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Original Article

Transplanted adult human hepatic stem/progenitor cells prevent histogenesis of advanced hepatic fibrosis in mice induced by carbon tetrachloride

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Abstract: Transplantation of adult human hepatic stem/progenitor cells (hHSPCs) has been considered as an alternative therapy, replacing donor liver transplantation to treat liver cirrhosis. This study assessed the antifibrotic effects of hHSPCs in mice with fibrosis induced by carbon tetrachloride (CCl_4) and examined the actions of hHSPCs on the fibrogenic activity of human hepatic stellate cells (HSCs) in a coculture system. Isolated hHSPCs expressed stem/progenitor cell phenotypic markers. Mice were given CCl_4 (twice weekly for 7 weeks) and hHSPC transplantation weekly. CCl_4 induced advanced fibrosis (bridging fibrosis and cirrhosis) in mice, which was prevented by hHSPC transplantation. The liver of hHSPC-transplanted mice showed only occasional short septa and focal parenchymal fibrosis, and a 50% reduction in hepatic collagen, assessed by Sirius red stain histomorphometry. Moreover, the proteins for α-smooth muscle actin (α-SMA) and collagen I were decreased. While α-SMA, collagen α1(I), and tissue inhibitor of metalloproproteinase-1 mRNAs were decreased, matrix metalloproteinase (MMP)-1 mRNA was increased, consistent with decreased fibrogenesis. MMP-2 and transforming growth factor-β were not affected. Alanine aminotransferase and aspartate aminotransferase were lower, suggesting improvement of liver function/damage. In coculture, hHSPCs elicited changes of α-SMA and fibrogenic molecules in HSCs similar to those observed *in vivo*, providing evidence for a functional link between hHSPCs and HSCs. A decreased HSC proliferation was noted. Thus, transplantation of hHSPCs prevents histogenesis of advanced liver fibrosis caused by CCl_4. hHSPCs mediate downregulation of HSC activation coincident with modulation of fibrogenic molecule expression, leading to suppression of fibrogenesis both *in vivo* and *in vitro*.

Keywords: Adult human hepatic stem/progenitor cells, cell transplantation, hepatic fibrosis

Introduction

Hepatic fibrosis is a chronic liver disease that results from wound healing in response to liver injury. The fibrosis will progress to cirrhosis, the end stage of liver fibrotic disease. Cirrhosis affects hundreds of millions of people worldwide and causes over 1 million deaths annually [1]. In the US, there are 17000 adults and children awaiting liver transplantation (Data from American Liver Foundation). Every year more than 1500 people die waiting for a donor liver to become available. Currently, orthotopic liver transplantation is the only treatment that improves the survival rate in patients with live cirrhosis [2, 3]. Unfortunately, the availability of liver transplantation is limited due to the shortage of donor livers, serious complications, immunological suppression and high costs. Therefore, searching for an effective alternative treatment for advanced liver fibrosis is essential. Cell therapy using hepatocyte transplantation presents a promising alternative to donor liver transplant in patients with metabolic dis-

eases as well as acute and chronic liver failure [3, 4]. However, the supply of viable hepatocytes is limited by the shortage of liver donors and their low proliferative rate both *in vitro and in vivo* [3, 5]. Because of their great capacity to proliferate and ability to differentiate into mature hepatocytes, human hepatic stem/progenitor cells (hHSPCs) from adult liver [6] that reside in the canals of Hering [7] may have potential therapeutic effects against end-stage liver cirrhosis.

Accordingly, in the present study we undertook to assess the antifibrotic effects of adult hHSPCs in experimental fibrosis *in vivo* and *in vitro*. We first assessed the effects of hHSPC transplantation on the histogenesis of advanced fibrosis, including bridging fibrosis and cirrhosis, in the carbon tetrachloride (CCl_4) mouse model of fibrosis, and then examined the actions of hHSPCs on the fibrogenic activity of hepatic stellate cells (HSCs)-the principal extracellular matrix producing of the liver [8] in a co-culture system. The ultimate goal is to establish a preclinical model of adult hHSPC transplantation therapy for hepatic fibrosis that could be translated into clinical medicine.

