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Pretreatment with growth differentiation factor 15 augments cardioprotection by mesenchymal stem cells in myocardial infarction by improving their survival

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Abstract

Background The clinical application of mesenchymal stem cells (MSCs) in myocardial infarction (MI) is severely hampered by their poor survival. Pretreatment is a key strategy that has been adopted to promote their therapeutic efficacy. This study aimed to investigate the benefit of growth differentiation factor 15-pretreated MSCs (GDF15-MSCs) in enhancing cardiac repair following MI and to determine the underlying mechanisms.

Methods MSCs with or without GDF15 pretreatment were exposed to serum deprivation and hypoxia (SD/H) challenge. Apoptosis of MSCs was assessed by TUNEL staining. The conditioned media (CM) of MSCs and GDF15-MSCs was collected by centrifugation. MSCs and GDF15-MSCs were transplanted into the peri-infarct region in a mouse model of MI. Cardiac function, fibrosis and MSC survival were examined 4 weeks after MSC transplantation.

Results Pretreatment with GDF15 greatly reduced SD/H-induced apoptosis of MSCs via inhibition of reactive oxygen species (ROS) generation by attenuating mitochondrial fission. Mechanistically, GDF15 pretreatment ameliorated mitochondrial fission of MSCs under SD/H challenge by activating the AMPK pathway. These effects were partially abrogated by AMPK inhibitor. Pretreatment with GDF15 also promoted paracrine effects of MSCs in vitro, evidenced by improving tube formation of HUVECs, and inhibited the apoptosis of cardiomyocytes induced by SD/H. At 4 weeks after transplantation, compared with MSCs, GDF15 pretreatment strongly promoted the survival of MSCs in the ischemic heart with consequent enhanced cardiac function, reduced cardiac fibrosis and increased angiogenesis.

Conclusions Our study showed that pretreatment with GDF15 promoted the cardioprotective effects of MSCs in MI via regulation of pro-survival signaling and paracrine actions. GDF15 pretreatment is an effective approach to enhance the therapeutic efficacy of MSCs in ischemic heart disease.

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Keywords Mesenchymal stem cells, Myocardial infarction, Growth differentiation factor 15, Cell survival, Mitochondrion

Introduction

Myocardial infarction (MI) is one of the major causes of morbidity and mortality worldwide with millions of patients affected each year. Although advanced therapies including pharmacotherapy and surgical interventions significantly reduce MI mortality, they are ineffective for myocardial regeneration to compensate for the lost cardiomyocytes [1]. Over the past decades, numerous animal and clinical studies have shown that therapy with mesenchymal stem cells (MSCs) is a promising strategy to treat MI due to their unique properties of easy isolation, immunomodulatory ability and multipotency [2–4]. Nevertheless poor survival of engrafted MSCs in the hostile microenvironment of the infarcted heart has limited their cardioprotective benefits. It has been reported that more than 90% of MSCs disappear in the first 24 h post transplantation into ischemic heart tissue and fewer than 2% survive 4 weeks post transplantation [5–7]. It has been reported that only 6% and 3% respectively of MSCs survived 14 days after transplantation via intracoronary infusion or endocardial injection into ischemic female swine hearts [8]. Therefore, exploring a novel approach to promote MSC survival may improve the effectiveness of cell therapy for MI. There is accumulating evidence, including that from our group, that pretreatment with pharmacologic agents can prime MSCs into a pro-survival state, leading to improved cardioprotective effects for MI [9–13].

Growth differentiation factor 15 (GDF15), also known as macrophage inhibitory cytokine 1, is a member of the transforming growth factor-β (TGF-β) super family that regulates a variety of biological processes [14]. It is established that GDF15 plays a protective role in cell viability under various stress conditions. GDF15 treatment inhibits rotenone-induced mitochondrial apoptosis in SH-SY5Y (human neuroblastoma cell line) cells via regulation of the PI3K/Akt/mTOR signaling pathway [15]. GDF15 loss promotes oxidative stress and decreases cellular viability in hyperoxia-treated pulmonary endothelial and epithelial cells [16]. In addition, GDF15 has recently been reported to stimulate the proliferation of human umbilical vein endothelial cells (HUVECs), and thus promote blood vessel growth [17]. More importantly, as a cardiac-derived endocrine hormone, GDF15 plays an important role in mediating cardioprotective effects by inhibiting cardiomyocyte apoptosis, hypertrophy and cardiac fibrosis [18–20]. Administration of recombinant GDF15 has been shown to improve heart function following infarction in mice by repressing polymorphonuclear leukocytes [21]. Nonetheless whether GDF15 pretreatment improves MSC survival in the ischemic heart has not been determined. We hypothesized that GDF15 pretreatment can promote the survival of MSCs in the ischemic heart and enhance their therapeutic potential for MI. We show that GDF15 promoted the cardioprotective effects of MSCs in MI by improving their survival and paracrine actions.

Materials and methods

Cell culture

MSCs were routinely cultured in DMEM-high glucose (Gibco, 11965084) supplemented 10% fetal bovine serum (FBS, Life Technologies, 16000) with 5 ng/mL endothelial growth factor (EGF, PeproTech, AF-100-15) and 5 ng/mL basic fibroblast growth factor (bFGF, PeproTech, 100-18B) as described previously [9]. All cell cultures in this study were reviewed and approved by the Ethics Committees of Tongji University (Approved project: mesenchymal stem cell-based therapy for myocardial infarction, Approval No. 2016-050, Date of approval: Sep 18, 2016). Written informed consent was obtained from patients and their family for participation in the study and the use of samples. MSCs at passage 3 to 5 were used in the current study. Neonatal mice cardiomyocytes (NCMs) were isolated from heart tissue of 0- to 1-day-old neonatal mice and routinely cultured at 37 °C on Claycomb Medium (51800, Sigma) containing 10% FBS.

GDF15 pretreatment

For GDF15 (MedChemExpress, HY-P75170) pretreatment, MSCs were cultured on fresh completed medium supplemented with GDF15 (10, 20, 50 and 100 ng/mL) under normoxia (95% air and 5% CO_2) for 24 h and then exposed to serum deprivation and hypoxia (SD/H, 1% oxygen, 5% carbon dioxide and 94% nitrogen) for 48 h to mimic the ischemic conditions in vitro.

Cell viability analysis

A total of 5×10^3 cells per well was cultured on 96-well plates, with or without GDF15. At indicative time points, cell viability was evaluated using a Cell Counting Kit-8 (CCK-8) assay kit (APExBIO, K1018) according to the protocol. After incubation with CCK-8 reagent for 1–4 h in a cell incubator, the absorbance was determined at 450 nm by a microplate reader (Tecan, Austria).

Table 1 Antibody information

No.	Name	Catalogue/product	Supplier	Dilution	Reference
1	Anti-GDF15	ab180929	Abcam	1/1000	https://doi.org/10.1186/s12959-023-00547-7
2	Anti-p-Drp1-ser616	3455	Cell signaling	1/1000	https://doi.org/10.1007/s00395-024-01072-y
3	Anti-Drp1	ab184247	Abcam	1/1000	https://doi.org/10.1038/s41380-023-02372-w
4	Anti-Mfn1	ab221661	Abcam	1/1000	https://doi.org/10.1111/febs.16793
5	Anti-Mfn2	ab124773	Abcam	1/1000	https://doi.org/10.3390/ijms241512148
6	Anti-p-AMPK	AF3423	Affinity	1/500	https://doi.org/10.1016/j.redox.2021.101856
7	Anti-AMPK	10929-2-AP	Proteintech	1/2000	https://doi.org/10.1038/s41586-020-2928-y
8	Anti-GFRAL	CSB-PA751020LA01HU	Cusabio	1/500	https://doi.org/10.1016/j.peptides.2023.171112
9	Anti-GAPDH	60004-1-Ig	Proteintech	1/10000	https://doi.org/10.1038/s41586-019-1722-1
10	Anti-human mitochondrion	ab92824	Abcam	1/800	https://doi.org/10.1038/s41698-023-00466-w
11	Anti-Troponin	ab209813	Abcam	0.5–5 μg/mL	https://doi.org/10.1038/s41598-023-32941-6
12	Anti-CD31	77699	Cell Signaling	1/50	https://doi.org/10.1172/jci.insight.171371
13	Anti-α-smooth muscle actin (α-SMA)	ab5694	Abcam	1/50	https://doi.org/10.1016/j.bioactmat.2023.09.019
14	Anti-COXIV	ab202554	Abcam	1/2000	https://doi.org/10.1161/JAHA.122.026160
15	Anti-CD45	GB113886	Servicebio	1/500	https://doi.org/10.1186/s12964-024-01554-5

TUNEL staining

A total of 5×10^4 cells per well was seeded on 96-well plates and cultured for 24 h with or without GDF15, then exposed to SD/H for 48 h. Next, a TUNEL Kit (RIBOBIO, C11026-1) was used to assess apoptotic cells following the manufacturer's protocol. Finally, cells were mounted with DAPI and randomly photographed and the percentage of TUNEL positive MSCs counted. The experiments were repeated at least three times.

