Human Placental Mesenchymal Stem Cells Ameliorate Liver Fibrosis in Mice by Upregulation of Caveolin1 in Hepatic Stellate Cells

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Research

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Abstract

Background: Liver fibrosis (LF) is a common pathological process characterized by the activation of hepatic stellate cells (HSCs) and accumulation of extracellular matrix. Severe LF causes cirrhosis and even liver failure, a major cause of morbidity and mortality worldwide. Transplantation of human placental mesenchymal stem cells (hPMSCs) has been considered as an alternative therapy, replacing liver transplantation in clinical trials, to treat LF. However, the underlying mechanisms and the appropriate time window for hPMSC transplantation are not well understood.

Methods: We established mouse models of CCL4-injured LF, and administered hPMSCs once a week for 2 weeks. The therapeutic effect of hPMSCs on LF was investigated, according to histopathological and blood biochemical analyses. What's more, the culture supernatant of hPMSCs were isolated, and the effect of the culture supernatant on the inhibition of activated HSCs was also assessed. RNA sequencing (RNA-seq) analysis, Real-time PCR array, and western blotting were performed to explore possible signaling pathways involved in treatment of IF with hPMSCs.

Results: hPMSC treatment can alleviate experimental hepatic fibrosis, restore liver function, and inhibit inflammation. Furthermore, the therapeutic effect of hPMSCs against mild to moderate LF was significantly greater than against severe disease. Mechanistic dissection studies suggested ameliorating LF using hPMSCs occur partly by restoring Caveolin-1 (*Cav1*) in activated hepatic stellate cells (HSCs). Upregulation of *Cav1* can inhibit the TGF-β/Smad signaling pathway, mainly by reducing Smad2 phosphorylation, resulting in the inhibition of activated HSCs, whereas this effect could be abated if *Cav1* was silenced in advance by siRNAs.

Conclusions: Our findings suggest that hPMSCs could provide multifaceted therapeutic benefits for the treatment of LF, and the TGF-β/*Cav1* pathway might act as a therapeutic target for hPMSCs in the treatment of LF.

Significance Statement

hPMSCs may be used to reduce LF due to their tissue repair potential and immunomodulatory properties. Despite having a demonstrated antifibrotic effect in a model of hepatic fibrosis, the mechanisms involved in these processes remain unclear.

This study reveals that human placental mesenchymal stem cells (hPMSCs) have a therapeutic effect on liver fibrosis (LF) in a mouse model by suppressing the activation of hepatic stellate cells, and hPMSC treatment in the early stage of LF is more effective. To our knowledge, this is the first report that the TGF-β/*Cav1* pathway mediated by hPMSC treatment plays a key role in relieving LF. The present findings are extended the knowledge of the antifibrotic effects of hPMSCs and their mechanisms, and advance their potential use for treating liver diseases associated with fibrosis.

Background

Hepatic fibrosis is a reversible wound-healing response caused by many chronic liver diseases, such as viral infection, alcohol abuse, and autoimmune hepatitis. It is characterized by activation of HSCs and excessive accumulation of extracellular matrix (ECM) in the liver (1, 2). HSCs are a resident mesenchymal cell type located in the subendothelial space of Disse, and usually display a quiescent state (3). Following liver injury, HSCs are activated and transdifferentiated into the fibrogenic myofibroblasts, the major cell type that causes fibrosis and collagen synthesis. If the source of the injury is sustained, HSC activation and accumulation of ECM persist (4). Advanced LF can develop into irreversible cirrhosis, portal hypertension, and liver failure, and correlates with an increased risk of hepatocellular carcinoma (5). The available treatment methods mainly focus on inhibiting HSC activation and/or promoting ECM degradation. Although there are many drugs to treat LF, the effect is very limited and may also aggravate liver damage (1, 6). Therefore, new strategies to delay or prevent the progression of LF are urgently required.

Cell therapy is a promising approach for the treatment of liver disease, hepatocyte-based therapies have been shown to be very effective in experimental animals, but limited cell sources and low proliferation have restricted their large-scale application (7, 8). Recently, many studies have demonstrated the therapeutic potential of mesenchymal stem cells (MSCs) in liver disease (9, 10). It has recently been shown that MSCs isolated from the bone marrow, umbilical cord, adipose tissue or placenta have the capacity to migrate toward sites of injury and differentiate into hepatocyte-like cells (11, 12). Moreover, several studies have shown that MSCs can also secrete various cytokines in a paracrine manner to regulate inflammatory responses, hepatocyte apoptosis, and fibrosis, and finally restore liver function after acute injury or chronic fibrogenesis (13, 14). Along with their properties of high self-renewal, multipotent differentiation capacity, and immunosuppressive qualities (15–17), MSCs are considered to be the most suitable source of cells for cell-based therapy for LF.

The placenta is another promising source of MSCs. In contrast to autologous MSCs, including bone marrow and adipose mesenchymal stem cells, human placental mesenchymal stem cells (hPMSCs) can be easily obtained in massive numbers by a simple and painless procedure (18, 19). Furthermore, they exhibit greater self-renewal, multilineage differentiation capacity, and strong immunologic privileges (20, 21). More importantly, many studies indicate that hPMSCs have therapeutic potential in liver diseases, A previous study proved the therapeutic potential of hPMSCs in miniature pigs model of acute liver failure (22). Additional studies have shown that hPMSCs exert an anti-fibrotic effect when transplanted into rats with carbon tetrachloride (CCl_4)-injured livers by promoting hepatic regeneration via increased autophagy (23). Although the use of hPMSCs has been studied in animal models of LF, the number of MSCs transplanted and the appropriate time window as well as the mechanisms responsible for liver repair by hPMSCs are not well understood.

The aim of the present study was to investigate whether transplantation of hPMSCs reduces fibrosis in a CCl_4-injured mouse liver, and to perform a comparative analysis of the important factors involved in MSC-based cell therapy. We further analyzed the involvement of the TGF-β/*Cav1* pathway in hPMSC-mediated

anti-fibrosis activity *in vitro* and *in vivo*. Our results provide further support that hPMSCs could provide a new avenue for the treatment of LF.

Methods

Isolation of Human Placental-Derived Mesenchymal Stem Cells

Placental tissue was obtained from three health donors in the Sichuan Maternal and Child Health Hospital, upon consent of its donor according to procedures approved by the Medical Ethics Committee, Sichuan University (K2018109-1). hPMSCs were isolated and purified, the immunophenotype and differentiation potential of hPMSCs were then determined according to reported procedures (18, 21). hPMSCs were cultured in mesenchymal stem cell basal medium (DAKEWE, Beijing, China) supplemented with 5% UltraGROTM (HPCFDCRL50, Helios), and cells between passage 3 and 6 were used for all experiments.

HSCs Culture and in vitro Study

human primary hepatic stellate cells (HSCs) were provided by ScienCell Research Laboratories and cultured in Stellate Cell Medium (SteCM, San Diego, CA) supplemented with growth supplement (SteCGS). The TGF-β1 (a well-known pro-

fibrotic ligand) (20 ng/mL) mediated HSCs activation were induced after growing in DMEM with only 0.2% FBS for 24 hours, and determined by western blot analysis (Supplemental Fig. 3). To investigate the effect of hPMSCs *in vitro*, hPMSCs secretomes were harvested and concentrated 15-fold with ultrafiltration tubes (millipore), activated HSCs were cultured with concentrated hPMSCs secretomes in a gradient ratio (10%, 20%, 40%). Additionally, to exclude the affect of medium compositions, concentrated MSC complete medium also treated in the same manner, and the results were compared to the hPMSCs supernatant. Unactivated and activated HSCs without extra treatment were used as controls.

CCl_4-Injured Mice Liver Fibrosis and hPMSCs Transplantation

To induce liver fibrosis, 8 weeks old C57BL/6 mice (20 ± 2 g) were intraperitoneally injected with CCl_4 (0.5 mL/kg body weight, dissolved in olive oil, 1:9; Sigma-Aldrich) twice a week for 6 weeks (n = 20). Five mice were sacrificed every two weeks for histopathological examination and liver function test. The liver tissue sections from CCl_4-treated mice exhibited focal fibrosis, confirming the successful establishment of an animal model of liver fibrosis. No animals died during the experiments after CCl_4 administration. All experimental procedures involving animal experiments were approved by the Sichuan University Medical Ethics Committee (K2018109-2).

