Original Paper

Exosomes from Human-Induced Pluripotent Stem Cell-Derived Mesenchymal Stromal Cells (hiPSC-MSCs) Protect Liver against Hepatic Ischemia/Reperfusion Injury *via* Activating Sphingosine Kinase and Sphingosine-1-Phosphate Signaling Pathway

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Key Words

Induced pluripotent stem cells derived mesenchymal stromal cells • Exosomes • Hepatic ischemic reperfusion injury • Sphingosine-1-phosphate • Sphingosine kinase • Proliferation

Abstract

Background/Aims: This study aimed to evaluate the effects of exosomes produced by human-induced pluripotent stem cell-derived mesenchymal stromal cells (hiPSC-MSCs-Exo) on hepatic ischemia-reperfusion (I/R) injury, as well as the underlying mechanisms. **Methods:** Exosomes derived from hiPSC-MSCs were isolated and characterized both biochemically and biophysically. hiPSC-MSCs-Exo were injected systemically into a murine ischemia/reperfusion injury model via the inferior vena cava, and then the therapeutic effects were evaluated. The serum levels of transaminases (aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as well as histological changes were examined. Primary hepatocytes and human hepatocyte cell line HL7702 were used to test whether exosomes could induce hepatocytes proliferation *in vitro*. In addition, the expression levels of proliferation markers (proliferation cell nuclear antigen,

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PCNA; Phosphohistone-H3, PHH3) were measured by immunohistochemistry and Western blot. Moreover, SK inhibitor (SKI-II) and S1P1 receptor antagonist (VPC23019) were used to investigate the role of sphingosine kinase and sphingosine-1-phosphate-dependent pathway in the effects of hiPSC-MSCs-Exo on hepatocytes. **Results:** hiPSCs were efficiently induced into hiPSC-MSCs that had typical MSC characteristics. hiPSC-MSCs-Exo had diameters ranging from 100 to 200 nm and expressed exosome markers (Alix, CD63 and CD81). After hiPSC-MSCs-Exo administration, hepatocyte necrosis and sinusoidal congestion were markedly suppressed in the ischemia/reperfusion injury model, with lower histopathological scores. The levels of hepatocyte injury markers AST and ALT were significantly lower in the treatment group compared to control, and the expression levels of proliferation markers (PCNA and PHH3) were greatly induced after hiPSC-MSCs-Exo administration. Moreover, hiPSC-MSCs-Exo also induced primary hepatocytes and HL7702 cells proliferation *in vitro* in a dose-dependent manner. We found that hiPSC-MSCs-Exo could directly fuse with target hepatocytes or HL7702 cells and increase the activity of sphingosine kinase and synthesis of sphingosine-1-phosphate (S1P). Furthermore, the inhibition of SK1 or S1P1 receptor completely abolished the protective and proliferative effects of hiPSC-MSCs-Exo on hepatocytes, both *in vitro* and *in vivo*. **Conclusions:** Our results demonstrated that hiPSC-MSCs-Exo could alleviate hepatic I/R injury via activating sphingosine kinase and sphingosine-1-phosphate pathway in hepatocytes and promote cell proliferation. These findings represent a novel mechanism that potentially contributes to liver regeneration and have important implications for new therapeutic approaches to acute liver disease.

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Introduction

Hepatic ischemia and reperfusion (I/R) is a major clinical problem that affects liver transplantation and other hepatic surgeries. Hepatic I/R frequently leads to injury in remote organs, including kidney, lung, and heart. In particular, acute kidney injury (AKI) after major liver IR is very common (40–85% incidence) and greatly increases patient mortality and morbidity during perioperative period [1–5]. Therefore, reducing the adverse effects of I/R injury can significantly increase the patients' lifespan after liver transplantation.

Mesenchymal stem cells (MSCs) have been shown to be able to ameliorate the severity of graft-versus-host disease, autoimmune conditions, musculoskeletal injuries and cardiovascular diseases [6–10]. However, the number of MSCs that can be obtained from a single donor is very limited, while most of these clinical therapeutic applications require substantial cell quantities. In recent years, the MSCs derived from human-induced pluripotent stem cells (hiPSCs) has been used in pre-clinical studies and showed better performance compared to the adult MSCs in terms of cell proliferation, immunomodulation, cytokines profiles, production of microenvironment modulating exosomes, and secretion of bioactive paracrine factors [11, 12]. It has been shown that hiPSC-MSCs can prevent I/R damage in the kidney, liver and heart [13–19]. However, the underlying mechanism of the protective effect of hiPSC-MSCs is still unclear.

