Original Article
Suppression of hedgehog signaling regulates hepatic stellate cell activation and collagen secretion

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Abstract: Hepatic stellate cells (HSCs) play an important role in liver fibrosis. This study investigates the expression of hedgehog in HSC and the role of hedgehog signaling on activation and collagen secretion of HSC. Liver ex vivo perfusion with collagenase IV and density gradient centrifugation were used to isolate HSC. Expression of hedgehog signaling components Ihh, Smo, Ptc, Gli2 and Gli3 in HSC were detected by RT-PCR. Hedgehog siRNA vectors targeting Ihh, Smo and Gli2 were constructed and transfected into HSC respectively. Suppression of hedgehog signaling were detected by SYBR Green fluorescence quantitative RT-PCR. Effects of hedgehog signaling inhibition on HSC activation and collagen I secretion were analyzed. Hedgehog signaling components Ihh, Smo, Ptc, Gli2 and Gli3 were expressed in HSC. siRNA vectors targeting Ihh, Smo and Gli2 were successfully constructed and decreased target gene expression. Suppression of hedgehog signaling significantly decreased the expression of α-SMA in HSC ($P<0.01$). Collagen type I secretion of HSC were also significantly decreased ($P<0.01$). In summary, HSC activation and collagen secretion can be regulated by hedgehog signaling. Hedgehog may play a role in the pathogenesis of liver fibrosis.

Keywords: Hepatic stellate cell, hedgehog, liver fibrosis, activation, collagen secretion

Introduction

Hepatic fibrosis is a common result of chronic liver injury and may progress to liver cirrhosis and portal hypertension. It has been proved that hepatic stellate cells (HSCs) play a key role in hepatic fibrosis and portal hypertension [1-8]. HSCs reside in the space of Disse, in close contact with sinusoidal endothelial cells and hepatocytes. A characteristic feature of HSC is that they possess long, branching cytoplasmic processes. In normal liver, HSCs are responsible for the transport and storage of retinoids and for the production of basement membrane components. After liver injury, HSCs are activated. The maker of HSCs activated phenotype is the expression of the intracellular microfilament protein α-smooth muscle actin (α-SMA) [9-13]. Activated HSCs lose retinoids, produce increased level of extracellular matrix (ECM) e.g. collagen, vimentin, et al., and express cytokins and its receptors. Because HSC play a role in both hepatic fibrosis and por-

tal hypertension, they are postulated therapeutic target in the prevention and treatment the complications of chronic liver disease.

Hedgehog signaling pathway is known to regulate critical cellular responses including proliferation, apoptosis, migration, and differentiation. It also modulates wound healing responses in a number of adult tissues and organs, including the liver during progression of chronic liver diseases [14]. Normal liver usually expresses quite low levels of hedgehog signaling pathway components. Moreover, hedgehog signaling has been reported to be progressively increased during the process of liver injury [15, 16]. Hedgehog signaling may be contributes to the process of HSC activation and their ability to synthesize ECM components.

In this study, we examined the expression of hedgehog pathway components in HSC. And hedgehog signaling was suppressed in HSC, to observe the activation and collagen secretion of HSC.

Table 1. Antibodies used for immunocytochemistry and Western blot

Primary antibody	Source	Company, Country
Desmin	Mouse, monoclonal	DAKO, Denmark
Gliad fibrillary acidic proteins (GFAP)	Mouse, monoclonal	DAKO, Denmark
α-smooth muscle actin (α-SMA)	Mouse, monoclonal	Santa Cruz, CA, USA
Collagen type I	Rabbit, polyclonal	Santa Cruz, CA, USA
α-tubulin	Goat, polyclonal	Santa Cruz, CA, USA

Table 2. RT-PCR primers and products lengths

Primers	Sequences	Length of the Products
Ihh	5-AAACTCGTGCCTCTTGCCTA-3 5-TGACAGAGATGGCCAGTGAG-3	229 bp
Smo	5-AATTGGCCTGGTGCTTATTG-3 5-CTGAAGGTGATGAGCACGAA-3	183 bp
Ptc	5-ATTTCTTGCCCTTGGTGTTG-3 5-GAAGGCAGTGACATTGCTGA-3	166 bp
Gli2	5-ACGCTAAGTGGCAGTCCTGT-3 5-TGGGGCAGCGAGACTAAATA-3	246 bp
Gli3	5-GGGGACAAAGATGAAAGCAA-3 5-GCTTTGAACGGTTTCTGCTC-3	194 bp
β-actin	5-TGGGACGATATGGAGAAGAT-3 5-ATTGCCGATAGTGATGACCT-3	523 bp

