

# The Anti-fibrotic Effect of Human Fetal Skin-derived Stem Cell Secretome on the Liver Fibrosis

**Xia Yao**

First Affiliated Hospital to Changchun University of Chinese Medicine

**Jing Wang**

First Affiliated Hospital to Changchun University of Chinese Medicine

**Jiajing Zhu**

The Third Hospital of Jilin University

**Xiaoli Rong** (✉ [rongxl16@mails.jlu.edu.cn](mailto:rongxl16@mails.jlu.edu.cn))

Affiliated Hospital of Changchun University of Chinese Medicine

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## Research

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# Abstract

**Background:** Liver fibrosis resulting from chronic liver injury is one of the major causes of mortality worldwide. Stem cells-secreted secretome has been evaluated for overcoming the limitations of cell-based therapy in hepatic disease, while maintaining its advantages.

**Methods:** In this study, we investigated the effect of human fetal skin-derived stem cells (hFFSCs) secretome in the treatment of liver fibrosis. To determine the therapeutic potential of the hFFSCs secretome in liver fibrosis, we established the CCl<sub>4</sub>-induced rat liver fibrosis model, and administered hFFSCs secretome in vivo. Moreover, we investigated the anti-fibrotic mechanism of hFFSCs secretome in hepatic stellate cells (HSCs).

**Results:** Our results showed that hFFSCs secretome effectively reduced collagen content in liver, improved the liver function and promoted liver regeneration. Interestingly, we also found that hFFSCs secretome reduced liver fibrosis through suppressing the epithelial-mesenchymal transition (EMT) process. In addition, we found that hFFSCs secretome inhibited the TGF- $\beta$ 1, Smad2, Smad3, and Collagen I expression, however, increased Smad7 expression.

**Conclusions:** In conclusions, our results suggest that hFFSCs secretome treatment could reduce CCl<sub>4</sub>-induced liver fibrosis via regulating the TGF- $\beta$ /Smad signal pathway.

## Introduction

Liver fibrosis is a wound healing response generated against chronic or iterative liver injury[1]. Recent evidence suggests that stem cell-based liver fibrosis treatment can be mediated through paracrine effects[2, 3]. The exclusive use of stem cell-secreted secretome, has been evaluated for overcoming the limitations of cell-based therapy, while maintaining its advantages to their parent cells[4]. It included that extracellular vesicles, and other soluble proteins or biologically active molecules. In addition, previous studies have indicated that human bone marrow mesenchymal stem cells-derived exosomes and other stem cells-derived secretome can recued liver fibrosis[5, 6].

Previous studies have indicated that the features of fetal tissue cells facilitate engraftment in vivo and may provide preferred effects against diseases difficult to treat[7]. Since 1928, hundreds of clinical trials using various types of fetal transplants have been performed worldwide[8-10]. Moreover, recently studies have been demonstrated that human fetal stem cells have a great growth promoting potential, which benefited to the tissue regeneration and cell therapy[11, 12]. In comparison to other mesenchymal stem cell (MSCs), fetal stem cells (FSCs) are easier to culture and more readily proliferate, and less likely to be rejected by transplant recipients, as these cells are less antigenic[8]. Although there remain still ethical and social issues with respect to the clinical use of fetal tissue, fetal stem cell secretome transplantation may overcome these problems and have more perspectives on hepatic disease treatment.

In our previous study, we have successfully isolated and identified hFSSCs[13]. Interestingly, we found hFSSCsecretome has great ability to control and balance the collagen formation in skin tissue[13]. Therefore, we hypothesis that hFSSC have a potential to reduce the collagen formation in liver fibrosis, whereas liver fibrosis is caused by over-abundance of collagen. We further analyzed the effects of hFSSCsecretome on live fibrosis in vitro, and the investigated the mechanism of hFSSCsecretome and TGF- $\beta$ /Smad pathway involvement on anti-fibrosis. Our preliminary results provide the first evidence that hFFSCssecretome effectively reduce liver fibrosis through the TGF- $\beta$ /Smad pathway. We believe that the hFFSCssecretome as an acellular regenerative therapy and approaches can provide great potential for the treatment of liver fibrosis.

## Materials And Methods

### Cell culture

hFSSCs and human umbilical cord mesenchymal stem cell (hUCMSCs) were provided and extracted by our previous study[13]. HSCs were purchased from the Chinese Academy of Medical Sciences, China. In brief, hFSSCs, hUCMSCs and HSCs were cultured in DMEM (Gibco, Grand island, U.S.) supplemented with 500 U/ml penicillin and 500  $\mu$ g/ml streptomycin (Invitrogen, Shanghai, China), and 10% FBS (Gibco, Grand island, U.S.) at 37°C, with saturated humidity and 5% CO<sub>2</sub>. hFSSCs and hUCMSCs at the P5 were used for this study, and hFSSCs and hUCMSCs secretome was collected as reported in our previous study[13]. Briefly, cells were cultured and reached 70~80% confluence, placed in serum-free medium (SFM; Invitrogen, Shanghai, China), and incubated in 5% CO<sub>2</sub> in a humidified condition. After cultured 24h, the conditioned medium (CM) was collected and centrifuged to purify for 10 min at 4 °C, 4000g. Next, 10 ml conditioned medium was re-centrifuged with Amicon Ultra Centrifugal Filters (Millipore Corp, Billerica, MA, USA) at 4 °C, 4000g, 2 h. At last, 300~500  $\mu$ l supernatant solution was collected as cell-free secretome each time. The protein content was measured using the BCSA kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instruction.

