Hepatic stellate cells-specific Loxl1 deficiency abrogates hepatic inflammation, fibrosis, and corrects lipid metabolic abnormalities in non-obese NASH mice

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Research Article

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Abstract

Background & Aims: Lysyl oxidase-like-1 (LOXL1), a vital crosslinking enzyme in extracellular matrix (ECM) protein maintenance, is well established in fibrosis via mediating ECM stabilization. However, the potential role of LOXL1 in the pathogenesis of nonalcoholic steatohepatitis (NASH) has not been previously studied.

Methods: We generated $Loxl1^{fl/fl}$ mice to selectively delete Loxl1 in hepatic stellate cells (HSCs) ($Loxl1^{fl/fl}Gfap^{cre}$; $Loxl1^{fl/fl}$ as littermate controls) and then examined liver pathology and metabolic context in $Loxl1^{fl/fl}Gfap^{cre}$ fed a choline-deficient L-amino acid-defined (CDAA) diet or an isocaloric control diet for 16 weeks. We confirmed study findings in 23 patients with biopsy-proven NAFLD.

Results: LOXL1 was significantly increased in CDAA induced non-obese NASH compared with control diet. Here, utilizing a HSCs-specific deletion of Loxl1 model, we found that Loxl1 deficient in HSCs ameliorated CDAA-induced inflammation and fibrosis, with reduced expression of pro-inflammation and pro-fibrogenic genes. Interestingly, CDAA-fed Loxl1 deficient mice was associated with improved body weight and attenuated hepatic steatosis and to an up-regulation of leptin in adipose tissue and in serum, without changes in hepatic lipogenesis gene expression, compared with CDAA-fed control mice. Most importantly, analyses of serum LOXL1 and leptin levels from NAFLD patients revealed that LOXL1 was positively correlated with histological fibrosis progression, whereas was inversely correlated with leptin levels, especially in non-obese NAFLD patients.

Conclusion: In a mouse model of CDAA-induced non-obese NASH, selective deletion of Loxl1 from HSCs attenuated steatohepatitis, hepatic fibrosis and improved lipid metabolic abnormalities. Hence, LOXL1 inhibition may serve as a new therapeutic strategy for NASH.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease around the worldwide. The epidemiology and demographic characteristics of NAFLD vary worldwide, usually parallel to the prevalence of obesity, but a substantial proportion of patients are lean (1). NAFLD display a wide spectrum of pathologies ranging from steatosis, steatohepatitis (nonalcoholic steatohepatitis, NASH) and fibrosis to cirrhosis and/or hepatocellular carcinoma (HCC) (2). Among the various phenotypes of NAFLD, NASH is highly likely to progress to development of end-stage liver disease and cardio-metabolic disease, resulting in liver-related and non-liver-related mortality (3). Therefore, pharmacotherapy is typically not indicated for patients with simple steatosis but focused on histological improvement of NASH and fibrosis (4).

Significant steps in the progression from NASH to cirrhosis and liver failure involve extracellular matrix (ECM) remodeling and fibrosis, which is characterized by the abundant production of ECM proteins (consisting of collagens, elastin and other proteins) and incomplete fibrinolysis (5). Activated hepatic stellate cells (HSCs) and myofibroblasts are responsible for producing the majority of the ECM. A key step

in ECM deposition requires cross-linking of ECM to form an insoluble matrix, controlled by the copper dependent amine oxidase, lysyl oxidase (LOX) and lysyl oxidase like proteins (LOXL1-4), which determined mechanical properties of the ECM, thus making it less prone to degradation (6–8).

Although the LOX family members share high homology, each of the genes are located on a different chromosome associated with multifunction apart from organ fibrogenesis. Of note, among five LOX family members, LOX and LOXL1 mRNA expression are markedly up-regulated in fibrotic liver tissues across humans and mice (8). Thus, a marked increase in the liver expression of LOX and LOXL1 in fibrosis may significantly contribute to regulated fibrosis. Our prior study had shown that only Loxl1 increased expression is paralleled by increased type I procollagen and tropoelastin expression during later stages of liver fibrogenesis, and adeno-associated viral vector encoding Loxl1 shRNA prevented collagen and elastin deposition in CCl_4 induced-liver fibrosis (9). Deposition of ECM in liver is not only the mainly features of fibrosis, but also have powerful an impact on cell proliferation, differentiation, death, and lipid metabolism (10, 11). Recently, Romani *et al.* provides evidence that cell-matrix adhesions delivered mechanical signals linked with metabolic pathway regulating lipid synthesis and accumulation by shifting the homeostatic SREBP1/2 activity (12). In liver, Dongiovanni *et al*. showed that lipotoxicity in hepatocytes enhance LOXL2 production by HSCs thereby facilitating extracellular matrix stabilization (13), implicating a reciprocal crosstalk between ECM remodeling and lipid metabolism.

Considering the critical role of HSCs derived LOXL1 for ECM crosslinking which enables fibrosis development, and the relationship between ECM remodeling and lipid metabolism, we postulated that there could be a potential links between LOXL1 and progression of NAFLD. In this report, we generated HSC-specific Loxl1 knockout mice and determined the potential of Loxl1 deficient to ameliorate the progression of NASH in mice fed choline-deficient L-amino acid-defined (CDAA) diet. CDAA diet model in mice has been shown to mimic human NASH by sequentially producing steatohepatitis, liver fibrosis, and liver cancer without any body weight loss and thus was called the “non-obese NASH” model (14, 15). Further studies explored the potential mechanisms of LOXL1 regulation fibrosis and lipid metabolism. Finally, we assessed expression of LOXL1 in NAFLD patients to confirmed the experimental results.

Materials And Methods

Study design of animal experiment

This study was approved by the Institutional Animal Care and Usage Committee of the Beijing Friendship Hospital, Capital Medical University, Beijing, China. Male 6-8 weeks mice were subjected to 16 weeks of a choline deficiency amino acid (CDAA) diet (M10530i; Moldiets) or control diet (choline supplemented amino acid, CSAA) (M10530Ci; Moldiets).

The body weight of each mouse was measured weekly. At the end of the experiment, blood was collected by cardiac puncture, epididymal white adipose tissue as well as the liver were rapidly dissected out,

weighed, frozen in liquid nitrogen, and stored at -80°C until being analyzed. A portion of each liver was fixed in 4% formalin for histology.