Measurement of reactive oxygen species (ROS) generation

The intracellular level of ROS and mitochondrial ROS in MSCs with or without GDF15 treatment under SD/H challenge was determined by 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Invitrogen, C369) and a Mito-Sox staining kit (Invitrogen, M36008), respectively. Briefly, MSCs were cultured on a 24-well dish with glass cover slips and different treatments applied. Next, MSCs from different groups were incubated with 10 μM DCFH-DA solution or 5 μM Mito-Sox solution for 15 min at 37 °C in the dark. Finally, the sample was randomly captured and fluorescence intensity analyzed for five different view fields of each group using ImageJ software. Experiments were repeated at least three times.

Mitochondrial membrane potential measurement

The TMRM staining was carried out to evaluate mitochondrial membrane potential (MMP) following the protocol (Invitrogen, T668). Briefly, MSCs with different treatments were cultured on 24-well plates with glass cover slips and stained with 50 nM TMRM solution for 15 min at 37 °C in the dark. Next, the sample was imaged randomly and fluorescence intensity analyzed from five view fields of each group by ImageJ software.

RNA sequencing and bioinformatics analysis

RNA sequencing (RNA-seq) was performed for MSCs with or without GDF15 pretreatment under SD/H challenge. Total RNA from each group was extracted in Trizol (ThermoFisher Scientific) and the purity, quantity, and integrity of RNA determined. Next, the sequencing was carried out by the BGISEQ-500 platform (BGI, Guangzhou, China). The differentially expressed genes (DEGs) between any two groups were evaluated using linear models with absolute fold change ≥ 1.5 and adjusted p value < 0.05 as a threshold.

Preparation of conditioned medium (CM)

The CM from MSCs was prepared as described previously [19]. Briefly, 3×10^6 MSCs were cultured in a 15-cm culture dish with or without GDF15 for 24 h. Next, the medium was replaced with 15 mL serum- and antibiotic-free DMEM and cultured for a further 48 h. Finally, the CM from MSCs or GDF15-MSCs was harvested, centrifuged, and concentrated 20-fold using ultrafiltration tubes (Millipore, UFC9050). The concentrated CM was subsequently quantified using a BCA protein assay and stored at − 80 °C for future use.

Tube formation assay

The angiogenic effects of MSC-CM or GDF15-MSC-CM were determined by capillary tube formation assays. Briefly, 3×10^4 HUVECs were cultured on 96-well plates coated with growth-factor-reduced Matrigel

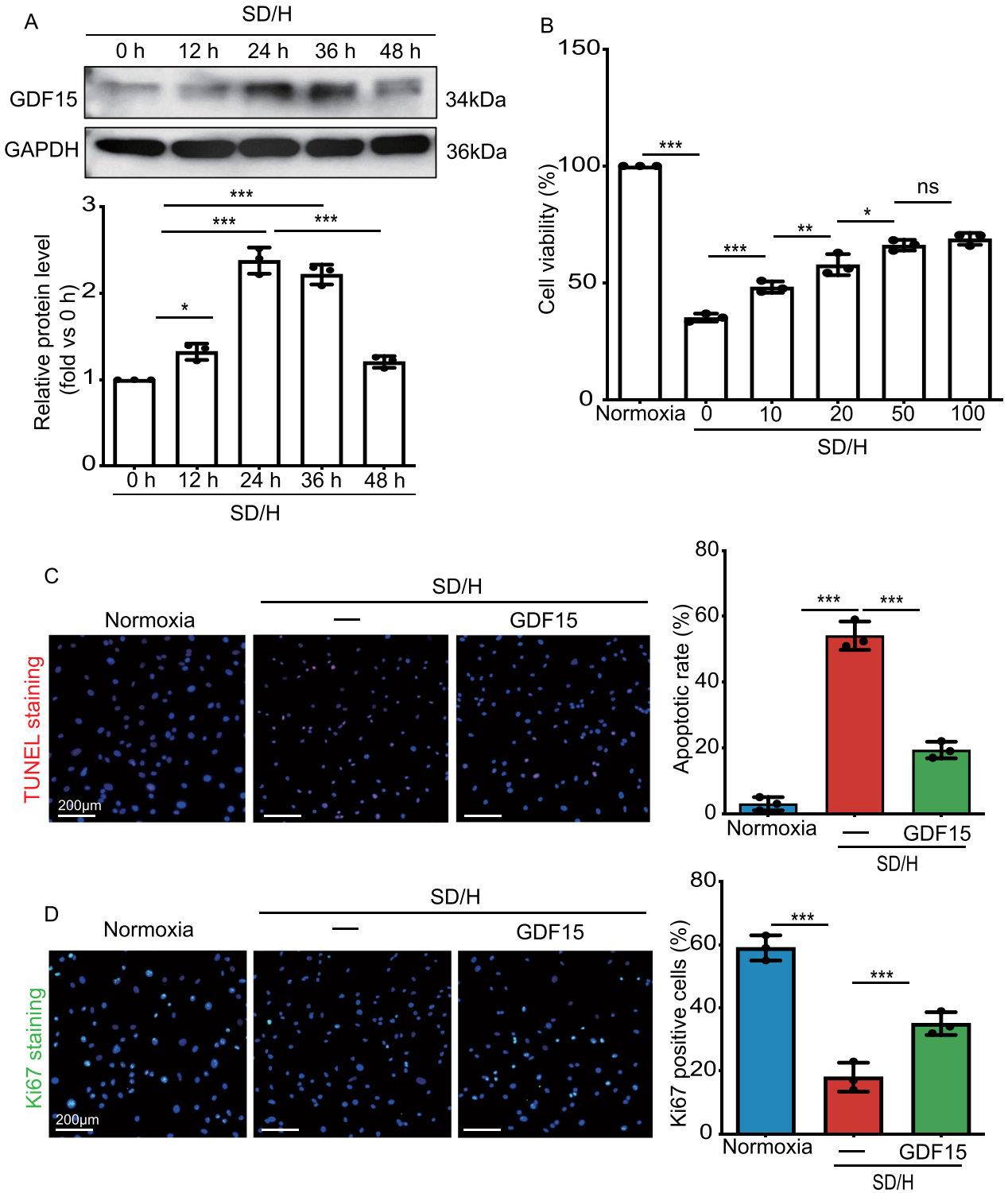

Fig. 1 GDF15 pretreatment protected MSCs against SD/H-induced injury. **A** Western blotting and quantitative analysis of the protein level of GDF15 in MSCs under normoxic and SD/H conditions at specified times. Full-length blots/gels are presented in Supplementary Fig. 3A. **B** CCK-8 assay showed the cell viability of MSCs with or without GDF15 (10, 20, 50 and 100 ng/mL) pretreatment for 24 h under normoxic or SD/H conditions. **C** Representative images of TUNEL staining and quantitative analysis of apoptosis in MSCs and GDF15-pretreated MSCs under normoxic or SD/H conditions. **D** Representative images of Ki67 staining and quantitative analysis of Ki67 positive cells in MSCs and GDF15-pretreated MSCs under normoxic or SD/H conditions. Scale bar = 200 μm. N = 3 biological replicates for each group. Data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ns, not significant

(CORNING, 354234). Next, cells were treated with MSC-CM or GDF15-MSC-CM for 6 h and capillary-like tube formation randomly captured. Tube length was analyzed by ImageJ software.

