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Research Article

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Macrophage migration inhibitory factor protects bone marrow mesenchymal stem cells from hypoxia/ischemia-induced apoptosis by regulating lncRNA MEG3

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Abstract: Objective: This research was performed to explore the effect of macrophage migration inhibitory factor (MIF) on the apoptosis of bone marrow mesenchymal stem cells (BMSCs) in ischemia and hypoxia environments. Methods: The cell viability of BMSCs incubated under hypoxia/ischemia (H/I) conditions with or without pretreatment with MIF or triglycidyl isocyanurate (TGIC) was detected using cell counting kit-8 (CCK-8) analysis. Plasmids containing long noncoding RNA (lncRNA) maternally expressed gene 3 (MEG3) or β-catenin small interfering RNA (siRNA) were used to overexpress or downregulate the corresponding gene, and the p53 signaling pathway was activated by pretreatment with TGIC. The influences of MIF, overexpression of lncRNA MEG3, activation of the p53 signaling pathway, and silencing of β-catenin on H/I-induced apoptosis of BMSCs were revealed by western blotting, flow cytometry, and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining. Results: From the results of CCK-8 assay, western blotting, and flow cytometry, pretreatment with MIF significantly decreased the H/I-induced apoptosis of BMSCs. This effect was inhibited when lncRNA MEG3 was overexpressed by plasmids containing MEG3. The p53 signaling pathway was activated by TGIC, and β-catenin was silenced by siRNA. From western blot results, the expression levels of β-catenin in the nucleus and phosphorylated p53 (p-p53) were downregulated and upregulated, respectively, when the lncRNA MEG3 was overexpressed. Through flow cytometry, MIF was also shown to significantly alleviate the increased reactive oxygen species (ROS) level of BMSCs caused by H/I. Conclusions: In summary, we conclude that MIF protected BMSCs from H/I-induced apoptosis by downregulating the lncRNA MEG3/p53 signaling pathway, activating the Wnt/β-catenin signaling pathway, and decreasing ROS levels.

Key words: Macrophage migration inhibitory factor (MIF); Long noncoding RNA (lncRNA); Maternally expressed gene 3 (MEG3); Bone marrow mesenchymal stem cells (BMSCs); β-Catenin; Apoptosis

1 Introduction

Osteonecrosis of the femoral head (ONFH), also known as avascular necrosis of the femoral head, is a multi-factor-induced disabling injury (George and Lane, 2022) and is one of the most common diseases leading to disability in young adults (Hines et al., 2021). Since the success rate of hip preservation in the late stage of ONFH is low, timely intervention is likely the best way to reduce the rate of hip arthroplasty and improve the quality of life of patients. Bone marrow mesenchymal stem cells (BMSCs), a subgroup of cells derived from the mesoderm, can be isolated from bone marrow tissue (Zhao et al., 2022). They are spindle-shaped and can differentiate into organs and tissues such as bone, cartilage, and muscle (Zeng et al., 2015; Jiao et al., 2021). With the development and maturity of regenerative stem cell medicine, the clinical application of hematopoietic and mesenchymal stem cells has gradually increased. With their advantages of low immunogenicity, easy cultivation and amplification, and multilineage differentiation potential, the use of BMSCs in the treatment of ONFH has become popular in basic experiments and clinical trials (Xu et al., 2021). Previous studies have shown that ischemic and anoxic microenvironments are the main factors causing

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apoptosis of transplanted BMSCs during regenerative treatment of ONFH (Zhu et al., 2006; Fan et al., 2015). Improving the apoptosis resistance of BMSCs and promoting osteogenic differentiation, thereby delaying or reversing the pathological process of early steroid-induced avascular necrosis of femoral head (ESANFH), may be the key to improving the efficacy of regenerative treatment in patients with ONFH.

Long noncoding RNAs (lncRNAs) are endogenous RNA strands with a length of more than 200 bp that cannot encode proteins (Jarroux et al., 2017). Previous studies have shown that lncRNAs are involved in biological processes such as cell proliferation, apoptosis, and migration by regulating downstream signaling pathways (Yu and Li, 2020; Yuan et al., 2022). In recent in vivo and in vitro experiments, lncRNA maternally expressed gene 3 (MEG3) was reported to be upregulated and to play an important role in the cerebral ischemic/hypoxic environment (Yan et al., 2016). LncRNA MEG3 is able to regulate energy metabolism, endoplasmic reticulum stress, and DNA damage, which are closely related to the proliferation and apoptosis of BMSCs (Xia Y et al., 2015; Chen et al., 2016; Qiu et al., 2016). However, the specific mechanism by which lncRNA MEG3 affects apoptosis in BMSCs has not been reported. Exploring and revealing this mechanism may assist in finding a target to inhibit apoptosis in transplanted BMSCs.

Macrophage migration inhibitory factor (MIF) was firstly found to be a proinflammatory factor involved in the regulation of a variety of signaling pathways (Calandra et al., 1994; Wirtz et al., 2021). It can be found in monocytes, vascular smooth muscle cells, and cardiomyocytes. Conversely, MIF can promote cell proliferation and survival, and inhibit apoptosis (Jung et al., 2021). There is a close relationship between MIF and apoptosis. In ischemic and hypoxic BMSCs, the expression of MIF is significantly decreased and the rate of apoptosis increased. Also, an additional supply of exogenous MIF can significantly reduce the apoptosis of BMSCs (Xia and Hou, 2016; Zhang YL et al., 2019). However, the potential mechanism is still unclear. Regulating the expression of MIF may be an effective way to interfere with the apoptosis of transplanted cells.

It has been reported that MIF can affect apoptosis through the p53 signaling pathway (Fukaya et al., 2016; Yoshihisa et al., 2016; Song et al., 2020). In addition, many signaling pathways activated or inhibited by p53 can also be activated or inhibited by lncRNA MEG3 (Chen et al., 2016; Yan et al., 2016). It has also been revealed that MIF can significantly promote the proliferation and differentiation of stem cells and reduce the apoptosis of cells by regulating the Wnt/β-catenin signaling pathway (Xia et al., 2018). In addition, lncRNA MEG3-mediated apoptosis is related to oxidative stress (Qiu et al., 2016). However, the relationships among MIF, lncRNA MEG3, p53 and Wnt/β-catenin signaling pathways, and oxidative stress have not been described in detail.

Therefore, this study was designed to explore the effect of MIF on the apoptosis of BMSCs in ischemic and hypoxic environments based on lncRNA MEG3, p53 and Wnt/β-catenin signaling pathways, and oxidative stress.

2 Materials and methods

2.1 Isolation of BMSCs

BMSCs were harvested from the bone marrow of C57BL/6 mice. Briefly, bone marrow cells were flushed out and collected from the femur and tibia of mice, plated in 10-cm cell culture dishes, and cultured overnight in an incubator at 37 °C with 5% CO_2. Then, nonadherent cells were removed by rinsing twice with phosphate-buffered saline (PBS; Gibco, Grand Island, USA). Adherent cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% (0.1 g/mL) fetal bovine serum (FBS; Gibco), 2 mmol/L GlutaMAX (Gibco), and 1% (0.01 g/mL) penicillin and streptomycin (PS; Gibco).

