Original Paper

Mesenchymal Stem Cells Modified with Heme Oxygenase-1 Have Enhanced Paracrine Function and Attenuate Lipopolysaccharide-Induced Inflammatory and Oxidative Damage in Pulmonary Microvascular Endothelial Cells

Xuxin Chen^a Yinliang Zhang^b Wenjing Wang^c Zhenqian Liu^a
Jiguang Meng^a Zhihai Han^a

^aDepartment of Respiratory Medicine, Navy General Hospital of the PLA, Beijing, ^bDepartment of Respiratory Medicine, Xi'an XD Group Hospital, Xi'an, ^cCardiothoracic Surgery ICU, Anqing Municipal Hospital, Anqing, China

Key Words

Mesenchymal Stem Cells (MSCs) • Acute lung injury (ALI) • Lipopolysaccharide (LPS) • Heme Oxygenase-1 (HO-1) • Paracrine • Inflammation • Oxidative Stress

Abstract

Background/Aims: Bone marrow-derived mesenchymal stem cell (BM-MSC) transplantation has therapeutic effects on endothelial damage during acute lung injury (ALI). Heme oxygenase-1 (HO-1) can restore homeostasis and implement cytoprotective defense functions in many pathologic states. Therefore, we explored whether transduction of *HO-1* into BM-MSCs (MSCs-HO-1) would have an increased beneficial effect on lipopolysaccharide (LPS)-induced inflammatory and oxidative damage in human pulmonary microvascular endothelial cells (PVECs). **Methods:** MSCs were isolated from rat bone marrow and transfected with the *HO-1* gene by a lentivirus vector. The phenotype and multilineage differentiation of MSCs were assessed. MSCs or MSCs-HO-1 were co-cultured with PVECs using a transwell system, and LPS was added to induce PVEC injury. The production of reactive oxygen species (ROS), and the activities of lipid peroxide (LPO), malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in PVECs were determined by flow cytometry and colorimetric assays, respectively. The levels of human PVEC-derived tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β and IL-6 in the supernatants of the co-culture system, and the activity of nuclear transcription factor- κ B and NF-E2-related factor 2 (Nrf2) in PVECs were examined by enzyme-linked immunosorbent assay (ELISA). The mRNA expression of TNF- α , IL-1 β and IL-6. X. Chen, Y. Zhang and W. Wang contributed equally to this work.

Prof. Jiguang Meng
and Prof. Zhihai Han

Department of Respiratory Medicine, Navy General Hospital of the PLA
No. 6 Fucheng Road, Beijing, 100037 (China)
Tel. +86 10 6695 7589, E-Mail mjg2016@tom.com; zhihaihanddoctor@163.com

in PVECs was detected by quantitative real-time polymerase chain reaction (qRT-PCR), HO-1 expression and enzymatic activity in PVECs and the influence of zinc protoporphyrin (ZnPP) or HO-1 small interfering RNA on the above inflammatory and oxidative stress markers were evaluated. In addition, the expression of rat MSC-derived hepatocyte growth factor (HGF) and IL-10 was determined by ELISA and qRT-PCR. **Results:** MSCs showed no significant changes in phenotype or multilineage differentiation after transduction. LPS strongly increased the production of inflammatory and oxidative stress indicators, as well as decreased the levels of antioxidant components and the activity of Nrf2 in PVECs. MSC co-cultivation ameliorated these detrimental effects in PVECs and MSCs-HO-1 further improved the damage to PVECs induced by LPS when compared with MSCs alone. The beneficial effects of MSCs-HO-1 were dependent on HO-1 overexpression and may be attributed to the enhanced paracrine production of HGF and IL-10. **Conclusion:** MSCs-HO-1 have an enhanced ability to improve LPS-induced inflammatory and oxidative damage in PVECs, and the mechanism may be partially associated with the enhanced paracrine function of the stem cells. These data encourage further testing of the beneficial effects of MSCs-HO-1 in ALI animal models.

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Introduction

Acute lung injury (ALI) and its advanced stage, acute respiratory distress syndrome (ARDS), are clinical syndromes characterized by acute respiratory dysfunction, decreased pulmonary compliance, and severe imbalance of ventilation and blood flow ratio resulting from pulmonary inflammation, noncardiogenic pulmonary edema, and disruption of the endothelium and epithelium [1, 2]. Although substantial progress has been made in the treatment of ALI/ARDS, the mortality rate among ALI/ARDS patients remains high [1, 2].

ALI/ARDS results in an excessive inflammatory response and oxidative stress [3, 4]. Moreover, oxidative stress and inflammation are closely linked and have an important role in the pathogenesis of ALI/ARDS. Oxidative stress causes recruitment of inflammatory cells leading to inflammation, while inflammatory cells cause induction of oxidative stress [5]. Diffuse injury of pulmonary endothelial cells is characteristic of ALI/ARDS and leads to an increase in alveolar-capillary permeability and alveolar pulmonary edema [6]. Lipopolysaccharides (LPS), important components of the outer wall of Gram-negative bacteria, are highly proinflammatory molecules that cause pulmonary endothelial injury through induction of inflammatory and oxidative damage [7]. The lung endothelium is the first barrier preventing cells and proteins in the blood from infiltrating into the interstitial space and alveoli of the lungs. Therefore, damage to pulmonary endothelial cell can contribute to the development of ALI/ARDS and several treatments targeting pulmonary endothelial cell dysfunction have shown validity and efficacy for the prognosis of ALI/ARDS [8-11].

Mesenchymal stem cells (MSCs) have multidirectional differentiation potential and can differentiate into osteoblasts, chondrocytes, and adipocytes [12, 13]. MSCs are capable of self-renewal and can be obtained from multiple sources, including bone marrow, adipose tissue, umbilical cord blood, skin, tendon, muscle, and dental pulp [6, 12-14]. Their relative ease of isolation and enormous expansion potential in culture make them attractive therapeutic candidates [15]. Bone marrow-derived mesenchymal stem cells (BM-MSCs) are one of several types of stem cells, and considerable evidence has demonstrated that BM-MSCs hold promise for the treatment of ALI/ARDS [16-19]. However, the underlying mechanism by which BM-MSCs attenuate ALI have not yet been fully clarified. This attenuation might be associated with paracrine functions of BM-MSCs, with moderation of the inflammatory reaction that accompanies ALI, with variable anti-bacterial effects, or by adoption of the phenotype of lung cells [20-23]. In addition, BM-MSCs can serve as cellular vehicles for gene delivery and can target protective genes to injured lung tissues, which enhances their therapeutic effects [20, 22].

Heme oxygenase-1 (HO-1) is an inducible stress protein that responds to multiple stressors, including hypoxia, hyperoxia, acidosis, shear stress, and reactive oxygen species

[24]. HO-1 activity restores homeostasis and implements cytoprotective defense functions in many pathologic states by the anti-oxidant activity of biliverdin and its metabolite, bilirubin, as well as the anti-inflammatory effects of carbon monoxide (CO) [25, 26]. Accumulated research has demonstrated that HO-1 upregulation protects against endotoxin shock and allograft rejection, and attenuates hyperoxia and post-liver transplantation ALI [24, 26-28]. Further, HO-1 deficiency is related to a chronic inflammatory state and increased leukocyte recruitment in *HO-1* gene-deficient mice [29, 30]. Therefore, sustained and local overexpression of HO-1 protein in lung tissue can attenuate injury to pulmonary endothelial cells in ALI/ARDS through exerting anti-inflammatory and anti-oxidant effects.

Taken together, these observations indicate that both BM-MSc engraftment into the lung and HO-1 overexpression can protect against ALI/ARDS. Moreover, BM-MSCs are excellent cellular vehicles for gene delivery. With this in mind, we hypothesized that treatment with sustained highly expressed HO-1 protein, delivered by BM-MSCs, could target injured endothelial cells to achieve an optimal therapeutic effect and endothelial repair. Therefore, the present study was performed to investigate the effects of genetically engineered BM-MSCs on pulmonary endothelial cells in *in vitro* experiments, by combining these two protective approaches, BM-MSc treatment and HO-1 overexpression. To elucidate the potential effects of *HO-1* gene-transduced BM-MSCs (MSCs-HO-1) on human pulmonary microvascular endothelial cells (PVECs), oxidative stress markers and antioxidant components, expression of human PVEC-derived pro-inflammatory cytokines, and the activity of nuclear transcription factor- κ B (NF- κ B) and NF-E2-related factor 2 (Nrf2) in PVECs were examined following LPS challenge. Additionally, potential mechanisms of MSCs-HO-1 protection against LPS-induced endothelial cell damage were explored.

Materials and Methods

Cell lines and cell culture

Human PVECs (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% FBS (Gibco) and 1% antibiotics (streptomycin and penicillin; Gibco) at 37°C in a 5% CO₂ incubator overnight.

Isolation and expansion of MSCs

Preparation of rat BM-MSCs was conducted following a previously published method, the bone marrow adherence selection method [16]. Adult Wistar rats (8 weeks old) were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences (Certificate no. SCXK-Army-2012-0004, Beijing, China). All rats used in this research were raised and used in accordance with the ARRIVE (Animal Research: Reporting of *In vivo* Experiments) Guidelines on the Use of Laboratory Animals [31] and the care and housing of rats were approved by the Animal Care and Use Committee of the Navy General Hospital (Approval no. SCXK-Army-2012-0012). Briefly, Wistar rats were sacrificed with a supraphysiological dose of pentobarbital sodium (100 mg/kg) and the long bones of the limbs were aseptically dissected. The bone marrow cells were acquired by flushing the femur and tibia cavities under sterile conditions and the cell suspension was precipitated by centrifugation at $1,000 \times g$ for 5 min (4°C). The isolated cells were then suspended in MSC medium, which consisted of DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% antibiotics (streptomycin and penicillin; Gibco), and 2 mmol/L L-glutamine (Gibco) in 75 cm² tissue culture flasks at 37°C in a humidified chamber with 5% CO₂. Three days later, non-adherent cells were removed and the adherent cells that had grown to 90% confluence were considered as passage 0 MSCs. For expansion, cells were diluted in at a ratio of 1:3 per passage and continuously cultured in MSC medium. Passages 3–6 were used for the subsequent experiments. An inverted microscope (Olympus Co., Ltd, Tokyo, Japan) was used to monitor the morphology of the cells.

