Lentiviral vector-mediated down-regulation of IL-17A receptor in hepatic stellate cells results in decreased secretion of IL-6

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Abstract

AIM: To investigate the mechanism of interleukin (IL)-6 secretion through blocking the IL-17A/IL-17A receptor (IL-17RA) signaling pathway with a short hairpin RNA (shRNA) in hepatic stellate cells (HSCs) in vitro.

METHODS: HSCs were derived from the livers of adult male Sprague-Dawley rats. IL-6 expression was evaluated using real-time quantitative polymerase chain reaction and enzyme linked immunosorbent assay. The phosphorylation activity of p38 mitogen activated protein kinases (MAPK) and extracellular regulated protein kinases (ERK) 1/2 upon induction by IL-17A and suppression by IL-17RA shRNA were examined using Western blotting.

RESULTS: IL-6 expression induced by IL-17A was significantly increased compared to control in HSCs (P < 0.01 in a dose-dependent manner). Suppression of IL-17RA using lentiviral-mediated shRNA inhibited IL-6 expression induced by IL-17A compared to group with only IL-17A treatment (1.44 ± 0.17 vs 4.07 ± 0.43, P < 0.01). IL-17A induced rapid phosphorylation of p38 MAPK and ERK1/2 after 5 min exposure, and showed the strongest levels of phosphorylation of p38 MAPK and ERK1/2 at 15 min in IL-17A-treated HSCs. IL-6 mRNA expression induced by IL-17A (100 ng/mL) for 3 h exposure was inhibited by preincubation with specific inhibitors of p38 MAPK (SB-203580) and ERK1/2 (PD-98059) compared to groups without inhibitors preincubation (1.67 ± 0.24, 2.01 ± 0.10 vs 4.08 ± 0.59, P < 0.01). Moreover, lentiviral-mediated IL-17RA shRNA 1 inhibited IL-17A-induced IL-6 mRNA expression compared to random shRNA in HSCs (1.44 ± 0.17 vs 3.98 ± 0.68, P < 0.01). Lentiviral-mediated IL-17RA shRNA 1 inhibited phosphorylation of p38 MAPK and ERK1/2 induced by 15 min IL-17A (100 ng/mL) exposure.

CONCLUSION: Down-regulation of the IL-17RA receptor by shRNA decreased IL-6 expression induced by IL-17A via p38 MAPK and ERK1/2 phosphorylation in HSCs. Suppression of IL-17A expression may be a strategy to reduce the inflammatory response induced by IL-17A in the liver.

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Key words: Interleukin 17A; Interleukin 6; Hepatic stellate cells; Liver fibrosis

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INTRODUCTION

Hepatic stellate cells (HSCs), also known as fat-storing cells, are a major cell type involved in liver fibrosis. Activation and proliferation of HSCs are associated with liver injury[8]. Activated HSCs accumulate excess extracellular matrix and produce a variety of pro-inflammatory cytokines, including macrophage inflammatory protein-2, monocyte chemo-attractant protein-1, interleukin (IL)-6, IL-8, and transforming growth factor-β (TGF-β) [9,10]. These pro-inflammatory cytokines can eventually lead to liver fibrosis.

IL-17A is the founding member of the IL-17 cytokine family, which now includes six major isoforms, IL-17A, -B, -C, -D, -E, and -F[11]. The original “IL-17” has been designated IL-17A and is considered a T cell (Th17) cell-specific cytokine[12]. The IL-17A receptor (IL-17RA) is expressed on many cell types in the human body[13]. IL-17A exerts pro-inflammatory, pro-apoptotic, and pro-mitogenic effects via binding to IL-17RA[14].

In this study, we constructed a highly efficient lentiviral short hairpin RNA (shRNA) targeting IL-17RA to study the level of IL-6 secretion in the absence of IL-17A in activated HSCs and the underlying mechanism(s). Our results showed that the silencing effect of IL-17RA shRNA eliminated IL-6 secretion in activated HSCs. We suggest that secretion of IL-6 involves the mitogen activated protein kinases (MAPK) signaling pathway through phosphorylation of p38 MAPK and extracellular regulated protein kinases (ERK) 1/2. Our finding may provide a novel interventional therapy against hepatic inflammatory responses and hepatic fibrosis.