Patient study

Human serum samples were obtained from biopsy-confirmed NAFLD in Beijing Friendship Hospital, Capital Medical University. The Ethics Committee of Beijing Friendship Hospital approved study (No.: 2018-P2-228-02). Fibrosis is staged 0 to 4 by liver biopsy using the METAVIR scoring system, F0 = no fibrosis, F1 = portal fibrosis, F2 = periportal fibrosis, F3 = bridging fibrosis, F4 = cirrhosis. The subjects were divided into two groups: mild fibrosis (F= 0-1, n = 12), and significant fibrosis (F ≥ 2-4, n = 11). The clinical characteristics (gender, age, BMI, AST, ALT, PLT, GGT, TG, TC, HDL-C, LDL-C, LSM, NAS) of NAFLD patients are provided in the Supporting Table S2.

Statistical analysis.

Two-group comparisons were performed using a two-tailed unpaired Student's t-test (data with normal distribution) and Mann-Whitney U test (data with non-normal distribution). Multiple groups were subjected to analysis of variance (ANOVA) with Bonferroni post-hoc test comparison. The correlations of serum LOXL1 with leptin were analyzed using Spearman rank correlation tests. P values < 0.05 were considered statistically significant.

Results

1. Selectively depletion of Loxl1 in HSCs attenuated the CDAA induced LOXL1 up-regulation in the whole liver

Based on our previously findings supporting LOXL1 expression in HSCs was strongly associated with liver fibrosis progression(9). To assess the crucial role of LOXL1 in HSCs during NAFLD development, we generated HSCs-specific Loxl1 deletion (Loxl1$^{fl/fl}$Gfap^{cre}) by crossing Loxl1$^{fl/fl}$ mice to mice bearing a Gfap-Cre transgene. Loxl1$^{fl/fl}$ mice were used as control in the following experiments.

Successful deletion of Loxl1 in Loxl1$^{fl/fl}$Gfap^{cre} mice was confirmed by qPCR analysis using isolated primary HSCs from the livers, accompanied by decreased Col1α1 and α-SMA expression (Fig. 1A). Six to Eight-week-old male Loxl1$^{fl/fl}$Gfap^{cre} mice and their littermate control mice (Loxl1$^{fl/fl}$) were then subjected to CSAA or CDAA diet for 16 weeks (Fig. 1B). As expected, CDAA-fed the control mice led to significant increased LOXL1expression both in mRNA and protein levels in the whole liver compared with CSAA-fed control mice. However, this increase was abrogated in CDAA-fed Loxl1$^{fl/fl}$Gfap^{cre} mice (Figure C&D). In order to confirm deletion of LOXL1 in activated HSCs, we performed co-localization immunofluorescence of LOXL1 with α-SMA in CDAA-fed Loxl1$^{fl/fl}$Gfap^{cre} mice and Loxl1$^{fl/fl}$ mice. We found that LOXL1 expression in activated HSCs was significantly diminished in Loxl1$^{fl/fl}$Gfap^{cre} mice; interestingly, we found that LOXL1 also positioned between the plates of hepatocytes (Fig. 1E).

Altogether, these data suggested that increased LOXL1 was associated with CDAA-induced NAFLD progression, and we generated HSCs-specific Loxl1 deficiency mice with highly efficient disruption of LOXL1 in HSCs.

2. Selectively depletion of Loxl1 in HSCs decreased steatosis, ballooning and liver injury

To confirm the function of Loxl1 in HSCs during NAFLD development, we first investigated hepatic histopathological features in Loxl1$^{fl/fl}$Gfap^{cre} and Loxl1$^{fl/fl}$ mice challenged with CSAA or CDAA diet. In accord with previous observations(14), we found that CSAA-fed mice displayed nonpathological liver histology regardless of the genotype, whereas CDAA-fed mice developed NASH with remarkable hepatic steatosis, inflammation, and ballooning (Fig. 2A). In addition, we found that Loxl1$^{fl/fl}$Gfap^{cre} mice developed histologic features of NASH with a significantly lower nonalcoholic fatty liver disease activity score (NAS) with respect to control mice, including less steatosis (2.60 ± 0.16 *vs.* 1.60 ± 0.22, $P < 0.05$), less inflammation (2.40 ± 0.16 *vs.* 1.60 ± 0.22, $P < 0.05$) and less hepatocyte ballooning (2.10 ± 0.23 *vs.* 1.40 ± 0.16, $P < 0.05$) (Fig. 2.B). The liver weight/body weight ratio or spleen/body weight ratio was significantly increased in CDAA-fed control mice compared with CSAA-fed control mice, and this increase was slight decreased in CDAA-fed Loxl1$^{fl/fl}$Gfap^{cre} mice ($P > 0.05$) (Fig. 2C).

Consistent with histological observations, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were elevated in the CDAA-fed as compared to CSAA-fed control mice, however Loxl1 deficiency in HSCs markedly attenuated CDAA induced-elevation of ALT and AST activities by ~ 40–55% ($p < 0.05$, Fig. 2C&D), indicating a protective role of Loxl1 deficient against CDAA-induced NASH.

3. Selectively depletion of Loxl1 in HSCs reduced fibrosis in CDAA-induced NASH

To examine the NAFLD progression, we analyzed hepatic fibrosis using histological staining. Sirius red staining showed that CDAA feeding for 16 weeks induced typical pericellular fibrosis. Quantification of hepatic collagen deposition, indicated by SR and Collagen I staining, was markedly increased in CDAA-fed the control mice compared with CSAA-fed the control mice, whereas this rise was abrogated in CDAA-fed Loxl1$^{fl/fl}$ Gfap^{cre} mice (Fig. 3. A-C). Consistent with histological evaluation, measurement of hydroxylproline level confirmed that liver fibrosis was less in CDAA-fed Loxl1$^{fl/fl}$ Gfap^{cre} mice than in control mice (Fig. 3D). Furthermore, this resolved fibrosis in CDAA-fed Loxl1$^{fl/fl}$ Gfap^{cre} mice was associated with decreased expression of fibrosis-related genes, such as α-SMA, Col1α1, Lox, Fbln5, Timp1, Mmp2, Mmp9, Mmp12, Mmp13, Tgfb1, Pai1, and Pdgfrb, as assessed by qPCR analysis, compared with CDAA-fed the control mice (Fig. 3E). Together, these findings suggested that CDAA-fed HSCs-specific Loxl1 deficiency mice exhibited less collagen deposition with decreased pro-fibrosis genes expression compared with CDAA-fed control mice.

