

# Mechanical force and tensile strain activated hepatic stellate cells and inhibited retinol metabolism

Su-Hong Yi · Yi Zhang · Dan Tang · Liang Zhu

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## Abstract

**Objectives** Hepatic stellate cells (HSCs), the principal producers of extracellular matrix proteins, play a major role in the development of liver fibrosis which is accompanied with elevated sinusoidal pressure and portal hypertension. We have isolated primary rat HSCs and investigated the effect of mechanical pressure and tensile strain on retinol metabolism in the cells.

**Results** Mechanical force and tensile strain significantly increased the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and collagen I, and notably inhibited the expression of cellular retinol-binding protein I (CRBP-I), lecithin-retinol acyltransferase (LRAT), retinyl ester hydrolase (REH), retinoic acid receptor- $\beta$  (RAR- $\beta$ ) and retinoid X receptor- $\alpha$  (RXR- $\alpha$ ). Such effects were partially reversed by the retinoid X receptor antagonist, HX531, and the retinoic acid receptor antagonist, LE135.

**Conclusion** Mechanical force and tensile strain significantly activate HSCs by regulating the retinoid metabolic pathway. Activation of HSCs can therefore be manipulated through mechanical force and tensile strain in vitro.

Su-Hong Yi and Yi Zhang contributed equally to this paper.

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**Keywords** Hepatic stellate cells · Liver fibrosis · Mechanical force · Retinol metabolism · Tensile strain

## Introduction

Progressive activation of hepatic stellate cell (HSC) is associated with the formation and development of cirrhotic portal hypertension (Reynaert et al. 2002; Rockey 1997, 2006). When changes happen in this mechanical environment, HSCs are activated or trans-differentiated to myofibroblast-like phenotype lacking intracytoplasmic lipid droplets. Activation and proliferation of HSCs are central to the development of hepatic fibrosis (Moreira 2007).

HSC is a non-parenchymal cell that distributes homogeneously throughout the perisinusoidal space of Disse and accounts for 15 % of liver cells (Friedman 2008). HSCs in hepatic sinusoids are mainly exposed to two mechanical stimuli: the microvascular pressure between Disse sinus and hepatic sinusoids, and the tensile strain generated by the change in sinusoidal pressure.

Stellate cells are in a resting-state in the normal liver and play a key role in the lipid storage and vitamin A metabolism (Friedman 2008). About 80 % of the total body vitamin A (retinoyl esters) is stored as lipid droplets in the cytoplasm (Higashi and Senoo 2003). The lipid composition of these droplets maintains homeostasis that depends on the regulation of cellular retinol-binding protein I (CRBP-I), lecithin-retinol acyltransferase (LRAT) and retinyl ester hydrolase (REH). Degradation of the lipid droplet in activated HSCs is caused by an imbalance of retinoid metabolism which can produce a mechanical barrier in nucleus, can promote release of composition factor in nucleus and signal transduction outside the cell, and can enhance proliferation of HSCs. Moreover, as substrates of retinyl esters, retinoic acid and retinol play important roles in the activation process of HSC (Azais Braesco et al. 1995). The metabolic status of retinoic acid, retinol and retinyl esters is therefore closely related to HSC activation.

Retinoid species outside the cells can influence the metabolism of retinyl ester, retinol and retinoic acid inside the cells. However, they are not the only regulators and many studies have shown that mechanical strain can influence cell metabolism. The effects of mechanical factors on endothelial cells, bone cells and hepatocytes have been extensively investigated (Koike et al. 2005; Liu and Agarwal 2010; Sponkel et al. 1996). HSCs are exposed to direct pressure from Disse and tensile strain from sinusoids (Liu and Agarwal 2010). The effects of these mechanical factors on the lipid composition of lipid droplets and retinoid metabolism of HSCs have not been reported.

The present study was aimed to investigate the effects of mechanical factors on the retinoid metabolism of HSCs during the process of HSCs activation. Mechanical forces, tensile strain or pressure was applied on rat HSCs and then the activation and the metabolic response of HSCs were evaluated. In the

present study, we found that forces such as pressure and tensile strain can promote the activation of HSCs and reduce the expression levels of retinoid metabolic factors. Relevant studies indicated that these forces are associated with the development of cirrhotic portal hypertension and implied that retinoid species in the HSCs were probably involved in the signal pathway regulating HSCs activation. We then inhibited the binding of retinol and retinoic acid to their receptors by using retinoid antagonists. Our data demonstrated that the effects of these mechanical forces on HSCs activation and retinoid metabolism were partially reversed by pre-treatment with retinoid antagonists.