### CCl<sub>4</sub>-induced liver fibrosis in rats

Liver fibrosis was induced in Sprague Dawley (SD) rats (8-week old, female, 200g). All protocols and procedures were approved by the Animal Experiment Ethic Committee of Changchun University of Traditional Chinese Medicine (Approval NO. XW201903167). Detailed procedures for CCl<sub>4</sub>-induced have been described in our published studies[6]. Briefly, rats were administered with an intraperitoneal injection of 30% CCl<sub>4</sub>, 3ml/kg body weight twice weekly in olive oil. After eight weeks, CCl<sub>4</sub> treated rats were randomly assigned into three groups (n=10 rats, tail vein injection/weekly): PBS group (1ml); hUCMSCsecretome group (250 $\mu$ g, 1ml); hFSSCsecretome group (250 $\mu$ g, 1ml). After 4 weeks, liver tissue and serum were collected. Livers were divided into two parts of preservation in 10% formalin and freezing at -80 °C.

### Histopathological analysis

Liver tissues were processed for paraffin embedding by slicing into 4µm sections. Liver sections were stained with hematoxylin & eosin (H&E) and Masson and Sirius red according to standard protocols. We selected the liver section fields randomly to analyze the liver fibrosis. The percentage of collagen stained area was calculated via Image-Pro Plus. Immunohistochemistry (IHC) was measured with the Kit (Maixin KIT-9710, Fuzhou, China) in accordance with the manufacturer's instructions. In brief, the liver sections were deparaffinized, rehydrated, and incubated in a 99 °C water bath for 15 minutes. Then, the slide was incubated with 3% H<sub>2</sub>O<sub>2</sub> for 15 minutes, and blocked with 10% normal goat serum for 1 h at 37 °C. Following with the incubation of primary antibody against PCNA (ab15497, 1:500 dilution, Abcam, Cambridge, UK), α-SMA (ab5694, 1:500 dilution, Abcam, Cambridge, UK), and HNF-4α (ab219610, 1:500 dilution, Abcam, Cambridge, UK) overnight at 4 °C. Next, slides were incubated with biotinylated goat-anti-rabbit IgG antibody. Add diaminobenzidine solution for 15 minutes at 37 °C, then incubated with avidin peroxidase reagent, and hematoxylin for counterstaining. Lastly, slides were photographed using an optical microscope (Olympus, Tokyo Metropolitan, Japan). We used 10 random fields per section and 10 sections in total (n=10 rats) for quantification of IHC results. The IHC results were calculated via Image-Pro Plus.

### **Biochemical analysis**

The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), total bilirubin (TBIL), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (γ-GT) were assessed using the Automated Biochemical Analyzer (AU-680, Beckman, California, U.S.) according to the procedure. Liver homogenate (10%, w/v) was prepared by homogenizing the right lobe of liver on ice in 150 mM Tris-HCl buffered saline (pH 7.2) using a polytron homogenizer (PT3100D; Kinematical, Lucerne, Switzerland). The levels of Malondialdehyde (MDA) and Hydroxyproline (Hyp) in liver tissue were measured using kits (NanJingJianCheng Bio., Nanjing, China) according to the manufacturer's instructions.

### **Quantitative real-time PCR (qRT-PCR)**

HSCs were co-cultured with either PBS, hUCMSC secretome, or hFFSC secretome (5ng/ml) for 48h before samples were collected for mRNA extraction. Total RNA was isolated from HSCs using Trizol reagent (Invitrogen, Shanghai, China) according to the manufacturer's protocol. Then, 1µg total RNA was reverse-transcribed to give cDNA, which was used as the template, and combined with standard SYBR premix Ex Taq (Invitrogen, Shanghai, China) on the Real-Time PCR Detection System (Roche, Basel, Switzerland), and experiments were conducted in triplicate. The levels of EMT related genes (E-cadherin, Snail1, Vimentin, FSP1 and α-SMA), TGF-β/Smad signaling pathway related genes (TGF-β1, Smad2, Smad3, Smad7 and Collagen I), and the internal standard GAPDH mRNA were measured by qRT-PCR. The primers are listed in Table S1, and GAPDH served as the internal control. All reactions were performed in triplicate and the data were analyzed using the  $2^{-\Delta\Delta Ct}$  method.

### **Immunofluorescence (IF) staining**

When HSCs reached 60~70% confluence on 24-well plates, they were cultured with either PBS, hUCMSCsecretome, or hFFSCsecretome (5ng/ml) for 48h. Next, HSCs were incubated with 4% paraformaldehyde at room temperature for 10 minutes, and then incubated with 1% bovine serum albumin (BSA, Biosharp, Wuhan, China) for 30 minutes. Cells were incubated with a primary antibody against  $\alpha$ -SMA (ab5694, 1:100 dilution, Abcam, Cambridge, UK) for 1h, followed by incubation with a secondary antibody (goat anti-rabbit IgG, ab15007, 1:500 dilution, Abcam, Cambridge, UK) for 30 minutes at room temperature. Rhodamine phalloidin (Thermo Scientific, Waltham, U.S.) was stained for cytoskeleton. The nuclei were labeled with DAPI (Thermo Scientific, Waltham, U.S.). Fluorescent images were captured using an EVOS Cell Imaging System (Thermo Scientific, Waltham, U.S.).

## Western blotting

HSCs were co-cultured with either PBS, hUCMSCsecretome, or hFFSCsecretome (5ng/ml) for 48h before samples were collected for protein extraction. Protein samples were mixed with SDS sample buffer and heated to 95 °C for 10 minutes, followed by separation on SDS-polyacrylamide gels. Resolved proteins were electro-blotted onto nitrocellulose membrane and probed with antibodies against TGF- $\beta$ 1 (ab92486), Smad2 (ab40855), Smad3 (ab40854), Smad7 (ab216428), Collagen I (ab90395) and  $\beta$ -actin (ab5694), (1:1000 dilution, Abcam, Cambridge, UK) overnight at 4°C (1:1000 dilution, Abcam, Cambridge, UK). Nitrocellulose membranes were then incubated with a secondary antibody, HRP-conjugated goat anti-rabbit IgG (ab15007), at room temperature for 2h, and visualized by chemiluminescent detection according to the manufacturer's instructions (Immobilon western chemiluminescent HRP substrate, Millipore, Massachusetts, U.S.).

## Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 6. One-way ANOVA with Dunnett's multiple comparisons test was used to test for statistically significant differences. All quantitative data are expressed as mean  $\pm$  SD for at least three independent experiments, and  $p < 0.05$  was considered to be statistically significant.

## Results

### hFFSCsecretome reduced CCl<sub>4</sub>-induced liver fibrosis in rats.

To explore the effect of hFFSCsecretome on liver fibrosis, we used CCl<sub>4</sub>-induced a liver fibrosis model in rats (Fig. 1a). Compared to the PBS group, gross morphology changes obviously in hFFSCsecretome group, including less fibrous nodular and more ruddy on the surface, more uniform surface and soft texture (Fig. 1b). After 4 weeks treatment, histopathologic analysis using Masson and Sirius red staining indicated that the collagen area percentage in hFFSC group (9.2%) was significantly reduced, compared to the other two control groups (24.3% in PBS group and 14.9% in hUCMSCsecretome group, Fig. 1b and 1c,  $p < 0.05$ ). Furthermore, we detected the MDA (a marker for oxidative stress and liver cell injury) and Hyp (a main component in collagen tissue) content in the liver tissue. We found that the level of MDA and Hyp in

hFSSCsecretome group was significantly lower than the other two control groups (Fig.1d and 1e  $p < 0.01$ ). These findings suggest that hFSSCsecretome effectively reduced CCl<sub>4</sub>-induced liver fibrosis in rats.

### **hFSSCsecretome reduced liver fibrosis through suppressing the EMT**

To further verify the roles of hFSSCsecretome in the pathogenesis of liver fibrosis, we performed immunofluorescence staining of TGF- $\beta$  in HSCs. As TGF- $\beta$ 1 is considered as a crucial mediator in tissue fibrosis, and HSCs are one of the major effector cells in liver fibrosis. We found that hFSSCsecretome group reduced fluorescence intensity observably, compared to the other two control groups (Fig.2a). In future study, we explore the effect of hFSSCsecretome on EMT, and qRT-PCR analysis was used to examine the expression of EMT-related indicators (E-cadherin, Snail1, Vimentin, FSP1 and  $\alpha$ -SMA in HSCs). Interestingly, our results found that hFSSCsecretome treatment increased the epithelial marker of E-cadherin expression, while decreased the transcription factors of Snail and mesenchymal markers (Vimentin, FSP1 and  $\alpha$ -SMA) expression, compared to the PBS group (Fig. 2b,  $p < 0.05$ ). Meanwhile, we also examined hFSSCsecretome increased E-cadherin, and decreased FSP1 and  $\alpha$ -SMA expression compared to the hUCMSCsecretome group (Fig. 2b,  $p < 0.05$ ). These results suggest that hFSSCsecretome reduced liver fibrosis through suppressing the EMT.

### **hFSSCsecretome improved liver functionality and promoted liver regeneration**

To explore the effect of hFSSCsecretome on liver functionality, we performed the biochemical analyses. In comparison to the PBS group, hFSSCsecretome group significantly reduced serum levels of ALT, AST, TBIL,  $\gamma$ -GT and ALP (Fig. 3a-3e,  $p < 0.05$ ). However, the serum level of TP in hFSSCsecretome group was higher than that in PBS group (Fig. 3f,  $p < 0.05$ ). In addition, hFSSCsecretome group significantly reduced the serum levels of TBIL and  $\gamma$ -GT compared to hUCMSCsecretome group (Fig. 3c and 3d,  $p < 0.05$ ). These results suggest that hFSSCsecretome effectively improved liver functionality.

Next, we performed IHC to assess the effects of the hFSSCsecretome on the liver.  $\alpha$ -SMA is an important indicator of the occurrence and development of hepatic fibrosis. IHC results showed that the percentage of  $\alpha$ -SMA positive area in hFSSCsecretome group (0.82%) was significantly decreased compared to the PBS group (5.51%, Fig. 4a and 4b,  $p < 0.001$ ). PCNA and HNF-4a are two crucial indicators of hepatocyte proliferation. IHC results showed that the percentage of PCNA positive area in hFSSCsecretome group (4.13%) was significantly increased compared to the PBS group (7.48%, Fig. 4a and 4c,  $p < 0.01$ ). Consistent with the above results, the percentage of HNF-4a positive area was significantly increased in hFSSCsecretome group (12.1%), compared to the PBS group (4.5%,  $p < 0.01$ ) as well as hUC-MSCs group (8.2%, Fig. 4a and 4d,  $p < 0.05$ ). The histological results indicated that hFSSCsecretome effectively delayed the progression of liver fibrosis, and promoted the liver regeneration.

### **hFSSCsecretome regulate the TGF- $\beta$ /Smad signal pathway**

To investigate the underlying mechanism of the effect of hFSSCsecretome on liver fibrosis, we performed the Western blot and RT-qPCR to analyze the expression of TGF- $\beta$ 1, Smad2, Smad3, Smad7 and Collagen

I in HSCs, as it is one of the major effector cells in liver fibrosis. We found that TGF- $\beta$ 1, Smad2, Smad3, and Collagen I expression was significantly decreased in hFSSCsecretome group, compared that of PBS group (Fig. 5a and 5b,  $p < 0.001$ ). However, we detected the Smad7 was significantly increased in hFSSCsecretome group, compared that of the other two control groups (Fig. 5a and 5b,  $p < 0.01$ ). Smad7 serves as a negative feedback regulator of TGF- $\beta$ 1/Smad pathway, thereby protects against TGF- $\beta$ 1-mediated fibrosis (Fig. 6). These results suggest that hFSSCsecretome effectively reduced liver fibrosis via regulating the TGF- $\beta$ /Smad signal pathway (Fig 5).