4. Selectively depletion of Loxl1 in HSCs ameliorated liver inflammation and macrophage infiltration in CDAA-induced NASH

Next, we aimed to determine whether HSCs- specific Loxl1 deletion affected hepatic inflammation. We found that macrophage infiltration, indicated by CD68 staining, was much higher in the CDAA-fed control mice than that in the CSAA-fed the control mice, however this rise was abrogated in Loxl1$^{fl/fl}$Gfap^{cre} mice (Fig. 4A-B). Concordantly, this finding was supported by the decrease in hepatic mRNA expressions of the pro-inflammatory cytokines, such as Mcp1 and Tnfa expression in CDAA-fed Loxl1$^{fl/fl}$Gfap^{cre} mice compared with CDAA-fed control mice. A tendency towards a reduced expression was also observed for Il6, whereas no change was detected in Il10 mRNA (Fig. 4C). Collectively, these findings suggested that CDAA-fed HSCs-specific Loxl1 deficiency mice exhibited less macrophage infiltration with decreased pro-inflammation genes expression compared with CDAA-fed control mice

5. Selectively depletion of Loxl1 in HSCs reversed the metabolic abnormalities in CDAA-induced NASH

The above results suggested that deletion Loxl1 could reduce liver injury, inflammation and fibrosis in NASH. Furthermore, we aimed to detect whether Loxl1 deficiency in HSCs altered the metabolic phenotype or the steatosis in CDAA-fed mice.

We assessed the metabolic status of each group of mice. The initial body weight of all mice was comparable. Body weight during the whole study period was similar between CSAA-fed control mice and CSAA-fed Loxl1$^{fl/fl}$Gfap^{cre} mice, but was lower in CDAA-fed control mice in comparison with CSAA-fed mice. Notably, CDAA-fed Loxl1$^{fl/fl}$Gfap^{cre} mice reversed body weight loss to normal level (Fig. 5A). In line with body weight, epididymal fat to body weight ratio was higher approximately 20% in CDAA-fed Loxl1$^{fl/fl}$Gfap^{cre} compared with control mice (Fig. 5B).

Histologically hepatic steatosis, observed by Oil-Red O staining, was greater in CDAA-fed mice than CSAA-fed mice; in contrast, relatively low-level lipid deposition occurred in Loxl1$^{fl/fl}$Gfap^{cre} mice of CDAA feeding (Fig. 5C&D). This was also confirmed by biochemical analysis of hepatic triglycerides and NEFA (Fig. 5E), in contrast serum TG was increased in Loxl1$^{fl/fl}$Gfap^{cre} mice of CDAA feeding (Fig. 5F).

To evaluate the mechanisms by which selective Loxl1 deletion could influence the steatotic process, we detected several key lipogenic genes (ChREBP/Mlxipl, Srebp1c, Fasn, Scd1, Acc, Ppara). Consistent with a previous study, we also found that CDAA diet significantly down-regulated the expression of genes involved in fatty acid synthesis. These findings may be explained by compensatory hepatic uptake of serum lipids or by impairment in VLDL secretion from the live (16). Interestingly, a tendency towards a reduced expression was observed for these lipogenic markers between CSAA-fed Loxl1$^{fl/fl}$Gfap^{cre} mice and control mice, whereas no change was detected between CDAA-fed Loxl1$^{fl/fl}$Gfap^{cre} mice and control mice (Fig. 5G). Collectively, these findings suggested that selective deletion of Loxl1from HSCs reversed the metabolic disorder without effect of hepatic fatty acid synthesis.

6. Loxl1 deficiency in liver affected adipose tissue function in CDAA-induced NASH

Progressive adipose tissue dysfunction is key events in NASH development, and adipose transplantation could reverse the metabolic abnormalities associated with lipoatrophy(17), supporting the existence of an adipose tissue-liver crosstalk. Considerate Loxl1 deficient increase body weight and epididymal fat weight, we hypothesize that Loxl1 may affect the function of adipose tissue. To evaluate selective deletion of Loxl1 in liver crosstalk with adipose tissue, we performed the main adipikine (leptin, IL6, adiponectin) and the key lipogenic genes (Srebp1c, Mlxipl) in adipose tissue.

As expected, CDAA-fed the control mice caused an increase in Col1a1 in comparison with the control mice fed with CSAA diet. However, this indicator of ECM was lower in CDAA-fed $Loxl1^{fl/fl}Gfap^{cre}$ mice than CDAA-fed the control mice (Fig. 6A).

Consistent with Srebp1c expression in liver, adipose tissues expression of Srebp-1C was significantly increased in CDAA-fed $Loxl1^{fl/fl}Gfap^{cre}$ compared with CDAA-fed control mice, however no differences Mlxipl expression was observed ($P > 0.05$) (Fig. 6C). Interestingly, gene expression adipokines including leptin and IL6 was increased in CDAA-fed $Loxl1^{fl/fl}Gfap^{cre}$ mice compared with CDAA-fed the control mice, a tendency towards an increased expression was also observed for adiponectin (Fig. 6.B). Serum leptin levels confirm this funding. These data suggested that HSCs-specific Loxl1 depletion reversed the metabolic disorder by recovery adipose tissue function characterized by increased fat mass, enhanced adipikine (leptin, IL6) releasing, and adipose tissue remodeling (Fig. 6.D).

7. LOXL1 levels were negatively associated with leptin levels in non-obesity NASH patients.

To assess the clinical relevance of our findings, we examined LOXL1 and leptin levels in NAFLD patients with no or mild or significant fibrosis stage (Table S2). Consistent with the mice data, we observed that serum LOXL1 level was significant higher in patients with no or mild fibrosis than those with significant fibrosis in NAFLD patients (F0/1 *vs.* $F \geq 2$, 30.0 (21.9, 38.0) *vs.* 66.6 (31.0, 79.7) pg/ml, $P < 0.05$), a tendency of lower serum leptin levels in patients with no or mild fibrosis than those with significant fibrosis (F0/1 *vs.* $F \geq 2$: 197.5(130.0, 251.0) *vs.* 132(90.0, 218.0) ng/ml, $P > 0.05$). A small sample size which may lead to results that potentially failed to be significant. Correlation analyses showed significantly reverse association between serum LOXL1 and Leptin in NAFLD patients (Fig. 7C). Furthermore, we noted that the prevalence of histologically detected with significant fibrosis was significantly higher among patients with non-obese NALFD (BMI < 25 kg/m^2) (7/9, 77.8%) compared to those obese NAFLD (BMI ≥ 25 kg/m^2) (4/14; 8.6%; $p = 0.027$). Together, our results demonstrated LOXL1 was associated with fibrosis during NAFLD development, meanwhile there will be a potential connection between LOXL1 and leptin in NAFLD, especially in non-obese NAFLD (Fig. 7D).