2.2 Hypoxia/ischemia treatment

Hypoxia was achieved using a CO_2 water-jacketed incubator (Baocheng, Shenzhen, China), which could deplete the concentration of O_2 down to 0.5%. BMSCs were placed in the incubator for 24 h. Control plates were kept in normoxic conditions for the corresponding time. An ischemic condition was achieved by replacing culture medium with DMEM without glucose or serum.

2.3 Cell culture and treatment

The BMSCs were cultured in complete medium containing 10% FBS. Cells from the second passage

were used for further experiments, and the cytoprotective effect of MIF (MCE, New Jersey, USA) was detected following pretreatment for 24 h. The lowest effective drug concentrations were determined using the cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan). BMSCs were transfected with pcDNA3.1-MEG3/β-catenin small interfering RNA (siRNA) (RiboBio, Guangzhou, China) to overexpress or downregulate the corresponding gene at a concentration of 50 nmol/L using Lipofectamine 3000 (Invitrogen, California, USA). To activate the p53 signaling pathway in BMSCs, triglycidyl isocyanurate (TGIC; MCE) (Kim et al., 2018) was used at a concentration of 20 μmol/L.

2.4 Cell viability assay

CCK-8 analysis was performed to detect the effect of different drugs on the viability of BMSCs according to the operator's instructions, and to investigate the viability of BMSCs incubated under hypoxia/ischemia (H/I) conditions for different time. Briefly, 1×10^4 cells were seeded and cultured in each well of a 96-well plate for 24 h before stimulation with different concentrations of drugs for a certain period. Then, each well was refreshed with 100 μL of medium containing 10 μL CCK-8 solution and incubated in a cell incubator at 37 ℃ for another 2 h. Finally, the absorbance of each well at 450 nm was measured using an enzyme-labeling meter (Tecan, Mannedorf, Switzerland). Each group had three duplicates.

2.5 RNA isolation and qRT-PCR

The total RNA of the BMSCs was extracted using TRIzol reagent (Beyotime, Shanghai, China). According to the manufacturer's instructions, complementary DNA (cDNA) was obtained by reverse transcription in a 20-μL reaction system using qRT Master Mix (TOROIVD, Shanghai, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with a total of 20 μL solution containing cDNA, SYBR Green (MCE), and the forward and reverse primers using qRT Master Mix. The sequences of primers were listed in Table 1. Thermal cycling conditions consisted of 60 s at 95 ℃, and followed by 40 cycles of 95 ℃ for 10 s and 60 ℃ for 30 s. The expression of related messenger RNAs (mRNAs) was analyzed by the $2^{-\Delta\Delta C_T}$ method with β-actin (*ACTB*) as the internal reference control.

Table 1 Primer sequences for qRT-PCR

Gene	Primer sequence
MEG3	F: 5'-GAGTGTTTCCCTCCCCAAGG-3'
	R: 5'-GCGTGCCTTTGGTGATTCAG-3'
ACTB	F: 5'-TGTTACCAACTGGGACGAGA-3'
	R: 5'-CTTTTCACGGTTGGCCTTAG-3'
Wnt	F: 5'-TGCCGGACTCTCATGAAC-3'
	R: 5'-GTGTGGTCCAGCACGTCTTG-3'

qRT-PCR: quantitative real-time polymerase chain reaction; *MEG3*: maternally expressed gene 3; *ACTB*: β-actin; F: forward; R: reversed.

2.6 Protein isolation and western blot analysis

After intervention, the BMSCs in six-well plates were washed with PBS 2–3 times and lysed with 80 μL radio immunoprecipitation assay (RIPA) lysate containing 1% phenylmethanesulfonyl fluoride (PMSF) at 4 ℃ for 15–20 min. The lysate was collected and centrifuged at 12 000 r/min for 15 min at 4 ℃. To isolate the proteins in the nucleus, the cells were washed and collected with ethylenediamine tetraacetic acid (EDTA) solution. The protein in the cytoplasm was first collected using the supplied cytoplasmic protein extractors A and B, and the residual cell precipitate was lysed with the supplied nuclear protein extraction reagent (Beyotime). The concentration of the protein in the collected supernatant was measured using the bicinchoninic acid (BCA; Beyotime) method according to the manufacturer's instructions. After loading buffer was added, 40 μg of protein from each group was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% (0.05 g/mL) nonfat milk at room temperature for 2–3 h, the membrane was incubated with primary antibodies: anti-phospho-p53 (1/1000–1/5000 (mass ratio, the same below); Beyotime, Cat. No. AF1402), anti-B-cell lymphoma-2 (Bcl-2)-associated X (Bax) (1/1000–1/10 000; Abcam, Boston, USA, Cat. No. ab32503), anti-Bcl-2 (1/1000; Abcam, Cat. No. ab32124), anti-caspase-3 (CASP3) (1/5000; Abcam, Cat. No. ab32351), anti-β-catenin (1/5000–1/10 000; Abcam, Cat. No. ab32572), and anti-β-actin (1/200, Abcam, Cat. No. ab115777) at 4 ℃ overnight. After washing with Tris-buffered saline with Tween-20 (TBST) three times, the bands were incubated with the respective secondary antibodies at room temperature for 1 h, followed by visualization with an

electrochemiluminescence (ECL)-plus detection kit (New Cell & Molecular Biotech, Suzhou, China). Expression was quantified by densitometry using Quantity One software (Bio-Rad, California, USA).

2.7 Detection of apoptosis

Apoptosis of the treated BMSCs was detected by flow cytometry with Annexin V/propidium iodide (PI) double staining (MULTI SCIENCES, Hangzhou, China). Briefly, the BMSCs were collected and washed with PBS and resuspended in 100 μL of staining buffer with 5 μL of Annexin V and 10 μL of PI dyes at 37 °C for 10 min. Fluorescence was detected by flow cytometry (BD Biosciences, New Jersey, USA) within 1 h.

2.8 Immunofluorescence staining

The treated cells were washed with PBS and fixed with 4% (0.04 g/mL) paraformaldehyde for 30 min. After blocking with 1% (0.01 g/mL) bovine serum albumin (BSA) for 1 h, the BMSCs were incubated with primary antibodies overnight at 4 °C and then with the secondary antibody (labeled with green) for 1 h at a dilution of 1:50 (volume ratio). Then, the cells were washed with PBS and stained with 4',6-diamidino-2-phenylindole (DAPI; Beyotime) at a concentration of 1.43 μmol/L. Finally, the cells were observed by fluorescence microscopy (Leica, Wetzlar, Germany).