## Discussion

In this study, our results demonstrated that hFSSCsecretom reduce liver fibrosis both in rats. Moreover, our research illustrated that hFSSCsecretom reduce liver fibrosis through suppressing the EMT and regulating the TGF- $\beta$ /Smad signal pathway in HSCs.

Recent evidence indicates that the mesenchymal stem cell secretome as an acellular regenerative therapy for liver disease [5, 14]. The use of MSC secretome has been shown to have anti-fibrotic effects [14]. Moreover, IV injection of hucMSC-EVs decreased liver fibrosis, reduced apoptosis and mitigated liver damage induced by CCl<sub>4</sub> in mice [15, 16]. In our study, we found that hFSSCsecretom effectively reduced CCl<sub>4</sub>-induced liver fibrosis, and improved liver functionality in rats (Fig. 1). Moreover, hFFSCssecretome treatment is more effective than of hUCMSCssecretome in resolving fibrosis, such as some indicators included collagen area, MDA, Hyp, TBIL, and  $\gamma$ -GT. The reasons that differences found between hUCMSCs and hFFSCssecretome may be reported that hFFSCssecretome contains more biological activity factors than hUCMSCssecretome [13]. Our studies indicated that hFSSCsecretom is an attractive emerging option for therapeutic applications as a therapeutic strategy for liver fibrosis.

Previous study that mesenchymal stem cell secretome inhibit HSC activation and promoted liver regeneration [17-19]. HSCs stimulate the production of a large amount of collagen fibers to form liver fibrosis. Moreover, positive expression of  $\alpha$ -SMA can serve as a marker for HSCs activation [20]. Our results demonstrate that hFSSCsecretom decreased  $\alpha$ -SMA expression. PCNA and HNF-4a is two crucial indicator of hepatocyte proliferation [15]. Our study demonstrate that the percentage of PCNA and HNF-4a positive area was significantly increased after hFSSCsecretom treatment. These results confirmed that hFSSCsecretom promotes liver regeneration while reducing fibrosis. Despite our results confirming that hFSSCsecretome had significantly reduced liver fibrosis and caused no detectable immunological responses, their exact mechanisms of action need further exploration. Moreover, study has reported that type 2 EMT is associated with tissue regeneration and organ fibrosis. After CCl<sub>4</sub> injury in liver, inflammatory cytokines, or chemical substances stimulate EMT to produce new collagen fibroblast pools to repair injury [5]. In present study, we demonstrated that hUCMSCsecretome reduced liver fibrosis through suppressing the EMT process.

The MSC secretome secreted some growth factors and cytokines, such as hepatocyte growth factor (HGF), transforming growth factor beta isoform 3 (TGF- $\beta$ 3), and tumor necrosis factor-alpha (TNF- $\alpha$ ), and

IL-10 can modulate cell signaling and processes involved in fibrogenesis and can attenuate liver fibrosis[5, 21]. Recently, fetal mesenchymal stem cells functional secretome analysis illustrates that 737±80 protein identifications was obtained from the amniotic fluid-mesenchymal stem cells (AF-MSCs) secretome, interestingly, it reveals that Annexin-A1 as important paracrine factor in hepatic regeneration[22]. However, previous study demonstrated that hFSSC presented with the characteristics of both MSC and embryonic stem cell (ESC)[13]. These may be the partial reason that hFSSCsecretome can reduce liver fibrosis, andhas better effects than hUCMSCsecretome in some indicators.

TGF- $\beta$ 1/Smad pathway is an important pathogenic mechanism in tissue fibrosis[23, 24]. Studies have shown that TGF- $\beta$ 1 is considered as a crucial mediator in tissue fibrosis and causes tissue scarring largely by activating its downstream small mother against decapentaplegic (Smad) signaling[25]. However, different TGF- $\beta$  signalings play different roles in fibrogenesis[24]. TGF- $\beta$ 1 directly activates Smad signaling which triggers pro-fibrotic gene overexpression[26]. Increasing studies have demonstrated that dysregulation of TGF- $\beta$ 1/Smad pathway was an important pathogenic mechanism in tissue fibrosis[27]. Smad2 and Smad3 are the two major downstream regulator that promote TGF- $\beta$ 1-mediated tissue fibrosis, while Smad7 serves as a negative feedback regulator of TGF- $\beta$ 1/Smad pathway thereby protects against TGF- $\beta$ 1-mediated fibrosis[24]. Our findings proved that hFSSCsecretome effectively reduced TGF- $\beta$ 1, Smad2, Smad3, and Collagen I expression, moreover, increased Smad7 expression. It indicated that hFSSCsecretome effectively reduced liver fibrosis via regulating the TGF- $\beta$ /Smad signal pathway (Fig. 6).

In conclusion, we successfully investigated the role of hFSSCsecretome on cutaneous liver fibrosis. Our results demonstrated that hFSSCsecretomecan exert promoting effect of liver fibrosis via regulating the TGF- $\beta$ /Smad signal pathway.

## Abbreviations

hFSSC: Human fetal skin-derived stem cell; MSCs: mesenchymal stem cells; hUCMSCs: human umbilical cord mesenchymal stem cell; HSCs: Hepatic stellate cells; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin;  $\gamma$ -GT: Gamma glutamyl transpeptidase; ALP: Alkaline phosphatase; TP: Total protein; Hyp: Hydroxyproline; MDA: Malonaldehyde; qRT-PCR: Quantitative real-time PCR;  $\alpha$ -SMA: Alpha-smooth muscle actin;HGF: Hepatocyte growth factor; EMT: Epithelial to mesenchymal transition; TNF- $\alpha$ :Tumor necrosis factor-alpha; ESC: Embryonic stem cell; FSP1: fibroblast-specific protein 1

## Declarations

### Ethics approval and consent to participate

All the protocols and procedures were approved by the Animal Experiment Ethics Committee of the Changchun University of Traditional Chinese Medicine, China (Approval NO. XW201903167). The

informed written consent form was approved by the Clinical Research Ethics Committee and signed by the donor before sample collection.

