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Hedgehog-mediated paracrine interaction between hepatic stellate cells and marrow-derived mesenchymal stem cells

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ABSTRACT

During liver injury, bone marrow-derived mesenchymal stem cells (MSCs) can migrate and differentiate into hepatocytes. Hepatic stellate cell (SC) activation is a pivotal event in the development of liver fibrosis. Therefore, we hypothesized that SCs may play an important role in regulating MSC proliferation and differentiation through the paracrine signaling pathway. We demonstrate that MSCs and SCs both express hedgehog (Hh) pathway components, including its ligands, receptors, and target genes. Transwell co-cultures of SCs and MSCs showed that the SCs produced sonic hedgehog (Shh), which enhanced the proliferation and differentiation of MSCs. These findings demonstrate that SCs indirectly modulate the activity of MSCs in vitro via the Hh pathway, and provide a plausible explanation for the mechanisms of transplanted MSCs in the treatment of liver fibrosis.

Mesenchymal stem cells (MSCs) are thought to be pluripotent cells that can differentiate into a variety of cell types, and can be an ideal resource for transplantation therapy. Recent studies have demonstrated that MSCs can be used to prevent fibrotic lesions in several organs (such as in pulmonary fibrosis after bleomycin challenge) [1], and to protect cardiac function after a myocardial infarction [2]. MSCs have been shown by in vivo transplantation to differentiate into hepatocytes [3]. Systemic delivery of MSCs prior to and during the induction of experimental liver fibrosis significantly inhibited tissue remodeling, as assessed by liver histology, and improved liver function [4,5], but the mechanisms have yet to be elucidated.

During liver injury, MSCs can migrate and differentiate into hepatocytes. This may be due to paracrine signaling, which regulates MSC implantation, differentiation, and proliferation. Paracrine signaling is important for tissues during embryonic development, as well as for the regulation of the tissues' cell lineages in adults. Although many of the specific paracrine signals involved remain to be elucidated, evidence shows that MSCs can exert a protective role through paracrine signaling to hepatic stellate cells (SCs) [6]. To investigate the interaction between MSCs and SCs, a co-culture experiment was developed using human MSCs (hMSCs) and a human hepatic stellate cell (HSC) line. Hepatocyte-specific protein and gene expression in co-culture was analyzed. In a co-culture system, HSCs enhanced the proliferation of hMSCs.

We tried to determine if HSCs are involved in creating a microenvironment to facilitate stem cell differentiation and proliferation. RT-PCR demonstrated that hMSCs and HSCs both express ligands, receptors, and target genes of the hedgehog (Hh) pathway, which is known to regulate the viability and differentiation of various types of progenitors [7–9]. Transwell co-cultures of HSCs and hMSCs showed that both cell types produced and responded to Hh ligands. In addition, antibody neutralization studies indicated that the HSCs produced Hh ligands that enhanced the proliferation and differentiation of hMSCs. We demonstrated that HSCs indirectly modulate the activity of hMSCs in vitro via the Hh pathway. These findings may explain the beneficial effects of transplanted MSCs in various models of acute and chronic liver injuries.

Materials and methods

Cell isolation and cell culture. Liver cells were isolated from normal human liver as previously described [10,11]. Briefly, after digestion of the liver with pronase (Boehringer Mannheim,

Abbreviations: MSC, marrow-derived mesenchymal stem cell; SC, hepatic stellate cell; HSC, human hepatic stellate cell; hMSC, human mesenchymal stem cell; RT-PCR, reverse-transcriptase polymerase chain reaction; mRNA, messenger ribonucleic acid; DMEM, Dulbecco's modified Eagle's medium; IL, interleukin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline; BrdU, bromodeoxyuridine; Hh, Hedgehog; Ihh, Indian hedgehog; Ptc, patched; Shh, sonic hedgehog; AFP, Alpha fetal protein.