Materials and methods

Induction of hepatic fibrosis in mice by CCl_4

Male C57BL6 mice weighing 22-25 g (purchased from Beijing Weitong Lihua Company, China) were maintained according to National Institute of Health guidelines for the care and use of animals, approved by the Institutional Animal Care and Use Committee of Jining Medical University Affiliated Hospital. Mice were kept in the Animal Care Facility of Jining Medical University with a 12-h light-dark cycle at constant temperature. Mice had free access to tap water during the study period. Mice were divided into 2 groups with 10 per group. They were administered CCl_4 intraperitoneally (IP) at a dose of 0.3 ml/kg in olive oil twice a week for 7 weeks. For cell transplantation, the CCl_4-mice were given IP either phosphate buffer saline (PBS) or 2×10^6 hHSPCs (infra vide), weekly for 7 weeks. The mice tolerated the treatments well and no overt adverse effects were observed. At the time of sacrifice, blood was collected in EDTA containing microfuge tubes and pelleted at 5,000 rpm for 10 min. Serum samples were obtained for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurement. Liver was removed and divided into portions as follows: formalin fixation for Sirius red staining for collagens; snap frozen in liquid nitrogen for mRNA extraction; and homogenization in protein buffer for protein analysis by Western blotting.

Sirius red stain for collagens and histomorphometry of hepatic fibrosis

Liver tissue was fixed in 10% formalin, embedded in paraffin and sectioned according to the standard methods. The sections were stained for collagens-histological marker of fibrosis-with 0.1% Sirius red and 0.01% fast green in saturated picric acid (Sigma-Aldrich, St. Louis, MO, USA). Three Sirius red-stained sections from each mouse were used for histological evaluation of fibrosis. In each section, six images of the liver lobules were taken at a magnification of × 100, providing a total of 18 images per animal. These were used for collagen quantification by histomorphometry using a computerized Bioquant Life Science® morphometry system.

Isolation of human HSCs

Wedge sections of normal human live in selected patients undergoing hepatic resection for primary benign tumors or for a single metastasis from colon cancer were used as a source of HSCs. Cells were isolated as a previously described [9]. Immediately after hepatectomy, a piece of liver tissue was placed in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA). The liver was washed and an intrahepatic vein cannulated for *in situ* digestion with collagenase B and pronase (Roche Applied Science, Indianapolis, IN, USA). The digested liver was subject to density gradient centrifugation to separate the HSC fraction. The cells were collected and plated on a plastic dish for culturing, designated as primary human HSCs. The protocol was approved by the Human Ethics Committee at Jining Medical University Affiliated Hospital. Informed consents were obtained from the guardians.

Isolation and culture of hHSPCs

Isolation of hHSPCs was performed as follows: Human liver specimens (20 to 50 g) which was obtained from Jining Medical University Affi-

Table 1. SYBR Green Real-Time Quantitative PCR Primer Sequences

Target gene	Forward primer	Reverse primer
TGF-β	5'-CAAGGGCTACCATGCCAACT-3'	5'-AGGGCCAGGACCTTGCTG-3'
α-SMA	5'-GTCCCAGACATCAGGGAGTAA-3'	5'-CAGCACCGCCTGGATAGCC-3'
Collagen Iα1	5'-GGCTTCCCTGGTCTTCCTGG-3'	5'-CCAGGGGGTCCAGCCAAT-3'
TIMP-1	5'-GTGGGAAATGCCGCAGAT-3'	5'-TGATGTGCAAGAGTCCACC-3'
MMP-1	5'-CTGGCCACAACTGCCAAATG-3'	5'-CTGTCCCTGAACAGCCCAGTACTTA-3'
MMP-2	5'-CCCCAAGCTCATCGCAGAT-3'	5'-CTTGTTGCCCAGGAAAGTGAAG-3'
GADPH	5'-TTCACCACCATGGAGAAGGC-3'	5'-GGCATGGACTGTGGTCATGA-3'
AFP	5'-GCAAAGCTGAAAATGCAGTTGA-3'	5'-GGAAAGTTCGGGTCCCAAAA-3'
CK7	5'-TGAATGATGAGATCAACTTCCTCAG-3'	5'-TGTCGGAGATCTGGGACTGC-3'
CK8	5'-TCATAGACAAGGTACGGTTCC-3'	5'-GCCTAAGGTTGTTGATGTAGC-3'
CK19	5'-TCGACAACGCCCGTCTG-3'	5'-CCACGCTCATGCGCAG-3'
ALB	5'-CTGAGCAAAGGCAATCAACA-3'	5'-CACAGTCTGCTGAGGTTGGA-3'
MMP-13*	5'-AAGATGTGGAGTGCCTGATG-3'	5'-AAGGCCTTCTCCACTTCAGA-3'

*mouse gene.