Identification of MSCs

After 4 passages in culture, MSCs were identified by phenotypic markers using flow cytometry (BD Biosciences, San Jose, CA, USA). Phycoerythrin (PE)-conjugated antibodies (CD29, CD73, CD105, CD34, and CD11b) and fluorescein isothiocyanate (FITC)-conjugated antibodies (CD90, CD45 and CD14) (all from Abcam, Cambridge, UK) were used for detecting the MSC surface markers. Otherwise, MSCs were cultured with adipogenic, chondrogenic or osteogenic induction medium (Cyagen Biosciences, Guangzhou, China) until they reached 90% confluence. After 2 weeks, MSCs were stained with Oli Red O, Alcian Blue or Alizarin Red S. All specimens were analyzed by microscopy (Olympus).

Lentivirus preparation and transduction into MSCs

A lentivirus was generated that contained a single open reading frame encoding rat *HO-1* (NM_012580). First, cDNA coding for rat *HO-1* was synthesized by GenePharma Co. Ltd. (Shanghai, China) and cloned into pGLV3/H1/GFP/Puro containing green fluorescent protein (GFP) and puromycin (GenePharma Co. Ltd.) to generate the plasmid pGLV3/H1/GFP/Puro-*HO-1*. Recombinant lentiviruses (Lenti-*HO-1*) were generated by using 293 packaging cells co-transfected with the expression plasmid and the other 3 packaging plasmids, pLV/helper-SL3, pLV/helper-SL4, and pLV/helper-SL5 (GenePharma Co. Ltd.). A control vector containing only the *GFP* gene (Lenti-GFP) was constructed using the same method (GenePharma Co. Ltd.). Titers of recombinant lentiviruses were determined by the fluorescence labeling method and the titers of Lenti-*HO-1* and Lenti-eGFP were 2×10^9 transfection units (TU)/ml and 1×10^9 TU/ml, respectively. The MSCs were transfected with control lentivirus (Lenti-eGFP) or lentivirus expressing *HO-1* (Lenti-*HO-1*) at a multiplicity of infection of 20. After infection for 12 h, the medium was changed and the MSCs were further cultured in MSC medium for 96 h. The lentivirus transfection efficiency was evaluated using fluorescence microscopy or western blotting. Otherwise, puromycin (1.5 µg/ml) was used for approximately 7–14 days to purify the MSCs carrying both the *HO-1* and *GFP* genes (MSCs-*HO-1*). The puromycin-resistant cells were collected and passaged, and the *HO-1* expression of puromycin-resistant cells was detected using fluorescence microscopy and western blotting.

Determination of MSC and MSC-*HO-1* growth curves

MSCs and MSCs-*HO-1* at passage 4 were plated as monolayers in 24-well plates at a density of 1×10^4 cells/well at day 0. Cells were harvested by trypsinization and centrifugation, and total cell numbers were obtained by counting cells grown for 1 to 10 d with a Coulter Counter (Beckman Coulter, Fullerton, CA).

Western blot analysis of *HO-1*

Following appropriate treatment, cells were lysed in lysis buffer (30 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany)). The protein concentrations were measured by the bicinchoninic acid (BCA) assay method (Beyotime Institute of Biotechnology, Haimen, China). Equal amounts of protein (30 µg) were separated using SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membranes were then blocked and incubated overnight at 4°C with anti-rHO-1 (Abcam; 1:500) and anti-β-actin antibodies (Abcam; 1:500) in Tris buffered saline with Tween-20 (TBST). After extensive washing with TBST, the membranes were incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:1,000) in TBST (Beyotime) at room temperature for 120 min. The signals were detected by enhanced chemiluminescence following the manufacturer's instructions (Beyotime). Membranes were photographed using a Gel Documentation and Analysis System (GBOX-HR, Syngene, US) and band intensities were measured with Adobe Photoshop Version 7.0.1 software (Adobe Systems, US) and normalized to the expression of β-actin for quantitative analysis.

Co-culture of PVECs with MSCs and experimental design

The stem cells (MSCs or MSCs-*HO-1*) and PVECs were co-cultured using the transwell system (Corning Inc., NY, USA) (Fig. 4) as described previously [20]. Briefly, 5×10^4 PVECs per well were seeded in 24-well culture plates in 400 µl of DMEM/F12 supplemented with 10% FBS. Then, 1×10^4 MSCs were plated in the upper chamber of the transwell inserts with 200 µl of the culture medium and were co-cultured with the PVECs for 24 h at 37°C in a 5% CO₂ incubator. The co-cultures were then treated with 100 ng/ml of LPS

(*Escherichia coli* 0111: B4; Sigma-Aldrich) for 6 h. PVECs without stem cells were negative controls and PVECs cultured alone and treated with LPS were positive controls.

For other experiments, MSCs and MSCs-HO-1 were pretreated with 10 μ mol/l of zinc protoporphyrin (ZnPP) (Sigma, St. Louis, MO, USA) for 1 h or transfected with HO-1 small interfering RNA (siRNA) (Santa Cruz Biotechnology, Dallas, TX, USA) before co-culture experiments. Stem cells were transfected with a non-targeting siRNA (Santa Cruz) as a negative control. Briefly, PVECs were seeded in the lower compartment of the transwell system as described above; 3×10^5 MSCs or MSCs-HO-1 per well were seeded in 6-well culture plates and left untreated, were treated with 10 μ mol/l of ZnPP or were transfected with HO-1 or negative control siRNA when the cells reached 80% confluence. After treatment with ZnPP for 1 h or transfection with HO-1 or negative control siRNA for 48 h, the stem cells were routinely trypsinized and collected for detection of HO-1 expression and activity or used in successive experiments. The stem cells were plated in the upper chamber of the transwell inserts and were co-cultured with PVECs as described above. The co-cultures were treated with 100 ng/ml of LPS for 6 h.

After exposure to LPS, the supernatants were collected for use in enzyme-linked immunosorbent assays (ELISA) for human PVEC-derived TNF- α , IL-1 β and IL-6, as well as rat MSC-derived hepatocyte growth factor (HGF) and IL-10. The PVECs were harvested for assays of reactive oxygen species (ROS), lipid peroxide (LPO), malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx), as well as total RNA and nuclear protein extraction. In addition, stem cells in the co-culture system were also collected for detection of mRNA expression levels of HGF and IL-10. Each experiment was repeated three times, and the sample size in each experiment ranged from six to eight.

Measurement of PVEC and stem cell viability

PVECs or stem cells (MSCs and MSCs-HO-1) were seeded in 96-well plates at 2×10^3 cells per well and a cell viability assay was performed using a CCK8 kit (Beyotime) according to the manufacturers' protocol. After treatment with 100 ng/ml of LPS for 6 h or 10 μ mol/l of ZnPP for 1 h, CCK8 solutions (10 μ l) were added for 1 h at 37°C in a 5% CO₂ incubator. The optical density (OD) was then measured with a microplate reader at the wavelength of 450 nm (BioTek Instruments, Inc., Vermont, USA).

Measurement of oxidative stress markers (ROS, LPO, MDA, SOD, and GPx) in PVECs

After exposure to LPS, oxidative stress markers in PVECs were assessed. The intracellular accumulation of ROS was measured using a commercial kit (Beyotime) according to the manufacturer's instructions; 1×10^6 cells were collected and washed twice with PBS, then cells were loaded with medium containing 10 μ mol/l DCFH-DA for 20 min at 37°C. The cells were shaken slightly every 5 min to thoroughly mix cells with probes. After removing the medium and washing the cells with serum-free culture medium, the cells were collected and the fluorescence intensity of each sample was examined using flow cytometry (BD Biosciences). The fluorescence intensity was monitored with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

After appropriate treatment, the PVECs were washed twice and resuspended in PBS, sonicated for 30 s on ice, and centrifuged at $1,000 \times g$ for 15 min, according to the manufacturer's instructions. The LPO, MDA, SOD, and GPx activities in PVECs were determined by using commercial kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

ELISA for human PVEC-derived pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6)

After exposure to LPS, the supernatants of the co-culture system were collected, centrifuged at $1,000 \times g$ for 20 min, and assayed by ELISA for human PVEC-derived TNF- α , IL-1 β and IL-6 (Cusabio, Wuhan, China), strictly following the protocols provided by the manufacturer.

Quantitative real-time polymerase chain reaction (qRT-PCR) for TNF- α , IL-1 β and IL-6 mRNA in PVECs

After exposure to LPS, total RNA was extracted from the PVECs with TRIzol Reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Reverse transcription of RNA samples was performed using the Takara Primescript RT Reagent Kit (Takara, Dalian, China). Then, qRT-PCR was performed using the iTaq Universal SYBR Green Supermix (Takara), according to the manufacturer's instructions. Rat β -actin was selected as an internal control. The primers sequences were designed as follows: TNF- α , 5'-CTGCCTGCTGCACTTTGGA -3' (sense) and 5'-TTGAAGAGGACCTGGGAGTAGAT-3' (antisense);

IL-1 β , 5'-GCCGTGTCAGTTGTTGTAGC-3' (sense) and 5'-TGAAGGGAATCAAGGTGCTC -3' (antisense); IL-6, 5'-TACATCCTCGACGGCATCTC-3' (sense) and 5'-GCCATCTTTGGAAGGTTTCAG-3' (antisense); β -actin, 5'-GGAGATTACTGCCCTGGCTCCTA-3' (sense) and 5'-GACTCATCGTACTCCTGCTTGCTG-3' (antisense). All reactions were performed in triplicate and reports were generated by Rotor-Gene Real-Time Analysis Software 6.0 (Corbett Research, Australia). The relative expression of each target gene was calculated by the $2^{-\Delta\Delta Ct}$ method [32].

Detection of Nrf2 and NF- κ B activities in PVECs

After exposure to LPS, nuclear extracts of PVECs were prepared using a commercially available extraction kit (Active Motif, Carlsbad, Calif), according to the manufacturer's instructions. The protein concentrations of the nuclear extracts were quantified by the BCA method (Beyotime). The DNA binding activities of Nrf2 and NF- κ B p65 in the isolated nuclear extracts were assessed by performing ELISAs using the TransAM™ NF- κ B Transcription Factor Assay kit and TransAM™ Nrf2 Transcription Factor Assay kit according to the manufacturer's protocol (Active Motif) [1, 33]. Each sample was assayed in triplicate. The binding of the activated transcription factor to the consensus sequence was quantified by colorimetric assay.