Exosomes are membrane nanovesicles (50–200 nm) released by cells into the extracellular environment upon fusion with multivesicular bodies. Exosomes contain membrane components, proteins, micro-RNAs and mRNAs [20–26]. Recently, exosomes secreted from hiPSC-MSCs have been shown to play a critical role in protection against I/R injury in the liver, kidney and heart [13, 18, 27–30].

Here, we investigated the hepatoprotective effect of hiPSC-MSCs derived exosomes on hepatic I/R injury, as well as the underlying mechanisms. We demonstrated that exosomes produced by hiPSC-MSCs could reduce hepatic I/R injury and promote hepatocyte proliferation both *in vitro* and *in vivo*. Moreover, we found that the activating of sphingosine kinase and sphingosine-1-phosphate pathway played an important role in this effect. Our study represents a potential mechanism for liver regeneration and has important implications for new therapeutic approaches to acute liver diseases.

Materials and Methods

Animals

Male C57Bl/6 mice, age of 6-8 weeks, were purchased from the Slac Laboratory Animal Center (Shanghai Slac Laboratory Animal Co. Ltd., China). The experimental protocols were approved by the institutional Animal Care and Use Committee of the Transplantation Center of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University. All the animals and samples were assigned a number that did not reveal the group treatment in order to perform blind analysis.

Murine Model of Hepatic IR

The partial hepatic ischemia lasts for 60 min and results in a segmental (B70%) hepatic ischemia. But the right liver lobe is left out, so the mesenteric venous congestion is prevented by allowing portal decompression through the right and caudate liver lobes. Sham-operated mice were subjected to laparotomy and identical liver manipulations without the vascular occlusion. Plasma as well as liver tissues were collected at 24 h after liver IR injury. For the administration of exosomes, hiPSC-MSCs derived exosomes (2.5×10^{12} particles) were suspended in 500 μ L PBS (Exo+ I/R group) and injected in to mice immediately after the initiation of reperfusion via the inferior vena cava. The control group was only injected with 500 μ L PBS.

Serum aminotransferase activities

To estimate the degree of hepatocyte damage after I/R injury, serum levels of ALT and AST were measured using a Hitachi 7600 automatic analyzer (Hitachi, Ltd., Tokyo, Japan).

Histology

Liver tissue samples were fixed in 10% formalin for 24 h and embedded in paraffin. The sections were then stained with hematoxylin and eosin. The stained sections were examined by a pathologist using an Olympus light microscope (Olympus Optical Co., Tokyo, Japan), and were graded according to the criteria from Suzuki et al. In this classification, three liver injury indices- sinusoidal congestion (score: 0-4), hepatocyte necrosis (score: 0-4), and ballooning degeneration (score: 0-4)-are graded and summed up to a total score (0-12). No congestion, necrosis or ballooning is scored 0, while severe congestion/ballooning and > 60% lobular necrosis is scored 12. The histological changes were evaluated in randomly chosen fields at 200 $\times$ magnification.

For hepatocyte proliferation examination, tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. The sections were then stained for proliferating cell nuclear antigen (PCNA) and phosphorylated histone H3 (PHH3). PCNA staining was performed as previously described, and PHH3 staining was performed according to the manufacturer's instructions (Ser10; dilution of 1:200, Cell Signaling Technology, Danvers, MA). Sections were counterstained with hematoxylin. The positive nuclei were quantified and its percentage was calculated (for each mouse, 400-600 hepatocytes from 4-6 fields at 200 $\times$ magnification were counted).

Human iPSCs derived MSCs

Human iPSCs were provided by Dr. Xiao from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences [29]. The iPSCs were cultured in mTESR1 medium (StemCell Technologies) to 90% confluency. Then mTESR1 was replaced by MSC medium (Dulbecco's Modified Eagle Medium (DMEM)-low glucose supplemented with 10% fetal bovine serum (FBS) and 2 mmol/L L-Glutamine). After 2 weeks of culturing in MSC medium, cells were trypsinized and expanded in 0.1% gelatin-coated dishes in MSC medium. Cells were passaged until they developed the fibroblast-like morphology. Then these cells were ready to be used in the MSC phenotypic characteristics analysis and further experiments.

