Bone marrow-derived mesenchymal stem cells attenuate acute liver injury and regulate the expression of fibrinogen-like-protein 1 and signal transducer and activator of transcription 3

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Abstract. In recent years, bone marrow-derived mesenchymal stem cells (BMSCs) have been demonstrated to exert extensive therapeutic effects on acute liver injury; however, the underlying mechanisms of these effects have remained to be elucidated. The present study focused on the potential anti-apoptotic and pro-regenerative effects of BMSCs in D-galactosamine (D-Gal) and lipopolysaccharide (LPS)-induced acute liver injury in rats. An experimental rat acute liver injury model was established by intraperitoneal injection of D-Gal (400 mg/kg) and LPS (80 μg/kg). BMSCs and an identical volume of saline were administered via the caudal vein 2 h after the D-Gal and LPS challenge. Subsequently, the serum samples were collected to detect the levels of alanine aminotransferase and aspartate aminotransferase. Hematoxylin and eosin staining, terminal deoxynucleotidyl transferase-mediated nick-end labeling assay and immunohistochemical staining were performed to determine apoptosis, regeneration and histological changes of liver sections. Western blotting and reverse transcription-quantitative polymerase chain reaction were performed to detect the protein and mRNA expression levels of fibrinogen-like-protein 1 (FGL1), phosphorylated signal transducer and activator of transcription 3 (p-STAT3), STAT3 and B-cell lymphoma 2 (Bcl-2) and Bcl-2 associated X protein (Bax) in liver tissue samples. The results indicated that intravenous transplantation of BMSCs significantly decreased the levels of alanine aminotransferase and aspartate aminotransferase, and reduced hepatocellular necrosis and inflammatory cell infiltration. Additionally, a terminal deoxynucleotidyl transferase-mediated nick-end labeling assay and immunohistochemical staining revealed that BMSC treatment reduced hepatocyte apoptosis and enhanced liver regeneration. Furthermore, Bcl-2 expression was increased, whilst the protein expression of Bax was reduced. The expression of FGL1 and p-STAT3 were elevated concurrently with the improvement of liver function. These results demonstrated that BMSCs may provide a promising potential agent for the prevention of acute liver injury via inhibition of hepatocyte apoptosis and acceleration of liver regeneration. The mechanism may be, a least in part, a consequence of the upregulation of FGL1 expression and the induction of STAT3 phosphorylation.

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Introduction

The liver is vulnerable to tissue injury due to its anatomical position as well as its function, which involve exposure to various aberrant factors containing hepatotropic viruses, ethanol (1) and medicines (2), and may result in liver injury. Acute liver injury is characterized by liver dysfunction and may progress into liver failure in the absence of intervention at an early stage (3). Once this dysfunction is aggravated and exacerbated into liver failure, it becomes a disease associated with high rates of mortality, often requiring liver transplantation (4). However, liver transplantation is limited due to the critical shortage of liver donors, high economic costs and risk of transplant rejection (5). In recent years, cellular therapy has been demonstrated to be a prospective treatment for acute liver injury, in animal experiments and cellular level investigations, through resisting hepatocyte apoptosis and promoting liver tissue regeneration (6,7).

Bone-marrow-derived mesenchymal stem cells (BMSCs) have been reported to be able to treat numerous types of disease in animals, including acute kidney failure (8), ischemic heart disease (9), lung injury (10) and intracerebral hemorrhage (11), based on their biological characteristics. Multiple studies have demonstrated that BMSCs are able to migrate to impaired

tissues in response to damage, including necrosis (12), hypoxia (13) and ischemia (14). The mechanisms by which BMSCs alleviate acute liver injury involve challenges. It has primarily been reported that BMSCs are able to differentiate into hepatocyte-like cells and thereby promote liver tissue recovery and healing (15). However, there is increasing awareness of the contribution of BMSC-induced generation of trophic molecules by paracrine action in the alleviation of acute liver injury. BMSCs migrate to the injured tissue site and secrete a broad range of cytokines, growth factors and chemokines, which exert anti-necrotic, anti-apoptotic and anti-inflammatory effects under injury conditions (16-18). However, the specific molecular mechanisms underlying the involvement of BMSCs in acute liver injury have not been sufficiently explored.

Recently, Xagorari *et al* (19) demonstrated that mesenchymal stem cell-conditioned medium enhanced the expression of fibrinogen-like-protein 1 (FGL1) in cultured mouse hepatoycytes, following the induction of acute liver injury. FGL1 (also termed FREP1 or hepassocin) is a protein secreted by hepatocytes, which is a member of the fibrinogen family (20). Studies have shown that FGL1 is implicated in liver injury as a mitogenic growth factor and may facilitate damaged liver tissue regeneration and downregulate the expression of proapoptotic factors (21-23). In the present study, it was hypothesized that intravenous transplantation of BMSCs may increase the expression of FGL1 in the injured rat liver and rescue the liver following acute injury. By determination of dynamic changes in B cell lymphoma 2 (Bcl-2) and Bcl-2 associated X protein (Bax) expression, as well as hepatocyte apoptosis, the effect of BMSCs on apoptosis following acute rat liver injury was investigated. The expression of proliferating cell nuclear antigen (PCNA) in liver tissues was also evaluated in order to determine the role of BMSCs in liver regeneration. To further investigate the mechanism of BMSCs in the treatment of acute liver injury, FGL1, phosphorylated signal transducer and activator of transcription 3 (p-STAT3) and STAT3 protein expression levels were detected. The results of the present study may lay the foundation for further exploration of the mechanisms underlying BMSC transplantation in the treatment of acute liver injury.

