## Original Article
# Transplantation of mesenchymal stem cells expressing TIMP-1-shRNA improves hepatic fibrosis in CCl$_4$-treated rats

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**Abstract:** This study was to investigate the therapeutic effect of intravenous transplantation of TIMP-1-silencing mesenchymal stem cells (MSCs) in a rat model of liver fibrosis. MSCs were transduced with a lentiviral vector expressing tissue inhibitor of metalloproteinase 1 (TIMP-1)-shRNA, and the liver cirrhosis model was established by injection of CCl$_4$ (1 ml/kg body weight twice a week for 4 weeks) in Sprague Dawley rats. The survived 36 rats were randomly divided into 3 groups: control group, MSCs group, and TIMP-1-shRNA group. At 4 weeks after establishment of animal model, $3 \times 10^6$ MSCs were intravenously injected. In TIMP-1-shRNA group, MSCs expressing TIMP-1-shRNA were transplanted. Animals were sacrificed 4 weeks later. Blood was collected for the detection of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The livers were harvested for histological examination. At 5 days after transfection, strong fluorescence was detectable in each group. TIMP-1-shRNA group had the lowest TIMP-1 expression. Following MSCs transplantation, serum ALT and AST reduced in rats with hepatic cirrhosis, and histology showed less fibrotic areas and collagens, as compared to control group. These improvements were more obvious in the TIMP-1-shRNA group. Our study indicates that transplantation of MSCs expressing TIMP-1-shRNA is able to inhibit the progression of liver fibrosis and possibly restore the liver function in a rat model.

**Keywords:** Tissue inhibitor of matrix metalprotease-1, bone marrow mesenchymal stem cells, liver fibrosis, RNA interference

## Introduction

Liver cirrhosis is characterized by the excess accumulation of collagens and other extracellular matrix (ECM) proteins in pathology and by the clinical impairment of liver function [1]. Currently, the most effective therapy for acute liver failure and advanced hepatic cirrhosis is liver transplantation, but its use is limited because of organ donor shortage, financial considerations, and requirement for lifelong immunosuppression. Stem cell transplantation as an alternative approach has been found to be effective for the therapy of hepatic diseases [2]. Among the stem cells, mesenchymal stem cells (MSCs) in particular have practical advantages in the regenerative medicine due to their high capacity for selfrenewal, multipotent differentiation, and low immunogenicity [3].

Systemic administration of MSCs in different animal models has been found to ameliorate liver fibrosis, although the specific mechanisms involved are largely unknown. It has been proposed that the use of MSCs as vehicles of therapeutic genes might result in an enhanced amelioration of liver fibrosis [4]. In the same context, it has been described that transplantation of MSCs at multiple doses in athymic mice lead to a better outcome when compared with MSCs at a single dose [5].

The progression of liver fibrosis is a wound healing response to liver injury. Liver injury may cause the differentiation of hepatic stellate cells (HSCs), which is associated with the loss of quiescent phenotype corresponding functionally to vitamin A storage. During the hepatic fibrosis, the activated HSCs undergo continu-

ous proliferation as reflected by the expression of activation marker α-SMA. The excess ECM proteins are secreted by HSCs. Along with the change in phenotype of activated HSC; the most important roles of these cells in the hepatic fibrosis are the increased synthesis of procollagen type I, together with elevated proliferation, migration, and contractility [6-8].

ECM degradation is mediated by matrix metalloproteinases (MMPs), a class of zinc- and calcium-dependent enzymes that are secreted as zymogens and activated by the cleavage of their propeptides. Once secreted, MMPs activity is regulated by their specific inhibitors, tissue inhibitor of metalloproteinases (TIMPs) [9]. MMPs can form complexes with TIMP, which regulates the biological activities of the proteases [10]. TIMP-1 is an essential member of TIMP family and participates in a lot of pathophysiological processes by inhibiting the activation of MMPs [11]. Under the physiological condition, there is a balance between MMPs and TIMP-1, and any increase in TIMP-1 with or without reduction in MMP may result in an increase in ECM [12].