Immunofluorescence staining

For immunofluorescence staining, cultured hiPSCs and hiPSCs-MSCs were fixed with 4% buffered paraformaldehyde in PBS, and incubated with blocking solution (1% BSA and 0.1% Triton-X in PBS) at room temperature for 2 h. Then cells were incubated with primary antibodies against SOX2 and CD90 diluted in PBS overnight at 4°C. After that, appropriate secondary antibodies (Abcam) were added and incubated with cells for 1 h at room temperature. The nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI;

Beyotime, Shanghai, China) for 20 min at room temperature. Immunofluorescence was examined under a fluorescence microscope (Nikon 80i, Otawara, Tochigi, Japan).

Flow cytometry

Flow cytometry was used to analyze the characteristics of cultured hiPSC-MSCs. Briefly, cells were incubated with 1% bovine serum albumin for 30 min (BSA; Gibco) in PBS to block nonspecific antigens. Conjugated monoclonal antibodies (PE-CD34, PE-CD45, APC-HLA-DR-PE, FITC-CD29, FITC-CD90 and FITC-CD105; Biolegends) were used according to the manufacturer's instructions. The samples were analyzed using an Epics xL flow cytometer (Beckman Coulter, High Wycombe, UK).

Multipotential of hiPSC-MSCs

Osteogenesis and adipogenesis were examined to determine the multipotential of hiPSC-MSCs. For induction, 1×10^5 hiPSC-MSCs were seeded in 6-well plates until they reached 90% confluency, at which point the medium was replaced with osteogenesis medium or adipogenesis medium (ThermoFisher). The cells were fixed with 4% paraformaldehyde after 21 days and stained in alizarin red or oil red O for observation under an optical microscope (Zeiss).

Exosome isolation

Exosomes were collected using differential centrifugation. Briefly, cells were grown to 80% confluency and then cultured for 48 h. The condition medium (CM) was obtained and filtered by 0.45- μ m PVDF filter (Millipore, Billerica, MA) to further eliminate cellular debris. ExoQuick Exosome Precipitation Solution (SBI System Biosciences, Mountain View, Calif) was added to the supernatant and mixed well, then the mixture was precipitated by staying in 4°C for 30 min. Exosomes were harvested after centrifuging at 4°C 1,500 $\times$ g for 30 min. Exosome pellets were re-suspended with PBS and their total protein concentration was determined by bicinchoninic acid (BCA) assay (Thermo Fisher).

Exosome characterization

Exosome morphology was visualized by transmission electron microscopy. Briefly, exosomes were diluted to 1 mg/ml with PBS. Then, a specimen of exosomes was spotted onto a glow-discharged copper grid on the filter paper and dried for 20 min under infrared lamp. Finally, the grid was stained with 2% uranyl acetate at pH 7.0 for 40 s and air-dried at room temperature. Exosomes were examined under transmission electron microscopy (H-600 HITACHI microscope, Japan) at 80 keV. The size of the exosome was determined using a Zetasizer Nano (Malvern Instruments, Malvern, UK), and the purity was assessed by Western blot with exosome markers (Alix, CD63, and CD81). The number of exosomes was assessed by the CD81-antigen ELISA kit (ThermoFisher).

Hepatocyte culture

Hepatocytes were isolated from mice as previously described [31]. 2×10^6 cells/5 ml were cultured in 50 mm plates with Williams media supplemented with 5% fetal bovine serum (Gibco). The human hepatocyte cell line HL7702 was obtained from American Type Culture Collection (Manassas, VA, USA). They were plated in 100-mm culture dishes in RPMI1640 (ThermoFisher) medium supplemented with 20% fetal bovine serum, 100 U/mL penicillin, 2 mM glutamine, 100 μ g/mL streptomycin, and 1 mM HEPES buffer. The cells were cultured at 37°C, 5% CO₂ in humidified incubator. The medium was changed every other day.