ELISA

The concentration of EGF, VEGF and bFGF in the MSC-CM and GDF15-MSC-CM was examined using a human EGF ELSIA kit (CUSABIO, CSB-E08027h), human VEGF ELSIA kit (CUSABIO, CSB-E11718h), and a human bFGF ELSIA kit (CUSABIO, CSB-E08000h), respectively.

Seahorse assay

The mitochondrial function of MSCs with or without GDF15 pretreatment under SD/H conditions was determined using a Seahorse Extracellular Flux Analyzer XF96 (Seahorse Bioscience, USA) according to the protocol.

Western blotting

The protein of MSCs with different treatments was extracted using RIPA buffer (Beyotime, P0013B) and the concentration measured. A total of 25 μg protein was separated on SDS-PAGE gel and transferred to PVDF membranes. Next, the membranes were washed with tris-buffered saline with 0.1% Tween-20 and blocked with 5% fat-free milk in TBS. Subsequently, the membranes were incubated at 4 °C overnight with primary antibodies (see Table 1). After washing with TBS with 0.1% Tween-20 three times, the membranes were incubated with secondary antibodies for 1 h at room temperature and exposed using enhanced chemiluminescence (ECL plus) (Amersham).

Establishment of an MI model and MSC transplantation

All animal procedures were performed in accordance with the ARRIVE guidelines and approved by the Committee on the Use of Live Animals in Teaching and Research of Tongji University for Laboratory Animal Medicine (Approved project: The therapeutic effects of growth differentiation factor 15 pretreated mesenchymal Stem Cells on myocardial infarction, Approval No. TJBB04724101, Date of approval: Feb 28, 2024). C57/B6J mice (6–8 weeks old, 25–30 g weight) were used to establish a model of MI by ligation of the left anterior descendent coronary artery (LAD) with an 8–0 Prolene suture as described previously [22]. All mice were housed in a controlled environment with a 12/12 h light/dark cycle and provided with food and water. To create the MI model, mice were anesthetized by 1.5% isoflurane/air mixture inhalation. Mice who underwent surgery without LAD ligation served as a sham group (Sham group, n = 6). Following LAD ligation, all MI mice received an intramuscular injection at four sites around the border zone of the infarcted heart of one of the following treatments: (1) PBS (MI group, n = 12); (2) 3×10^5 MSCs (MSCs group, n = 10); (3) 3×10^5 GDF15-MSCs (GDF15-MSCs group, n = 12). The animal experiment utilized block randomization to allocate mice to the sham, MI or treatment groups. All procedures were performed by a skilled researcher who was blinded to treatment allocation and unable to influence whether an animal would receive PBS, MSCs or GDF15-MSC treatment. The animal temperature during surgery was maintained using a heat pad. After surgery, carprofen was administered immediately and daily for three days to relieve inflammation and pain. Heart function in the different groups was examined by transthoracic echocardiography (Ultramark 9; Soma Technology) at baseline (before MI) and 28 days following MI under anesthesia with 1.5% isoflurane and heart rate maintained at 400–500 bpm. Left ventricle fractional shortening (LVFS) and ejection fraction (LVEF) were calculated.

Masson's staining

After echocardiographic assessment at 4 weeks following MSC transplantation, all mice were euthanized by intraperitoneal administration of 2 mL pentobarbital (200 mg/mL, Vetoquinol, UK) and heart tissue harvested. Hearts

(See figure on next page.)

Fig. 2 GDF15 pretreatment protects MSCs against SD/H-induced injury via attenuation of ROS generation. **A** Volcano plots illustrating differentially expressed genes identified in SD/H versus normoxia group and SD/H + GDF15 versus SD/H group. **B** The upper Venn diagram showing the overlap between significantly upregulated genes in SD/H versus normoxia groups and significantly downregulated genes in SD/H + GDF15 versus SD/H groups. The lower Venn diagram showing the overlap between significantly downregulated genes in SD/H versus normoxia groups and significantly upregulated genes in SD/H + GDF15 versus SD/H groups. **C** The top enriched terms of the genes that were upregulated in SD/H versus normoxia and downregulated in SD/H + GDF15 versus SD/H analyzed by Gene Ontology biological processes (GO-BP) (left). The top enriched terms of the genes that were downregulated in SD/H versus normoxia and upregulated in SD/H + GDF15 versus SD/H (right). **D** Representative images of DCFH-DA staining and quantitative analysis of intracellular ROS in MSCs and GDF15-pretreated MSCs under normoxic or SD/H conditions. **E** Representative images of Mito-Sox staining and quantitative analysis of mitochondria ROS in MSCs and GDF15-pretreated MSCs under normoxic or SD/H conditions. **F** Representative images of TMRM staining and quantitative analysis of MMP in MSCs and GDF15-pretreated MSCs under normoxic or SD/H conditions. MFI, mean fluorescence intensity. Scale bar = 200 μm. N = 3 biological replicates for each group. Data are expressed as mean ± SEM. ****p < 0.001*

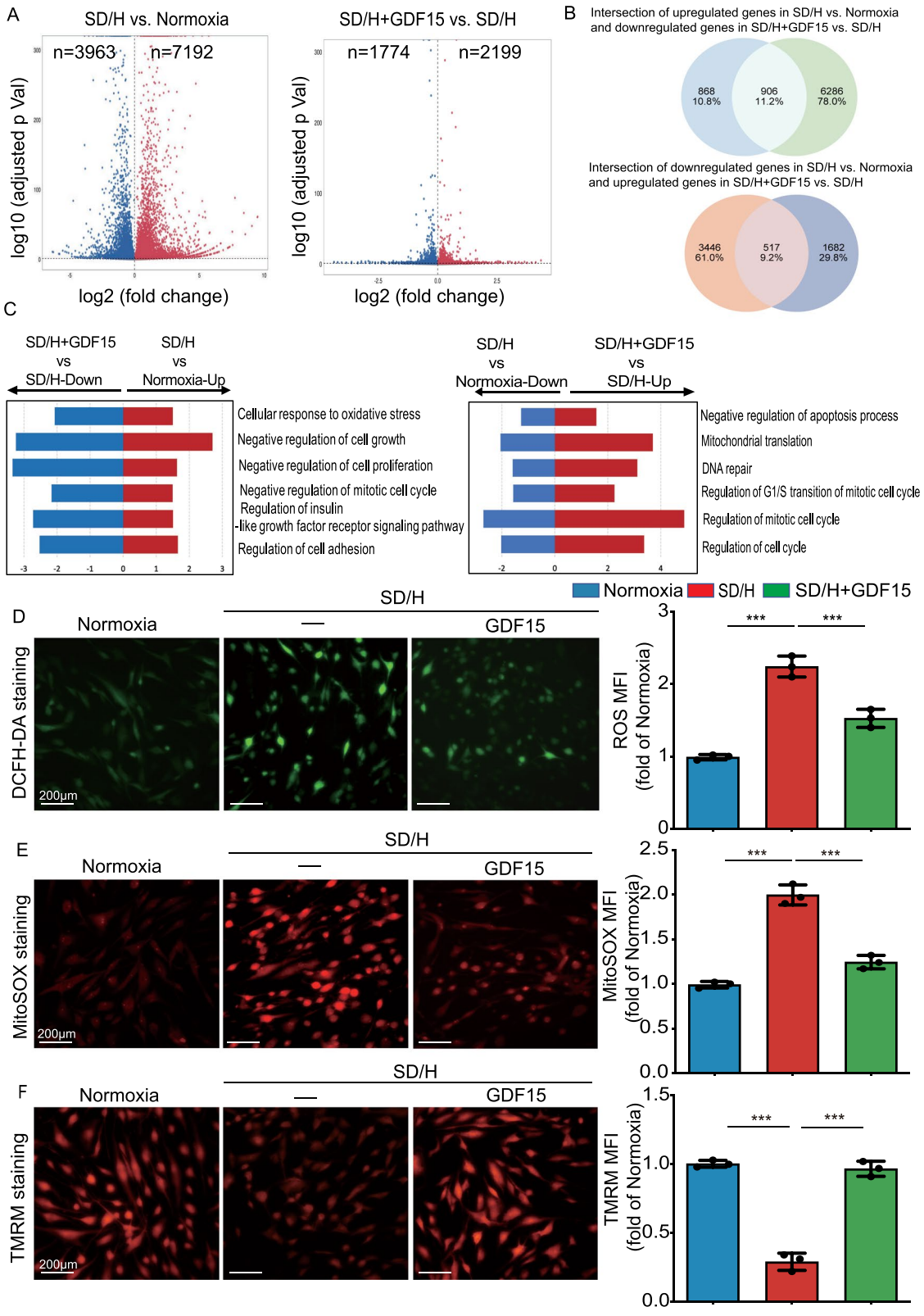

Fig. 2 (See legend on previous page.)

were fixed, embedded with paraffin and cut into 5 μm sections. Masson's staining was performed according to the manufacturer's protocol to detect fibrosis (Sigma, HT15). The percentage infarct size was evaluated as the ratio of fibrotic area to total left ventricular area × 100%.