liated Hospital were first perfused *in situ* for 10 minutes with 200 to 300 mL of EGTA buffer and collagenase type IV (0.05%) in DMEM until the tissue became soft and showed signs of dissolution. The tissue was minced and the cell suspension was centrifuged twice at 50 × *g* for 2 min at 4°C. The supernatant was collected and centrifuged at 150 × *g* for 8 min at 4°C. The resultant cell pellet was resuspended in DMEM and centrifuged at 150 × *g* for 5 min at 4°C. Finally, the pelleted cells containing crude hHSPCs were suspended in PBS for purification in density gradients made of 50% Percoll (Sigma-Aldrich), 20% Percoll and cell suspension. The preparation was centrifuged at 350 × g for 20 min at 4°C. The interface between the 20% and 50% Percoll was decanted to a tube and centrifuged at 350 × g for 5 min. The purified hHSPCs were collected and used for culture in 6-well plates in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. These were cultured for 2-3 weeks with the medium changed twice a week. When colonies became visible, they were encircled with cloning rings and subcultured to an individual well of a 6-well plate. The expanded cells were taken for assessment of markers of hepatic stem cells. Thereafter, cells were transferred to T-75 flasks. At confluence, cells were taken for experiments.

Co-culture of hHSPCs and human HSCs

This was performed in 6-wells plates with transwell inserts containing microporous membrane (3 μm pore; Falcon, Becton Dickinson, NJ, USA). Primary human HSCs (50,000 cells/well) were seeded into the wells of the plates (lower compartments), while hHSPCs were seeded into the transwell inserts (upper compartments) at either 16,000 cells or 150,000 cells in a 1/3 or 3/1 proportion relative to HSCs, respectively. hHSPCs and HSCs were co-incubated at 37°C in a 5% CO_2-air humidified atmosphere. For controls, HEK-293 cells (human embryonic kidney cells) were used in place of hHSPCs in the co-culture protocol. The experiments were conducted in triplicates. After co-cultured for 24 hr and 48 hr, HSCs were taken for qRT-PCR (quantitative Real Time-Polymerase Chain Reaction), Western blot analyses and cell proliferation measurement.

RT-PCR

Total RNA was extracted from 50 mg of the mouse liver and primary human HSCs that had been co-cultured with hHSPCs as described above. The RNAs were purified using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). RNA (3 μg) was reverse transcribed into cDNA using SprintTM RT Complete-RNA to cDNA EcoDryTM Premix (Double Primed) tubes (Clontech, Mountain View, CA, USA) and analyzed in triplicate by qRT-PCR using SYBR green qPCR Master Mix (Roche Applied Science) on the LightCycler 480 Real-Time PCR System (Roche Applied). Data are presented as the relative expression of fibrogenic genes after normalized to GAPDH. The sequences for the primers are listed in **Table 1**.

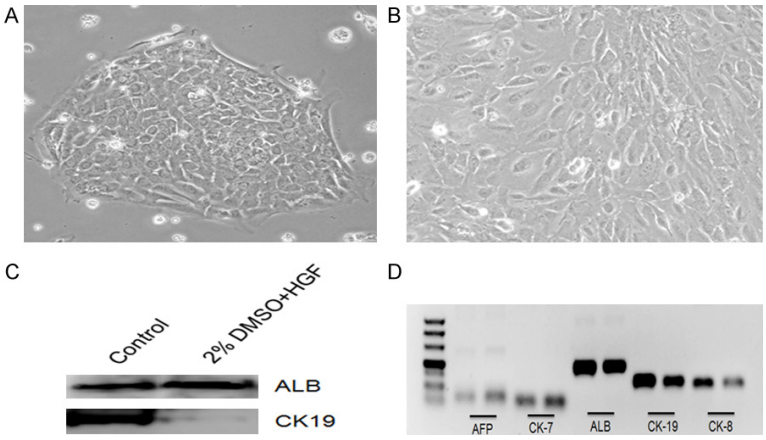

Figure 1. Phase contrast microscopy (A, B) and expression of hepatocyte and biliary epithelial cell markers (C, D) of hHSPCs. (A) During the first 10-20 days in culture, the cells that are attached on the dish occur in cluster and appear round. (B) In subculture, the cells are spreading and look flattened and larger. Original magnification 100 ×. (C) Western blots demonstrate expression of albumin and CK19 by hHSPCs. Upon exposure to DMSO and HGF, albumin expression is elevated while CK19 expression is lost. (D) RT-PCR shows that hHSPCs express the hepatocyte marker genes Albumin, CK8 and weakly AFP, as well as the cholangiocyte marker genes *CK7* and *CK19*.