### Consent for publication

Not applicable.

### Availability of supporting data

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

### Competing interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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### Authors' Contributions

X.Y. carried out the cell culture and animal studies, and helped to draft the manuscript. J.W. carried out animal studies and qRT-PCR. J.Z. carried out WB and performed the statistical analysis. X.R. conceived of the study, and participated in its design and coordination and helped to draft the manuscript.

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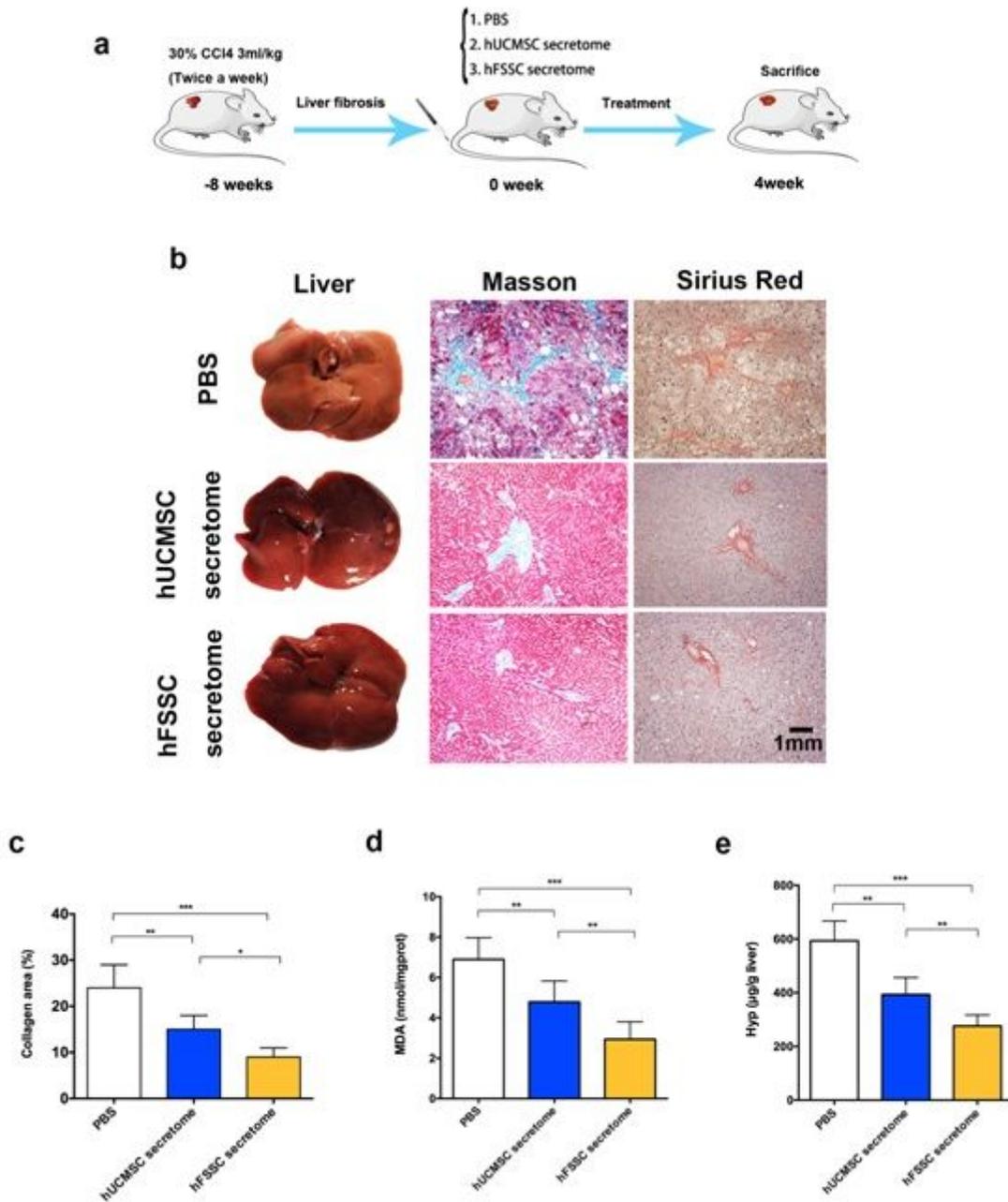
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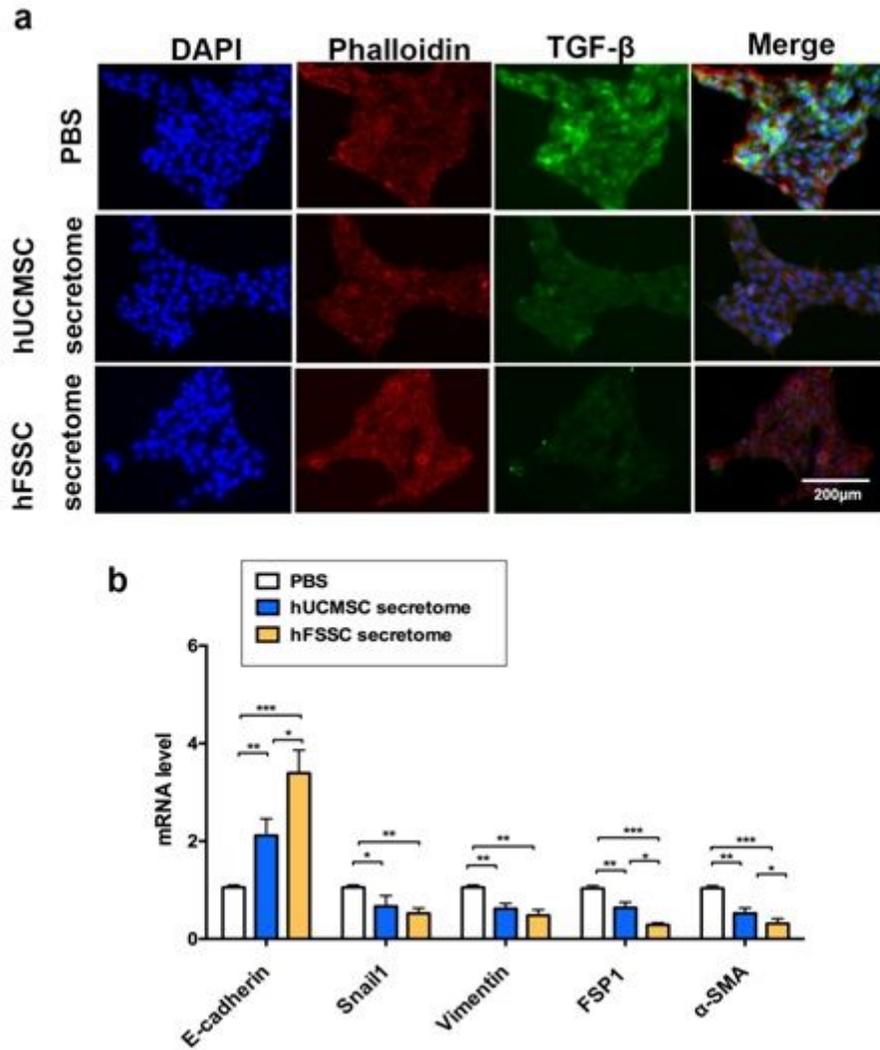
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## Figures



