1 **Liver X Receptor β Controls Hepatic Stellate Cell Activation via Hedgehog** 2 **Signaling**

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14 approves the final version of the submitted manuscript and the submission to
15 *JOURNAL OF CELL SCIENCE*.

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17 Competing interests: The authors declare no conflict of interest

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19 **Running title:** LXR β Controls Hepatic Stellate Cell Activation

20 **Key words:** hepatic fibrosis; hepatic stellate cell activation; LXR β ; Hedgehog
21 signaling

22 23 24 **Abstract**

25 Liver X receptors (LXR) α and β serve important roles in cholesterol homeostasis,
26 anti-inflammatory processes and the activation of hepatic stellate cells (HSCs).
27 However, the development of therapies for liver fibrosis based on LXR agonists have
28 been hampered due to side-effects such as liver steatosis. In this study, we
29 demonstrated that HSCs expressed high levels of LXR β , but not LXR α , and that

overexpression of LXR β suppressed fibrosis and HSC activation in a carbon tetrachloride (CCl₄)-induced fibrosis mouse model, without resulting in liver steatosis. Furthermore, Hedgehog (Hh)-regulated proteins, markedly increased in the CCl₄-affected liver and mainly expressed in activated HSCs, were repressed under conditions of LXR β overexpression. In addition, LXR β knockout led to activation of Hh signaling and triggering of HSC activation, while overexpression of LXR β led to the inhibition of the Hh pathway and suppression of HSC activation. These results suggest that LXR β suppresses the activation mechanism of HSCs by inhibiting Hh signaling. In conclusion, LXR β , by restoring the differentiation of HSCs, may be a promising therapeutic target for liver fibrosis without the adverse side-effects of LXR α activation.

Abbreviations

LXR α , liver X receptor α ; LXR β , liver X receptor β ; HSCs, hepatic stellate cells; α -SMA or Acta2, smooth muscle alpha actin; collagen I, collagen, mainly of type I; HCs, hepatocytes; siRNA, small interfering RNA; Hh, Hedgehog; CCl₄, carbon tetrachloride.

Introduction

Fibrosis of the liver, triggered by chronic liver injury, is the overproduction of fibrillar collagen and remodeling of extracellular matrix. Left untreated, hepatic fibrosis can lead to scarring of the liver, known as cirrhosis, which is associated with significant morbidity and mortality rates. Fibrosis occurs primarily due to the activation of hepatic stellate cells (HSCs), which maintain a quiescent phenotype in the normal liver and store the majority of the retinyl esters, triglycerides and cholesterol esters of the body in lipid droplets [1-3]. Under conditions of liver injury, HSCs are activated, lose their lipid content, and undergo a transformation giving rise to a fibrogenic myofibroblastic phenotype [4,5]. Activated HSCs exhibit high proliferative activity and overexpress smooth muscle α actin (Acta2) and collagen, mainly of type I (collagen I) [6,7].

HSC activation and the conversion to a myofibroblastic phenotype are associated with a marked decrease in the expression of several adipogenic transcription factors, including the liver X receptors (LXRs) [8]. LXRs, occurring as two isoforms (α and β), function as sensors of cholesterol levels, prompting reverse cholesterol transport and its excretion into bile. Previous studies have revealed certain beneficial effects of these proteins on inflammation, atherosclerosis, and diabetes [9,10], making them promising therapeutic targets for these conditions. LXRs also play an important role in HSC activation and susceptibility to liver fibrosis [11,12]. Nevertheless, activation of LXRs leads to enhanced hepatic triglyceride synthesis and may give rise to liver steatosis and hypertriglyceridemia, hampering therapeutic development based on LXR agonists [13]. However, LXR α and LXR β exhibit distinct distributions, and certain studies have indicated that it is LXR α , predominantly expressed in hepatocytes (HCs), that specifically mediates the increase of triglyceride synthesis [14,15]. Although LXRs have already been proven to influence the activation of HSCs, the specificity of the two isoforms and their molecular mechanisms remain unresolved. The aims of this research, therefore, were to investigate the antifibrogenic role of LXR β in the process of HSC activation *in vitro* and *in vivo*. If confirmed, LXR β -selective agonists may be a potential therapeutic target to avoid HSC activation-associated fibrosis, simultaneously avoid undesirable LXR α -associated side-effects.

