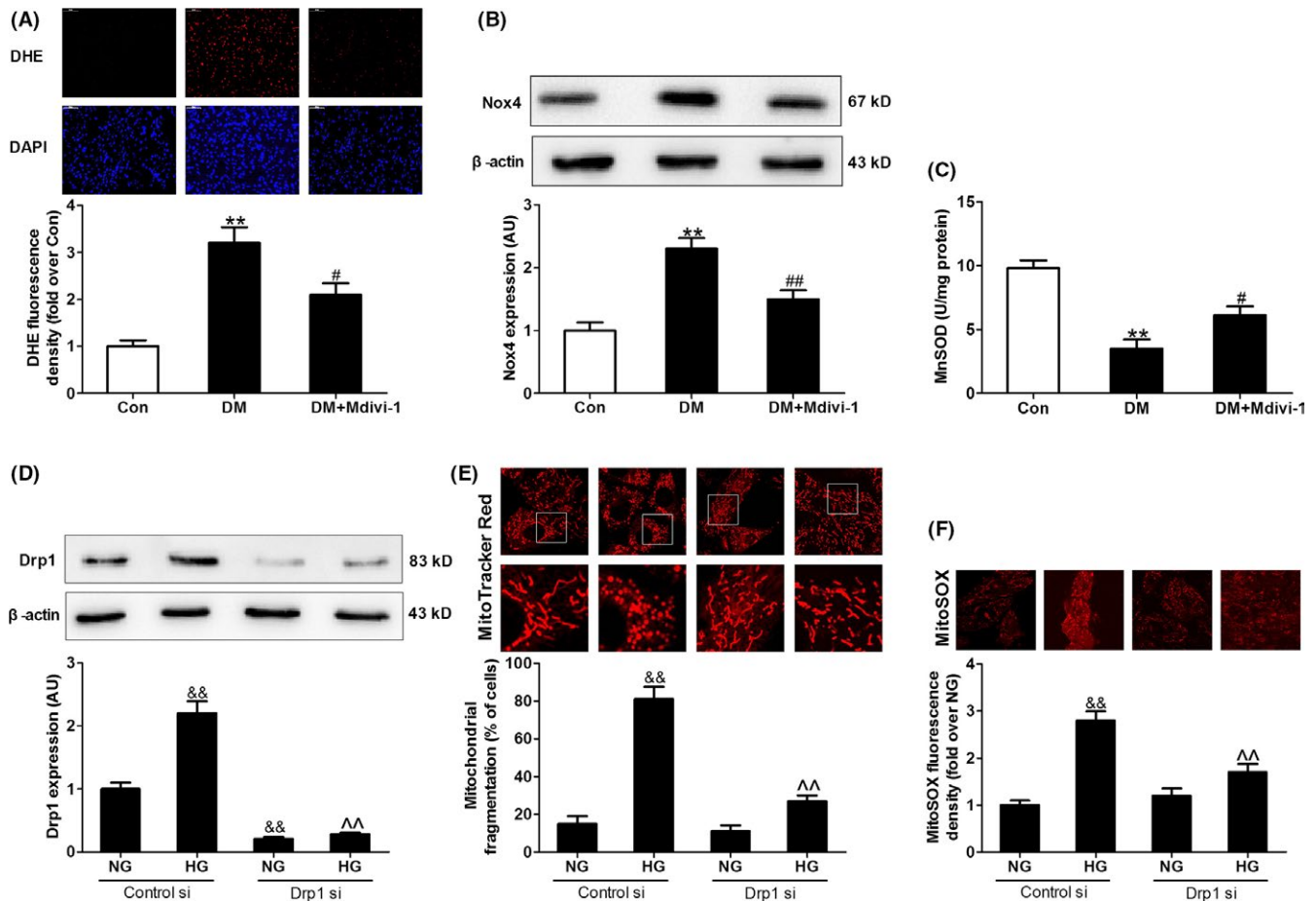
**FIGURE 9** Inhibition of mitochondrial fission with mdivi-1 improved mitochondrial function and cardiac function and reduced cardiomyocyte apoptosis in diabetic mice. (A) Representative transmission electron microscopic images of the myocardium (major finding is B,C). (B) Mean size of mitochondria. (C) The number of mitochondria per  $\mu\text{m}^2$ . (D) Mitochondrial complex activity (percentage of Con). (E) Normalized ATP levels. (F) Left ventricular ejection fraction (LVEF). (G) Representative photomicrographs of TUNEL-stained and DAPI-stained heart sections. Original magnification  $\times 400$ . (H) Apoptosis index. (I) Myocardial caspase-3 activity (fold over Con). Presented values are means  $\pm$  SEM. DM, diabetes mellitus.  $n = 8$  in each group. \* $P < .05$ , \*\* $P < .01$  vs Con. #  $P < .05$ , ##  $P < .01$  vs DM