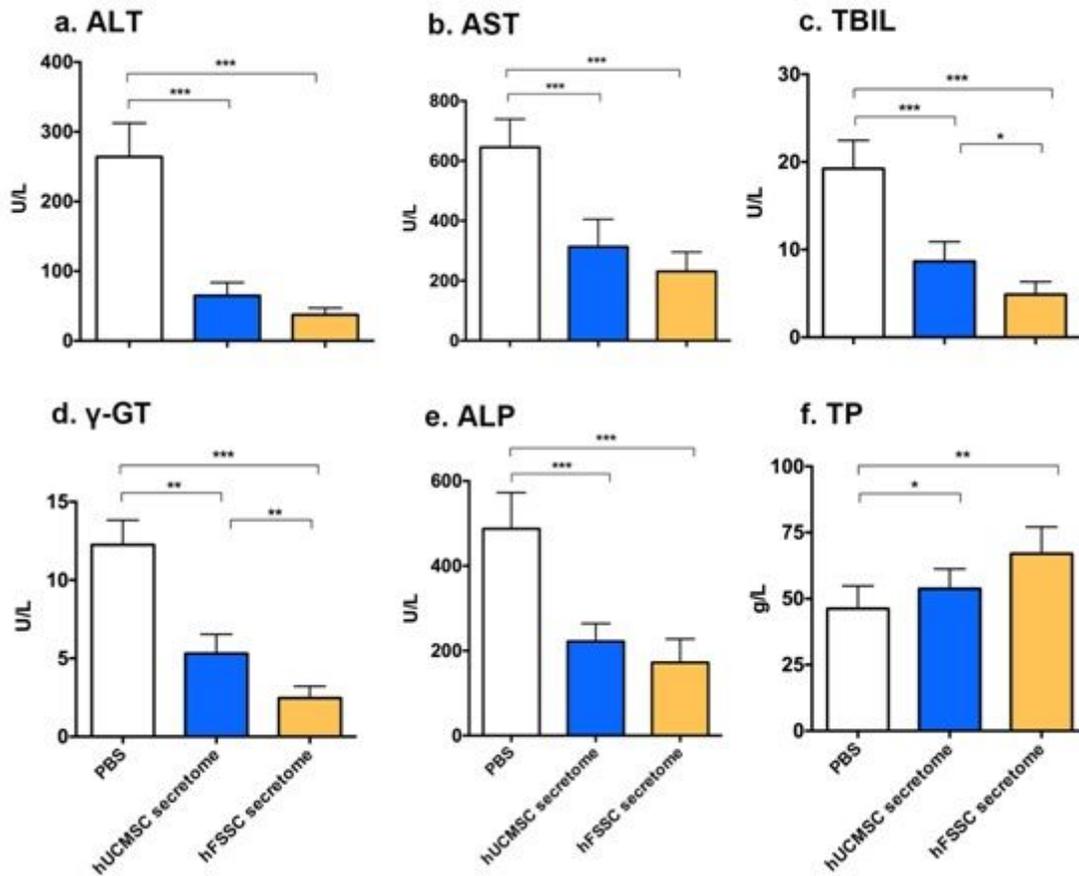
**Figure 1**

hFSSC secretome reduced liver fibrosis in rats. a. Experimental design. b. The representative images of gross morphology, Masson and Sirius red staining analysis of liver. Bar = 1 mm), n = 10 rats. c. Quantitative analysis of the collagen area percentage at 4 weeks. d. Quantitative analysis of hepatic MDA and Hyp content. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . n = 10; mean  $\pm$  SD.