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Table 1
Primers sequences

Gene	Forward sequence	Reverse sequence	Product size (bp)
18S	5′-ACATCCAAGGAAGGCAGCAG-3′	5′-TTCGTCACTACCTCCCCGG-3′	357
Shh	5′-ACTGGGTGTACTAGTCCAAGG-3′	5′-AAAGTGAGGAACTCGTAGAGC-3′	356
Ihh	5′-CTACGCCCCGCTCACAAAG-3′	5′-GGCAGAGGAGATGGCAGGAG-3′	376
Ptc	5′-TTCTCACAACCCTCGGAACCCA-3′	5′-CTGCAGCTCAATGACTTCCACCTT-3′	265
Smo	5′-ATCTCCACAGGAGAGACTGGTTCGG-3′	5′-AAAGTGGGGCCTTGGGAACATG-3′	263
Gli1	5′-ACTGGAGACCTCTCCAGC-3′	5′-GCTGACAGTATAGGCAGA-3′	156
Gli2	5′-TGGCCGCTTCAGATGACAGATGTTG-3′	5′-CGTTAGCCGAATGTCAGCCGTGAAG-3′	241
Gli3	5′-GCTCTTCAGCAAGTGGTTCC-3′	5′-CTGTCGGCTTAGGATCTGTTG-3′	146
Albumin	5′-GCTGTCATCTCTTGTGGGCTGT-3′	5′-ACTCATGGGAGCTGCTGGTTC-3′	245
AFP	5′-CTCTTCCAGAAACTAGGAGAA-3′	5′-CTCTTCAGCAAAGCAGACTT-3′	282
β-Actin	5′-ACAATGAGCTGCGTGTGGCT-3′	5′-TCTCCTTAATGTCACGCACGA-3′	344

Indianapolis, IN) and collagenase (Crescent Chemical, Hauppauge, NY), dispersed cell suspensions were layered on a discontinuous density gradient of 8.2% and 15.6% Accudenz (Accurate Chemical and Scientific, Westbury, NY). The resulting upper layer consisted of >95% SCs. The viability of all cells was verified by phase contrast microscopy, as well as by their ability to exclude propidium iodide. The viability of all cell cultures utilized for this study was >95%. Isolated stellate cells were seeded at a density of 3×10^2 cells/mm^2 with DMEM supplemented with 10% fetal bovine serum, 100 units/mL of streptomycin, and 100 units/mL of penicillin. The hMSC line was derived from the bone marrow of normal human donors after informed consent, and the HSC line was generated from normal human liver tissues as previously described [12,13].

To determine if hMSCs can respond to exogenous Shh, hMSCs was transfected with a Gli1-luciferase reporter construct [14] and treated with recombinant Shh (20 μM) for 24 h. The luciferase assay system (BD Pharmingen, San Diego, CA) was used to measure Gli1 transcriptional induction according to the manufacturer's protocol. All measurements of luciferase activity (relative light units) were normalized to the protein concentration. For BrdU proliferation assy, hMSCs were seeded at a density of 5000 cells/well in 96-well plates in growth medium containing 10% FBS. After 24 h, the medium was changed to growth medium containing 0.2% FBS for 24 h, and the cells were pre-treated with vehicle or cyclopamine (Toronto Research Chemicals, Toronto, Canada) for 30 min. Subsequently, Shh was added and the cells were incubated in medium containing recombinant Shh (20 μM) for 48 h with BrdU present during the last 24 h.

Immunoblot. Cells were homogenized in 150 μL of Dignam C buffer [15] containing protease and phosphatase inhibitors as described [16]. The homogenized samples were rotated on a tumbler for 30 min at 4 °C, and then centrifuged at 14,000 rpm for 5 min at 4 °C. The protein concentrations of samples were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Proteins (50 μg from whole liver; 10 μg from cell) were separated by SDS–PAGE and then transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) in a buffer containing 20 mmol/L Tris, pH 8.3, 150 mmol/L glycine, 0.01% SDS, and 20% methanol. Equal loading of the gel was confirmed by staining nitrocellulose membranes with Ponceau S. After blocking with 5% nonfat milk (Carnation, Swampscott, MA) in Tris-buffered saline (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl) containing 0.1% Tween 20 (TBS-T) for 1 h, nitrocellulose membranes were incubated with the anti-Shh primary antibody (Santa Cruz, Santa Cruz, CA) or β-actin antibody (Sigma, St. Louis, MO), both diluted 1:1000 in 5% nonfat milk, for 1 h (or in the case of immunoblotting involving whole liver extracts, for 12 h), and then washed 3 times in TBS-T. Secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG from Amersham, UK), was incubated with the nitrocellulose membranes at a dilution of 1:1000 in 5% nonfat milk for 30 min. After 4 washes in TBS-T, antibody complexes were detected using the Amersham ECL system in the linear range.