The expression of inducible TIMP-1 is fibrosis dependent and TIMP-1 may modulate the profile of fibrosis-related genes. There is evidence showing that TIMP1 is involved in hepatic fibrosis. TIMP-1 over-expression may exacerbate liver fibrosis, and hepatic fibrosis may mediate fibrosis through the TIMP-1 dependent signaling pathway [13]. The activated HSCs not only secret excess type I collagen, but markedly increase TIMP expression, leading to a shift toward excess ECM synthesis and fibrogenesis [14]. In the present study, an HIV-based lentiviral vector was used to transfect bone marrow MSCs of rats to silence TIMP-1 expression stably, and then the therapeutic effects of MSCs expressing TIMP-1-shRNA were investigated in a rat liver fibrosis model.

## Materials and methods

### Animals

Sprague Dawley rats aged 2-3 weeks were purchased from the Laboratory Animal Center of Soochow University. All animal experiments in this study were performed in according with the Guide for the Care and Use of Laboratory Animals and this study was approved by the Institutional Animal Care Committee of Soochow University.

### Preparation of bone marrow MSCs

Bone marrow was harvested by flushing the tibiae and femurs of 6-week-old Sprague Dawley rats with Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen Corp. Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone). Nucleated cells were isolated by density gradient centrifugation (Ficoll, Pharmacia) and suspended in complete medium (CM) consisting of DMEM (Gibco-Invitrogen) supplemented with 10% FBS (HyClone), and 100 U/ml penicillinestreptomycin (Gibco, Invitrogen Corp). Cells were maintained at 37°C and 5% $CO_2$ in a humidified atmosphere, and the medium was refreshed once or twice weekly. When large colonies were present (80-90% confluence), cells were washed twice with phosphate buffer saline (PBS) and digested with 0.25% trypsin-EDTA (Gibco, Invitrogen Corp. Carlsbad, CA, USA) for approximately 5 min at 37°C. After centrifugation at 2,400 rpm for 20 min, cells were suspended with CM and incubated in a culture flask Falcon.

### Detection of cell-surface markers

Cells after trypsinization were aliquoted (1×10$^5$ cells) into FACS tubes. Following FITC and phycoerythrin (PE) conjugated monoclonal antibodies were added to these cells: CD34-FITC, CD90-PE, CD73-FITC, and HLA-DR-FITC (BD Bioscience, San Jose, CA, USA), CD45-PE and CD105-FITC (Biolegend). FITC- or PE-conjugated rat IgGs were used as the control isotypes. Then, cells were incubated at room temperature in dark for 20 min. The fluorescence intensity was determined by flow cytometry (Becton & Dickinson, USA). A minimum of 10$^4$ gated events was acquired from each sample for analysis, and data were analyzed using the CellQuest program (Becton & Dickinson, USA).

### Construction of TIMP-1-shRNA lentiviral expression vector

The lentiviral vector system (Genepharma, Shanghai, China) was composed of three parts for viral packaging: LV-3-shNC (LV-3-TIMP-1-rat-246, LV-3-TIMP-1-rat-142, and LV-3-TIMP-1-rat-373). Vectors that have the correct sequence and the lentiviral packaging plasmid
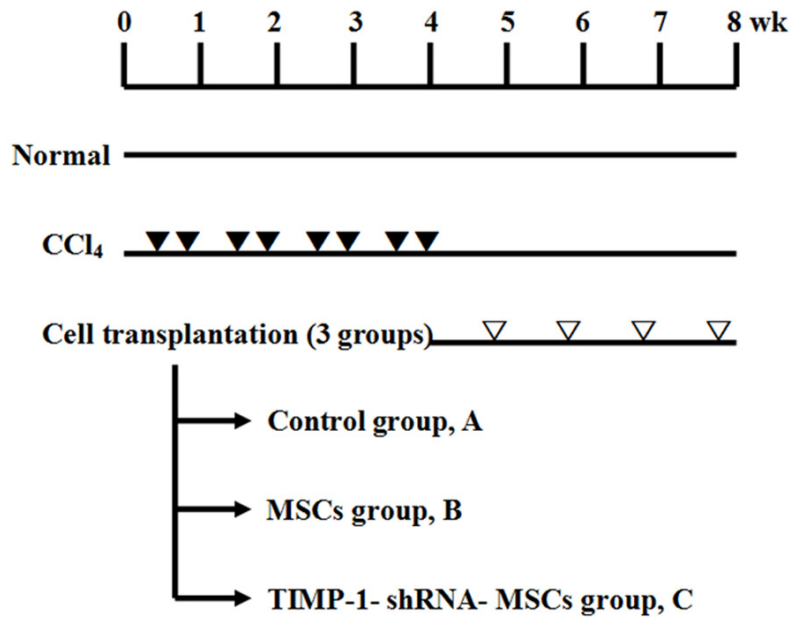
**Figure 1.** Experimental design of MSCs injection.

