# Role of lincRNA-p21 in the protective effect of macrophage inhibition factor against hypoxia/serum deprivation-induced apoptosis in mesenchymal stem cells

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**Abstract.** Stem cell transplantation is a promising clinical strategy for curing ischemic cardiomyopathy. However, its efficacy is impaired by low cell survival following transplantation, partly caused by insufficient resistance of the transplanted stem cells to severe oxidative stress at the injury site. In the current study, it was demonstrated that the small-molecule macrophage migration inhibitory factor (MIF) enhanced the defense of bone marrow-derived mesenchymal stem cells (MSCs) against hypoxia/serum deprivation (SD)-induced apoptosis *in vitro*. MIF significantly suppressed apoptosis and caspase family activities through inhibition of long intergenic noncoding (linc) RNA-p21 to maintain activation of the Wnt/β-catenin signaling pathway. The regulatory loop between MIF and the lincRNA-p21-Wnt/β-catenin signaling pathway was identified to be associated with the inhibition of oxidative stress. The involvement of the lincRNA-p21-Wnt/β-catenin signaling pathway in the effects of MIF in MSCs by overexpression of lincRNA-p21and silencing β-catenin using small interfering RNA was also demonstrated, both of which abolished the anti-apoptotic and anti-oxidative effects of MIF in MSCs under hypoxia/SD conditions. In conclusion, MIF protected MSCs from hypoxia/SD-induced apoptosis by interacting with lincRNA-p21, leading to activation of the downstream Wnt/β-catenin signaling pathway and decreased oxidative stress. Thus, treatment with MIF may have important therapeutic implications in improving MSC survival and therapeutic efficiency.

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## Introduction

Ischemic cardiomyopathy arising from myocardial ischemia is the leading cause of morbidity and mortality worldwide (1). There are numerous therapeutic options for ischemic cardiomyopathy, including the potential use of mesenchymal stem cells (MSCs) for tissue repair, as demonstrated in clinical trials for intractable diseases (2,3). However, the therapeutic efficacy of MSCs has been hindered by the low survival rate of transplanted cells. The survival and retention of MSCs following transplantation are adversely affected by the harmful ischemic microenvironment (4), and myocardial oxidative stress restricts the therapeutic effects of MSCs on cardiac repair (5). It is therefore important to develop novel strategies to promote donor cell survival to improve the efficacy of stem-cell-based therapy for ischemic cardiomyopathy.

Macrophage migration inhibitory factor (MIF) is a widely expressed pleiotropic cytokine and is considered to be an important therapeutic target for treating cardiovascular disease (6). MIF regulates cellular activities through transcriptional regulation of inflammatory gene products, modulation of cell proliferation, cell cycle control and metabolism, and by inhibition of apoptosis (7). MIF protects against myocardial ischemia/reperfusion injury primarily through preventing redox stress (8). MIF is a good anti-apoptotic factor candidate, and in an ischemia/reperfusion injury model, MIF was also demonstrated to protect against oxidative stress-mediated cardiomyocyte apoptosis (9). In the current study, the protective effects of exogenous MIF were determined in MSCs exposed to hypoxia/serum deprivation (SD) to mimic the ischemic environment.

Long non-coding RNAs (lncRNAs) are RNA transcripts >200 bp in length with no apparent protein-coding ability (10). Increasing evidence suggests that lncRNAs affect numerous cellular functions, and participate in diverse physiological and pathological processes (11), including development, differentiation, stem cell pluripotency and apoptosis (12). Long intergenic noncoding RNA-p21 (lincRNA-p21) is a p53-dependent transcriptional target gene involved in proliferation, the cell cycle, metabolism and apoptosis (13). Previous studies have demonstrated that lincRNA-p21 is associated with oxidative-stress-induced apoptosis (14,15), and participates in

senescence-induced cellular injury through the induction of oxidative stress (16). MIF inhibited the p21-dependent death signaling in keratinocytes (17). We therefore hypothesized that lincRNA-p21 may be a target gene inhibited by MIF, subsequently preventing hypoxia/SD-induced injury.

Wnt/β-catenin signaling is known to serve essential roles in cell growth, survival and apoptosis (18). As a target gene of lincRNA-p21, β-catenin is associated with cellular apoptosis, proliferation and oxidative stress (19). lincRNA-p21 was reported to inhibit hepatic stellate cell proliferation through inactivation of the Wnt/β-catenin signaling pathway (20). Furthermore, lincRNA-p21negatively regulates β-catenin translation at the post-transcriptional level and contributes to glioma stem cell apoptosis (15). In addition, inhibition of Wnt/β-catenin signaling induced by lincRNA-p21 contributes to cellular senescence in MSCs by inhibiting cellular oxidative stress (16). However, the role of lincRNA-p21-associated inhibition of the Wnt/β-catenin pathway in hypoxia/SD-induced MSC apoptosis remains unclear.

We proposed that exogenous MIF prevents hypoxia/SD-induced apoptosis and inhibits oxidative stress. Thus, the effect of MIF on hypoxia/SD-induced apoptosis of MSCs and its associated signaling pathways were examined in the present study.

## Materials and methods

*Reagents*. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GE Healthcare Life Sciences (HyClone; Logan, UT, USA), TRIzol® reagent was obtained from Thermo Fisher Scientific, Inc. (Invitrogen; Waltham, MA, USA), and the Transcriptor First Strand cDNA Synthesis kit, FastStart Universal SYBR®-Green Master (Rox) and X-tremeGENE HP DNA transfection reagent were purchased from Roche Diagnostics (Basel, Switzerland). The Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit was obtained from BD Biosciences (BD Pharmingen; Franklin Lakes, NJ, USA). Rabbit monoclonal antibodies against β-catenin (#8480; 1:1,000) and β-actin (#4970; 1:1,000) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA) and horseradish peroxidase-conjugated anti-rabbit secondary antibodies (#7074; 1:2,000) were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Small interfering RNAs (siRNAs) targeting lincRNA-p21 and β-catenin transcripts were purchased from Thermo Fisher Scientific, Inc. The ELISA kit for MIF was purchased from Abcam (#ab7207; Cambridge, UK), and the Mitochondrial Membrane Potential assay kit with JC-1 (#C2006) and Reactive Oxygen Species (ROS) assay kit (#S0033) were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). The Superoxide Dismutase (SOD) Activity Colorimetric assay (#ab211096), Lipid Peroxidation (malondialdehyde; MDA) assay kits (#ab118970) and mouse recombinant MIF were purchased from Abcam.