Exosome-hepatocyte fusion

Exosomes were labeled with 2 μ M PKH67 (Sigma-Aldrich) for 5 min. After washing, they were incubated with cultured hepatocytes or HL7702 cells for 24 h. Then the samples were washed and counterstained with PE labelled anti-E-cadherin antibody and DAPI, followed by analyzing with confocal microscopy.

Cell proliferation

The proliferation rates of primary hepatocyte and HL7702 cells *in vitro* were determined by 5-bromo-2-deoxyuridine (BrdU) incorporation and CCK8 assay. Hepatocytes were treated with hepatocyte-derived exosomes for 24 h prior to BrdU incorporation. The results were normalized by the amount of viable cells and compared with medium alone control. A commercial BrdU proliferation ELISA kit (Abcam, Cambridge,

UK) was used for this assay. For CCK8 assay, primary hepatocytes and HL7702 cells were treated with CCK8 (10 μ l/well, Sigma, USA) for an additional 2 h. Then the absorbance at 450 nm were measured using a microplate absorbance reader (Tecan, Safire II, Switzerland).

SK activity assay

SK activity was measured as described by Vessey et al. [32]. Briefly, enzyme preparations from primary hepatocyte (50 μ g total protein) or HL7702 cells (150 μ g total protein) were incubated in assay buffer (0.05% Triton X-100, 250 mM KCl, 0.05 μ M [3H]sphingosine (20 Ci/mmol; American Radiolabeled Chemicals), 5 mM ATP, 10 mM MgCl₂, 100 mM Tris pH 8.0} at 20°C for 30 min, in a total volume of 100 μ l. Different incubation times yielded a linear relationship with SK activity (data not shown). Then, [3H]S1P was extracted using 1.2 ml of methanol:chloroform: trisodium EDTA (pH=9) (1:2:1). After vortexing and centrifuging for 5 min at 2,700 g, the upper aqueous phase was collected and counted in a liquid scintillation counter (Packard BioScience, Meriden, CT). To verify the product of our extraction, aliquots of the upper aqueous phases were resolved on silica 60 A° TLC plates (Whatman, Florham Park, NJ) using 1-butanol:methanol:acetic acid:ddH₂O (80:20:10:20). The lanes were scraped in 1-cm increments and counted in a liquid scintillation counter. We found that the peaks from our extraction had the same relative mobility (Rf) as purified [3H] S1P (data not shown).

Measurement of [3H]S1P synthesis in primary hepatocytes and HL7702 cells

Cellular [3H]S1P formation in primary hepatocytes and HL7702 cells was measured using a method described by Lavie et al. [32]. Confluent cells in six-well plates were cultured in serum-free media containing 0.02 μ M [3H]sphingosine (20 Ci/mmol) overnight to allow for equilibration. Then the media were removed and the cells were scraped in 100- μ l lysis buffer. [3H]S1P was extracted from the cell lysates as described above and counted.

Western Blot

Liver tissues or cells were lysed in ice-cold RIPA lysis buffer (Solarbio, Beijing, China) containing 0.1 mM PMSF and protease inhibitor (Roche) for 30 min on ice. Samples were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with the following primary antibodies: anti-PCNA (Abcam, Cambridge, UK), anti-PHH3 (Abcam), anti-SK1 (Abcam) and anti-GAPDH (Santa Cruz Biotechnology). After four washes with PBS-Tween 20, horseradish peroxidase-conjugated secondary antibodies were added. The signals were detected with Pierce® ECL Western blotting substrate (Pierce, Rockford, IL, USA) and developed on X-ray films (Kodak, Rochester, NY, USA).

Statistical Analysis

Continuous variables are presented as mean $\pm$ S.E.M. Analysis of variance (ANOVA) and post hoc Bonferroni analysis was conducted for multiple comparisons by GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). P-values < 0.05 is considered statistically significant.