Detection of HO-1 protein expression and activity in PVECs

After exposure to LPS, total proteins were prepared from PVECs as described above, and HO-1 expression was detected by western blot analysis, also as described above. Anti-human-HO-1 (1: 1, 000), anti-human- β -actin primary antibodies (1: 1, 000), and corresponding horseradish peroxidase-labeled secondary antibody (1: 1, 000) were purchased from Beyotime Institute of Biotechnology. In addition, HO-1 activity in PVECs was determined as described previously [34, 35]. PVECs were collected and homogenized in 4°C homogenization buffer (1 M Tris-Cl, 250 mM sucrose), then centrifuged at $12,000 \times g$ for 30 min at 4°C. The cell supernatants were collected and added to a reaction mixture (heme (18 μ M), biliverdin reductase (30 U/ml) and NADPH (2.75 mM)). The reaction was performed in the dark for 1 h at 37°C, and the amount of bilirubin extracted with chloroform was calculated by the difference in absorbance. HO-1 activity was expressed as pmol bilirubin min⁻¹ mg protein⁻¹. Protein content level was also determined by the BCA assay method (Beyotime).

Detection of HO-1 protein expression and activity in stem cells

After pretreatment with ZnPP or transfection with HO-1 or negative control siRNA, stem cells (MSCs-HO-1, MSCs, MSCs-HO-1+ZnPP, MSCs+ZnPP, MSCs+siRNA or MSCs-HO-1+siRNA) were collected and HO-1 protein expression and activity were detected as described above.

ELISA for rat MSC-derived paracrine factors (HGF and IL-10)

Before treatment with LPS and at 6 h following the addition of LPS, supernatants of the co-culture system were collected, centrifuged at $1,000 \times g$ for 20 min and used in ELISAs for rat MSC-derived HGF and IL-10 (R&D Systems, Inc., Minneapolis, MN, USA), strictly following the protocols provided by the manufacturer.

QRT-PCR for HGF and IL-10 mRNA in MSCs

Before treatment with LPS and at 6 h following the addition of LPS, total RNA from the stem cells (MSCs-HO-1, MSCs, MSCs-HO-1+ZnPP, MSCs+ZnPP, MSCs-HO-1+siRNA, MSCs+siRNA, MSCs-HO-1+Ctrl siRNA or MSCs+Ctrl siRNA) was prepared as described above, reverse transcribed into cDNA and qRT-PCR was performed as described above, using β -actin as an internal standard. The primers sequences were as follows: HGF, 5'-GCACCTCCTCTGCTTCC-3' (sense) and 5'-CCAAACCCTTTTTCCTACTCCA-3' (antisense); IL-10, 5'-GCTCAGCACTGCTATGTTGC-3' (sense) and 5'-TGTTGTCCAGCTGGTCCTTC-3' (antisense); and β -actin, 5'-GGAGATTACTGCCCTGGCTCCTA-3' (sense) and 5'-GACTCATCGTACTCCTGCTTGCTG-3' (antisense). The relative expression of each target gene was calculated by the $2^{-\Delta\Delta Ct}$ method [32].

Statistical analysis

All experimental results were analyzed using SPSS 13.0 statistical software. All data are presented as means $\pm$ SEM. Comparison of multiple means was performed by a one-way analysis of variance (ANOVA), and the Student-Newman-Keuls (SNK) test was used to compare multiple groups. Comparisons between two groups were performed using a two-tailed Student's t-test. Statistical significance was determined at $p < 0.05$.

Results

MSC primary culture and transduction efficiency with lentivirus vector

The isolated MSCs expanded extensively in the culture medium from single cell-derived colonies under optimal conditions. Examples of MSC morphology from passage 0 and 4 are shown in Fig. 1A and B. After obtaining enriched MSCs (passage 3–5), we transduced the cells with Lenti-HO-1 (carrying the HO-1 gene and GFP gene) or Lenti-GFP (only carrying the GFP gene). An Example of MSC morphology 96 h after Lenti-HO-1 transduction under phase contrast and fluorescence microscopy is shown in Fig. 1C and D. The transduction efficiency was assessed by western blot, which showed high HO-1 protein expression in the Lenti-HO-1 group ($p < 0.05$) compared with the Lenti-GFP group (Fig. 1E). However, there was no significant difference between PBS and Lenti-GFP groups (Fig. 1E). An example of the morphology from Lenti-HO-1 transduced MSCs (MSCs-HO-1) at passage 30 under phase contrast and fluorescence microscopy is shown in Fig. 1F and G. The lentiviral vector-mediated HO-1 gene transduction of MSCs was well maintained. Western blots showed high levels of HO-1 protein expression in MSCs-HO-1 ($p < 0.05$) compared with MSCs at passage 30 (Fig. 1H). The cell growth curves of MSCs and MSCs-HO-1 were determined by cell counting (Fig. 1I) and indicated that the growth rate of MSCs-HO-1 was similar to that of normal MSCs.

Phenotypes of MSCs-HO-1 and MSCs

MSCs have many markers [36, 37]. To identify the phenotypes of MSCs and MSCs-HO-1, the cell markers CD29, CD90, CD73, CD105, CD34, CD45, CD14, and CD11b were identified by

Fig. 1. Transduction of HO-1 into MSCs using a lentivirus vector. (A, B) Examples of normal MSC morphology at passage 0 (A) and passage 4 (B) under phase contrast ($\times 100$). (C, D) Examples of MSC morphology 96 h after Lenti-HO-1 transduction (multiplicity of infection=20) under phase contrast (C) and fluorescence microscopy (D). (E) The expression of HO-1 protein in MSCs was measured by western blotting 96 h after Lenti-HO-1 or Lenti-GFP transduction. Data are expressed as means $\pm$ SEM of three independent experiments; * $p < 0.05$ vs. Lenti-GFP; # $p > 0.05$ vs. PBS. Examples of transduced MSC-HO-1 morphology at passage 30 under phase contrast (F) and fluorescence microscopy (G) ($\times 100$). (H) The expression of HO-1 protein in MSCs and MSCs-HO-1 was measured by western blotting. Data are expressed as means $\pm$ SEM of three independent experiments; * $p < 0.05$ vs. MSCs. (I) Cell growth curves of MSCs and MSCs-HO-1 at passage 4.

flow cytometry. MSCs expressed the phenotypic markers CD29, CD90, CD73, and CD105, but not CD34, CD45, CD14 or CD11b (Fig. 2). The phenotype of MSCs-HO-1 showed no difference from that of MSCs (Fig. 2), suggesting that the gene transfer procedure did not alter the MSC phenotype.

Multilineage differentiation of MSCs and MSCs-HO-1

To evaluate their multilineage differentiation capacity, MSCs and MSCs-HO-1 were incubated in osteogenic, adipogenic or chondrogenic media, then osteogenesis, adipogenesis and chondrogenesis were detected by Alizarin Red S, Oil Red O, and Alcian Blue, respectively. As expected, both MSCs (Fig. 3A-C) and MSCs-HO-1 (Fig. 3D-F) could form Alizarin-Red-positive mineral deposits, Oil Red O-positive lipid droplet deposition and Alcian-Blue-positive chondrocytes. Therefore, all results suggested that the multilineage differentiation capacity of MSCs was unaffected by the lentivirus transduction process.

LPS had no influence on the cell viability of MSCs, MSCs-HO-1 or PVECs

The stem cells (MSCs or MSCs-HO-1) and PVECs were co-cultured in an indirect co-culture model system (Fig. 4A). The results of the CCK8 assay demonstrated that 100 ng/ml of LPS did not suppress the cell viability of MSCs or MSCs-HO-1 compared with MSCs and MSCs-HO-1 in normal medium (Fig. 4B) ($p > 0.05$). Further, as shown in Fig. 4C, LPS (100 ng/ml) did not influence the cell viability of PVECs ($p > 0.05$ vs. untreated PVECs).

MSCs-HO-1 attenuated LPS-induced oxidative stress in PVECs

The level of ROS was measured to test whether oxidative stress plays a role in LPS-

Fig. 2. Flow cytometry identification of MSC phenotype. Cells were immunophenotyped with antibodies for CD29, CD73, CD90, CD105, CD14, CD11b, CD34, or CD45 using FACS flow cytometry; (A-F) represent the phenotype of MSCs and (G-L) represent the phenotype of MSCs-HO-1. Representative plots from 3 samples are shown. No significant differences between MSCs and MSCs-HO-1 were found.

induced injury. Exposure of PVECs to LPS (LPS-exposed PVECs) caused a remarkable increase in intracellular ROS compared with PVECs alone (PVEC) ($p < 0.05$). Co-culture with MSCs inhibited the production of ROS in PVECs after LPS stimulation ($p < 0.05$ vs. LPS-exposed PVECs). However, co-culture with MSCs-HO-1 further decreased the production of ROS in PVECs after LPS stimulation ($p < 0.05$ vs. LPS-exposed PVECs + MSCs) (Fig. 5A and B). LPO and MDA are indicators of lipid peroxidation [15]. LPS exposure increased the levels of LPO and MDA in PVECs compared with PVECs alone ($p < 0.05$). Co-culture with MSCs inhibited the production of LPO and MDA in PVECs after LPS stimulation ($p < 0.05$ vs. LPS-exposed PVECs). In the MSCs-HO-1 treated group, the LPS-induced increase of LPO and MDA was further suppressed compared with the MSC-treated group ($p < 0.05$) (Fig. 5C and D). SOD and GPx are important anti-oxidant enzymes involved in the protection against LPS-induced ALI [15, 38]. The activities of SOD and GPx in PVECs were suppressed by LPS compared with PVECs alone ($p < 0.05$). Co-culture with MSCs partially recovered the activities of SOD and GPx in PVECs ($p < 0.05$ vs. LPS-exposed PVECs). However, co-culture with MSCs-HO-1 further improved the activities of SOD and GPx in PVECs after LPS stimulation ($p < 0.05$ vs. LPS-exposed PVECs + MSCs) (Fig. 5E and F).

Fig. 3. Multilineage differentiation of MSCs and MSCs-HO-1. Multilineage differentiation capacity assays of rat mesenchymal stem cells were performed to confirm MSC and MSC-HO-1 phenotypes, including osteogenic differentiation stained with Alizarin Red S (A, D), adipogenic differentiation stained with Oli Red O (B, E), and chondrogenic differentiation stained with Alcian Blue (C, F) ($\times 400$). No differences in multilineage differentiation capacities between MSCs and MSCs-HO-1 were found.

