Liver X receptor α is essential for the capillarization of liver sinusoidal endothelial cells in liver injury

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Liver X receptors (LXRs) play essential roles in lipogenesis, anti-inflammatory action and hepatic stellate cells (HSCs) activation in the liver. However, the effects of LXRs on the capillarization of liver sinusoidal endothelial cells (LSECs) in liver fibrosis remain undetermined. Here, we demonstrated that LXRα plays an important role in LSECs capillarization in a manner that involved Hedgehog (Hh) signaling. We found that LXRα expression in LSECs was increased in the carbon tetrachloride (CCL4)-induced fibrosis model. LXRα deletion markedly exacerbated CCl4-induced lesions assessed by histopathology, as well as inflammation and collagen deposition. Furthermore, capillarization of the sinusoids was aggravated in CCl4-treated LXRα-deficient mice, as evidenced by increased CD34 expression, the formation of continuous basement membranes and aggravation of the loss of fenestrae. In vitro, LXR agonist could maintain freshly isolated LSECs differentiation on day 3. Furthermore, LXRα deletion led to increased expression of Hedgehog (Hh)-regulated gene in LSECs in the injured liver. Conversely, the LXR agonist could inhibit the Hh pathway in cultured LSECs. These responses indicated that LXRα suppressed the process of LSECs capillarization by repressing Hh signaling. Overall, our findings suggest that LXRα, by restoring the differentiation of LSECs, may be critical for the regression of liver fibrosis.
in vitro. As Hedgehog (Hh) signaling has been shown to play a crucial role in regulating LSECs capillarization and to be regulated by the LXRs\textsuperscript{17}, the involvement of Hh signaling underlying the protective effects by LXR\textsubscript{α} was also explored.

**Results**

**LXR\textsubscript{α} expression in LSECs increases in response to CCl\textsubscript{4} administration.** Double immunostaining for CD31 (a marker of capillarised LSECs\textsuperscript{10}) and LXR\textsubscript{α} was used to detect the change of LXR\textsubscript{α} expression in LSECs from WT mice after CCl\textsubscript{4} administration. The number of CD31/ LXR\textsubscript{α} double positive sinusoidal cells was negligible in the vehicle-treated mice, but double-positive sinusoidal cells were increased in the CCl\textsubscript{4}-treated group (p < 0.001; Fig. 1a,b). These results indicate that LXR\textsubscript{α} may be related to the capillarization of LSECs and hepatic fibrosis.

**Aggravation of liver injury and fibrogenesis in LXR\textsubscript{α} KO (knockout) mice after CCl\textsubscript{4} treatment.** We investigated hepatic histopathology after chronic CCl\textsubscript{4} treatment by hematoxylin and eosin (HE) staining. By histology, neither wild-type (WT) nor LXR\textsubscript{α}/−/− livers were affected by the injection of vehicle and they presented a normal architecture. In the CCl\textsubscript{4}-treated WT mice, the livers showed hepatocytes necrosis and extensive disorganization with the beginning of septa formation. CCl\textsubscript{4}-treated LXR\textsubscript{α}/−/− livers showed more septa formation, which was characterized by more severe hepatocytes necrosis, inflammatory cells infiltration and congestion (Fig. 2a).

F4/80 staining was used to observe the infiltration of macrophages and activation of Kupffer cells in the liver after chronic injury. The population of F4/80\textsuperscript{+} cells was greatly increased in the CCl\textsubscript{4}-treated LXR\textsubscript{α}/−/− mice compared with that in the CCl\textsubscript{4}-treated WT mice (p < 0.01; Fig. 2b,e). Furthermore, there was no significant difference in the number of F4/80\textsuperscript{+} cells in the liver between vehicle-treated WT and vehicle-treated LXR\textsubscript{α}/−/− mice (p > 0.05; Fig. 2b,e).

We next evaluated the effect of LXR\textsubscript{α} on hepatocyte proliferation following chronic CCl\textsubscript{4} administration. Proliferating cell nuclear antigen (PCNA)-positive hepatocytes in WT mice were significantly increased after CCl\textsubscript{4} injection. Compared with the CCl\textsubscript{4}-treated WT mice, PCNA-positive hepatocytes were dramatically increased in the CCl\textsubscript{4}-treated LXR\textsubscript{α}/−/− mice (p < 0.001; Fig. 2c,f). Very few PCNA-positive hepatocytes could be observed in both vehicle-treated WT and vehicle-treated LXR\textsubscript{α}/−/− mice (p > 0.05; Fig. 2c,f). These results suggest that the proliferative capability of hepatocytes after CCl\textsubscript{4} administration is enhanced by the genetic deletion of LXR\textsubscript{α}.