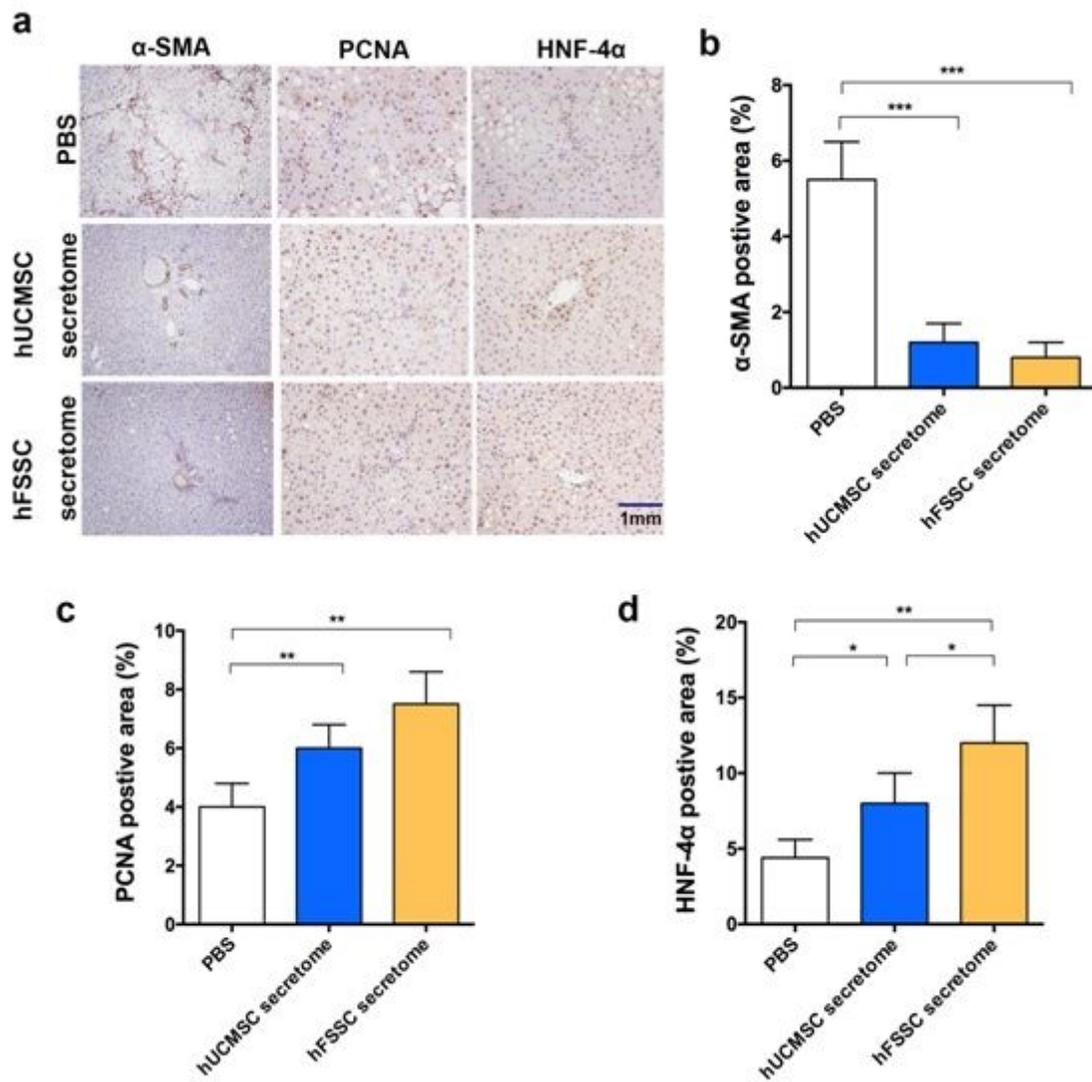
**Figure 2**

hFSSC secretome reduced liver fibrosis through suppressing the EMT process. a. Representative images of immunofluorescence staining performed for TGF- $\beta$  in HSCs. Bar = 200 $\mu$ m. b. Relative mRNA expression levels of EMT related gene, included of E-cadherin, Snail1, Vimentin, FSP1 and  $\alpha$ -SMA in HSCs. Note that EMT: epithelial–mesenchymal transition; FSP1: fibroblast-specific protein 1;  $\alpha$ -SMA: alpha smooth muscle actin. \* $p$ <0.05, \*\* $p$ <0.001, \*\*\* $p$ <0.0001.  $n$  =10, mean  $\pm$  SD.