Fig. 4. Stem cell and PVEC co-culture system and cell viability analysis. (A) An indirect co-culture model system was used to investigate the therapeutic effects of MSCs and MSCs-HO-1 on inflammatory and oxidative damage in PVECs induced by LPS. (B) Effect of LPS on the cell viability of MSCs and MSCs-HO-1. MSCs and MSCs-HO-1 were treated with 100 ng/ml of LPS for 6 h. (C) Effect of LPS on the cell viability of PVECs. PVECs were treated with 100 ng/ml of LPS for 6 h. Data are expressed as means $\pm$ SEM of three independent experiments. No significant differences in the cell viability of each group were found.

Fig. 5. MSCs-HO-1 attenuated LPS-induced oxidative stress in PVECs. (A, B) MSCs-HO-1 decreased the production of ROS in PVECs induced by LPS. Intracellular ROS were measured by flow cytometry using an oxidation-sensitive fluorescent probe, DCFH-DA, which is oxidized to DCF in the presence of ROS. (A) Representative examples of flow cytometry analysis for the DCF intensity in each group. (B) The DCF intensity analyses of all groups. Data are expressed as means $\pm$ SEM of three independent experiments; * $p < 0.05$ vs. LPS-exposed PVECs, # $p < 0.05$ vs. LPS-exposed PVECs + MSCs. (C, D, E, F) The intracellular LPO (C), MDA (D), SOD (E), and GPx (F) activities in PVECs. Data are expressed as means $\pm$ SEM of three independent experiments; * $p < 0.05$ vs. LPS-exposed PVECs, # $p < 0.05$ vs. LPS-exposed PVECs + MSCs.

MSCs-HO-1 decreased LPS-induced inflammation in PVECs

The influence of MSCs-HO-1 on the expression of TNF- α , IL-1 β and IL-6 was measured by ELISA and qRT-PCR in LPS-stimulated PVECs. The ELISA results showed that the levels of TNF- α , IL-1 β and IL-6 were very low in the cell culture supernatant of control (LPS-unstimulated) cells. However, in response to LPS, there was a significant increase in their levels ($p < 0.05$ vs. unstimulated PVECs). Co-culture with MSCs decreased the levels of inflammatory cytokines (TNF- α , IL-1 β and IL-6) in the cell culture supernatants after LPS treatment ($p < 0.05$ vs. LPS-exposed PVECs). However, in the MSC-HO-1-treated group, the regulatory effect of stem cells on inflammatory cytokines was further enhanced compared with the MSC-treated group ($p < 0.05$) (Fig. 6A-C). Further, qRT-PCR showed that the mRNA levels of TNF- α , IL-1 β and IL-6 in PVECs reflected the protein levels of TNF- α , IL-1 β and IL-6 in the supernatants of the co-culture system (Fig. 6D-F).

MSCs-HO-1 modulated the activities of Nrf2 and NF- κ B in PVECs

Nrf2 has been reported to play a central role in antioxidant and anti-inflammatory responses [5, 34]. Therefore, we examined the activity of Nrf2 by using ELISA. As shown in Fig. 7A, LPS exposure increased the Nrf2 DNA binding activity in PVECs. Both MSCs and MSCs-HO-1 further improved the LPS-induced Nrf2 activity in nuclear extracts of PVECs. However, compared with the MSC group, co-culture with MSCs-HO-1 further significantly increased Nrf2 activation induced by LPS ($p < 0.05$) (Fig. 7A). NF- κ B is considered a critical transcription factor required for the maximal expression of many cytokines [1]. We also detected the activity of NF- κ B in each group. Both MSCs and MSCs-HO-1 suppressed the LPS-induced increase in the amount of translocated, active NF- κ B in nuclear extracts of PVECs. However, compared with the MSC group, co-culture with MSCs-HO-1 further significantly attenuated the NF- κ B activation induced by LPS ($p < 0.05$) (Fig. 7B).

MSCs-HO-1 promoted HO-1 expression and enzymatic activity in PVECs

Nrf2 is a key regulator of HO-1 expression [39]. In light of the results regarding Nrf2 activity in PVECs, we investigated the influence of co-cultureing PVECs with MSCs or MSCs-HO-1 on HO-1 expression and enzymatic activity. Co-culture with MSCs or MSCs-HO-1

Fig. 6. MSCs-HO-1 modulated LPS-induced inflammation in PVECs. (A, B, C) MSCs-HO-1 decreased LPS-induced production of the human PVEC-derived pro-inflammatory factors TNF- α (A), IL-1 β (B), and IL-6 (C) in the supernatants of the co-culture system. (D, E, F) MSCs-HO-1 decreased the LPS-induced mRNA expression of TNF- α (D), IL-1 β (E), and IL-6 (F) in PVECs. Data are expressed as means $\pm$ SEM of three independent experiments; * p <0.05 vs. LPS-exposed PVECs, # p <0.05 vs. LPS-exposed PVECs + MSCs.

Fig. 7. MSCs-HO-1 modulated the activities of Nrf2 and NF- κ B in PVECs. MSCs-HO-1 enhanced LPS-induced Nrf2 activation in PVECs. (A) ELISA for Nrf2 activation after LPS stimulation. Data are expressed as means $\pm$ SEM of three independent experiments; \$ p <0.05 vs. PVECs, # p <0.05 vs. LPS-exposed PVECs + MSCs. MSCs-HO-1 inhibited LPS-induced NF- κ B activation in PVECs. (B) ELISA for NF- κ B activation after LPS stimulation. Data are expressed as means $\pm$ SEM of three independent experiments; * p <0.05 vs. LPS-exposed PVECs, # p <0.05 vs. LPS-exposed PVECs + MSCs.

promoted LPS-induced HO-1 expression in PVECs, as demonstrated by western blot (p < 0.05, vs. LPS-exposed PVECs) (Fig. 8A and B). Moreover, co-culture with MSCs-HO-1 significantly upregulated LPS-induced HO-1 protein expression in PVECs compared with the MSC group (p < 0.05) (Fig. 8A and B). HO-1 activity displayed a similar tendency to HO-1 expression (Fig. 8C).

HO-1 expression and enzymatic activity in stem cells

As shown in Fig. 9A, MSCs-HO-1 expressed HO-1 at high levels compared with MSCs ($p < 0.05$). However, the HO-1 inhibitor, ZnPP, and HO-1 siRNA markedly decreased the protein expression of HO-1 ($p < 0.05$ vs. MSCs-HO-1 and MSCs) (Fig. 9A). In the MSC-HO-1 group, HO-1 activity was relatively high ($p < 0.05$ vs. MSCs), but the HO-1 inhibitor ZnPP and HO-1 siRNA significantly suppressed the HO-1 activity ($p < 0.05$ vs. MSCs-HO-1 and MSCs) (Fig. 9B), while negative control siRNA had no influence on the expression and activity of HO-1 in MSCs or MSCs-HO-1 ($p > 0.05$ vs. MSCs-HO-1 or MSCs) (Fig. 9A and B). In addition, 10 $\mu\text{mol/l}$ of ZnPP did not decrease the viability of MSCs and MSCs-HO-1, compared with untreated cells in normal medium ($p > 0.05$) (Fig. 9C).

Effect of the HO-1 inhibitor ZnPP and HO-1 siRNA on the antioxidant action of MSCs-HO-1

Co-culture with MSCs-HO-1 inhibited the production of ROS, LPO and MDA in PVECs after LPS stimulation. However, the antioxidant efficacy of MSCs-HO-1 was diminished by ZnPP and HO-1 siRNA. As presented in Fig. 10A-D, in MSCs-HO-1 pretreated with ZnPP or HO-1 siRNA, the levels of ROS, LPO and MDA from PVECs were significantly higher than those of the MSC-HO-1 group after LPS stimulation ($p < 0.05$). Co-culture with MSCs-HO-1 enhanced the activities of SOD and GPx in PVECs after LPS stimulation. However, the enhancement of SOD and GPx activities induced by MSCs-HO-1 was attenuated by ZnPP or HO-1 siRNA pretreatment ($p < 0.05$) (Fig. 10E and F). Pretreatment with ZnPP or HO-1 siRNA had a similar effect on the antioxidant action of MSCs, significantly impairing their

Fig. 8. MSCs-HO-1 promoted HO-1 protein expression and enzymatic activity in PVECs. (A, B) MSCs-HO-1 upregulated HO-1 protein expression in LPS-stimulated PVECs. (A) Representative examples of HO-1 bands. (B) Densitometric analyses of HO-1 bands. (C) MSCs-HO-1 enhanced HO-1 enzymatic activity in LPS-stimulated PVECs. Data are expressed as means $\pm$ SEM of three independent experiments; \$ $p < 0.05$ vs. PVECs, # $p < 0.05$ vs. LPS-exposed PVECs + MSCs.

Fig. 9. HO-1 inhibitor ZnPP and HO-1 siRNA inhibited HO-1 expression and enzymatic activity in MSCs-HO-1. (A) ZnPP and HO-1 siRNA pretreatment suppressed the protein expression of HO-1. (B) ZnPP and HO-1 siRNA pretreatment inhibited the enzymatic activity of HO-1. Ctrl siRNA, a non-targeting siRNA (negative control). Data are expressed as means $\pm$ SEM of three independent experiments; * $p < 0.05$ vs. MSCs-HO-1, # $p < 0.05$ vs. MSCs. (C) Effect of ZnPP on the cell viability of MSCs and MSCs-HO-1. MSCs and MSCs-HO-1 were treated with 10 $\mu\text{mol/l}$ of ZnPP for 1 h. Data are expressed as means $\pm$ SEM of three independent experiments. No significant differences in the cell viability of each group were found.