*Cell culture and treatment*. Bone marrow-derived mesenchymal stem cells (MSCs) were isolated using a standard protocol, as described previously (16). A total of 12 male mice (mean age, 6 months; mean weight, 22.85±2.62 g) were purchased from the Laboratory Animal Center of Wenzhou Medical University (Wenzhou, China). Mice were kept to a 12 h light/12 h dark cycle at 21±2˚C with 30-70% relative humidity. Food and water was freely available throughout. All animal procedures were approved by the Wenzhou Medical University Institutional Animal Care and Use Committee (Wenzhou, China). Briefly, bone marrow was isolated from mouse femurs and tibias by flushing with PBS. Adherent MSCs were cultured at 37˚C and 5% $CO_2$ in high-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Third-passage MSCs were used for experiments.

Apoptosis was induced *in vitro* by hypoxia and SD to mimic the *in vivo* conditions of the ischemic myocardium, as previously reported (21). Apoptosis was induced by incubating MSCs in serum-free DMEM in a glove box (model no. 855-AC; Plas Labs, Inc., Lansing, MI, USA) with a regulated atmosphere (anaerobic chamber) to scavenge free oxygen (hypoxia/SD group). For MIF treatment, cells were cultured with DMEM containing 100 ng/ml recombinant MIF and incubated at 37˚C for various periods of time, as reported previously (22). Untreated cells were used as the control group throughout.

*Flow cytometric analysis of cell apoptosis*. The effects of MIF on apoptosis were determined by detecting phosphatidylserine exposure on cell plasma membranes using an Annexin V-FITC Apoptosis Detection kit, according to the manufacturer's protocol. Briefly, cells were harvested (4˚C, 5 min, 12,000 x g) and washed in ice-cold PBS, resuspended in 300 $\mu$l binding buffer, and incubated with 5 $\mu$l Annexin V-FITC solution for 30 min at 4˚C in the dark, followed by further incubation in 5 $\mu$l propidium iodide for 5 min at 4˚C. The cells were then analyzed immediately using bivariate flow cytometry with a BD FACSCanto II equipped with BD FACSDiva software (version 8.0.1; BD Biosciences).

*Calculation of caspase 3/7/8 activities*. Caspase 3/7/8 activities in MSCs were determined as described previously (23). Briefly, activities of caspases 3/7/8 in cell lysates of MSCs, MSCs under hypoxia/SD conditions and MSCs treated with MIF under hypoxia/SD conditions were measured using a Cell Meter Caspase 3/7/8 Activity Apoptosis assay kit (AAT Bioquest, Sunnyvale, CA, USA) according to the manufacturer's protocol. Results were read at 520 nm with a microplate reader and expressed as the fold change in caspase 3/7/8 activity compared with the control.

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. Expression levels of several genes were analyzed using RT-qPCR. Briefly, total cellular RNA was isolated using TRIzol reagent and reverse transcribed using a Transcriptor First Strand cDNA Synthesis kit according to the manufacturer's protocol. qPCR was performed using the Fast Start Universal SYBR Master and the Applied Biosystems Step One Plus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. The threshold number of cycles (Cq) was set within the exponential phase of the PCR. The ΔCq value for each target gene was calculated by subtracting the Cq value for GAPDH (internal control) from that of the

Table I. Primer sequences.

| Genes | Sequences |
|---|---|
| lincRNA-p21 | |
| F | 5'-CCT GTC CAC TCG CTT TC-3' |
| R | 5'-GGA ACT GGA GAC GGA ATG TC-3' |
| β-catenin | |
| F | 5'-TAG TGT GAC AAG CTG AGT ATG CGA-3' |
| R | 5'-CTG GAG CGT CTG ATG AG-3' |
| GAPDH | |
| F | 5'-GGA GCC AAA AGG GTC ATC AT-3' |
| R | 5'-GTG ATG GCA TGG ACT GTG GT-3' |
| siRNA-LincRNA-p21 | UGA AAA GAG CCG UGA GCU A |
| siRNA-β-catenin | CTC ACT TGC AAT AAT TAC AAA |
| siRNA-NT | CTC UCC GAA CGU GUC ACG UTT |

siRNA, small interfering RNA; linc, long intergenic noncoding; NT, non-targeting; F, forward; R, reverse.

target gene. Relative gene expression levels were calculated by comparing the ΔCq values between the control and experimental conditions for each target PCR using the following equation: $2^{-(\Delta Cq\ sample-\Delta Cq\ control)}$ (24). The primer pairs used to detect the mRNA levels of target genes are listed in Table I.

*Western blot analysis*. MSCs were lysed with ice-cold lysis buffer (Beyotime Institute of Biotechnology) to obtain total protein, then β-catenin and β-actin expression levels were evaluated using western blotting. Cellular extracts were prepared according to the manufacturer's protocol. Protein samples were quantified and separated by SDS-PAGE. Western blotting was performed as described previously (25). Quantity One software (version 4.5.2; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for densitometric analysis.