Materials and methods

Animals. Seventy-six male specific pathogen-free (SPF) Sprague-Dawley (SD) rats (8 weeks old; 250-300 g) were acquired from the Shanghai Laboratory Animal Center (Shanghai, China). All rats were fed under SPF conditions and had free access to food and water in a standardized laboratory. The rats were housed at 20-26˚C and 40-70% relative humidity, and underwent a 12 h/12 h light/dark cycle. Experiments and procedures were approved by the Institutional Animal Committee of Wenzhou Medical University (Wenzhou, China).

Isolation and culture of BMSCs. The isolation of BMSCs was conducting using the adherence method (24,25). Four healthy male SPF SD rats (8 weeks old) were sacrificed by intraperitoneal injection of 10% chloral hydrate (3 ml/kg; Shanghai Chemical Reagent Co., Ltd., Shanghai, China) and placed in 70% alcohol for sterilization prior to the dissection of the bilateral femur and tibia. The adjunctive muscle and soft tissue were stripped under axenic conditions and the marrow cavity was flushed slowly with a 10-ml injection syringe filled with α-minimum essential medium (α-MEM; Thermo Fisher Scientific, Waltham, MA, USA). The mixture was filtered through a 200-mesh screen filter (Shanghai Rego Biotechnology Co., Ltd, Shanghai, China) and the filtered solution was centrifuged at 206 x g for 5 min (Thermo Fisher Scientific). The supernatant was discarded and cells were resuspended in liquid culture medium with α-MEM containing 10% fetal bovine serum (Gibco Life Technologies, Grand Island, NY, USA). Cells were seeded in 50-cm^2 flasks (Corning Inc., Corning, NY, USA) at 37˚C in a 5% CO_2 humidified atmosphere. Following 48 h of incubation, cells which were not adherent were washed away with sterile saline solution. Subsequently, the medium was changed every 3-4 days. The cultured cells were digested with 0.25% trypsin (Gibco Life Technologies) with a passage ratio of 1:2 and the third generation of BMSCs was sent to the Institute of Medical Sciences of The First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) for flow cytometric analysis (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). The forth generation of BMSCs were digested by 0.25% trypsin (Gibco Life Technologies) and used to generate a cell suspension in 9% sodium chloride solution (Shanghai Chemical Reagent Co., Ltd.) with a density of $1.0x10^7$ cells/ml for experimental use.

Flow cytometric analysis. The third generation of BMSCs were harvested and incubated with the following phycoerythrin-conjugated antibodies: CD45 (cat. no. 202207), CD29 (cat. no. 102207), CD11b (cat. no. 101207), and CD90 (cat. no. 205903) (BioLegend, Inc., San Diego, CA, USA) at room temperature for 15 min. The cells were subsequently washed twice with PBS (Gibco Life Technologies) and were sent to the Institute of Medical Sciences of The First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) for flow cytometric analysis (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Groups and experimental design. The remaining 72 SD rats were randomly divided into two groups (the control group and the BMSCs treatment group; n=36 per group, 12 per time point). The rats of the two experimental groups were injected with lipopolysaccharide (LPS; 80 ug/kg; Sigma-Aldrich, St. Louis, MO, USA) and D-galactosamine (D-Gal; 400 mg/kg; Sigma-Aldrich), and two hours following this injection, rats in the BMSC treatment group were injected with 0.5 ml ($5.0x10^6$ cells) BMSC suspension via the tail vein and the control group were injected with an identical volume of saline. At 24, 72 and 120 h following the injection of BMSCs or saline, rats were sacrificed by intraperitoneal injection of 10% chloral hydrate (3 ml/kg), blood samples and liver tissues were prepared for further experiments.

Detection of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). In order to determine the degree of liver injury, the levels of serum transaminase were measured. Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (1.5 ml/kg), and 2 ml blood was collected

via the inferior vena cava. Following standing for 3 min, the samples were centrifuged at 2,400 x g for 10 min and the upper serum was kept and stored in a -80˚C deep-freezer (Thermo Fisher Scientific). The levels of AST and ALT in the serum were measured with an automatic biochemistry analyzer (KT6HB560; Abbott Laboratories, Chicago, IL, USA).

Histological analysis. Liver tissues were fixed in 4% neutral formaldehyde solution (Shanghai Sangon Biotechnology Co., Ltd., Shanghai, China) embedded in paraffin (Shanghai Sangon Biotechnology Co., Ltd.), cut into 5-μm sections and viewed under an optical microscope (Nikon Eclipse; Nikon Corp., Tokyo, Japan) to observe pathological changes following staining with hematoxylin and eosin (Shanghai Chemical Reagent Co., Ltd).

Detection of apoptotic hepatocytes in liver tissues. A terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Roche Diagnostics, Basel, Switzerland) was used to detect the apoptosis of hepatocytes in stained tissue slices. According to the operating instructions, tissue sections embedded with paraffin were routinely dewaxed, rehydrated and pretreated with proteinase-K. Following immersion in methanol, the endogenous peroxidase activity was blocked. Subsequently the TUNEL reaction mixture and converter-peroxidase were added. Following staining of the tissue slices with 3,3'-diaminobenzidine (DAB), the number of apoptotic cells was calculated in five randomly selected areas under x400 magnification. Photographs were captured using a digital image-capture system (Nikon Eclipse LV100D).