Time-point and cell-dose are two important parameters for cell-based regeneration medicine. 4 weeks or 6 weeks after CCl_4 treatment are two important time point for hPMSC engraftment in most previous studies (24, 25). In this study, these two time points were chosen as treatment 1 (T1) and treatment 2 (T2) respectively, and a comparative study was conducted. hPMSCs were injected into the tail vein of mice in the liver fibrosis model after 4 or 6 weeks of CCl_4 treatment, once a week for 2 weeks. Meanwhile, the different doses of hPMSCs (5×10^6 cells/kg or 2×10^6 cells/kg) were also tested in each group, as shown in Fig. 1A. The liver fibrosis group mice were injected with PBS alone. Untreated mice were treated as control. Serum and liver tissue samples were collected after hPMSCs transplantation for 2 weeks.

Western blot analysis

Total proteins were extracted from HSCs with different treatments, equal amounts of soluble protein were separated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis using 10% Tris-glycine mini-gels and transferred onto a nitrocellulose membrane (Bio-Rad).The primary antibodies were listed in supplementary Table S1, GAPDH mAb (Santa Cruz, Biotechnology) was used as an internal control. Following incubation with horseradish peroxidase-conjugated secondary antibody (Zsbio, Beijing, China) for 2 hours at room temperature, the bands were then tested by a chemiluminescent substrate ECL kit (Merck Millipore).

Flow cytometry and Immunofluorescence Staining

Immunophenotype of hPMSCs and intrahepatic macrophages were detected by flow cytometry. The single-cell suspensions were filtered, fixable Viability Stain 620 (BD Biosciences) were used to discriminate live and dead cells. The cells were then blocked with Fc-block (BD Biosciences) and stained with fluorochrome-labeled mAbs. Data were acquired on a NovoCyte flow cytometer. The primary antibodies were listed in supplementary Table S1.

For immunofluorescence staining, the cells were fixed in 4% paraformaldehyde for 20 min. The fixed cells were blocked with goat serum and subsequently incubated with primary antibodies at 4°Covernight. After thorough washing, secondary antibodies were used. Nuclei were visualized with DAPI (Roche Basel, Switzerland). The primary antibodies were listed in supplementary Table S1.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from liver tissue and other cells using TRIzol reagent (Invitrogen, USA). qRT-PCR was performed using Step-One Real-Time PCR system (Takara) according to the manufacturer's instructions. The expression of genes was normalized to GAPDH. All reactions were repeated in triplicate, and the primer sequences were listed in supplementary Table S2 and supplementary Table S3.

Microarray analysis

Total RNA of HSCs or liver tissues obtained from different groups was prepared with Trizol reagent (Invitrogen, Carlsbad, CA, USA). The products were sequenced by an Illumina HiSeq2500 instrument in Shanghai Majorbio Biopharm Technology Co. Ltd. (Shanghai, China). Data were extracted and

normalized according to the manufacturer's standard protocol (26, 27). Differentially expressed genes were identified using the nbinomTest and DESeq (2012) functions estimate Size Factors. Genes displaying twofold or greater changes ($P < 0.05$, t test) in expression level between control group and test group were selected to generate the heatmap and for GO term enrichment analysis.

Liver Function Tests and Histological Analysis

Liver function and fibrotic degree were assessed by analyzing the levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin (ALB) and hydroxyproline using UniCel DxC 800 Synchron (Beckman Coulter) according to the manufacturer's instructions.

For histopathological and immunohistochemical analysis, formalin-fixed, paraffin-embedded liver samples were cut into 4-μm-thick sections and stained with hematoxylin-eosin (H&E), Sirius red and Masson staining. At least 3 animals per group were examined. The primary antibodies were listed in supplementary Table S1.

Enzyme-Linked Immunosorbent Assay

The serum of the mice in each group was separated and detected according to the manufacturer's instructions of Xinbosheng's QuantiCyto® Mouse ELISA kits. IL-6, TNF-α and IFN-γ were tested.

Transfection of Cav1 Small Interfering RNA in HSCs

To investigate the role of *Cav1* in inhibiting the activation of HSCs, we designed three small interfering RNA (siRNAs), and transfected into HSCs with the Invitrogen's Lipofectamine™ 3000 reagent. The down-regulation of *Cav1* in HSCs were determined by western blot analysis and qRT-PCR. siRNA negative control (siRNA-NC) and untransfected HSCs were treated as controls. The sequences of siRNAs were listed in supplementary Table S4.

Statistical analyses

For statistical analysis, the experimental data were analyzed by SPSS software version 17.0 statistical software. Multivariate data were compared using analysis of variance. After statistical significance, pairwise comparisons were performed by Sidak's multiple comparisons test. Statistical analyses were performed with Prism 6.0 (GraphPad). $p \leq .05$ was considered significant.

Results

hPMSC transplantation alleviated CCl_4-injured liver fibrosis in mice

hPMSCs exhibited characteristics typical of MSCs. They had obvious fibroblastic morphology and were positive for the known cell surface markers CD105, CD73, CD90, and CD166, but negative for CD11b, CD34, and CD45. hPMSCs also exhibited low immunogenicity, with low or almost no expression of HLA-

DR (Supplemental Fig. 1A-B). Additionally, hPMSCs showed multipotency when cultured under osteogenic, adipogenic, and chondrogenic differentiation conditions (Supplemental Fig. 1C).

We next tested the efficiency of hPMSC engraftment in experimental LF by CCl_4 administration, as shown in the modeling process (Supplemental Fig. 2A). After CCl_4 treatment for 2 weeks, normal liver lobules were destroyed and the fibrous connective tissue in the portal area was significantly increased. Four weeks later, the fibrous tissue further increased, extending to adjacent liver lobules, and dividing the liver tissue. Six weeks later, the increased collagen fibers formed linear fibrous septa, and pseudolobules formed, according to Sirius Red staining, Masson staining, and α-SMA staining (Supplemental Fig. 2B and 2C). AST, ALT, ALB and hepatic hydroxyproline content were also elevated in CCl_4-treated mice, with a rising trend consistent with histopathological examinations (Supplemental Fig. 2D).

Time-point and cell-dose are two important parameters for cell-based therapy. In previous studies, MSCs were usually transplanted in vivo at the 4th week or 6th week after CCl_4 administration (24, 25). They reflect the mild to moderate stage of LF and the severe stage of LF, respectively. In this study, these two time points were chosen as treatment 1 (T1) and treatment 2 (T2) respectively, and a comparative study was conducted, as shown in Figure. 1A. Two doses of hPMSCs, including conventional cell doses (2×10^6 cells/kg) and high cell doses (5×10^6 cells/kg) were analyzed as well, mice from fibrosis group received CCl_4 followed by PBS injection were served as control (Fig. 1A). Compared with the fibrosis group, biochemical parameters of liver function, including ALT, AST, and ALB levels were reduced in all hPMSC treatment groups, especially in the T1 groups (Fig. 1B). Moreover, we found that the level of hydroxyproline, the main component in collagen tissue, was also reduced in all hPMSC treatment groups (Fig. 1B).

Histopathological examination using Sirius Red staining and Masson staining was performed to quantify the degree of LF. Compared to the fibrosis group, the fibrous area of liver tissue was significantly reduced in the hPMSC treatment groups (Fig. 2C). In addition, immunostaining showed α-SMA expression was decreased in hPMSC treatment groups, (Fig. 2D). Furthermore, the expression of fibrosis-related genes, including *Acta2*, *Col1a1*, and *Vimentin* was decreased upon hPMSC treatment, and downregulation of these genes was greater in T1 groups compared with T2 groups, as determined by qRT-PCR analysis. (Fig. 1E). These results suggest that hPMSC treatment could improve liver function and alleviate LF in CCl_4-treated mice.