2.9 Reactive oxygen species detection

The production of intracellular reactive oxygen species (ROS) was detected by 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime). Briefly, the treated cells were resuspended in 1 mL of diluted DCFH-DA and incubated in a cell incubator at 37 °C for 20 min. Then, the cells were washed three times with serum-free medium to remove the residual DCFH-DA and detected by flow cytometry (BD Biosciences) or observed by fluorescence microscopy.

2.10 TUNEL staining

The treated cells were washed once with PBS, fixed with 4% paraformaldehyde for 30 min, and then washed once with PBS. The cells were incubated at room temperature for 5 min following the addition of 0.1% (1 g/L) Triton X-100. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) detection solution was prepared according to the manufacturer's instructions (Beyotime). After 50 μL TUNEL detection solution was added, the BMSCs were incubated at 37 °C for 60 min away from light. The cells were finally observed by fluorescence microscopy.

2.11 Data analysis

The data were expressed as mean±standard deviation (SD) and were analyzed using SPSS 22 (IBM, New York, USA). The measurement data between groups were compared by *t*-tests or one-way analysis of variance (ANOVA). The count data were processed using a χ^2 test or continuity correction χ^2 test. The histograms were made using GraphPad 7.0 (GraphPad Software, Santiago, USA). A *P*-value less than 0.05 was considered significant.

3 Results

3.1 Effect of MIF on H/I-induced apoptosis of BMSCs

The CCK-8 results showed that exposure to H/I conditions for 24 h significantly reduced the viability of BMSCs ($P<0.001$; Fig. 1a), and MIF exhibited a cytoprotective effect at concentrations greater than 100 ng/mL ($P<0.001$; Fig. 1b). Western blotting and flow cytometry analysis consistently showed that incubation under H/I conditions for 24 h increased the expression of apoptosis-related proteins Bax and cleaved CASP3 and the proportion of apoptotic BMSCs, and decreased the expression of Bcl-2, which were all alleviated by pretreatment with MIF at 100 ng/mL for 24 h (Figs. 1c–1f). Pretreatment with MIF also downregulated the expression of lncRNA MEG3, as shown by qRT-PCR analysis, and phosphorylated p53 (p-p53), as shown by western blotting, in BMSCs under H/I conditions (Figs. 1c and 1g).

3.2 Effect of overexpression of MEG3 on H/I-induced apoptosis of BMSCs

Transfection with plasmids expressing lncRNA MEG3 significantly increased the lncRNA MEG3 expression level in BMSCs (Fig. 2a). Compared to the BMSCs exposed to H/I conditions, pretreatment with MIF and plasmids expressing the non-targeting mRNA (vector) significantly downregulated the expression of apoptosis-related proteins Bax and cleaved CASP3 and