Discussion

In this study, for the first time, we demonstrated that Loxl1 deficiency in hepatic stellate cells (HSCs) attenuated liver fibrosis, inflammation, meanwhile improved lipid metabolic abnormalities in CDAA fed

non-obesity NASH model, suggesting LOXL1 in HSCs play an important role in the pathogenesis of non-obese NAFLD.

LOXLl appears to be specifically required for cross-linking of soluble tropoelastin into insoluble elastin during formation, stabilization, maintenance, and remodeling(18), however, up- to-date knowledge of a cell specific function of LOXL1 in NAFLD is very limited. Given that activated HSCs and myofibroblasts are a major cellular source of LOXL1 in in fibrotic areas, therefore, we generated HSCs-specific Loxl1 depleting mice to investigate the role of LOXL1 in mice NASH model. In CDAA-induced NASH model, our first observations indicated that LOXL1 were strongly expressed in activation HSCs and also in endothelial cells positioned between the plates of hepatocytes, suggested that abnormal expression of LOXL1 was different from the CCl_4 induced fibrosis model. Many reports indicate that LOX or LOXL2 predominantly expressed in HSCs, but also expressed in injured hepatocytes or endothelial cells under pathological conditions (13, 19–21), especially under metabolic disorders condition. We speculated that different etiology of the liver diseases would display differences in the expression patterns of LOXL1. It will thus be of great interest to further explore the role of LOXL in endothelial-specific biologic functions during NAFLD.

Not surprisingly, due to LOXL1 is an extracellular enzyme responsible for cross-linking collagen and elastin molecules to form a stable extracellular matrix, we observed that selective HSCs loss of LOXL1 significantly reduced hepatic collagen deposition and attenuated liver fibrosis in a CDAA feeding NASH model. In a previous study of our group showed that LOXL1 significantly increased in cirrhosis stage compared with normal and fibrosis, LOXL1 inhibition could limit liver fibrosis progression and inhibit HSCs activation. Our results are consistent with previous reports in idiopathic pulmonary fibrosis, showing LOXL1 deficiency prevented fibrosis progression through reducing lung stiffness and inhibiting TGFβ1 activation, likely by limiting collagen crosslinking at a late stage (22). Indeed, growing evidence indicates that LOX/LOXL1/LOXL2 plays a crucial role in the maintenance of extracellular matrix stability and markedly related with fibrosis, LOX enzyme inhibition had a strikingly positive effect in vary experiment fibrosis mice model (8). However, a recent phase II clinical trial targeting the isoform LOXL2 with the humanized monoclonal antibody simtuzumab failed to show an effect on its primary endpoint, the improvement of progression-free survival (23–25). While unexpected and disappointing, it is unclear whether the antibody really reached the fibrotic compartment in the study population. Meanwhile, this negative result highlights the need for a deep understanding of the function of LOX family regulated ECM crosslinking. Under physiological conditions, formation of ECM crosslinking play an important role in maintaining tissue structure and regulating cellular function. For example, LOX knockout mice died perinatal due to aortic aneurysms, cardiovascular dysfunction and diaphragmatic ruptures (26, 27); LOXL1 deficiency induces a strong phenotype in mice with disturbance of elastic fiber (28–30); general LOX inhibitor usually decreases the amount of several collagen and elastin cross-links, however it is not always able to restore tissue architecture as shown in a hyperoxia-based mouse model of bronchopulmonary dysplasia associated with aberrant late lung development(31). Recently, using Loxl1 knockout mice, Li *et al*. found that living Loxl1 knockout mouse eyes, ocular compliance in Loxl1

knockout mice was lower than wild-type littermates (32). This was suggested that improper crosslinking of elastin and collagen was compensated by other mechanisms, for example, increased collagen content, or compensated increased the expression of other cross-linking enzymes to maintaining tissue structure. Therefore, it is necessary to evaluate the tissue structure, cellular function and the safety of long term use of LOX family members inhibitors.

Beyond its role in ECM maturation, our study provided some cues indicating that LOXL1 was involved in lipid metabolism. Indeed, LOX and LOXL2 exhibit a pathologically relevant role in the metabolic dysfunction. In high-fat diet model, LOX mRNA levels are increased in adipose tissue samples (27, 33). In the MCD model of NASH, LOXL2 expression was positively correlated with both steatosis and fibrosis (13). Besides the animal models, elevated expression and activity of LOX family members are also found in sera of patients with NAFLD (13, 34). Mechanically, ECM mechanical cues control lipid synthesis through direct regulation of SREBP1/2 activity (12, 35), and LOX family members are a major player in ECM maturation, which could be in part explain LOX family involved in regulation of lipid metabolism directly. In contrast, Martinez *et al*. reported LOX inhibitor (BAPN) reduced body weight gain and improved the metabolic profile in diet-induced obesity in rats (36). This inconsistency between results may be due to two animal models (CDAA and HFD) of fatty liver disease have different characteristics with varying degrees of inflammation, fibrosis, steatosis, weight gain, insulin resistance, and cell death (16, 37). Our data showed that depletion LOXL1 in HSCs protects against steatosis in liver, whereas lipogenesis related genes in liver were not changed significantly. Further we noted that depletion LOXL1 in liver improved whole body lipid metabolism abnormalities in CDAA-diet induced NASH model, including normalized the decrease in body weight, promoted hepatic TG transport from the liver (increase in serum TG levels), and increased fat mass weight. These results prompted us to study the role of selective LOXL1 deletion in liver in the control of adipose tissue function, given the crosstalk between liver and adipose tissue in the control of whole-body metabolism during the progression of NAFLD.

Interestingly, we found that Loxl1 deficient in liver could regulate the function of adipose tissue, including reduction of col1a1 in the adipose tissue, promoting expression of leptin and IL6. Although adipose tissue is currently considered to be a major contributor to fatty liver, inflammation, and fibrosis in the progression of NAFLD (38), inhibition of adipocyte cell death in Bid knockout mice protected against development of insulin resistance and hepatic steatosis associated with diet-induced obesity (39). Leptin is the main adipokine predominantly made by adipose tissue that involved in the regulation of hepatic lipid metabolism (40). Notably, replacement therapy with recombinant leptin potently reverses hepatic steatosis in lean rats, but not in diet-induced obesity rats(41), as well as transplantation adipose tissue treatment. Moreover, recently, Hackl *et al.* found CNS leptin signaling both promotes hepatic TG export and decreases de novo lipogenesis in the liver (42). Our study also demonstrated that depletion LOXL1 in liver promoted TG export and reduced hepatic steatosis, we believe that leptin, which is chiefly secreted in proportion to body fat stores, regulates hepatic lipid content and improves lipid metabolic abnormalities.