Materials and methods

Isolation and culture of primary rat HSCs

Rat HSCs were isolated from normal Wistar rats by liver ex vivo perfusion of collagenase type IV and density gradient centrifugation methods [17-20]. Isolated HSCs were seeded on uncoated plastic tissue culture dishes and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, New York, USA) supplemented with 20% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). Cell viability was determined by Trypan blue exclusion staining. Cells were counted under fluorescence microscopy for auto-fluorescence at a wavelength of 328 nm. HSC purity was identified by immunocytochemistry detection of the expression of either desmin or glial fibrillary acidic proteins (GFAP). Details of the primary antibodies are shown in **Table 1**. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Peking University People's Hospital (Permit Number: RDB2012-11). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Expression of hedgehog signaling pathway in HSC

Total RNA of HSC was isolated from subconfluent cells using Trizol Reagent (Gibco BRL). Hedgehog signaling pathway components Ihh, Smo, Ptc, Gli2 and Gli3 expression were detected by RT-PCR. Primer sequences and product lengths are shown in **Table 2**. β-actin was used as a control. All PCR reaction was performed in a total volume of 25 μl. PCR mixture contained 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl, 0.01% gelatin, 200 pmol each primer, 2 U Taq DNA polymerase, and 0.5 μl cDNA. Amplification was performed by denaturation at 94°C for 2 min, followed by 28 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 5 min. PCR products were electrophoresed on 2% agarose gel.

Construction of hedgehog signaling siRNA vector

According to the RT-PCR results of hedgehog signaling pathway components expression, Ihh, Smo and Gli2 were selected for RNA interference. Using Qiagen software to design targeting sequences. Each interference gene designs 3 to 5 interference targets. Targeting sequences were synthesized and then connected into the linear eukaryotic expression vector. The connected products were transfected into the prepared JM109 bacterial competent cells, to prepare recombinant plasmid pEGFP-N1 (purchased from Shanghai GeneChem Co., Ltd.) containing hedgehog signaling siRNA sequence. The clonal was confirmed by sequencing.

Suppression of hedgehog signaling in HSC

Recombinant hedgehog siRNA vectors were transfected into HSCs. Transfection steps were in accordance with the Lipofectamine 2000 manufacturer's instructions. Suppression of hedgehog signaling were detected by SYBR Green fluorescence quantitative RT-PCR. Total cellular RNA was extracted using RNeasy Mini Kits (Qiagen, Hilden, GA) and reverse tran-

Table 3. Sequence of siRNA

Target		Loop	
Ihh	5'-ACCTTCAGCGATGTGCTCAT	TTCAAGAGA	ATGAGCACATCGCTGAAGGT-3'
Smo	5'-TACCAAGAAGCCCATTCCTGA	TTCAAGAGA	TCAGGAATGGGCTTCTTGGTA-3'
Gli2	5'-AGCAGCAACTGTATAAGTGA	TTCAAGAGA	TCACTTATACAGTTGCTGCT-3'

analyses included the univariate variance analysis and t-test. $P<0.05$ was considered statistically significant.

Results

scribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed via ABI PRISM 7500 Real-Time PCR Systerm (Applied Biosystems) with 1× SYBR Green Universal PCR Mastermix (Takara). Quantitative PCR was performed by pre-denaturation at 95°C for 1 min, followed by 40 cycles at denaturation 95°C for 15 s, annealing at 60°C for 15 s, and extending at 72°C for 20 s. Transcript levels were calculated according to the ΔΔCt method. According to quantitative PCR results, each interference gene selects one interference target (**Table 3**).