## Materials and methods

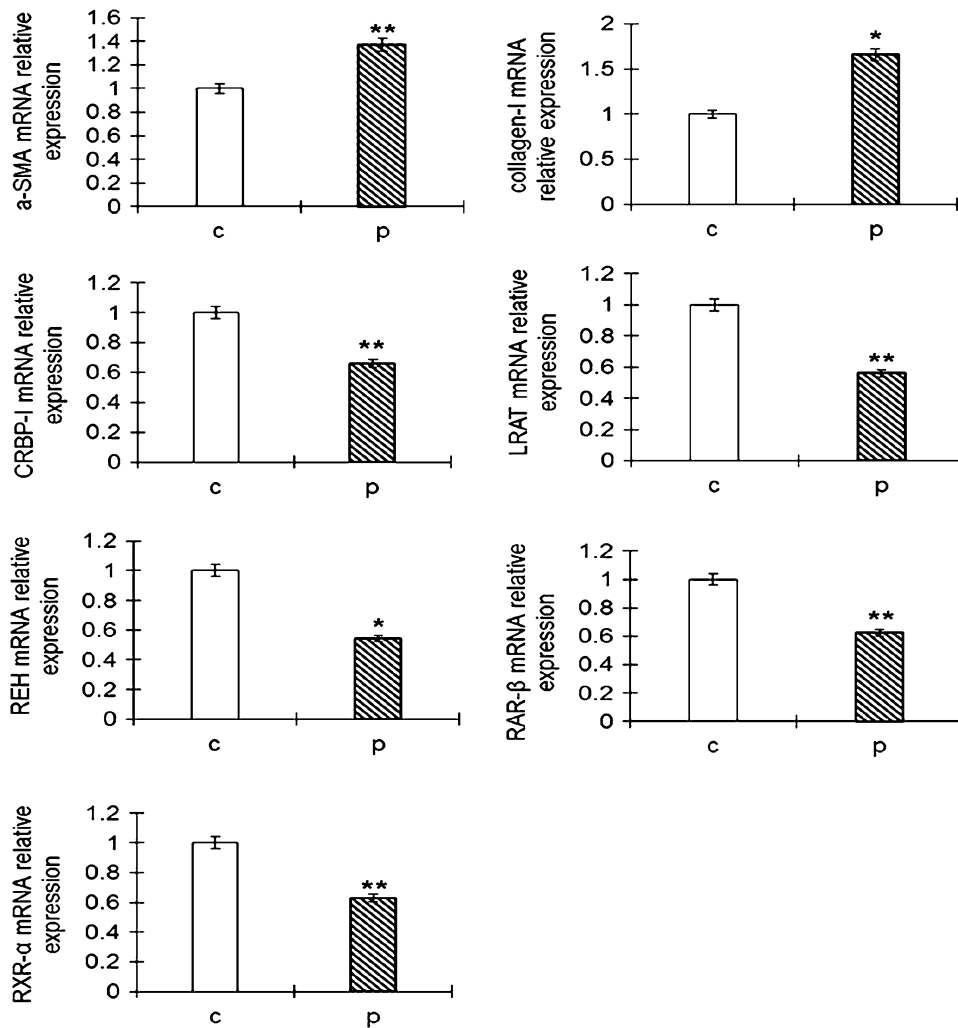
### Hepatic satellite cell (HSC) isolation and culture

HSCs were isolated from the livers of healthy male Sprague–Dawley rats by *in situ* perfusion with pronase and collagenase and cultured in DMEM containing 10 % (v/v) fetal bovine serum (FBS) and 0.5 % (v/v) penicillin and streptomycin. Before performing pressure loading or tensile strain assay, cells were incubated in DMEM medium without FBS for 24 h. In some experiments, after serum starvation, cells were treated with LE135 or HX531 inhibitor for 1 h before pressure loading or tensile strain assay.

The study protocol was approved by the local institutional review board. All animal experiments were performed under the guidelines by the Animal Care and Use Committee of the Second Military Medical University.

### Pressure loading

The pressure-loading assay was based on our previous studies (Wu et al. 2010). Before load pressure, the apparatus was transferred to CO<sub>2</sub> incubator for 30 min. To generate pressure, compressed He was released into the chamber and the pressure was monitored by a sphygmomanometer. Tissue cultures used for experiments were placed in the chamber inside the incubator. After 24 h serum starvation, the cultured cells were exposed to pressure at 10 mm Hg for 1 h.



**Fig. 1** Effects of mechanical pressure on the mRNA levels of fibroblast markers and retinoid metabolic factors. Hepatic stellate cells (HSCs) were serum starved for 24 h, a pressure of 10 mm Hg was applied for 1 h and then they were harvested for RNA extraction. mRNA expression levels of  $\alpha$ -SMA,

collagen I, LRAT, CRBP-I, REH, RAR- $\beta$  and RXR- $\alpha$  were assessed by real-time PCR. *P* HSCs were applied to the pressure at 10 mm Hg for 1 h, *C* control group without pressure ( $N = 3$ ), \* $P < 0.05$ , \*\* $P < 0.01$  compared with control group