Immunohistochemical analysis of PCNA. Paraffin-embedded slices were dewaxed, rehydrated and treated with 3% hydrogen peroxide (Shanghai Sangon Biotechnology Co., Ltd.). Following 10 min of incubation, the slices were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) in Tris-buffered saline Tween (TBST; Shanghai Sangon Biotechnology Co., Ltd.), and incubated with the primary rabbit monoclonal antibody against PCNA (1:16,000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C overnight. Subsequently, the samples were incubated with peroxidase-conjugated secondary monoclonal antibody (PV-9001; dilution 1:10,000; Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China) at 37˚C for 20 min. DAB then served as the chromogen and hematoxylin as the counterstain. PBS was used as the negative control, instead of the primary antibody. Sections were examined under a microscope, and nuclei exhibiting a brown color (regardless of staining intensity) were considered to be positive. The number of apoptotic cells was calculated in five randomly selected areas under a microscope (Nikon Eclipse LV100D; magnification, x400).

Protein isolation and western blot analysis. Total protein was isolated using lysis buffer (1 ml radioimmunoprecipitation pyrolysis liquid, 10 μl phenylmethanesulfonylfluoride and 10 μl sodium orthovanadate activating agent; Roche Diagnostics) accompanied by a protease inhibitor cocktail obtained from Roche Diagnostics. Samples were placed in an ice bath for 30 min and centrifuged at 16,000 x g for 10 min, the supernatant was subsequently collected and stored at -80˚C for preservation, and the protein concentration was measured using a bicinchoninic acid protein assay kit (ProteinTech, Wuhan, China). The samples (8 μg protein each) were subjected to 10% SDS-PAGE following heat denaturation at 95˚C for 5 min. Subsequently, the target proteins in the gel were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the membranes were blocked with 5% BSA for 1.5 h at room temperature. The membranes were incubated with the following primary antibodies: Rabbit polyclonal immunoglobulin G (IgG) anti-FGL1 (sc-55957-R; dilution 1:1,000), mouse monoclonal anti-Bax (sc-7480; dilution 1:200), mouse monoclonal anti-Bcl-2 (sc-7382; dilution 1:200), mouse monoclonal anti-p-STAT3 (sc-8059; dilution 1:1,000), rabbit polyclonal anti-STAT3 (sc-482; dilution 1:1,000) and mouse monoclonal anti-GAPDH (sc-365062; dilution 1:5,000) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C overnight. Subsequently, the membranes were washed four times with TBST for 7 min. Following incubation with the HRP-conjugated goat anti-rabbit and anti-rat IgG secondary antibodies (Santa Cruz Biotechnology, Inc.) at room temperature for 1.5 h, the membranes were washed as mentioned above and visualization was performed. Briefly, enhanced chemiluminescence substrate (Pierce Biotechnology, Inc., Rockford, IL, USA) was added to the PVDF membranes. The images were then captured on X-ray film (Eastman Kodak, Rochester, NY, USA), and protein quantification was determined using Gel Pro Analyzer 4 image analysis software (Media Cybernetics, Inc., Rockville, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (qPCR) analysis of FGL1 mRNA expression in liver tissues. Total RNA was extracted using RNAiso Plus reagent (Aidlab Biotechnologies Co., Beijing, China) according to the manufacturer's instructions. To measure the concentration and purity of the RNA, optical density at 260/280 nm was assessed. The primer design was based on the National Institutes of Health Biotechnology Information Center Primer BLAST set (www.ncbi.nlm.nih.gov/tools/primer-blast/) and was conducted by Beijing Keno Leip Biological Technology (Beijing, China). In order to synthesize cDNA, the Power RT kit (Bioteke Corp., Beijing, China) was used, and RT-PCR was conducted on the ABI 7500 Real-Time PCR System (Applied Biosystems Life Technologies, Carlsbad, CA, USA) with SYBR Green fluorescent dye (Toyobo Co., Ltd., Osaka, Japan). The reaction conditions for qPCR were as follows: 95˚C for 2 min, followed by 40 cycles at 95˚C for 20 sec, 60˚C for 30 sec and 72˚C for 30 sec, and a final extension step at 72˚C for 10 min. The relative mRNA abundance was determined using the standard curve method (26,27). The data were analyzed using the $2^{-\Delta\Delta CT}$ method (26,27). The primers used for RT-qPCR were as follows: FGL1 (144 bp) forward, 5'-GGTGGGCTAGTCACCAAACA-3' and reverse, 5'-CCTTGGTAGTACACGCCGTT-3'; β-actin (207 bp) forward, 5'-CACCCGCGAGTACAACCTTC-3' and reverse, 5'-CCCATACCCACCATCACACC-3'.

Statistical analysis. SPSS 19.0 statistical software (IBM SPSS, Armonk, NY, USA) was used for statistical analyses