In the present study, we isolated HSCs from mice and specifically silenced LXR α or LXR β in order to evaluate the distinct roles of the isoforms in the activation of HSCs. We discovered that HSCs predominantly express LXR β . The silencing of LXR β by small interfering RNA (siRNA) significantly inhibited LXR target gene expression and promoted the activation of HSCs *in vitro*. Notably, LXR α knockdown had no significant effect on the expression of LXR target genes or the activation of HSCs. Moreover, the HSCs in LXR β -overexpressing mice were resistant to carbon tetrachloride (CCl₄)-induced activation. Hedgehog (Hh) signaling has been revealed to be important in the promotion of HSC activation and conversion to the myofibroblastic phenotype [16,17]. In chronic liver injury, Hh signaling plays a major

role in liver fibrogenesis [18], therefore, its association with the protective function of LXR β was also investigated. The results of the present study demonstrate that LXR β regulates the activation of HSCs and prevents CCl₄-induced fibrosis via Hh signaling, simultaneously avoiding undesirable LXR α -associated liver steatosis side-effects. Specific activators of LXR β may be used as potential therapeutic agents against liver fibrosis.

Materials and Methods

1. Animals and diets

Male C57/BL6 mice were obtained from Chongqing Medical University. All mice were housed individually in plastic cages with free access to standard chow and water. Chronic liver injury in the mice was induced by intraperitoneal (IP) injections of a 10% CCl₄ solution in olive oil (0.5 μ l pure CCl₄/g body weight) 2 times per week for 5 weeks. Alternatively, mice were administered IP injections of LXR agonist T0901317 (50 mg/kg body weight; dissolved in DMSO at 50 mg/ml) every 3 days. The animals in the control groups received equivalent doses of DMSO and vehicle olive oil. To determine the antifibrotic effects of LXR β , adenovirus vector (Genechem, Shanghai, China) encoding the cDNA of murine LXR β (Ad-LXR β) or empty control vector (Ad-control) were injected via the tail vein (1×10^8 virus particles/mouse). The animal experimental procedures were performed according to the National Institutes of Health Guidelines for the Use of Experimental Animals and approved by the Ethics Committee and the Medicine Animal Care Committee of Chongqing Medical University.

2. Cell isolation and culture

HCs and HSCs were isolated from adult male C57 mice as previously described [19]. Briefly, the mice livers were perfused *in situ* with collagenase IV (0.5 mg/ml, Gibco; Thermo Fisher Scientific, Inc., USA) for approximately 20 min until the liver became smooth and soft. The liver was excised and placed in a dish containing Gey's balanced salt solution (GBSS) with 0.5 mg/ml collagenase IV and 20 μ g/ml DNase I.

120 The liver capsule was opened using forceps and the cell suspension was gently
121 dissociated using a plastic pipette. The cell suspension was filtered through a 200- μ m
122 gauze to remove undigested tissue, and centrifuged at 50 x g for 2 min at 4°C in a
123 50-ml tube . HCs are main components of the resulting cell pellet, while HSCs are
124 light and remain in the supernatant. The pelleted HCs were further resuspended in 48%
125 Percoll and purified by centrifugation at 50 x g for 10 min. The aforementioned
126 supernatant containing the HSCs was transferred to a new 50-ml tube and centrifuged
127 at 500 x g for 7 min at 4°C to pellet the HSCs, which were then resuspended and lysed
128 in GBSS with 0.5 mg/ml pronase E and 20 μ g/ml DNase I for 20 min at 37°C to lyse
129 the HSCs. The mixture was centrifuged at 500 x g for 7 min at 4°C, the cell pellet was
130 resuspended in 12 ml 15% Optiprep diluted in GBSS, and 12 ml 11.5% Optiprep was
131 carefully transferred onto the cell suspensions, followed by 12 ml of GBSS.
132 Following centrifugation at 1,420 x g for 20 min, the HSCs were removed from the
133 top of the 11.5% Optiprep layer. The freshly isolated cells were cultured in DMEM
134 with 10% FBS and maintained at 37°C in a humidified 5% CO₂ atmosphere. The
135 viabilities of the HCs and HSCs were assessed by trypan blue exclusion and the purity
136 were tested using glial fibrillary acidic protein (GFAP) (quiescent HSC marker; 1:100;
137 16825-1-AP, Proteintech Group, China), Acta2 (activated HSC marker; 1/100; ab7817;
138 Abcam, Cambridge, UK) and cytokeratin 18 (HC marker; 1:100; 10830-1-AP,
139 Proteintech Group) (Supplementary Fig. 1). To further confirm the effects of LXRs
140 and Hh signaling on HSC activation, after 3 days of isolation, primary HSCs were
141 treated with LXR agonist T0901317 (5 μ M) or antagonist SR9238 (10 μ M), and Hh
142 agonist Purmorphamine (2 μ M) or antagonist GDC0449 (5 μ M) (MedChemExpress,
143 Monmouth Junction, NJ, USA). The siRNA targeting mouse LXR α and LXR β
144 sequences, constructed by Genescript (Shanghai, China), were transfected according
145 to the manufacturer's instructions. The siRNA sequences were as follows: LXR α
146 sense, 5'-GGCAACACUUGCAUCCUATT-3'; and antisense,
147 5'-UAAGGAUGCAAGUGUUGCCTT-3'; LXR β sense,
148 5'-CAUCCACCAUCGAGAUAUTT-3'; and antisense,
149 5'-AUGAUCUCGAUGGUGGAUGTT-3'; control scramble sense,

150 5'-UUCUCCGAACGUGUCACGUTT-3'; and antisense,
151 5'-ACGUGACACGUUCGGAGAATT-3'.