To examine the influence of LXR\textsubscript{α} expression on liver fibrosis, chronic CCl\textsubscript{4} treatment was used. Collagen α1 (1) immunohistochemical staining showed that the deposition of collagen-I was increased in the portal tracts, septa and perisinusoidal spaces in WT mice treated with CCl\textsubscript{4}; whereas LXR\textsubscript{α}/−/− mice treated with CCl\textsubscript{4} showed aggravated collagen accumulation in the liver (p < 0.01; Fig. 2d,g). Together, these data show that LXR\textsubscript{α} deletion aggravates inflammation and fibrosis in the liver injury.

**Exacerbation of hepatic sinusoidal capillarization in LXR\textsubscript{α} KO mice after chronic CCl\textsubscript{4} administration.** CD34 is not expressed by endothelial cells of normal liver sinusoids, but is expressed after liver sinusoidal capillarization has developed. CD34 expression in areas of sinusoidal capillarization (capillary microvessel density [MVD]) was examined according to Weidner et al.\textsuperscript{18}. In the CCl\textsubscript{4}-treated WT mice, there were areas where sinusoidal endothelial cells were diffusely positive for CD34 (Fig. 3a). More CD34-positive cells were
found in the CCl4-treated LXRα−/− mice (p < 0.01; Fig. 3a,b). No CD34 expression was detected in either vehicle-treated WT or LXRα−/− mice (Fig. 3a,b).

Transmission electron microscopy (TEM) of the sinusoidal areas of hepatic tissues showed that in both groups of vehicle-treated mice, the sinusoids contained numerous fenestrae with a discontinuous basement membrane that could be observed on the basal side of LSECs. In the LXRα−/− mice treated with CCl4, the deposition of extracellular matrix proteins in Disse’s spaces was found, with continuous basement membranes evident on the basal side of LSECs. However, CCl4-induced changes in sinusoidal areas could be ameliorated in WT mice (Fig. 3c).

Scanning electron microscopy (SEM) was used to evaluate the sinusoidal lining. As shown in Fig. 3d, in sinusoidal areas of livers from WT mice treated with CCl4, fenestrae of LSECs were markedly decreased in number. In the CCl4-treated LXRα−/− mice, LSECs fenestrae had completely disappeared, whereas in both groups of vehicle-treated mice, a thin sinusoidal lining containing many fenestrae could be observed (p < 0.01; Fig. 3d,e).
Collectively, these data indicate that LXRα deletion increases susceptibility to liver injury, which is at least in part, triggered by LSECs capillarization.

In addition, TEM results revealed changes in mitochondrial structure and the shape of hepatocytes. LXRα−/− mice displayed mitochondrial swelling and loss of cristae compared with mitochondria from the WT mice. CCl4 induced more serious damage in mitochondria from WT mice. Thus, hepatocytic mitochondria injuries were aggravated in the CCl4-treated LXRα−/− mice, including aggravated mitochondrial swelling, loss of cristae and vacuole-like changes (Fig. 3f). These findings reveal that LXRα play an important role in mitochondrial injuries, which is closely associated with the liver injuries.

LXRα inhibits LSECs capillarization via Hh signaling in vivo. Hh ligands are scarce in healthy adult livers, but liver injury increases the production of Hh ligands. Thus, Hh signaling becomes dramatically activated in injured livers. It has been reported that Hedgehog signaling might be an important factor that is involved in liver injury, liver fibrosis and LSECs capillarization. Negative regulation of Hh signaling by LXR has been demonstrated. The role of LXR in the regulation of liver fibrosis-associated capillarization of the sinusoids may exert via Hedgehog signaling. To examine this hypothesis, Hh pathway activity was investigated in liver injury induced by CCl4. Double immunofluorescence for Gli2 and CD31 (a marker of capillarised LSECs) was used to assess the expression of the Hh target gene Gli2 in LSECs. LSECs in WT mice treated with CCl4 consistently expressed Gli2, but treatment of LXRα−/− mice with CCl4 significantly increased the number of Gli2-positive cells in LSECs (p < 0.001; Fig. 4a,b), indicating that LXRα may modulate the Hh pathway in the process of LSECs capillarization.
LXR agonist inhibits LSECs capillarization via Hh signaling in vitro. To more directly test the hypothesis that we have proposed above, we isolated LSECs from WT mice and cultured with or without T0. We examined the beneficial effects that LXR agonist may have exerted on LSECs in vitro. Fenestration of LSECs was rapidly lost after 2 days of culture, but fenestration was maintained in the presence of 5 μM T0 on day 3 (p < 0.01; Fig. 5a,b). Accordingly, mRNA expression of ET-1, a capillarization-associated gene, was demonstrated in cultured LSECs. ET-1 mRNA levels decreased by approximately 25% and 27% after treatment with T0 compared with vehicle on day 1 and day 3 of culture, respectively (p < 0.05; Fig. 5c).