Effect of hedgehog signaling inhibition on expression of α-SMA in HSC

HSC cells were allocated into five groups, group 1 as control, group 2: the blank plasmid (Non) group, group 3: siRNA-Ihh group, group 4: siRNA-Smo group, and group 5: siRNA-Gli2 group. Group 2 was transfected with blank plasmid not containing targeting sequence. Group 3 to 5 were transfected with siRNA vectors containing Ihh, Smo and Gli2 targeting sequences respectively. After 72 h transfection, cells were collected and cellular total proteins were extracted, expression of α-SMA was detected by Western blot. The gray scale ratio of α-SMA/α-tubulin represents the relative expression amount.

Effect of hedgehog signaling inhibition on secretion of collagen type I in HSC

Grouping method as mentioned, effect of Ihh, Smo and Gli2 suppression on secretion of type I collagen in HSCs were measured. Cell culture supernatant were collected after 72 h transfection, ELSIA method was used to determination of the content of collagen type I, differences were compared between groups.

Statistical analysis

The final value of data was expressed as mean ± standard deviation ($\bar{x}$ ± s) and statistical

Primary culture of rat HSC

The yield rate of HSC was $2.82×10^7$ with the cell viability exceeding 98%. The purity of HSC identified by double staining with antibodies against desmin and GFAP was higher than 97%. (**Figure 1**).

Hedgehog signaling pathway components expressed in HSC

Hedgehog signaling pathway components, Ihh, Ptc, Smo, Gli2 and Gli3 gene were expressed in HSC, as detected by RT-PCR and shown in **Figure 2A**.

Down-regulation of hedgehog signaling inhibit HSC activation and collagen secretion

After RNA interference, the hedgehog signaling expression of Ihh, Smo and Gli2 decreased. Fluorescence quantitative RT-PCR results showed that, when the expression of non-interference group was set to 1, the expression of Ihh, Smo and Gli2 gene were 0.261 ± 0.103, 0.247 ± 0.115 and 0.228 ± 0.098 respectively in siRNA-Ihh, siRNA-Smo and siRNA-Gli2 groups (**Figure 2B**). Differences between non-interference and interference groups were significant ($P<0.01$).
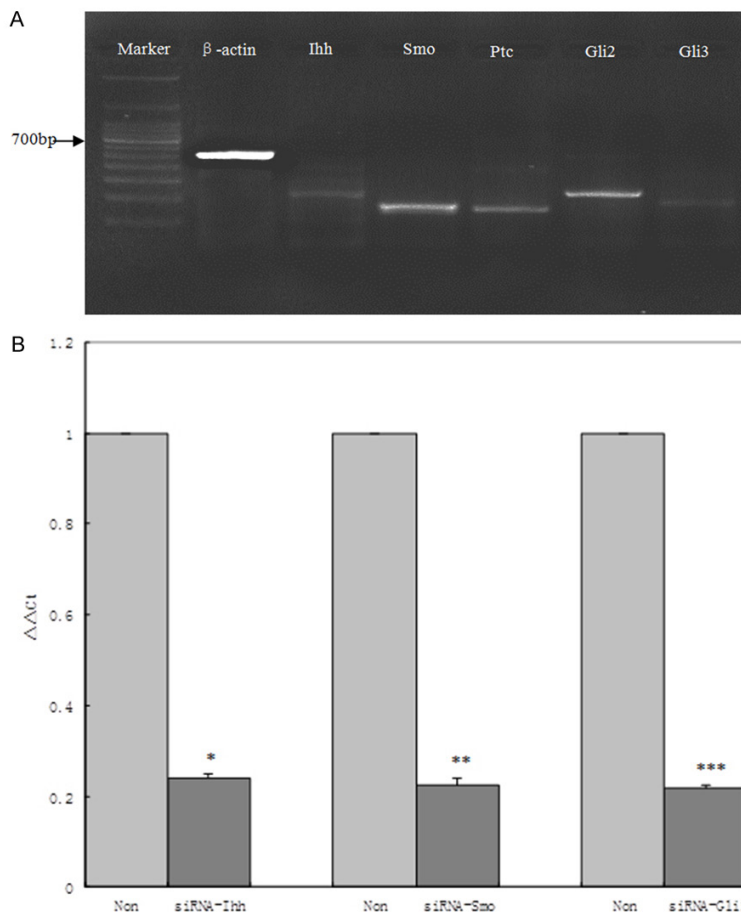
When hedgehog signaling was suppressed in HSC, expression of α-SMA was inhibited (**Figure 3A**). The scale ratio of α-SMA/α-tubulin in siRNA-Ihh, siRNA-Smo and siRNA-Gli2 group were 0.203 ± 0.009, 0.298 ± 0.017 and 0.096 ± 0.013 respectively, significant lower than that of the control and blank group (**Figure 3B**, 1.069 ± 0.018 and 1.352 ± 0.009 respectively, $P<0.01$).