ase inhibitor cocktails. Protein samples were separated on 8-15% Bis-Tris gels in sodium dodecyl sulfate (SDS) and transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories, U.S.A.). The membrane was blocked in 5% skim milk/TBST (Tris buffered saline with Tween 20) for 1.5 h, and then incubated overnight at 4°C with a rat monoclonal anti-TIPM-1 antibody (1:1000; R&D). After washing in TBST thrice, the membrane was incubated with peroxidase-conjugated goat anti-rat IgG (1:2000, Proteintech), and washed in TBST thrice. Immunoreactivity was detected using an enhanced electrochemiluminescence (ECL) kit (Pierce, U.S.A.) and visualized on Kodak Omat X-ray films (Kodak, U.S.A.). The membrane was stripped and re-probed with a rabbit polyclonal anti-β-tubulin antibody (1:2000, Proteintech), as an internal reference.

*Establishment of a rat liver fibrosis model*

Hepatic cirrhosis model was established by injection of $CCl_4$ (1 ml/kg body weight twice weekly for 4 weeks) in olive oil (1:1) in Sprague Dawley rats. The survived 40 rats were randomly divided into 4 groups: normal group (n = 4), control group (A, n = 12), MSCs group (B, n = 12), and TIMP-1-shRNA- MSCs group (C, n = 12). Group A: rats received no treatment; Group B: rats were injected with MSCs ($3 \times 10^6$ cells per rat) in PBS via the tail vein; Group C: rats were injected with TIMP-1-shRNA-MSCs ($3 \times 10^6$ cells per rat) in PBS via the tail vein. All the rats were housed for 4 weeks [15] (**Figure 1**).