LSECs capillarization was inhibited by T0 after 2 days of culture, and this process was accompanied by inactivation of the Hh pathway. The mRNA expression levels of Shh ligand, the cell surface receptor for Hh ligand Patched1 (Ptch1) and the Hh target gene Gli2 were assessed in LSECs on day 1 or 3 of culture. T0 treatment inhibited the mRNA expression of Ptch1 and Gli2 on day 1 (Shh: p > 0.05; Ptch1: p < 0.01; Gli2: p < 0.05) and inhibited Shh, Ptch1 and Gli2 mRNA level on day 3 (Shh: p < 0.05; Ptch1: p < 0.05; Gli2: p < 0.01), indicating that LXR regulated the Hh pathway activity while capillarization progressed (Fig. 5d–f). The protein levels of Ptch1 and Gli2 were further confirmed by western blot. Decreased levels of Ptch1 and Gli2 on day 3 were induced by T0 treatment (Ptch1: p < 0.05; Gli2: p < 0.01), but there was no obvious difference on day 1 (Ptch1: p > 0.05; Gli2: p > 0.05; Fig. 5g–i).

To further confirm that the effects of T0 on LSECs capillarization were mediated by Hh signaling, primary LSECs were treated with T0 along with 300 nM Hh agonist SAG (a potent agonist of Hh signaling that activates the Smoothened (Smo) protein function)20. The effect of T0-induced down-regulation expression of LSECs capillarization markers (inducible nitric oxide synthase (iNOS), ET-1 and CD31) was blocked by SAG (Fig. 5j–l).

Therefore, these results show that the LXR plays an important role in the maintenance of LSECs fenestration by regulating Hh pathway activity.

LXR agonist attenuates CCl₄-induced liver injury, but induces the exacerbation of hepatic steatosis in vivo. To evaluate the possible protective effects afforded by a LXR agonist in CCl₄-induced liver injury, T0 pretreatment in vivo was employed. HE staining showed that the control DMSO and olive oil-treated mice had livers with a normal architecture, while the T0-treated group showed some lipid droplets in the liver. The livers of mice treated with CCl₄ showed necrotic areas that were characterized by severe hepatocytes necrosis and inflammatory cells infiltration. Although these findings could also be observed in the T0 and CCl₄-treated group, the incidence and severity of histopathological lesions were reduced compared with those in the CCl₄-treated group. However, lipid droplets were also observed in T0 and CCl₄-treated group (Fig. 6a).

In the livers of mice treated with CCl₄, the population of F4/80+ cells was greatly increased compared with that in the control DMSO and olive oil-treated mice, and T0-pretreatment decreased the number of F4/80+ cells after CCl₄ injection (p < 0.05; Fig. 6b,e). Furthermore, there was no significant difference in the number of F4/80+ cells in the liver between the control and T0-pretreated group (p > 0.05; Fig. 6b,e).

Collagen-I was not expressed in the control or T0-treated groups. Deposition of collagen-I could be observed in the livers of mice treated with CCl₄. By contrast, the expression of collagen-I was significantly decreased in the T0 and CCl₄-treated group (p < 0.05; Fig. 6c,f).
Figure 5. T0 inhibits LSECs capillarization via Hh signalling in vitro. (a) SEM of LSECs cultured with and without T0 for 2 days showed loss of fenestrae in sieve plates in vitro in the absence of T0 and maintenance of fenestrae with T0. Scale bar, 5µm. (b) Porosity of liver sections was quantified. Data are presented as mean ± SD (**p < 0.01, n = 3, Student’s t-test). (c) Quantitative Real-Time PCR analysis of ET-1 expression changes during culture-induced capillarisation with and without T0. Data are presented as mean ± SD (*p < 0.05, **p < 0.01, n = 3). (d–f) Quantitative Real-Time PCR analysis of the expression change of Shh ligand, the cell surface receptor for Hh ligand Ptc1 and Hh target gene Gli2. Data are presented as mean ± SD (*p < 0.05, **p < 0.01, n = 3, Student’s t-test). (g–i) Western blot and graphic analysis of the expression of Ptc1 and Gli2 in the cultured LSECs with
and without T0. The levels of the Ptc unh1 and BLBP protein are expressed relative to the levels of the β-actin. Data are presented as mean ± SD (**p < 0.01, n = 3, Student's t-test). Abbreviations: d, day; (j–l) LSECs were treated with T0 for 24 hours and then treated with either DMSO or SAG for 24 hours. Quantitative Real-Time PCR analysis of the gene expression changes of iNOS, ET-1 and CD31. Data are presented as mean ± SD (**p < 0.05, ***p < 0.001, n = 3, Student's t-test).