Compared with the T2 groups, the therapeutic effects of hPMSC transplantation were more profound in T1 groups according to the results of the blood biochemical indices, collagen area, and fibrosis-related genes. Moreover, doubling the cell doses did not further improve the therapeutic effect of hPMSCs, indicating that conventional cell doses (2×10^6 cells/kg) are sufficient to play a therapeutic role in experimental LF.

hPMSCs Transplantation Has an Anti-inflammatory Effect in CCl_4-Injured Mice Liver

Inflammation is vital to the initiation and progression of LF (1). To investigate the potential anti-inflammatory effects of hPMSCs *in vivo*, we examined the differences in intrahepatic macrophages and inflammatory cytokines in liver tissue isolated from different mice. Compared to normal liver tissue, a large number of infiltrating macrophages ($F4/80^{+}$) were found in fibrotic livers according to immunohistochemical staining, while the number of macrophages reduced with hPMSC treatment (Fig. 2A). To explore the source of infiltrating macrophages, we then examined the proportion of mononuclear/macrophage cells using FACS analysis. The results showed that there was no statistical difference in the proportion of monocyte-derived macrophages (MoMF, $CD11b^{high}F4/80^{low}$) (28) from different liver tissues. Interestingly, the proportion of Kupffer cells ($CD11b^{low}F4/80^{high}$) (28) was 17.44 ± 3.18% in the hPMSC group, which was significantly lower than that in the fibrosis group (34.64 ± 2.12%) (Fig. 4B-C). These results suggested that hPMSC treatment could suppress the infiltration of macrophages, mainly by reducing the number of Kupffer cells.

We then detected the expression of inflammatory cytokines in the serum of mice by ELISA. As expected, the expression levels of inflammatory factors, including IL-6 and TNF-α, were lower in the hPMSC treatment group than in the LF group (Fig. 4D). However, there was no significant change ($P = 0.07$) in the expression level of IFN-γ, which may be related to the alleviation of inflammation in mice after treatment. These results were further confirmed by qRT-PCR analysis (Fig. 4E). Our findings indicated that hPMSC treatment contributes to the improvement of CCl_4-injured mouse liver, at least in part through anti-inflammatory processes

hPMSCs inhibit TGF-β1 -induced HSCs activation in vitro.

HSC activation is an indispensable component in the initiation and progression of LF (3). We then investigated whether hPMSCs could regulate the activity of the HSCs *in vitro*. In the presence of TGF-β1, the number of activated HSCs that expressed the myogenic marker α-SMA was markedly increased, as determined by western blot analyses (Fig. 3A; Supplemental Fig. 3). Activated HSCs became elongated, with a dendritic-like shape, compared with unactivated HSCs (Fig. 3B). Activated HSCs were then cultured with secretomes in a gradient ratio of 10%, 20% and 40%. The source of the secretomes was the culture supernatant from hPMSCs, which was concentrated 15-fold in advance. The use of secretomes, but not hPMSCs was aimed at reducing the interference of cell components. Unactivated and activated HSCs without extra treatment were used as controls (Fig. 3A). Interestingly, the expression of α-SMA protein in activated HSCs was downregulated after treatment with 10% hPMSC secretomes, accompanied by the partial recovery of cell morphology. Furthermore, this inhibitory effect was more pronounced as the concentration of secretomes was increased to 40%, which indicated that the hPMSC secretomes inhibited HSC activation in a concentration-dependent manner (Fig. 3A and Fig. 1B). Additionally, to exclude the effect of MSC medium composition, MSC complete medium was also concentrated and tested in the same manner. Compared with the hPMSC supernatant, the concentrated MSC medium exhibited limited effects on the inhibition of HSC activation (Fig. 3A and Fig. 3B).

The suppression of HSC activation was also confirmed by qRT-PCR analysis, which revealed a downregulation of fibrosis-related genes in the hPMSC secretomes group compared with unactivated HSCs. In particular, *Acta2*, the α-SMA coding gene, was significantly downregulated compared with the activated HSCs, while the expression of *Timp1*, an anti-fibrotic gene was increased with hPMSC treatment (Fig. 3C). These results demonstrate that HSC activation can be inhibited by treatment with hPMSC secretomes.

Cav1 is a potential target for hPMSC treatment in liver fibrosis

To further investigate the mechanism of relieving LF with hPMSC treatment, we performed RNA sequencing (RNA-seq) analysis of liver tissues obtained from normal C57 mice (Control group), mouse models with hepatic fibrosis (Fibrosis group), and hPMSC-treated fibrosis mice (hPMSCs group). The RNAseq raw expression files and details have been deposited in NCBI GEO under accession nos. SRR12777460, SRR12777461, and SRR12777462. RNA-Seq analyses showed that the gene expression profiles of the hPMSC group was more closely resembled those seen in control liver tissues, and were significantly different from those fibrosis group (Fig. 4A). Furthermore, the genes included in three key functional clusters, including fibrosis, cytoskeleton, and inflammation-related factors were analyzed. The results revealed a significant change in the expression of these genes in fibrotic liver tissues, and they can be restored after hPMSC treatment (Fig. 4B-D).

In addition, the top 10 GO biological process terms are listed in Fig. 4E. The inclusion of the terms related to TGF-β signaling pathway, SMAD protein signal transduction and SMAD protein phosphorylation in this list suggests that regulation of TGF-β/Smad signaling may be a potential mechanism in the treatment of LF with hPMSCs. We also performed qRT-PCR analyses to check the expression of ten important fibrosis-related genes (Fig. 4F). Among them, *Cav1* revealed the most significant differences between fibrotic group and hPMSCs group. Importantly, previous studies have shown that *Cav1* can participates in regulating TGF-β/Smad signaling pathway in many situations (29), indicating that *Cav1* may be a potential target for hPMSC treatment in LF.

To further confirm the above findings, we performed RNA-seq analyses on different cells, including activated HSCs (TGF-β1 group), unactivated HSCs (blank group), and activated HSCs, which were then treated with hPMSC secretomes (hPMSCs group) and medium (medium group), respectively. The RNAseq raw expression files and details have been deposited in NCBI GEO under accession nos. SRR12806194, SRR12806195, SRR12806196, and SRR12806197. The gene expression profiles of the hPMSC group was more closely resembled those seen in blank group, and were significantly different from those TGF-β1 group and medium (Fig. 5A). Furthermore, the expression of fibrosis-related genes as well as the ECM-associated genes similar to findings in liver tissue. It appears that, hPMSC secretomes can restore the expression of genes that had changed in the TGF-β1 group or medium group to some extent (Fig. 5B). In addition, the top GO biological process terms related to the proteinaceous ECM, and ECM suggest that hPMSC secretomes may reduce the formation of ECM by inhibiting the activation of HSCs, a key factor in alleviating LF (Fig. 5C). We also performed qRT-PCR analyses to check the expression of ten important

fibrosis-related genes (Fig. 5D). The expression of *Cav1* was significantly upregulated in activated HSCs when cultured with hPMSC secretomes, further supporting the finding of vivo analysis, indicating that *Cav1* might be a potential target for hPMSC treatment in LF.

Downregulation of Cav1 is associated with activation of HSCs

To test this hypothesis, we investigated the effect of *Cav1* on HSC activation. We measured the expression of *Cav1* and α-SMA in liver tissues by immunofluorescence staining. Results showed that *Cav1* was expressed at a low level both in normal liver tissues and in fibrotic liver tissues, where α-SMA was greatly upregulated after CCl_4 administration. However, after treatment with hPMSCs, the expression of Cav1 was upregulated compared with that in fibrotic liver tissues, accompanied by the reduction of α-SMA in the hepatic lobular margin (Fig. 6A). To further illustrate the relationship between *Cav1* downregulation and HSC activation, we also tested the expression of *Cav1* and α-SMA in activated HSCs. Compared to unactivated HSCs, α-SMA was significantly increased while *Cav1* was decreased in activated HSCs. After treatment with hPMSC secretomes, α-SMA levels were greatly attenuated, while *Cav1* levels were partially restored in activated HSCs (Fig. 6B). These data demonstrated the involvement of *Cav1* in HSC activation.