MATERIALS AND METHODS

Reagents

Recombinant murine IL-17A was purchased from PeproTech (Princeton Business Park, NJ). Anti-IL-17R (sc-1902, dilution factor 1:200) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PD-98059 (inhibitor of MEK1) and SB-203580 (inhibitor of p38 MAPK) were obtained from Sigma-Aldrich (Saint Louis, MO). The p38 MAPK antibody (dilution factor 1:200), phospho-p38 MAPK rabbit mAb (dilution factor 1:200), ERK1/2 MAPK rabbit mAb (dilution factor 1:250), and phospho-ERK1/2 MAPK rabbit mAb (dilution factor 1:250) were obtained from Cell Signaling Technology (Beverly, MA).

shRNA design and plasmid constructs

Candidate sequences targeting rat IL-17RA mRNA (GenBank Accession NM 001107883) were designed using the Dharmacon siDESIGN Center procedure. Two complementary single-strand oligonucleotides containing the target sequences were synthesized chemically and annealed. The double-stranded oligonucleotides were inserted between AgeI and EcoRI restriction sites in the pGCL-green fluorescent protein (GFP) small interfering RNA (siRNA) vector that contains a cytomegaloivirus-driven enhanced green fluorescent protein reporter gene. The ligated plasmid was transformed into Escherichia coli DH5α competent cells for pGCSIL/IL-17RA shRNA plasmid amplification.

shRNA lentivirus transduction

Plasmids containing the IL-17RA shRNA lentivirus were transfected into 293T cells using the ViraPower packaging mix (pGCSIL/IL-17RA shRNA plasmids, pHelper 1.0, and pHelper 2.0) and Lipofectamine 2000 (Invitrogen). After 48 h, the harvested viral supernatant was used to infect HSCs at a multiplicity of infection of 10 for 24 h. Cells with the GFP label were harvested after 48 h, and total RNA was extracted. The interference efficiency of IL-17RA shRNA was determined using real-time quantitative polymerase chain reaction (qPCR), and the most efficient silencing sequence of IL-17RA shRNA was selected for subsequent studies.

Isolation and culture of rat HSCs

Adult male Sprague-Dawley rats (body weight, 400-500 g) were used for HSC isolation as described previously[18]. The liver tissues were digested with collagenase IV (0.5 g/L) and deoxyribonuclease I (0.03 g/L) before fractionation on a discontinuous gradient of iodixanol. HSCs were harvested from the 11.5% medium interface, washed, and seeded in tissue culture plates. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, United States) with 10% fetal bovine serum (Sijiqing Bio. Co. Hangzhou, China), 100 U/mL penicillin, and 100 μg/mL streptomycin. Culture medium was changed every third day. All experiments were performed with cells from passage numbers 3-6.

Identification of primary HSCs and activated HSCs

The harvested primary HSCs were studied at days 1, 2, and 5 after isolation. Primary d1 HSCs showed intrinsic fluorescence of lipid droplet and desmin expression (Boster, Wuhan, China) under a fluorescence microscope. Nuclei cells were stained with DAPI. After the first subculture passage, activated HSC purity was assessed using α-smooth muscle actin (α-SMA, dilution factor 1:100; Boster) and immunoocytochemical staining.

IL-6 secretion

Cells cultured in 6-well plates were exposed to different concentrations of IL-17A for 24 h. The amount of IL-6...
in supernatants was determined using enzyme linked immunosorbent assay kits purchased from R and D Systems (Minneapolis, MN).

Western blotting analysis
Cells exposed to IL-17A in the presence or absence of inhibitors for the indicated time period were extracted with lysis buffer containing a phosphatase inhibitor cocktail and a protease inhibitor cocktail. The protein concentration was measured using the Bradford assay. Proteins from each sample were loaded equally and separated on a 6% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Proteins were electrothermally transferred onto polyvinylidene fluoride membranes, which were then incubated with primary antibodies at 4 °C overnight. On the following day, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at 37 °C for 2 h and then signals were detected using chemiluminescence (ECL Plus, GE Healthcare). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); dilution factor 1:2000, Santa Cruz Biotechnology) was used as a loading control.