*Biochemical analysis and histopathologcal examination*

The blood was collected from the abdominal aorta using a 25-gauge needle and centrifuged at 3,000 rpm for 15 min. The serum was collected and processed for the measurement of ALT and AST with an automated biochemical analyzer (Beckman Coulter Power Processer,
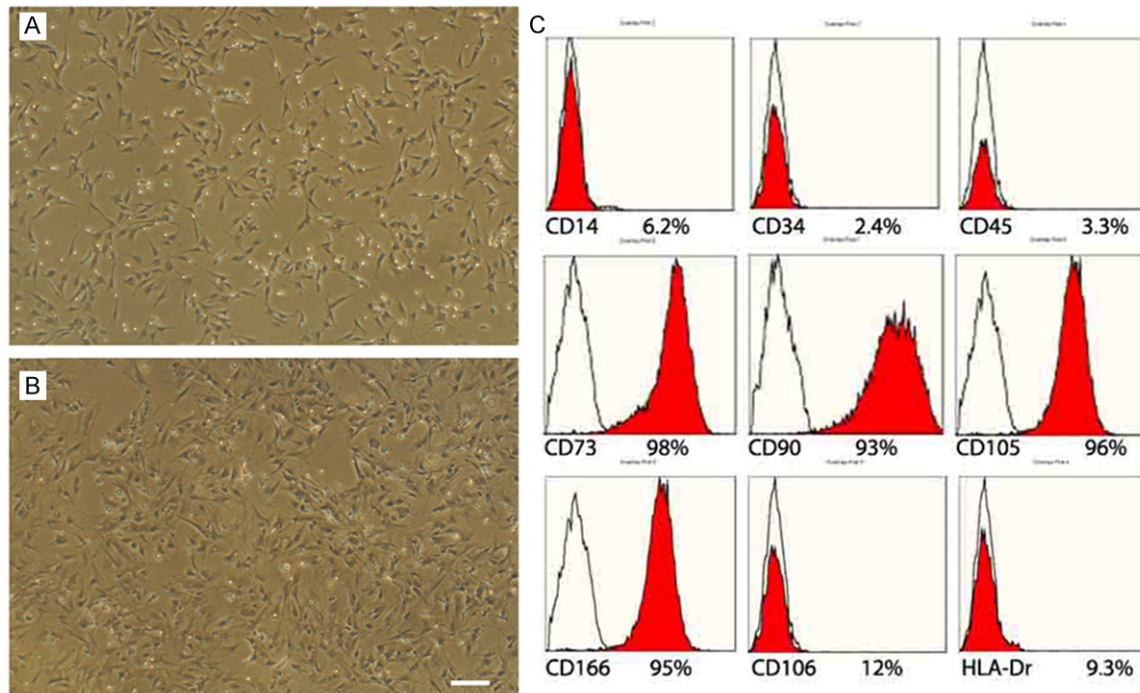
(SBI, Shanghai, China) were co-transfected into 293T cells in the presence of Lipofectamine 2000 (Invitrogen, Carlsbad CA, USA). Then, virus particles were collected from the cell supernatant following concentration, purified and concentrated. LV-3-shNC and enhanced green fluorescent protein (GFP) were constructed and packaged by Genepharma Gene Technology (Genepharma, Shanghai, China) at titers of $1 \times 10^{12}$ and $2 \times 10^{12}$ vector genomes per milliliter (v.g/mL), after purification.

*Infection of MSCs with Lentiviral Vectors*

After passaging thrice, the MSCs medium was removed and then, MSCs were incubated with LV-3-shNC or LV-GFP in special polybrene-containing (5 mg/ml) growth medium. On the day of infection, cells were plated along with LV-3-shNC or LV-GFP at different multiplicities of infection (MOI) in serum-free growth medium containing 5 mg/ml polybrene. Serum-containing growth medium was added 4 h later and the medium was refreshed 48 h later. Subsequently, at 4-5 days post-infection, reporter gene expression was examined by fluorescence microscopy. MSCs were plated in T-75 flasks and prepared for cell transplantation.

*Western blot analysis*

The total protein was extracted from the co-transfected MSCs with lysis buffer and prote-

**Figure 2.** Characterization of MSCs. A. Mixed population of epithelial cells and fibroblast-like cells were seen in MSCs in early stage (7 days). A. Fibroblast-like morphology of MSCs in late stage. B. Scale bar: 50 μm. C. Flow cytometry of undifferentiated MSCs of the third passage for CD73, CD14, CD90, CD105, CD106, CD34, CD45, CD166 and HLA-DR. Red curves indicate positive staining in the sorted population. Empty curves indicate negative background staining.

Beckman Coulter Inc. USA). At the same time, the liver was rapidly removed by dissection, washed in ice-cold physiological saline and dried. Cryostat sections (5-mm) were obtained. The liver specimens were fixed in 10% formaldehyde, embedded, sectioned and stained with hematoxylin and eosin (HE) for routine histologcal examination.

*Statistical analysis*

Data are presented as means ± standard deviation (SD). To test the significance of differences among groups, a one-way ANOVA was employed for statistical analysis. A value of $P < 0.05$ was considered statistically significant.

## Results

*MSCs have a unique morphological and molecular signature*

In the early stages of MSCs culture, two morphologically distinct populations were observed. While one consisted of fibroblastoid, spindle-shaped cells, the other was comprised of cells with an epithelioid, polygonal morphology

(**Figure 2A**). After passaging with trypsin, cells formed a monolayer of homogenous bipolar spindle like cells with a whirlpool-like array (**Figure 2B**). To characterize the isolated cells, flow cytometry was performed for cell-surface markers. Results showed that MSCs were consistently positive for CD90, CD105, CD166 and CD73, but negative for hematopoietic surface markers CD34, CD104, CD14 and CD45, as well as HLA-DR (**Figure 2C**).