Immunofluorescence staining

After hydration and retrieval with antigens, heart tissue slices from different groups were permeabilized for 30 min with PBS containing 0.1% Triton X-100. Next, the slices were blocked with 5% bovine serum albumin (BSA, Sigma, B2064) for 1 h and incubated overnight at 4 °C with primary antibodies (see Table 1). After washing with TBS and 0.1% Tween-20 three times, the sections were incubated for 30 min at room temperature with fluorescent secondary antibodies. Subsequently, the slices were mounted with DAPI and randomly photographed. The capillary or arteriolar density was analyzed by the average number of CD31- or α-SMA-positive blood vessels per field, and immune cells quantified by counting CD45-positive cells.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analyses were performed by GraphPad Prism. Comparison between two groups was analyzed by unpaired Student's t-test and among more than two groups by one-way-ANOVA followed by the Bonferroni test. A p value < 0.05 was considered statistically significant.

Results

GDF15 pretreatment protected MSCs against SD/H-induced apoptosis

To evaluate the protective effect of GDF15 on MSCs, we first examined the expression of GDF15 in MSCs under SD/H challenge. As shown in Fig. 1A, the protein level of GDF15 was significantly up-regulated in the first 24 h but significantly down-regulated at 48 h under SD/H (Fig. 1A). Next, to determine whether GDF15 pretreatment could protect MSCs under SD/H challenge, we pretreated MSCs with different concentrations of GDF15 (10, 20, 50 and 100 ng/mL) for 24 h and then exposed cells to SD/H for 48 h. The CCK-8 assay demonstrated that GDF15 pretreatment had a dose-dependent protective effect on MSCs under SD/H challenge, with the highest cell viability reached at a concentration of 50 ng/mL (Fig. 1B). Therefore, we used 50 ng/mL GDF15 to pretreat MSCs for 24 h in subsequent studies. More importantly, we found that GDF15 pretreatment significantly inhibited apoptosis (Fig. 1C) and improved the proliferative capacity of MSCs under SD/H challenge (Fig. 1D). We also examined the expression of GDF15 receptor GFRAL in MSCs with or without GDF15 pretreatment under SD/H challenge. The expression of GFRAL was increased in MSCs under SD/H challenge and further enhanced by GDF15 pretreatment (Supplementary Fig. 1). Collectively, these data revealed that GDF15 pretreatment exerted protective effects on MSCs under SD/H conditions.

GDF15 pretreatment protected MSCs against SD/H-induced injury via attenuation of ROS generation

To explore the potential mechanisms whereby GDF15 pretreatment protected MSCs against SD/H-induced injury, we collected MSCs from the SD/H group and SD/H + GDF15 group, as well the normoxia group, and performed RNA-seq analysis. Compared with the normoxia group, 7192 upregulated genes and 3963 downregulated genes were identified in the SD/H group (Fig. 2A). Meanwhile, 2199 genes were upregulated and 1774 genes were downregulated in the SD/H + GDF15 group compared with the SD/H group (Fig. 2A). Importantly, 906 upregulated genes and 517 downregulated genes in the SD/H group were reversed by GDF15 pretreatment (Fig. 2B). Next, we performed gene ontology (GO) enrichment analysis of the genes rescued by GDF15 pretreatment using gene ontology biological processes (GO-BP). The upregulated pathways in the SD/H + GDF15 group were primarily associated with DNA repair, regulation of cell cycle and mitochondrial translation (Fig. 2C). The downregulated pathways were closely related to cellular response to oxidative stress, and negative regulation of cell growth and cell proliferation

(See figure on next page.)

Fig. 3 GDF15 pretreatment downregulates ROS generation via inhibition of mitochondrial fission in MSCs under SD/H challenge. **A** Representative images and quantitative measurement of the fragmented mitochondria in normoxia, SD/H, SD/H + GDF15 and SD/H + GDF15 + FCCP-treated MSCs. **B** Western blotting and quantitative analysis of the protein level of p-Drp1, Mfn1 and Mfn2 in normoxia, SD/H, SD/H + GDF15 and SD/H + GDF15 + FCCP-treated MSCs. Full-length blots/gels are presented in Supplementary Fig. 3B. **C** Western blotting and quantitative analysis of the protein level of p-AMPK, p-Drp1, Mfn1 and Mfn2 in normoxia, SD/H, SD/H + GDF15 and SD/H + GDF15 + Compound C-treated MSCs. Full-length blots/gels are presented in Supplementary Fig. 3C. **D** Representative images and quantitative measurement of the fragmented mitochondria in normoxia, SD/H, SD/H + GDF15 and SD/H + GDF15 + Compound C-treated MSCs. Scale bar = 10 μm. N = 3 biological replicates for each group. Data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

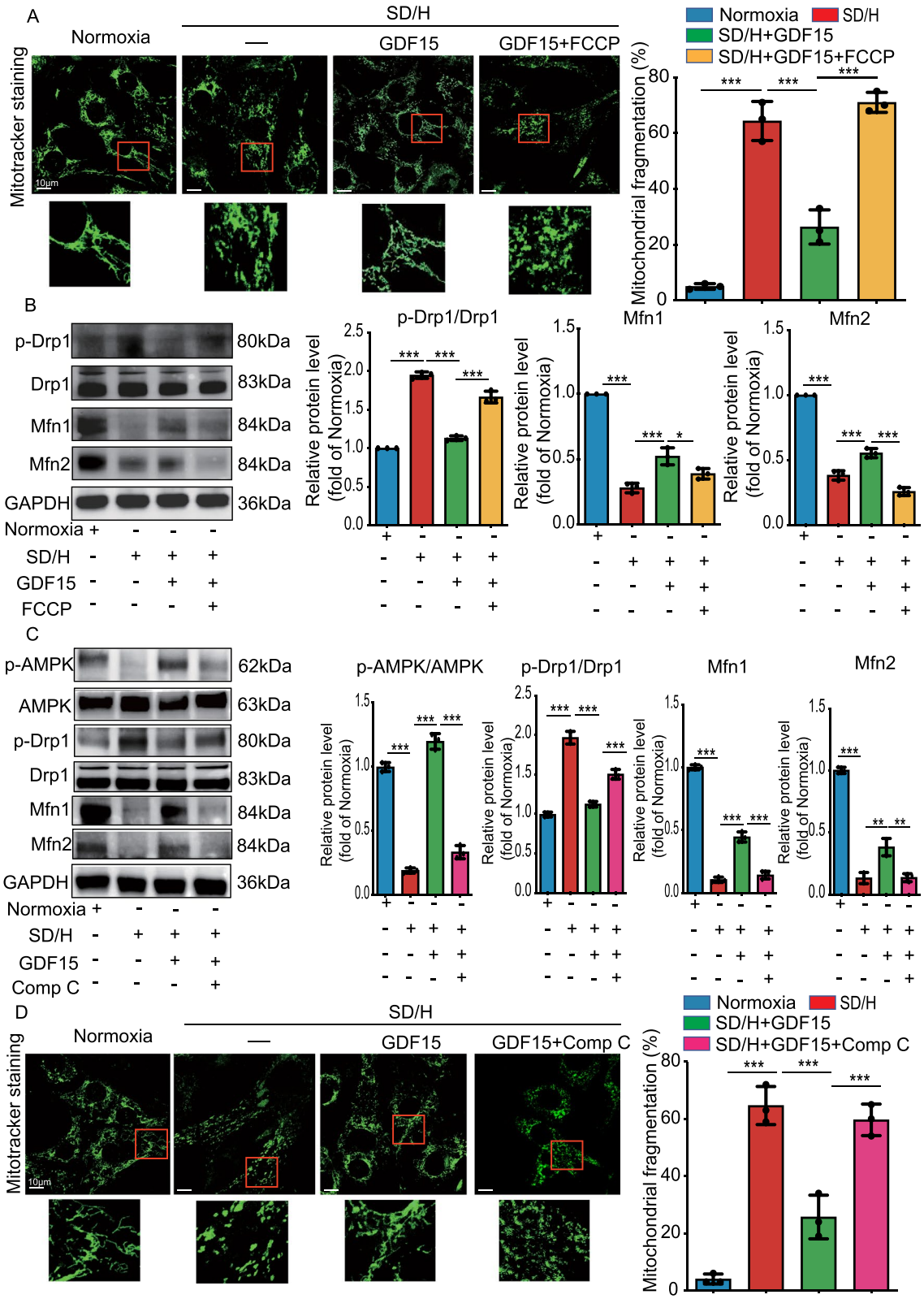

Fig. 3 (See legend on previous page.)