Collagen type I secretion of HSC was also inhibited by hedgehog signaling pathway suppression. As shown in **Figure 4**, collagen type I content of cell culture supernatant in siRNA-Ihh, siRNA-Smo and siRNA-Gli2 group were 20.88 ± 1.5 ng/mL, 17.93 ± 0.9 ng/mL and 18.69 ± 1.2
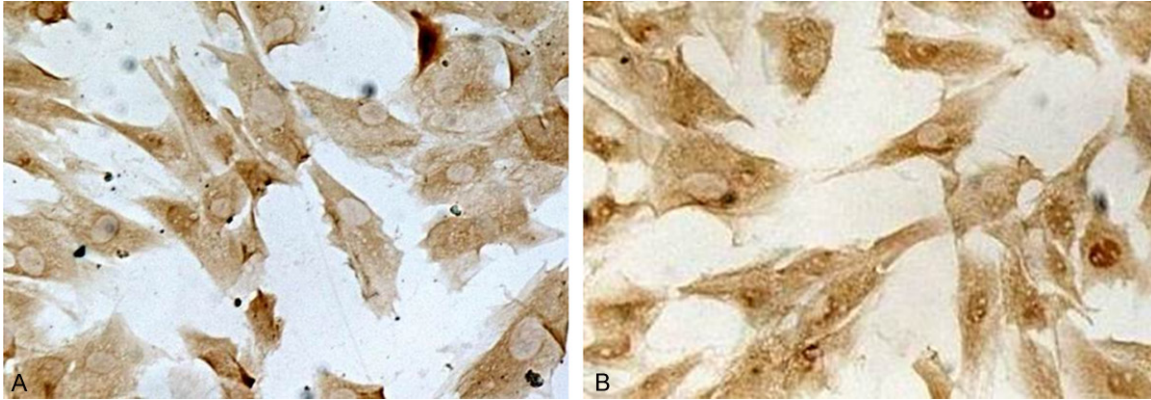
Figure 1. Primary HSC identified by immunocytochemistry staining of desmin and GFAP. A: HSC were positive of desmin (original magnification, ×200); B: HSC were positive of GFAP (original magnification, ×200).

Figure 2. A: Hedgehog signaling components, Ihh, Ptc, Smo, Gli2 and Gli3 gene were expressed in HSC, marker: 100bp DNA ladder. B: After RNA interference, the expression of Ihh, Smo and Gli2 decreased. Compared with non-interference group, *,**,***P<0.01.

mL and 51.75 ± 2.5 ng/mL respectively, *P*<0.01).

Discussion

Research on HSCs has made great advances since Knook [17] successfully isolated and cultured HSC in 1982. Subsequently, numerous studies have shown that HSC plays a fundamental role in liver fibrogenesis [1-8]. HSC has become a focus in understanding the mechanisms involved in hepatic fibrogenesis.

There is emerging evidence that hedgehog, a master developmental regulator [21, 22], becomes reactivated during adult wound healing [23]. Hedgehog signaling is initiated by the interaction of a family of ligands (Sonic hedgehog-Shh, Indian hedgehog-Ihh, and Desert hedgehog-Dhh) which interact with the Patched (Ptc) specific cell surface receptor that is expressed on the plasma membrane of responsive cell. Ligand-receptor interaction derepresses activity of another molecule, Smoothened (Smo), responsible for the propagation of the signal that leads to nuclear translocation of members of Glioblastoma (Gli1, Gli2,