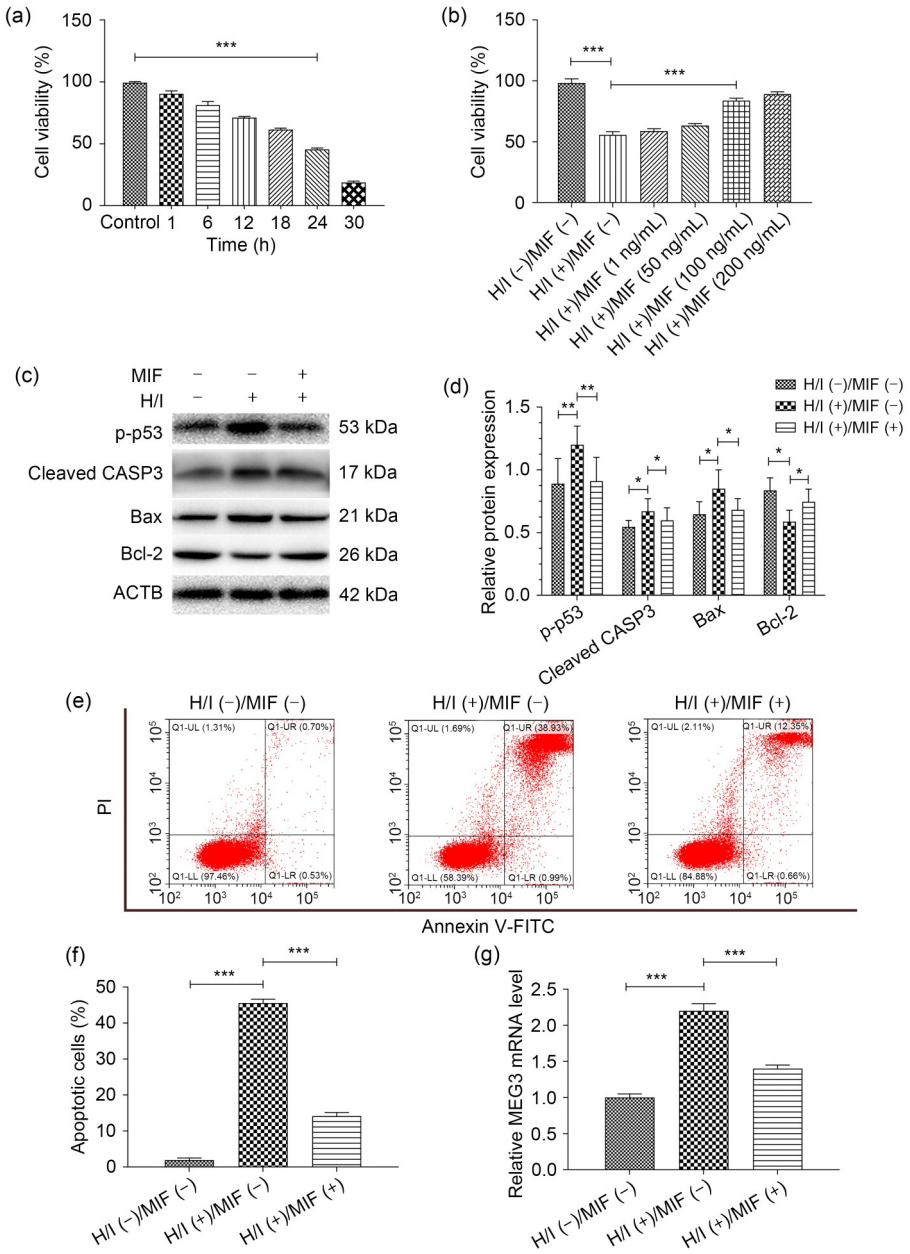

Fig. 1 MIF protected BMSCs against H/I-induced apoptosis. (a) CCK-8 assay was performed to investigate the viability of BMSCs incubated under H/I conditions for different time points (1, 6, 12, 18, 24, or 30 h). (b) CCK-8 assay was performed to investigate the viability of BMSCs pretreated with MIF (0, 1, 50, 100, or 200 ng/mL) for 24 h followed by exposure to H/I conditions for 24 h. (c) The expression of p-p53 and apoptosis-related proteins was detected by western blotting in BMSCs with or without exposure to H/I conditions and those pretreated with MIF before exposure to H/I conditions. (d) Quantitation of the data in panel (c) was shown. (e) The percentages of apoptotic BMSCs with or without exposure to H/I conditions and those pretreated with MIF before exposure to H/I conditions were detected by flow cytometry with Annexin V/PI double staining. (f) Quantification of the percentages of apoptotic BMSCs in panel (e) was shown. The percentage of Annexin-V-positive BMSCs was obtained from three separate experiments. (g) The expression of lncRNA MEG3 was detected by qRT-PCR in BMSCs with or without exposure to H/I conditions and those pretreated with MIF before exposure to H/I conditions. All experiments were carried out three times and the data are expressed as mean±SD. * P<0.05, ** P<0.01, *** P<0.001. MIF: macrophage migration inhibitory factor; BMSCs: bone marrow mesenchymal stem cells; H/I: hypoxia/ischemia; CCK-8: cell counting kit-8; p-p53: phosphorylated p53; PI: propidium iodide; LncRNA: long noncoding RNA; MEG3: maternally expressed gene 3; qRT-PCR: quantitative real-time polymerase chain reaction; SD: standard deviation; CASP3: caspase-3; Bcl-2: B-cell lymphoma-2; Bax: Bcl-2-associated X; ACTB: β-actin; FITC: fluorescein isothiocyanate; UL: upper left; UR: upper right; LL: lower left; LR: lower right.

upregulated the expression of Bcl-2, as shown by western blotting, and reduced the number of apoptotic BMSCs, as shown by flow cytometry. These effects were attenuated when lncRNA MEG3 was overexpressed (Figs. 2b−2e). In addition, overexpression of lncRNA MEG3 in BMSCs significantly upregulated p-p53 (Fig. 2b).

3.3 Effect of activation of the p53 signaling pathway on H/I-induced apoptosis of BMSCs

The CCK-8 results showed that, at a concentration of 20 μmol/L, TGIC significantly decreased the viability of BMSCs treated with MIF and exposed to H/I conditions (P<0.01; Fig. 3a). TGIC significantly

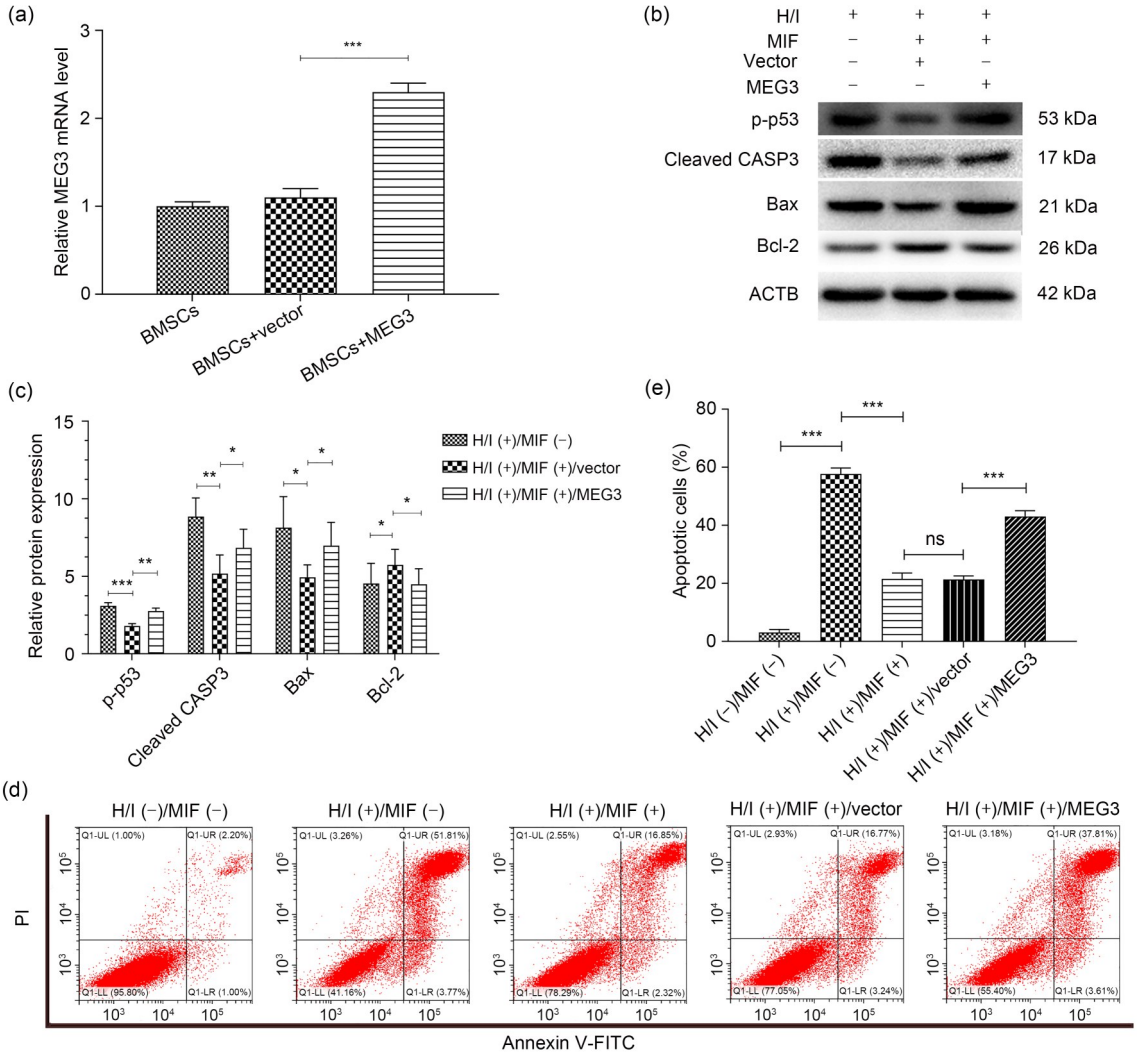

Fig. 2 Overexpression of lncRNA MEG3 attenuated the cytoprotective effect of MIF. (a) The expression of MEG3 in BMSCs with or without transfection of plasmids expressing lncRNA MEG3 or the non-targeting mRNA (vector) was measured by qRT-PCR. (b) The expression of p-p53 and apoptosis-related proteins in BMSCs pretreated with or without a combination of MIF and transfection of plasmids expressing lncRNA MEG3 or vector before exposure to H/I conditions was detected by western blotting. (c) Quantification of the data in panel (b) was shown. (d) Apoptotic BMSCs pretreated with or without a combination of MIF and transfection with plasmids expressing lncRNA-MEG3 or vector before exposed to H/I conditions were analyzed by flow cytometry with Annexin V/PI double staining. (e) Quantification of the proportion of apoptotic BMSCs measured in panel (d) was shown. The experiments were carried out three times and the data are expressed as mean±SD. * P<0.05, ** P<0.01, *** P<0.001. No significance is noted as "ns." LncRNA: long noncoding RNA; MEG3: maternally expressed gene 3; MIF: macrophage migration inhibitory factor; BMSCs: bone marrow mesenchymal stem cells; mRNA: messenger RNA; qRT-PCR: quantitative real-time polymerase chain reaction; H/I: hypoxia/ischemia; p-p53: phosphorylated p53; CASP3: caspase-3; Bcl-2: B-cell lymphoma-2; Bax: Bcl-2-associated X; ACTB: β-actin; PI: propidium iodide; FITC: fluorescein isothiocyanate; UL: upper left; UR: upper right; LL: lower left; LR: lower right; SD: standard deviation.