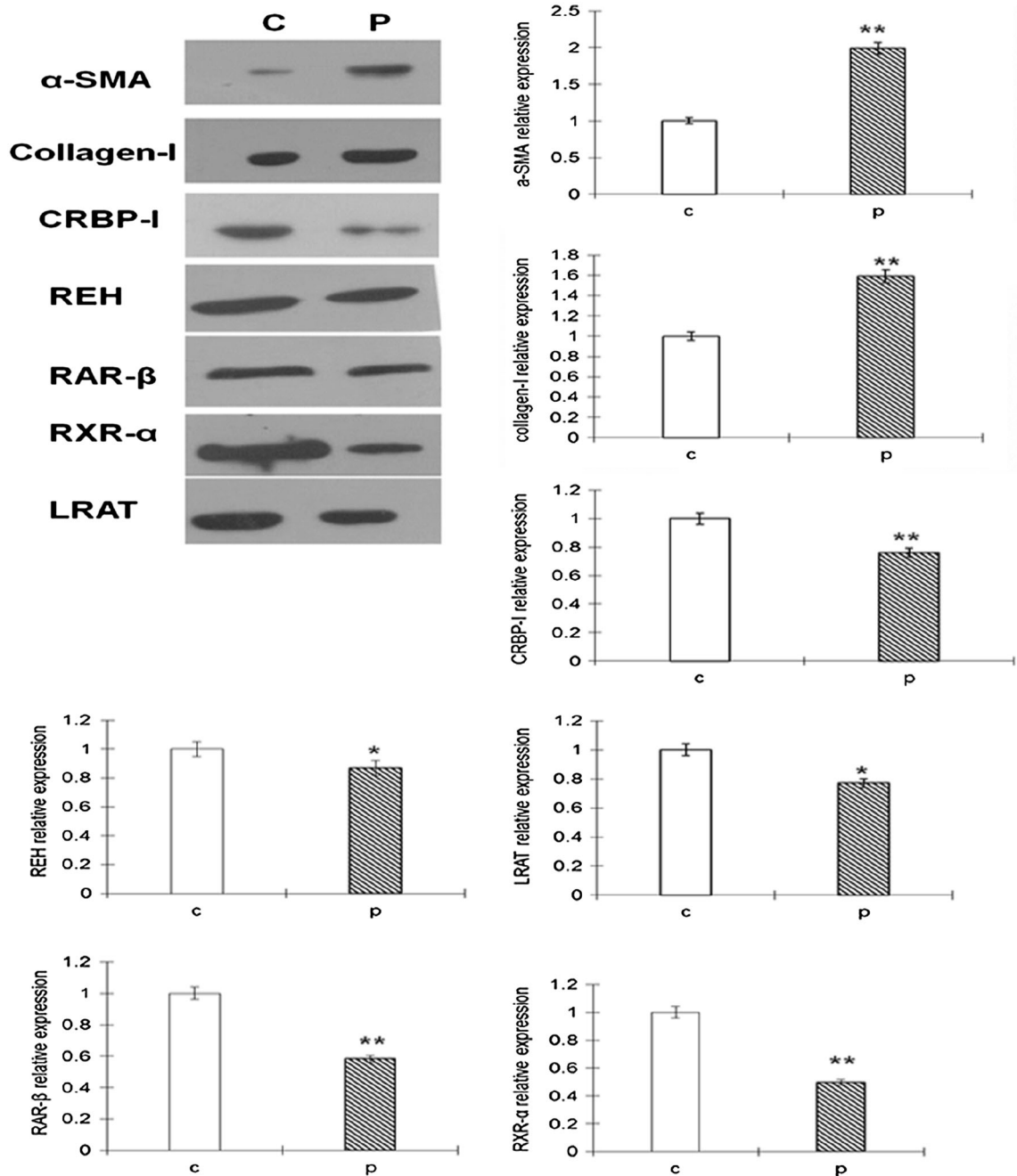
### Tensile strain

For tensile strain experiments, cultured HSCs were plated into BioFlex amino-culture plates coated with collagen (Flexcell International, McKeesport, PA) in DMEM without FBS. Cultures were maintained in a humidified atmosphere of 5 %  $\text{CO}_2$  at 37 °C and subjected to mechanical tensile strain for variable periods of time in a vacuum-operated cyclic strain-

providing instrument (FlexerCell FX-2000 System; Flexcell International, McKeesport, PA).

### Quantitative RT-PCR measurements

Total RNA was extracted from cells using Trizol and mRNA levels of indicated genes were determined using real-time PCR with primer sets obtained from Shanghai Bioengineering Co. Ltd (Shanghai, China).



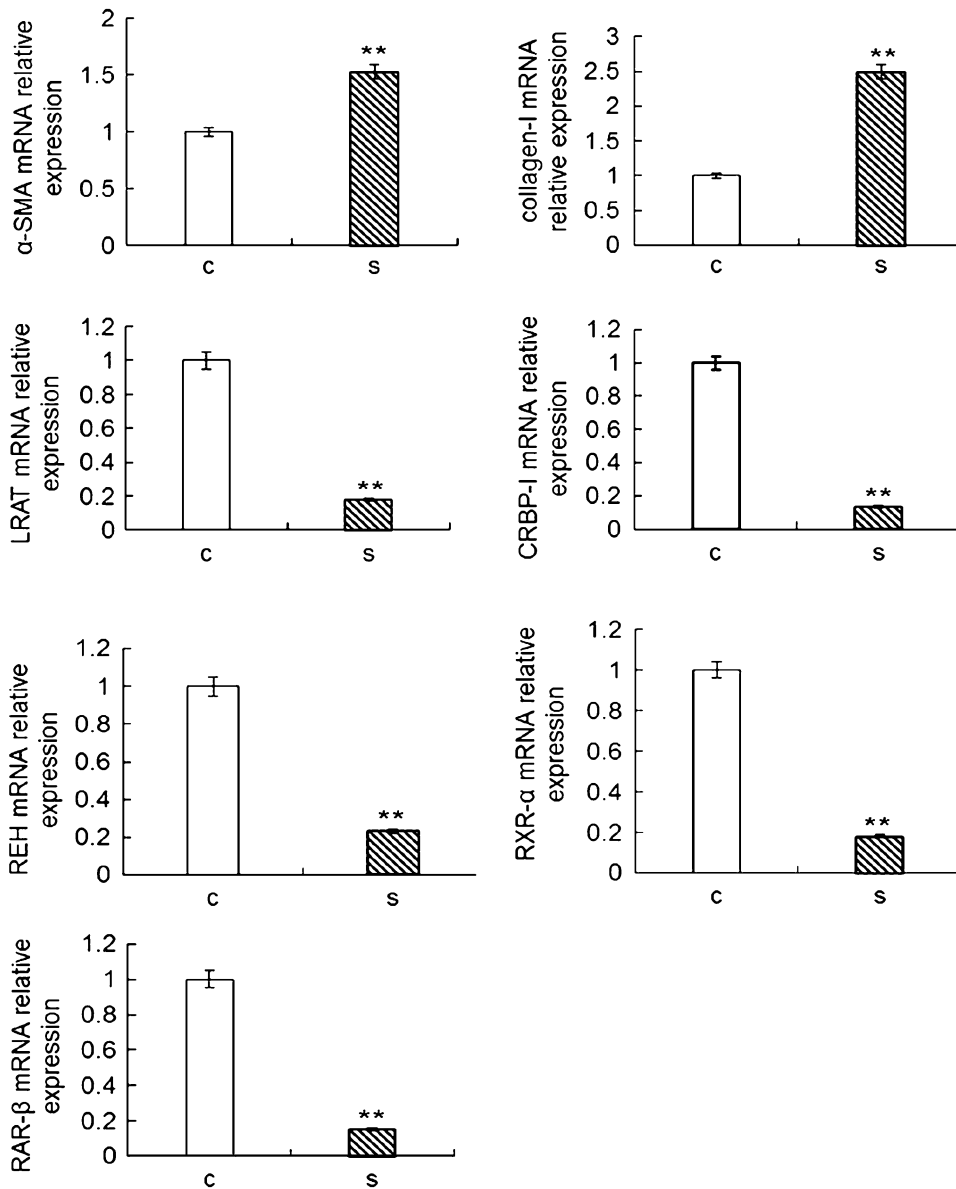
**Fig. 2** Effects of mechanical pressure on the protein levels of fibroblast markers and retinoid metabolic factors. HSCs were serum starved for 24 h, subjected to a pressure of 10 mm Hg for 1 h and then harvested for protein extraction. Protein levels of  $\alpha$ -

SMA, collagen I, LRAT, CRBP-I, REH, RAR- $\beta$  and RXR- $\alpha$  were assessed by western blotting. *P* HSCs were subjected to a pressure of 10 mm Hg for 1 h, *C* control group without pressure ( $N = 3$ ), \* $P < 0.05$ , \*\* $P < 0.01$  compared with control group

The data was analyzed by ABI Prism 7300 SDS Software. The sequences of primers are available in the supplemental data section (see Supplementary Table 1).