*Stable TIMP-1 silencing in MSCs following transfection with lentiviral vector carrying TIMP-1-shRNA*

Three lentiviral expression vectors expressing different TIMP-1-shRNAs were independently transfected into MSCs (SH1, SH2 and SH3 groups), and cells transfected with an empty vector served as a negative control. Cells were observed before and after cell transfection. After 5-d culture, GFP (green) was observed under a fluorescence microscope (**Figure 3**). Total protein was extracted, and Western blot assay was carried out to detect TIMP-1 expression. TIMP-1 expression in SH3 group was lower than in SH1 and SH2 groups (**Figure 4**).
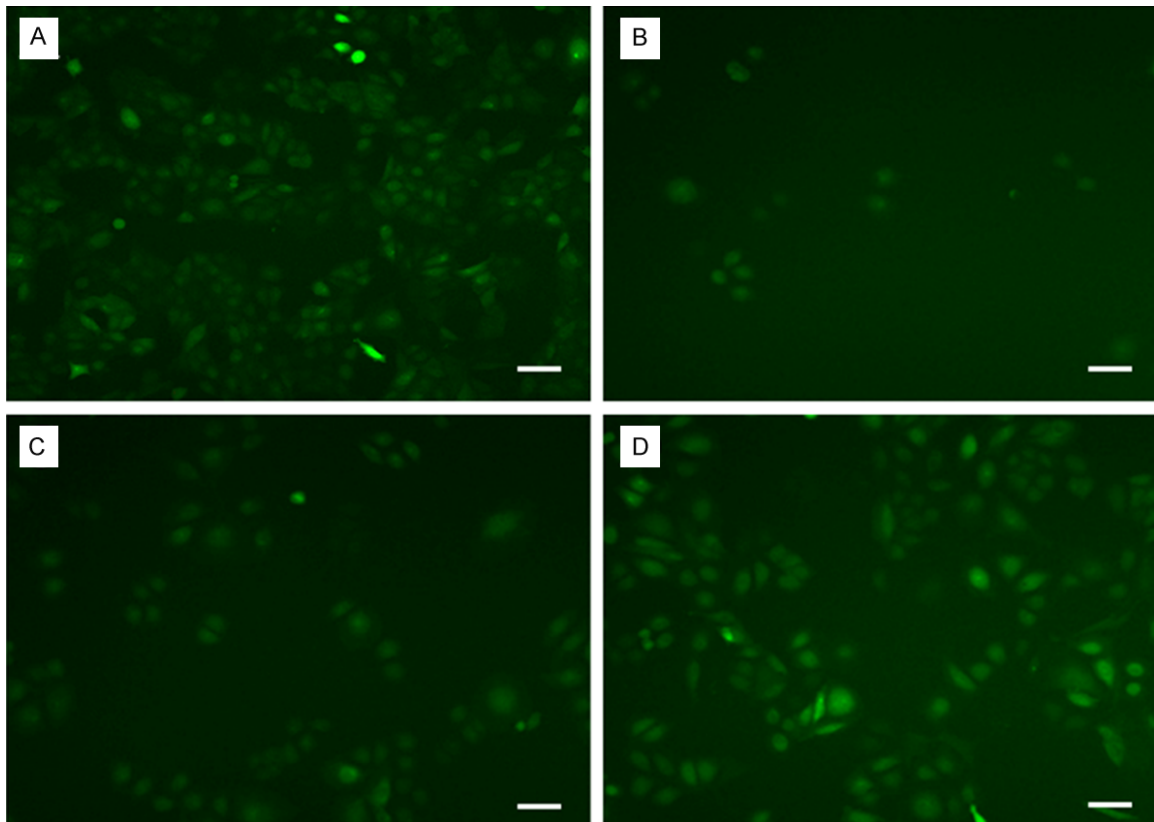
**Figure 3.** MSCs transfected with lentiviral vector expressing TIMP-1-shRNA. Lentiviral vector carrying TIMP-1-shRNA and green fluorescent protein (GFP) were transfected into MSCs (MOI = 20), and cells were observed under a fluorescence microscope. The green fluorescence was relatively strong on 7th day. A. SH1 group (LV-3-TIMP-1-rat-246); B. SH2 group (LV-3-TIMP-1-rat-373); C. SH3 group (LV-3-TIMP-1-rat-142); D. Negative control group (LV-3-shNC). (Bar = 50 μm)
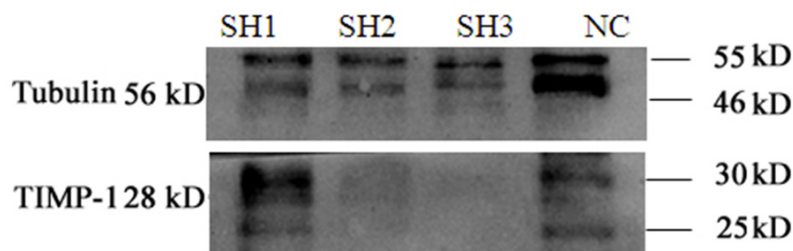