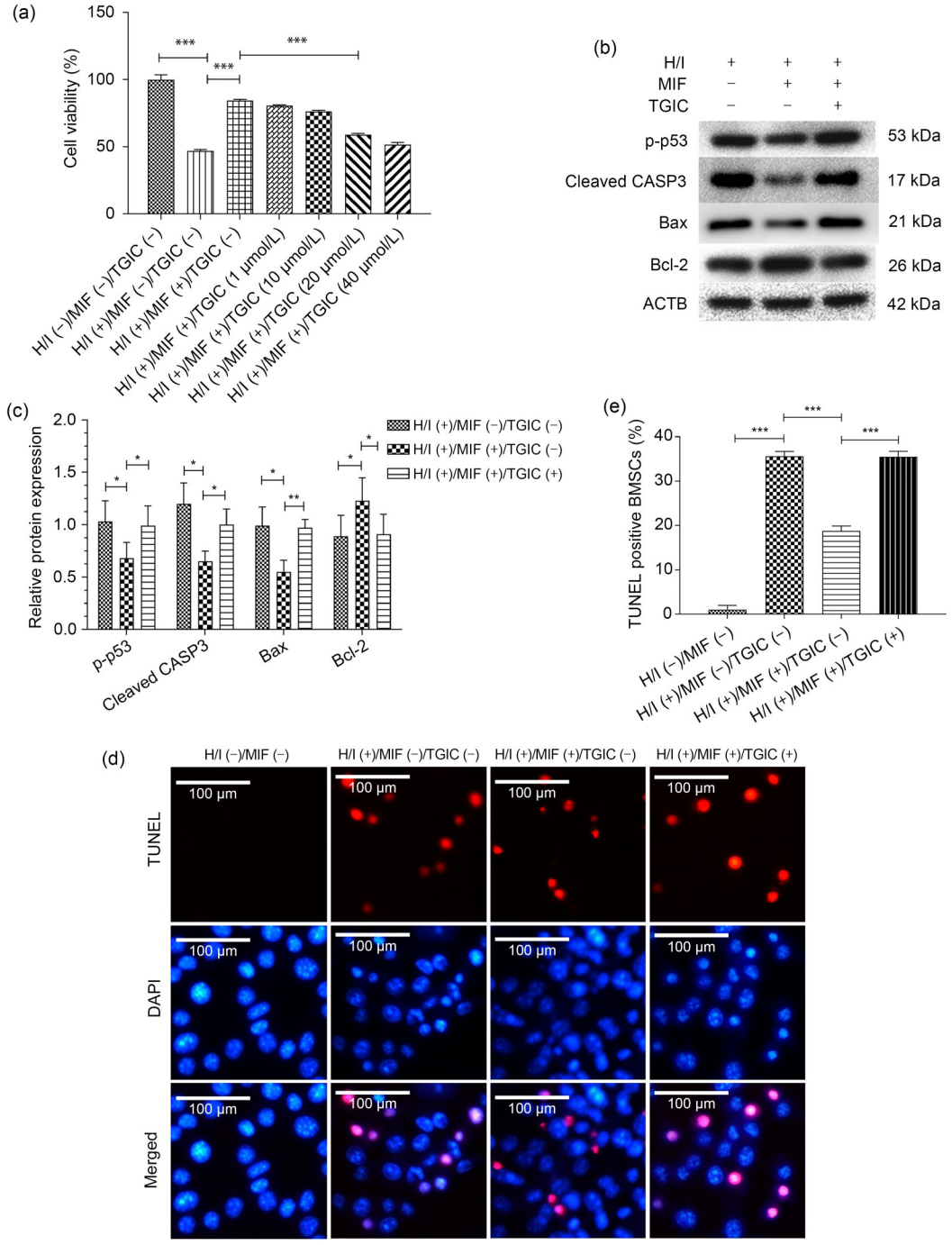

Fig. 3 Activation of the p53 signaling pathway inhibited the protective effect of MIF. (a) The influence of TGIC (0, 1, 10, 20, or 40 μmol/L) on the viability of BMSCs pretreated with MIF before exposure to H/I conditions was detected by the CCK-8 test. Each group was tested in triplicate. (b) The expression of p-p53 and apoptosis-related proteins in BMSCs with or without pretreatment with MIF or a combination of MIF and TGIC before exposure to H/I conditions was detected by western blotting. (c) Quantification of the data shown in panel (b). (d) The percentage of apoptotic BMSCs with or without pretreatment with MIF or a combination of MIF and TGIC before exposure to H/I conditions was detected by TUNEL fluorene staining and observed under a fluorescence microscope. (e) Quantification of the data in panel (d) was shown. The experiments were carried out three times and the data are expressed as mean±SD. * P<0.05, ** P<0.01, *** P<0.001. MIF: macrophage migration inhibitory factor; BMSCs: bone marrow mesenchymal stem cells; TGIC: triglycidyl isocyanurate; H/I: hypoxia/ischemia; CCK-8: cell counting kit-8; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; SD: standard deviation; p-p53: phosphorylated p53; CASP3: caspase-3; Bcl-2: B-cell lymphoma-2; Bax: Bcl-2-associated X; ACTB: β-actin; DAPI: 4',6-diamidino-2-phenylindole.

upregulated the expression of p-p53 and inhibited the protective effect of MIF on H/I-induced upregulation of Bax and cleaved CASP3 and downregulation of Bcl-2, as shown by western blot analysis (P<0.05; Figs. 3b and 3c). The results of TUNEL staining showed the proportion of apoptotic cells with red fluorescence in BMSCs pretreated with MIF before exposure to H/I conditions was significantly lower than that of those with or without pretreatment of a combination of TGIC and MIF before exposure to H/I conditions (P<0.001; Figs. 3d and 3e).