We then carried out loss-of-function experiments by transfecting an siRNA targeting human *Cav1*, which effectively reduced *Cav1* expression in HSCs. *Cav1* was not knocked down in siRNA negative control (siRNA NC)-transfected HSCs. Untransfected HSCs served as control cells (blank). HSC mRNAs from different groups were collected and subjected to qRT-PCR testing. Compared to control cells, the expression of pro-fibrotic genes, such as *Acta2*, and *Col1a1* were upregulated by 1.5 – 3-fold in *Cav1*-silenced HSCs, accompanied by the attenuation of *Timp1*, an antifibrotic gene (Fig. 6C), indicating a vital role of *Cav1* in HSC activation and collagen production. To explore the molecular mechanism of the antifibrotic effects of *Cav1*, we detected the regulation of Smad activation by *Cav1* in HSCs using loss-of-function experiments. It is noteworthy that Smad genes, including *Smad2* and *Smad4*, which are related to the TGF-β signaling pathway, were upregulated in *Cav1*-silenced HSCs, but not in negative control group and control cells (Fig. 6D). These data reveal the involvement of the TGF-β/Smad signaling pathway in *Cav1*-mediated HSC activation.

hPMSCs inhibit HSCs activation by restoring Cav1 function

To explore the interplay between hPMSC and Cav1 in HSC activation, we prepared siRNA-transfected HSCs, cells were activated by TGF-β1 and then treated with hPMSC secretomes (columns 3–6). Untransfected but activated HSCs served as control (column 2). Additionally, unactivated HSCs were also tested (column 1). The detailed operation is shown in Supplemental Table S5. As shown in Fig. 7A, the expression of *Cav1* was reduced in HSCs in the presence of TGF-β1. It is noteworthy that the decreased *Cav1* in activated HSCs can be upregulated by hPMSC secretomes. Knockdown of *Cav1* in activated HSCs, however, alleviated the effect of hPMSC secretomes on the upregulation of *Cav1* (Fig. 7A). Furthermore, the relative expression of the pro-fibrotic genes *Acta2*, *Col1a1*, and *Desmin* were also

measured and normalized to *β-actin*. The data showed that the trend of changes in pro-fibrotic gene expression in different groups was opposite to that of *Cav1* (Fig. 7A). These results indicate that hPMSC secretomes inhibit HSC activation by restoring *Cav1* function in activated HSCs.

We then collected protein from HSCs of different groups and validated the results by western blot analysis. Consistent with the results from the qRT-PCR assay, *Cav1* was reduced in activated HSCs that were induced by TGF-β1 (Fig. 7B). In contrast, hPMSC treatment upregulated *Cav1* expression in activated HSCs and inhibited HSC activation, as indicated by the reduction of α-SMA production. However, *Cav1* knockdown in activated HSCs attenuated the inhibitory effect of hPMSCs in activated HSCs (Fig. 7B). Importantly, after hPMSC treatment, HSCs showed reduced TGF-β/Smad signaling, as reflected by a significantly smaller amount of phosphorylation of Smad2 and α-SMA expression compared to that in the activated HSCs. However, knockdown of *Cav1* by siRNA increased Smad-2 phosphorylation and α-SMA expression in activated HSCs even after treatment with hPMSC secretomes (Fig. 7B). In summary, hPMSC treatment restored the function of *Cav1*, and elevated *Cav1* was sufficient to inhibit HSC activation and collagen production, partly by regulating the TGF-β/Smad signaling pathway.

Discussion

In this study, we demonstrated that transplantation of hPMSCs can reduce LF in a mouse model, with changes including the improvement of liver function, inhibition of inflammation, and a reduction in ECM deposition. Moreover, the therapeutic effects of hPMSCs against mild to moderate LF were significantly greater than those in severe fibrotic cases. Furthermore, our *in vitro* and *in vivo* data indicated that the therapeutic effects of hPMSCs are achieved partly through inhibition of the TGF-β/Smad signaling pathway via upregulation of *Cav1* in activated HSCs, which resulted in inhibited HSC activation and alleviated LF.

In recent years, hPMSC-based cell therapy in regenerative research has gained a broad interest owing to its great potential for self-renewal, differentiation, and the immunomodulatory properties (20, 21). Compared with the well-investigated autologous MSCs, including bone marrow and adipose mesenchymal stem cells, the number of studies using hPMSCs in the treatment of liver disease is relatively small, and the underlying molecular mechanisms have not yet been fully elucidated. In this study, we used mouse models of CCl_4-injured LF to explore the therapeutic value of hPMSCs. Our findings indicated that hPMSC transplantation not only enhanced general hepatic function, as indicated by increased levels of liver functional index, including ALT, AST, and ALB, but also alleviated LF, as demonstrated by reductions in collagen fiber regions in liver tissues. This effect occurred concomitantly with a reduction in activated HSCs and downregulation of fibrosis-related genes. This is consistent with previous studies in a miniature pigs and rat models of CCl_4-injured liver (22, 23). Therefore, our findings provide further proof of the potential therapeutic effects of hPMSCs in LF.

Choosing an appropriate time window is a key factor for hPMSC transplantation. Although the effects of hPMSCs in LF have been reported in previous studies (30, 31), to date, little is known about the optimal of therapeutic procedures in terms of time windows. In previous studies, MSCs were transplanted at 4 weeks after CCl_4 administration (25). However, in a few studies, MSCs were administered at 6 weeks after CCl_4 administration, at the more severe stage of the LF according to liver function tests and histopathological examination (24). In this study, two representative time points were chosen and comparative research was performed. We found that the therapeutic effects of hPMSCs at the early stage of LF were significantly greater than those of hPMSCs in advanced LF. These results suggest that earlier intervention with hPMSCs, may afford better therapeutic effects. These findings will need to be considered in the design of future clinical studies.

In the present study, we also investigated the possible mechanisms involved in relieving LF by hPMSCs. In summary, the following mechanisms may account for them: (1) Transplanted hPMSCs inhibit the secretion of multiple cytokines that otherwise promote inflammation and impair liver restoration (6, 17, 32). Serum IL-6 and TNF-α levels were significantly lower in hPMSC-treated mice than in untreated fibrotic mice. Furthermore, the number of Kupffer cells in the fibrotic liver also decreased after hPMSC treatment. These results provide evidence supporting the immunomodulatory roles of hPMSCs, which is beneficial to the improvement of liver function as well as the inhibition of LF (2). In addition, the therapeutic potential of hPMSCs in LF stems mainly from inhibiting HSC activation. According to RNA-seq analysis of liver tissues and GO enrichment analysis (Supplemental Fig. 4), we found that hPMSC treatment contributed to the upregulation of *Cav1* in activated HSCs, which then helped in the inhibition of HSC activation by regulating the TGF-β1/Smad signaling pathway.

CAV1 is a fatty acid- and cholesterol-binding protein that constitutes the major structural protein of caveolae (29, 33). Previous studies have confirmed that CAV1 can exercise a homeostatic function in the process of fibrosis by regulating TGF-β and its downstream signaling (34, 35). Moreover, *Cav1* knockout mice have revealed impaired wound healing and profound fibrosis in the lungs, heart, and liver (36–38). However, it remains unclear whether *Cav1*-mediated signaling pathways plays an important role in relieving LF by hPMSC treatment. In this study, we found that mRNA and protein levels of *Cav1* were decreased in activated HSCs. Importantly, *Cav1* upregulation can be achieved after hPMSC treatment. *Cav1* influenced the activity of Smad2, Smad3 and Smad4. In particular, it influenced the phosphorylation of Smad2, which then inhibits the TGF-β1/Smad signaling pathway as well as HSC activation. While endogenous inhibition of *Cav1* by siRNA alleviated the effect of hPMSC secretomes on the upregulation of *Cav1*, based on immunofluorescence staining and qRT-PCR assays. The results from *in vitro* assays were consistent with the findings in our animal models.