It is worthy of note that not only serum LOXL1 level was remarkably correlated with advanced fibrosis, but also inversely correlated with leptin production in NAFLD patients, which further confirmed the mice

results. More interestingly, the proportion of BMI less than 25 in patients with progression fibrosis was significantly higher than in patients without fibrosis, suggesting non-obese NASH patients have serious fibrosis than obese NASH. A recently published meta-analysis and study also confirmed that patients with lean-NASH had higher fibrosis (43). To our best knowledge, this is the first report studying the link between LOXL1 and leptin in NAFLD patients. This is surprising in light of the hypothesis of the lower leptin level and the increased tissue fibrosis found in non-obesity NAFLD. Paster *et al.* found that Lox activity in circulation could be correlated with BMI. Interestingly, when treating adipose tissue explants with leptin, they measured a significant decrease in Lox expression(44). The role of LOXL1 in the control of adipocyte function has been poorly characterized, further study should be clarified that the mechanisms of LOXL1 in liver how to modulate adipose tissue function.

Given the complexity of the pathophysiology of NASH, it will take the engagement of several targets and pathways to improve the results of pharmacological intervention. The treatment target should be individualized based on numerous factors, such as female, age, life expectancy, comorbid conditions, and risk of adverse consequences. Although the pathological lean-NASH is similar to obesity-NASH, the mechanism is unclear. According to our study, perhaps the treatment that inhibition LOXL1 does not apply to NASH patient who are associated with elevated leptin with obesity. Since we documented this only for one animal model, future studies are needed to evaluate whether this mechanism is also at work in other animals.

To conclude, in the current study we demonstrated that selectively depletion of Loxl1 in HSCs prevented CDAA diet-induced inflammation and fibrosis. In addition, LOXL1 deficiency in liver improved lipid metabolic abnormalities in CDAA non-obese NAFLD model. These effects were likely due to promote leptin secretion in adipose tissue to protect from ectopic lipid accumulation in the liver by stimulating TG secretion. Notably, LOXL1 levels were negatively associated with leptin levels in non-obesity NASH patients. Therefore, LOXL1 inhibition might be an interesting therapeutic target for drug development in NASH, which may provide new insights into clinical therapy for non-obesity related fibrosis and metabolic diseases.

Declarations

Author contributions

A.Y, and H.Y. conceived the study. A.Y, X.Y, X.F, Y,S, T.H, W.L, W.C, J.J, and H.Y performed experiments or analyzed the data. A.Y drafted the manuscript, and all authors read or revised the manuscript.

Conflicts of interest

The authors disclose no conflicts.

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Animal Research: This study was approved by the Institutional Animal Care and Usage Committee of the Beijing Friendship Hospital, Capital Medical University (No.:20-2002).

Consent to Participate: The study was conducted in accordance with the principles enshrined in the Declaration of Helsinki and the Good Clinical Practices. The Ethics Committee of Beijing Friendship Hospital approved study (No.: 2018-P2-228-02).

Consent to Publish: All authors had access to the study data and reviewed and approved the final manuscript.