*lincRNA-p21 and β-catenin siRNA knockdown*. MSCs were transfected using X-treme GENE HP DNA Transfection reagent according to the manufacturer's protocol. Briefly, MSCs ($1x10^5$ cells/well) were cultured in 6-well plates for 24 h and then treated with the transfection reagent (siRNA weight ratio of 3:1) for 20 min. This was followed by the addition of a mixture containing 100 nM siRNA and incubation in 2 ml DMEM for 48 h at 37˚C. Scrambled non-targeting siRNA (siRNA-NT) was used as a negative control. The knockdown efficiency was determined by RT-qPCR, as aforementioned. The sequences are listed in Table I.

*Plasmid transfection*. Adenoviral vectors expressing lincRNA-p21 (Ad-lincRNA-p21 group) and control scrambled sequence (Ad-ctrl group) were designed and synthesized by Shanghai GeneChem Co., Ltd. (Shanghai, China). MSCs were transfected using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration of 100 nM.

*Evolution of mitochondrial transmembrane potential*. Cells were cultured in complete DMEM in 96-well microtiter plates at 37˚C for 1 day to achieve $1x10^5$ cells/well. The cells were then washed with PBS and incubated with 5 μg/ml JC-1 at 37˚C for 15 min. Following two washes with PBS, time-dependent JC-1 fluorescence was recorded using an ELISA plate reader. The fluorescent probe was excited at 490 nm and the emission was read alternately at 530 and 590 nm.

*ROS measurement*. Levels of intracellular ROS were determined using 2,7-dichlorodihydrofluorescein diacetate (Beyotime Institute of Biotechnology), following the manufacturer's protocol. The fluorescence intensity of the cells was measured using a fluorescence spectrophotometer, with excitation and emission wavelengths of 488 and 525 nm, respectively.

*SOD activity*. SOD activity in MSCs was determined using a SOD Activity Colorimetric assay according to the manufacturer's protocol. Briefly, protein was isolated from MSCs using lysis buffer, and SOD activity was measured in 10 μg of total protein extract. Absorbance was measured at 450 nm.

*Lipid peroxidation assays*. Lipid peroxidation was monitored using an assay kit to measure the formation of MDA, according to the manufacturer's protocol. Briefly, MSCs ($1x10^6$ cells) were homogenized on ice in 300 μl of MDA lysis buffer (with 3 μl of 100X butylated hydroxytoluene), and then centrifuged (4˚C, 13,000 x g, 10 min) to remove insoluble material. The supernatant (200 μl) was added to 600 μl of thiobarbituric acid and incubated at 95˚C for 60 min. The samples were cooled to room temperature in an ice bath for 10 min, and the absorbance at 532 nm was measured spectrophotometrically.

*Statistical analysis*. Data are expressed as the mean ± standard deviation following three repeats. Differences among groups were analyzed using one-way analysis of variance followed by Tukey's multiple comparisons test, and comparisons between two groups were evaluated by Student's t-tests using SPSS software (version 19.0; IBM, Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