TEM of the sinusoidal areas of livers showed that in the control and T0-treated groups, sinusoids contained numerous fenestrae, with a discontinuous basement membrane, could be observed on the basal side of LSECs. In the CCl4-induced sinusoidal areas, deposition of extracellular matrix proteins in the Disse's spaces was found, with continuous basement membranes evident on the basal side of LSECs. However, the CCl4-induced changes in the sinusoidal areas could be ameliorated by T0 pretreatment (Fig. 6d).

Double immunostaining for Gli2 and CD31 (a marker of capillarised LSECs) was performed to assess the effects of T0 on the expression of the Hh target gene Gli2 in LSECs. In the mice treated with CCl4, consistent expression of Gli2 in capillarised LSECs was observed. T0 pretreatment could reduce the number of Gli2 and CD31 double-positive cells in the liver (p < 0.001; Fig. 6g,h).

Theses results indicate that activation of LXRα attenuate CCl4-induced liver inflammation, fibrosis and hepatic sinusoidal capillarization by inhibiting Hh signaling. However, LXR activation can induce hepatic lipogenesis.

Discussion

LXRα plays a crucial role in hepatic lipogenesis and steatosis. The mRNA and protein levels of LXRα as well as its downstream lipogenic genes are aberrantly increased in NAFLD, hepatic fibrosis and HCV patients15,16. Our results also confirmed that LXRα is related to hepatic fibrosis in an experimental animal model. LXRα activation can inhibit HSCs activation; whereas LXRα/β deletion enhances the activation of HSCs and exacerbates CCl4-induced liver fibrosis12. In addition, LXRα inhibit skin fibrosis by suppressing of macrophage infiltration and decreasing release of the pro-fibrotic cytokine IL-621. LXRα have been identified as a novel target for antifibrotic therapy. Although our findings support the antifibrotic role of LXRα, we may have established an indirect antifibrotic mechanism of LXRα that involves LSECs capillarization associated-fibrosis. In this study, we demonstrated that LXRα deletion aggregated liver injury, fibrogenesis and LSECs capillarization in vivo, while T0 treatment resulted in the inhibition of LSECs capillarization in vitro. We also found that LXRα regulated Hh signaling in the process of LSECs capillarization.

LSECs provide a porous barrier that facilitates access of hepatocytes to oxygen and small molecules in the microcirculation. LSECs clear colloids and macromolecules, promote HSCs quiescence, and induce immune tolerance. Both in vitro and in vivo studies have shown that LSECs maintain HSCs quiescence and induce the reversion of activated HSCs to quiescence9,10. When LSECs dedifferentiate to a defenestrated "capillarized" phenotype, HSCs become activated. LSECs capillarization precedes fibrosis in both human chronic liver diseases and experimental animal models. Moreover, reversal of capillarization promotes reversion of HSCs quiescence and amelioration of fibrosis2. We demonstrated that LXRα deletion aggravated fibrosis-associated sinusoidal capillarization in mice treated with CCl4, providing further evidence of a link between LSECs and the progression of liver fibrosis in vivo.

In this present study, hepatocyte proliferation was enhanced in LXRα knockout mice compared with WT mice after CCl4-induced liver injury, suggesting that LXRα is involved in hepatocyte proliferation. This was determined by the increased immunohistochemical staining of PCNA in hepatocytes. Consistent with previous studies, Zhang X. et al. found that cytosolic sulfotransferase 2B1b (SULT2B1b) and its product, 5-cholesten-3S-25-diol-3-sulfate (25HC3S) as LXRα antagonist, induced hepatocytes proliferation by inactivating oxysterol/LXR signaling22,23. Furthermore, activation of LXR in the regenerating liver in response to partial hepatectomy reduced hepatocyte proliferative capability24. Collectively, our data suggest that LXRα plays a physiological role in hepatocyte proliferation.