in diabetic hearts.<sup>16,17</sup> The present work indicates that melatonin-inhibited mitochondrial fission may be a novel mechanism underlying its cardioprotective effect in diabetes. Moreover, melatonin treatment increased SIRT1 expression in both diabetic hearts and hyperglycemia-incubated cells. Previous findings have reported a rise in the expression of SIRT1 in cells and animal models after melatonin treatment.<sup>46,47</sup> The SIRT1 inhibitor was used to verify that SIRT1 plays a pivotal role in several functions of melatonin including protecting against kidney injury and myocardial ischemia-reperfusion injury.<sup>23,48</sup> However, it is still largely unknown whether melatonin-induced up-regulation of SIRT1 is responsible for its inhibitory effect on mitochondrial fission. In the current study, we found that over-expression of SIRT1 reduced Drp1 expression and mitochondrial fragmentations. In contrast, SIRT1 knockout or knockdown blocked the inhibitory effect of melatonin on mitochondrial fission in response to high glucose both in vivo and in vitro, suggesting that the regulated effect of

melatonin on mitochondrial dynamics may be dependent on the action of SIRT1.

SIRT1 knockout also blocked the inhibitory effect of melatonin on oxidative stress in diabetic hearts, while it has been reported that melatonin has direct anti-oxidative protection including directly scavenging free radicals and enhancing the activity of the antioxidant enzyme and indirect anti-oxidative protection dependent on its interaction with ROR $\alpha$ .<sup>16,49</sup> Interestingly, recent study has found that melatonin increases SIRT1-dependent NF- $\kappa$ B deacetylation through a ROR $\alpha$ -dependent mechanism,<sup>50</sup> suggesting the existence of a melatonin-ROR $\alpha$ -SIRT1 connection may be involved in the inhibition of ROS. Moreover, melatonin and SIRT1 have a long evolutionary history and exert cooperative biological effects in some conditions. For example, melatonin as well as some of its effects undergoes a circadian cycle, and SIRT1 is an accessory amplitude-enhancing component of cellular circadian oscillators.<sup>51,52</sup> It is possible that melatonin and SIRT1 have some interaction in



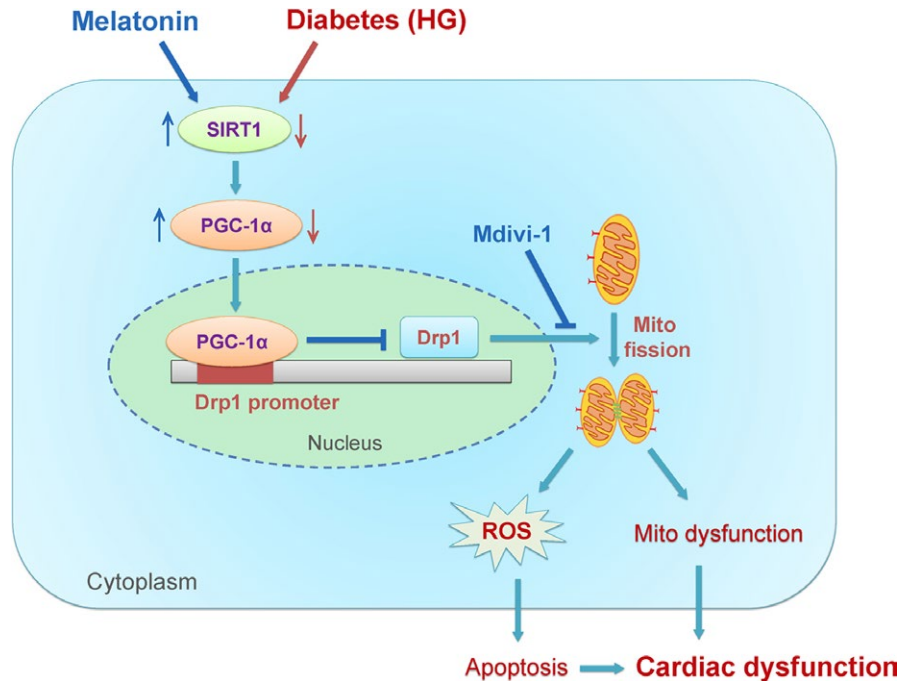


**FIGURE 10** Inhibition of mitochondrial fission with mdivi-1 or Drp1 siRNA suppressed hyperglycemia-induced oxidative stress. (A) Quantitative analysis of DHE fluorescence density in heart sections (fold over Con). (B) Protein expression of Nox4. (C) Mitochondrial manganese superoxide dismutase (MnSOD) activity. (D) Protein expression of Drp1. (E) The percentage of cells with fragmented mitochondria. (F) Quantitative analysis of MitoSOX fluorescence density (fold over NG with Control si). Presented values are means  $\pm$  SEM. NG, normal glucose (5.5 mmol/L); HG, high glucose (33 mmol/L glucose).  $n = 6-8$  in each group. \*\* $P < .01$  vs Con. #  $P < .05$ , ##  $P < .01$  vs DM. &&  $P < .01$  vs NG with Control si. ^^  $P < .01$  vs HG with Control si

a certain way. SIRT1 deficiency may disrupt the interaction and blunt direct anti-oxidative ability of melatonin. Then melatonin cannot exert its anti-oxidative protection in SIRT1 deficient mice. Further study is needed to clarify this interesting issue.