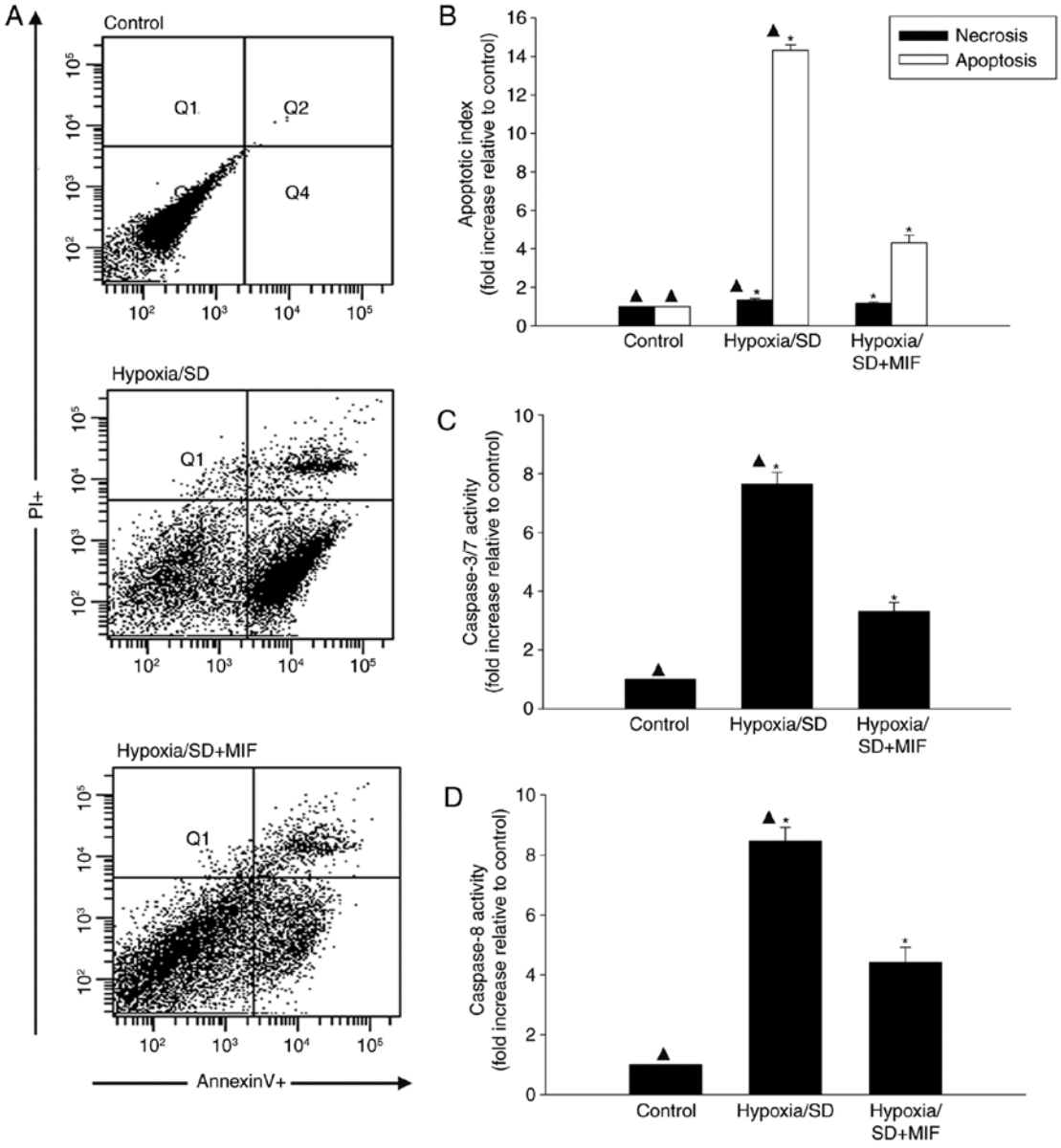


Figure 1. MIF ameliorated cell injury induced by hypoxia/SD in MSCs. MIF (100 ng/ml) was maintained in the incubation medium throughout the hypoxia/SD treatment period. (A) Representative flow cytometric dot plots of apoptotic cells following Annexin V/propidium iodide staining and (B) quantification of apoptosis. (C) Activities of caspases 3/7 and (D) caspase 8 in cell lysates measured using ELISA. Each column represents the mean ± standard deviation of three independent experiments; *P<0.05 vs. control; ▲P<0.05 vs. hypoxia/SD+MIF. MIF, macrophage migration inhibitory factor; MSC, mesenchymal stem cells; SD, serum deprivation.

## Results

*MIF ameliorates cell injury induced by hypoxia/SD in MSCs.* Hypoxia/SD induced MSC injury, with maximal injury at 24 h. To determine if MIF protects MSCs from hypoxia/SD-induced injury, MSCs were exposed to MIF (100 ng/ml) followed by hypoxia/SD for 24 h and apoptosis rates were determined by flow cytometry. MIF demonstrated a significant anti-apoptotic effect of the hypoxia/SD model, as demonstrated by Annexin V-FITCFACS analysis (Fig. 1A and B).

The anti-apoptotic effects of MIF were further examined by measuring the effects of MIF pretreatment on changes in caspases 3/7 and caspase 8 following hypoxia/SD induction. MIF pretreatment significantly reduced the stress-induced increases in caspase activities (Fig. 1C and D).

*MIF protects MSCs from hypoxia/SD-induced apoptosis by inhibiting the expression of lincRNA-p21.* lincRNA-p21 has been reported to be associated with cellular apoptosis (26). To determine if lincRNA-p21 was involved in hypoxia/SD-induced apoptosis, its expression was evaluated in MSCs exposed to hypoxia/SD for 24 h. lincRNA-p21 was significantly increased in MSCs following hypoxia/SD treatment, as demonstrated by RT-qPCR analysis (Fig. 2A). Furthermore, the hypoxia/SD-induced increase was significantly attenuated by exogenous MIF treatment (Fig. 2A).

The role of MIF-induced inhibition of lincRNA-p21in protecting MSCs from hypoxia/SD-induced apoptosis was further examined. MIF was added to MSCs prior to hypoxia/SD treatment and apoptosis was measured by flow cytometry. In a parallel experiment, MSCs were transfected