#### Western blotting

Cells were lysed in RIPA solution and homogenized on ice. The supernatants with equal amounts of protein

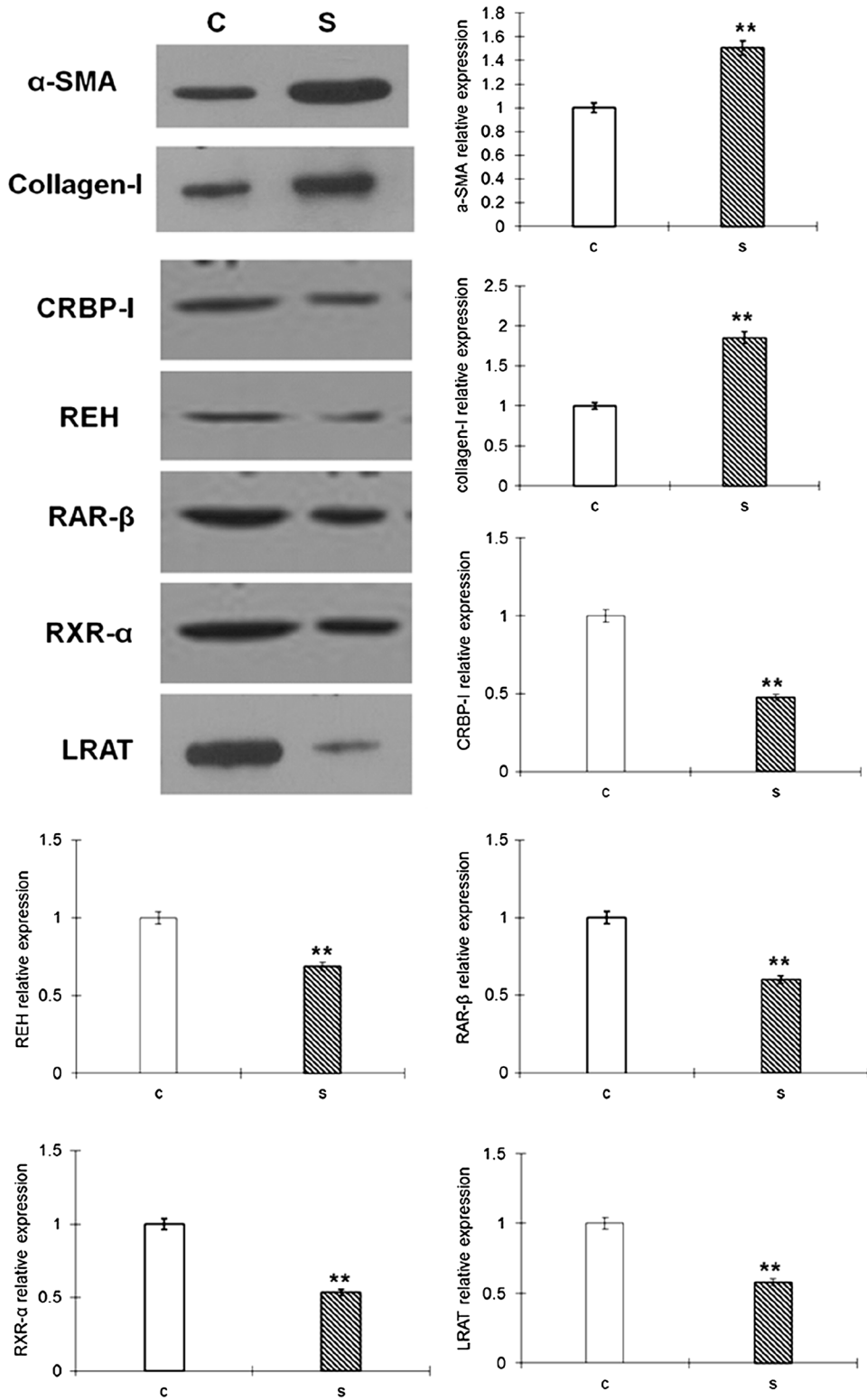


**Fig. 3** Effects of tensile strain on the mRNA levels of fibroblast markers and retinoid metabolic factors. HSCs were serum starved for 24 h, subjected to a 10 % tensile strain, 0.5 Hz, for 24 h and then harvested for RNA extraction. mRNA expression levels of  $\alpha$ -SMA, collagen I, LRAT, CRBP-I, REH, RAR- $\beta$  and

RXR- $\alpha$  were assessed by real-time PCR. *S* HSCs subjected to a 10 % tensile strain, 0.5 Hz, for 24 h, *C* control group without tensile strain (N = 3), \* $P < 0.05$ , \*\* $P < 0.01$  compared with control group

were mixed with sample buffer, heated to 95 °C for 5 min, and resolved by SDS-PAGE followed by western blotting. Western blotting was conducted using antibodies against lecithin-retinol acyltransferase (LRAT), cellular retinol-binding protein I

(CRBP-1), retinoid X receptor- $\alpha$  (RXR- $\alpha$ , Santa Cruz Biotech., Santa Cruz, CA, USA), retinoic acid receptor- $\beta$  (RAR- $\beta$ ), retinyl ester hydrolase (REH),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA, Abcam, Cambridge, MA), collagen-I antibody (Sigma-Aldrich) or  $\beta$ -actin



◀ **Fig. 4** Effects of tensile strain on the protein levels of fibroblast markers and retinoid metabolic factors. HSCs were serum starved for 24 h, subjected to a 10 % tensile strain, 0.5 Hz, for 24 h and then harvested for protein extraction. Protein levels of  $\alpha$ -SMA, collagen I, LRAT, CRBP-I, REH, RAR- $\beta$  and RXR- $\alpha$  were assessed by western blotting. *S* HSCs subjected to a 10 % tensile strain, 0.5 Hz, for 24 h, *C* control group without tensile strain (N = 3), \**P* < 0.05, \*\**P* < 0.01 compared with control group

(CST Biotech., Danvers, MA, USA) and following horseradish peroxidase-conjugated secondary antibody.

#### Statistical analysis

The data was presented as mean  $\pm$  SD. Statistical analysis was performed using SPSS 13.0 software and the *p* value is significant at the 0.05 level.