mRNA quantitation by real-time RT-PCR. mRNAs were quantified by real-time RT-PCR per the manufacturer's specifications (Stratagene, Mx3000P™ Real-Time PCR). The sequences of primers are listed in Table 1.

Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA). One microgram of RNA was reverse-transcribed using a random primer and Superscript RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA). Samples were incubated at 20 °C for 10 min, and at 42 °C for 30 min; reverse transcriptase was inactivated by heating at 99 °C for 5 min and cooling at 5 °C for 5 min. Amplification reactions were performed using a SYBR Green PCR Master Mix (Applied Biosystems). Five microliters of diluted cDNA samples (1:5 dilution) were used for quantitative two-step PCR (a 10-min step at 95 °C, followed by 50 cycles of 15 s at 95 °C and 1 min at 65 °C) in the presence of 400 nM specific forward and reverse primers, 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris buffer (pH 8.3), 200 μM dATP, dCTP, dGTP, and 400 μM dUTP, and 1.25 U of AmpliTaq Gold DNA polymerase (Perkin Elmer Applied Biosystems, Foster City, CA). Each sample was analyzed in triplicate. PCR products were analyzed by electrophoresis in a 2% agarose gel. In all experiments, PCR products were sequenced to confirm specificity.

Bromodeoxyuridine proliferation assay. The hMSC line was seeded at a density of 5000 cells/well in 96-well plates in growth medium containing 10% FBS with or without HSC co-culture for 48 h. In some experiments, the cells were also treated with recombinant Shh, anti-Shh neutralizing antibody, or control IgG at the same time. The bromodeoxyuridine (BrdU) assay was performed according to the manufacturer's protocol (Amersham, Little Chalfont, England). Each experiment was performed in triplicate, and repeated at least 3 times.

Caspase 3/7 activity. Apoptotic activity was assayed in parallel using the Apo-ONE Homogeneous Caspase 3/7 Apoptosis Assay (Promega, Madison, WI), according to the vendor's instructions. All the experiments were repeated at least 3 times. A FLUOstar OPTIMA microplate reader (BMG Labtech, Durham, NC) was used for all absorbance, luminescence, and fluorescence measurements.

Statistical analysis. Results are expressed as means ± SEM. Significance was established using the Student's *t*-test and analysis of variance when appropriate. Differences were considered significant when $p < 0.05$.

Results

Human mesenchymal stem cell and human hepatic stellate cell lines basally express hedgehog signaling pathway components and human hepatic stellate cell lines produce biologically active Shh

We first investigated the mRNA expression of Hh pathway components in hMSCs, HSCs, and hepatocytes. The hMSC line was derived from patients undergoing lower-extremity reconstructive surgery, while the HSC line was generated from normal human liver tissues as previously described. Our semi-quantitative RT-PCR