Western blot

Proteins from the mouse liver and co-cultured human primary HSCs cells were homogenized and extracted using RIPA lysis buffer (50 mM Tris-HCl, pH = 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) containing protease and protein phosphatase inhibitor mixtures (Roche Applied Science). The protein concentration was determined with a Bio-Rad DC kit (Bio-Rad, Hercules, CA, USA). Antibodies used were as follows: rabbit anti-collagen type I (1:3,000) (Rockland Immunochemicals, Gilbertsville, PA, USA), mouse anti-alpha-smooth muscle actin (αSMA) (1:500) (Millipore, Boston, MA, USA), and rabbit anti-GAPDH (1:2,000) (Santa Cruz, Santas Cruz, CA, USA).

Densitometric analysis

Enhanced chemiluminescent images on the immunoblots were analyzed by scanning densitometry and quantified with a BIOQUANT NOVA imaging system. All values were normalized to housekeeping protein and expressed as fold changes relative to the controls.

Cell proliferation assay

HSC cell proliferation was determined by incorporation of [^{3}H]-thymidine into DNA as previously described [9]. hHSPCs or HEK 293 cells (as control) were seeded into the upper compartments of the transwell inserts (upper compartments) and primary human HSCs were plated (20,000 cells per well) in the lower compartments of 24-wells plates. hHSPCs or HEK 293 cells were co-incubated with HSCs at 1:3 and 3:1 proportions for 24 and 48 hours. Then, 1 μCi/mL ^{3}H-thymidine (specific activity 6.7 Ci/mmole; PerkinElmer, Waltham, MA, USA) was added to the HSC culture and incubated for additional 12 hours before harvesting. HSCs were washed three times with ice-cold PBS and fixed in methanol for 30 min at 4°C. Cells were solubilized in 0.25% sodium hydroxide/0.25% sodium dodecyl sulfate. After neutralization with hydrochloric acid (1N), the radioactivity was measured in a liquid scintillation counter (Beckman Coulter).

Statistics

Results are expressed as means ± standard deviation. Statistical significance between groups was tested using an unpaired Student's *t*-test (SPSS software), and $P < 0.05$ indicated a significant difference.

Results

hHSPCs phenotypes

In culture, hHSPCs that were attached on culture plates appeared small and round, forming colonies at about first 15 days (**Figure 1A**). In subculture, the cells looked enlarged and more flattened, resembling hepatocytes (**Figure 1B**). These hHSPCs were found to express both CK19 (biliary epithelial cell marker) and albumin (hepatocyte lineage marker), as disclosed by Western blotting in **Figure 1C**. Exposure of the hHSPCs culture to 20 ng/ml hepatic growth factor (HGF) for 5 days, followed by treatment with 1.5% dimethyl sulfoxide (DMSO) in 10% fetal bovine serum (FBS) supplemented DMEM

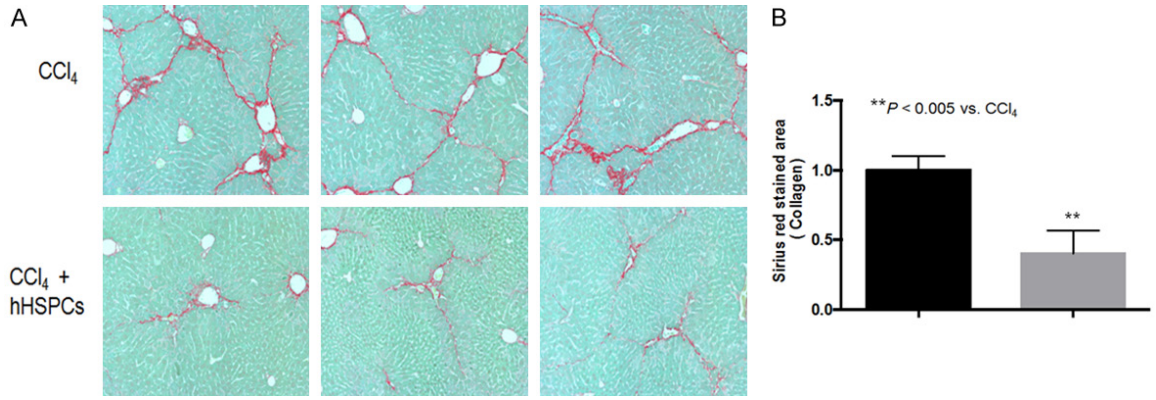

Figure 2. A: Liver fibrosis revealed by Sirius red staining for collagens. Top row: Representative images showing the development of advanced fibrosis induced by CCl_4, which includes formation of nodules (cirrhosis) and linking septa without apparent nodular formation (bridging fibrosis). Bottom row: Representative images showing fibrosis in mice treated with CCl_4 and were also given hHSPCs. Short septa and focal fibrotic tissue are visible. Original magnification 100 ×. B: Histomorphometry of Sirius red stained collagens. The amount of collagens in the liver parenchyma was halved in mice received the hHSPC transplantation. N = 10 mice/group.