Fig. 10. Inhibition of HO-1 diminished the antioxidant effect of MSCs-HO-1 on PVECs. (A, B) ZnPP and HO-1 siRNA pretreatment abolished the inhibitory effect of MSCs-HO-1 on ROS production induced by LPS in PVECs. (A) Representative examples of flow cytometry analysis for the DCF intensity in each group. (B) The DCF intensity analyses of each group. (C, D) ZnPP and HO-1 siRNA pretreatment abolished the inhibitory effect of MSCs-HO-1 on LPO (C), and MDA (D) activities induced by LPS in PVECs. (E, F) ZnPP and HO-1 siRNA pretreatment abolished the positive effect of MSCs-HO-1 on SOD (E), and GPX (F) activities induced by LPS in PVECs. Ctrl siRNA, a non-targeting siRNA (negative control). Data are expressed as means $\pm$ SEM of three independent experiments; * $p < 0.05$ vs. MSCs-HO-1 + LPS, # $p < 0.05$ vs. MSCs + LPS.

antioxidant activity on PVECs through inhibiting the expression and activity of HO-1 ($p < 0.05$ vs. MSCs) (Fig. 10). In contrast, transfection with negative control siRNA had no effect on the antioxidant efficacy of MSCs-HO-1 or MSCs ($p > 0.05$) (Fig. 10).

Effect of the HO-1 inhibitor ZnPP and HO-1 siRNA on the anti-inflammatory action of MSCs-HO-1

Co-culture with MSCs-HO-1 decreased the LPS-induced release of TNF- α , IL-1 β and IL-6 from PVECs. However, the efficacy of the anti-inflammatory effects of MSCs-HO-1 was diminished by ZnPP or HO-1 siRNA. Levels of TNF- α , IL-1 β and IL-6 from PVECs with ZnPP- or HO-1 siRNA-pretreated MSCs-HO-1 were significantly higher than those in the MSC-HO-1 group after LPS stimulation ($p < 0.05$) (Fig. 11A-C). Furthermore, ZnPP and HO-1 siRNA also diminished the inhibitory effects of MSCs-HO-1 on the mRNA expression of TNF- α , IL-1 β and IL-6 in PVECs ($p < 0.05$) (Fig. 11D-F) and the regulatory effects of MSCs on PVEC-derived inflammatory cytokines were also counteracted by ZnPP or HO-1 siRNA ($p < 0.05$ vs. MSCs) (Fig. 11 D-F). Negative control siRNA did not interfere with the modulatory effect of MSCs-HO-1 and MSCs on the mRNA expression and secretion of TNF- α , IL-1 β and IL-6 ($p > 0.05$ vs. MSCs-HO-1 or MSCs) (Fig. 11).

Effect of the HO-1 inhibitor ZnPP and HO-1 siRNA on the regulatory functions of MSCs-HO-1 for Nrf2 and NF- κ B

As shown in Fig. 12, ZnPP or HO-1 siRNA impaired the regulatory effect of MSCs-HO-1 on the activities of Nrf2 and NF- κ B in PVECs following LPS stimulation. Compared with the MSC-HO-1 group, the Nrf2 DNA-binding activity of PVECs with ZnPP- or HO-1 siRNA-pretreated MSCs-HO-1 was significantly downregulated after LPS stimulation ($p < 0.05$) (Fig. 12A). Conversely, the NF- κ B DNA-binding activity was opposite to the change of Nrf2 DNA-binding activity in PVECs following ZnPP or HO-1 siRNA pretreatment ($p < 0.05$) (Fig. 12B).

ZnPP and HO-1 siRNA also impaired the regulatory functions of MSCs on the activities of Nrf2 and NF- κ B in PVECs ($p < 0.05$ vs. MSCs) (Fig. 12). However, transfection with negative control siRNA did not influence the regulation of Nrf2 and NF- κ B by MSCs-HO-1 and MSCs ($p > 0.05$ vs. MSCs-HO-1 or MSCs) (Fig. 12).

Effect of the HO-1 inhibitor ZnPP and HO-1 siRNA on the regulation of HO-1 expression and activity by MSCs-HO-1

Co-culture with MSCs-HO-1 promoted HO-1 protein expression and enzymatic activity in PVECs under LPS-stimulation. However, the promotive effect of MSCs-HO-1 could be weakened by ZnPP or HO-1 siRNA. The protein expression and enzymatic activity of HO-1 in PVECs were significantly lower following co-culture with ZnPP- or HO-1 siRNA-pretreated MSCs-HO-1 than in co-culture with MSCs-HO-1 after LPS stimulation ($p < 0.05$) (Fig. 13). Furthermore, the regulatory effects of MSCs on HO-1 expression and activity were also counteracted by ZnPP or HO-1 siRNA ($p < 0.05$ vs. MSCs) (Fig. 13). In contrast, negative control siRNA had no effect on the modulatory effect of MSCs-HO-1 and MSCs on HO-1 expression and activity ($p > 0.05$ vs. MSCs-HO-1 or MSCs) (Fig. 13).

HO-1 overexpression promoted MSCs to secrete HGF and IL-10

HGF and IL-10 are important paracrine factors derived from MSCs following exposure to inflammatory stimuli [40]. Therefore, we compared the production of HGF and IL-10 in different MSC types (MSCs-HO-1, MSCs, MSCs-HO-1+ZnPP, MSCs+ZnPP, MSCs-HO-1+siRNA, MSCs+siRNA, MSCs-HO-1+Ctrl siRNA and MSCs+Ctrl siRNA) using ELISA and qRT-PCR. ELISA showed that the levels of rat MSC-derived HGF and IL-10 in the supernatants of the co-culture system were similar among these different MSC types before LPS stimulation

Fig. 11. Inhibition of HO-1 counteracted the anti-inflammatory effect of MSCs-HO-1 on PVECs. (A, B, C) ZnPP and HO-1 siRNA pretreatment abolished the inhibitory effect of MSCs-HO-1 on LPS-induced human PVEC-derived TNF- α (A), IL-1 β (B) and IL-6 (C) release in the supernatants of the co-culture system. (D-F) ZnPP and HO-1 siRNA pretreatment abolished the inhibitory effect of MSCs-HO-1 on the LPS-induced mRNA expression of TNF- α (D), IL-1 β (E) and IL-6 (F) in PVECs. Ctrl siRNA, a non-targeting siRNA (negative control). Data are expressed as means $\pm$ SEM of three independent experiments; * $p < 0.05$ vs. MSCs-HO-1 + LPS, # $p < 0.05$ vs. MSCs + LPS.

($p > 0.05$) (Fig. 14A and B). Moreover, the results of qRT-PCR indicated that there was no significant difference in mRNA expression of HGF and IL-10 among these different MSC types before LPS stimulation ($p > 0.05$) (Fig. 14C and D). However, following LPS stimulation, the concentration of rat HGF and IL-10 in the supernatant of the MSC-HO-1/PVEC co-culture system was significantly higher than that of that of the MSC/PVEC co-culture system ($p < 0.05$) (Fig. 14E and F) and the HO-1 inhibitor (ZnPP) and HO-1 siRNA decreased HGF and IL-10 production from MSCs-HO-1 and MSCs ($p < 0.05$) (Fig. 14E and F). In addition, LPS upregulated the mRNA expression of HGF and IL-10 in MSCs, which was more prominent in MSCs-HO-1 ($p < 0.05$) (Fig. 14G and H). ZnPP and HO-1 siRNA also inhibited the transcription of HGF and IL10 in MSCs-HO-1 and MSCs ($p < 0.05$) (Fig. 14G and H), while negative control siRNA did not influence the paracrine secretion of HGF or IL-10 by MSCs-HO-1 or MSCs ($p > 0.05$ vs. MSCs-HO-1 or MSCs) (Fig. 14).

Discussion

The major findings of the present study can be summarized as follows. (1) MSCs transduced with the *HO-1* gene by the lentiviral vector had a high transduction efficiency and steadily overexpressed HO-1 protein in the long term. Moreover, the phenotype and multilineage differentiation capacity of the MSCs were unaffected by the lentiviral vector transduction processes. (2) Compared with MSCs, MSCs-HO-1 were more effective against inflammatory and oxidative damage induced by LPS in PVECs and this was counteracted by the HO-1 inhibitor ZnPP and HO-1 siRNA. (3) HO-1 overexpression enhanced the release of HGF and IL-10 from MSCs, which might be the mechanism of MSC-HO-1 protection against LPS-induced endothelial damage. To our knowledge, this is the first study to demonstrate that *HO-1* gene-

Fig. 12. Inhibition of HO-1 counteracted the regulatory effect of MSCs-HO-1 on the activities of Nrf2 (A) and NF-κB (B) induced by LPS in PVECs. Ctrl siRNA, a non-targeting siRNA (negative control). Data are expressed as means $\pm$ SEM of three independent experiments; * $p < 0.05$ vs. MSCs-HO-1 + LPS, # $p < 0.05$ vs. MSCs + LPS.

Fig. 13. Inhibition of HO-1 counteracted the regulatory effect of MSCs-HO-1 on HO-1 expression (A and B) and activity (C) in LPS-stimulated PVECs. (A) Representative examples of HO-1 bands. (B) Densitometric analyses of HO-1 bands. (C) Assay of HO-1 enzymatic activity in PVECs after coculture with stem cells following LPS-stimulation. Ctrl siRNA, a non-targeting siRNA (negative control). Data are expressed as means $\pm$ SEM of three independent experiments; * $p < 0.05$ vs. MSCs-HO-1 + LPS, # $p < 0.05$ vs. MSCs + LPS.

Fig. 14. HO-1 overexpression promoted MSCs to secrete HGF and IL-10 in response to LPS stimulation. The secretion levels of HGF (A) and IL-10 (B) in the supernatants of the co-culture system without LPS stimulation were measured using appropriate ELISA kits. The mRNA levels of HGF (C) and IL-10 (D) in MSCs, MSCs-HO-1, MSCs-HO-1+ZnPP, MSCs+ZnPP, MSCs-HO-1+HO-1 siRNA, MSCs+HO-1 siRNA, MSCs-HO-1+Ctrl siRNA and MSCs+Ctrl siRNA without LPS stimulation were determined by qRT-PCR. The secretion levels of HGF (E) and IL-10 (F) in the supernatants of the co-culture system following LPS stimulation were measured using appropriate ELISA kits. The mRNA levels of HGF (G) and IL-10 (H) in MSCs, MSCs-HO-1, MSCs-HO-1+ZnPP, MSCs+ZnPP, MSCs-HO-1+HO-1 siRNA, MSCs+HO-1 siRNA, MSCs-HO-1+Ctrl siRNA and MSCs+Ctrl siRNA following LPS stimulation were determined by qRT-PCR. Ctrl siRNA, a non-targeting siRNA (negative control). Data are expressed as means $\pm$ SEM of three independent experiments; * p <0.05 vs. MSCs-HO-1, # p <0.05 vs. MSCs.

transduced BMSCs (MSCs-HO-1) have increased anti-inflammatory and antioxidant effects on LPS-induced endothelial damage in PVECs, and this may be a result of enhancing the paracrine activity of MSCs.