**Figure 4.** TIMP-1 protein expression. Western blot assay was employed for the detection of TIMP-1 protein expression in SH1 group (LV-3-TIMP-1-rat-246), SH2 group (LV-3-TIMP-1-rat-373), SH3 group (LV-3-TIMP-1-rat-142) and negative control group (LV-3-shNC). Tubulin served as an internal control.

*Protective effects of TIMP-1-shRNA-MSCs on hepatic fibrosis*

After transplantation, the liver was harvested for histological examinations. Green fluorescence of different degrees was observed (**Figure 5A**). HE staining showed the compromised vacuolar degeneration of the hepato-

cytes in TIMP-1-shRNA-MSCs transplanted mice as compared to control group (**Figure 5D**). The perilobular regions were the main areas affected by $CCl_4$ hepatotoxicity while the centrilobular regions seemed to be less affected. In TIMP-1-shRNA-MSCs group, the rat liver showed significantly compromised hyperplasia of collagen fibers; although the false lobules still existed, the perilobular and interlobular collagen fibers significantly reduced in the hepatic lobules, and liver ECM and their arrangement became nearly normal. **Figure 5B-D**. There were significant differences in the serum ALT and AST among healthy rats, hepatic fibrosis rats, MSCs-treated rats, and TIMP-1-shRNA-MSCs treated rats (**Figure 6**).