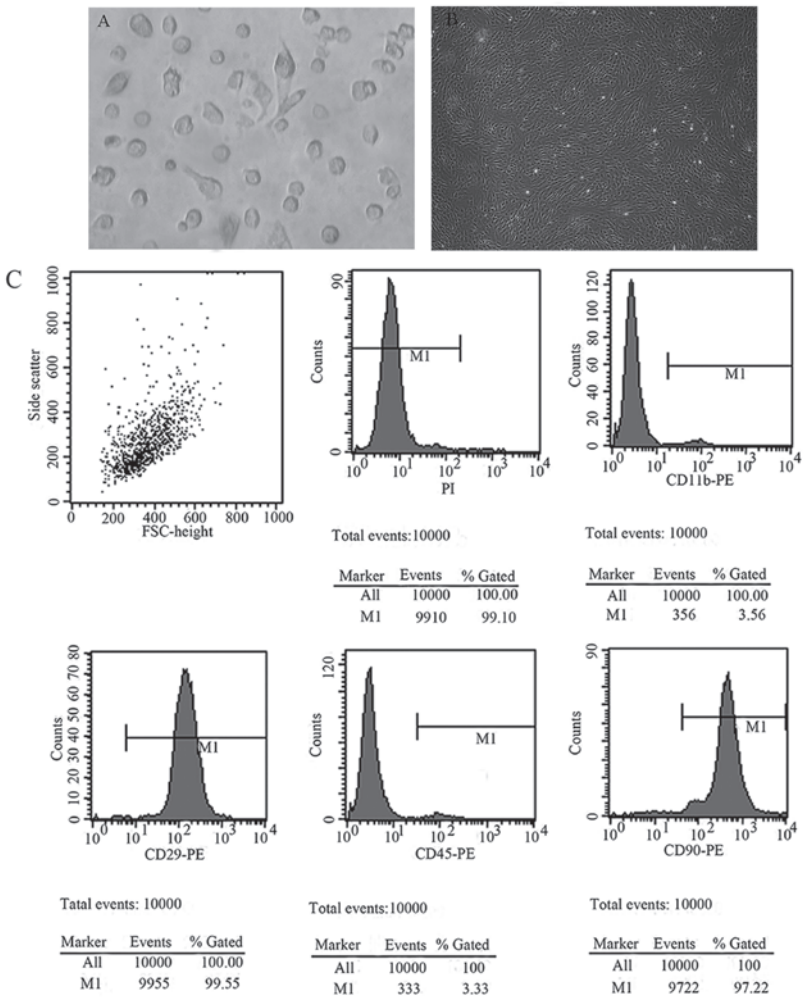

Figure 1. Characterization of BMSCs. (A) BMSCs following three days of culture (F0; magnification, x400). (B) BMSCs in the third generation (F3; magnification, x100). (C) Characterization of rat BMSCs by flow cytometric analysis. BMSCs, bone marrow-derived mesenchymal stem cells; PE phycoerythrin.

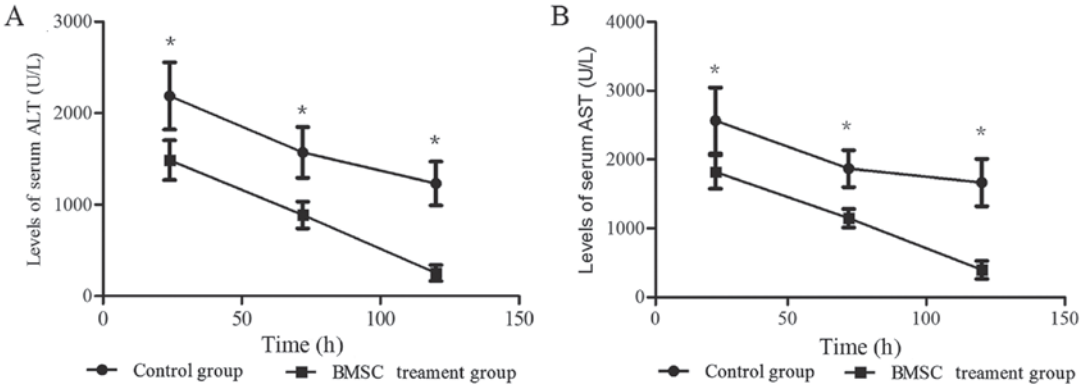

Figure 2. Levels of serum ALT and AST in the control and BMSC treatment groups. (A) ALT levels in peripheral blood samples. (B) AST levels in peripheral blood samples. *P<0.05. Values are presented as the mean ± standard deviation. BMSC, bone marrow-derived mesenchymal stem cell; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

and values are expressed as the mean ± standard deviation. One-way analysis of variance was employed for statistical comparisons amongst all groups. P<0.05 was considered to indicate a statistically significant difference. The comparative C (T) method (26,27) was used to estimate the gene expression fold-change.

Results

Characterization and identification of BMSCs. The extraction of BMSCs was conducted using the adherence method. Following cultivation of primary BMSCs for three days, a small number of cells had adhered to the culture flasks, which

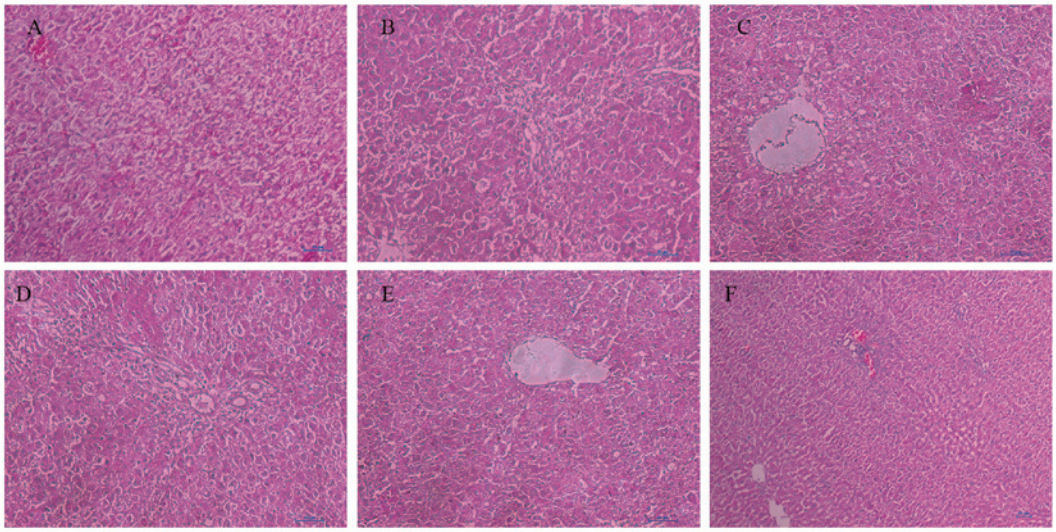

Figure 3. Histological evaluation of liver sections by hematoxylin and eosin staining. (A-C) Control group following 24, 72 and 120 h of incubation, respectively. (D-F) Bone marrow-derived mesenchymal stem cell treatment group following 24, 72 and 120 h of incubation, respectively. Magnification, x200.