Results

Generation of hiPSC-MSCs and characterization of hiPSC-MSCs secreted exosomes

After one-step induction, hiPSCs were successfully induced into hiPSCs-MSCs and grew in a monolayer with large spindleshaped morphology at the colony border (Fig. 1A). Immunofluorescence staining was used to assess the surface antigens of hiPSCs (SOX2) and hiPSCs-MSCs (CD90) before and after induction. Flow cytometry was also used to identify the surface antigens in differentiated hiPSCs-MSCs. The results showed that hiPSC-MSCs were negative for CD34 and CD45, but positive for CD29, CD73, CD90 and CD105 (Fig. 1B). Upon analyzing the effect of osteogenetic and adipogenic inductions on hiPSCs-MSCs, hiPSC-MSCs could fulfill a multipotential role by differentiating into cells staining positive with alizarin red and oil red O (Fig. 1C). Furthermore, the exosomes secreted from hiPSCs-MSCs were isolated and subjected to biochemical and biophysical analyses. Electron microscopy analysis on exosomes exhibited expected cup-shaped morphology (Fig. 1D). The exosome

Fig. 1. Characterization of hiPSC-MSCs and hiPSC-MSCs derived exosomes. (A) Morphological changes in hiPSCs during MSC induction, examined by immunofluorescence staining; (B) Flow cytometric analysis of surface markers of hiPSC-MSCs. (C) Evaluation of the differentiating capacity of hiPSC-MSCs: (Up) alizarin red staining for osteocytes; (Down) oil red O staining for adipocytes (scale bars, 200 μ m). (D) Left: TEM images of exosomes derived from hiPSC-MSCs (scale bar, 100nm); middle: the sizes of hiPSC-MSCs derived exosomes (mean: 135 nm); right: western blot for Alix, CD80 and CD63 in hiPSC-MSCs derived exosomes. Medium alone is the control. (E) A standard curve indicated the relation between the protein content and the exosome number.

size was quantified by a Zetasizer Nano and the mean vesicle diameter was 135 nm (Fig. 1D). Biochemical analysis of exosomes showed positive expression of the exosome proteins Alix, CD63, and CD81 (Fig. 1D). We also evaluated the relation between the protein content and the exosome number and found that there are about 5×10^9 particles of exosome in 1 μ g exosome (Fig. 1E).

Exosomes secreted from hiPSC-MSCs ameliorated hepatic I/R injury

To evaluate the effect of hiPSC-MSCs derived exosomes on hepatic I/R injury, exosomes were injected into the murine I/R injury model. Compared with control (PBS alone injection), administration of hiPSC-MSCs-Exo significantly improved the liver health (Fig. 2A). After surgery, a larger area of necrosis was observed in the livers of PBS + I/R group compared to sham group, (Fig. 2A) together with sinusoidal congestion and cell swelling. However, in hiPSC-MSCs-Exo treated group, the hepatocyte necrosis and sinusoidal congestion were

Fig. 2. The protective effect of hiPSC-MSCs derived exosomes on hepatic I/R injury. hiPSC-MSCs derived exosomes (2.5×10^{12} particles) were suspended in 500 μ L PBS (Exo+ I/R group) and injected in to mice immediately after the initiation of reperfusion via the inferior vena cava. The control group was only injected with 500 μ L PBS. (A) Representative histopathologic images of liver sections harvested at 24 h after reperfusion (H&E staining; scale bars, 100 μ m). The damage degree was graded using Suzuki criteria; (B) Blood samples were collected to determine ALT and AST levels (U/L). Data is presented as means $\pm$ SEM, n = 6 mice per group; statistical significance: *p < 0.05; **p < 0.01, ***p < 0.001.

markedly reduced. Pathologists scored the histology of these livers based on Suzuki criteria in a single-blinded way, and the hiPSC-MSCs-Exo treated group showed lower scores than the corresponding control group (Fig. 2A). Moreover, we also measured the ALT and AST levels in these mice, since these are common biomarkers for hepatocellular damage. We found that the levels of ALT and AST were significantly lower in hiPSC-MSCs-Exo treated group than control at 1, 3, 6, 12, 24 h after reperfusion (Fig. 2B). Collectively, these results demonstrate that hiPSC-MSCs-Exo treatment could ameliorate hepatic I/R injury.

hiPSC-MSCs-Exo treatment promote hepatocyte proliferation in vivo and in vitro

Liver regeneration is a compensatory process to replace the lost liver mass after injury or disease [33]. To investigate whether hiPSC-MSCs-Exo treatment induces hepatocyte proliferation in the murine I/R injury model, immunohistochemical staining and Western blot were used to examine hepatocytes proliferation markers at 48 h after reperfusion, including proliferating cell nuclear antigen (PCNA) and phosphorylated histone H3 (PHH3). The PCNA and PHH3 levels were similar between I/R group and sham group, but were significantly increased in hiPSC-MSCs-Exo treated group (Fig. 3A and 3B).