152

153 **3. Immunohistochemistry and immunofluorescence**

154 The mouse livers were fixed in 4% paraformaldehyde and embedded in paraffin
155 for hematoxylin-eosin (H&E), Masson, Sirius red and immunohistochemical staining.
156 For immunochemical staining, the paraffin-embedded sections were dewaxed with
157 xylene, rehydrated with alcohol and heated with citrate buffer for antigen retrieval.
158 The sections were incubated with 3% peroxide to block endogenous peroxidase and
159 then incubated in 0.5% Triton X-100 for 15 min. Following blocking with 5% goat
160 serum solution for 40 min at 37°C, the sections were stained with anti-Acta2 (1:400)
161 at 4°C overnight. The sections were further processed by the application of the
162 immunoperoxidase technique, using the Envision kit (Boster Biological Technology,
163 China). For immunofluorescence analysis, the samples were incubated with
164 anti-LXR α (1:100; ab3585, Abcam), anti-LXR β (1:100; ab228867, Abcam),
165 anti-Acta2 (1:100) and anti-nuclear transcription factor Gli family zinc finger (Gli)2
166 (1:100; bs-11564R; Bioss, China) overnight at 4°C, followed by incubation with
167 fluorescein isothiocyanate-labeled goat anti-rabbit and anti-mouse secondary antibody
168 (Alexa Fluor; Invitrogen; Thermo Fisher Scientific, Inc.). DAPI was applied to each
169 section for 10 min to counterstain the nuclei.

170

171 **4. Western blot analysis**

172 Cells or liver samples were lysed in radioimmunoprecipitation assay buffer with
173 protease inhibitors, and the concentration of the resulting protein extract was
174 measured using the bicinchoninic acid assay. The extracted proteins were separated
175 by SDS-PAGE (10% gel) and transferred to polyvinylidene difluoride membranes.
176 The membranes were blocked with 5% non-fat milk and immunological detection was
177 performed with the following primary antibodies: Acta2 (1:1,000), collagen I (1:500;
178 14695-1-AP; Proteintech, Rosemont, IL, USA), LXR α (1:1,000), LXR β (1:1000),
179 sterol regulatory element binding protein-1c (Srebp1-c; 1:1000; ab28481; Abcam),

ATP binding cassette subfamily A member 1 (Abca1; 1:500, Ag24118; Proteintech), Patched (Ptch; 1:1,000; PA5-18544, RRID AB_10983895; Thermo Fisher Scientific, Inc.) and sonic hedgehog homolog (Shh; 1:500, 20697-1-AP; Proteintech) overnight at 4°C, followed by incubation with a peroxidase-coupled secondary antibody for 1 h at 37°C. The blots were visualized using an enhanced chemiluminescence kit (Amersham, UK).

5. Quantitative Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted from the cells or tissue samples using the RNeasy Mini Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. Equal amounts of total RNA from each sample was prepared and reverse transcribed into complementary DNA. Supplementary Table 1 lists the specific oligonucleotide primers used. The PCR thermocycling conditions were as follows: 95°C for 30 s followed by 39 cycles of 95°C for 5 s and 56°C for 30 s using SyBr Green reagents (Biosystems, Foster City, CA). The gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the housekeeping gene GAPDH.

6. Statistical analysis

All data were analyzed using SPSS version 19.0 software. Quantitative data are expressed as the mean $\pm$ standard deviation of at least 5 independent experiments for animal studies, and at least 3 independent experiments for each cell experimental group. The groups were analyzed using Student's t-test and $P < 0.05$ was considered to indicate statistically significant differences.

Results

1. LXRs decreased during HSCs activation

Plating HSCs on plastic dishes can artificially cause their activation [20]. Therefore, in order to identify the underlying mechanisms of HSC activation, we tested gene expression in freshly isolated HSCs from healthy mice for 168 h. The

209 freshly isolated quiescent HSCs had a round, phase-dense cell appearance with large
210 numbers of refractile lipid droplets, which appeared garland-like by Oil red O staining.
211 The purity of the HSCs was approximatively 90%, as determined by quiescent HSC
212 marker GFAP and activated HSC marker Acta2 (Fig. 1A). Notably, collagen I and
213 Acta2, regarded as specific markers of HSC activation, exhibited a marked
214 upregulation during *in vitro* HSC culture (Fig. 1B). LXR α and LXR β , and their target
215 genes Srebp1 and Abca1 significantly decreased during HSC activation (Fig. 1C). The
216 protein levels of the indicators of HSC activation, LXRs and their target genes were
217 further confirmed by western blotting. The observed trend for each protein was
218 consistent with the corresponding mRNA expression (Fig. 1D). The results confirm
219 that both of LXR α and LXR β may play roles in HSCs activation.