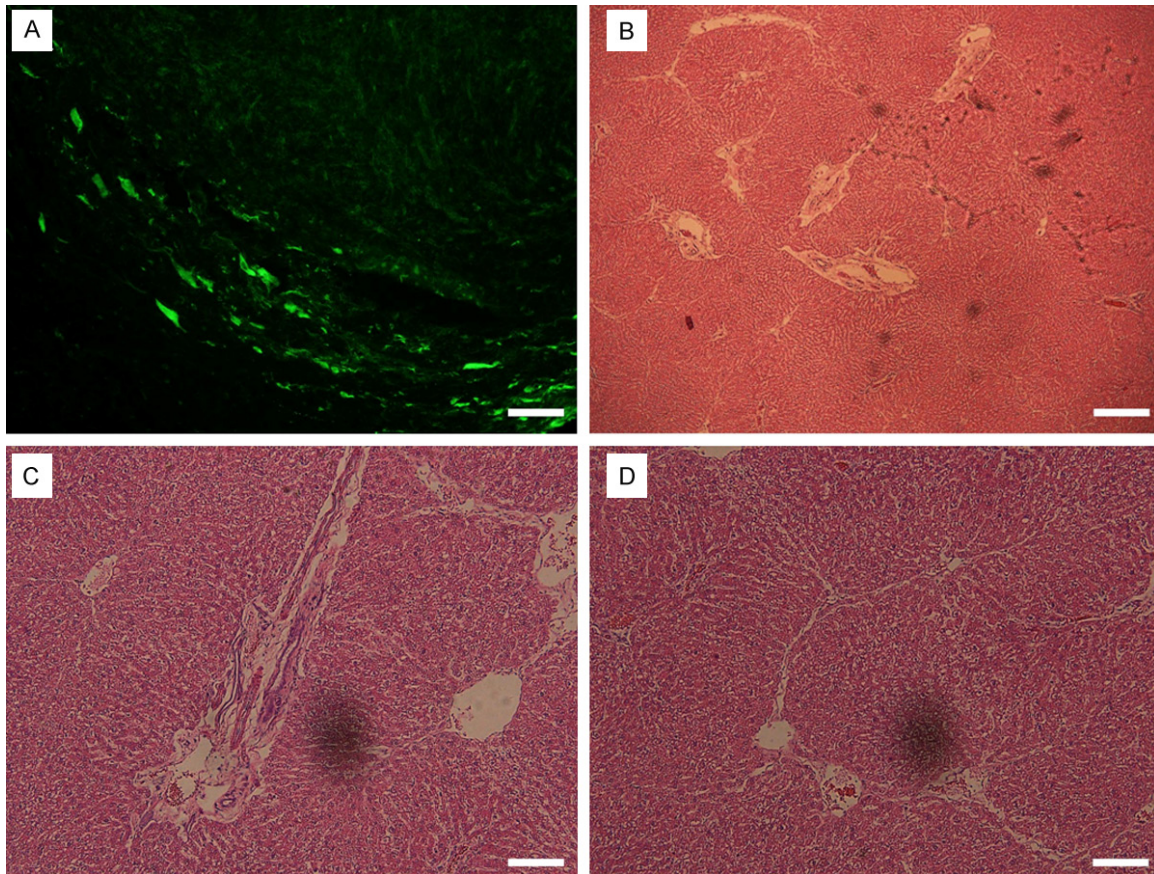
**Figure 5.** Histological examination of liver sections. Histological examination of the liver at 4 weeks after transplantation. A. MSCs expressing GFP were detectable in the rat liver at 4 weeks after transplantation in group C. B-D. HE staining of liver sections at 4 weeks after transplantation in groups A-C (A, B bar = 50 μm; C, D bar = 100 μm)
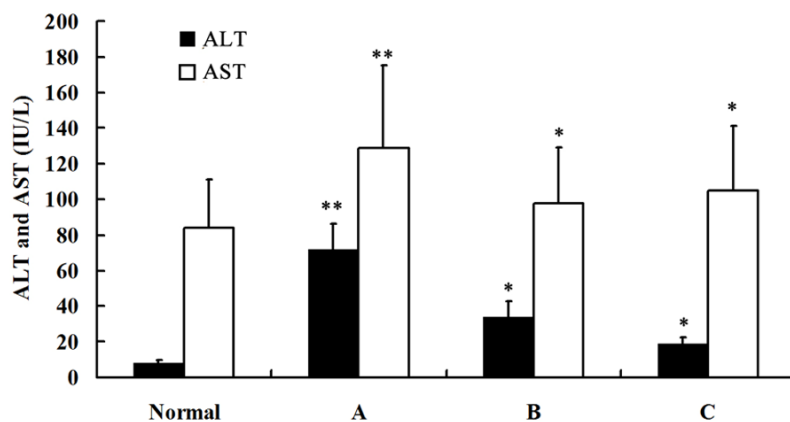


**Figure 6.** Serum ALT and AST in different groups. Serum ALT and AST were measured for the evaluation of liver function. $^{*}P < 0.05$ vs. control group; $^{**}P < 0.01$ vs. normal group. Data were from three independent detections (n ≥ 6 per group). Data are given as means ± standard deviation.

## Discussion

The present study aimed to evaluate the anti-fibrotic effects of bone marrow-derived MSCs expressing TIMP-1-shRNA in $CCl_4$-induced liver fibrosis rat model. MSCs were identified by their morphology, adherence and surface markers. Our results showed, following transfection with lentiviral vector carrying TIMP-1-shRNA, TIMP-1 expression was significantly silenced in MSCs. Moreover, transplantation with shRNA-TIMP-1-MSCs reversed the liver damage and decreased the toxicity characterized by the reduction of ALT and AST.