3.4 Effect of inhibition of the Wnt signaling pathway on H/I-induced apoptosis of BMSCs

Immunofluorescence examination and qRT-PCR analysis revealed that pretreatment with MIF significantly increased the expression of β-catenin in the nucleus and Wnt of BMSCs under H/I conditions. This was alleviated when lncRNA MEG3 was overexpressed (Figs. 4a–4c). After pretreatment with MIF before exposure to H/I conditions, transfection with β-catenin siRNA significantly decreased the expression

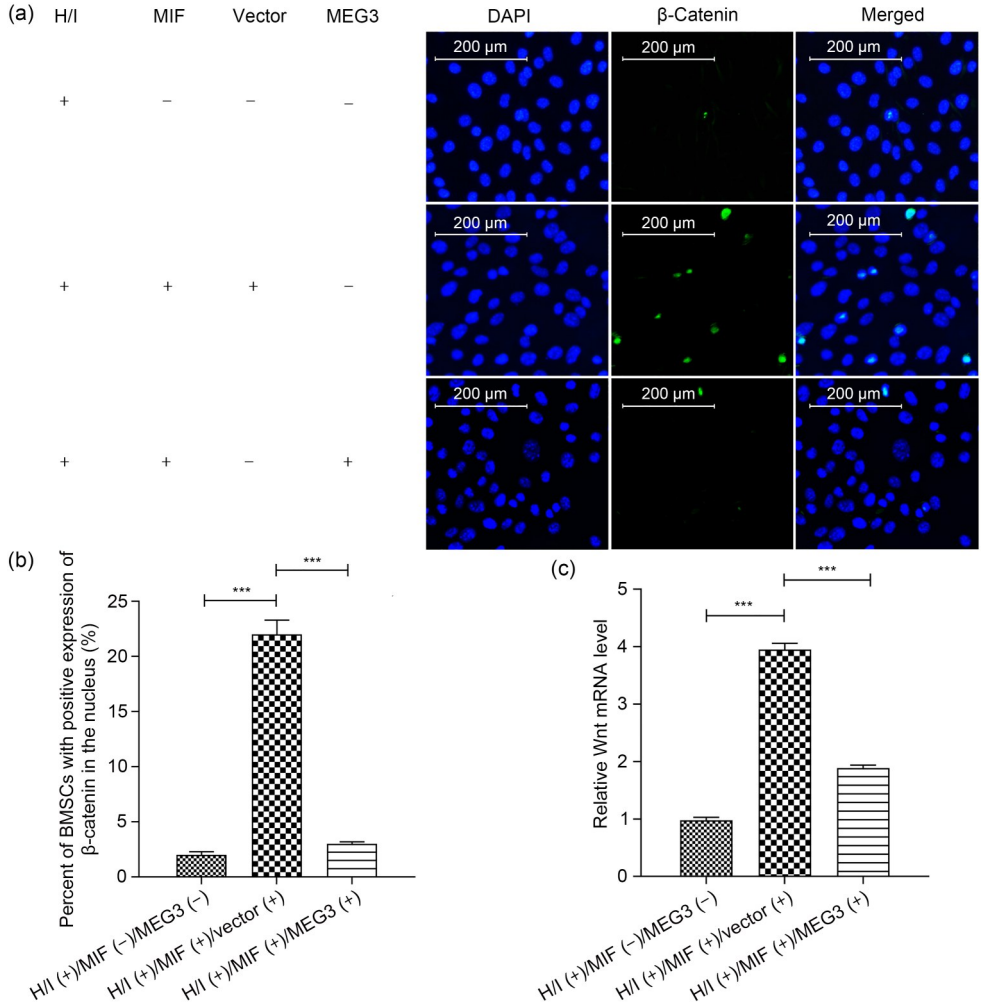

Fig. 4 MEG3 inhibited MIF-induced activation of the Wnt/β-catenin signaling pathway. (a) The influence of MIF with or without overexpression of lncRNA MEG3 on the expression of β-catenin in the nucleus of BMSCs exposed to H/I conditions was analyzed by immunofluorescence assays. The nucleus and β-catenin were stained with blue and green fluorescent dyes, respectively. (b) The percentage of BMSCs with positive expression of β-catenin in the nucleus from the data in panel (a) was compared. (c) The influence of MIF with or without overexpression of lncRNA MEG3 on the expression of Wnt in BMSCs exposed to H/I conditions was analyzed by qRT-PCR. The experiments were carried out three times and the data are expressed as mean±SD. *** P<0.001. MEG3: maternally expressed gene 3; MIF: macrophage migration inhibitory factor; LncRNA: long noncoding RNA; BMSCs: bone marrow mesenchymal stem cells; H/I: hypoxia/ischemia; qRT-PCR: quantitative real-time polymerase chain reaction; SD: standard deviation; DAPI: 4', 6-diamidino-2-phenylindole (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

of β-catenin in the nucleus of BMSCs (Figs. 5a and 5c). Besides, the expression of apoptosis-related proteins Bax and cleaved CASP3 was increased, and that of Bcl-2 was decreased (Figs. 5a and 5b). The proportion of apoptotic BMSCs pretreated with β-catenin siRNA before treatment with MIF and exposed to H/I