RNA extraction and gene expression analysis
Real-time qPCR was used to examine the expression of IL-17RA and IL-6 in HSCs. Total RNAs were isolated from HSCs using the Trizol reagent following the manufacturer’s protocol and reverse-transcribed using a cDNA synthesis kit (Promega). SYBR Green detection was used and the values were normalized using GAPDH. Real-time qPCR was performed with a DNA Engine (ABI 7500) using SYBR GREENER qPCR UNIVERSAL. Primer sequences were: IL-17RA (forward) 5'-TG-GCGGTTTCTCCTGCACTGC-3' and IL-17RA (reverse) 5'-CGGTGTAGTCTCATCTTACCTGC-3', IL-6 (forward) 5'-CGTTTCACCTGGAACTTTGTG-3' and IL-6 (reverse) 5'-ATTAGGAGGACTTGGAAGTGGG-3', and GAPDH (forward) 5'-TTCACCCGACAGTCAAGG-3' and GAPDH (reverse) 5'-CTCACCCGACAGCATCACC-3'. Relative expression levels of each primer set were normalized to GAPDH expression.

Statistical analysis
Data were expressed as mean ± SD. The statistical significance of changes was determined using the t-test. P values < 0.05 were considered to indicate statistical significance.

RESULTS

**IL-17RA shRNA lentiviral construction and transduction**
The sequences of IL-17RA shRNAs 1, 2, 3, and 4 and random shRNA are shown in Table 1. The recombinant plasmid of pGCSIL/IL-17RA shRNA 1 was confirmed by sequence analysis (Figure 1A) and titers were approximately 1 × 10⁷ TU/mL. The interference efficiency of IL-17RA shRNAs 1, 2, 3, and 4 were shown in Figure 1B. The relative expression of IL-17RA mRNA for IL-17RA shRNAs 1, 2, 3, and 4 were 0.253 ± 0.011, 0.643 ± 0.022, 0.673 ± 0.051, and 0.444 ± 0.043, respectively. IL-17RA shRNAs 1, 2, 3, and 4 were 0.253 ± 0.011, 0.643 ± 0.022, 0.673 ± 0.051, and 0.444 ± 0.043, respectively. IL-17RA shRNA 1 exhibited a significant silencing effect, with 74.7% interference efficiency. Figure 1C shows HSC morphology with successfully transduced GFP-labeled IL-17RA shRNA 1 using a fluorescence or light microscope (× 200) after 72 h.

**Isolation and culture of rat HSCs**
Primary HSC culture after isolation on days 1, 2, and 5 are shown in Figure 2A. The cultured HSCs were assessed for intrinsic fluorescence of lipid droplet and desmin expression (Boster; Figure 2B). The purity of activated HSCs was confirmed using immunocytochemical staining for α-smooth muscle actin (Boster; Figure 2C); the purity reached > 98%.

**IL-17A induces IL-6 expression**
HSCs were incubated with increasing concentrations of IL-17A for 24 h, and the levels of IL-6 secretion in the supernatant were measured by ELISA. As shown in Figure 3A, IL-17A-induced IL-6 secretion increased in a dose-dependent manner. The expression level of IL-6 mRNA by IL-17A was quantified using real-time qPCR (Figure 3B). A rapid increase in IL-17A-induced IL-6 mRNA expression reached a maximum level at 3 h of IL-17A exposure. However, the level of IL-6 mRNA expression decreased gradually after 3 h exposure.

**IL-17A induces activation of MAPKs**
In various cells, the MAPK family has been shown to

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**Table 1 Interleukin-17A receptor short hairpin RNA and random short hairpin RNA sequences**