HO is an enzyme that catalyzes the degradation of heme to generate biliverdin, iron and carbon monoxide (CO) [41]. The HO enzyme system consists of three isoforms, HO-1, HO-2 and HO-3 [41]. Unlike the other HO isoforms, which are constitutively expressed, HO-1 is inducible and can be upregulated by oxidants and inflammatory cytokines [39]. Endogenous HO-1, which is ubiquitously expressed in various cell types, plays an important role in host defense against excessive inflammation and oxidation-related diseases, such as neurodegenerative disease, pulmonary disease, systemic autoimmune disease, and cancer [34, 42]. Overexpression of HO-1 inhibits the production of cytokines and ROS, thereby reducing neutrophil infiltration [34]. Therefore, upregulation of HO-1 represents one of the most critical cytoprotective mechanisms and has been confirmed to offer cytoprotection in various models of lung injury and disease, including ALI, ischemia-reperfusion-induced lung injury, lung cancer and asthma [28]. However, the current protocols for inducing HO-1 overexpression in animal research are not suitable for patients with inflammation and oxidation-related disorders. The severe side effects of protoporphyrins and hemin limit their clinical application [34]. Therefore, it is warranted to find a novel, effective and safe approach to upregulate HO-1 expression for clinical use [43].

BM-MSCs have low immunogenicity, can differentiate into different types of cells, secrete paracrine soluble factors and release microvesicles [6, 44]. Importantly, BM-MSCs may be used as potential cell vectors or factories of desirable paracrine factors or other protective gene products for gene therapy [45]. MSCs are capable of homing to and acting as a vehicle for delivery of protective genes to the injury site, which may not only enhance their therapeutic effects but also promote local lung repair [20]. Delivery of a protective gene by MSCs can overcome the limitations of transient gene expression, host immunoinflammatory responses, and nonspecific cell targeting by classic viral or nonviral vectors [46]. Therefore, we chose

BM-MSCs as the vehicle to upregulate HO-1 expression in an LPS-induced inflammatory microenvironment. We found that MSC-based HO-1 gene therapy had increased effectiveness for decreasing LPS-induced inflammatory and oxidative damage in PVECs compared with MSC treatment alone. HO-1-modified MSCs had more significant inhibitory effects on LPS-induced oxidative stress in PVECs when compared with MSCs alone. Moreover, for the LPS-induced inflammatory response in PVECs, the modulatory effect of MSCs-HO-1 was stronger than that of MSCs.

Nrf2 is a key transcription factor that mediates the cellular antioxidant defenses and maintains redox homeostasis [34]. Moreover, NF- κ B is a critical transcription factor required for the maximal expression of many proinflammatory cytokines [47]. The present results demonstrated that MSCs can increase the LPS-induced activation of Nrf2 in PVECs. However, MSCs-HO-1 elicited further improvement of Nrf2 activation compared with MSCs alone. Correspondingly, MSCs-HO-1 also displayed a more significant suppressive effect on the LPS-induced activation of NF- κ B compared with MSCs alone. These changes in activities of Nrf2 and NF- κ B suggest that both MSCs-HO-1 and MSCs might regulate LPS-induced inflammatory responses and oxidative stress via inhibiting the activation of NF- κ B and promoting the activation of Nrf2 in PVECs. However, MSCs-HO-1 were more effective at regulating inflammation and oxidative stress. In addition, Nrf2 has been shown to regulate HO-1 expression [39, 48]. Once Nrf2 is activated, it translocates into the nucleus and binds to the antioxidant response element of the HO-1 promoter [39, 49]. Our results showed that MSCs and MSCs-HO-1 could promote LPS-driven HO-1 expression and activity in PVECs. Moreover, MSCs-HO-1 further upregulated LPS-induced HO-1 protein expression and activity in PVECs, compared with MSCs. These results suggested that enhanced endothelial HO-1 expression and activity also contributed to the beneficial effects of co-culture with MSCs-HO-1. Multiple kinase signaling pathways, such as phosphatidylinositol 3-kinase/p38 and extracellular-signal-regulated kinase/c-Jun N-terminal kinase pathways, have been reported to be involved in HO-1 inducible expression [35, 39]. However, further investigations are still needed to reveal which kinase signaling pathways are involved the promotive effect of MSCs-HO-1 on endothelial HO-1 expression and activity following LPS-simulation.

The main focus of this study was the HO-1 modification of MSCs. MSCs-HO-1 showed significant superiority in decreasing the release of proinflammatory cytokines and pro-oxidant factors, inhibiting the activation of NF- κ B, as well as promoting the production of anti-oxidant factors, the activation of Nrf2 and the expression and enzymatic activity of HO-1 compared with MSCs alone, which might further attenuate inflammatory and oxidative damage in PVECs. The detailed molecular mechanism of these effects is worth exploration and discussion. As we found that the cell growth curve of MSCs was similar to that of MSCs-HO-1 it suggests that the additional protective effect of MSCs-HO-1 was not associated with cell viability. Accumulating evidence using different preclinical animal models, including endotoxin-induced, live bacteria-induced, sepsis-associated, and pancreatitis-associated lung injury, has shown that cell-based therapy with MSCs is a potentially attractive option for treating ALI/ARDS [18]. The mechanisms of MSC protection against ALI/ARDS include multilineage differentiation potential (e.g., cell replacement and tissue regeneration), paracrine (e.g., secretion of antimicrobial and repair factors), and cell-cell contact-dependent (e.g., transfer of nucleic acids and organelles) mechanisms [17]. Currently, it is believed that paracrine activity plays a predominant role in the therapeutic effects of MSCs [46]. MSCs have the ability to release multiple soluble factors, such as growth factors, anti-inflammatory cytokines, and antimicrobial peptides, which can improve endothelial and epithelial permeability, modulate innate and adaptive immunity, and protect against oxidative damage-induced apoptosis [50]. In our present study, we also found that the stem cells had a protective effect on endothelial cells, in line with previous reports [12, 17, 18, 20, 51]. Because the indirect co-culture system was adopted during our experiment, we think that the immunomodulatory effect of MSCs on inflammation and oxidative stress can be ascribed to a paracrine mechanism. Our results also revealed that overexpression of HO-1 enhanced the protective effects of MSCs on PVECs. We presume that this process might be associated with

the enhancement of paracrine activity following overexpression of HO-1. Among paracrine factors, HGF has been considered to be closely related to endothelial barrier function, and IL-10 can act as an important anti-inflammatory cytokine to protect endothelial cells against injury during ALI [6, 40, 52]. Our results showed that overexpression of HO-1 enhanced the production of rat MSC-derived HGF and IL-10 following LPS stimulation, which simulates ALI in an *in vitro* model. This finding suggested that enhanced expression of HO-1 in MSCs might protect PVECs from LPS-induced injury through increasing the secretion of HGF and IL-10 in an inflammatory environment. The increased paracrine secretion of HGF and IL-10 by MSCs-HO-1 might be associated with the MSCs-HO-1-mediated amelioration of inflammation and oxidation in PVECs. IL-10 is the best-studied of these paracrine soluble factors, and accumulating evidence has confirmed that MSC-derived IL-10 plays important roles in the anti-inflammatory and immunomodulatory effects of MSCs [53, 54]. Burchfield et al. [53, 54] reported that IL-10-deficient MSCs had an inferior cardio-protective effect after myocardial infarction. However, IL-10-transduced MSCs could further inhibit the graft-versus-host response, attenuate collagen-induced arthritis and attenuate ischemia reperfusion-induced ALI [54, 55]. Moreover, antibodies to IL-10 or its receptor eliminated the beneficial effect of MSCs on sepsis-related mortality in mice [56]. These findings confirmed that the protective effect of MSCs on tissue and organ damage is at least partly dependent on IL-10 secretion [53, 55]. Based on these previous studies, we speculated that the additional effect of MSCs-HO-1 might be impaired by neutralizing or silencing IL-10 activity or expression. We also showed that HGF secretion, as well as IL-10, was increased in HO-1 modified MSCs under inflammatory conditions. Yang et al. [52] reported that MSC-secreted HGF and vascular endothelial growth factor had a synergistic effect on stabilizing endothelial barrier function upon LPS stimulation. These results suggested that the paracrine mechanism of MSCs involved various soluble factors and complex cytokine networks. However, the main focus of the present study was to confirm that MSCs-HO-1 had a superior protective effect on LPS-induced endothelial injury, and that this advantage was dependent on HO-1 overexpression, and further studies are therefore needed to identify the main paracrine factor responsible for the protective effect of MSCs-HO-1 in LPS-injured PVECs. Moreover, the paracrine network of MSCs is complex and involves factors other than IL-10, and further more extensive studies of this topic are planned in the future. Notably, there was no difference in the levels of HGF and IL-10 in co-culture supernatants and their mRNA expression between MSCs and MSCs-HO-1 without LPS-stimulation. This finding suggested that the paracrine function of MSCs was not activated under the normal resting state, in line with previous reports [52, 57]. Certainly, numerous paracrine factors are secreted by MSCs and whether HO-1 overexpression can increase the secretion of other paracrine factors besides HGF and IL-10 remains to be investigated in future studies. A protein array analysis or microarray analysis could be used to illuminate this issue in the future.