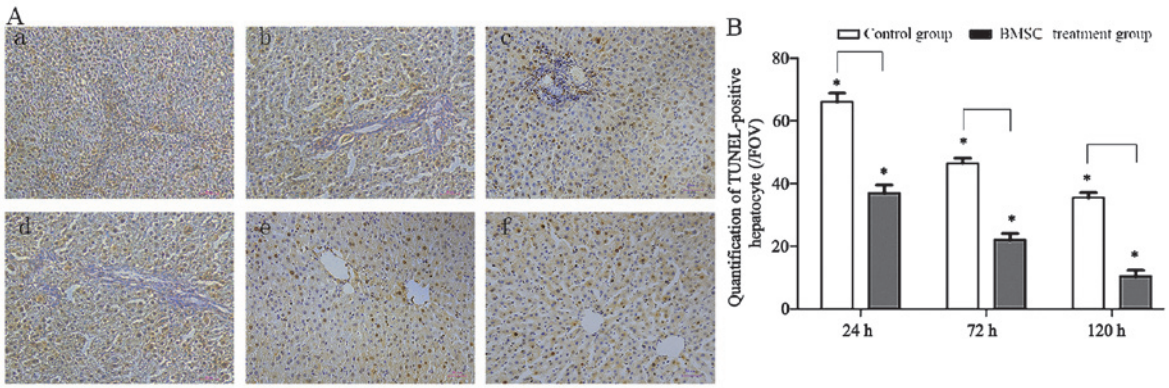

Figure 4. Analysis of apoptosis by TUNEL assay. (A) TUNEL assay of the liver tissues: (a-c) Tissues of the control group 24, 72 and 120 h following treatment, respectively; (d-f) tissues of the BMSC treatment group 24, 72 and 120 h following treatment, respectively. Magnification, x200. (B) Quantification of TUNEL activated cells. Values are presented as the mean ± standard deviation; *P<0.05. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; BMSCs, bone marrow-derived mesenchymal stem cells; FOV, field of view.

mainly exhibited a short spindle facade (Fig. 1A). Following seven days of culture, cells continuously extended and fused into a single-layered appearance, and most cells displayed a long fusiform or polygon appearance when observed under a microscope. Upon extension to the third generation (Fig. 1B), the morphology of the cells and the light-admitting quality of the cytoplasm were altered and the cells appeared similar to fibroblasts, exhibiting a spiral exterior. The third generation of BMSCs was evaluated by flow cytometry, the results of which indicated that the cells highly expressed CD90 and CD29, whereas the expression levels of CD11b and CD45 were low, demonstrating that BMSCs with a high purity status had been produced (Fig. 1C).

BMSCs inhibit the release of liver enzymes and reduce liver injury. When the population of dead liver cells overcome the regenerating cells during acute liver injury, the liver function becomes out of balance. The levels of ALT and AST released into the peripheral blood indicate the degree of liver damage. In the two experimental groups, the levels of ALT (Fig. 2A) and AST (Fig. 2B) peaked at 24 h. However, the levels of ALT and AST were significantly decreased in the BMSC treatment group at all time-points (Fig. 2), and the minimum levels of ALT and AST in the BMSC treatment group were markedly decreased by 79% (P<0.05) and 76% (P<0.05) compared with that of the control group at 120 h, respectively. These results indicated that treatment with BMSCs induced a marked decrease in liver enzyme release and demonstrated that the intravenous transplantation of BMSCs may ameliorate the function of the liver in an acute liver injury rat model.

In order to observe the potential effects of BMSC transplantation, histological changes in transplanted and control liver tissues were evaluated by HE staining. The results indicated that the liver tissues of the control group exhibited pronounced congestion and pericentral necrosis of a large area, characteristic of lobular disorder, which was accompanied by significant inflammatory cell infiltration (Fig. 3A). In addition, it was observed that the liver tissues of the control group presented time-dependent injury recovery (Fig. 3B and C); this may be due to the unique regenerative capacity of the liver,