| Target sequence | Double strand DNA oligo sequence |
|-----------------|---------------------------------|
| IL-17RA shRNA 1 | CAAGTCCAAGACCACCTCTTA 5'-ccggcaCAAGTCCAAGACCATCTTAAttcaagagaTGTGACATTGGATCGCTGGtgtttttg-3' |
| IL-17RA shRNA 2 | CACCAGCATCCAATGTCACA 5'-ccggcaCACCAGCATCCAATGTCACAttcaagagaATCATGTTCATGGCTGCTGtgtttttg-3' |
| IL-17RA shRNA 3 | CGACCCGATAGACCAACATGAT 5'-ccggcaGGAAGAAAGTGGAGTGGTAttcaagagaTACCACTCCACTTTCTTCCtctttttg-3' |
| IL-17RA shRNA 4 | GGAAGAAAGTGGAGTGGTA 5'-ccggTTCTCCGAACGTGTCACGTttcaagagaACGTGACACGTTCGGAGAA-3' |
| Random shRNA | CAAGTCCAAGACCATCTTA 5'-ccggcaCAAGTCCAAGACCATCTTAAttcaagagaTAAGATGGTCTTGGACTTGtgtttttg-3' |
| | TTCTCCGAACGTGTCACGT 5'-ccggTTCTCCGAACGTGTCACGTttcaagagaACGTGACACGTTCGGAGAA-3' |

**shRNA:** Short hairpin RNA; **IL-17RA:** Interleukin-17A receptor.
play an important role in regulating gene expression in response to inflammatory mediators\(^7\). To investigate whether IL-17A induced activation of p38 MAPK and ERK1/2 in HSCs, phosphorylation of p38 MAPK and ERK1/2 were evaluated after IL-17A stimulation. IL-17A induced rapid phosphorylation of p38 MAPK and ERK1/2 after 5 min, and showed the strongest levels of phosphorylation of p38 MAPK and ERK1/2 at 15 min (Figure 3C). However, IL-17A did not affect the expression of total p38 MAPK or ERK1/2. This result indicated that IL-17A enhanced p38 MAPK and ERK1/2 phosphorylation in HSCs.

**MAPK inhibitors suppress IL-6 expression**

To further assess the phosphorylation of p38 and ERK1/2 induced by IL-17A, two specific inhibitors, SB-203580 (p38 MAPK) and PD-98059 (ERK1/2), were used. As shown in Figure 3D, SB-203580 inhibited p38 MAPK phosphorylation and PD-98059 blocked phosphorylation of ERK1/2 induced by IL-17A in HSCs. SB-203580 and PD-98059 were used to further evaluate the roles of p38 MAPK and ERK1/2 in IL-6 mRNA expression induced by IL-17A in HSCs. Both inhibitors reduced IL-17A-induced IL-6 mRNA expression significantly (Figure 3E).

**shRNA suppresses IL-17RA and IL-6 expression**

Western blotting and real-time qPCR were performed to study the silencing effect of lentiviral-mediated shRNA on IL-17RA-induced IL-6 expression in HSCs. The protein levels of IL-17RA in shRNA 1-treated HSCs were
reduced, compared with random shRNA and control (Figure 4A). HSCs treated with IL-17A alone or IL-17A with random shRNA showed increased IL-6 mRNA expression, whereas, IL-6 mRNA expression in HSCs pretreated with IL-17RA shRNA 1 was decreased significantly (Figure 4B).

**shRNA suppresses phosphorylation of p38 and ERK1/2**

IL-17A induced IL-6 expression via p38 MAPK and ERK1/2 phosphorylation. In Figure 4C, we show that HSCs treated with IL-17A transduced with shRNA 1 exhibited reduced protein levels of phosphorylation of p38 MAPK and ERK1/2, compared with IL-17A alone and IL-17A transduced with random shRNA group. Phosphorylation of p38 MAPK and ERK1/2 was blocked in the group treated with lentiviral-mediated IL-17RA shRNA 1, whereas there was almost no change in the IL-17A alone or IL-17A transduced with random shRNA groups.