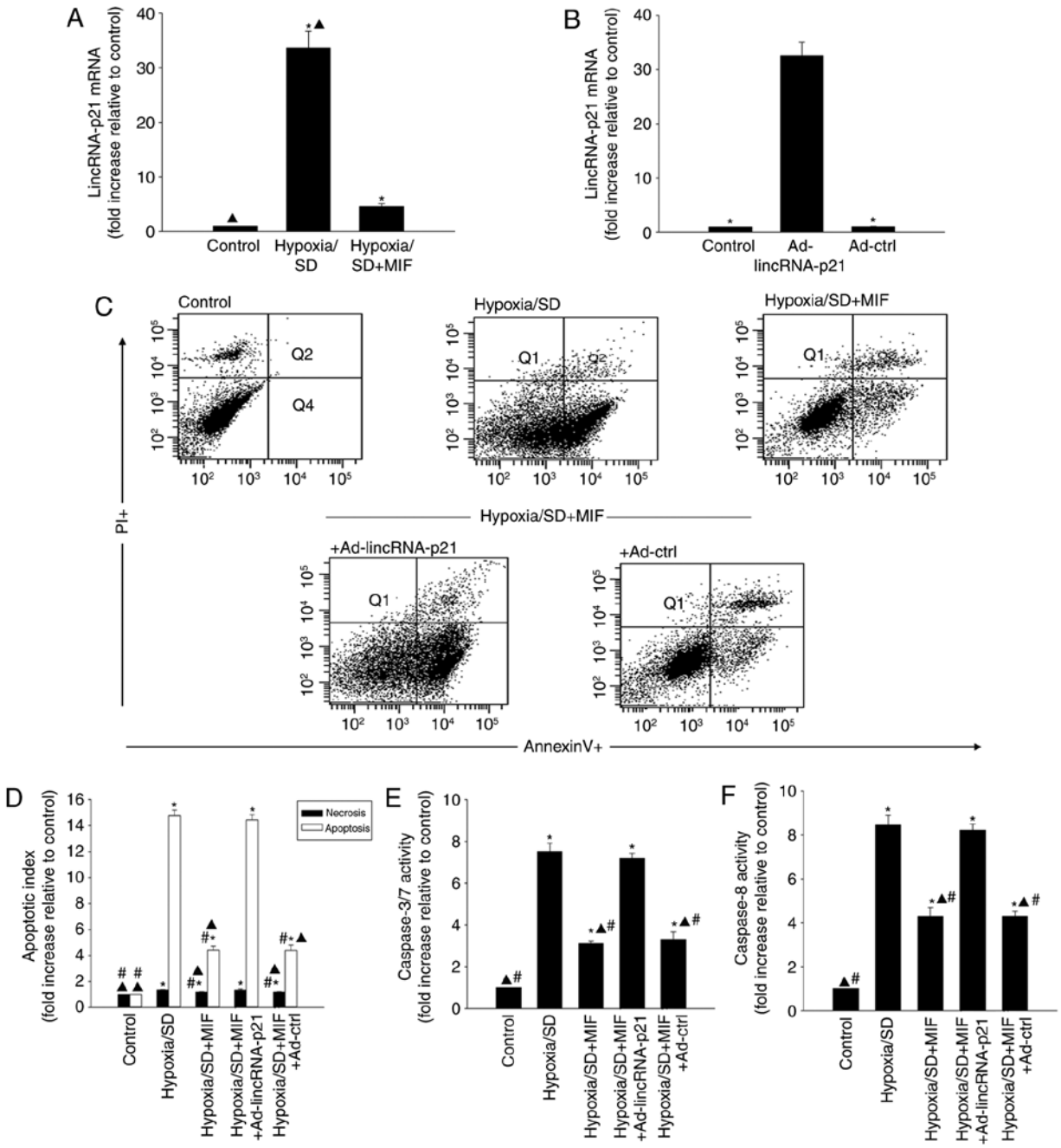


Figure 2. MIF protected MSCs against hypoxia/SD-induced apoptosis by inhibiting expression of lincRNA-p21. (A) LincRNA-p21 mRNA levels in MSCs cultured under hypoxia/SD conditions with or without MIF were analyzed by RT-qPCR; *P<0.05 vs. control; ▲P<0.05 vs. hypoxia/SD+MIF. (B) MSCs were transfected with Ad-lincRNA-p21 or Ad-ctrl. The transfection efficiency was demonstrated by RT-qPCR; *P<0.05 vs. Ad-lincRNA-p21. MSCs were then subjected to hypoxia/SD for 24 h. In parallel experiments, cells were treated with MIF (100 ng/ml) prior to hypoxia/SD, and MIF was maintained in the incubation medium throughout the hypoxia/SD treatment period. (C) Representative flow cytometric dot plots of apoptotic cells following Annexin V/propidium iodide staining and (D) quantification of apoptosis. (E) Activities of caspases 3/7 and (F) caspase 8 in cell lysates were measured by ELISA. Each column represents the mean ± standard deviation of three independent experiments; *P<0.05 vs. control; ▲P<0.05 vs. hypoxia/SD; #P<0.05 vs. hypoxia/SD+MIF+Ad-lincRNA-p21. MIF, macrophage migration inhibitory factor; MSC, mesenchymal stem cells; SD, serum deprivation; linc, long intergenic noncoding; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Ad-lincRNA-p21, adenoviral vectors expressing lincRNA-p21; Ad-ctrl, adenoviral vectors expressing control scrambled sequence.

with Ad-lincRNA-p21 prior to treatment with MIF (Fig. 2B) and cultured under hypoxia/SD conditions. MIF treatment significantly decreased cellular apoptosis (Fig. 2C and D), and reduced caspases 3/7 and caspase 8 activities compared with cells under hypoxia/SD treatment alone (Fig. 2E and F). In addition, these effects were significantly abolished by lincRNA-p21 overexpression (Fig. 2C-F).

*MIF restores the lincRNA-p21-Wnt/β-catenin signaling pathway altered by hypoxia/SD induction*. The Wnt/β-catenin signaling pathway is a known target of lincRNA-p21, and has been reported to be involved in cellular injury in MSCs (16). In the present study, β-catenin protein expression levels in MSCs were significantly decreased by hypoxia/SD compared with control cells, which was restored following

pre-treatment with MIF. Overexpression of lincRNA-p21by Ad-lincRNA-p21 transfection abolished the effect of MIF, while transfection with the control vector had no significant effect (Fig. 3A and B). The mechanism underlying the modulation of lincRNA-p21-Wnt/β-catenin signaling by MIF in hypoxia/SD-associated cellular apoptosis was examined by silencing β-catenin using siRNA. β-catenin mRNA expression levels were significantly reduced in cells transfected with siRNA-β-catenin compared with cells transfected with siRNA-NT control (Fig. 3C). Pretreatment with MIF protected MSCs from apoptosis induced by hypoxia/SD (Fig. 4A and B), and significantly decreased the activities of caspases 3/7 and caspase 8 (Fig. 4C and D). However, these effects were abolished by silencing β-catenin and not by transfection with siRNA-NT (Fig. 4).

*MIF enhances MSC survival via inhibition of oxidative stress*. Oxidative stress is associated with cellular apoptosis (27), thus, the feedback loop between oxidative stress and the lincRNA-p21-Wnt/β-catenin signaling pathway modulated by MIF was explored. Mitochondrial transmembrane potential, ROS generation, SOD activation and lipid peroxidation were examined (Fig. 5). Hypoxia/SD significantly decreased the mitochondrial transmembrane potential (Fig. 5A) and SOD activation (Fig. 5C), while increasing ROS generation (Fig. 5B) and MDA activation (Fig. 5D). Pretreatment with MIF significantly increased the mitochondrial transmembrane potential and SOD activation, and decreased ROS generation of ROS and MDA activation. These anti-oxidant effects of MIF were abolished by ectopic expression of lincRNA-p21 or by silencing β-catenin (Fig. 5A-D).