(Fig. 2C). To verify these findings, we examined the total cellular superoxide and mitochondrial ROS level among the different groups using DCFH-DA and Mito-SOX staining, respectively. The level of intracellular and mitochondrial ROS was greatly increased under SD/H challenge but reduced by GDF15 pretreatment (Fig. 2D, E). Moreover, TMRM staining revealed that GDF15 pretreatment significantly upregulated the MMP in SD/H-treated MSCs (Fig. 2F). More importantly, it has been reported that mitochondria can release GDF15 [23]. We first examined the expression of GDF15 in mitochondria of MSCs. We found that GDF15 was expressed in mitochondria in MSCs as well as in the cytoplasm (Supplementary Fig. 2A). Therefore, we examined the mitochondrial function of MSCs under SD/H challenge, with or without GDF15 pretreatment. The level of basal mitochondrial OCR, ATP production, and maximum mitochondrial respiratory function was greatly downregulated in MSCs under SD/H challenge but upregulated by GDF15 pretreatment (Supplementary Fig. 2B). These results suggested that GDF15 pretreatment improved mitochondrial function of MSCs under SD/H conditions. Increased mitochondrial function of MSCs may lead to GDF15 secretion, causing further upregulation of mitochondrial function, thus forming positive feedback loop. These results suggested that GDF15 pretreatment largely reversed ROS generation in MSCs under SD/H challenge.

GDF15 pretreatment downregulates ROS generation in MSCs under SD/H challenge via inhibition of mitochondrial fission by regulating the AMPK pathway

Accumulating evidence has shown that excessive mitochondrial fission contributes to ROS generation with consequent cell injury [24, 25]. Mito-tracker staining showed that GDF15 pretreatment significantly ameliorated SD/H-induced mitochondrial fragmentation in MSCs (Fig. 3A). Western blotting also revealed that GDF15 pretreatment downregulated the protein level of p-Drp1/Drp1 and upregulated that of Mfn1 and Mfn2 in SD/H-treated MSCs (Fig. 3B). Nevertheless the suppressive effects of GDF15 pretreatment on SD/H-induced MSC mitochondrial fragmentation were partially abrogated by FCCP, a mitochondrial fission activator (Fig. 3A, B). Our previous study showed that the AMPK pathway is involved in regulating mitochondrial dynamics in MSCs. We aimed to determine whether GDF15 pretreatment could inhibit SD/H-induced MSC mitochondrial fragmentation via regulation of the AMPK pathway. Western blotting analysis showed that GDF15 pretreatment greatly increased the downregulated expression of p-AMPK in SD/H-treated MSCs (Fig. 3C). Nonetheless the AMPK inhibitor, Compound C, reduced the enhanced p-AMPK, Mfn1 and Mfn2 and enhanced the reduced p-Drp1 in GDF15 pretreated-MSCs under SD/H challenge (Fig. 3C). More importantly, Compound C treatment partially abrogated the inhibitory effect of GDF15 pretreatment on mitochondrial fragmentation in SD/H-treated MSCs (Fig. 3D). These results showed that GDF15 pretreatment downregulated ROS generation in MSCs under SD/H challenge via inhibition of mitochondrial fission by regulating the AMPK pathway.

GDF15 pretreatment augments the paracrine effects of MSCs in vitro

To determine whether GDF15 pretreatment could improve the paracrine effects of MSCs, isolated MSC-CM and GDF15-MSC-CM was co-cultured with HUVECs or NCMs under SD/H, respectively (Fig. 4A). Compared with DMEM treatment, tube formation of HUVECs was greatly increased in those with MSC-CM treatment and further increased by GDF15-MSC-CM treatment (Fig. 4B, C). To further examine the angiogenic capacity of MSCs and GDF15-MSCs, the concentration of VEGF and EGF, and bFGF in MSC-CM and GDF15-MSC-CM was determined by ELISA assay. As shown in Fig. 4D, the concentration of VEGF and EGF, and bFGF was significantly increased in GDF15-MSC-CM compared with MSC-CM (Fig. 4D). When NCMs were co-cultured with MSC-CM or GDF15-MSC-CM under SD/H for 48 h, the number of apoptotic NCMs was significantly reduced compared with DMEM (Fig. 4E, F). More importantly, compared with MSC-CM treatment, GDF15-MSC-CM treatment further inhibited NCM apoptosis induced by SD/H (Fig. 4E, F). These data showed that GDF15 pretreatment improved the paracrine effects of MSCs.

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Fig. 4 GDF15 pretreatment augmented paracrine effects of MSCs. **A** Schematic showing the experiments for assaying the paracrine effect of MSCs with or without GDF15. **B** Representative images of tube formation in HUVECs treated with DMEM, MSC-CM or GDF15-MSC-CM. Scale bar = 100 μm. **C** Quantification of tube formation in HUVECs treated with DMEM, MSC-CM or GDF15-MSC-CM. **D** The concentration of VEGF, EGF and bFGF in MSC-CM and GDF15-MSC-CM was determined by ELISA assay. **E** Representative images of the apoptotic NCMs treated with DMEM, MSC-CM or GDF15-MSC-CM under SD/H condition. Scale bar = 200 μm. **F** Quantification of the apoptotic rate of NCMs treated with DMEM, MSC-CM or GDF15-MSC-CM under SD/H condition. Data are expressed as the mean ± SEM. N = 3 biological replicates for each group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