**DISCUSSION**

IL-17A is a pro-inflammatory cytokine secreted by a subset of T helper cells, named Th17 cells. IL-17A signals exert miscellaneous effects through binding to the IL-17A receptor, which is expressed on a variety of cells and tissues\(^9,10\). The IL-17A pathway plays important roles in the human inflammatory and autoimmune diseases and tumor progression\(^11,12\). Clinical studies have shown that high levels of IL-17A and other cytokines related to the IL-17A pathway are seen in sera or tissues of patients with diseases such as psoriasis, multiple sclerosis, systemic sclerosis, ankylosing spondylitis, and juvenile idiopathic arthritis\(^13-17\). The IL-17RA is found on hepatocytes, Kupffer cells, HSCs, biliary epithelial cells, and sinusoidal endothelial cells\(^18\). Thus, IL-17A function in liver diseases has attracted much attention. Previous studies indicated that IL-17A is elevated in various liver diseases, including liver autoimmunity and inflammatory diseases, alcoholic liver disease (ALD), and hepatocellular carcinoma\(^19\).
Studies based on acute hepatic injury (AHI) and hepatitis C virus (HCV) showed that patients with AHI and HCV have significantly higher serum IL-17A levels than controls [20-22]. Furthermore, percentages of circulating Th17 cells and Th17-associated cytokines, including IL-17A, IL-6, and IL-23, were increased markedly in peripheral blood and in liver tissues of chronic hepatitis B patients compared with controls [23,24]. Recent studies indicate that patients with ALD had significantly higher plasma levels of IL-17A compared with healthy subjects. Moreover, the number of liver Th17 cells was correlated positively with hepatocellular damage and the severity of the disease in a cohort study of patients with ALD [25].

HSCs are the major source of extracellular matrix in liver fibrosis [26] and participate in modulating liver inflammation during liver fibrogenesis [27]. IL-17RA is expressed on HSCs, and thus blockade of IL-17A/IL-17RA signal transduction in HSCs may be a strategy to interfere with hepatic inflammatory processes in chronic liver diseases.

In our study, we sought to investigate the secretion of IL-17A in hepatic stellate cells (HSCs) induced by interleukin 17A (IL-17A) was determined using enzyme-linked immunosorbent assay. IL-6 mRNA expression in HSCs induced by IL-17A (100 ng/mL) was measured by polymerase chain reaction. IL-6 mRNA expression induced by IL-17A (100 ng/mL) for 3 h exposure was inhibited by preincubation with SB-203580 (SB) and PD-98059 (PD) for the same time period as above. T: Total; P: Phosphorylation; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MAPK: Mitogen activated protein kinases; ERK: Extracellular regulated protein kinases.

Figure 3 Mitogen activated protein kinases pathway involved in interleukin 17A induced interleukin 6 expression. A: Secretion of interleukin (IL)-6 in hepatic stellate cells (HSCs) induced by interleukin 17A (IL-17A) was determined using enzyme-linked immunosorbent assay. B: IL-6 mRNA expression in HSCs induced by IL-17A (100 ng/mL) was measured by polymerase chain reaction. C: Phosphorylation of p38 MAPK and ERK1/2 induced by IL-17A (100 ng/mL) in HSCs was detected using Western blotting. D: Phosphorylation of p38 MAPK and ERK1/2 induced by IL-17A (100 ng/mL) for 15 min was blocked by preincubation with MAPKs inhibitors, SB-203580 (1 μmol/L in DMSO, 30 min) and PD-98059 (10 μmol/L in DMSO, 1 h). E: IL-6 mRNA expression induced by IL-17A (100 ng/mL) for 3 h exposure was inhibited by preincubation with SB-203580 (SB) and PD-98059 (PD) for the same time period as above.
IL-6, one of the major cytokines in various liver injuries, is stimulated by IL-17A in HSCs. Our data show that IL-17A induces a large amount of IL-6 secretion (68.96 ± 8.30 pg/mL) even at a low concentration of IL-17A treatment (1 ng/mL). With increasing concentrations of IL-17A, the secretion of IL-6 also increased. Surprisingly, a higher concentration of IL-17A (500 ng/mL) induced only moderate expression of IL-6 (109.16 ± 6.91 pg/mL). This may suggest that there is saturation of binding between IL-17A and IL-17RA at a particular concentration or that there may be a negative feedback response on IL-6 expression on IL-17A induction. Our data further show that the IL-6 mRNA expression detected in real-time qPCR reached a maximum level after 3 h exposure to IL-17A and declined thereafter. These results are based on data obtained from the expression of IL-6 protein in HSCs supernatants and indicate that IL-17A induces IL-6 expression in vitro. We made a lentivirus-mediated IL-17RA shRNA construct to repress endogenous IL-17RA in HSCs. As a result, we found that IL-6 mRNA expression was notably reduced. Our results further suggest that the MAPK pathway in IL-6 expression is induced by IL-17A in HSCs. We showed that the phosphorylation of p38 MAPK and ERK1/2 is increased after 15 min exposure to IL-17A, and lentivirus-mediated IL-17RA shRNA decreased the phosphorylation of p38 MAPK and ERK1/2. Moreover, use of specific inhibitors further revealed the importance of the MAPK pathway in IL-17A-induced IL-6 mRNA expression in HSCs. The imidazole compound SB-203580, a specific inhibitor of p38 MAPKs, caused a significant decrease in IL-17A-induced IL-6 mRNA expression. Additionally, PD-98059, a specific inhibitor of MEK1 that is the directly upstream protein kinase of ERK1/2, promotes marked suppression of IL-17A induced IL-6 mRNA expression. Thus, we conclude that the p38 MAPK and ERK1/2 pathways are involved in IL-6 mRNA expression induced by IL-17A in HSCs. Hot et al. indicated that IL-17A stimulated IL-6 secretion by inducing activation of all three MAPKs (ERK, p38, JNK). Inhibition of IL-17RA expression via siRNA lead to near complete abrogation of IL-6 expression mediated by IL-17A.