One effect of LXRα is to cause changes in mitochondrial morphology and the structure of hepatocytes. TEM examination revealed that LXRα deletion induced mitochondrial swelling and the disruption of normal cristae morphology. Moreover, in LXRα−/− mice, treatment with CCl4 aggravated mitochondrial injury. Consistent with this study, Qing et al. found that LXRα knockout mice exhibited exacerbated mitochondrial dysfunction25 and T0 attenuated high glucose-induced mitochondrial damage and apoptosis in cardiomyocytes26. Based on these studies, we may infer that the role of LXRα in mitochondrial morphology, structure and function is related to its effects on inflammation and metabolism, although this prediction need to be further confirmed in the future studies.

Hh signaling is crucial for repair of the adult liver27. In healthy adult livers, Hh ligands are scarce, but increase local production in liver injury. Hh signaling becomes greatly activated in injured livers, because several cell types, such as HSCs and various immune cells, produce and respond to Hh ligands28,29. In vitro study has confirmed that LSECs expressed and responded to Hh ligands and that capillarization development was regulated by Hh signaling17. Another study has confirmed the interaction between LXRs and Hh pathway in the differentiation of bone marrow stromal cells (MSCs). Activation of LXRs has been shown to suppress the expression of Hh-target genes, Ptc1, Gli1, and Gli-dependent transcriptional activity and regulated (MSCs) differentiation into osteoblasts19. Our present findings demonstrated that LXRα deletion increased the expression of Hh-target gene in vitro, while T0 inhibited upregulation of mRNA and protein levels of Hh signaling components in vitro. Furthermore, Hh agonist SAG blocked the inhibiting effect of LSECs capillarization induced by T0. Our results indicate that the molecular mechanisms underlying the LXR-Hh pathway show “cross talk” in LSECs capillarization.

LSECs capillarization not only precedes fibrosis, but also may be an integral part of the process of fibrosis. Restoring LSECs phenotype may be a beneficial approach to regress the development of fibrosis. Our findings indicate that LXRα regulates LSECs capillarization and reverses CCl4-induced fibrosis simultaneously and...
activation of LXRα maintains LSECs differentiated phenotype. We identified a new role for LXRα in LSECs capillarization. Considering the major side effect of LXR agonists, the translation our findings into clinical practice still has a long way to go.

Methods

Animals. Male LXRα−/− mice on a C57/BL6 background were provided by Jan-Ake Gustafsson. All animals were housed in the temperature-controlled room with a 12 hour light/dark cycle, pathogen-free conditions, and free access to water and standard chow. To induce chronic liver injury by carbon tetrachloride (CCl4) as described previously12, intraperitoneal injection of a 10% solution of CCl4 in sterile olive oil (0.5 μl pure CCl4/g body weight) was used biweekly for a month, with harvesting 72 hours after the last dose. Control groups received the same volume of vehicle. Pretreatment of T0 (dissolved in DMSO at 50 mg/ml) was intraperitoneal (i.p.) injected (50 mg/kg) daily for 7 days. Intraperitoneal injection of a 10% solution of CCl4 in sterile olive oil (0.5 μl pure CCl4/g body weight) was used biweekly for a month. The control groups received an equivalent dose of vehicle DMSO and/or olive oil. Mice were sacrificed 72 h after the last CCl4 or vehicle injection.

Figure 6. T0 attenuated CCl4-induced liver injury, but induced exacerbation of hepatic steatosis in vivo. (a) HE staining of livers in the groups: DMSO and olive oil; T0 and olive oil; DMSO and CCl4; T0 and CCl4. Scale bar, 50 μm. (b) F4/80 immunostaining of livers in the groups: DMSO and olive oil; T0 and olive oil; DMSO and CCl4; T0 and CCl4. Scale bar, 50 μm. (c) Collagen α1(I) immunohistochemical staining of livers in the groups: DMSO and olive oil; T0 and olive oil; DMSO and CCl4; T0 and CCl4. Scale bar, 1 μm. (d) TEM of hepatic sinusoids from the groups: DMSO and olive oil; T0 and olive oil; DMSO and CCl4; T0 and CCl4. White arrows indicate the change of basement membranes in hepatic sinusoids. Scale bar, 1 μm. (e) Statistical analysis of the number of F4/80 positive cells. Data are presented as mean±SD (*p < 0.05, n = 4, Student’s t-test). (f) Liver fibrosis score was quantified. Data are presented as mean±SD (*p < 0.05, n = 4, Student’s t-test). (g) Double immunostaining for the expression of the Hh-target gene, Gli2 (green), and the LSECs activation marker, CD31 (red). White arrows indicate the CD31/Gli2 double positive cells. Scale bar, 50 μm. (h) Statistical analysis of the number of Gli2/CD31 double-positive cells. Data are presented as mean±SD (**p < 0.001, n = 4, Student’s).
Ethical considerations. Animal maintenance and experimental procedures were performed in accordance with 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 the Third Military Medical University.