## Results

### Effects of mechanical pressure on retinoid metabolism and hepatic stellate cells (HSCs) activation

The effect of pressure on HSCs activation and retinoid metabolism was evaluated (Figs. 1, 2). The mRNA and protein levels of fibroblast markers,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and collagen I were significantly increased in HSCs under the pressure at 10 mm Hg for 1 h, compared with the control group without pressure. For the retinoid metabolic factors, the pressure at 10 mm Hg loading significantly inhibited the expression of cellular retinol-binding protein I (CRBP-I), lecithin-retinol acyltransferase (LRAT), retinyl ester hydrolase (REH), retinoic acid receptor- $\beta$  (RAR- $\beta$ ) and retinoid X receptor- $\alpha$  (RXR- $\alpha$ ) both in mRNA and protein levels. These results implied that mechanical pressure can promote HSCs activation and reduce the expression of retinoid metabolic factors.

### Effects of tensile strain on retinoid metabolism and HSCs activation

As shown Figs. 3 and 4, mechanical deformation applied at 10 % tensile strain, 0.5 Hz, for 24 h, significantly increased the mRNA and protein levels

of  $\alpha$ -SMA and collagen I, and notably inhibited the mRNA and protein levels of LRAT, CRBP-I, REH, RAR- $\beta$  and RXR- $\alpha$ . Our data suggested that tensile strain can similarly cause a great increase in HSCs activation and serve as a negative regulator in retinoid metabolism.

### Effects of retinoid antagonists on the activation of HSCs and downregulation of retinoid metabolic pathway by pressure loading

To further explore the effects of retinoid species on HSCs activation, we inhibited the binding of retinol and retinoic acid to their receptors by using retinoid antagonists before HSCs were applied to pressure loading. As shown in Figs. 5 and 6, the effects of pressure loading on the mRNA and protein levels of fibroblast markers and the retinoid metabolic factors were partially reversed by pre-treated with retinoid X receptor antagonist, HX531 and retinoic acid receptor antagonist, LE135.

### Effects of retinoid antagonists on the activation of HSCs and downregulation of retinoid metabolic pathway induced by tensile stress

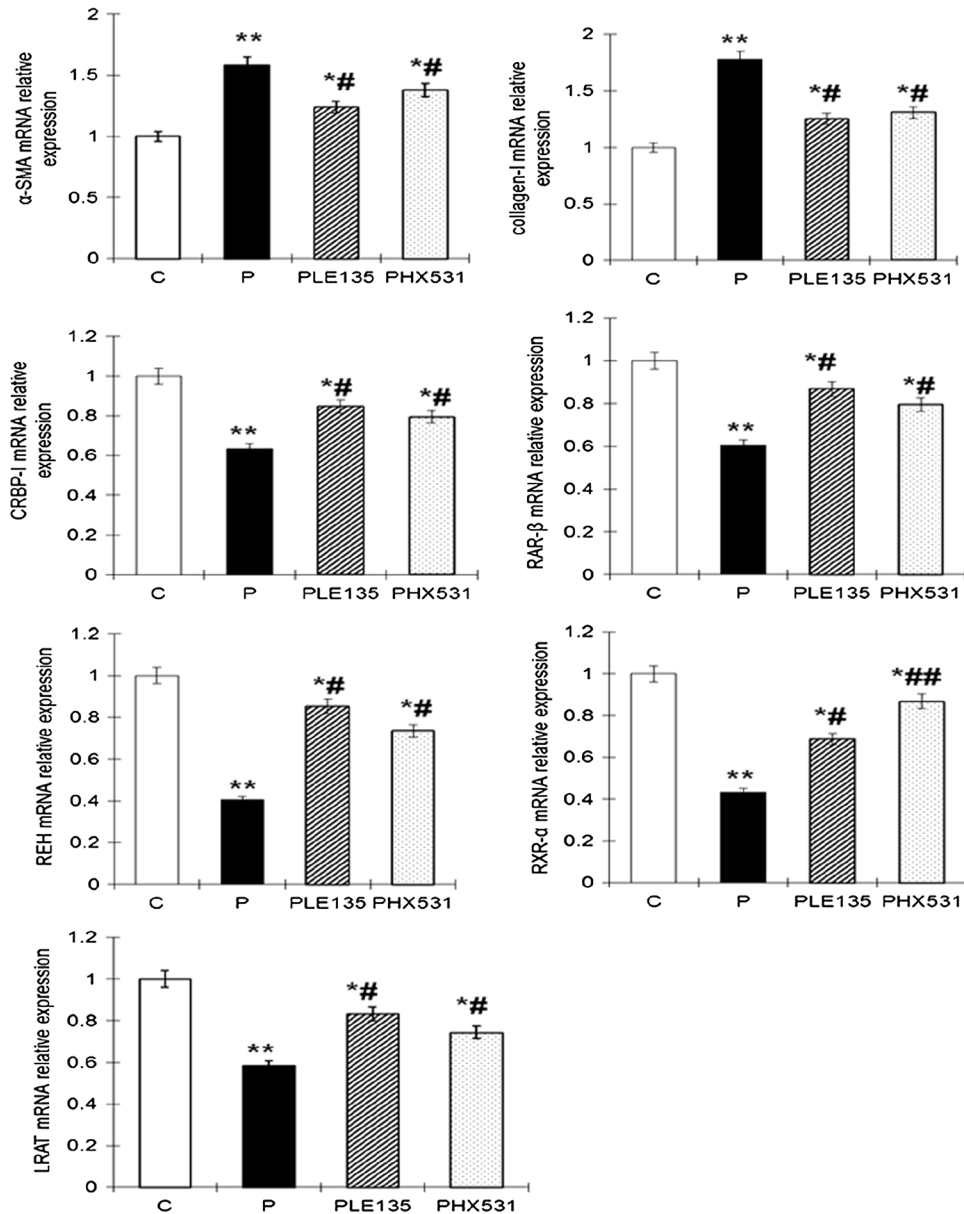
As shown in Figs. 7 and 8, the effects of tensile stress on the mRNA and protein levels of fibroblast markers and the retinoid metabolic factors were partially reversed by pre-treated with retinoid antagonists, HX531 and LE135.