220

221 **2. LXR β , but not LXR α , regulates HSCs activation in mice**

222 According to previous studies, primary isolated HSCs are activated and induce
223 high levels of activation markers Acta2 and collagen I *in vitro* following 72 h of
224 culture [21]. Therefore, in the present study, freshly isolated HSCs were tested
225 following culture for 72 h. Firstly, an LXR agonist and antagonist were tested to
226 confirm the regulatory effect of LXRs on the activation of mouse primary HSCs. As
227 expected, LXR agonist T0901317 led to a significant increase in the mRNA levels of
228 Abca1 and Srebp1, and suppressed the activation of the mouse primary HSCs. In
229 addition, LXR antagonist SR9238 decreased the expression of LXR target genes and
230 notably promoted the activation of HSCs ($P<0.05$; Fig. 2A). Subsequently, the
231 endogenous expression levels of LXR α and LXR β were measured in mice primary
232 HCs and HSCs, and normalized to the levels of the housekeeping gene GAPDH.
233 LXR α was mainly expressed in HCs, while, HSCs expressed high levels of LXR β
234 ($P<0.01$; Fig. 2B). Similarly, freshly isolated HSCs exhibited strong nuclear
235 expression of LXR β , but almost undetectable levels of LXR α after culture for 24 h
236 (Fig. 2C). Furthermore, we determined the effect of the knockdown of each LXR
237 isoform on the expression of major LXR target genes and the activation of HSCs.
238 After 72 h of culture, siRNA was used to silence LXR α or LXR β in mouse primary

HSCs. The cells in the control group were transfected with siRNA containing a scrambled sequence. Despite efficient silencing, no obvious effect by the LXR α knockdown was observed on Abca1 or Srebp1 on the transcription level, and no significant activation of HSCs was noted ($P>0.05$). In contrast, LXR β silencing led to a notable decrease in the expression levels of LXR target genes, and promoted HSCs activation, as measured by Acta2 and collagen I mRNA expression ($P<0.05$; Fig. 2D). The impact of LXR α and LXR β silencing was also investigated at the protein level. Compared with the mRNA results, similar changes in the protein levels were observed by western blot analysis (Fig. 2E). Additionally, HSCs in the LXR β -silenced group exhibited a high expression of Acta2 and displayed more fibrocyte morphological features, in contrast with the LXR α -silenced group (Fig. 2F). These results indicate that LXR β , but not LXR α , regulates the LXR target genes and suppresses the activation of mouse primary isolated HSCs.

252

253 **3. Overexpression of LXR β inhibits CCl₄-inducing liver fibrosis and HSCs** 254 **activation in mice**

CCl₄-induced chronic liver injury was adopted to investigate whether LXR β serves a role in HSC activation and fibrogenesis *in vivo*. After C57/BL6 mice received IP injections of CCl₄ for 5 weeks, hepatic histopathology was evaluated by H&E, Sirius Red and Masson staining. According to the histology, the mice in the control vehicle group presented normal liver architecture, whereas the livers of the Ad-control-CCl₄-treated mice, which received empty control adenovirus vector and CCl₄, displayed extensive structural disorganization, with the beginnings of septa formation between adjacent vascular structures, and evidence of fibrosis completely surrounding certain parenchymal nodules. The livers of the T0901317-CCl₄ treatment group displayed less septa formation, but were characterized by diffuse distribution of hepatic microvesicular steatosis, caused by the LXR α activation. Furthermore, the Ad-LXR β -CCl₄ treatment resulted in a significantly lower degree of septa formation compared with the Ad-control-CCl₄ treatment, and overexpression of LXR β did not induce the pathological features of liver steatosis (Fig. 3A). Subsequently, we

269 compared the effect of T0901317-stimulated and adenovirus-mediated LXR β
 270 overexpression on LXR target genes at the protein level by western blot analysis. The
 271 T0901317-treated group displayed a significant increase in Abca1 and Srebp1 levels,
 272 compared with the control groups (control vehicle and Ad-control-CCl₄). However,
 273 overexpression of LXR β in the mice resulted in just a moderate increase of the
 274 expression of Abca1 and Srebp1, suggesting that activation of LXR β does not
 275 significantly impact the lipid metabolism in the liver (Fig. 3C). Acta2 staining was
 276 performed to observe the infiltration of activated HSCs in the liver following chronic
 277 injury. The population of Acta2⁺ cells was greatly increased in the
 278 Ad-control-CCl₄-treated mice compared with those in the mice treated with the
 279 control vehicle, indicating that chronic liver injury can produce significant HSC
 280 activation (P<0.01; Fig. 3B and E). Conversely, the number of Acta2⁺ cells was
 281 markedly lower in the LXR β -overexpressed mice compared with that in the
 282 Ad-control-CCl₄-treated mice (P<0.05; Fig. 3B and D). These data suggest that LXR β
 283 suppresses the process of HSC activation and inhibits fibrogenesis in a fibrosis mouse
 284 model without resulting in liver steatosis.