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Figures

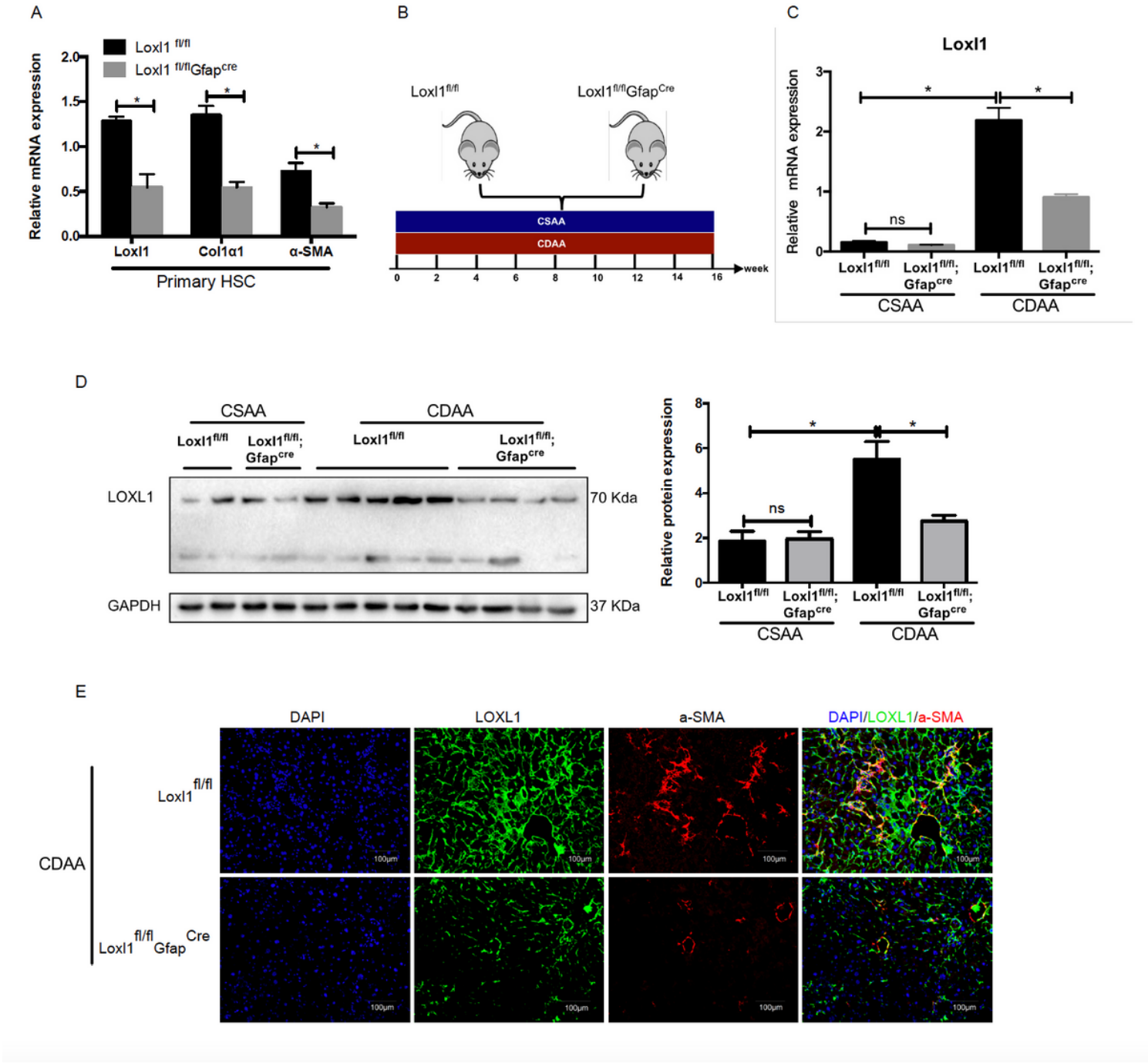

Figure 1

Efficient HSC-specific deletion of Loxl1 in Loxl1fl/flGfapcre mice. (A)The expression of Loxl1, Col1α1, and α-SMA in HSCs isolated from Loxl1fl/flGfapcre and their matched littermate control mice (Loxl1fl/f) were determined by qPCR analysis. (B) Schematic representation of experiment design. Loxl1fl/flGfapcre and their matched littermate control mice (n = 10 mice per subgroup) were fed a CSAA diet (n = 3 mice per subgroup) or CDAA diet (n = 10 mice per subgroup) for 16 weeks. (C) qPCR analysis of Loxl1 expression in the livers of Loxl1fl/flGfapcreand their control mice fed with a CSAA or CDAA. (D) Western blot analysis (left panel) and quantification (right panel) of LOXL1 expression in the livers of Loxl1fl/flGfapcre and their control mice fed with CSAA or CDAA for 16 weeks. (D) Immunofluorescence detection of LOXL1 and

a-SMA. Data in (A, C, D) are presented as the mean ± SEM. Statistical analysis was performed by unpaired two-tailed Student's t test and one-way ANOVA followed by Tukey post hoc test (*p < 0.05).

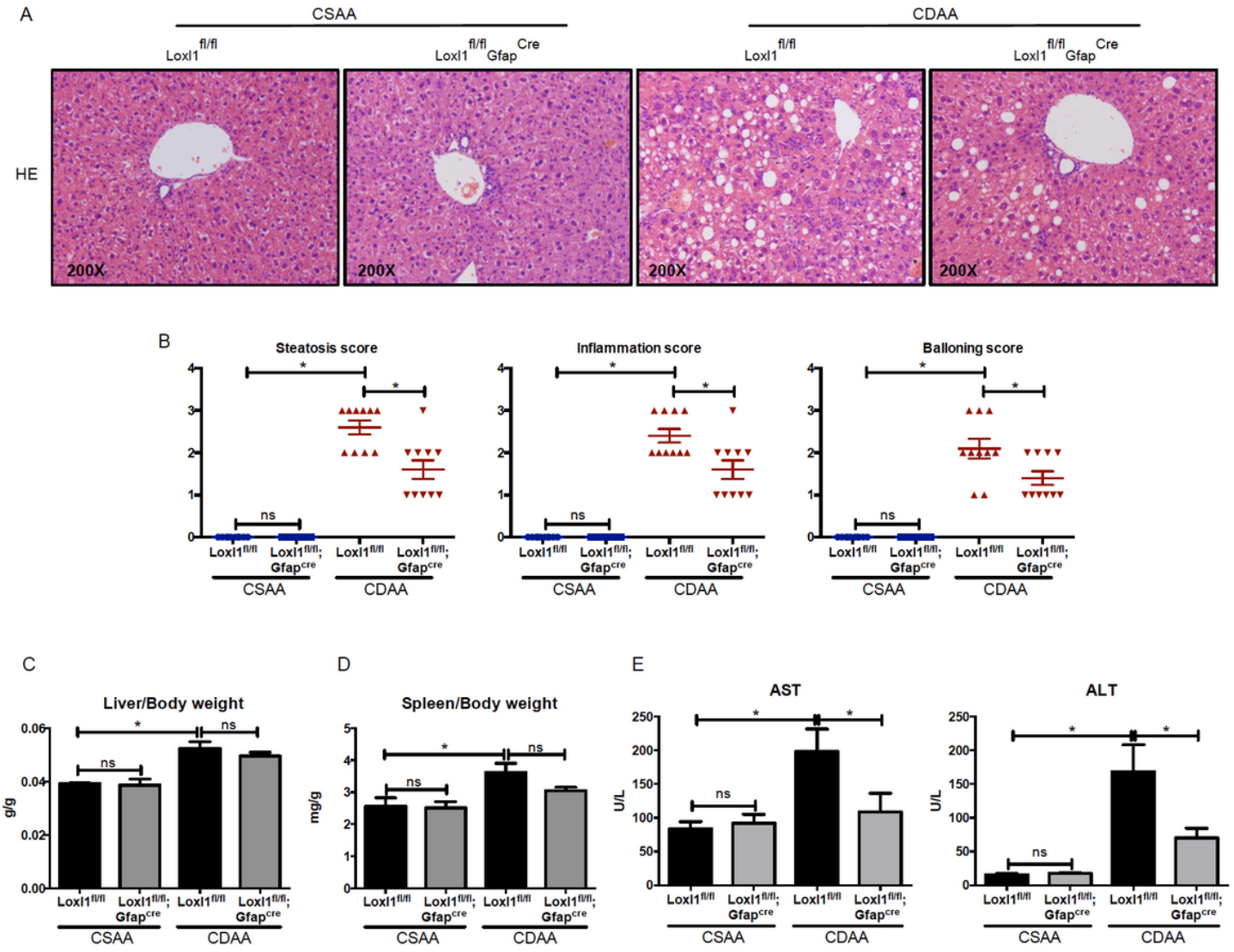

Figure 2

Characterization of HSCs -specific Loxl1 deletion mice fed with a CSAA or CDAA diet. (A) HE staining of representative liver sections in Loxl1fl/flGfapcre mice and their control mice after 16 weeks CSAA (n = 3 mice per genotype) or CDAA diet (n = 10 mice per genotype). (B) NAFLD activity score (NAS) of the four subgroups. (C) Liver/body weight ratios of the four subgroups. (D) Spleen/body weight ratios of the four subgroups at the end of CSAA (n = 3 mice per genotype) or CDAA feeding (n = 10 mice per genotype). (E-F) Serum AST (left panel) and ALT (right panel) levels of the four subgroups. Data in (B, C, D) are presented as the mean ± SEM. Statistical analysis was performed by unpaired two-tailed Student's t test and one-way ANOVA followed by Tukey post hoc test (*p < 0.05).