To determine if hiPSC-MSCs-Exo had direct effect on hepatocyte proliferation, hiPSC-MSCs-Exo was added to cultured primary hepatocytes and HL7702 cells. We first examined the transfer of exosome components with hepatocytes or HL7702 cells. The exosomes were labeled with cell membrane marker PKH67 prior to incubation with cells, which is counterstained with PE labelled anti-E-cadherin antibody and scanned by a confocal microscopy. The results showed that exosome were directly uptaken into the target cells (Fig. 4A). Moreover, the treatment of hiPSC-MSCs-Exo for 24 h resulted in an increase in hepatocytes and HL7702 cells' proliferation in a dose-dependent manner (Fig. 4B and 4C). Taken together, these results suggest that hiPSC-MSCs-Exo treatment can protect the liver in I/R injury by promoting hepatocytes proliferation.

Fig. 3. hiPSC-MSCs derived exosomes promote hepatocyte proliferation *in vivo*. (A) Immunohistochemistry staining of PCNA and PHH3 at 24 h after reperfusion, as well as the quantitative analysis. (B) Representative images and analysis data of the protein levels of PCNA and PHH3, assessed by Western blot at 24 h after reperfusion. Data is presented as means $\pm$ SEM, n = 6 mice per group; statistical significance: *p < 0.05; **p < 0.01, ***p < 0.001.

Fig. 4. hiPSC-MSCs derived exosomes directly fuse with target hepatocytes and get internalized, resulting in increased proliferation of primary hepatocytes and HL7702 cells *in vitro*. (A) hiPSC-MSCs derived exosomes were fluorescently labeled with cell membrane marker PKH67 prior to incubation with primary hepatocytes or HL7702 cells. After incubation for 24h, cells were washed and counterstained with PE labelled anti-E-cadherin antibody and DAPI and scanned by a confocal microscopy. The pictures show the representative results from four independent experiments; (B and C) hiPSC-MSCs derived exosomes were added to primary hepatocytes and HL7702 cells, and then cell proliferation was measured after 24 h by BrdU incorporation (B) and CCK8 assay (C). Data is presented as means $\pm$ SEM, n = 6 mice per group; statistical significance: *p < 0.05; **p < 0.01, ***p < 0.001.

hiPSC-MSCs-Exo treatment increase sphingosine kinase (SK) activity and [3 H] sphinganine-1-phosphate (S1P) formation in primary hepatocytes and HL7702 cells

It has been reported that exosomes secreted from hepatocytes could promote hepatocytes proliferation via activating sphingosine kinase and S1P-dependent pathway [34, 35].

Fig. 5. hiPSC-MSCs derived exosomes treatment increases sphingosine kinase (SK) activity and [^3H] sphinganine-1-phosphate (S1P) formation in primary hepatocytes and HL7702 cells. (A) The relative SK activity in target primary hepatocytes or HL7702 cells was measured by kinase assays at 24 h after treatment. Cells were either treated with 200 $\mu\text{g}/\text{ml}$ hiPSC-MSCs derived exosomes or media alone (control); (B) The relative cellular [^3H] S1P formation in target primary hepatocytes or HL7702 cells was measured at 24 h after treatment. (C) Western blot of SK1 in primary hepatocytes or HL7702 cells treated with 200 $\mu\text{g}/\text{ml}$ hiPSC-MSCs derived exosomes or media alone. SK1 expression was measured at 24 h after treatment and normalized to GAPDH. The quantitative analysis was performed. Data is presented as means $\pm$ SEM, $n = 6$ mice per group; statistical significance: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

Fig. 6. The proliferative effect of hiPSC-MSCs derived exosomes on primary hepatocytes and HL7702 cells was completely blocked by the inhibition of SK or S1P receptor 1. After pre-treatment of SKI-II or VPC23019, hiPSC-MSCs derived exosomes were added to primary hepatocytes and HL7702 cells. Cell proliferation was measured at 24 h later by BrdU incorporation (A) and CCK8 assay (B). Data is presented as means $\pm$ SEM, $n = 6$ mice per group; statistical significance: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