In summary, these combined mechanisms contribute significantly to the therapeutic potential of hPMSCs in the treatment of LF. To our knowledge, this is the first report to support the important role of *Cav1* in MSC-based therapy for LF. Two limitations were also present in this study. First, we did not verify our findings in clinical samples because LF tissue is scarce. Second, it remains unclear whether anti-fibrotic

factors or hPMSC-derived exosomes contribute to *Cav1* upregulation after hPMSC treatment and the underlying mechanisms. These points will be considered in further studies.

Conclusions

Collectively, we present further evidence demonstrating the potential of hPMSCs in reducing LF. Moreover, hPMSC-mediated upregulation of *Cav1* in activated HSCs plays a key role in deactivating HSCs via inhibition of TGF-β1/Smad signaling. These findings will contribute to the development of effective treatment for fibrotic liver diseases.

Abbreviations

MSCs, Mesenchymal Stem Cells; hPMSCs. human Placental Mesenchymal Stem Cells; TGF-β1. Transforming growth factor β1; L-DMEM, Low glucose-Dulbecco Modifed Eagle Medium; PBS, phosphate buffered saline; FBS, fetal bovine serum; ECM, Extracellular matrix; MSCBM, Mesenchymal Stem Cell Basal Medium; HSCs, Hepatic Stellate Cells; ELISA, Enzyme-Linked Immuno Sorbent Assay; PCR, polymerase chain raction; RT-PCR, Reverse transcription-polymerase chain reaction; q-PCR, Quantitati vereverse transcription-PCR; GAPDH, Glyceraldehyde – 3-phosphate dehydrogenase; CCl_4, Carbon tetrachloride ; TAA, Thioacetamide; α-SMA, alpha smooth muscle Actin; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; ALB, Albumin; IL-6, Interleukin-6; IL-10, Interleukin-10; IFN-γ, Interferon-γ; TNF-α, Tumor necrosis factor-α; Acta2, Actin alpha 2; Timp1, Tissue Inhibitor Of Metalloproteinases 1; Col1a1, Collagen Type I Alpha 1; PDGF, Platelet-derived growth factor; siRNA, Small interfering RNA; IHC, Immunohistochemistry; CAV1, Caveolin1; GO, Gene Ontology ; SOM, Self Organizing Maps; WB, Western Blot ; KC, Kupffer cells; MOMF, Monocyte derived macrophage

Declarations

Ethics approval and consent to participate:

Human embryonic tissue was obtained according to procedures approved by the Medical Ethics Committee, Sichuan University, (the registration number: K-2018109-1).

Consent for publication:

All authors provide consent for publication of this paper.

Availability of data and material:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

Competing interests:

There are no conflicts of interest to disclose by any of the authors.

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Authors' contributions:

Yunqi Yao, Zhemin Xia, Fuyi Cheng: Conception and design, Collection and assembly of data, Data analysis and interpretation, Manuscript writing; Qingyuan Jang, Jiao He, Cheng Pan: Provision of study material, Data analysis and interpretation; Yuan Wang, Lin Zhang, Yixin Ye, Shuang Chen, Dongsheng Su: Contributed to stem cell isolation and characterization, Provision of study material, Assembly of data; Xiaolan Su, Lin Cheng, Gang Shi, Lei Dai: Responsible for manuscript writing and revision of the manuscript; Hongxin Deng: Conception and design, Manuscript writing, Financial support, Final approval of manuscript. Hongxin Deng is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final manuscript.

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Figures

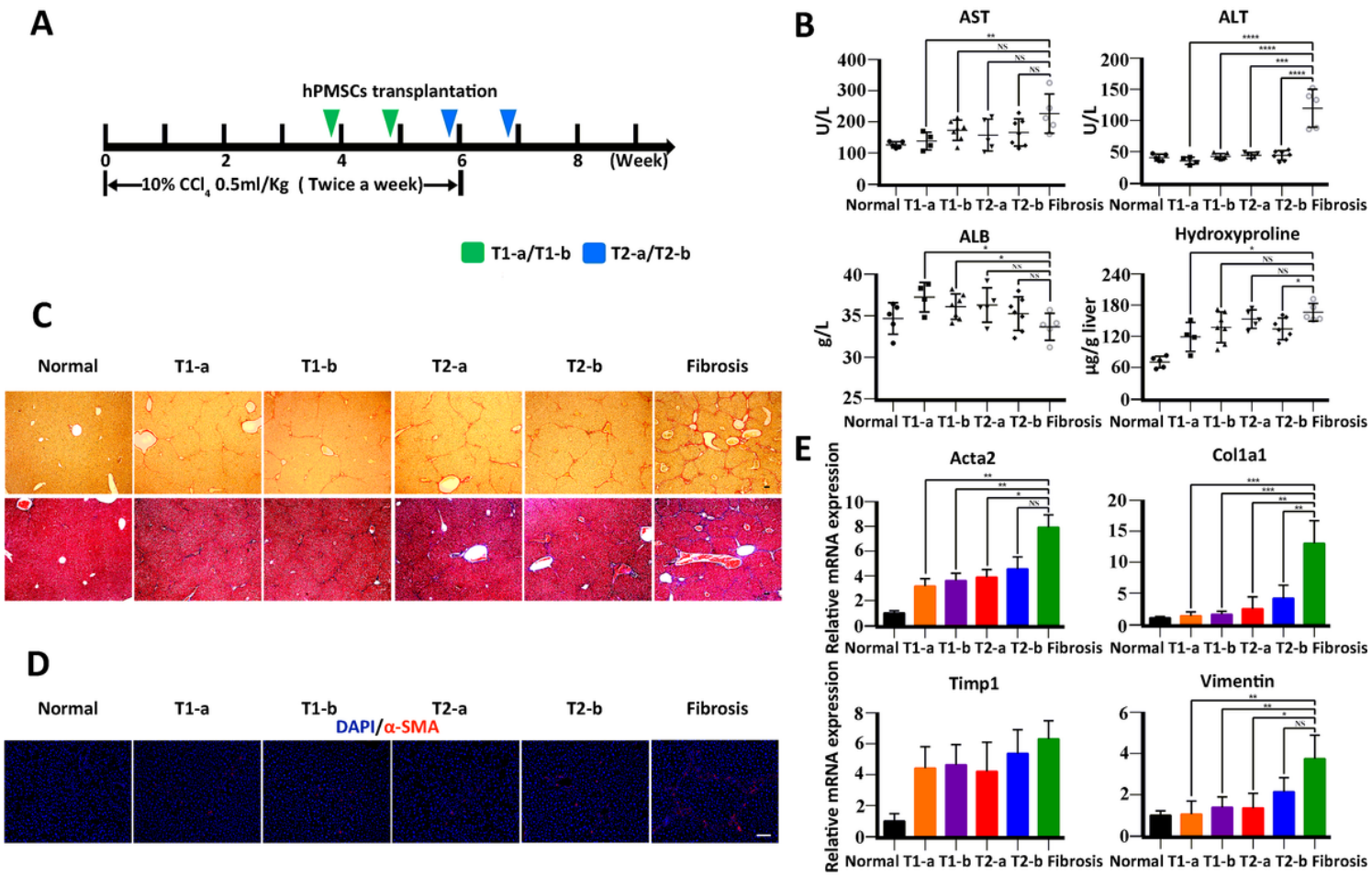

Figure 1

Two doses of hPMSCs, including conventional cell doses (2 × 106 cells/kg) and high cell doses (5 × 106 cells/kg) were analyzed as well, mice from fibrosis group received CCl4 followed by PBS injection were served as control (Fig. 1A). Compared with the fibrosis group, biochemical parameters of liver function, including ALT, AST, and ALB levels were reduced in all hPMSC treatment groups, especially in the T1 groups (Fig. 1B). Moreover, we found that the level of hydroxyproline, the main component in collagen tissue, was also reduced in all hPMSC treatment groups (Fig. 1B).