for an additional 5 days resulted in a loss of CK19 expression, concomitant with an enhancement of albumin expression. These data indicate differentiation of hHSPCs into hepatocyte-like cells in response to HGF and DMSO. Additionally, RT-PCR analysis revealed that hHSPCs strongly expressed hepatocyte marker genes *ALB* and *CK8*, while weakly expressed the gene for *AFP.* The cholangiocyte marker genes *CK7* and *CK19* were also expressed at a high level in hHSPCs (**Figure 1D**).

Anti-fibrotic effects of hHSPCs on hepatic fibrosis induced by CCl_4 in mice

As revealed by Sirius red staining for collagens, the livers of CCl_4-mice receiving PBS vehicle developed advanced fibrotic changes showing bridging fibrous septa (bridging fibrosis), incomplete nodule formation, and complete nodule formation (cirrhosis)-histological hallmarks of advanced fibrosis (**Figure 2A**). In striking contrast, no bridging septa and nodule formation were observed in the livers of the CCl_4-mice receiving hHSPC transplantation. In some of these livers, there were occasional short septa and focal parenchymal fibrosis present in the liver lobules. Histomorphometry revealed that the percent of Sirius red-stained collagen area in the lobules was reduced by more than 50% in the CCl_4-mice with hHSPC transplantation (**Figure 2B**).

Having demonstrated the beneficial effect of hHSPC transplantation against the development of advanced fibrosis induced by CCl_4, we examined expression of fibrogenic molecules in the mouse liver. As shown in **Figure 3A**, **3B**, the protein content for α-SMA or collagen I was significantly decreased (40%) by hHSPCs transplantation into the CCl_4-mice. Furthermore, the mRNA levels for αSMA, collagen α1(I), and tissue inhibitor of metalloproteinase-1 (TIMP-1) were significantly decreased (35%, respectively) in CCl_4-mice received the hHSPCs transplantation, while the mRNA for matrix metalloproteinase (MMP)-13 was increased by 40% (**Figure 3C**). The mRNAs for TGFβ1 and MMP-2, however, were not affected.

hSCPCs transplantation corrected the CCl_4-induced liver damage

Liver functions of mice were evaluated by measurement of plasma ALT and AST. Mice with CCl_4 plus hHSPCs transplantation had significantly lower ALT (320.6 ± 62.2 U/dl; $P < 0.05$, N = 10) and slightly lower AST (295.3 ± 135.2 U/dl) activity compared to those of mice treated with CCl_4 alone (ALT, 512.6 ± 92.3 U/dl and AST, 503.3 ± 61.2 U/dl). These data indicate the beneficial effect of hHSPCs transplantation in correcting liver damage/adverse liver functions caused by CCl_4.

Effects of hHSPCs on human primary HSCs in co-culture

To test the antifibrotic actions of hHSPCs on primary human HSCs in co-culture, we determined expression of fibrosis-related molecules in HSCs. **Figure 4A**, **4B** shows that hHSPCs significantly decreased the protein levels for