Liver cirrhosis is a chronic disease of the liver, characterized by the loss of functional hepatocytes and the increase in fibrous tissues, due to alcohol abuse, nutritional deprivation, or infection (especially the infection by hepatitis virus B and C [HBV and HCV]) [16]. Traditionally, $CCl_4$ is used to establish a rat

model of hepatic fibrosis. CCI$_4$ is bio-activated by cytochrome-P4502EI [17] to generate free radicals that trigger a cascade of events resulting in hepatic fibrosis. In rats with hepatic fibrosis, liver dysfunction is evident as reflected by a significant increase in serum parameters of liver function and a decrease in liver synthetic capacity.

Recently, stem cell-based therapy has been proposed as a promising alternative approach for the therapy of end-stage liver diseases. Stem cell therapies have shown promising benefits for the hepatic fibrosis in experimental and clinical studies [18-20]. MSCs originate from mesoblastic stem cells. They have self-renewal and can differentiate into various cell types, such as osteocytes, adipocytes, chondrocytes and myocytes, in different environments. MSCs are the main cell type involved in tissue repair [21]. Many studies have demonstrated that MSCs have considerable protective effects on liver injury [22, 23]. A number of mechanisms contribute to the therapeutic effects of MSCs: MSCs can differentiate into functional hepatic cells and then produce a series of growth factors and cytokines to ameliorate liver injury. In the present study, bone marrow MSCs were successfully isolated from rats, cultured and expanded in vitro, and then transplanted into hepatic fibrosis rats to investigate the protective effects of MSCs on hepatic fibrosis and the role of TIMP-1 in their protective effects. (**Figure 2**)

MSCs are multipotent adult progenitor cells used as vehicles of therapeutic genes. It has been proposed that the use of MSCs as vehicles of therapeutic genes may result in an enhanced amelioration of liver fibrosis [24]. In the present study, at 5 days after transfection with MSCs, strong fluorescence was detectable in each group; cell morphology remained unchanged after transfection. Western blot assay showed that SH3 group had the lowest TIMP-1 protein expression; transfection with lentiviral vector expressing TIMP-1-shRNA stably silenced TIMP-1 expression in MSCs (**Figures 3** and **4**). The hepatic mRNA expressions of TIMP-1 and procollagen type I have been reported to decrease significantly in the first week of spontaneous recovery from hepatic fibrosis in CCI$_4$-treated rats, which coincided with the rapid phase of collagen degradation [25]. In support of the role of TIMP-1 *in vivo*,

transgenic mice over-expressing human TIMP-1 showed deteriorated fibrosis in response to long-term CCI$_4$ administration [26]. TIMP-1 is also a contributory factor in the development of hepatic fibrosis as shown in animal models and patients [27]. TIMP-1 alone may not induce liver fibrosis, but it can significantly exacerbate the hepatic fibrosis [28].

MSCs have a significant inhibitory effect on the hepatic fibrogenesis through inhibiting the activation of HSCs. During the spontaneous recovery from hepatic fibrosis, there is a decrease in TIMP expression, an increase in collagenase activity, and an increase in apoptosis of HSCs. There is a correlation between reduction of TIMP expression and apoptosis of HSCs *in vivo*, demonstrating the crucial role of TIMP in regulating the HSCs survival. As shown in HE staining, TIMP-1-shRNA-MSCs group displayed a significant improvement of hepatic fibrosis as compared to untreated group (**Figures 5** and **6**). In addition, GFP positive transplanted cells were detectable in the injured liver tissues (**Figure 5A**). This issue is not trivial since many reports aimed at track MSCs *in vivo* have not been able to distinguish specific signals from auto fluorescence, a feature frequently observed in a fibrotic liver [29, 30]. In conclusion, MSCs may not only migrate into the injured liver tissues but differentiate into hepatocytes, contributing the regression of hepatic fibrosis. Intravenous delivery of TIMP-1-shRNA expressing MSCs will be effective to treat hepatic fibrosis.

### Acknowledgements

### Disclosure of conflict of interest

None.

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