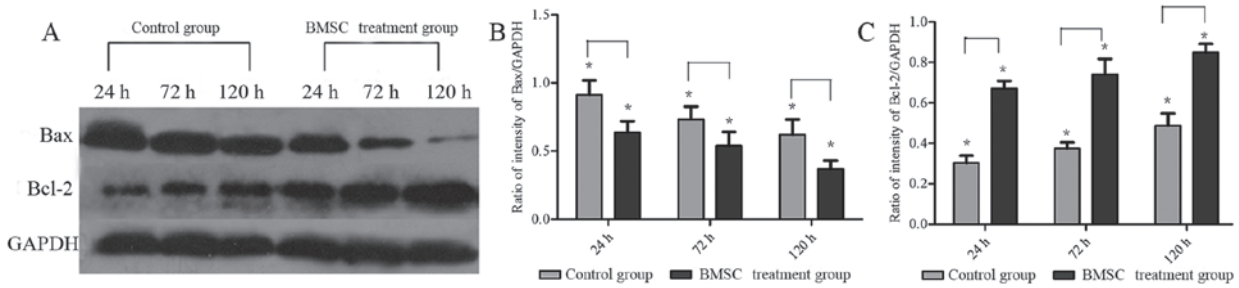

Figure 5. Protein expression of Bax and Bcl-2 in liver tissues. (A) The expression levels of Bax and Bcl-2 proteins were evaluated by western blotting. (B) Quantification of Bax protein expression. (C) Quantification of Bcl-2 protein expression. Values are presented as the mean ± standard deviation; *P<0.05. GAPDH was used as an internal control. BMSCs, bone marrow-derived mesenchymal stem cells; Bcl-2, B cell lymphoma-2; Bax, Bcl-2-like protein 4; FOV, field of view.

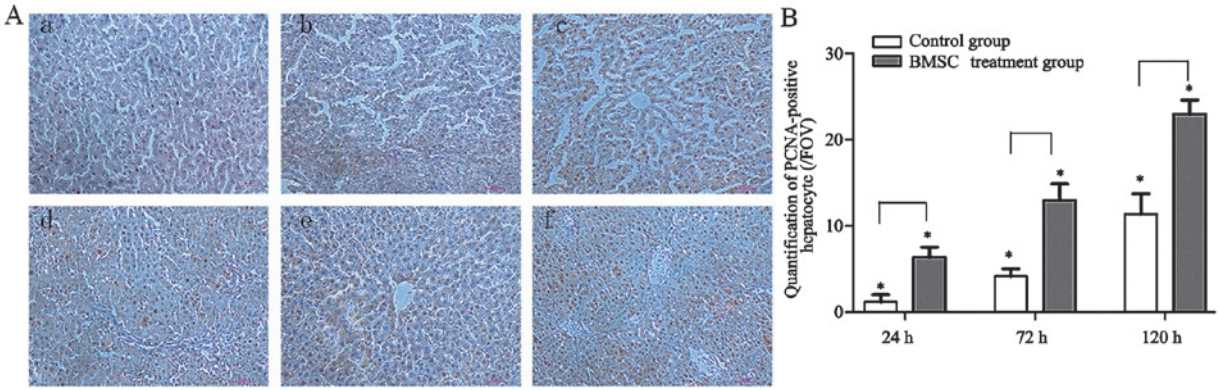

Figure 6. Immohistological staining for PCNA-active nuclei. (A) Immohistological staining of (a-c) control group following 24, 72 and 120 h of treatment, respectively; and (d-f) BMSC treatment group following 24, 72 and 120 h of treatment, respectively; (magnification, x200). (B) Quantification of PCNA-reactive hepatocytes. Values are presented as the mean ± standard deviation; *P<0.05. BMSCs, bone marrow-derived mesenchymal stem cells; FOV, field of view; PCNA, proliferating cell nuclear antigen.

meaning that the damaged liver may develop spontaneous recovery. However, these pathomorphological changes were further attenuated by intravenous transplantation of BMSCs, compared with those at the same time-point in the control group. Large tracts of land infiltration of inflammatory cells and necrosis were relieved following the BMSC treatment (Fig. 3D-F).

BMSCs inhibit hepatocellular apoptosis and regulate the expression of apoptosis-associated proteins. In order to verify whether treatment with BMSCs was able to reduce cell apoptosis, a TUNEL assay was performed. The hepatocyte nuclei tissue sections which reacted to TUNEL were calculated according to the aforementioned method. In the control group, numerous sections exhibiting large, apoptotic hepatocyte nuclei were detected (Fig. 4Aa-c); whereas, few were observed in the BMSC treatment group, and this number also significantly decreased with time (Fig. 4Ad-f). In addition, comparisons of the number of TUNEL-reactive hepatocytes between the two groups at the same time-point (Fig. 4B), revealed that the differences were significant at 24 (P<0.05), 72 (P<0.05) and 120 h (P<0.05). Calculation of the number of TUNEL-reactive hepatocyte nuclei 24 (37±2.5/FOV), 72 (22±2.0/FOV) and 120 h (10.5±1.8/FOV) following intravenous transplantation of BMSCs indicated that the number of apoptotic hepatocyte nuclei was reduced compared with that of the control group.

The expression levels of apoptosis-associated proteins were detected by western blotting (Fig. 5A). As shown in Fig. 5B, in the control group, the protein expression of Bax was decreased with time, and was significantly decreased following transplantation of BMSCs. Furthermore, the Bcl-2 expression levels were significantly higher in the BMSC treatment group, compared with those of the control group (Fig. 5C). Differences between the two groups were significant at 24, 72 and 120 h post-transplantation. These results indicated that BMSCs may exert protective effects against hepatocyte apoptosis following liver injury induced by LPS and D-Gal in a rat model.