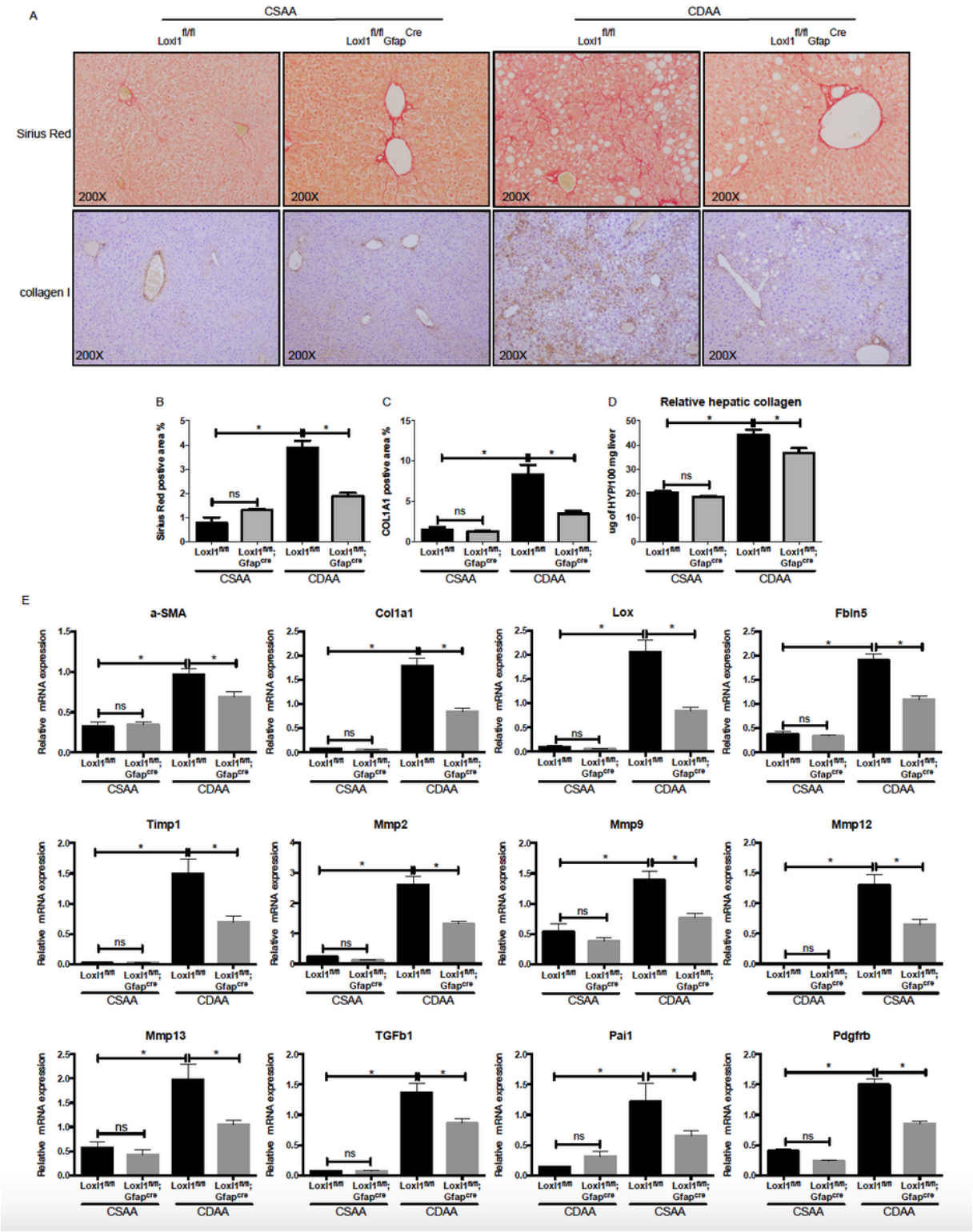

Figure 3

HSCs-specific Loxl1 deletion attenuated CDAA diet-induced liver fibrosis in murine NASH. (A) Sirius Red staining (up panel) and Collagen I IHC staining (bottom panel) of liver sections in the livers of Loxl1fl/flGfapcre and their control mice fed with CSAA or CDAA diet for 16 weeks. (B) Quantification of SR the positive staining area in the livers of the four subgroups. (C) Quantification of Collagen I the positive staining area in the livers of the four subgroups. (D) Relative collagen accumulation was

assessed by the measurement of hepatic hydroxyproline (HYP) content in the liver from Loxl1fl/flGfapcre+ and their control mice fed with CSAA or CDAA diet for 16 weeks. (E) qPCR analysis of fibrosis-related genes in the livers of the four subgroups. Data in (B, C, D) are presented as the mean ± SEM. Statistical analysis was performed by unpaired two-tailed Student's t test and one-way ANOVA followed by Tukey post hoc test (*p < 0.05).