To investigate whether this pathway is also involved in exosomes-induced hepatocyte proliferation, SK activity and [^3H] S1P formation were measured in primary hepatocytes and HL7702 cells treated with hiPSC-MSCs-Exo. The results showed that after hiPSC-MSCs-Exo treatment for 24 h, SK activity and [^3H] S1P formation were increased in a dose-dependent manner (Fig. 5A and 5B). Moreover, we also measured the protein level changes of SK1 after hiPSC-MSCs-Exo treatment in primary hepatocytes and HL7702 cells, and found a marked increase in SK1 protein levels in a dose-dependent manner (Fig. 5C). These results suggest that S1P-SK1 signaling pathway may be involved in the hepatoprotective and proliferative effects of hiPSC-MSCs-Exo.

Fig. 7. The hepatoprotective effect of hiPSC-MSCs derived exosomes against hepatic I/R injury was reversed by the inhibition of SK or S1P receptor 1. (A) Representative histopathologic images of liver sections harvested at 24 h after reperfusion (H&E staining; scale bars, 100 μ m). The damage degree was graded using Suzuki criteria; (B) Blood samples were collected to determine ALT and AST levels (U/L). Data is presented as means $\pm$ SEM, n = 6 mice per group; statistical significance: *p < 0.05; **p < 0.01, ***p < 0.001.

The proliferative effect of hiPSC-MSCs-Exo on primary hepatocytes and HL7702 cells was abolished by the inhibition of SK or S1P receptor 1

To directly test the hypothesis that activating S1P-SK1 pathway is critical for the proliferative effect of hiPSC-MSCs-Exo, we used a SK inhibitor (SKI-II, 5 μ M) and a S1P receptor 1 antagonist (VPC23019, 2 μ M) to block S1P-SK1 pathway. Both the BrdU incorporation assay and CCK8 assay showed that neither SK nor S1P receptor 1 inhibitor affect the level of proliferation (data not shown), while they both completely abolish the proliferative effect of hiPSC-MSCs-Exo on primary hepatocytes and HL7702 cells (Fig. 6A and 6B).

The hepatoprotective and proliferative effect of hiPSC-MSCs-Exo on hepatic I/R injury was abolished by inhibition of SK or S1P receptor 1

To further demonstrate that activating S1P-SK1 pathway is responsible for hepatocyte protection and proliferation *in vivo*, SK inhibitor or S1P1 receptor antagonist was given to mice that underwent I/R injury. The SK inhibitor SKI-II (50 mg/kg) was subcutaneously administered to mice at 15 min before ischemia. VPC23019, a specific S1P1/S1P3 receptor antagonist (10-fold more selective for S1P1 compared to S1P3), was intraperitoneally administered (10 mg/kg) to mice at 10 min before ischemia. Administration of SKI-II or VPC23019 alone in sham or I/R mice, the histopathologic score and ALT/AST levels were unchanged compared with corresponding control group. However, pre-treatment of SKI-II or VPC23019 abolished the hepatoprotective effect of hiPSC-MSCs-Exo in murine I/R injury model, demonstrated by changes of histopathologic score (Fig. 7A) and ALT/AST levels (Fig. 7B). Furthermore, the increased levels of proliferation markers (PCNA and PHH3) after hiPSC-MSCs-Exo treatment were also abrogated by the inhibition of SK or S1P1 receptor

Fig. 8. The proliferative effect of hiPSC-MSCs derived exosomes against hepatic I/R injury was reversed by the inhibition of SK or S1P receptor 1. (A) Immunohistochemistry staining of PCNA and PHH3 at 24 h after reperfusion, as well as the quantitative analysis. (B) Representative images and analysis data of the protein levels of PCNA and PHH3, measured by Western blot at 24 h after reperfusion. Data is presented as means $\pm$ SEM, n = 6 mice per group; statistical significance: *p < 0.05; **p < 0.01, ***p < 0.001.

Fig. 9. Proposed cellular mechanisms for the hepatic protection of hiPSC-MSCs derived exosomes after liver I/R injury. Based on our data, we propose that hiPSC-MSCs-Exo alleviate hepatic I/R injury by promoting hepatocytes proliferation via activating sphingosine kinase and sphingosine-1-phosphate pathway.