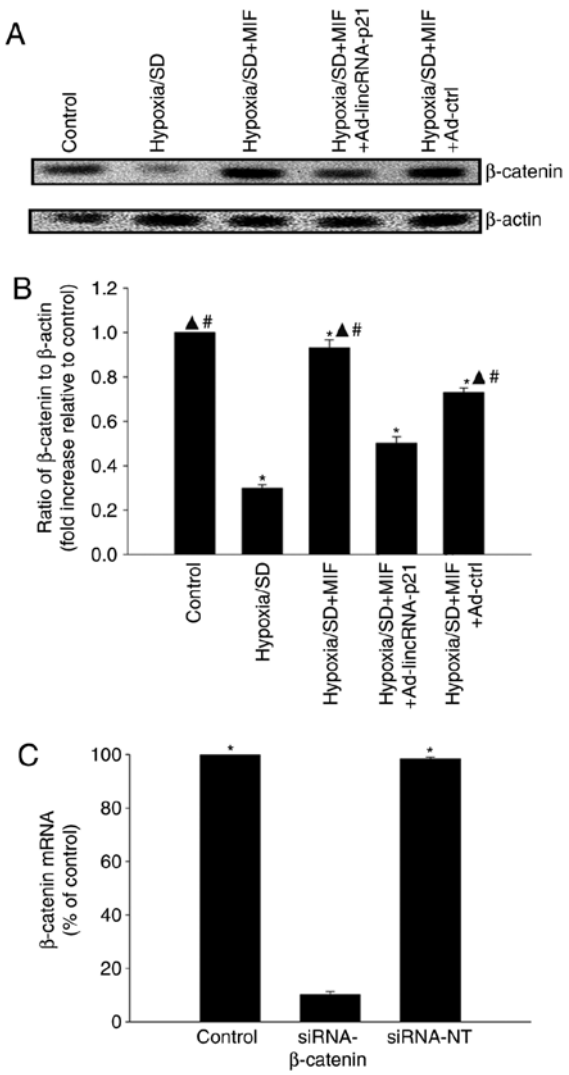


Figure 3. MIF modulated lincRNA-p21-Wnt/β-catenin signaling pathway. MSCs were transfected with Ad-lincRNA-p21 or Ad-ctrl followed by hypoxia/SD for 24 h. In parallel experiments, cells were treated with MIF (100 ng/ml) prior to hypoxia/SD. Untreated MSCs were used as a control. (A) Representative western blot and (B) quantification of β-actin and β-catenin in these groups. Each column represents the mean ± SD of three independent experiments; *P<0.05 vs. control; ▲P<0.05 vs. hypoxia/SD; #P<0.05 vs. hypoxia/SD+MIF+Ad-lincRNA-p21. MSCs were transfected with siRNA against β-catenin or control siRNA-NT. (C) The siRNA-mediated transfection efficiency was demonstrated by reverse transcription-quantitative polymerase chain reaction. Each column represents the mean ± standard deviation of three independent experiments; *P<0.05 vs. siRNA-β-catenin. MIF, macrophage migration inhibitory factor; MSC, mesenchymal stem cells; SD, serum deprivation; siRNA, small interfering RNA; NT, non-targeting; Ad-lincRNA-p21, adenoviral vectors expressing lincRNA-p21; Ad-ctrl, adenoviral vectors expressing control scrambled sequence.

## Discussion

MSCs are immune-protected and secrete a wide variety of growth factors that protect the heart from ischemic injury (28). The regenerative potency of these cells has thus been investigated in numerous preclinical and clinical studies, and MSC transplantation in the heart is considered to be a safe therapeutic option in patients with myocardial injury (29,30). However, numerous transplanted cells undergo apoptosis, which is considered to be one of the primary barriers limiting the effectiveness of cell therapy (31). In the present study, hypoxia/SD culture conditions were used to mimic the ischemic environment *in vitro*, in order to determine whether the survival and potency of transplanted MSCs increase following treatment with MIF.

Plasma MIF levels are increased in patients with myocardial infarction (32), and ischemia also increases MIF expression in rat hearts (33). Excessive MIF reduces cardiac contractility in isolated, perfused rat hearts (34), and has also been reported to enhance the survival of cultured cardiomyocytes (35). The present study revealed that MIF treatment significantly reduced apoptosis in hypoxia/SD-cultured MSCs *in vitro*, and this reduction was accompanied by inhibition of caspase 3/7 and caspase 8 activities, which are known to be involved in the cellular apoptosis process.

Mammalian genomes encode >10,000 lncRNAs, which are RNA molecules >200 nucleotides that do not appear to encode proteins (36). lncRNAs have emerged as important regulators in various cellular processes, including proliferation, survival, autophagy and apoptosis (37). A recent study demonstrated that lncRNAs regulate cell proliferation, apoptosis and autophagy in response to energy stress (38). lincRNA-p21 is a p53-dependent transcriptional target gene with roles in cell cycle arrest and apoptosis in response to DNA damage, and has been revealed to activate damage signaling and cell cycle arrest (39). MIF inhibited p53-dependent death signaling in keratinocytes and exerted a protective effect against cellular apoptosis (17). The results of this study demonstrated that