LSECs isolation and culture. LSECs were isolated from WT mice by collagenase digestion, immunomagnetic bead method as previously described with modifications. The mice were anesthetised by 10% chloral hydrate and the portal vein was exposed, cannulated with a 22-gauge intravenous catheter (BD Biosciences, Franklin Lakes, NJ, USA). The liver was pre-perfused via the portal vein with liver perfusion medium (Gibco-Invitrogen, Carlsbad, CA) for 10 min and then injected with 5 mg/ml collagenase IV (Sigma-Aldrich, St. Louis, MO) in buffer (pH 7.4) containing 135 mM NaCl, 5 mM KCl, 4.8 mM Glucose, 17.6 mM HEPES, 22 mM NaHCO3, 2 mM CaCl2, 2 mM MgCl2 (all chemicals obtained from Sigma-Aldrich, St. Louis, MO) for 10 min. After the two-step collagenase perfusion, the liver was disrupted and incubated in 5 mg/ml collagenase IV digestion for 15 minutes at 37°C. The undigested tissue was removed by 100-μm nylon mesh filter (Falcon; BD Biosciences). The filtered suspension was centrifuged (70 × g) for 1 min for three times to remove hepatocytes, and the supernatant was centrifuged (300 × g) for 10 min at 4°C. The pellet was resuspended in 90 μl of buffer prepared by MACS BSA stock solution (Miltenyi Biotec, Auburn, CA, USA) and autoMACS rinsing solution (Miltenyi Biotec, Auburn, CA, USA) and incubated with 10 μl anti-CD146 (LSEC) immunomagnetic beads (Miltenyi Biotec, Auburn, CA, USA) per 10^6 cells for 15 min at 4°C in the dark. The cells were washed and passed through a MACS column (Miltenyi Biotec, Auburn, CA, USA) according to manufacturers’ instructions. The freshly isolated cells were cultured on fibronectin (Sigma-Aldrich, St. Louis, MO)-coated culture flask in DMEM with 10% FBS, 100 IU/ml penicillin, 100 mg/ml streptomycin (Gibco-Invitrogen, Carlsbad, CA) and incubated in a CO2 incubator at 37°C. Cells were treated with vehicle (DMSO) or 1.5×10^6 cells/per mouse liver with a purity of approximately 92% fenestrated cells, as determined by SEM and fluorescence activated cell sorter (FACS) for CD31 and CD146 (Supplementary Figure S1).

Purity test by flow cytometry. The isolated LSECs were resuspended and modulated at a cell density of 5 × 10^6/ml. Followed by incubating with Fc block (anti-CD16/32, BD Biosciences), PE-conjugated anti-CD31 (clone:390) (Abcam, Cambridge, MA, USA) and FITC-conjugated anti-CD146 (clone:ME-9F1) (Miltenyi Biotec, Auburn, CA, USA) were added into 100 μl cell suspension. Then, the cell suspension was resuspended at 100 μl anti-CD146 (LSEC) immunomagnetic beads per 10^7 cells for 15 min at 4°C in the dark. After washed twice by PBS, fluorescence activated cell sorter (FACS) analysis was performed by BD FACS CanTo II and analyzed using FlowJo software (TreeStar).

Scanning electron microscopy and quantitative imaging. Fresh specimens were fixed in 2.5% glutaraldehyde, 0.1 M phosphate buffer (pH 7.4) for 1 h and post-fixed in 1% osmium tetroxide for 4 h. After dehydration with hexamethyldisilazane, sputter-coated with gold and examined using a SEM (HITACHHI, S-3400 N). The percentage of LSECs surface held by fenestrates which were counted manually by an investigator blinded to the identity of the slide using Zeiss Axiophot 3.0 system. Each average was taken from 10 images and three animals were tested at each group.

Transmission electron microscopy. Fresh specimens were fixed in 2.5% glutaraldehyde, 0.1 M phosphate buffer (pH 7.4) for 1 h and post-fixed in 1% osmium tetroxide for 4 h. After dehydration with a graded alcohol gradient, the samples were embedded in epoxy resin and cut into thin sections. Three samples per each group were examined with a TEM (FEI, TECNAI10) to observe changes in LSECs basement membranes and integrity of the space of Disse.