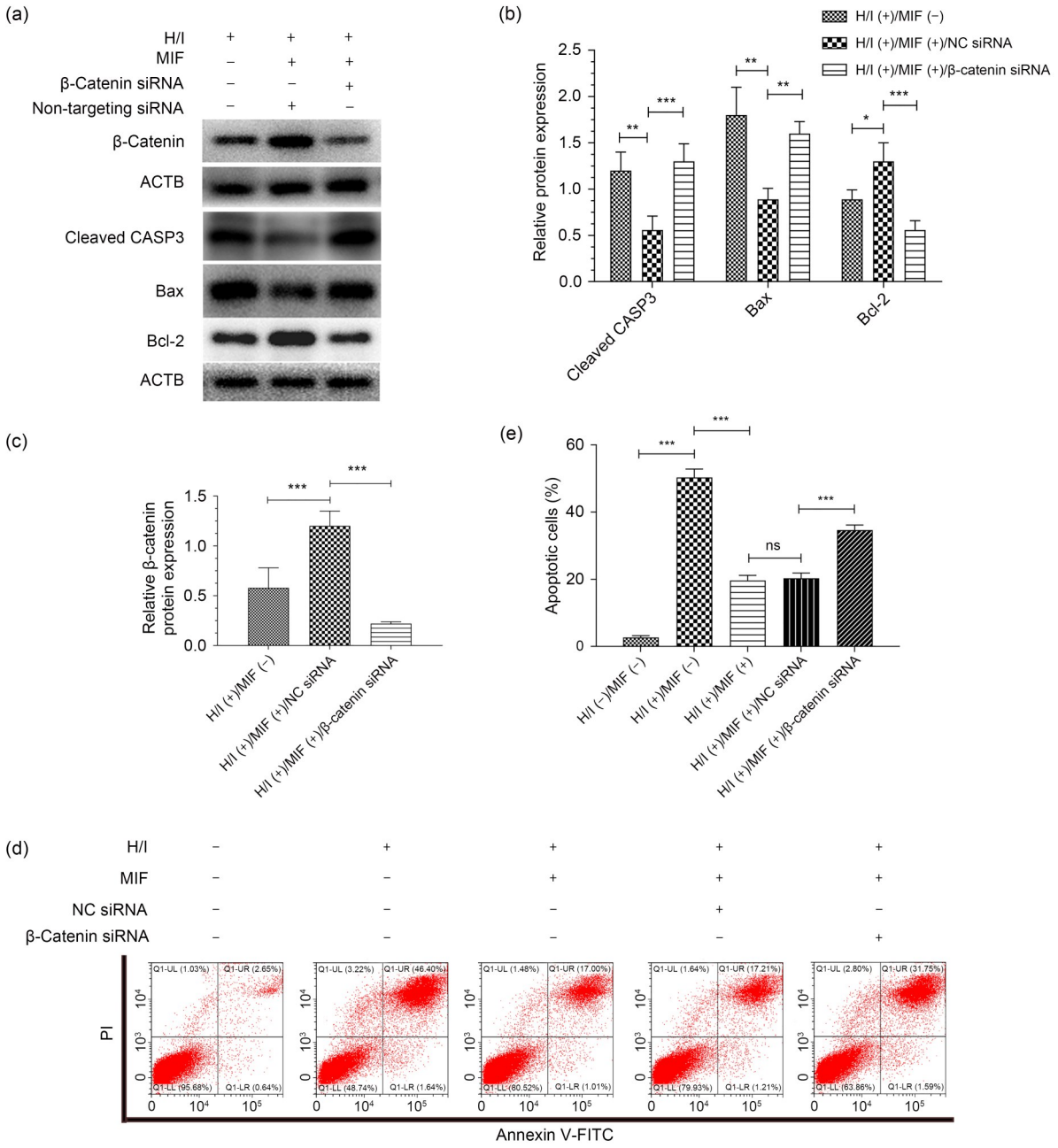

Fig. 5 Inhibition of the Wnt/β-catenin signaling pathway inhibited the effect of MIF. (a) The effect of MIF combined with transfection with β-catenin siRNA or NC siRNA on the expression of β-catenin in the nucleus and apoptosis-related proteins in BMSCs exposed to H/I conditions was detected by western blotting. (b, c) Quantification of the data in panel (a) was shown. (d) The effect of MIF combined with transfection with β-catenin siRNA or NC siRNA on H/I-induced apoptosis of BMSCs was detected by flow cytometry with Annexin V/PI double staining. (e) Quantification of the data in panel (d) was shown. The experiments were carried out three times and the data are expressed as mean±SD. * *P*< 0.05, ** *P*<0.01, * *P*<0.001. No significance is noted as "ns." MIF: macrophage migration inhibitory factor; siRNA: small interfering RNA; NC: non-targeting control; BMSCs: bone marrow mesenchymal stem cells; H/I: hypoxia/ischemia; PI: propidium iodide; SD: standard deviation; ACTB: β-actin; CASP3: caspase-3; Bcl-2: B-cell lymphoma-2; Bax: Bcl-2-associated X; FITC: fluorescein isothiocyanate; UL: upper left; UR: upper right; LL: lower left; LR: lower right.**

conditions were higher than those treated with MIF and exposed to H/I conditions (Figs. 5d and 5e).

3.5 Effect of MIF on H/I-induced oxidative stress of BMSCs

According to flow cytometry, the fluorescence intensity and proportion of BMSCs with high ROS levels increased as the apoptosis rate increased during exposure to H/I conditions. This effect was attenuated by pretreatment with MIF (Figs. 6a and 6b).

4 Discussion

Since the course of ONFH is long, it can be reversed before the late stage of degenerative hip arthritis if early treatment is applied to reduce damage to the blood supply. Inhibition of apoptosis is expected to delay its progression. With the development of tissue engineering technology, stem cells are the core component of regenerative medicine. However, the microenvironment of H/I in the early stage of ONFH is the main cause of apoptosis of transplanted BMSCs after tissue engineering treatment. Revealing the mechanism of stem cell apoptosis in an H/I environment may lead to enhanced apoptotic resistance of stem cells and improved efficacy of tissue engineering.

Previous studies have shown that in the nervous system, a microenvironment of H/I can lead to an increase in intracellular lncRNA MEG3 and apoptosis (Zhang Y et al., 2019). LncRNA MEG3 has been found in humans, orangutans, mice, rats, cows, and pigs (Uroda et al., 2019). It was initially reported as a tumor suppressor gene that can promote apoptosis of tumor cells by activating the p53 signaling pathway (Xu et al., 2018). However, the upregulation of lncRNA MEG3 is not always accompanied by activation of the p53 signaling pathway. Although lncRNA MEG3 has conserved domains in its secondary structure that can activate the p53 signaling pathway, there are three alternative splice forms of lncRNA MEG3, only some of which can activate the p53 signaling pathway (Uroda et al., 2019).

The role of lncRNA MEG3 in apoptosis of BMSCs induced by H/I has not been reported previously. When using stem cell transplantation to treat ONFH, ischemic and anoxic microenvironments are the main obstacles to stem cell proliferation and repair of osteonecrosis. We hypothesized that apoptosis of BMSCs caused by H/I might be related to lncRNA MEG3, and that regulating the expression of lncRNA MEG3 may promote the survival of transplanted BMSCs. In this study, we found that when BMSCs were incubated in an H/I microenvironment, the level of intracellular lncRNA MEG3 in BMSCs increased significantly, accompanied by upregulation of p-p53, cleaved CASP3, and Bax and an increase in the proportion of apoptotic BMSCs. Therefore, we concluded that the H/I condition induced apoptosis of the BMSCs via lncRNA-p53 signaling pathway.

Previous research showed that MIF, an inhibitor of macrophage migration, had an anti-apoptotic effect on BMSCs in H/I environments, but the potential mechanism was unknown (Xia WZ et al., 2015). In addition, it was pointed out that in an emphysema

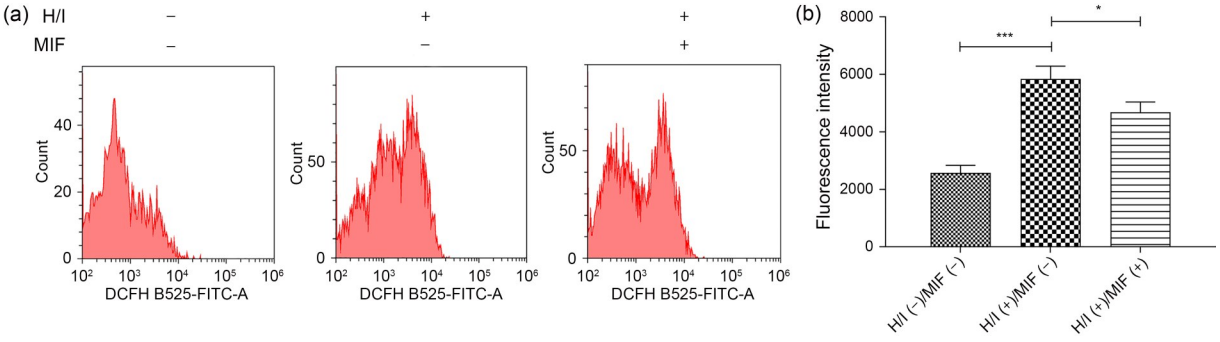

Fig. 6 MIF decreased the ROS level of BMSCs. (a) The ROS levels of BMSCs with or without exposure to H/I conditions and of those pretreated with MIF before exposure to H/I conditions were detected by flow cytometry with DCFH staining. (b) Quantification of the data in panel (a) was shown. The experiments were carried out three times and the data are expressed as the mean±SD. * P<0.05, *** P<0.001. MIF: macrophage migration inhibitory factor; ROS: reactive oxygen species; BMSCs: bone marrow mesenchymal stem cells; H/I: hypoxia/ischemia; DCFH: dichloro-dihydro-fluorescein diacetate; FITC: fluorescein isothiocyanate.