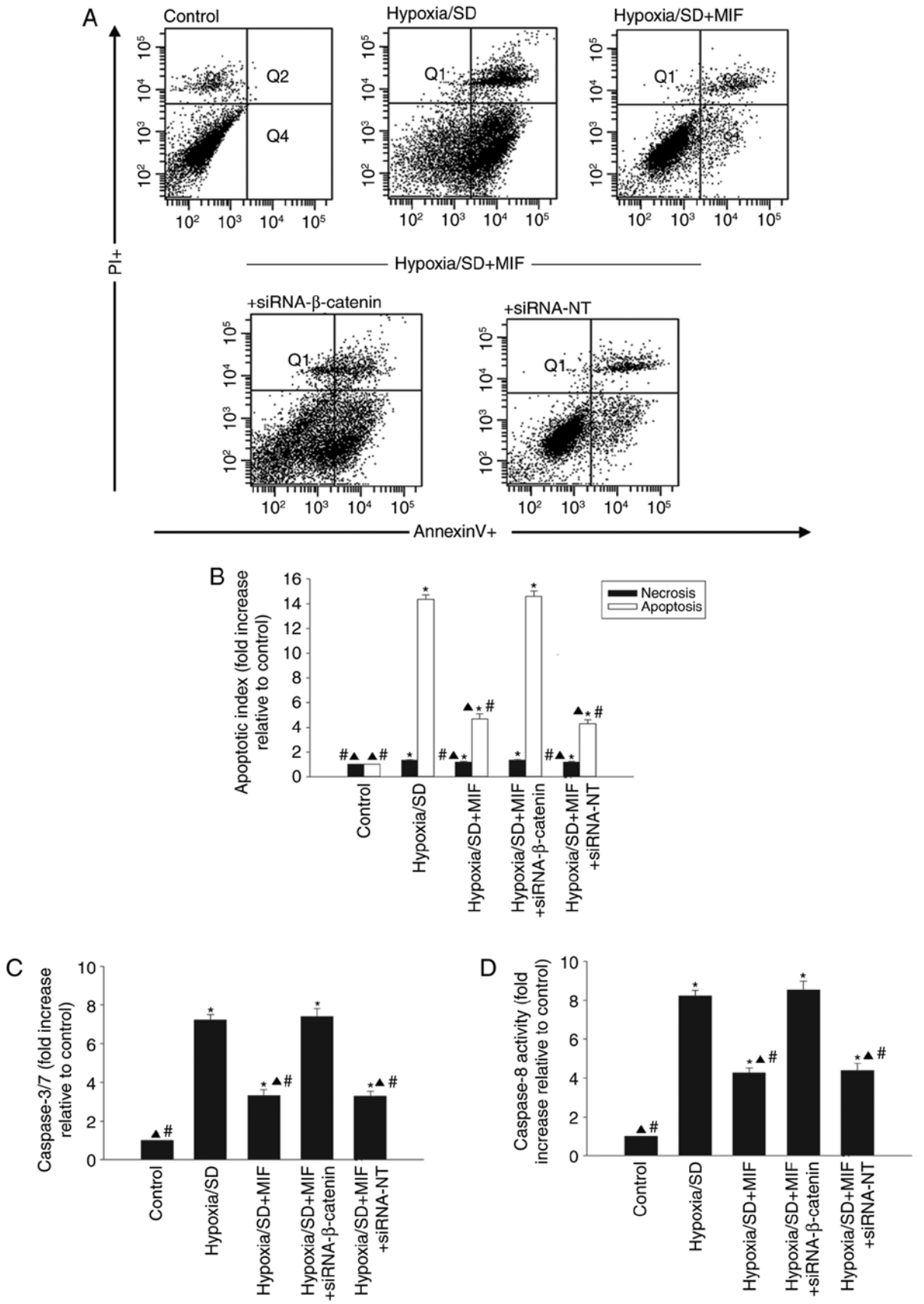


Figure 4. MIF restored the lincRNA-p21-Wnt/β-catenin signaling pathway altered by hypoxia/SD induction. MSCs were transfected with siRNA against β-catenin or control siRNA-NT and incubated under hypoxic/SD conditions for 24 h. In parallel experiments, cells were treated with MIF (100 ng/ml) in the incubation medium throughout the hypoxia/SD treatment period. (A) Representative flow cytometric dot plots of apoptotic cells following Annexin V/propidium iodide staining and (B) quantification of apoptosis. (C) Activities of caspases 3/7 and (D) caspase 8 in cell lysates were measured by ELISA. Each column represents the mean ± standard deviation of three independent experiments; *P<0.05 vs. control; ▲P<0.05 vs. hypoxia/SD; #P<0.05 vs. hypoxia/SD+MIF+siRNA-β-catenin. MIF, macrophage migration inhibitory factor; MSC, mesenchymal stem cells; SD, serum deprivation; siRNA, small interfering RNA; Linc, long intergenic noncoding; NT, non-targeting.