Immunohistochemistry and immunofluorescence. Livers were dissected and post-fixed in 4% paraformaldehyde overnight at 4°C. Tissues were embedded in paraffin and processed for sections (5μm). Hematoxylin and eosin staining was used to examine the histology of liver. Paraffin sections were deparaffinized in xylene, rehydrated through graded alcohol, and processed for antigen retrieval by boiling in 10 mM citrate buffer (pH 7.4) containing 135 mM NaCl, 5 mM KCl, 4.8 mM Glucose, 17.6 mM HEPES, 22 mM NaHCO3, 2 mM CaCl2, 2 mM MgCl2 (all chemicals obtained from Sigma-Aldrich, St. Louis, MO) for 10 min. After the two-step collagenase perfusion, the liver was disrupted and incubated in 5 mg/ml collagenase IV digestion for 15 minutes at 37°C. The undigested tissue was removed by 100-μm nylon mesh filter (Falcon; BD Biosciences). The filtered suspension was centrifuged (70 × g) for 1 min for three times to remove hepatocytes, and the supernatant was centrifuged (300 × g) for 10 min at 4°C. The pellet was resuspended in 90 μl of buffer prepared by MACS BSA stock solution (Miltenyi Biotec, Auburn, CA, USA) and autoMACS rinsing solution (Miltenyi Biotec, Auburn, CA, USA) and incubated with 10 μl anti-CD146 (LSEC) immunomagnetic beads (Miltenyi Biotec, Auburn, CA, USA) per 10^6 cells for 15 min at 4°C in the dark. The cells were washed and passed through a MACS column (Miltenyi Biotec, Auburn, CA, USA) according to manufacturers’ instructions. The freshly isolated cells were cultured on fibronectin (Sigma-Aldrich, St. Louis, MO)-coated culture flask in DMEM with 10% FBS, 100 IU/ml penicillin, 100 mg/ml streptomycin (Gibco-Invitrogen, Carlsbad, CA) and incubated in a CO2 incubator at 37°C. Cells were treated with vehicle (DMSO) or 1.5×10^6 cells/per mouse liver with a purity of approximately 92% fenestrated cells, as determined by SEM and fluorescence activated cell sorter (FACS) for CD31 and CD146 (Supplementary Figure S1).

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Membranes were blocked in 5% fat-free milk and primary antibodies were incubated overnight at 4 °C followed by acrylamide gel electrophoresis and electrically transferred onto a polyvinylidene difluoride transfer membrane. Western blot assay with BSA as standard. Protein was submitted to electrophoresis in a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and measured using the 2−ΔΔCt method. A p-value less than 0.05 were considered statistically significant.

Data analysis. Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The data were expressed as mean ± SD. Differences between groups were determined by two-tailed unpaired Student's t-test. A p-value less than 0.05 were considered statistically significant.