285

286 **4. LXR β may inhibit HSC activation *in vivo* via Hh signaling**

287 Hh signaling is crucial in developmental pattern formation, and stem cell growth
 288 and maintenance [22]. This signaling pathway is dormant in the livers of healthy
 289 adults, and is activated during liver injury, which triggers the production of Hh
 290 ligands and the expression of Hh target genes. It has been reported that Hh signaling
 291 is important for HSC activation and liver fibrogenesis [23,24]. As Hh signaling is
 292 negatively regulated by LXR [25,26], we hypothesized that the role of LXR β in the
 293 regulation of HSC activation and fibrogenesis is exerted via Hh signaling. In order to
 294 test this hypothesis, the Hh pathway activity was investigated in CCl₄-induced liver
 295 injury. Double immunofluorescence for Acta2 and Gli2, a main Hh target gene, was
 296 performed to assess Hh signaling in activated HSCs. Fig. 4A demonstrates the nuclear
 297 Gli2 localization in activated, Acta2⁺ HSCs in the livers of CCl₄-treated mice, and the
 298 low expression in the healthy livers of the control vehicle group. Notably, the livers

from the Ad-control-CCl₄-treated group exhibited markedly increased Gli2 nuclear staining in myofibroblastic cells located in the fibrotic areas (4.6-fold increase vs. control). However, the overexpression of LXR β significantly suppressed the number of Acta2⁺ cells as well as the nuclear expression of Gli2 (P<0.01; Fig. 4A and B). Furthermore, compared with the control vehicle group, the upregulation of Gli2 protein levels in CCl₄-induced fibrotic liver was accompanied by an increase in collagen I and Acta2 protein levels. These results indicate that Hh signaling is involved in HSC activation and fibrosis. The overexpression of LXR β in CCl₄-treated mice led to a decrease in the expression of Gli2, collagen I and Acta2 (P<0.05; Fig. 4C), suggesting that LXR β may affect Hh signaling and HSCs activation *in vivo*.

5. LXR β inhibits HSC activation via Hh signaling *in vitro*

Hh signaling activation occur at several levels of the signal transduction cascade [27,28,29]. Briefly, Hh signaling is initiated by Ptch, the cell surface receptor for the Hh ligand, binding of Shh Sonic hedgehog (Shh), a main mammalian Hh signaling ligand. This interaction permits the propagation of intracellular signals that culminate in the nuclear localization of Gli transcription factors, especially Gli2, and regulate the expression of Hh signaling target genes, including amplifications of Ptch and Gli. To further support the aforementioned hypothesis, mouse primary HSCs were isolated and cultured for 168 h. We found that the HSC activation process was accompanied by the activation of Hh signaling. Ptch and Gli2 were almost undetectable in freshly isolated, quiescent HSCs, but significantly upregulated during HSC activation (P<0.01; Fig. 5A and B). An Hh agonist and antagonist were then used to test the role of Hh signaling in the activation of HSCs. Accordingly, the mRNA expression levels of collagen I and Acta2 were upregulated by approximately 3.3- and 2.1-fold, respectively, following treatment with Hh agonist Purmorphamine compared with vehicle (P<0.01; Fig. 5C). In contrast, following treatment with Hh antagonist GDC0449, the levels of collagen I and Acta2 mRNA decreased by approximately 51 and 53%, respectively (P<0.05; Fig. 5C). These data demonstrate that Hh signaling is essential for HSC activation and fibrosis. To further confirm that Hh signaling