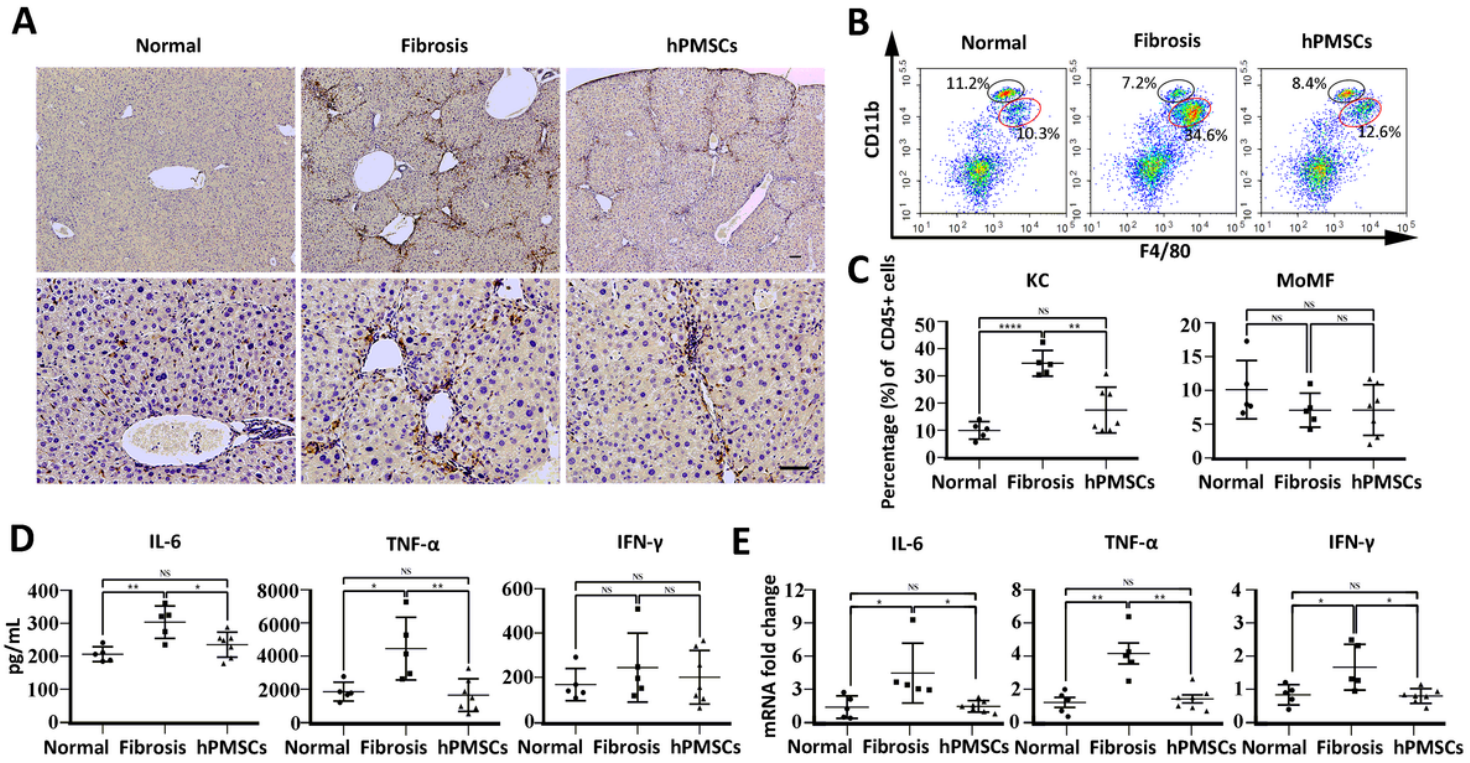

Figure 2

Histopathological examination using Sirius Red staining and Masson staining was performed to quantify the degree of LF. Compared to the fibrosis group, the fibrous area of liver tissue was significantly reduced in the hPMSC treatment groups (Fig. 2C). In addition, immunostaining showed α-SMA expression was decreased in hPMSC treatment groups, (Fig. 2D). Furthermore, the expression of fibrosis-related genes, including Acta2, Col1a1, and Vimentin was decreased upon hPMSC treatment, and downregulation of these genes was greater in T1 groups compared with T2 groups, as determined by qRT-PCR analysis.

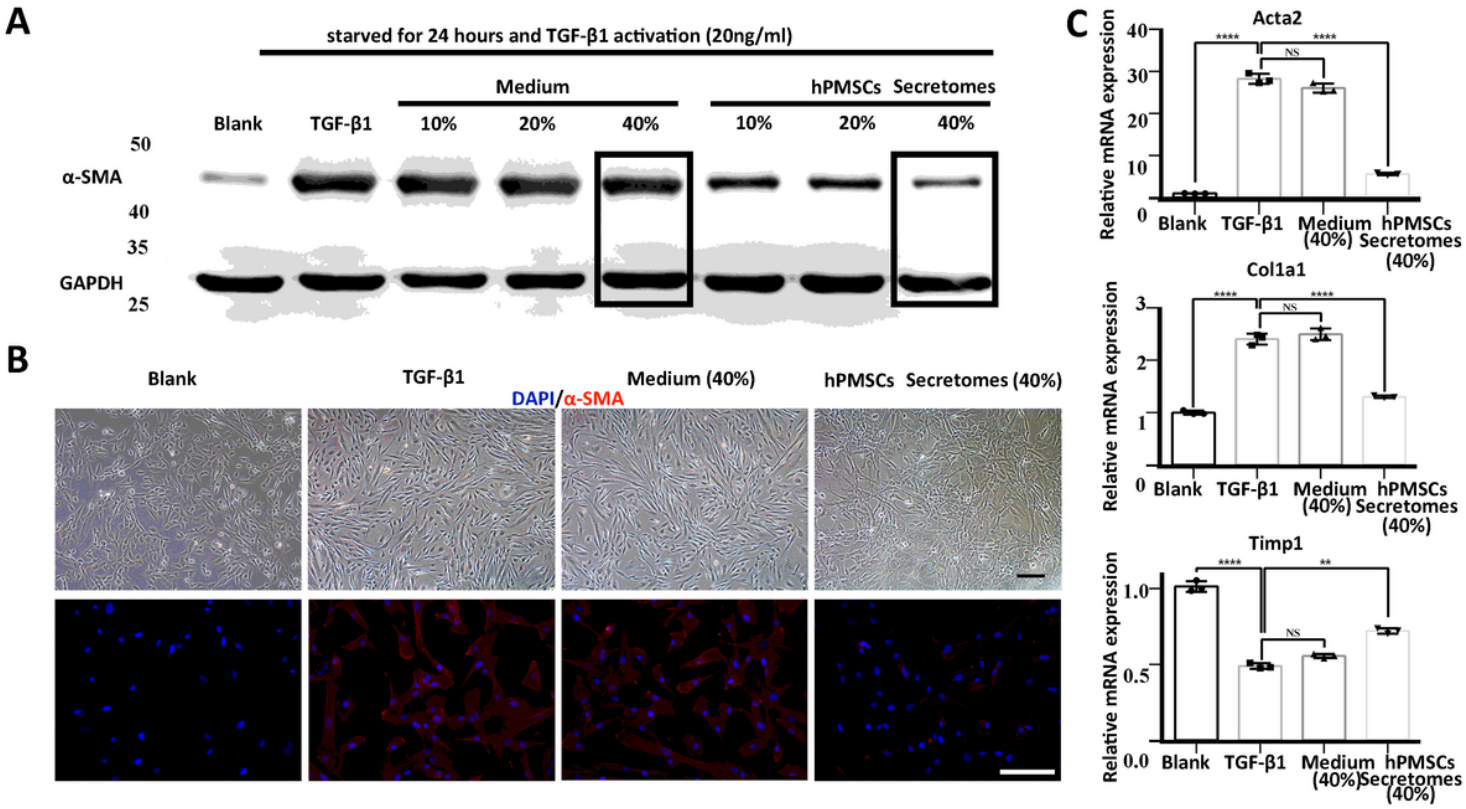

Figure 3

Activated HSCs became elongated, with a dendritic-like shape, compared with unactivated HSCs (Fig. 3B). Activated HSCs were then cultured with secretomes in a gradient ratio of 10%, 20% and 40%. The source of the secretomes was the culture supernatant from hPMSCs, which was concentrated 15-fold in advance. The use of secretomes, but not hPMSCs was aimed at reducing the interference of cell components. Unactivated and activated HSCs without extra treatment were used as controls (Fig. 3A).