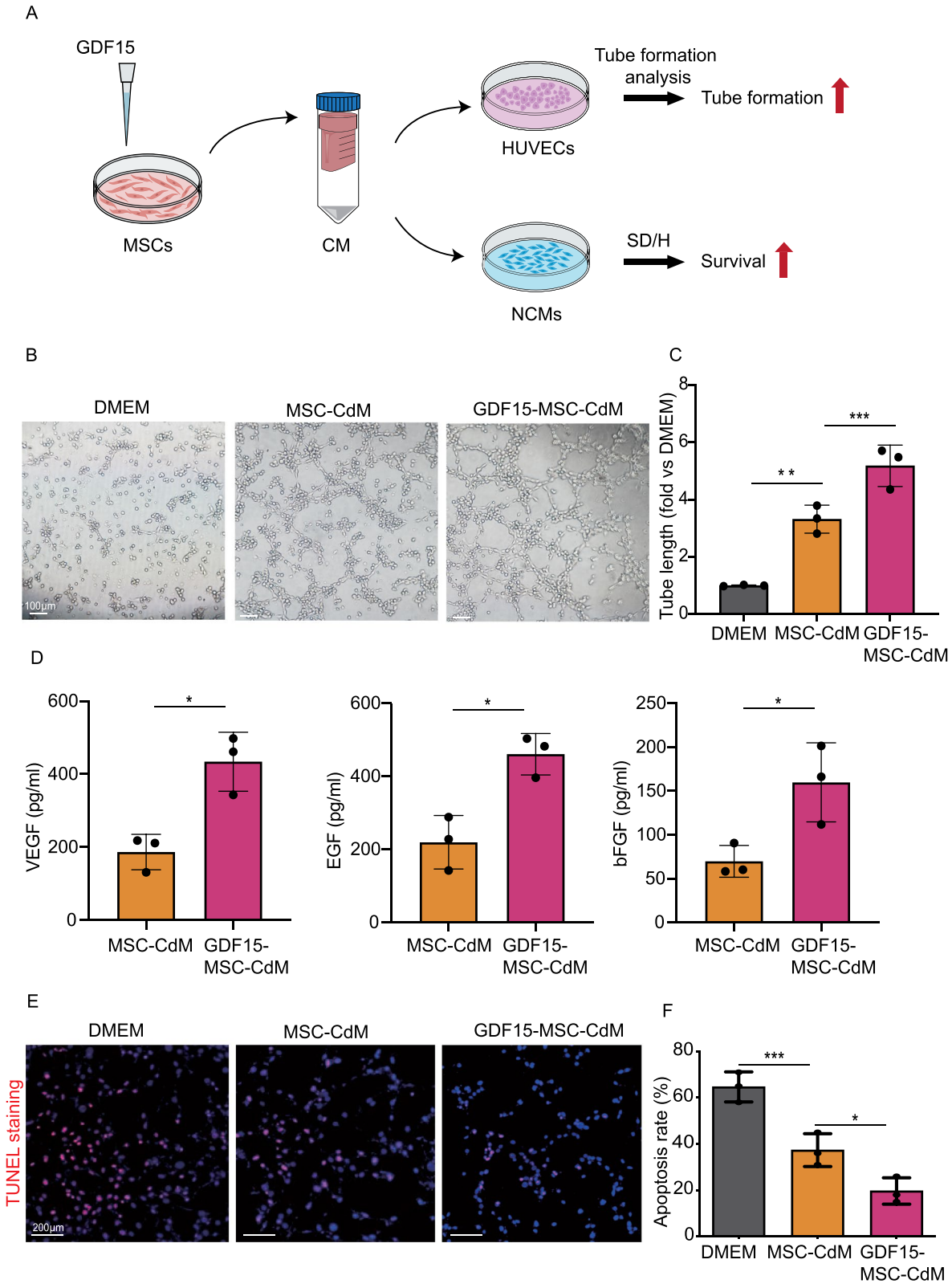

Fig. 4 (See legend on previous page.)

Transplantation of GDF15-MSCs improved cardiac function following infarction in mice

To determine whether GDF15 pretreatment could enhance the therapeutic effects of MSCs in MI, we transplanted MSCs and GDF15-MSCs into infarcted mouse hearts. Echocardiography was performed to examine cardiac function at baseline (before MI) and 28 days after MI. Representative echocardiographic images 28 days after MI in mice from different experimental groups are shown in Fig. 5A. Before MI, the LVEF and LVFS among the different groups were comparable (Fig. 5B). At 28 days after MI, compared with the sham group, LVEF and LVFS was greatly reduced in the MI group but significantly increased in the MSCs and GDF15-MSCs group, to a greater extent in the latter, suggesting that GDF15-MSCs exerted a more beneficial effect on MI (Fig. 5B). Cardiac fibrosis was examined by Masson's trichrome staining at 28 days after MI among the different groups (Fig. 5C). Compared with the sham group, the myocardial fibrotic area was severely enhanced in the MI group (Fig. 5D). Nonetheless MSC transplantation greatly reduced the myocardial fibrotic area in mice following infarction compared with the MI group (Fig. 5D). More importantly, GDF15-MSC treatment more significantly ameliorated the myocardial fibrotic area than MSC transplantation (Fig. 5D). The survival of MSCs in the ischemic hearts was examined by anti-human mitochondria at 28 days post-transplantation (Fig. 5E). Human mitochondria were detected in the MSCs and GDF15-MSCs group but not in sham or MI group (Fig. 5E). Moreover, compared with the MSC group, MSC survival was dramatically increased in the GDF15-MSC group, suggesting that GDF15 pretreatment notably improved MSC survival (Fig. 5F). Collectively, these results showed that GDF15 pretreatment robustly improved the cardioprotective effects of MSCs on MI in mice.

Transplantation of GDF15-MSCs inhibited cardiomyocyte apoptosis and enhanced angiogenesis in infarcted hearts

Cardiomyocyte apoptosis among the different groups was evaluated by TUNEL staining. Compared with the MI group, cardiomyocyte apoptosis was dramatically reduced in the MSC and GDF15-MSC group, to a further extent in the latter (Fig. 6A, B). We also assessed immune cell infiltration in ischemic heart tissue from the different groups using CD45 staining. In comparison to the sham group, the MI group displayed an increased degree of immune cell infiltration, a trend that was decreased in the MSC group and to an even greater extent in the GDF15-MSC group (Fig. 6C, D). To determine whether GDF15-MSC transplantation could improve angiogenesis in the ischemic mouse heart, we performed CD31 staining and α-SMA staining to determine the capillary and arteriolar density respectively in heart tissue from different groups. Compared with the MI group, the capillary density was greatly increased in all MSC-treated groups and further enhanced in the GDF15-MSC group (Fig. 6E, F). Similarly, transplantation of MSCs or GDF15-MSCs significantly increased the density of arterioles compared with that in MI hearts (Fig. 6E, G). The density of arterioles was much higher in the GDF15-MSC group than the MSC group (Fig. 6E, G). These data indicated that GDF15-MSC transplantation reduced cardiomyocyte apoptosis and enhanced angiogenesis in the ischemic heart of mice.

Discussion

This study highlighted several major findings. First, GDF15 pretreatment greatly reduced SD/H-induced MSC apoptosis via inhibition of reactive oxygen species (ROS) generation by attenuating mitochondrial fission. Second, GDF15 pretreatment ameliorated mitochondrial fission of MSCs under SD/H challenge by activating the AMPK pathway. Third, GDF15 pretreatment improved the paracrine effects of MSCs in vitro as manifested by increased tube formation of HUVECs and inhibition of cardiomyocyte apoptosis induced by SD/H. Fourth, compared with the MSCs, GDF15 pretreatment greatly promoted the survival of MSCs in ischemic heart tissue, leading to enhanced angiogenesis, decreased infarct size and improved heart function. Our study showed that pretreatment with GDF15 may be a promising strategy to enhance MSC-based therapy for MI.

(See figure on next page.)

Fig. 5 Transplantation of GDF15-MSCs improved cardiac function following infarction in mice. **A** Representative images of echocardiography taken at 28 days in Sham or mice with MI that received transplantation of PBS, MSCs, or GDF15-MSCs. **B** Quantitative analysis of LVEF and LVFS at baseline (before MI) and 28 days later in Sham or mice with MI that received transplantation of PBS, MSCs or GDF15-MSCs. **C** Representative Masson's trichrome staining images of hearts of Sham mice or mice with MI that received transplantation of PBS, MSCs or GDF15-MSCs. Scale bar = 2 mm. **D** Quantitative analysis of infarct size of heart from Sham mice or mice with MI that received transplantation of PBS, MSCs or GDF15-MSCs. **E** Representative images of anti-human mitochondria staining of heart tissue from mice with MI that received PBS, MSCs or GDF15-MSCs treatment and sham group. Scale bar = 50 μm. **F** Quantitative analysis of MSC survival in heart tissue of mice with MI that received transplantation of MSCs or GDF15-MSCs. Data are expressed as the mean ± SEM. N = 6. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