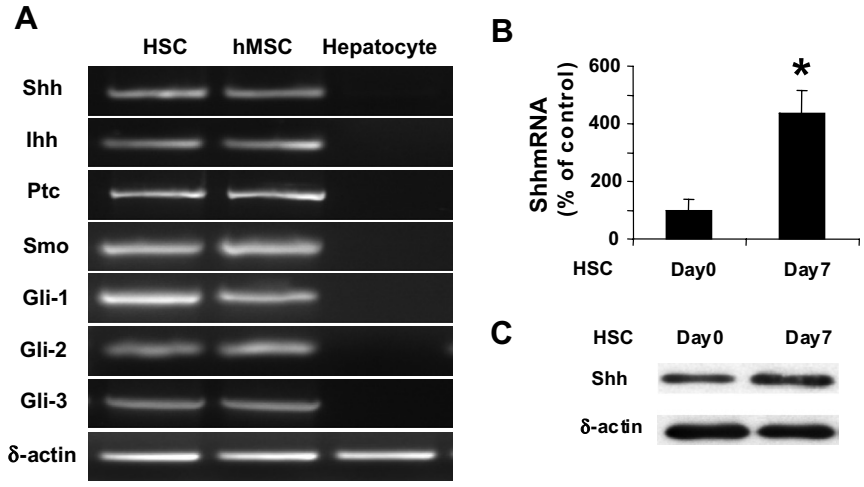

Fig. 1. The Hh pathway in human hepatic stellate cells (HSCs) and human mesenchymal stem cells (hMSCs). (A) Total RNA for HSCs, hMSCs, and hepatocytes was extracted as described in Materials and methods. RT-PCR was performed to measure the expression of Shh, Ihh, Ptc, Smo, Gli1, Gli2, and Gli3. β-Actin was used as a loading control. (B) Stellate cells were isolated and placed in culture; Shh mRNA expression was measured at the indicated time points in culture using real-time PCR ($^{*}p < 0.05$ compared with day 0, $n = 3$ for each day). (C) Shh was detected in stellate cells under the same conditions as in (B) by immunoblotting; β-actin was detected in the same lysates to highlight protein loading as an internal control. The immunoblot is representative of three independent experiments.

analysis demonstrated that hMSCs and HSCs express Hh ligands (Shh and Ihh), the Hh receptor (Ptc), the Hh co-receptor (Smo), and several Hh-responsive target genes (e.g., Gli1, Gli2, and Gli3). This was consistent with a previous report [17]. All of these Hh pathway components were easily detected within 21–28 RT-PCR amplification cycles, and readily detected on agarose gels of qRT-PCR products from the respective cell types (Fig. 1A). In contrast, Hh pathway components could not be detected in hepatocytes (Fig. 1A). Shh expression in HSCs was increased during culture activation, as determined by increases in both mRNA (Fig. 1B) and protein levels (Fig. 1C).

Recombinant Shh increases Gli1 promoter activity and Gli1 mRNA expression, stimulates proliferation, and inhibits apoptosis in a hMSC line

In order to verify the functional effect of exogenous Shh on hMSCs, we examined transcriptional activation in hMSCs after re-