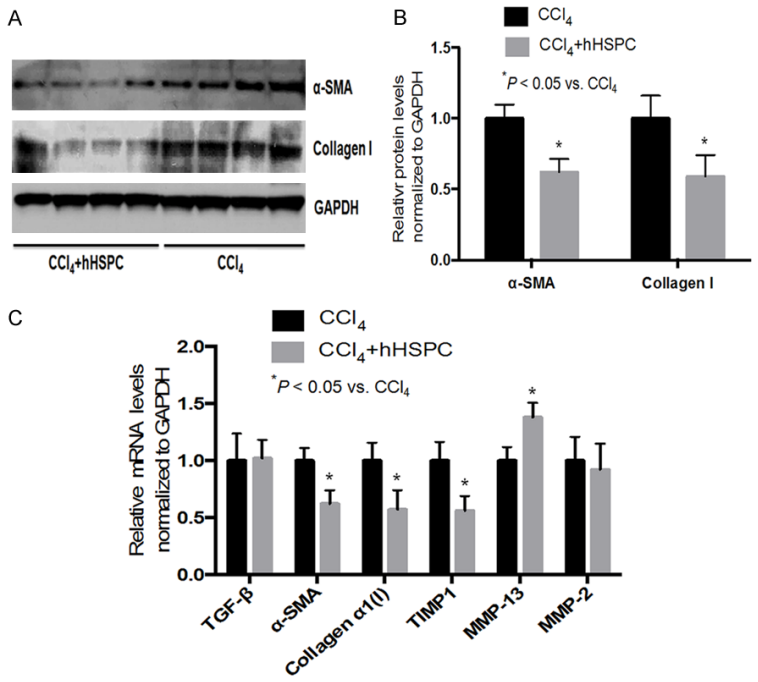

Figure 3. Expression of α-SMA and collagen I proteins (A, B) and changes in mRNA levels (C) in the liver of CCl_4 mice received hHSPC transplantation. (A, B) Upper panel: Immunoblots of four mice; lower panel: corresponding histograms of data of the blots. Both α-SMA and collagen I levels were reduced by nearly halves in the mice with the transplanted hHSPCs. Values are expressed as fold change relative to CCl_4-treated mice; N = 4 mice/group. (C) While α-SMA, collagen α1(I) and TIMP-1 mRNA levels were downregulated, MMP-13 mRNA level was upregulated. The gene expression for TGF-β and MMP-2 was not altered. Values are expressed relative to CCl_4-treated mice; N = 10 mice/group.

αSMA (60%) and collagen I (50%) in HSCs. Additionally, hHSPCs suppressed the mRNA levels for αSMA, α1(I) collagen, and TIMP-1, each by 30%, in human HSCs, while elevated mRNA for MMP-1 by 23% (**Figure 4C**). The gene expression for TGFβ1 and MMP-2 was not affected.

Figure 5 shows inhibition of primary HSC proliferation by hHSPC in the co-culture system by more than 25% at the ratio of 3:1 for hHSPCs:HSCs for 24 h, but not at the 1/3 ratio, suggesting a dose response. Also, the inhibition of proliferation seems to be transient as the effect disappeared in cells co-cultured for a longer duration of 48 hr.

Discussion

Experimental cell-based therapy for liver fibrosis and/or liver failure has been examined using stem cells from extrahepatic sources or from fetal and adult livers [3, 10]. Specifically, mesenchymal stem cells derived from human bone marrow were shown to diminish fibrosis and ameliorate chronic liver damage in rats caused by CCl_4 [11]. Infusion of human bone marrow-derived mesenchymal stem cells led to an improvement of CCl_4-induced cirrhosis in mice [12]. The antifibrotic effect was found to be associated with decreased expression of αSMA, TNF-α and TGF-β and conversely stimulation of MMP-9. Liver fibrosis can spontaneously regress upon the cessation of fibrogenic agents. To that effect, transplantation of bone marrow cells facilitates the resolution of liver fibrosis in mice, coincident with increased expression of the matrix protein degradation enzymes MMP-13 and MMP-9 [13]. The severity of cirrhosis caused by CCl_4 in rats was lessened by infusion of human umbilical cord blood-derived mesenchymal cells, accompanied by inhibition of TGF-β1, collagen I and α-SMA expression [14].

Transplantation of human fetal hepatic progenitor cells to a cohort of 25 patients with end-stage decompensated cirrhosis demonstrated a decrease in the Mayo's Model for End Stage Liver Disease (MELD) mean score in 6 months follow-up [15]. Along this line, fetal liver stem cells were found to contribute to the recovery of fulminant hepatic failure in rats [16].

Adult human hepatic stem cells were reported to contribute to the regeneration of liver parenchyma in severe-combined immunodeficient mice [17]. Additionally, the stem cells improved liver injury in a mouse model of fulminant liver failure, accompanied by reduction in animal mortality, decrease in apoptosis and enhancement of liver regeneration [18].