In addition, the present study also showed that pretreatment with the HO-1 inhibitor, ZnPP, reversed the positive effect of HO-1 overexpression on the paracrine function of MSCs. ZnPP has been reported to reduce HO-1 expression and suppress HO-1 activity [34, 35, 58, 59], and has been widely used for HO-1-related research [35, 58, 59]. Our present study showed that the expression and activity of HO-1 was downregulated by ZnPP pretreatment of MSCs-HO-1. In the ZnPP pretreatment group, levels of inflammatory cytokines and oxidative stress markers were significantly higher than those of the non-ZnPP-treated group, suggesting that the anti-inflammatory and anti-oxidant actions of MSCs-HO-1 were abolished by ZnPP. Our results also showed that the beneficial effect of MSCs-HO-1 on transcription factors (Nrf2 and NF- κ B), and HO-1 expression and activity in PVECs were impaired by ZnPP. We therefore presumed that the regulatory effect of MSCs-HO-1 on inflammation and oxidative stress in PVECs following LPS-stimulation was dependent on HO-1 overexpression. To further substantiate this viewpoint, we used HO-1 siRNA to downregulate the expression of HO-1 in MSCs and MSCs-HO-1 and demonstrated that the protective effects of MSCs and MSCs-HO-1 on PVECs were also abrogated. This finding further confirmed that the additional regulatory effect of MSCs-HO-1 on endothelial injury induced by LPS is dependent on HO-1

overexpression. Furthermore, we detected the influence of ZnPP and HO-1 siRNA on the levels of rat MSC-derived HGF and IL-10 in the supernatants of the co-culture system and the mRNA expression of HGF and IL-10 in MSCs; pretreatment with ZnPP or HO-1 siRNA decreased the release and mRNA expression of HGF and IL-10 induced by LPS in MSCs-HO-1, counteracting the synergistic effect of HO-1 overexpression on the paracrine functions of MSCs.

One limitation of our study should be noted. Our study suggested that MSCs-HO-1 had a greater protective role in LPS-induced endothelial cell damage and that this effect of MSCs-HO-1 is associated with the enhancement of paracrine functions of MSCs. However, the mechanism by which HO-1 overexpression enhanced the paracrine action of MSCs was not clearly defined. HO-1, as an anti-inflammatory and immunosuppressive molecule, has been reported to contribute to the immunomodulatory properties of adult rat and human MSCs [60]. HO-1 may mediate this effect through inhibition of toll-like receptor (TLR) activation and/or through cytoprotective effects [61, 62]. Whether the detailed mechanisms by which HO-1 enhanced the paracrine activity of MSCs are also associated with cytoprotective effects and inhibition of TLR activation remains to be investigated. Another limitation is that the present study used *in vitro* experiments. Whether MSCs-HO-1 have a similarly significant advantage in protection against LPS-induced endothelial cell injury in *in vivo* experiments needs further research. Further, in view of the low immunogenicity, lack of species differences and ready availability of rat-derived MSCs [63], rat-derived MSCs instead of human-derived MSCs were used in our study. Based on previous literature, we presume that human-derived MSCs-HO-1 should have a similarly protective effect on lung inflammation and injury. However, this question remains to be verified by separate future research.

In conclusion, the present study has shown that LPS activates oxidative stress and inflammatory responses in the endothelium. Bone marrow-derived MSCs modified with the HO-1 gene using a recombinant lentiviral vector stably overexpressed HO-1 protein over an extended period of time, maintained the phenotype and multilineage differentiation capacity of MSCs, and provided additional attenuation of LPS-induced inflammatory and oxidative damage in PVECs compared with MSCs alone. This effect was dependent on overexpression of the HO-1 protein and it occurred mainly via the enhanced release of HGF and IL-10 from MSCs-HO-1. These findings encourage the evaluation of the therapeutic value of HO-1 gene-modified MSCs in ALI animal models. MSC-based HO-1 gene therapy may provide a new therapeutic strategy against LPS-induced ALI in the future.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

References

- 1 Chen X, Tang L, Feng J, Wang Y, Han Z, Meng J: Downregulation of Paralemm-3 Ameliorates Lipopolysaccharide-Induced Acute Lung Injury in Rats by Regulating Inflammatory Response and Inhibiting Formation of TLR4/MyD88 and TLR4/TRIF Complexes. *Inflammation* 2017;40:1983-1999.

- 2 Chen X, Zhao Y, Wu X, Qian G: Enhanced expression of single immunoglobulin IL-1 receptor-related molecule ameliorates LPS-induced acute lung injury in mice. *Shock* 2011;35:198-204.
- 3 Zhu T, Wang DX, Zhang W, Liao XQ, Guan X, Bo H, Sun JY, Huang NW, He J, Zhang YK, Tong J, Li CY: Andrographolide protects against LPS-induced acute lung injury by inactivation of NF- κ B. *PLoS One* 2013;8:e56407.
- 4 Song X, Chen G, Luo X, You G, Yin Y, Wang Y, Zhao L, Zhou H: Early resuscitation with exendin-4 alleviates acute lung injury after hemorrhagic shock in rats. *J Surg Res* 2017;216:73-79.
- 5 Wu G, Dai X, Li X, Jiang H: ANTIOXIDANT AND ANTI-INFLAMMATORY EFFECTS OF RHAMNAZIN ON LIPOPOLYSACCHARIDE-INDUCED ACUTE LUNG INJURY AND INFLAMMATION IN RATS. *Afr J Tradit Complement Altern Med* 2017;14:201-212.
- 6 Hu S, Li J, Xu X, Liu A, He H, Xu J, Chen Q, Liu S, Liu L, Qiu H, Yang Y: The hepatocyte growth factor-expressing character is required for mesenchymal stem cells to protect the lung injured by lipopolysaccharide *in vivo*. *Stem Cell Res Ther* 2016;7:66.
- 7 Qian L, Zhao Y, Guo L, Li S, Wu X: Activating transcription factor 3 (ATF3) protects against lipopolysaccharide-induced acute lung injury via inhibiting the expression of TL1A. *J Cell Physiol* 2017;232:3727-3734.
- 8 Joshi AD, Barabutis N, Birmpas C, Dimitropoulou C, Thangjam G, Cherian-Shaw M, Dennison J, Catravas JD: Histone deacetylase inhibitors prevent pulmonary endothelial hyperpermeability and acute lung injury by regulating heat shock protein 90 function. *Am J Physiol Lung Cell Mol Physiol* 2015;309:L1410-1419.
- 9 Yin X, Liang Z, Yun Y, Pei L: Intravenous Transplantation of BMP2-Transduced Endothelial Progenitor Cells Attenuates Lipopolysaccharide-Induced Acute Lung Injury in Rats. *Cell Physiol Biochem* 2015;35:2149-2158.
- 10 Birukova AA, Meng F, Tian Y, Meliton A, Sarich N, Quilliam LA, Birukov KG: Prostacyclin post-treatment improves LPS-induced acute lung injury and endothelial barrier recovery via Rap1. *Biochim Biophys Acta* 2015;1852:778-791.
- 11 Zhou Z, You Z: Mesenchymal Stem Cells Alleviate LPS-Induced Acute Lung Injury in Mice by MiR-142a-5p-Controlled Pulmonary Endothelial Cell Autophagy. *Cell Physiol Biochem* 2016;38:258-266.
- 12 An X, Li L, Chen Y, Luo A, Ni Z, Liu J, Yuan Y, Shi M, Chen B, Long D, Cheng J, Lu Y: Mesenchymal Stem Cells Ameliorated Glucolipotoxicity in HUVECs through TSG-6. *Int J Mol Sci* 2016;17:483.
- 13 Qiao SK, Ren HY, Shi YJ, Liu W: Allogeneic Compact Bone-Derived Mesenchymal Stem Cell Transplantation Attenuates the Severity of Idiopathic Pneumonia Syndrome in a Murine Bone Marrow Transplantation Model. *Cell Physiol Biochem* 2016;40:1656-1669.
- 14 Li B, Li C, Zhu M, Zhang Y, Du J, Xu Y, Liu B, Gao F, Liu H, Cai J, Yang Y: Hypoxia-Induced Mesenchymal Stromal Cells Exhibit an Enhanced Therapeutic Effect on Radiation-Induced Lung Injury in Mice due to an Increased Proliferation Potential and Enhanced Antioxidant Ability. *Cell Physiol Biochem* 2017;44:1295-1310.
- 15 Shalaby SM, El-Shal AS, Abd-Allah SH, Selim AO, Selim SA, Gouda ZA, Abd EMDM, Zanfaly HE, El-Assar HM, Abdelazim S: Mesenchymal stromal cell injection protects against oxidative stress in Escherichia coli-induced acute lung injury in mice. *Cytotherapy* 2014;16:764-775.
- 16 Liang ZX, Sun JP, Wang P, Tian Q, Yang Z, Chen LA: Bone marrow-derived mesenchymal stem cells protect rats from endotoxin-induced acute lung injury. *Chin Med J (Engl)* 2011;124:2715-2722.
- 17 Horie S, Masterson C, Devaney J, Laffey JG: Stem cell therapy for acute respiratory distress syndrome: a promising future. *Curr Opin Crit Care* 2016;22:14-20.
- 18 Matthey MA: Therapeutic potential of mesenchymal stromal cells for acute respiratory distress syndrome. *Ann Am Thorac Soc* 2015;12:S54-57.
- 19 Masterson C, Jerkic M, Curley GF, Laffey JG: Mesenchymal stromal cell therapies: potential and pitfalls for ARDS. *Minerva Anesthesiol* 2015;81:179-194.
- 20 He HL, Liu L, Chen QH, Cai SX, Han JB, Hu SL, Chun P, Yang Y, Guo FM, Huang YZ, Qiu HB: MSCs modified with ACE2 restore endothelial function following LPS challenge by inhibiting the activation of RAS. *J Cell Physiol* 2015;230:691-701.
- 21 Zhao YF, Luo YM, Xiong W, Ding W, Li YR, Zhao W, Zeng HZ, Gao HC, Wu XL: Mesenchymal stem cell-based FGF2 gene therapy for acute lung injury induced by lipopolysaccharide in mice. *Eur Rev Med Pharmacol Sci* 2015;19:857-865.