ng/mL respectively, significant lower than that of the control and blank group (57.36 ± 3.1 ng/
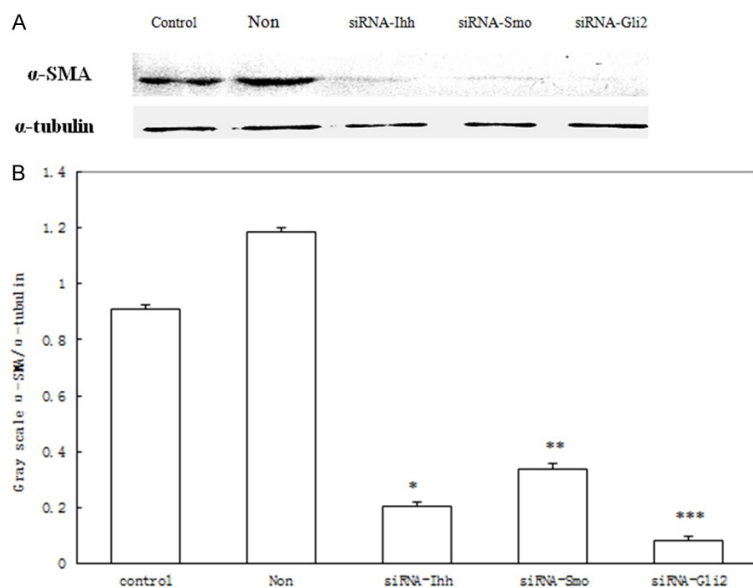
Figure 3. A: When hedgehog was suppressed in HSC, expression of α-SMA was inhibited. B: The scale ratio of α-SMA/α-tubulin in siRNA-Ihh, siRNA-Smo and siRNA-Gli2 group were significant lower than that of the control and blank group, *,**,***P<0.01.
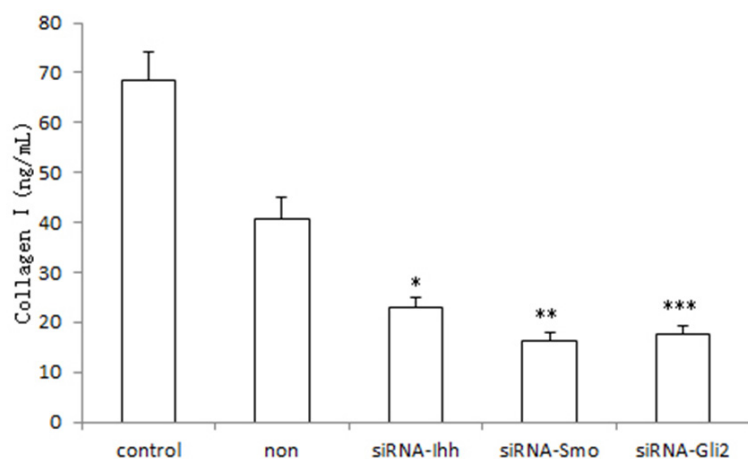
Figure 4. Collagen type I secretion of siRNA-Ihh, siRNA-Smo and siRNA-Gli2 group were significant lower than that of the control and blank group, *,**,***P<0.01.

Gli3) family transcription factors, that regulate the expression of a number of critical Gli-target genes. In the absence of hedgehog ligands, Ptc represses Smo and leads to Gli ubiquitination and subsequent proteasomal degradation. Although the exact role of hedgehog in adult tissue repair remains somewhat obscure, the pathway seems to control critical cell fate decisions that are required for reconstruction of healthy tissue because fibrosis typically result when hedgehog signaling becomes deregulat-

ed [21]. Like several of the key cell types involved in liver repair, HSCs are hedgehog responsive [22, 23]. The current study focused on HSC activation and collagen secretion. We found that hedgehog signaling pathway components were expressed in HSC. When hedgehog signaling was suppressed in HSC, expression of α-SMA and Collagen type I secretion of HSC were inhibited. Hedgehog signaling directly and significantly contributes to the process of HSC activation; drive quiescent HSC towards the activated phenotype. Our results suggested that the activation of HSC can be regulated by hedgehog signaling. The activation of hedgehog signaling in HSCs maybe contributes and sustains liver fibrogenesis. Hedgehog signaling promotes the ability of HSC synthesis and secretion of ECM [21].

In summary, our results suggested that HSC express hedgehog signaling pathway components. HSC activation and collagen secretion can be regulated by hedgehog signaling.

Disclosure of conflict of interest

None.

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