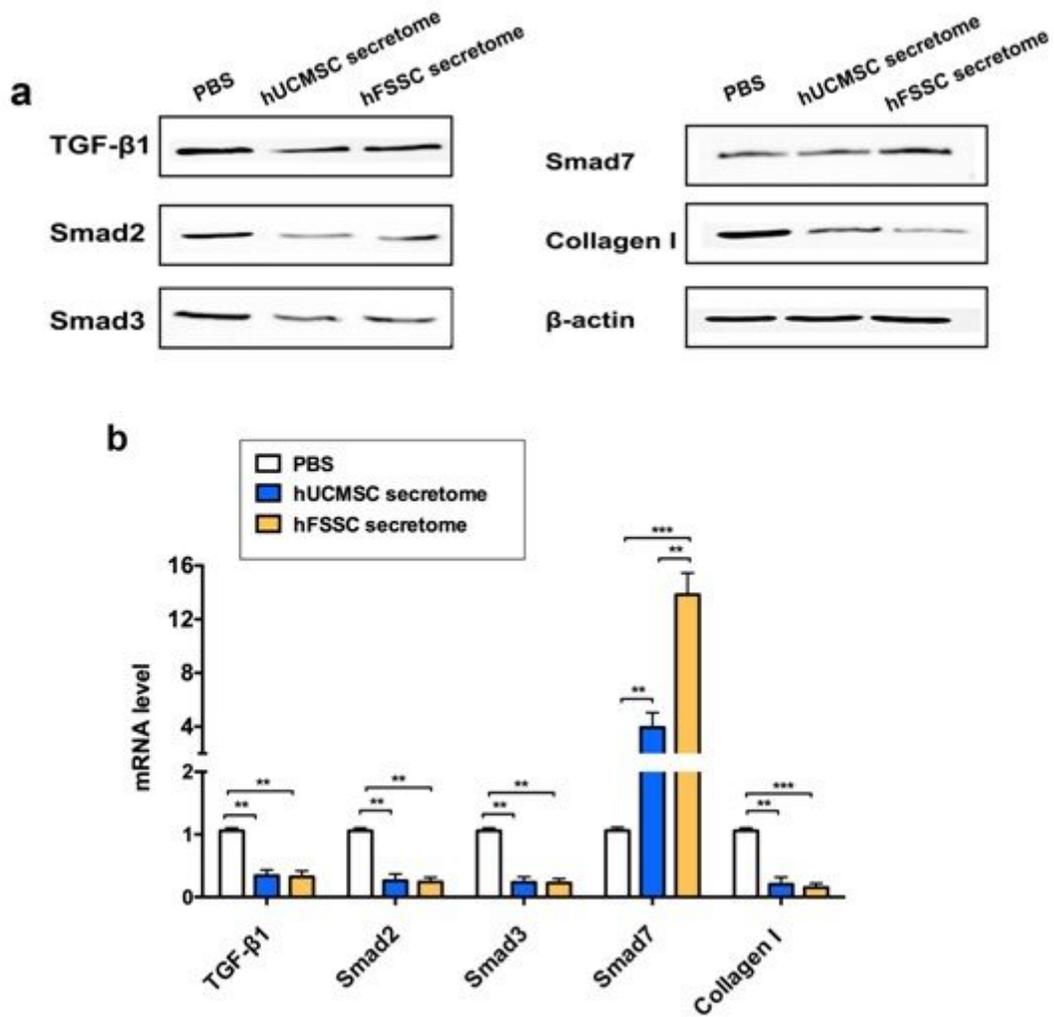
**Figure 3**

hFSSC secretome improves liver function in serum biochemical parameters. a. ALT: Alanine aminotransferase. b. AST: Aspartate aminotransferase. c. TBIL: Total bilirubin. d. γ-GT: Gamma glutamyl transpeptidase. e. ALP: Alkaline phosphatase. f. TP: Total protein. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. n = 10, mean ± SD.



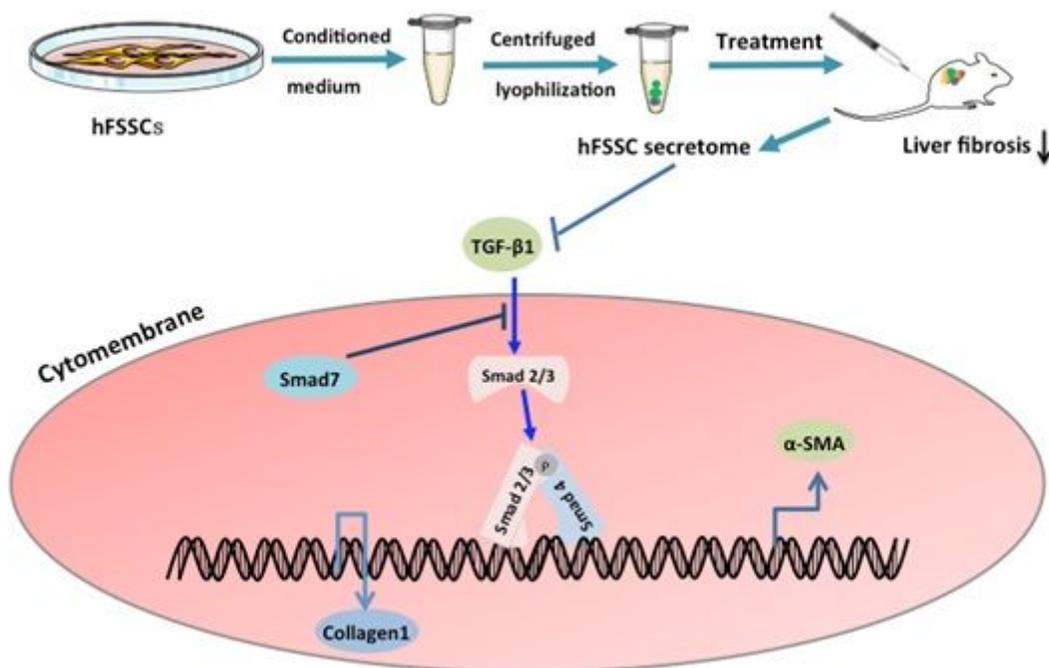
**Figure 4**

Histological analysis of hFSSC secretome reduced liver fibrosis and promoted liver regeneration. a. Photomicrographs of liver tissue sections showing IHC staining for  $\alpha$ -SMA, PCNA, and HNF-4 $\alpha$ . Brown cells represent the positive expression, bar = 1mm. b-d. The quantification of  $\alpha$ -SMA, PCNA, and HNF-4 $\alpha$  positive cells area. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .  $n = 10$ , mean  $\pm$  SD.



**Figure 5**

hFSSC secretome inhibited the TGF- $\beta$ /Smad signaling pathway in HSCs. a. Representative western blotting analysis the expression of TGF- $\beta$ 1, Smad2, Smad3, Smad7 and Collagen I in HSCs. b. Relative mRNA expression levels of TGF- $\beta$ 1, Smad2, Smad3, Smad7 and Collagen I in HSCs. \*\* $p < 0.001$ , \*\*\* $p < 0.0001$ .  $n = 3$ , mean  $\pm$  SD.



**Figure 6**

hFSSC secretome reduced liver fibrosis by regulating the TGF- $\beta$ /Smad signaling pathway. hFSSC secretome inhibited TGF- $\beta$ 1 expression, and activated Smad7 expression which are activated by the binding of the TGF- $\beta$  super family to the cell surface receptors. TGF- $\beta$ 1-mediated tissue fibrosis via Smad2/Smad3 which the two major downstream regulator, while Smad7 serves as a negative feedback regulator of TGF- $\beta$ 1/Smad pathway, thereby protects against TGF- $\beta$ 1-mediated fibrosis. Furthermore, hFSSC secretome decreased the collagen I expression, thereby reduce liver fibrosis.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterial.docx](#)