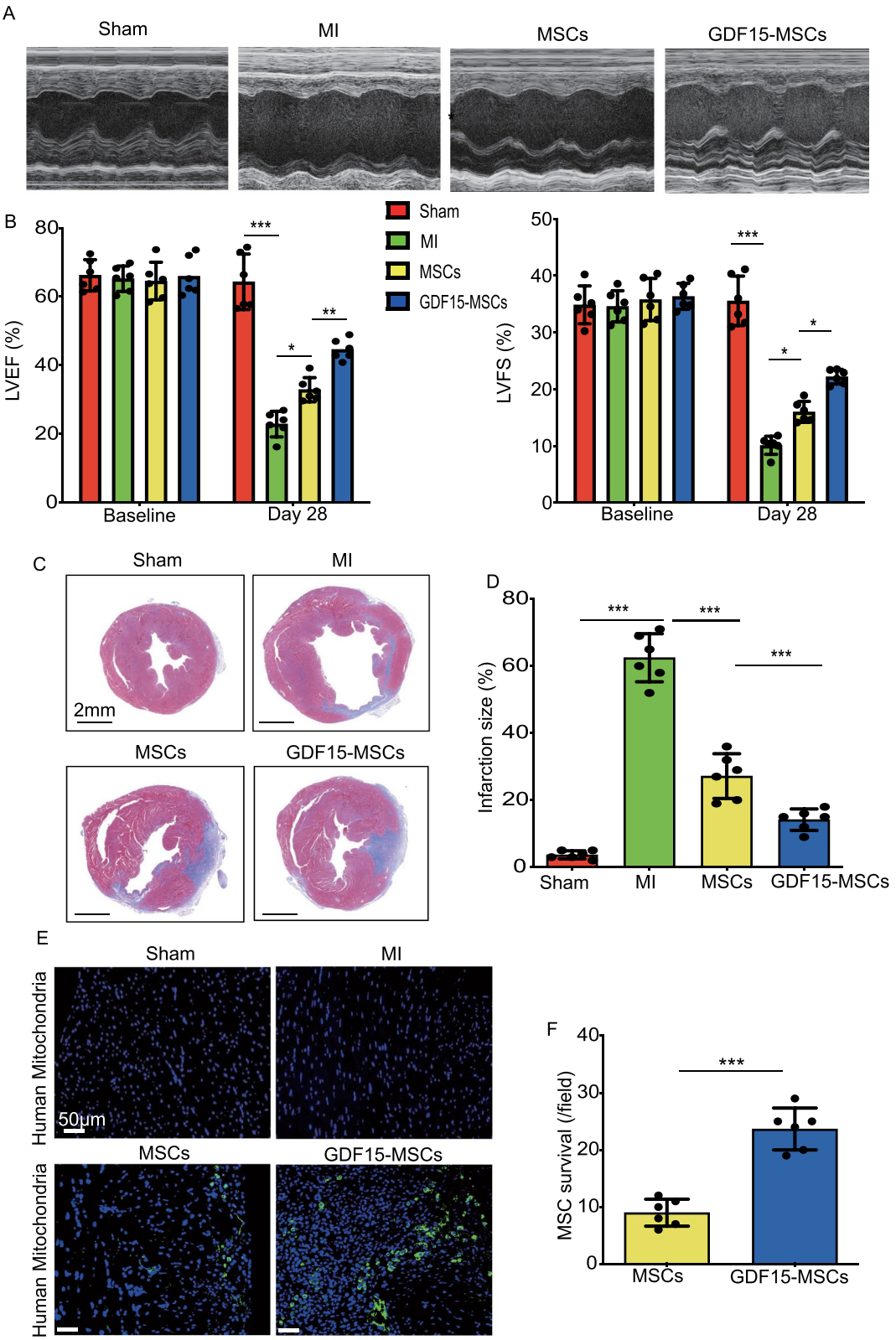

Fig. 5 (See legend on previous page.)

MI, caused by insufficient blood flow to the heart, results in an irreversible loss of one billion cardiomyocytes in a short time, finally leading to heart failure [26]. Due to its limited regenerative ability, the heart cannot replenish the lost cardiomyocytes. Therefore, MSC-based therapy has been proposed as a promising approach for MI treatment to replace the cardiomyocytes lost due to ischemia [27, 28]. MSC transplantation significantly reduces infarct size and improves cardiac function via multiple potential mechanisms including their paracrine effects, differentiation potential and immunomodulation capacity [29, 30]. Nevertheless their low survival and engraftment rates in the harsh and hostile environment of ischemic heart tissue severely hamper their therapeutic effects [31, 32]. Faced with this major limitation of MSC-based therapy for MI, one potential strategy is to pretreat MSCs before transplantation. Many pharmacological and biological substances have been shown to be efficacious in regulating cell function and MI. This prompted us to consider whether pretreatment of MSCs with these factors could improve their therapeutic efficacy for MI treatment. Many animal experiments have proven that preconditioning MSCs with various drugs, biological agents or growth factors greatly improves their survival in the heart following infarction and ultimately leads to improved cardiac function [9, 33, 34]. It has been reported that GDF15 plays a critical role in the regulation of various cellular processes including proliferation, apoptosis and aging [35]. GDF15 treatment has been shown to effectively ameliorate the ferroptosis of neurons post spinal cord injury via mediation of the p62-Keap1-Nrf2 signaling pathway [36]. Nonetheless the function of GDF15 in regulating MSC survival under ischemic conditions remains unclear. In the current study, we first found that the protein level of GDF15 was significantly increased during the first 24 h of SD/H challenge, and then reduced at 48 h. These results showed that hypoxic conditions can stimulate MSCs to release GDF15 and hypoxic conditions continuously induced MSC death. This led to downregulation of GDF15 secretion indicating that loss of GDF15 protein in hypoxic MSCs may be secondary to cell death. We believe that the initial release of GDF15 during the first 24 h remains therapeutically relevant. Upon MSC transplantation into the ischemic heart following MI, the hypoxic environment will stimulate MSCs to release GDF15 and other beneficial factors during the early stages. This early release may contribute to the observed improvement in heart function. Moreover, these results also indicated the GDF15 regulates MSC survival. Next, we used GDF15 to pretreat MSCs and then expose them to SD/H. Pretreatment with GDF15 significantly improved MSC survival in vitro. In vivo, compared with control MSCs, GDF15 pretreatment significantly enhanced the survival and engraftment of MSCs in ischemic heart tissue and improved cardiac function in a mouse model of MI. More importantly, GDF15 pretreatment enhanced the paracrine effects of MSCs as evidenced by improved tube formation of HUVECs in vitro and enhanced new blood vessel formation in vivo, as well as reduced cardiomyocyte apoptosis in vitro and in vivo. Therefore, after MSC transplantation into the heart following infarction, the ischemic environment stimulated MSCs to release some factors in the first 24 h, including GDF15, that contributed to heart function improvement. Nonetheless the mechanisms underlying these benefits have not been explored.

To gain further insight into the protective effects of GDF15 pretreatment on MSCs under SD/H challenge, we performed RNA-seq analysis of MSCs from SD/H + GDF15 group and SD/H group as well as the normoxia group. GO enrichment analysis revealed that the downregulated pathways in the SD/H + GDF15 group compared with the SD/H group were involved mainly in cellular response to oxidative stress. Indeed, DCFH-DA and Mito-SOX staining revealed that GDF15 pretreatment significantly downregulated ROS generation in SD/H-treated MSCs. Increasing evidence emphasizes that dysregulation of mitochondrial dynamics induces mitochondrial dysfunction, thereby causing overproduction of ROS with consequent cellular injury including senescence or apoptosis [37, 38]. It has been reported that lipopolysaccharide-induced mitochondrial fission mediated by Drp1 significantly enhanced the apoptosis of airway epithelial cells via upregulation of ROS generation. Inhibition of Drp1-mediated mitochondrial fission attenuated airway epithelial cell apoptosis [39]. In the

(See figure on next page.)