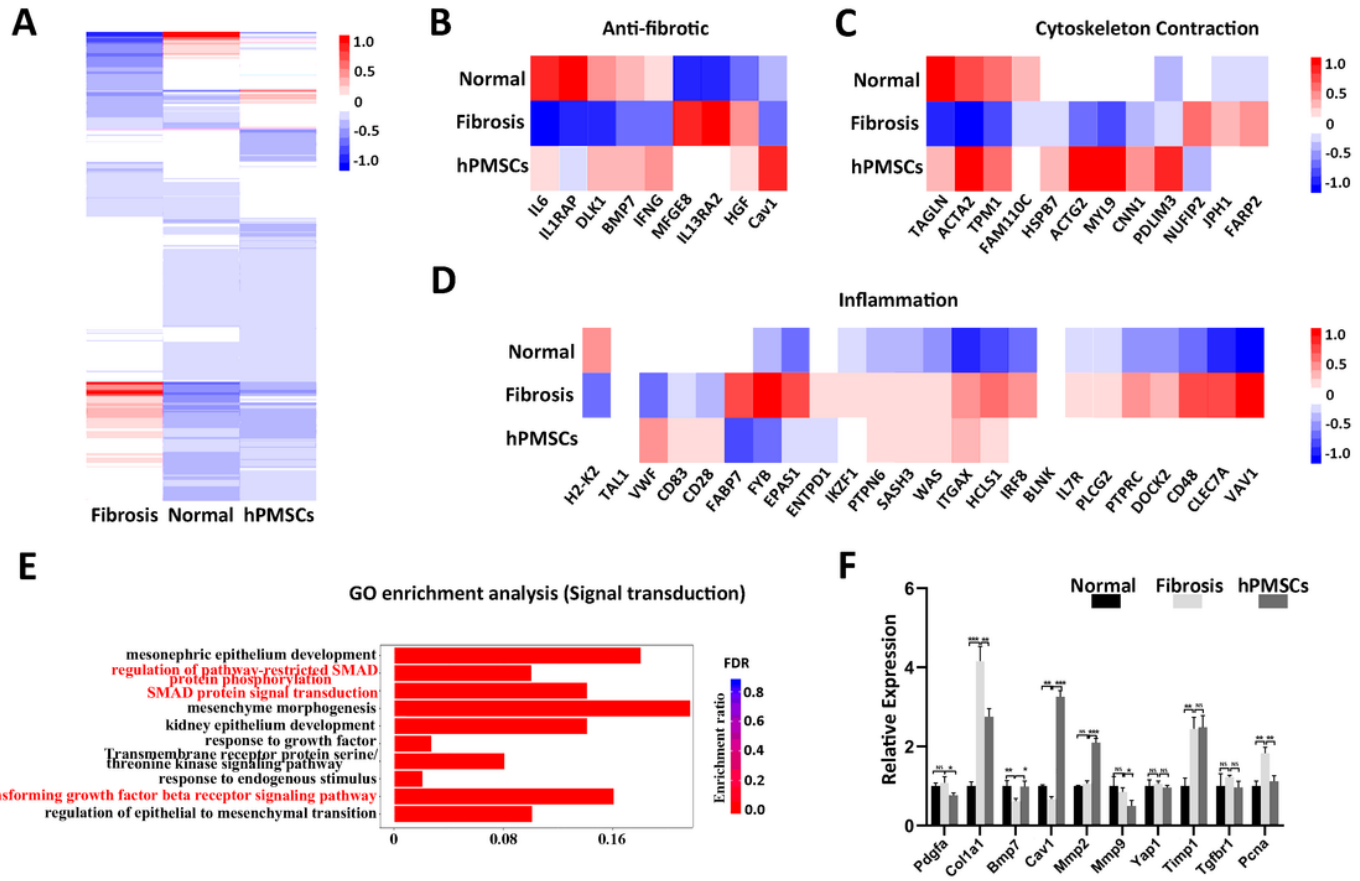

Figure 4

Furthermore, the genes included in three key functional clusters, including fibrosis, cytoskeleton, and inflammation-related factors were analyzed. The results revealed a significant change in the expression of these genes in fibrotic liver tissues, and they can be restored after hPMSC treatment (Fig. 4B-D).