controls HSC activation and is mediated by LXR β , we transfected siRNA targeting LXR α or LXR β , or adenovirus-mediated vectors overexpressing LXR α or LXR β into HSCs, and observed their effects on Hh signaling. Silencing LXR β in HSCs led to a marked upregulation of Hh target genes Gli2 and Ptch, and HSC activation markers collagen I and Acta2. Additionally, we observed that overexpression of LXR β decreased the expression of Gli2 and Ptch in HSCs, and suppressed their activation, as measured by collagen I and Acta2 protein levels ($P < 0.05$; Fig. 5D). Conversely, in the LXR α overexpression or knockdown groups, no significant differences were observed in the expression of Hh target genes or the levels of the HSC activation marker proteins, compared with the control group ($P > 0.05$; Fig. 5D). This supports the conclusion that LXR β is the main LXR isoform regulating HSC activation. Furthermore, double immunostaining for Gli2 and Acta2 was performed to assess the effects of LXR β on Hh signaling in HSCs. Compared with the control vector-transfected group, the HSCs displayed higher Gli2 expression and more mesenchymal phenotype characteristics with fibroblast-like features under LXR β knockdown conditions. On the other hand, HSCs transfected with Ad-LXR β exhibited suppressed nuclear Gli2 expression and maintained a more quiescent phenotype (Fig. 5E). Finally, to further determine the plausible mechanisms of LXR β inhibits Hh signaling, we transfected Ad-LXR β in the presence of Hh agonist Purmorphamine or siRNA targeting LXR β in the presence of Hh antagonist GDC0449 in HSCs. As shown in Fig. 5F, purmorphamine significantly induced the protein expression of Hh target genes Ptch and Gli2, and upregulated HSC activation markers collagen I and Acta2 in HSCs. Overexpression of LXR β did not block purmorphamine-stimulated induction of Ptch and Gli2 but partially inhibited the upregulation of HSC activation markers. In contrast, GDC0449 significantly inhibited the protein levels of Ptch, Gli2, collagen I and Acta2 in HSCs, while silencing LXR β partially rescued the inhibiting effects of GDC0449. Thus, Hh signaling is only a part of complex mechanisms for LXR β -influenced HSC activation. Additionally, Hh ligands Shh was drastically upregulated by purmorphamine treatment, but completely abolished while overexpression of LXR β in HSCs, indicating that LXR β influences the Hh signaling

359 by inhibiting Hh ligands production. Together, the results reveal that LXR β plays an
360 important role in inhibiting HSC activation through regulating Hh signaling.

361

362 **Discussion**

363 Hepatic fibrosis cause mass mortality in the worldwide [30], but effective
364 antifibrotic therapies are yet to be developed. Despite the fact that HSCs are a
365 universal source for myofibroblasts in the liver, with an important function in viral,
366 toxic, biliary and fatty liver diseases, the detailed molecular mechanisms of their
367 activation are only partially understood, highlighting the need for further research [31].
368 LXRs are key regulators of hepatic lipogenesis and cholesterol homeostasis, and
369 display anti-inflammatory and antifibrotic effects in HSCs [11,32]. Several
370 pharmaceutical companies have been actively researching LXR agonists to activate
371 LXR α and LXR β , and efficiently induce the expression of LXR target genes,
372 including Abca1 and Srebp1 [33]. However, the activation of LXR α leading to
373 enhanced hepatic triglyceride synthesis and adverse side effects, including liver
374 steatosis and hypertriglyceridemia, have hindered therapeutic development. Therefore,
375 it is important to ascertain the relative contributions of the two LXR isoforms in the
376 process of HSC activation.

377 Notably, immortalized HSC lines, such as Lx2 and Hsc-T6, are poor model
378 systems for the study of HSC activation, because these cells are already activated and
379 resistant to transformation. For this reason, purified primary HSCs were used in the
380 present study, which confirmed that the expression levels of the LXRs and their target
381 genes, Srebp1 and Abca1, were significantly decreased during HSC activation.
382 Quiescent HSCs store large amounts of lipid and 80% of the body's total vitamin A,
383 but rapidly lose lipid droplets and induce the expression of activation markers when
384 they differentiate into myofibroblasts upon stimulation. Therefore, LXRs as the key
385 regulators of the significant changes that lipogenesis undergoes during HSC activation
386 [12]. In contrast to HCs, in which LXR α plays a major role, HSCs mainly express
387 high levels of LXR β . In HSCs, LXR β is the main regulatory isoform for the LXR
388 target genes; knockdown of LXR β resulted in significant decrease in Srebp1 and

Abca1 expression, and enhanced the activation of HSCs *in vitro*. On the other hand, no obvious effect of LXR α silencing was observed on the expression of LXR target genes or the activation of HSCs (Fig. 2C). Based on this result, activated LXR β can inhibit HSC activation, while avoiding any effect on HCs, in which LXR α exerts a dominant regulatory function. Indeed, in a CCl₄-induced liver fibrosis mouse model, overexpressed LXR β led to the inhibition of HSC activation and fibrogenesis (Fig. 3A, B and E). Although similar findings were revealed in the T0901317-treated group, H&E staining demonstrated a diffused distribution of microvesicular steatosis in the liver (Fig. 3A). As HCs, liver sinusoidal endothelial cells and Kupffer cells, in which LXR α serves a dominant role, constitute approximately 60, 19 and 10% of the liver cell population, respectively, compared with HSCs, which mainly express LXR β and account for just 8% [34,35,36]. In T0901317-treated mice, the two LXR isoforms were activated in parallel and led to marked upregulation of Srebp1 and Abca1, the main regulators of triglyceride synthesis and cholesterol metabolism [37]. Overexpression of LXR β in mice resulted in the expression of Srebp1 and Abca1 being only moderately increased, suggesting that activation of LXR β does not significantly affect liver lipid metabolism (Fig. 3D). Therefore, the results of the present study verify the antifibrotic role of LXRs, and establish an antifibrotic mechanism for LXR β , avoiding HSC activation-associated fibrosis and undesirable side-effect of LXR α activation simultaneously. Furthermore, we found that LXR β regulates Hh signaling in HSC activation.