References
1. Racanelli, V. & Rehermann, B. The liver as an immunological organ. *Hepatology* **43**, S54–S62 (2006).
2. Wu, J. et al. Toll-like receptor-induced innate immune responses in non-parenchymal liver cells are cell type-specific. *Immunology* **129**, 363–374 (2009).
3. Schaffner, F. & Poper, H. Capillarization of hepatic sinusoids in man. *Gastroenterology* **44**, 239–242 (1963).
4. Mori, T. et al. Defenestration of the sinusoidal endothelial cell in a rat model of cirrhosis. *Hepatology* **17**, 891–897 (1993).
5. Xu, B. et al. Capillarization of hepatic sinusoid by liver endothelial cell-reactive autoantibodies in patients with cirrhosis and chronic hepatitis. *Am J Pathol* **163**, 1275–1289 (2003).
6. Martinez-Hernandez, A. & Martinez, J. The role of capillarization in hepatic failure: studies in carbon tetrachloride-induced cirrhosis. *Hepatology* **14**, 864–874 (1991).
7. DeLeve, L. D., Wang, X. D., Kanel, G. C., Atkinson, R. D. & McCuskey, R. S. Prevention of hepatic fibrosis in a murine model of metabolic syndrome with nonalcoholic steatohepatitis. *American Journal of Pathology* **173**, 993–1001 (2008).
8. Pasarin, M. et al. Sinusoidal endothelial dysfunction precedes inflammation and fibrosis in a model of NAFLD. *Plos One* **7**, e32785 (2012).
9. Xie, G. et al. Role of differentiation of sinusoidal endothelial cells in progression and regression of hepatic fibrosis in rats. *Gastroenterology* **142**, 918–927 e6 (2012).
10. DeLeve, L. D., Wang, X. D. & Guo, Y. M. Sinusoidal endothelial cells prevent rat stellate cell activation and promote reversion to quiescence. *Hepatology* **48**, 920–930 (2008).
11. Jakobsen, T., Treuter, E., Gustafsson, J. A. & Steffensen, K. R. Liver X receptor biology and pharmacology: new pathways, challenges and opportunities. *Trends in Pharmacological Sciences* **33**, 394–404 (2012).
12. Beaven, S. W. et al. Liver X Receptor Signaling Is a Determinant of Stellate Cell Activation and Susceptibility to Fibrotic Liver Disease. *Gastroenterology* **140**, 1052–1062 (2011).
13. Aubeuf, D. et al. Tissue distribution and quantification of the expression of mRNAs of peroxisome proliferator-activated receptors and liver X receptor-alpha in humans: no alteration in adipose tissue of obese and NIDDM patients. *Diabetes* **46**, 1319–1327 (1997).
14. Repa, J. J. & Mangelsdorf, D. J. The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. *Annu Rev Cell Dev Biol* **16**, 459–481 (2000).
15. Lima-Cabral, E. et al. Enhanced expression of pro-inflammatory mediators and liver X-receptor-regulated lipogenic genes in non-alcoholic fatty liver disease and hepatitis C. *Clinical Science* **120**, 239–250 (2011).
16. Ahih, S. B., Jang, K., Jun, D. W., Lee, B. H. & Shin, K. J. Expression of Liver X receptor correlates with intrahepatic inflammation and fibrosis in patients with nonalcoholic fatty liver disease. *Dig Dis Sci* **59**, 2975–2982 (2014).
17. Xie, G. H. et al. Hedgehog signalling regulates liver sinusoidal endothelial cell capillarisation. *Gut* **62**, 299–309 (2013).
18. Weidner, N., Semple, J. P., Welch, W. R. & Folkman, J. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *N Engl J Med* **324**, 1–8 (1991).
19. Kim, W. K. et al. Negative regulation of Hedgehog signaling by liver X receptors. *Mol Endocrinol* **23**, 1532–1543 (2009).
20. Bragina, O. et al. Smoothened agonist augments proliferation and survival of neural cells. *Neuroscience Letters* **482**, 81–85 (2010).
21. Beyer, C. et al. Activation of liver X receptors inhibits experimental fibrosis by interfering with interleukin-6 release from macrophages. Ann Rheum Dis 74, 1317–1324 (2015).
22. Zhang, X. et al. Cytosolic sulfotransferase 2B1b promotes hepatocyte proliferation gene expression in vivo and in vitro. Am J Physiol Gastrointest Liver Physiol 303, G344–G355 (2012).
23. Zhang, X. et al. Cholesterol metabolite, 5-cholesten-3beta-25-diol-3-sulfate, promotes hepatic proliferation in mice. J Steroid Biochem Mol Biol 132, 262–270 (2012).
24. Lo Sasso, G. et al. Down-regulation of the LXR transcriptome provides the requisite cholesterol levels to proliferating hepatocytes. Hepatology 51, 1334–1344 (2010).
25. He, Q. et al. Activation of liver-X-receptor alpha but not liver-X-receptor beta protects against myocardial ischemia/reperfusion injury. Circ Heart Fail 7, 1032–1041 (2014).
26. Cheng, Y. et al. Synthetic liver X receptor agonist T0901317 attenuates high glucose-induced oxidative stress, mitochondrial damage and apoptosis in cardiomyocytes. Acta Histochem 116, 214–221 (2013).
27. Ochoa, B. et al. Hedgehog signaling is critical for normal liver regeneration after partial hepatectomy in mice. Hepatology 51, 1712–1723 (2010).
28. Pereira Tde, A. et al. Viral factors induce Hedgehog pathway activation in humans with viral hepatitis, cirrhosis, and hepatocellular carcinoma. Lab Invest 90, 1690–1703 (2010).
29. Choi, S. S. et al. Hedgehog pathway activation and epithelial-to-mesenchymal transitions during myofibroblastic transformation of rat hepatic cells in culture and cirrhosis. Am J Physiol Gastrointest Liver Physiol 297, G1093–G1106 (2009).
30. Katz, S. C., Pillarisetty, V. G., Bleier, J. I., Shah, A. B. & DeMatteo, R. P. Liver sinusoidal endothelial cells are insufficient to activate T cells. J Immunol 173, 230–235 (2004).
31. Ishak, K. et al. Histological grading and staging of chronic hepatitis. J Hepatol 22, 696–699 (1995).
32. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408 (2001).

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Author Contributions
Y.X. and Y.Z.W. conceived and designed the experiments. Y.X., T.T.Z. and X.Y.G carried out the experiments. Y.X. analyzed the data. Y.Z.W. supervised the project. Y.X. wrote the manuscript. All authors reviewed the manuscript.

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