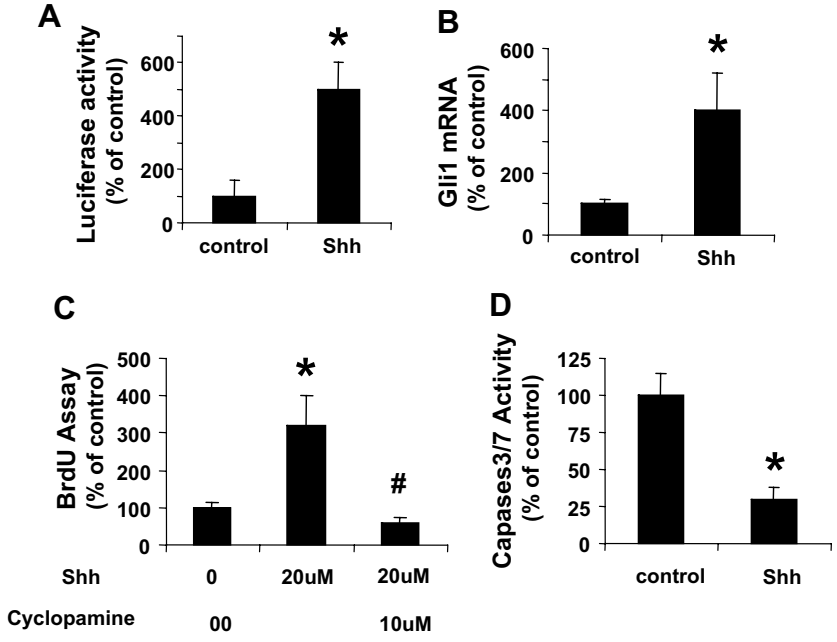

Fig. 2. Shh stimulates Gli1 expression, stimulates proliferation, and inhibits apoptosis in hMSCs. (A) Gli1 promoter activity is significantly increased in recombinant Shh (20 μM)-treated hMSCs, as shown by a transient transfection experiment with the Gli1-luciferase construct (described in Materials and methods). (B) hMSCs were serum-starved for 24 h, and then treated with 20 μM Shh for 6 h. Total RNA was isolated and Gli1 mRNA levels were assessed by real-time PCR. (C) hMSCs were seeded at a density of 5000 cells/well in 96-well plates in growth medium containing 10% FBS. After 24 h, the medium was changed to 0.2% FBS for 24 h, and the cells were pre-treated with vehicle or cyclopamine for 30 min. Subsequently, Shh was added and the cells were incubated in medium containing human Shh (20 μM) for 48 h with BrdU present during the last 24 h. DNA synthesis was assessed by BrdU incorporation. (D) hMSCs were seeded at a density of 5000 cells/well in 96-well plates in growth medium containing 10% FBS. After 24 h, the medium was changed to 0.2% FBS, and the cells were treated with Shh (20 μM) for 48 h. Caspase 3/7 activity was measured ($^{*}p < 0.05$ compared to control, $n = 3$ experiments).

combinant Shh treatments. In transfection experiments using a Gli1-luciferase reporter construct, recombinant Shh increased fivefold the Gli1 promoter activity (Fig. 2A). We used the known expression of Gli1 as a marker for Hh pathway activation [18–20]. Exposure of stellate cells to Shh for 6 h substantially increased Gli1 mRNA expression (Fig. 2B). Next, we investigated the functional effect of Shh pathway activation on hMSCs. Cell proliferation, as measured by the BrdU assay, was increased approximately fourfold after cells were exposed to Shh, and the effect was completely abrogated by cyclopamine (Fig. 2C). Additionally, the apoptosis rate of hMSCs was inhibited 70% by Shh (Fig. 2D).

Effects of co-culture with HSCs on hMSC proliferation and apoptosis

To assess potential paracrine signaling between HSCs and hMSCs, these cells were placed in transwell co-cultures. Cell proliferation (BrdU incorporation) and apoptosis (caspase 3/7 activity) of the co-cultured hMSCs and HSCs were compared to those of monocultures of the corresponding cells. For hMSCs, co-culture increased proliferation (Fig. 3A), decreased apoptosis (Fig. 3B), and improved overall growth (Fig. 3A–B). Gli1 mRNA expression was also measured as a marker of Hh pathway activation. After co-culture with HSC, Gli1 mRNA expression in hMSCs significantly increased 2.5-fold, compared with that of the mono-culture (Fig. 3C). Conversely, HSCs were not affected by being co-cultured with hMSCs (Fig. 3D–E), according to the measures of comparison used here.

Shh neutralizing antibody inhibits hMSC proliferation induced by co-culture with HSCs

To determine if the autocrine production of HSC-derived Hh ligands contributed to the beneficial effects of co-culture on hMSC growth, anti-Shh neutralizing antibody was used as described previously [17]. hMSCs were co-cultured with HSCs, and nonimmune IgG or anti-Shh neutralizing antibody was added to the culture medium. DNA synthesis was assessed by BrdU incorporation after 48 h.

Compared to the mono-culture and to control IgG treatment, neutralizing Hh ligand treatment in the HSC-conditioned medium inhibited the hMSC proliferation induced by HSC co-culture (Fig. 4A). This demonstrates that paracrine Hh-mediated signaling between hMSCs and HSCs promotes hMSC growth.