Hh signaling, a crucial developmental regulator during embryogenesis, is inactive in the healthy adult liver, but becomes reactivated during liver injury. Under conditions of liver damage, increased levels of Hh ligands activate cell surface receptor Ptch and upregulate the expression of nuclear transcription factor Gli2 [38]. During this process, Hh signaling promotes the transition of quiescent HSCs to activated myofibroblastic HSCs, which produce large amounts of collagen and extracellular matrix [39]. And activated HSCs also secrete Hh ligands, which then through autocrine and paracrine role to further activate Hh signaling in a positive feedback loop. In the present study, the expression of Gli2 was low in the control

group, but greatly increased in CCL₄-treated mice, and importantly, Gli2 was mainly located in the fibrotic areas and localized in activated Acta2⁺ HSCs. According to this result, HSCs may be the main responsive cell in which Hh signaling occurs during the process of liver damage and fibrosis. Subsequently, overexpression of LXR β significantly decreased the number of Acta2⁺ cells and their nuclear expression of Gli2 in CCL₄-treated mice (Fig. 4A and B). Using cultured mouse primary HSCs, we verified the contribution of Hh signaling to HSC activation *in vitro* (Fig. 5A, B and C). We further confirmed the underlying molecular mechanisms between LXR β and Hh signaling in HSC activation. Despite LXR β suppressing Hh signaling and regulating the expression of Hh target genes Gli2 and Ptch, which are closely associated with HSC activation, no influence of LXR α on Hh signaling or HSCs activation was observed (Fig. 5D). Although it is reported that LXR α and LXR β co-regulate several genes, and can compensate for one another in many pathological conditions, in our present study, LXR β deletion or overexpression was not compensated by LXR α . Finally, we primarily explored the possible mechanism of HSC activation that LXR β seems to suppress the Hh signaling by inhibiting Hh ligands production (Fig. 5F). As Hh ligands production to induce Hh signaling by at least two mechanisms, whether increasing Hh ligand expression or prolonging its half-life. Thus, much more researches need be done to explore the molecular mechanisms between LXR β and Hh signaling.

HSCs are the most relevant source of hepatic myofibroblasts in liver fibrosis, and activated HSCs secrete several inflammatory factors that exacerbate liver injury [40]. Suppressing HSC activation and restoring the quiescent phenotype may be a promising strategy for the development of therapies against fibrosis. The results of the present study demonstrate that LXR β regulates the activation of HSCs and prevents CCL₄-induced fibrosis via Hh signaling, simultaneously avoiding undesirable LXR α -associated liver steatosis side-effects. Specific activators of LXR β may be used as potential therapeutic agents against liver fibrosis.

Conflict of interest

449 The authors have no potential conflicts of interest to declare.

450

451 **Acknowledgements**

452 This study was supported by the National Natural Science Foundation of China
453 [grant number 81400612, 81270494 and 81500443]

454

455 **References**

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562

563 **Figure legends**

564

565 **Figure 1: LXRs decrease during HSC activation.** (A) a, Images of freshly isolated,
566 quiescent HSCs cultured for 24 h; HSCs appear as round, phase-dense cells
567 containing refractile lipid droplets. b, Oil red O staining of HSCs cultured for 24 h;
568 HSCs displayed abundant garland-like lipid droplets in the cytoplasm. c,
569 Immunohistochemical staining of HSCs cultured for 24 h; HSCs were positive for the
570 quiescent HSC marker GFAP. d, Immunohistochemical staining of HSCs cultured for
571 168 h; HSCs were positive for the activated HSC marker Acta2. (B) Relative mRNA
572 expression of collagen I and Acta2 during HSC activation. (C) Relative mRNA
573 expression of LXRs and downstream target genes Abca1 and Srebp1 during HSC
574 activation. (D) Protein expression of collagen I, Acta2, LXR α , LXR β , Srebp1 and
575 Abca1 during HSC activation was investigated by western blotting, using β -actin as a
576 loading control. DAPI was used to stain the nucleus. $n \geq 3$, * $P < 0.05$, ** $P < 0.01$.