BMSCs enhance liver regeneration. The induction of endogenous repair programs is a protective mechanism of BMSCs against acute liver injury. The number of PCNA-reactive hepatocytes in the BMSC treatment group and the control group was determined in order to evaluate the proliferative effect of BMSCs in the liver. The number of PCNA-positive hepatocytes increased in a time-dependent manner in the control and treatment groups (Fig. 6Aa-c). However, fewer PCNA-positive hepatocytes were expressed in the control group at 24, 72 and 120 h, compared with that of the BMSC treatment group (Fig. 6A and B). As indicated in Fig. 6B, the number of PCNA-positive hepatocytes reached their peak 120 h following BMSC treatment. The number of proliferating hepatocytes was increased 1.71- and 1.97-fold in the BMSC

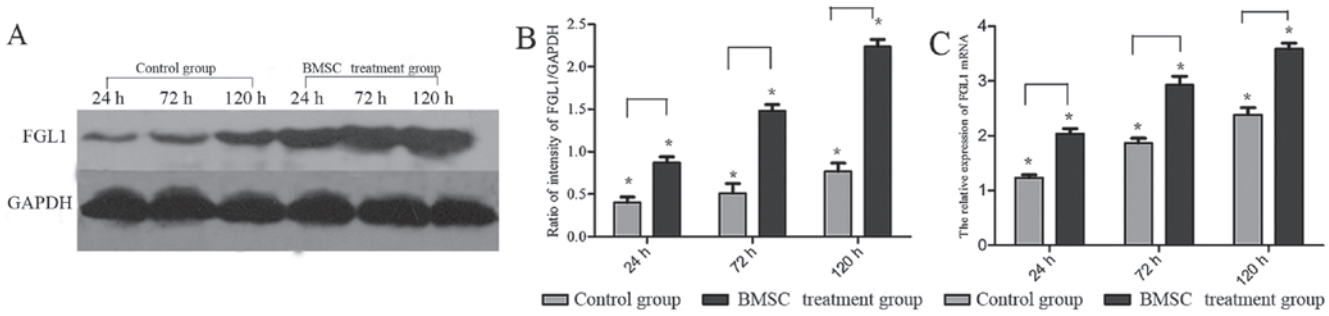

Figure 7. FGL1 protein and mRNA expression in the liver. (A) Protein expression levels of FGL1 were determined by western blotting. (B) Quantification of levels of FGL1 protein expression. (C) Relative FGL1 mRNA expression in the liver determined by reverse transcription-quantitative polymerase chain reaction. GAPDH was employed as an internal control. Values are expressed as the mean ± standard deviation; *P<0.05. FGL1, fibrinogen-like-protein 1; BMSCs bone marrow-derived mesenchymal stem cells; mRNA, messenger RNA.

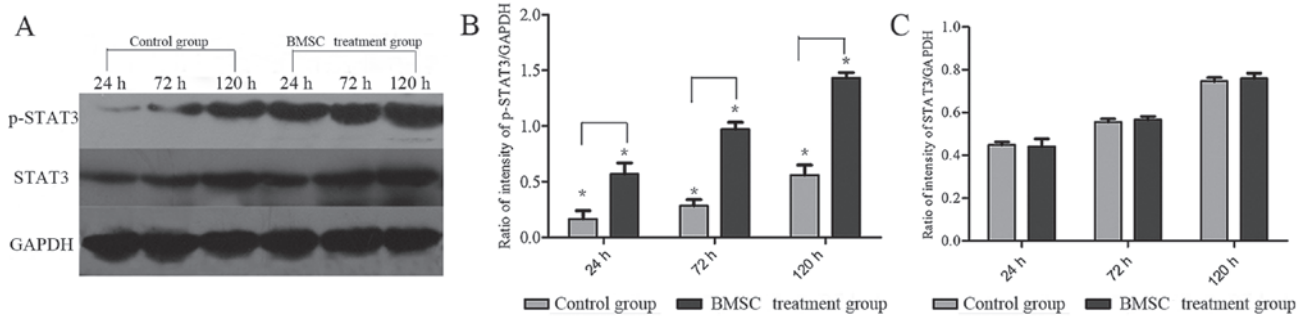

Figure 8. Levels of p-STAT3 and total STAT3 in liver tissues. (A) Protein expression levels of p-STAT3 and STAT3 were determined by western blotting. (B) Quantification of levels of p-STAT3 protein expression. (C) Quantification of levels of STAT3 protein expression. GAPDH was employed as an internal control. Values are presented as the mean ± standard deviation; *P<0.05. STAT3, signal transducer and activator of transcription 3; p-, phosphorylated; BMSC, bone marrow derived stem cell; FOV, field of view.

treatment group, compared with that of the control group at 72 and 120 h post-treatment, respectively. Furthermore, these differences were demonstrated to be statistically significant. This result indicated that BMSCs were able to enhance liver regeneration following acute liver injury in a rat model.

BMSCs enhance FGL1 mRNA and protein expression in the liver. The expression levels of FGL1 protein (Fig. 7A and B) and mRNA (Fig. 7C) were detected by western blotting and qPCR analysis, respectively. The expression of FGL1 was elevated in the control group, indicating that FGL1 activity was induced at an early stage of liver damage and elevated by spontaneous regeneration; however, the BMSC treatment group exhibited significantly higher expression of FGL1 than that of the control group (Fig. 7B) at identical time-points (24, 72 and 120 h). As revealed in Fig. 7C, following treatment with BMSCs, the level of FGL1 mRNA was significantly augmented, compared with that of the control group at the same time-point. The degrees of growth were 1.75- ($P<0.05$), 2.1- ($P<0.05$) and 2.3-fold ($P<0.05$) greater at 24, 72 and 120 h post-treatment, respectively.

BMSCs enhance p-STAT3 expression in liver tissue. To further elucidate the protective mechanisms of BMSC administration, the protein expression levels of STAT3 and p-STAT3 were evaluated by western blot analysis (Fig. 8A). The results revealed that the expression of p-STAT3 was significantly elevated in the BMSC treatment group, compared with that of the control group at the same time-point ($P<0.05$; Fig. 8B); whereas no significant difference was detected in the expression of STAT3 between the two groups (Fig. 8C).