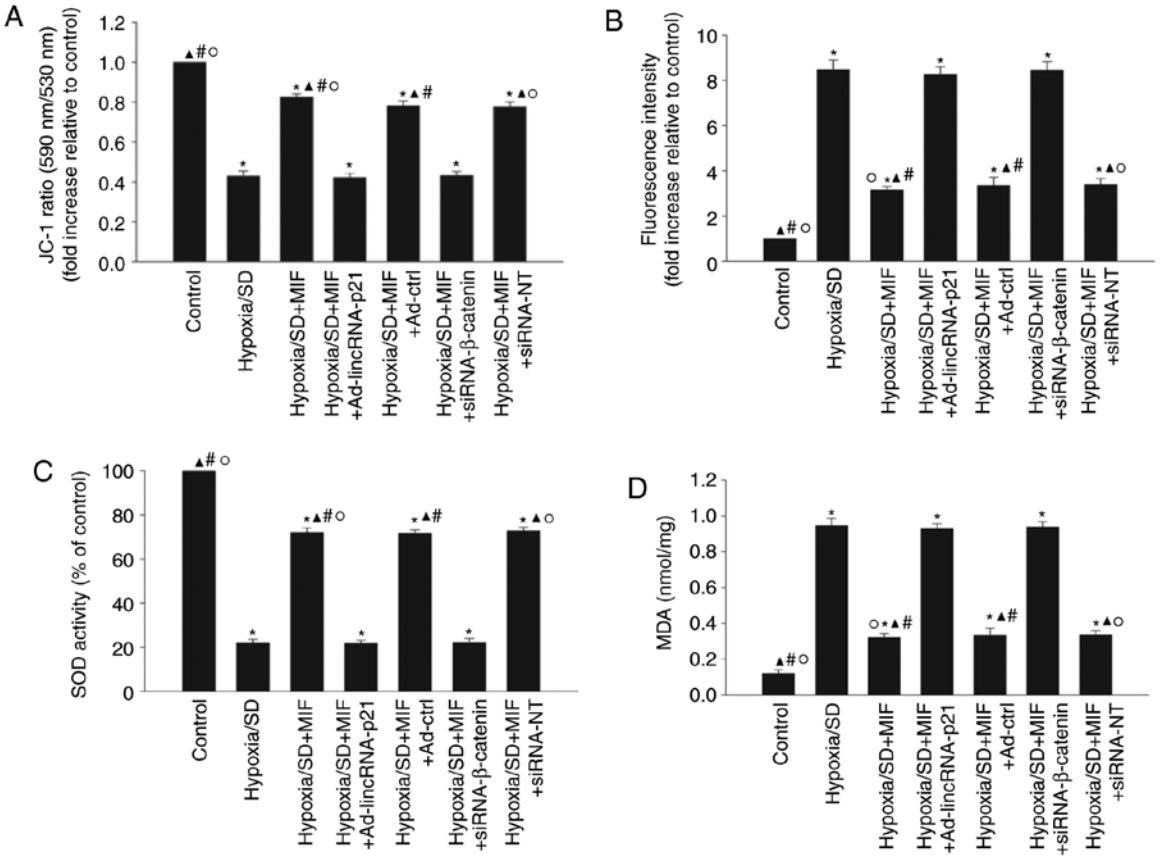


Figure 5. MIF enhanced MSC survival via inhibition of oxidative stress. MSCs were incubated under hypoxic/SD conditions for 24 h. In parallel experiments, cells were transfected with Ad-lincRNA-p21, Ad-ctrl, siRNA-β-catenin, or siRNA-NT before exposure to hypoxia/SD, and then treated with MIF. MIF (100 ng/ml) was added at the beginning of exposure to hypoxia/SD. Untreated MSCs were used as a control. (A) Mitochondrial membrane potential was measured using JC-1 stain. (B) Intracellular reactive oxygen species production was analyzed by fluorescence spectrophotometry. (C) Superoxide dismutase activity was evaluated by colorimetric assay. (D) Lipid peroxidation was evaluated by malondialdehyde formation. Data represent mean ± standard deviation from three independent experiments; *P<0.05 vs. control, ▲P<0.05 vs. hypoxia/SD; #P<0.05 vs. hypoxia/SD+MIF+Ad-lincRNA-p21; ○P<0.05 vs. hypoxia/SD+MIF+siRNA-β-catenin. MIF, macrophage migration inhibitory factor; MSC, mesenchymal stem cells; SD, serum deprivation; siRNA, small interfering RNA; linc, long intergenic noncoding; NT, non-targeting; Ad-lincRNA-p21, adenoviral vectors expressing lincRNA-p21; Ad-ctrl, adenoviral vectors expressing control scrambled sequence.

hypoxia/SD induced apoptosis in MSCs, accompanied by induction of lincRNA-p21, while MIF decreased lincRNA-p21 expression and protected MSCs from injury induced by hypoxia/SD. Overexpression of lincRNA-p21 abolished the anti-apoptotic effect of MIF, confirming that this anti-apoptotic effect was due to inhibition of lincRNA-p21 by MIF.

Wnt/β-catenin is a stress signaling kinase pathway and key regulator of energy-generating and consuming pathways. It serves as an essential sensor of cellular energy status and is activated under energy stress conditions, to protect cells against hypoxic injury and death (18). The Wnt/β-catenin signaling pathway also regulates organelle-compartmentalized protein folding, and is relevant in the cross-talk between mitochondria and endoplasmic reticulum, thus protecting against oxidative stress (40). β-Catenin is an important target gene of lincRNA-p21, and lincRNA-p21was demonstrated to inhibit β-catenin signaling, thereby attenuating the viability, self-renewal and glycolysis of cancer stem cells *in vitro* (41). Furthermore, MIF enhances the proliferation of neural stem/progenitor cells and promotes neuronal differentiation by activating the Wnt/β-catenin signal pathway (42). The current results suggest that the Wnt/β-catenin signaling pathway was inhibited by hypoxia/SD and reactivated by exogenous MIF, and this restoration was abolished by lincRNA-p21 overexpression. The relevance of MIF regulation of β-catenin in hypoxia/SD-induced apoptosis was confirmed by silencing β-catenin, which attenuated the anti-apoptotic effect of MIF.

Excess oxidative stress causes cell death in the heart (43), and excessive oxidative stress below physiological levels may not only induce myocardial injury in response to ischemia/reperfusion, but may also cause apoptosis of transplanted stem cells (44). lncRNAs are involved in cellular oxidative stress, and recent research revealed that the lncRNAFOXD3-AS1was involved in hypoxia/ROS-induced injury (45). lincRNA-p21was also demonstrated to inhibit HepG2 cell growth by activating endoplasmic reticulum stress (14). A recent study revealed that lincRNA-p21 participated in cellular senescence in MSCs through inducing the oxidative process (16). In addition, MIF-knockout exacerbated doxorubicin-induced mortality and cardiomyopathy, accompanied by cellular apoptosis and ROS generation (46). The results of the present study demonstrated that hypoxia/SD-induced oxidative stress was accompanied by decreased mitochondrial transmembrane potential and activation of SOD, and increased generation of ROS and MDA activation, and these effects were reversed by MIF treatment. The anti-oxidant effects of MIF

were in turn abolished by overexpression of lincRNA-p21 or silencing of β-catenin.

In conclusion, the results of this study suggest that MIF acts as an anti-apoptotic factor to counteract hypoxia/SD-induced apoptosis, via a mechanism involving rebalancing the lincRNA-p21-Wnt/β-catenin signaling pathway and decreasing oxidative stress.

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**Availability of data and materials**

All data generated or analyzed during the present study are included in the published article.

**Authors' contributions**

WX and LZ made substantial contributions to the acquisition of data, analysis and interpretation of data; and MH was involved in conception and design, drafting the manuscript and revising it critically for important intellectual content.

**Ethics approval and consent to participate**

All animal procedures were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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