(Fig. 8A and 8B). Collectively, these results showed that S1P and SK1 pathway played an important role in the protective and proliferative effects of hiPSC-MSCs-Exo on hepatic I/R injury.

Discussion

In this paper, we describe a novel and significant function of hiPSC-MSCs secreted exosomes. Our results show that the exosomes released by hiPSC-MSCs can protect liver against hepatic I/R injury in a murine model. Moreover, the hiPSC-MSCs-Exo can directly fuse with hepatocytes and promote their proliferation both *in vitro* and *in vivo*. Mechanistically, we found hiPSC-MSCs-Exo markedly increased S1P formation and SK activity, which was

shown to be required for hiPSC-MSCs-Exo induced hepatocyte proliferation (Fig. 9). These findings largely extend our current understanding of hiPSC-MSC-derived exosomes, and suggest that they may function to restore liver homeostasis after injury, dysfunction or disease. Furthermore, our data suggests that hiPSC-MSC-derived exosomes represent a novel therapeutic approach for liver disease.

Recently, stem cell implantation has been an alternative option for the treatment of many tissues and showed hepatoprotective effects in hepatic I/R injury [36]. Currently, MSCs are defined by their plastic adherent growth which displays fibroblast-like cellular colonies, as well as their capacity to differentiate towards at least three lineages, such as adipogenic, osteogenic and chondrogenic lineages [6, 8]. Many researchers suggest that the plasticity and immunomodulatory capability of MSCs indicate unique therapeutic potentials. Since the commonly used adult MSCs derived from tissues have limited abilities in proliferation, secreting bioactive factors and immunomodulation, an alternate source of MSCs is in need. The hiPSC-derived MSCs was found to be a potential source of MSCs because the cells exhibited better survival, proliferation and differentiations potentials [11, 12, 14-16]. More importantly, several studies showed that hiPSCs-MSCs had hepatoprotective effects in hepatic I/R injury [13, 36]. However, the direct use of hiPSCs-MSCs is limited due to the problems like potential immunological rejection, chromosomal variation, and so on. As exosomes are considered to be an important mediator of hiPSCs-MSCs, the therapeutic effects of hiPSCs-MSCs derived exosomes have also been investigated extensively in various disease models, and the results revealed that they could reduce the size of myocardial infarctions and the hepatic damage after I/R injury [13, 18, 28, 34]. Recently, Kate et al. showed that administration of hiPSC-MSCs-Exo immediately after reperfusion exhibited hepatoprotective effects [13]. Similarly, in our study, we also observed a protective effect of hiPSC-MSCs-Exo on hepatic I/R injury. Furthermore, we found that hiPSC-MSCs-Exo could promote hepatocytes proliferation. Consistently, another report also showed that exosomes isolated from mesenchymal stem cells increased hepatocytes proliferation after liver injury induced by carbon tetrachloride [37]. Thus, both results suggest that hiPSC-MSCs derived exosomes are able to trigger homeostatic liver protection after injury.

Our results demonstrate that the hepatoprotective and proliferative effects of hiPSC-MSCs-Exo are mediated by increased S1P formation and activated SK signaling pathway. Importantly, our results suggest that hiPSC-MSCs-Exo can fuse with hepatocytes and activate S1P-SK pathway intracellularly. In contrast, some other studies found that adding extracellular S1P to hepatocytes resulted in cell death *in vitro* and liver regeneration *in vivo*. But intracellular S1P has also been shown to promote cell proliferation in a variety of cell types [38-41]. These studies found that hiPSC-MSCs derived exosomes activated the synthetic machinery for S1P production in target hepatocytes and promoted their proliferation, which is consistent with our results.

In conclusion, we are the first to describe the hepatoprotective and proliferative effects of hiPSC-MSCs derived exosomes on hepatocytes after I/R injury, both *in vitro* and *in vivo*. Furthermore, we investigated the molecular mechanisms of these effects and found that exosomes could intracellularly activate the generation of S1P and SK1 activity in target hepatocytes. These findings suggest a novel mechanism for liver protection against I/R injury, and indicate a potential therapeutic approach for a variety of liver diseases and liver transplantation.

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Disclosure Statement

All authors declare no conflicts of interest.

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