## Discussion and conclusions

The mechanisms of HSCs activation are crucial for the understanding of the development of cirrhotic portal hypertension and liver fibrogenesis. HSCs, located in the subendothelial space of Disse between hepatocytes and sinusoidal endothelial cells, are exposed to direct pressure from Disse to tensile strain from sinusoids. The effect of shear stress on the HSCs can be ignored because of the very low fluid flow rate in the Disse's space. An imbalance of retinoid metabolism may contribute to HSCs activation. Therefore, fading of retinyl ester-containing lipid droplets is considered as one of the hallmarks of HSCs activation. Previous studies on the effects of mechanical forces on HSC activation and retinoid metabolism provide a hint to

the molecular mechanisms by which these mechanical factors regulate the development of cirrhotic portal hypertension and liver fibrogenesis, but experiment evidence are urgently needed.

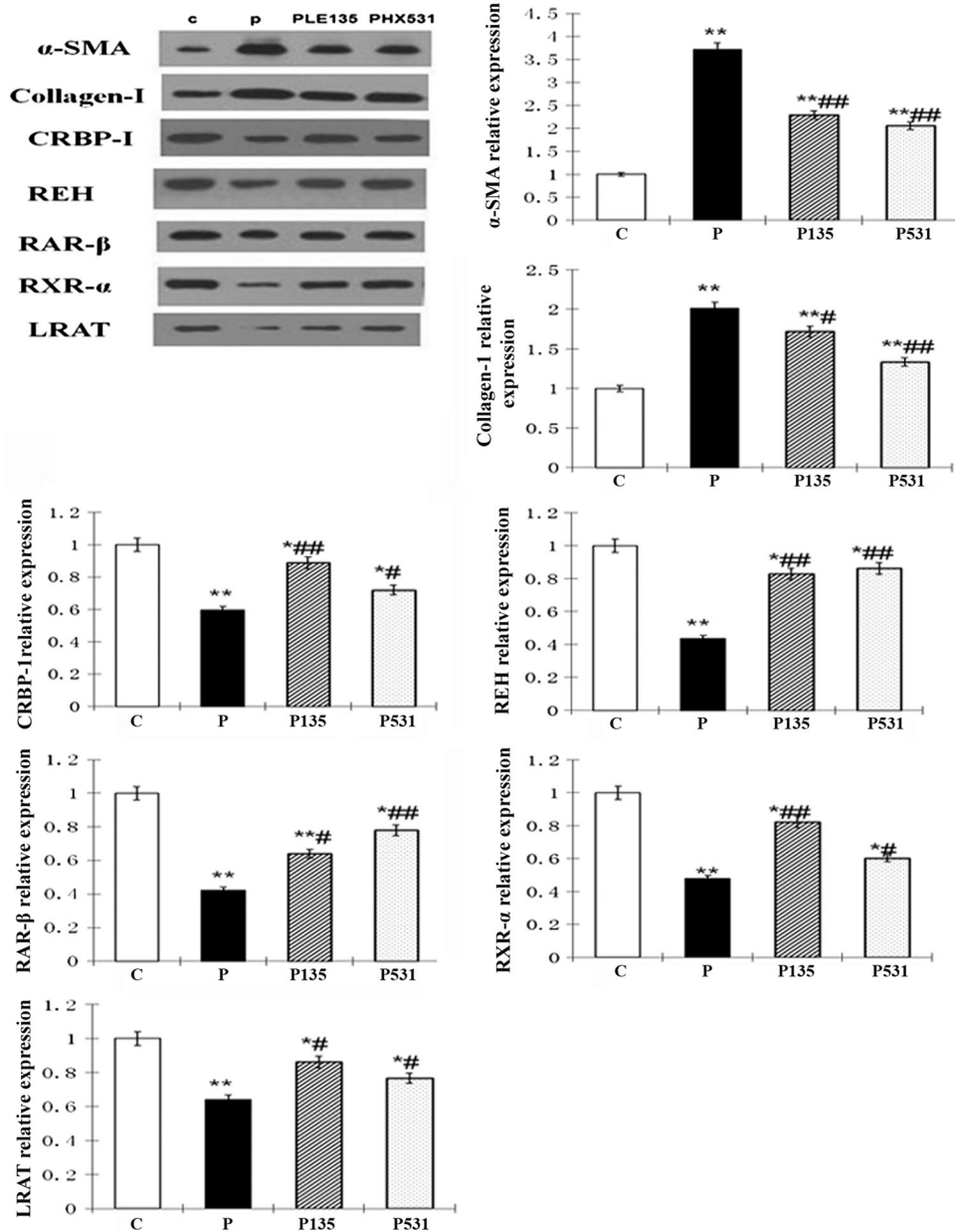
In this study, we used mechanically-active culture systems to stimulate the HSCs by the in vivo mechanical forces and to investigate the effects of these forces on HSCs activation and retinoid metabolism.



**Fig. 5** Effects of retinoid antagonists on the mRNA levels of fibroblast markers and retinoid metabolic factors in HSCs with pressure loading. HSCs were serum starved for 24 h and treated with an retinoid antagonist, LE135 or HX531, for 1 h. HSCs were subjected to a pressure of 10 mm Hg for 1 h and then harvested for RNA extraction. mRNA expression levels of  $\alpha$ -SMA, collagen I, LRAT, CRBP-I, REH, RAR- $\beta$  and RXR- $\alpha$

were assessed by real-time PCR. *P* HSCs were subjected to a pressure of 10 mm Hg for 1 h, *C* control group without pressure, *PLE135* HSCs were treated with LE135 and the pressure was then applied, *PHX531* HSCs were treated with HX531 and the pressure then applied (N = 3), \* $P < 0.05$ , \*\* $P < 0.01$  compared with control group; # $P < 0.05$ , ### $P < 0.01$  compared with pressure applied group