It is noteworthy that the above mentioned studies were aimed at the reduction of either established cirrhosis or chronic liver injury/failure in animal models, or treatment of end-stage cirrhosis in humans, using extrahepatic mesen-

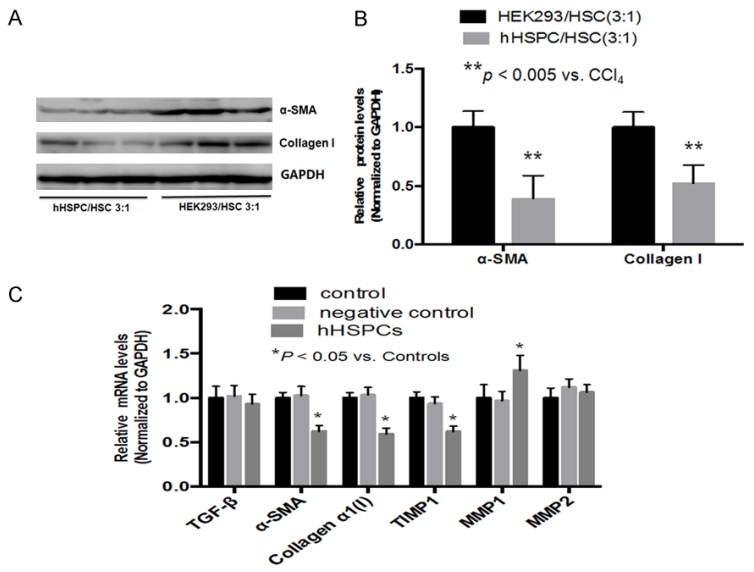

Figure 4. Expression of α-SMA and collagen I proteins (A, B) and changes in mRNA levels (C) in HSCs co-cultured with hHSPCs. (A, B) Upper panel: Immunoblots of three separate cultures; lower panel: corresponding histograms of data of the blots. Both α-SMA and collagen I levels in HSCs were reduced by halves in the presence of hHSPCs. Values are expressed relative to the control HEK293/HSC culture. (C) Control: HSCs were cultured alone in the bottom compartment of the Transwell, while the upper compartment contained only the medium. Negative control: HSCs in the bottom compartment were co-cultured HEK293 cells in the upper compartment. hHSPC: HSCs were co-cultured with hHSPCs in the upper compartment. Gene expression for α-SMA, collagen α1(I) and TIMP-1 in HSCs were downregulated by the presence of hHSPCs. The mRNA level of MMP-1 was higher, while the levels of TGF-β and MMP-2 were not altered. Values are expressed relative to HSC control.

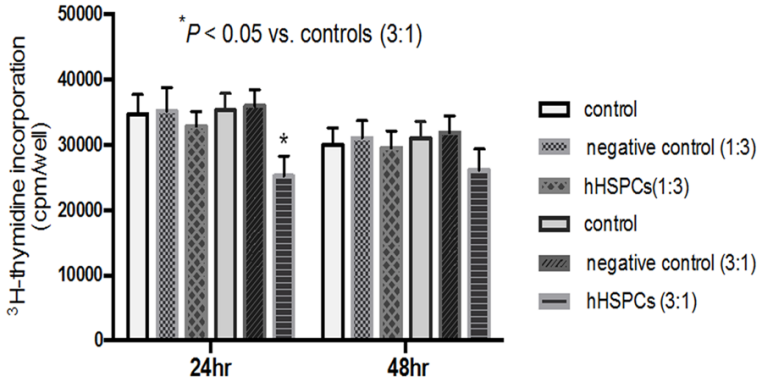

Figure 5. Effects of hHSPCs on proliferation of HSCs. Control: HSCs were cultured alone in the bottom compartment of the Transwell, while the upper compartment contained only the medium. Negative control: HSCs co-cultured with HEK293 cells. hHSPCs: hHSPCs were co-cultured with HSCs. hHSPCs when co-cultured with HSCs at the ratio of 3/1 elicited a 25% inhibition of HSC proliferation. Values are expressed relative to HSC control.

chymal cells, or fetal and adult liver stem cells. In the present study, we sought to test the efficacy of hHPSCs derived from adult human liver in the prevention of advanced liver fibrosis development in CCl_4-mice. Admittedly, our experimental design is at variance with the protocols employing cell therapy in the treatment of established cirrhosis in either animal models or patients in the clinical settings. Nonetheless, our study discovered a novel action of hHSPCs against the histogenesis of liver fibrosis to advanced stage fibrosis, namely bridging fibrosis and cirrhosis. The hHSPC's antifibrotic action involves downregulation of HSC activation and modulation of expression of critical fibrogenic molecules that mediate the production of collagen I and hence fibrosis.