Drp1 is a cytosolic protein that initiates mitochondrial fission by binding to Fis1 over the mitochondrial surface in mammalian cells. The regulation of Drp1 properties, such as mRNA transcription, protein expression, and mitochondrial translocation, is important for the modulation of Drp1 function. Previous studies have shown that high glucose increases Drp1 protein expression in several types of cells including cardiomyocytes,<sup>53</sup> hippocampal neurons,<sup>54</sup> and pancreatic  $\beta$ -cells.<sup>55</sup> In this study, we found that SIRT1 reduced HG-induced Drp1 expression in both mRNA and protein levels, suggesting that SIRT1 modulated the expression of Drp1 at the level of transcription. As SIRT1 is not a transcription factor in the nucleus, it is possible that SIRT1 regulates the

transcription of Drp1 through its downstream transcription factor. PGC-1 $\alpha$  is a well-known transcription factor, which is considered as a major downstream target of SIRT1. In our study, over-expression of SIRT1 increased PGC-1 $\alpha$  expression as expected, while SIRT1 knockout or knockdown blocked the up-regulation effect of melatonin on PGC-1 $\alpha$  expression. Importantly, we have further found that PGC-1 $\alpha$  modulated Drp1-mediated mitochondrial fission directly by binding to its transcription promoter. Interestingly, PGC-1 $\alpha$  also plays a pivotal role in the regulation of mitochondrial biogenesis. Previous studies have demonstrated that melatonin increased the expression of PGC-1 $\alpha$  and promoted mitochondrial biogenesis in pathological conditions.<sup>56,57</sup> Therefore, the effects of melatonin on mitochondria function might be multiple, well beyond its regulation on mitochondrial dynamics. Mitochondrial dynamics and mitochondrial biogenesis might both be involved in the melatonin-mediated mitochondrial adaptations. Overall, our results provide the



**FIGURE 11** Schematic figure illustrating that melatonin prevents against diabetes-induced mitochondrial fission and cardiac dysfunction through SIRT1-PGC-1 $\alpha$ -Drp1 pathway. SIRT1 positively regulates the expression of PGC-1 $\alpha$ , which negatively regulates the expression of Drp1 directly by binding to its promoter. Diabetes (hyperglycemia, HG) reduced the expression of SIRT1 and PGC-1 $\alpha$  (red arrows) and then increased the expression of Drp1. Drp1 triggers mitochondrial fission and subsequently leads to mitochondria-derived ROS production and mitochondrial dysfunction, which results in the development of cardiac dysfunction. Treatment with melatonin increased the expression of SIRT1 and PGC-1 $\alpha$  (blue arrows) and then prevented Drp1-mediated mitochondrial fission. Mdivi-1 prevented diabetes-induced mitochondrial fission by inhibiting Drp1 activity. As a result, melatonin or mdivi-1 suppressed mitochondria-derived ROS production, alleviated mitochondrial dysfunction, reduced cell apoptosis, and protected against diabetes-induced cardiac dysfunction. Mito, mitochondrial; ROS, reactive oxygen species

first clue for understanding the exact regulatory mechanisms of melatonin on mitochondrial fission in diabetes.

There are still some limitations in our study. First, our *in vivo* experiments were exclusively performed in STZ-induced insulin-deficient type 1 diabetic mice. Whether the research findings can be applied to insulin-resistance type 2 diabetic models needs further investigation. Second, the conclusion that melatonin prevents Drp1-mediated mitochondrial fission through SIRT1-PGC-1 $\alpha$  pathway was verified using cardiac-specific SIRT1-knockout mice and SIRT1/PGC-1 $\alpha$  siRNA. The use of PGC-1 $\alpha$ -knockout mice will be very helpful in clarifying the role of PGC-1 $\alpha$  in Drp1-mediated mitochondrial fission. Despite these limitations, we believe that this study has provided important new information for the understanding of effects of melatonin on mitochondrial dynamics.

In summary, our study demonstrates that diabetes results in impaired cardiac function by triggering Drp1-mediated mitochondrial fission. Melatonin prevents mitochondrial fission and improves cardiac function in mice with diabetes through SIRT1-PGC1 $\alpha$  pathway, which negatively regulates the expression of Drp1 directly. These findings identify melatonin-modulated mitochondrial fission as a potential target for treating cardiac dysfunction and other cardiac complications in diabetes.

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## AUTHORS' CONTRIBUTIONS

Feng Fu and Jianming Pei conceived and designed the study. Mingge Ding, Zeyang Li, Yuemin Wang, and Feng Fu performed the animal experiments. Mingge Ding, Daishi Tang, and Na Feng carried out the cell experiments. Na Feng, Daishi Tang, Jiahao Feng, Zhenhua Liu, and Min Jia performed the molecular biology experiments. Mingge Ding, Daishi Tang, and Feng Fu analyzed the data. Mingge Ding drafted the manuscript. Xiaoming Gu, Feng Fu, and Jianming Pei revised and edited the manuscript. All authors have read and approved the final version of this manuscript.

## COMPETING INTERESTS

The authors declare that they do not have any competing interests.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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