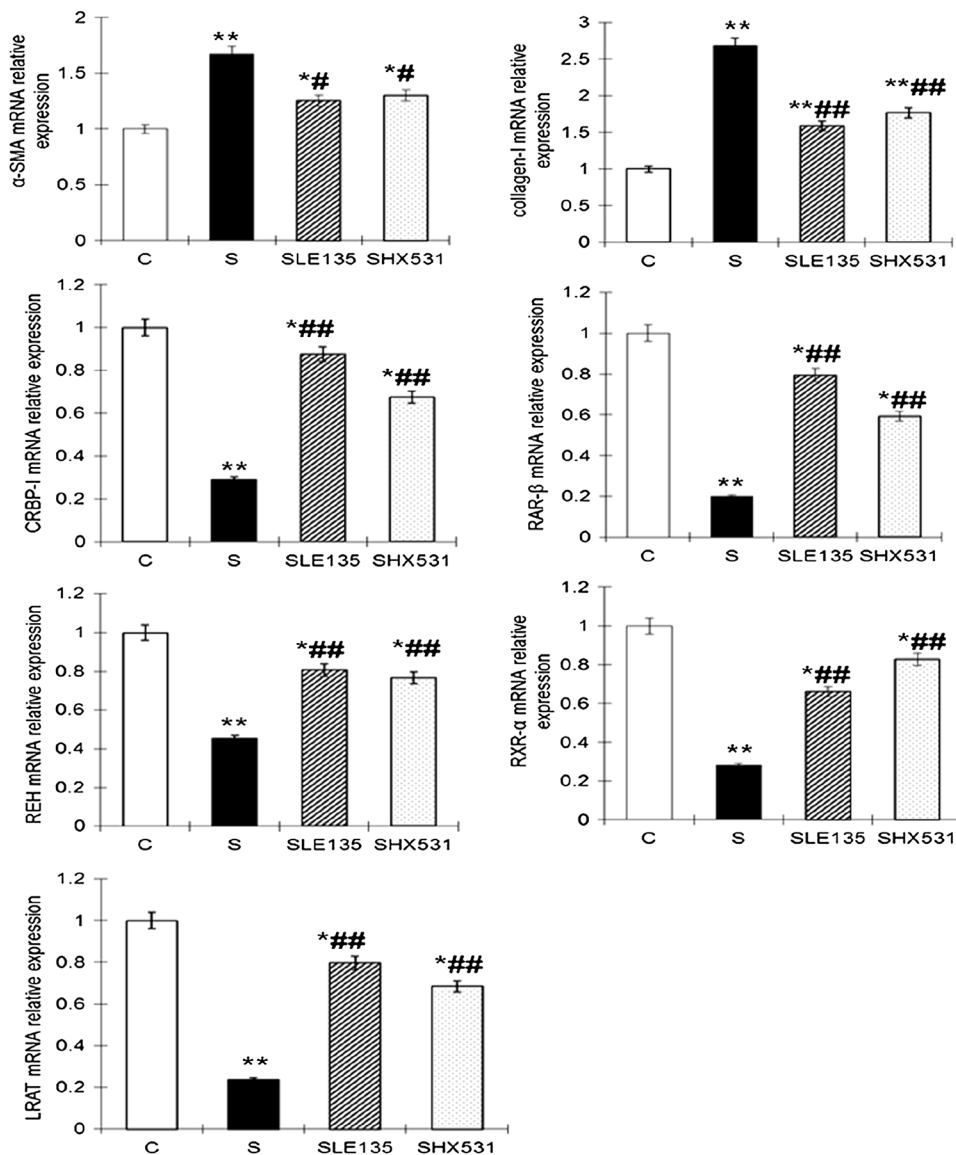


**Fig. 6** Effects of retinoid antagonists on the protein levels of fibroblast markers and retinoid metabolic factors in HSCs with pressure loading. HSCs were serum starved for 24 h and treated with a retinoid antagonist, LE135 or HX531, for 1 h. HSCs were subjected to a pressure of 10 mm Hg for 1 h and then harvested for protein extraction. Protein levels of  $\alpha$ -SMA, collagen I, LRAT, CRBP-I, REH, RAR- $\beta$  and RXR- $\alpha$  were assessed by

western blotting. *P* HSCs were subjected to a pressure of 10 mm Hg for 1 h, *C* control group without pressure, *PLE135* HSCs were treated with LE135 and the pressure was then applied, *PHX531* HSCs were treated with HX531 and the pressure applied (N = 3), \**P* < 0.05, \*\**P* < 0.01 compared with control group; #*P* < 0.05, ###*P* < 0.01 compared with pressure applied group

The pressure of 10 mm Hg for 1 h was applied using a gas-controlled pressure-loading system, which was previously reported able to control the variation of

pressure in the chamber to less than 1 %. An earlier study from our group verified that our pressure-loading system did not change the growth of the HSCs