HSCs residing in the space of Disse [19] produce the principal extracellular matrix proteins in the liver, collagen I included [8]. HSCs express α-SMA, which is a phenotypic marker of human HSCs [20]. Additionally, HSCs synthesize MMP-1 (in humans) or MMP-13 (in rodents), MMP-2, and TIMP-I in response to soluble signals released from profibrotic factors [21, 22]. Of these genes, α-SMA, collagen I, MMP-2, TIMP-1 are upregulated during progressive liver fibrosgenesis and in culture-induced activation of HSCs [22]. Therefore, expression of these transcripts is related to stellate cell activation. On the other hand, MMP-1 mRNA expression, which is reduced in liver fibrosis, is enhanced in early primary HSC culture but then decreases in late culture. Thus, MMP-1 expression is associated with downregulation of stellate cell activation phenotype, coincident with decreased fibrogenic activity. Notably, the activity

of MMP-1 is regulated by TIMP-1, its physiological inhibitor. As a result, collagen deposition in liver fibrosis is the outcome of an imbalance between the production of MMP-1 and its inhibitor TIMP-1 [23].

Our study showed that transplantation of hHSPCs prevents the development of advanced fibrosis (bridging fibrosis and cirrhosis) in CCl_4-mice. Instead, the liver of hHSPC-transplanted mice displays only occasional short septa and sporadic focal fibrotic tissue. The histopathology was corroborated by histomorphometry, revealing a greater than 50% reduction in the amount of collagen deposition in the liver of CCl_4-mice received the transplanted hHSPCs. Additionally, biochemical analysis disclosed that the hepatic α-SMA and collagen I proteins were significantly decreased with a corresponding decrease in their gene expression. Concomitantly, gene expression for α-SMA, collagen α1(I), and TIMP-1 was suppressed while that of MMP-13 was enhanced. The downregulation of α-SMA likely reflects the change in the activation of HSCs to a less activated state-or a more quiescent phenotype. Because MMP-1 is the major proteinase that degrades collagen I and its enzymatic activity is inhibited by TIMP-1, the imbalanced production of MMP-1 relative to TIMP-1 promotes the degradation of collagen I, thereby resulting in diminished collagen deposition. These changes could explain the decrease in fibrosis in the liver with transplanted hHSPC *in vivo*. We also found that the transplanted hHSPCs are beneficial in correcting the liver damage caused by CCl_4 treatment based on the decreased values of AST and ALT.

We then tested whether hHSPCs interact with HSCs, promoting the downregulation of the fibrogenic activities of HSCs. To that end, hHSPCs were co-cultured with HSCs in a transwell system separated by a porous membrane filter. We found that α-SMA expression was lowered at both the protein and mRNA levels, indicating the acquisition of a phenotype with a diminished fibrogenic activity. Consequently, the levels collagen I mRNA level and protein were both decreased, coincident with increased expression of MMP-1 while decreasing that of TIMP-1. The changes are consistent with findings *in vivo* (*vide supra*) and provide an explanation for the suppressed fibrogenesis at the cellular level, i.e. HSCs.

TGF-β and MMP-2 are known to involve in the development of liver fibrosis [24]. However, our data did not show that the expression of these factors was altered under our experimental conditions, suggesting that their regulation is independent of the antifibrotic actions of hHSPCs. Our study also showed that the proliferation of HSCs in coculture with hHSPCs is inhibited although transiently. Because we did not determine HSC proliferation *in vivo,* the significance of inhibition of HSC proliferation by hHSPCs remains to be evaluated.

In conclusion, we have isolated hHSPCs from adult human liver and characterized their stem/progenitor cellular phenotypes. Our study documented the striking ability for transplanted hHSPCs to prevent the histogenesis of advanced liver fibrosis in mice induced by CCl_4. The antifibrotic action of hHSPCs *in vivo* appears to involve downregulation of HSC activation and modulation of fibrogenic-related molecule expression, and these effects *in vivo* were replicated in coculture comprising hHSPCs and human HSCs. Thus, our *in vivo* and *in vitro* culture findings together provide evidence for a functional link between hHSPCs and HSCs, which could explain the antifibrotic action of the transplanted hHSPCs. It is likely that hHSPCs secrete soluble molecules that modulate the fibrogenic activities of HSCs via a paracrine mechanism. It will be our continuous efforts to identify and characterize these paracrine factors released by hHSPCs. The current investigation is a new starting point for our goal to establish a preclinical model of adult hHSPC transplantation for therapeutic intervention of the histogenesis of advanced hepatic fibrosis in humans.

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Disclosure of conflict of interest

None.

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