Discussion

The results of the present study revealed that intravenous BMSC transplantation exerted a protective effect against acute liver injury through increasing proliferation and decreasing hepatocyte apoptosis. The underlying mechanism may be in part due to the enhanced expression of FGL1 and activation of STAT3 signal transduction induced by BMSCs, which may alleviate the functional deterioration of the liver induced following acute injury.

Acute liver injury, induced by viruses (28), ethanol (29) and toxicity (30), is a growing health problem worldwide, and is associated with numerous complications throughout disease progression. Accumulating evidence suggests that liver injury is characterized by sequential, extensive activation of proteins and molecules (31,32), and that maintenance of the apoptosis-regeneration balance has a vital role in modulating the process of liver recovery (33).

The potential therapeutic effects of BMSC transplantation have been verified by promising results observed in the treatment of acute liver injury (34,35). Recently, studies have demonstrated that BMSCs reinforce liver regeneration and have significant suppressive effects on hepatocyte apoptosis (36-38). BMSCs suppress proapoptotic protein expression

and enhance anti-apoptotic protein expression, consequently attenuating hepatocyte degeneration and subsequent apoptosis, as well as accelerating hepatocyte regeneration (39). Novel therapeutic approaches may be developed for the treatment of acute liver injury based on the results of the present study, which indicated that BMSCs suppressed the elevation of proapoptotic factor Bax, whilst upregulating the expression of anti-apoptotic factor Bcl-2, indicating that BMSCs were able to inhibit hepatocyte apoptosis. Furthermore, it was confirmed that BMSCs significantly enhanced the expression of FGL1, which possesses mitogenic activity in hepatocytes.

FGL1, a liver-specific protein found in humans and rats, was initially discovered in a rat model and is associated with the fibrinogen family (20). Research has indicated that FGL1 exerts multiple hepatic protective functions by promoting hepatocyte proliferation and decreasing the rate of apoptosis. A previous study indicated that administration of FGL1 to animals following D-Gal treatment significantly reversed liver injury by promoting hepatocyte proliferation and reducing liver apoptosis, while knockdown of endogenous FGL1 expression exacerbated liver injury (22,23). Concurrent with these results, the present study indicated that the level of FGL1 was enhanced 72 h following the induction of liver injury, indicating that FGL1 was activated upon induction of spontaneous liver regeneration. Compared with that of the control group, the expression of FGL1 in the BMSC treatment group at the identical time-point (24, 72 and 120 h) was significantly higher, suggesting that BMSCs may have a role in the elevation of endogenous FGL1 expression. In addition, this elevation of FGL1 was accompanied by a decrease in the level of apoptosis and enhanced PCNA expression following liver injury, which revealed that FGL1 may attenuate the effects of liver injury by preventing hepatocyte apoptosis and enhancing liver regeneration. It was therefore postulated that the intravenous transplantation of BMSCs may exert protective effects by enhancing the expression of FGL1. Furthermore, it was previously reported that FGL1 was only able to influence normal liver cells (40), likely due to the existence of the relevant receptor in normal liver cells. Hence, intravenous transplantation of BMSCs may enhance the number and/or affinity of FGL1 receptors, although this hypothesis requires further investigation. Overall, it was demonstrated that BMSCs upregulated the expression of FGL1, which has a significant role in the elevation of liver regeneration and inhibition of hepatocyte apoptosis, and ultimately attenuated acute liver injury. However, further research is required in order to elucidate the exact mechanisms underlying these effects.

Previous studies have indicated that the STAT3 signal transduction pathway is involved in the liver injury regeneration and apoptosis mechanisms (40-42). Lou *et al* (43) revealed that hepatocyte-specific STAT3-deficient mice suffered more severe liver damage in a warm ischemia/reperfusion rat model. In order to evaluate the potential mechanisms by which BMSCs ameliorate liver injury, the protein expression levels of STAT3 and p-STAT3 were examined. STAT3 and p-STAT3 expression levels were increased in the BMSC treatment and control groups with time, demonstrating that STAT3 and p-STAT3 participated in LPS- and D-Gal-induced liver injury. However, the data revealed no significant differences in the expression levels of STAT3 between the two groups, suggesting that STAT3 may not be directly associated with the therapeutic effect of BMSCs. Notably, statistical differences in the expression levels of p-STAT3 were detected between the two groups, indicating that p-STAT3 had a role in the prevention of liver damage by BMSCs. p-STAT3 is the activated form of STAT3, a result of the phosphorylation of STAT3, which forms a dimer and is transferred to the nucleus in order to modulate the expression of anti-apoptotic gene Bax, Bcl-2 and cell cycle regulatory genes, including c-fos, c-myc and cyclin, and induce the progression of the cell cycle from G_1 to S phase, indicating its role in cell proliferation and apoptosis (44). Notably, the determination of the 5' flanking region of FGL1 in humans and rats indicated that there are DNA binding sites for STAT3 which are associated with the acceleration of promoter activity of FGL1 (45). It has been demonstrated that one of the pro-proliferative mechanisms of FLG1 is to maintain STAT3 phosphorylation; however, the specific underlying cross-talk remains to be elucidated and therefore further research is required.

In conclusion, the results of the present study demonstrated that BMSCs were therapeutically effective in the treatment of acute liver injury via positive regulation of the expression of endogenous FGL1 and activation of the STAT3 signaling pathway. These properties of BMSCs merit further clinical research for the development of novel therapeutic strategies for the treatment of acute liver injury.

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