model, MIF could significantly inhibit the p53 signaling pathway and reduce the apoptosis of pulmonary artery epithelial cells (Xia and Hou, 2016). In brain tumors, MIF can also promote anti-apoptosis by regulating the p53 signaling pathway, but the specific mechanism has not been clarified (Fukaya et al., 2016). In recent years, increasing evidence has implied that there is a close relationship between MIF and lncRNA MEG3. For example, MIF can influence gene methylation, chromosome modification and recombination by regulating the expression of lncRNAs, thereby promoting the occurrence and development of melanoma (Sarkar et al., 2015). In our study, we showed that pretreatment with MIF downregulated lncRNA MEG3, inhibited the activation of the p53 signaling pathway, and improved the apoptosis of BMSCs induced by H/I condition. The effect of MIF was attenuated in BMSCs overexpressing lncRNA MEG3 or with activation of the p53 signaling pathway. In summary, MIF protected BMSCs from H/I-induced apoptosis through lncRNA MEG3/p53 signaling pathway.

The Wnt/β-catenin signaling pathway was previously reported to be involved in stem cell regeneration and tissue renewal (Böttcher et al., 2021). In elderly patients with severe periodontitis, MIF may affect the periodontal tissue structure through the Wnt/β-catenin signal pathway and participate in the occurrence and development of cognitive dysfunction (Du et al., 2020). Previous research revealed that lncRNA MEG3 might bind to and inhibit the expression of β-catenin, which promotes apoptosis of chemotherapy-resistant lung cancer cells and enhances their sensitivity to chemotherapy (Xia Y et al., 2015). It was also reported that lncRNA MEG3 can significantly block the Wnt/β-catenin signaling pathway and inhibit esophageal squamous cell carcinoma cell proliferation, migration, and invasion (Ma et al., 2019). Therefore, it was speculated that MIF may also activate the Wnt signaling pathway to protect BMSCs from apoptosis under H/I condition. In this study, we confirmed that MIF protected BMSCs from H/I-induced apoptosis by activating the Wnt/β-catenin signaling pathway.

LncRNA MEG3 is closely related to cellular oxidative stress and DNA damage. In intracerebral hemorrhage (ICH)-induced brain injury, downregulation of lncRNA MEG3 relieves ICH by inhibiting oxidative stress and inflammation in a microRNA-dependent manner (Xie et al., 2021). Previous studies confirmed that oxidative inductions had a negative impact on mitochondrial function (Wajner et al., 2020). Under stress conditions, the mesenchymal stem cell (MSC) mitochondrial membrane potential decreases significantly, leading to apoptosis, but when the mitochondrial membrane potential is increased, apoptosis is attenuated (Hou et al., 2014). In this study, we measured the intracellular ROS level of BMSCs and found that MIF inhibited H/I-induced apoptosis of BMSCs by decreasing their ROS level.

In summary, here we reported for the first time that MIF can inhibit H/I-induced apoptosis of BMSCs by inhibiting lncRNA MEG3/p53, activating Wnt/β-catenin, and decreasing intracellular ROS (Fig. 7). It

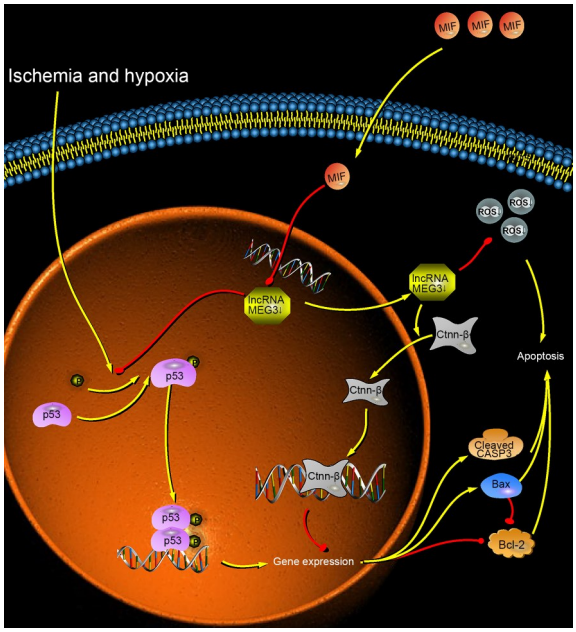

Fig. 7 MIF protected BMSCs from H/I-induced apoptosis by downregulating the lncRNA MEG3/p53 signaling pathway, activating the Wnt/β-catenin signaling pathway, and decreasing ROS level. Yellow arrows represent a promoting effect, red arrows represent an inhibiting effect, while a downward arrow represents downregulation. The H/I environment induced apoptosis of BMSCs by increasing the phosphorylation (P) of p53, which was alleviated by MIF-induced downregulation of lncRNA MEG3. Downregulation of lncRNA MEG3 also decreased the ROS level and activated the Wnt/β-catenin signaling pathway by increasing nuclear translocation. MIF: macrophage migration inhibitory factor; BMSCs: bone marrow mesenchymal stem cells; LncRNA: long noncoding RNA; MEG3: maternally expressed gene 3; ROS: reactive oxygen species; H/I: hypoxia/ischemia; Bcl-2: B-cell lymphoma-2; Bax: Bcl-2-associated X; CASP3: caspase-3; Ctnn-β: β-catenin (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

was revealed that lncRNA MEG3 may be a potential target to inhibit apoptosis in transplanted BMSCs and improve the efficacy of regenerative treatments in patients with ONFH.

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Author contributions

All authors contributed to the study conception and design. Zhibiao BAI and Kai HU performed the experimental research and data analysis, wrote and edited the manuscript. Jiahuan YU and Yizhe SHEN created the figures. Chun CHEN contributed to data analysis and discussion. All authors have read and approved the final manuscript, and therefore, have full access to all data relevant to the study and take responsibility for the integrity and security of such data.

Compliance with ethics guidelines

Zhibiao BAI, Kai HU, Jiahuan YU, Yizhe SHEN, and Chun CHEN declare that they have no conflict of interest.

This study was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China (No. wydw2020-0361).

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