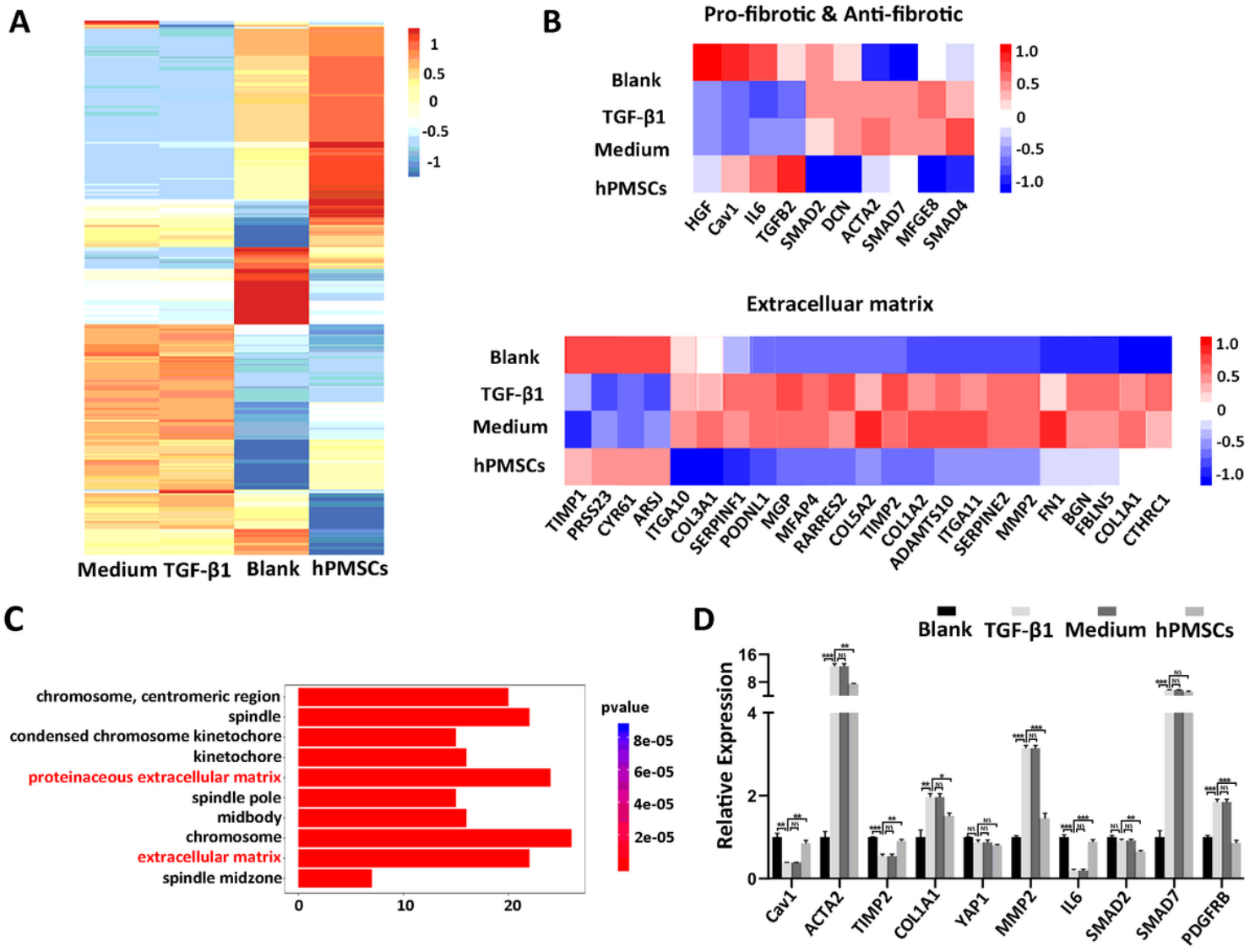

Figure 5

Furthermore, the expression of fibrosis-related genes as well as the ECM-associated genes similar to findings in liver tissue. It appears that, hPMSC secretomes can restore the expression of genes that had changed in the TGF-β1 group or medium group to some extent (Fig. 5B). In addition, the top GO biological process terms related to the proteinaceous ECM, and ECM suggest that hPMSC secretomes may reduce the formation of ECM by inhibiting the activation of HSCs, a key factor in alleviating LF (Fig. 5C). We also performed qRT-PCR analyses to check the expression of ten important fibrosis-related genes (Fig. 5D). The expression of Cav1 was significantly upregulated in activated HSCs when cultured with hPMSC secretomes, further supporting the finding of vivo analysis, indicating that Cav1 might be a potential target for hPMSC treatment in LF.

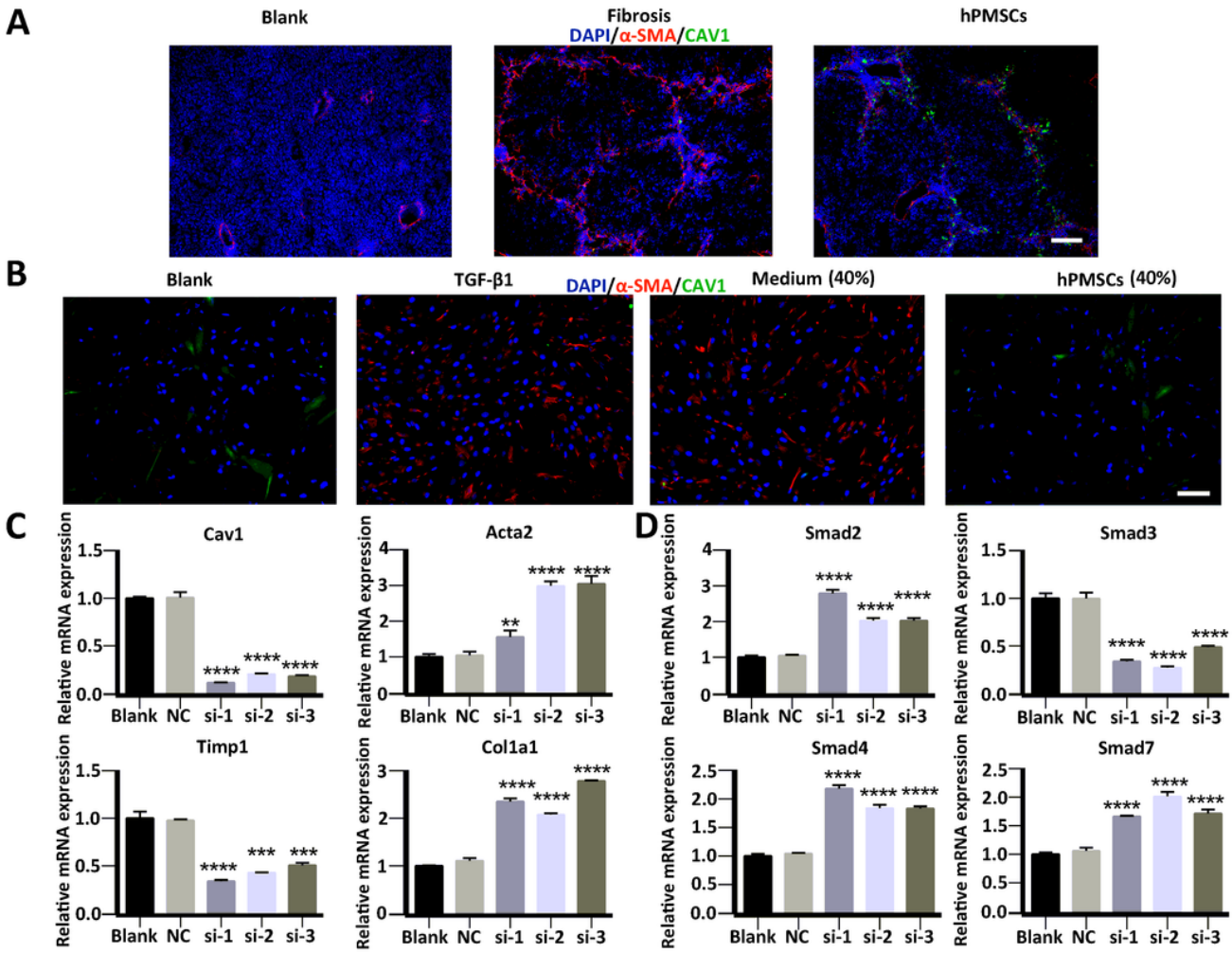

Figure 6

To further illustrate the relationship between Cav1 downregulation and HSC activation, we also tested the expression of Cav1 and α-SMA in activated HSCs. Compared to unactivated HSCs, α-SMA was significantly increased while Cav1 was decreased in activated HSCs. After treatment with hPMSC secretomes, α-SMA levels were greatly attenuated, while Cav1 levels were partially restored in activated HSCs (Fig. 6B). These data demonstrated the involvement of Cav1 in HSC activation.

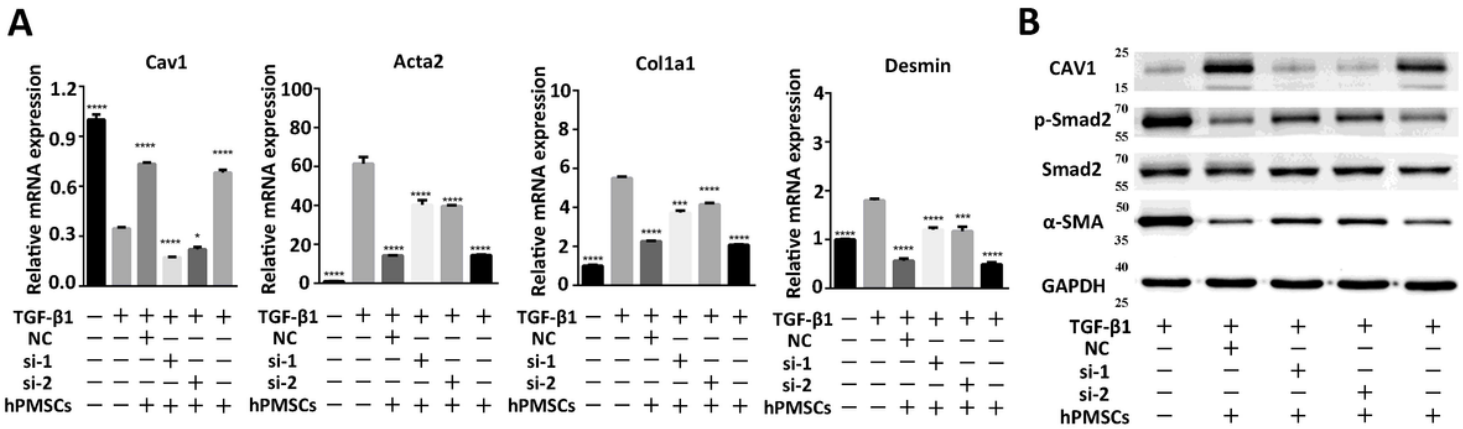

Figure 7

Furthermore, the relative expression of the pro-fibrotic genes Acta2, Col1a1, and Desmin were also measured and normalized to β-actin. The data showed that the trend of changes in pro-fibrotic gene expression in different groups was opposite to that of Cav1 (Fig. 7A). These results indicate that hPMSC secretomes inhibit HSC activation by restoring Cav1 function in activated HSCs.

Supplementary Files

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