577

578 **Figure 2: Effect of LXR knockdown on the mRNA and protein levels of LXR**

579 **target genes and HSC activation.** (A) Relative mRNA expression of Abca1, Srebp1,
580 collagen I and Acta2. HSCs were treated with T0901317 (5 μ M), SR9238 (10 μ M) or
581 DMSO for 48 h. (B) Relative mRNA expression of endothelial LXR α and LXR β in
582 freshly isolated HSCs. (C) Expression of endothelial LXR α and LXR β was
583 investigated by immunofluorescence in freshly isolated HSCs. (D) Relative mRNA
584 expression of LXR α , LXR β , Srebp1, Abca1, collagen I and Acta2. HSCs were
585 transfected with control, LXR α or LXR β siRNA for 48 h. (E) Protein expression of
586 LXR α , LXR β , Srebp1, Abca1, collagen I and Acta2 was investigated, using β -actin as
587 a loading control. HSCs were transfected with control, LXR α or LXR β siRNA for 72
588 h. (F) HSCs were transfected with control, LXR α or LXR β siRNA for 72 h. Acta2
589 was investigated by immunofluorescence. DAPI was used to stain the nucleus. Data
590 are presented as mean $\pm$ standard deviation. $n \geq 3$, * $P < 0.05$, ** $P < 0.01$.

591

592 **Figure 3: Inhibition of HSC activation in LXR β -overexpressed mice with**
593 **CCl $_4$ -induced liver damage.** (A) a, H&E staining of liver samples with CCl $_4$ -induced
594 injury. Black arrows indicate hepatic microvesicular steatosis. b, Sirius red staining
595 was used to evaluate the hyperplastic state of collagen, indicated with red staining. c,
596 Masson staining was used to evaluate the fibrosis, indicated with blue staining. (B)
597 Acta2 immunohistochemical staining of liver samples with CCl $_4$ -induced injury. (C)
598 Protein expression of LXR α , LXR β , Srebp1 and Abca1 was investigated by western
599 blotting, using GAPDH as a loading control. (D) Statistical analysis of the number of
600 Acta2 $^{+}$ cells. Data are presented as mean $\pm$ standard deviation. $n = 5$, * $P < 0.05$,
601 ** $P < 0.01$.

602

603 **Figure 4: LXR β inhibits HSC activation via Hh signaling *in vivo*.** (A) Double
604 immunofluorescence staining for the Hh target Gli2 (red), and the HSCs activation
605 marker Acta2 (green). DAPI was used to stain the nucleus. (B) Statistical analysis of
606 the number of Gli2 $^{+}$ cells. (C) Protein expression of Gli2, collagen I and Acta2 was
607 investigated by western blotting, using GAPDH as a loading control. (D) Statistical
608 analysis of the protein expression of Gli2, collagen I and Acta2. $n \geq 3$, * $P < 0.05$,
609 ** $P < 0.01$.

610

Figure 5: LXR β inhibits HSC activation via Hh signaling *in vitro*. (A) Relative mRNA expression of Gli2 and Ptch during HSC activation. (B) Protein expression of Gli2 and Ptch during HSC activation, with β -actin as a loading control. (C) Relative mRNA expression of collagen I and Acta2. HSCs were treated with Purmorphamine (2 μ M), GDC0449 (5 μ M) or DMSO for 48 h. (D) Protein expression of Gli2, Ptch, Collagen I and Acta2 was investigated, using β -actin as a loading control. HSCs were transfected with control, LXR α or LXR β siRNA, LXR α - or LXR β -overexpressing adenovirus vector for 72 h. (E) Double immunofluorescence staining for the Hh target Gli2 (red) and the HSC activation marker Acta2 (green). HSCs were transfected with control, LXR β siRNA or LXR β -overexpressing adenovirus vector for 72 h. (F) Protein expression of Shh, Gli2, Ptch, collagen I and Acta2 was investigated, using β -actin as a loading control. HSCs were treated with Purmorphamine, GDC0449 and transfected with control, LXR β -overexpressing adenovirus vector or LXR β siRNA for 72 h. $n \geq 3$, * $P < 0.05$, ** $P < 0.01$.

Sequence,		
Gene	Forward (5'- 3')	Reverse (3'- 5')
Acta2	GGCATCCACGAAACACCTA	TAGTCGTTTGTCTTATGCTGC
Collagen I	TGGCGGTTATGACTTCAGCTT	TTCGACTTCAGTATTGGCGGT
LXR α	GGATGGTGGATGGAGACGTAG	GATGCAGAGGTAGGTGGTAGG
LXR β	CTCCTGAAGGCATCCACTATCG	ATGTCGTTCTGCTGAAGGTGG
Srebp	GCCACAGAGCTTCCCAGCCC	ACTTGACACACTGGGTCGGG
Abca1	GCACAATTCCACAAGAACCGC	CGCCAAGAACACCTTAACACG
Gli2	AGCTAGTCGTACCGAGGAGTAC	TCGATCAGCAGTGGCTCCTCATG
Ptch	CAGAGACTGGCTTCAGGGACTT	TGCTACCTCAGGAACGGATGTT
GAPDH	AACACGGAAGGCCATGCCA	GCATCCTGCACCACCAACTT

630 Table 1 Primer and probe sequences for real-time PCR.

631

A

C

B

D

A **B** **C** **D** **E** **F**

A **B** **C** **D**

A **B** **C** **D**

A

B

C

D

E

F