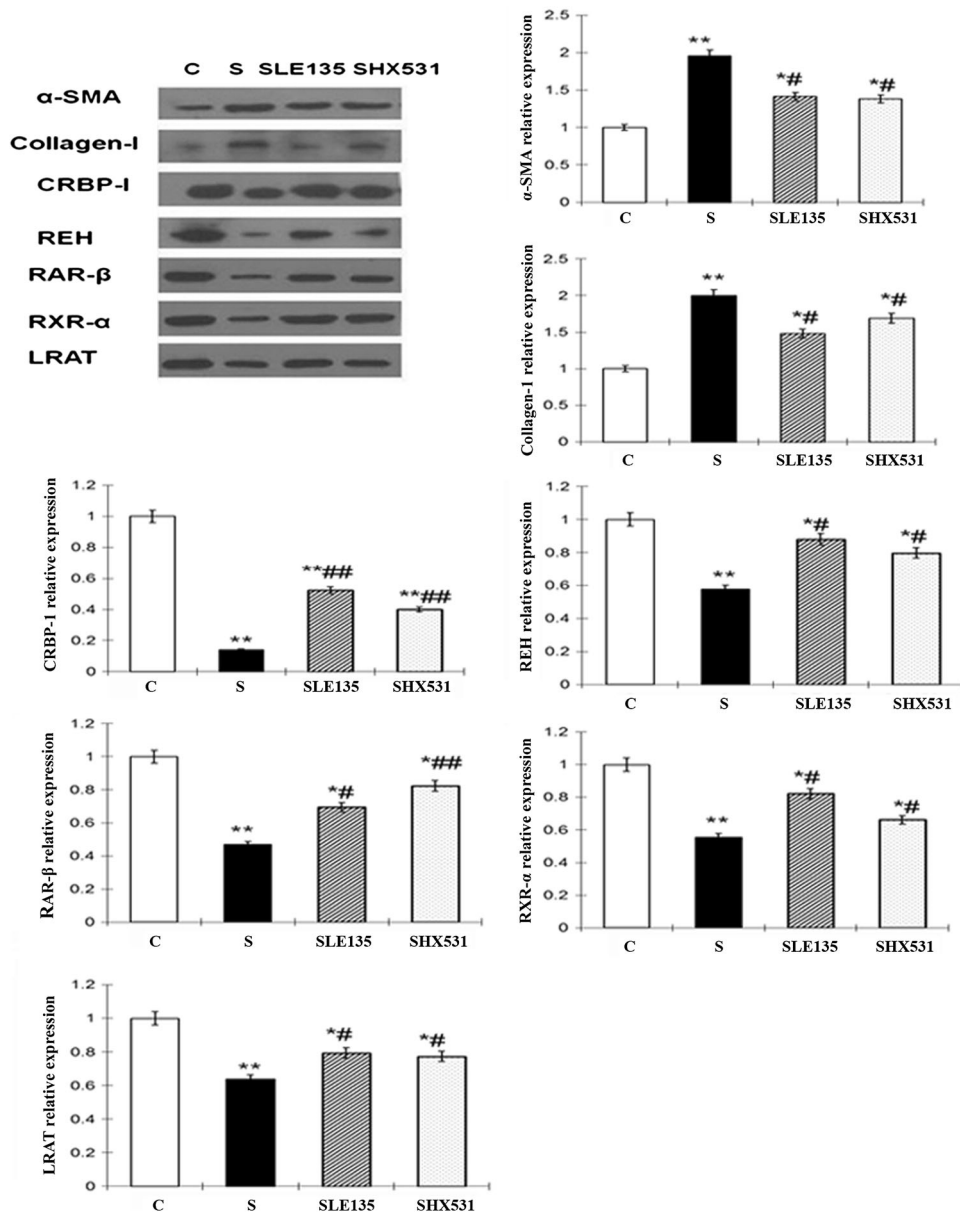
**Fig. 7** Effects of retinoid antagonists on the mRNA levels of fibroblast markers and retinoid metabolic factors in HSCs with tensile strain. HSCs were serum starved for 24 h and treated with a retinoid antagonist, LE135 or HX531, for 1 h. HSCs were subjected to a 10 % tensile strain, 0.5 Hz, for 24 h and then harvested for RNA extraction. mRNA expression levels of  $\alpha$ -SMA, collagen I, LRAT, CRBP-I, REH, RAR- $\beta$  and RXR- $\alpha$

were assessed by real-time PCR. *S* HSC subjected to 10 % tensile strain, 0.5 Hz, for 24 h, *C* control group without tensile strain, *SLE135* HSCs were treated with LE135 and subjected to the at tensile strain, *PHX531* HSCs were treated with HX531 and subjected to tensile strain (N = 3), \* $P < 0.05$ , \*\* $P < 0.01$  compared with control group; # $P < 0.05$ , ### $P < 0.01$  compared with tensile strain applied group

and suggested the pressure at 10 mm Hg for 1 h could significantly promote the activation, proliferation and migration of HSCs. Therefore, this level of pressure was used in the present study. The tensile strain was applied on HSCs using a computer-controlled Flexercell FX-4000T, a system that has been widely used in

the field of cell mechanics. It has been demonstrated that the level of tensile strain we used could remarkably accelerate the collagen secretion of HSCs while maintaining the HSCs in a stable status.

Our results show the pressure loading and tensile strain can significantly increase the expression of  $\alpha$ -



**Fig. 8** Effects of retinoid antagonists on the protein levels of fibroblast markers and retinoid metabolic factors in HSCs with tensile strain. HSCs were serum starved for 24 h and treated with a retinoid antagonist, LE135 or HX531, for 1 h. HSCs were then subjected to 10 % tensile strain, 0.5 Hz, for 24 h and harvested for protein extraction. Protein levels of  $\alpha$ -SMA, collagen I, LRAT, CRBP-I, REH, RAR- $\beta$  and RXR- $\alpha$  were

assessed by western blotting. *S* HSCs subjected to 10 % tensile strain, 0.5 Hz for 24 h, *C* control group without tensile strain, *SLE135* HSCs were treated with LE135 and subjected to the tensile strain, *PHX531* HSCs were treated with HX531 and subjected to the tensile strain (N = 3), \* $P < 0.05$ , \*\* $P < 0.01$  compared with control group; # $P < 0.05$ , ### $P < 0.01$  compared with tensile strain applied group

SMA and collagen I, while inhibit the expression of LRAT, CRBP-I, REH, RAR- $\beta$  and RXR- $\alpha$  (Figs. 1–4), which indicated that pressure loading and tensile strain can induce HSCs activation and inhibit retinoid metabolism. These results also suggested that retinoid

species in the HSCs may be involved in the regulation of HSCs activation.

To test this hypothesis, we used retinoid antagonists to inhibit the binding of retinol and retinoic acid to their receptors and to disturb the retinoid metabolic in

the HSCs. Our results showed that retinoid antagonists used in this study can partially reverse the promoted effects on the expression of  $\alpha$ -SMA and collagen I, and the inhibitory effects on the expression of LRAT, CRBP-I, REH, RAR- $\beta$  and RXR- $\alpha$  by the pressure loading and tensile strain (Figs. 5–8). These results suggest that retinoid metabolism is involved in the activation of HSCs and retinoid antagonists can partially inhibit HSCs activation. However, how cell membrane mechanical sensors, nuclear retinoic acid receptor pathway and retinoid X receptor pathway regulate HSCs activation is still unclear. Further studies are needed to explore the detailed mechanism.

In conclusion, our study investigated the effects of mechanical force on HSCs activation and retinoid metabolism. The results suggest that mechanical stress can promote HSCs activation through the regulation of retinoid metabolism. The findings may facilitate potential therapeutic approaches for hepatic fibrosis, because the methodology to reduce the activation of HSCs is becoming a key approach to block hepatic fibrogenesis.

**Supporting information** Supplementary Table 1—Primers used for quantitative RT-PCR measurements.

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