Co-culture with HSCs induces hMSC differentiation

To investigate if HSCs facilitate hMSC differentiation, hMSCs were cultured alone or co-cultured with HSCs for 7 days. hMSCs expressed no hepatocyte-specific genes in mono-culture. The expression of mRNA for liver-specific genes by hMSCs was examined on co-culture day 7. The expression of albumin and AFP as

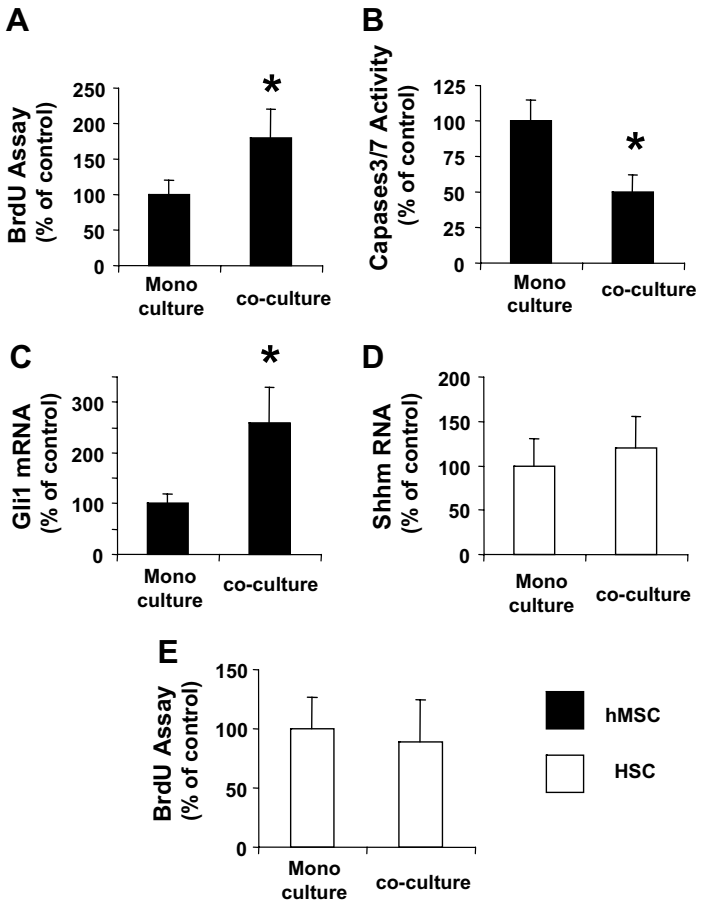

Fig. 3. Effects of co-culture with HSCs on hMSC proliferation and apoptosis. hMSCs (A–C) and HSCs (D and E) were cultured alone (mono-culture) or in co-culture systems. BrdU incorporation (A and E), caspase 3/7 activity (B), Gli1 mRNA expression (C), and Shh mRNA expression (D) were evaluated ($^{*}p < 0.05$ vs mono-culture).

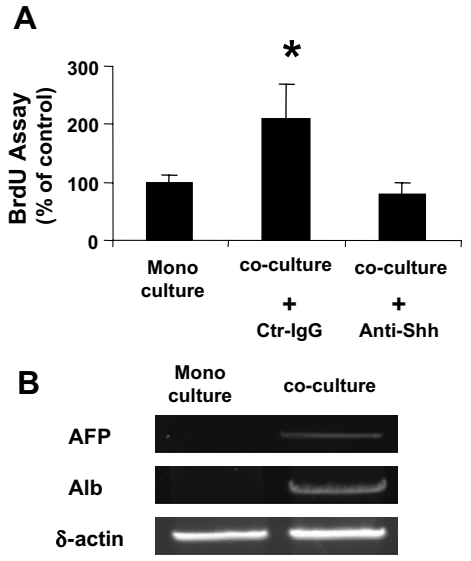

Fig. 4. Shh neutralizing antibody inhibits hMSC proliferation when co-cultured with HSCs. Co-culture with HSCs induces hMSC differentiation. (A) hMSCs were seeded at a density of 5000 cells/well in 96-well plates in growth medium containing 10% FBS. After 24 h, hMSCs were co-cultured with HSCs, and nonimmune IgG or anti-Shh neutralizing antibody was added to the culture medium. DNA synthesis was assessed by BrdU incorporation 48 h later ($^{*}p < 0.05$ vs co-culture with anti-shh). (B) hMSCs were cultured alone or co-cultured with HSCs in growth medium containing 10% FBS for 7 days. Total RNA from hMSCs was extracted as described in Materials and methods. RT-PCR was performed to measure the expression of AFP and albumin. β-Actin was used as a loading control.

hepatocyte markers in these cells was detected by RT-PCR (Fig. 4B). Thus, co-culture with HSCs induces hMSCs to differentiate into a hepatocyte-like lineage.