Fig. 6 Transplantation of GDF15-MSCs inhibited cardiomyocyte apoptosis and enhanced angiogenesis in a mouse model of MI. **A** Representative images of TUNEL and Troponin double staining of heart tissue from Sham mice or mice with MI that received transplantation of PBS, MSCs, or GDF15-MSCs. Scale bar = 100 μm. **B** Quantitative analysis of cardiomyocyte apoptosis in heart tissue from the different groups. **C** Representative images of CD45 staining of heart tissue from Sham mice or mice with MI that received transplantation of PBS, MSCs, or GDF15-MSCs. Scale bar = 100 μm. **D** Quantitative analysis of CD45 density in heart tissue from the different groups. **E** Representative images of CD31 and α-SMA staining of heart tissue from Sham mice or mice with MI that received transplantation of PBS, MSCs or GDF15-MSCs. Scale bar = 200 μm. **F** Quantitative analysis of CD31 density in heart tissue from the different groups. **G** Quantitative analysis of α-SMA density in heart tissue from the different groups. Data are expressed as the mean ± SEM. N = 6. ***p < 0.01; ***p < 0.001*

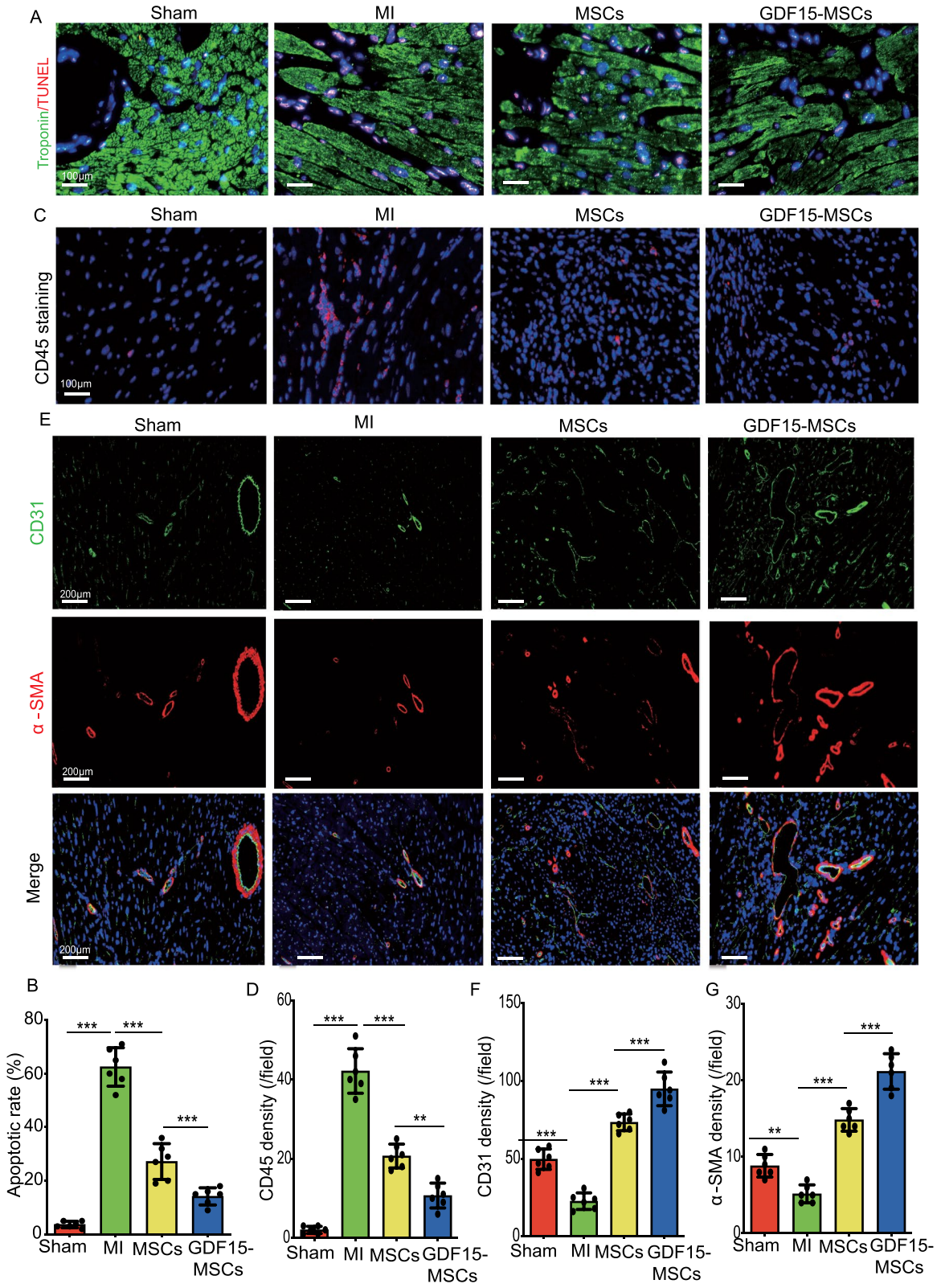

Fig. 6 (See legend on previous page.)

current study, we also found that GDF15 pretreatment dramatically alleviated mitochondrial fission in SD/H-treated MSCs as manifested by decreased mitochondrial fragmentation and protein level of p-Drp1^{ser616}. These effects were largely reversed by mitochondrial fission activator, indicating that GDF15 pretreatment protects against MSC apoptosis under SD/H challenge via inhibition of mitochondrial fission. There is accumulating evidence that the AMPK signaling plays an important role in maintaining mitochondrial dynamics and function [40, 41]. Previous study has shown that activation of AMPK attenuated hypoxia/reoxygenation-induced cardiomyocyte injury via inhibition of Drp1-mediated mitochondrial fission [42]. Here, we also found that GDF15 pretreatment significantly improved AMPK activation and reduced mitochondrial fission in SD/H-treated MSCs. In contrast, treatment with AMPK inhibitor Compound C partially abrogated these effects. These results showed that GDF15 pretreatment inhibited mitochondrial fission in MSCs under SD/H challenge via regulation of the AMPK pathway.

There are some limitations in the current study that we need to acknowledge. First, in addition to apoptosis, whether GDF15 pretreatment improves MSC survival under ischemic conditions by regulating other types of cell injury including ferroptosis or pyroptosis warrants exploration. Second, despite increased cardiac function in GDF15-MSC-treated mice following infarction at 28 days post-transplantation, whether GDF15 pretreatment can improve the long-term cardioprotective effects of MSCs was not determined. Third, although GDF15 pretreatment resulted in superior paracrine effects of MSCs, whether GDF15 pretreatment can also promote the therapeutic effects of MSC-derived extracellular vesicles on MI requires further investigation. Fourth, to the best of our knowledge, cardiovascular disease, especially MI, predominantly affects older individuals, so the cardioprotective effects of GDF15-MSCs on MI should be determined in a model of MI in aged mice to provide more clinically relevant insight. Finally, in addition to angiogenic cytokines, the important paracrine factors that contribute to the increased paracrine effects of GDF15 pretreated MSCs remain unclear. Future studies using protein arrays to identify potential factors are warranted.

Conclusions

These results revealed a crucial role of GDF15 in protecting MSCs against apoptosis under ischemic conditions via inhibition of mitochondrial fission by regulating the AMPK pathway and enhancing the paracrine function of MSCs. Our study identifies a pharmacological approach that may improve the cardioprotective efficacy of stem cell-based therapy for cardiovascular disease.

Abbreviations

α-SMA	α-Smooth muscle actin
CM	Conditioned medium
GDF15	Growth differentiation factor 15
Drp1	Dynamin-related protein 1
Mfn1	Mitofusin1
Mfn2	Mitofusin2
LVEF	Left ventricle ejection fraction
LVFS	Left ventricle fractional shortening
HUVECs	Human umbilical vein endothelial cells
MI	Myocardial infarction
MMP	Mitochondrial membrane potential
MSCs	Mesenchymal stem cells
ROS	Reactive oxygen species
SD/H	Serum deprivation and hypoxia

Supplementary Information

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Additional file 1.

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Author contributions

Y. Zhang, X. Li and W. Li designed the research, analyzed the data and wrote the manuscript. X. Huang, X. Liang and Q. Han performed the research, analyzed the data and wrote the manuscript. Y. Shen, J. Chen, Z. Li, Q. Jie, X. Gao, Y. Hong and F. Lin contributed to the collection of the data and the discussion. All authors have read and approved the manuscript for publication.

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Availability of data and materials

The datasets generated during the current study in regard to the bulk RNA-Sequencing is available in the figshare repository. Bulk RNA-Sequencing data: https://figshare.com/articles/dataset/Bulk-RNA-sequence_total_count_txt/26860873?file=48845077. Other data are included in the paper and supplement. Other datasets used in this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All cell cultures in this study were reviewed and approved by the Ethics Committees of Tongji University (Approved project: mesenchymal stem cell-based therapy for myocardial infarction, Approval No. 2016-050, Date of approval: Sep 18, 2016). Written informed consents were obtained from patients and their families for participation in the study and the use of samples. All animal procedures were performed in accordance with the ARRIVE guidelines and approved by approved by the Committee on the Use of Live Animals in Teaching and Research of Tongji University for Laboratory Animal Medicine (Approved project: The therapeutic effects of growth differentiation factor 15

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Consent for publication
All authors have read and approved the final version of the manuscript.

Competing interests
The authors declare that they have no competing interests.

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