IL-6 is an important cytokine in regulating different inflammatory responses in liver diseases and is a marker in the diagnosis of symptoms of liver cirrhosis. Previous studies have described that IL-6, in conjunction with TGF-β, promotes Th17 cell differentiation and drives IL-17A production. On the other hand, IL-17A can also stimulate IL-6 expression and IL-6 is a key downstream target gene for IL-17A in non-immune cells, such as fibroblasts. IL-17A triggers a positive feedback loop of IL-6 signaling and forms the “IL-6 amplifier.” IL-17A plays a pivotal role in immune and non-immune tissues. The IL-6 amplifier exerts its ef-
fected through activation and phosphorylation of both NF-κB and STAT3 proteins. It is also known that HSCs are involved in extracellular matrix degradation and maintenance, imbalances in which lead to liver fibrosis, as a result of unbalanced levels of metalloproteinases, inhibitors of fibrillar collagen, and tissue inhibitors of metalloproteinase-1 and -2. Thus, it is important to examine the role of the IL-17A/IL-6 pathway in HSCs. Zhao et al. indicated that IL-17A induced IL-6 expression via the MAPK signaling pathway in hepatocytes, which, in turn, may further stimulate Th17 cells and forms a positive feedback loop. They concluded that Th17 cells and the IL-17A signaling pathway played an important role in targets for AIH. Yan et al. demonstrated that acute Con-A induced liver injury was reduced in mice treated with an adenovirus vector encoding a soluble IL-17R immunoglobulin (IgG fusion (AdIL-17R:Fc) that neutralized the interaction between IL-17A and IL-17RA. This supported the key role of IL-17A/IL-17RA signaling in Con A-induced hepatitis. Blockade of the IL-17A/IL-17RA signaling pathway may provide a novel therapeutic target in human autoimmune-related hepatitis. Genovese et al. also reported that IL-17A neutralization can achieve positive results in rheumatoid arthritis.

Consistent with previous reports in other cells, our data indicate that IL-17A induces IL-6 expression in HSCs. Suppression of IL-17RA with lentivirus-mediated IL-17RA shRNA inhibited IL-6 expression and blocked IL-17A-triggered positive-feedback loop of IL-6 signaling. Additionally, phosphorylation of p38 MAPK and ERK1/2, induced by IL-17A, was blocked in the presence of IL-17RA shRNA. Negative regulation of MAPK signaling using two specific inhibitors (SB-203580 and PD-98059) for p38 MAPK and ERK1/2, respectively, significantly attenuated IL-6 mRNA expression. These results indicated that blocking IL-17RA expression may be an alternative strategy to reduce the inflammatory response induced by IL-17A in the liver. Further, this study allows us to further understand the function of IL-17RA in HSCs. We would like to examine more potential target substrates that may be affected by the IL-17A/IL-17RA signaling pathway in the future and to further examine the underlying mechanism of HSCs in liver fibrosis.

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