Discussion

Prior studies have shown that MSCs can be successfully transplanted via a peripheral vein into the liver of hepatic fibrosis/cirrhosis models to prevent histopathological changes [4,5,21]. These in vivo findings provide a rationale for the therapeutic benefit of MSCs, although the underlying mechanism has not been elucidated. Several reports showed that MSCs could successfully differentiate into hepatocytes, both in vivo and in vitro [22,23]. These results indicated that the damaged liver may contain some signaling molecules which play an important role in mediating MSC differentiation. The mechanisms for maintaining the pluripotency of stem cells, and promoting their differentiation, are complex, and involve many signaling pathways. The Hh pathway is known to regulate the viability and differentiation of various types of progenitors. The HSC is the primary extracellular matrix-producing cell type in the liver [24]. HSCs are the major source of collagen and other matrix proteins that characterize liver fibrosis, and have been show to increase Shh production after activation [25]. Several studies have previously demonstrated that Shh can stimulate stem cell proliferation [17,26]. Thus, in this study, we investigated the effects of HSC-secreted paracrine factors on MSCs to determine if HSCs were required for a microenvironment that facilitates MSC differentiation and proliferation.

We observed that HSCs promote the proliferation and differentiation of MSCs in a paracrine manner. HSCs and MSCs express the Hh receptor, Ptc. Ptc-expressing cells are targets for extracellular Hh ligands, such as Shh and Ihh. The interaction of Hh ligands with Ptc releases the Ptc co-receptor, Smoothened (Smo), from the inhibitory influence of Ptc. This permits intracellular propagation of Hh-initiated signals, and leads to the activation of Hh-regulated trans-activating factors, including the Gli family of transcription factors. Gli binding, in turn, regulates the expression of Hh target genes, which include ptc and gli family members themselves [8]. Both HSCs and MSCs express these Hh target genes and are thus capable of Hh transcriptional activity.

Activation of Hh signaling generally enhances the viability of Ptc (+), providing them with a selective growth advantage over neighboring cells that lack Hh receptors. This promotes the transient amplification of Hh-responsive cell populations whose ultimate fates are dictated by the local availability of various mitogen and differentiating factors [27]. Hepatic expression of Hh ligands and Hh target genes increases significantly in co-cultures of HSCs and MSCs. Immunohistochemistry demonstrates that Hh ligands, the Hh receptor, and Hh transcriptional targets localize in MSCs. The co-culturing of HSCs with MSCs also upregulates Hh ligand expression, induces the expression of Hh target genes, and promotes the viability and growth of both cell types. Addition of neutralizing antibodies to Hh ligands reverses these trophic effects in vitro, suggesting that Hh signaling may modulate the proliferation and differentiation of MSCs.

Several studies have previously documented the presence of host bone marrow-derived cells in the liver, and the mobilization of stem cell progenitors during liver fibrosis [28]. The timing and numbers of MSCs homing to the liver may be closely related to the presence of liver injury, which promotes the homing of MSCs through signaling molecules. We hypothesize that MSCs may be mobilized in response to injury, and that HSCs help to create a microenvironment that facilitates stem cell differentiation and proliferation. Our results show that HSCs, which have long been recognized as the major matrix-producing cells in injured livers, also produce Hh ligands that provide trophic signals for MSCs. HSCs trigger and stimulate the proliferation and differentiation of MSCs.

Our investigation reveals a one-way response of activated HSCs to MSCs, and provides the first mechanistic evidence that HSCs can facilitate MSC differentiation and proliferation through the Hh pathway. However, the exact mechanism of the anti-fibrotic effect of MSCs requires further study. These findings may account for the beneficial effects of transplanted MSCs in various models of acute and chronic liver injuries.

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