- 22 Xu J, Qu J, Cao L, Sai Y, Chen C, He L, Yu L: Mesenchymal stem cell-based angiopoietin-1 gene therapy for acute lung injury induced by lipopolysaccharide in mice. *J Pathol* 2008;214:472-481.
- 23 Ding Y, Zhao R, Zhao X, Matthay MA, Nie HG, Ji HL: ENaCs as Both Effectors and Regulators of MiRNAs in Lung Epithelial Development and Regeneration. *Cell Physiol Biochem* 2017;44:1120-1132.
- 24 Lee JW, Park JW, Shin NR, Park SY, Kwon OK, Park HA, Lim Y, Ryu HW, Yuk HJ, Kim JH, Oh SR, Ahn KS: *Picrasma quassioides* (D. Don) Benn. attenuates lipopolysaccharide (LPS)-induced acute lung injury. *Int J Mol Med* 2016;38:834-844.
- 25 Liang OD, Mitsialis SA, Chang MS, Vergadi E, Lee C, Aslam M, Fernandez-Gonzalez A, Liu X, Baveja R, Kourembanas S: Mesenchymal stromal cells expressing heme oxygenase-1 reverse pulmonary hypertension. *Stem Cells* 2011;29:99-107.
- 26 Yin H, Li X, Gong Q, Jin X, Gu H, Yuan B, Zhang B, Zheng F, Gong F, Zhu J: Heme oxygenase-1 upregulation improves lipopolysaccharide-induced acute lung injury involving suppression of macrophage migration inhibitory factor. *Mol Immunol* 2010;47:2443-2449.
- 27 Yamashita K, Ollinger R, McDaid J, Sakahama H, Wang H, Tyagi S, Csizmadia E, Smith NR, Soares MP, Bach FH: Heme oxygenase-1 is essential for and promotes tolerance to transplanted organs. *FASEB J* 2006;20:776-778.
- 28 Chi X, Guo N, Yao W, Jin Y, Gao W, Cai J, Hei Z: Induction of heme oxygenase-1 by hemin protects lung against orthotopic autologous liver transplantation-induced acute lung injury in rats. *J Transl Med* 2016;14:35.
- 29 Wiesel P, Patel AP, DiFonzo N, Marria PB, Sim CU, Pellacani A, Maemura K, LeBlanc BW, Marino K, Doerschuk CM, Yet SF, Lee ME, Perrella MA: Endotoxin-induced mortality is related to increased oxidative stress and end-organ dysfunction, not refractory hypotension, in heme oxygenase-1-deficient mice. *Circulation* 2000;102:3015-3022.
- 30 Kapturczak MH, Wasserfall C, Brusko T, Campbell-Thompson M, Ellis TM, Atkinson MA, Agarwal A: Heme oxygenase-1 modulates early inflammatory responses: evidence from the heme oxygenase-1-deficient mouse. *Am J Pathol* 2004;165:1045-1053.
- 31 Reynolds P, Wall P, van Griensven M, McConnell K, Lang C, Buchman T: Shock supports the use of animal research reporting guidelines. *Shock* 2012;38:1-3.
- 32 Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods* 2001;25:402-408.
- 33 Liu XM, Peyton KJ, Durante W: Physiological cyclic strain promotes endothelial cell survival via the induction of heme oxygenase-1. *Am J Physiol Heart Circ Physiol* 2013;304:H1634-1643.
- 34 Cao TH, Jin SG, Fei DS, Kang K, Jiang L, Lian ZY, Pan SH, Zhao MR, Zhao MY: Artesunate Protects Against Sepsis-Induced Lung Injury Via Heme Oxygenase-1 Modulation. *Inflammation* 2016;39:651-662.
- 35 Cao H, Feng Y, Ning Y, Zhang Z, Li W, Li Q: Edaravone protects rats and human pulmonary alveolar epithelial cells against hyperoxia injury: heme oxygenase-1 and PI3K/Akt pathway may be involved. *Exp Lung Res* 2015;41:404-414.
- 36 Choudhery MS, Badowski M, Muise A, Harris DT: Effect of mild heat stress on the proliferative and differentiative ability of human mesenchymal stromal cells. *Cytotherapy* 2015;17:359-368.
- 37 Chen Q, Shou P, Zheng C, Jiang M, Cao G, Yang Q, Cao J, Xie N, Velletri T, Zhang X, Xu C, Zhang L, Yang H, Hou J, Wang Y, Shi Y: Fate decision of mesenchymal stem cells: adipocytes or osteoblasts. *Cell Death Differ* 2016;23:1128-1139.
- 38 Zhang X, Chang N, Zhang Y, Ye M, Han Z, Li J, Zhang J: Bakuchiol Protects Against Acute Lung Injury in Septic Mice. *Inflammation* 2017;40:351-359.
- 39 Feng J, Zhang P, Chen X, He G: PI3K and ERK/Nrf2 pathways are involved in oleanolic acid-induced heme oxygenase-1 expression in rat vascular smooth muscle cells. *J Cell Biochem* 2011;112:1524-1531.
- 40 Ho MS, Mei SH, Stewart DJ: The Immunomodulatory and Therapeutic Effects of Mesenchymal Stromal Cells for Acute Lung Injury and Sepsis. *J Cell Physiol* 2015;230:2606-2617.
- 41 Kao MC, Yang CH, Chou WC, Sheu JR, Huang CJ: Cepharanthine mitigates lung injury in lower limb ischemia-reperfusion. *J Surg Res* 2015;199:647-656.
- 42 Wang F, Meng Y, Zhang Y, Zhao G, Zheng X, Xiao Q, Yu Y: Ketamine reduces lipopolysaccharide-induced high-mobility group box-1 through heme oxygenase-1 and nuclear factor erythroid 2-related factor 2/ p38 mitogen-activated protein kinase. *J Surg Res* 2015;194:599-613.
- 43 Motterlini R, Foresti R: Heme oxygenase-1 as a target for drug discovery. *Antioxid Redox Signal* 2014;20:1810-1826.

- 44 Cheng GS, Zhang YS, Zhang TT, He L, Wang XY: Bone marrow-derived mesenchymal stem cells modified with IGFBP-3 inhibit the proliferation of pulmonary artery smooth muscle cells. *Int J Mol Med* 2017;39:223-230.
- 45 Cheng G, Wang X, Li Y, He L: Let-7a-transfected mesenchymal stem cells ameliorate monocrotaline-induced pulmonary hypertension by suppressing pulmonary artery smooth muscle cell growth through STAT3-BMP2 signaling. *Stem Cell Res Ther* 2017;8:34.
- 46 He H, Liu L, Chen Q, Liu A, Cai S, Yang Y, Lu X, Qiu H: Mesenchymal Stem Cells Overexpressing Angiotensin-Converting Enzyme 2 Rescue Lipopolysaccharide-Induced Lung Injury. *Cell Transplant* 2015;24:1699-1715.
- 47 Chen XX, Tang L, Fu YM, Wang Y, Han ZH, Meng JG: Paralemmin-3 contributes to lipopolysaccharide-induced inflammatory response and is involved in lipopolysaccharide-Toll-like receptor-4 signaling in alveolar macrophages. *Int J Mol Med* 2017;40:1921-1931.
- 48 Joo CR, Cheng MS, Shik KY: Desoxyrhapontigenin up-regulates Nrf2-mediated heme oxygenase-1 expression in macrophages and inflammatory lung injury. *Redox Biol* 2014;2:504-512.
- 49 Kobayashi M, Yamamoto M: Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxid Redox Signal* 2005;7:385-394.
- 50 Wang L, Tu XH, Zhao P, Song JX, Zou ZD: Protective effect of transplanted bone marrow-derived mesenchymal stem cells on pancreatitis-associated lung injury in rats. *Mol Med Rep* 2012;6:287-292.
- 51 Li JW, Wu X: Mesenchymal stem cells ameliorate LPS-induced acute lung injury through KGF promoting alveolar fluid clearance of alveolar type II cells. *Eur Rev Med Pharmacol Sci* 2015;19:2368-2378.
- 52 Yang Y, Chen QH, Liu AR, Xu XP, Han JB, Qiu HB: Synergism of MSC-secreted HGF and VEGF in stabilising endothelial barrier function upon lipopolysaccharide stimulation via the Rac1 pathway. *Stem Cell Res Ther* 2015;6:250.
- 53 Burchfield JS, Iwasaki M, Koyanagi M, Urbich C, Rosenthal N, Zeiher AM, Dimmeler S: Interleukin-10 from transplanted bone marrow mononuclear cells contributes to cardiac protection after myocardial infarction. *Circ Res* 2008;103:203-211.
- 54 Meng X, Li J, Yu M, Yang J, Zheng M, Zhang J, Sun C, Liang H, Liu L: Transplantation of mesenchymal stem cells overexpressing IL10 attenuates cardiac impairments in rats with myocardial infarction. *J Cell Physiol* 2018;233:587-595.
- 55 Li J, Huang S, Wu Y, Gu C, Gao D, Feng C, Wu X, Fu X: Paracrine factors from mesenchymal stem cells: a proposed therapeutic tool for acute lung injury and acute respiratory distress syndrome. *Int Wound J* 2014;11:114-121.
- 56 Németh K, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, Robey PG, Leelahavanichkul K, Koller BH, Brown JM, Hu X, Jelinek I, Star RA, Mezey E: Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009;15:42-49.
- 57 Zhang W, Liu L, Huo Y, Yang Y, Wang Y: Hypoxia-pretreated human MSCs attenuate acute kidney injury through enhanced angiogenic and antioxidative capacities. *Biomed Res Int* 2014;2014:462472.
- 58 Kang JW, Lee SM: Melatonin inhibits type 1 interferon signaling of toll-like receptor 4 via heme oxygenase-1 induction in hepatic ischemia/reperfusion. *J Pineal Res* 2012;53:67-76.
- 59 Qi X, Zhang H, Xue T, Yang B, Deng M, Wang J: Down-regulation of cellular protein heme oxygenase-1 inhibits proliferation of avian influenza virus H9N2 in chicken oviduct epithelial cells. *J Gen Virol* 2018;99:36-43.
- 60 Chabannes D, Hill M, Merieau E, Rossignol J, Brion R, Soullou JP, Anegon I, Cuturi MC: A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stem cells. *Blood* 2007;110:3691-3694.
- 61 Chauveau C, Rémy S, Royer PJ, Hill M, Tanguy-Royer S, Hubert FX, Tesson L, Brion R, Berioux G, Gregoire M, Josien R, Cuturi MC, Anegon I: Heme oxygenase-1 expression inhibits dendritic cell maturation and proinflammatory function but conserves IL-10 expression. *Blood* 2005;106:1694-1702.
- 62 Shen XD, Ke B, Zhai Y, Gao F, Busuttil RW, Cheng G, Kupiec-Weglinski JW: Toll-like receptor and heme oxygenase-1 signaling in hepatic ischemia/reperfusion injury. *Am J Transplant* 2005;5:1793-1800.
- 63 Carroll SH, Wigner NA, Kulkarni N, Johnston-Cox H, Gerstenfeld LC, Ravid K: A2B adenosine receptor promotes mesenchymal stem cell differentiation to osteoblasts and bone formation *in vivo*. *J Biol Chem* 2012;287:15718-15727.