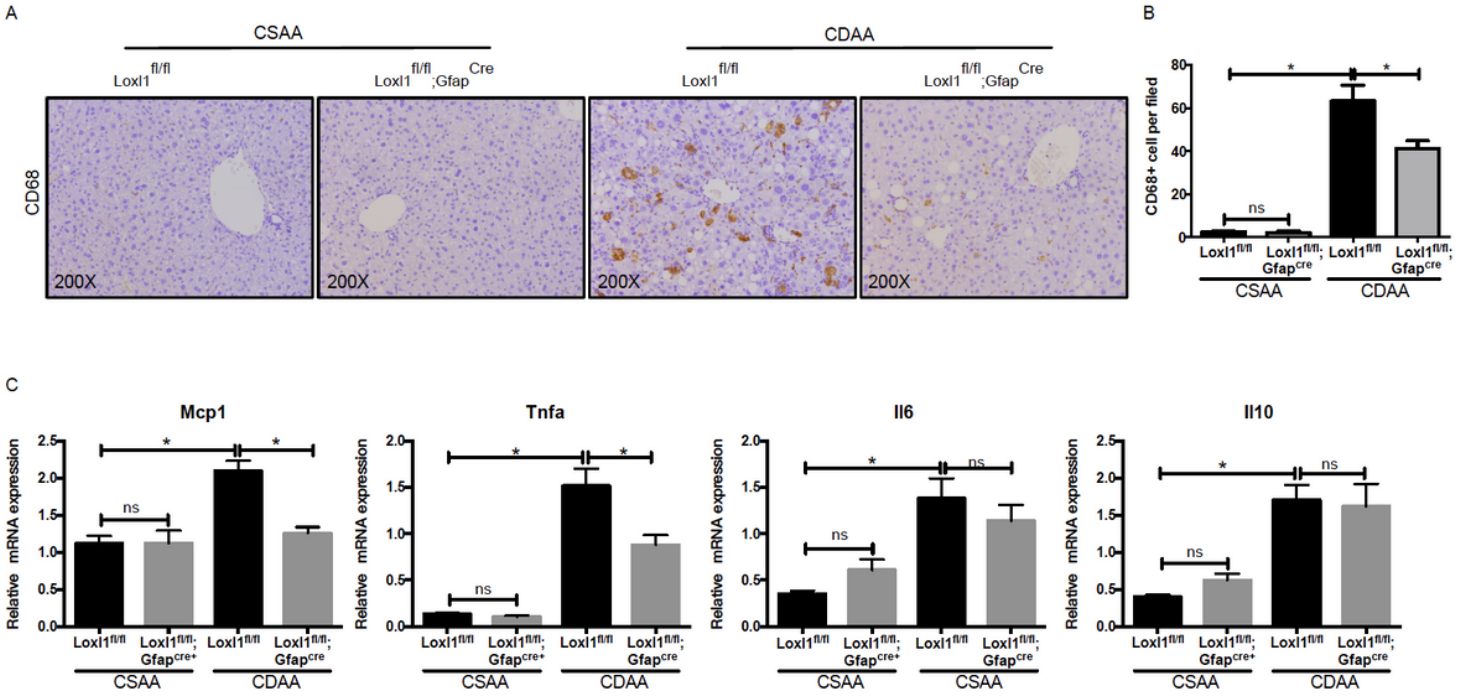

Figure 4

Hepatic stellate cell-specific Loxl1 deletion reduced CDAA diet-induced liver inflammation in murine NASH. (A) Representative CD68 immunohistochemical staining of liver sections in liver tissues of Loxl1fl/flGfapcre and control mice fed with CSAA or CDAA diet for 16 weeks. (B) The numbers of CD68-positive macrophage cells per high field were counted. (C)qPCR analysis of pro-inflammation related genes in the livers of Loxl1fl/flGfapcre and their control mice fed with CSAA or CDAA diet for 16 weeks. Data in (B, C) are presented as the mean ± SEM. Statistical analysis was performed by unpaired two-tailed Student's t test and one-way ANOVA followed by Tukey post hoc test (*p < 0.05).

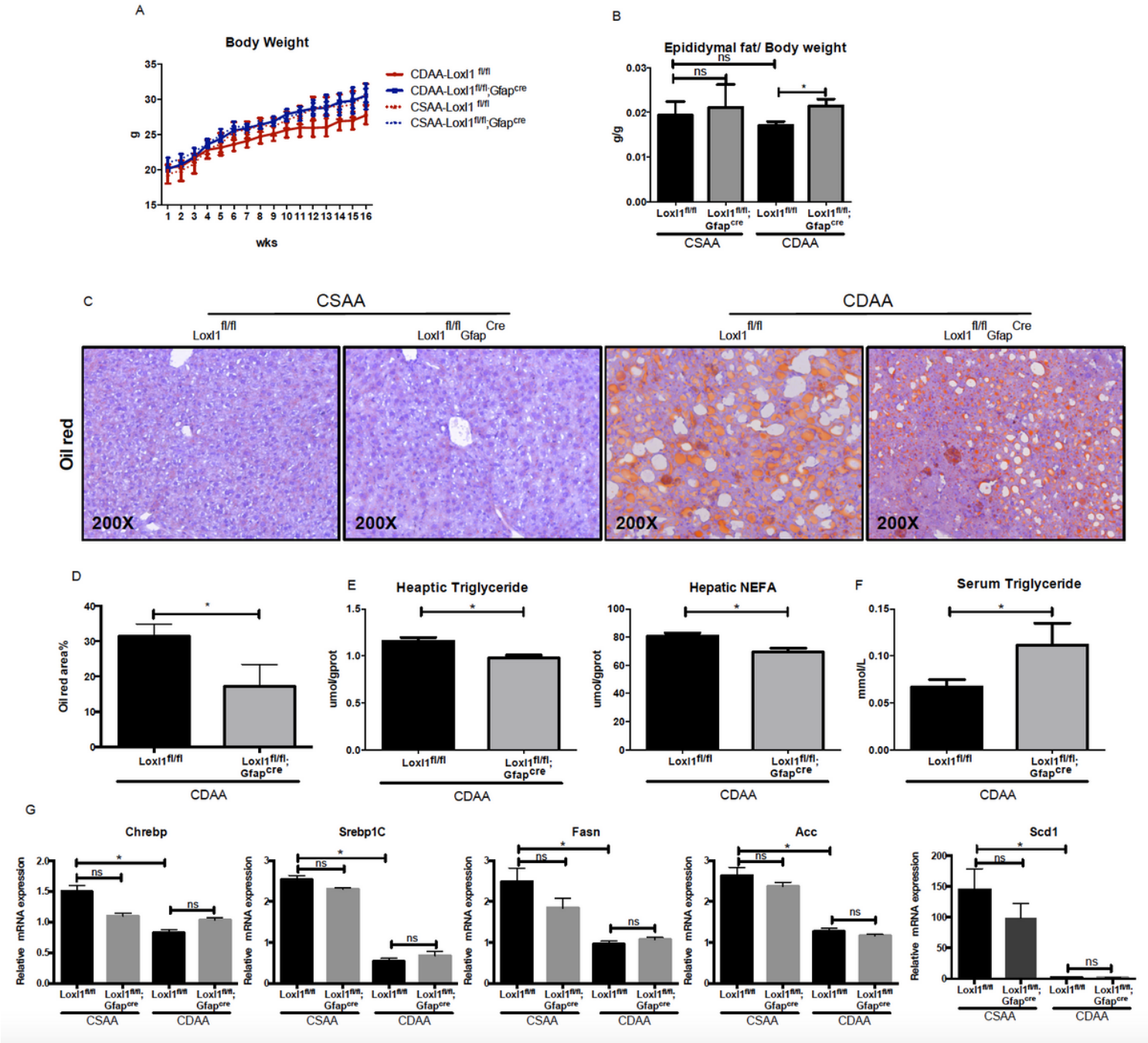

Figure 5

Hepatic stellate cell-specific Loxl1 deletion attenuated CDAA diet-induced liver steatosis in murine NASH. (A) Changes in body weight in Loxl1fl/flGfapcre mice and their control mice after 16 weeks CSAA (n = 3 mice per genotype) or CDAA diet (n = 10 mice per genotype). (B) Epididymal/body weight ratio at the end of CSAA or CDAA feeding. (C) Representative Oil Red O staining of liver sections and quantification of the positive staining area (D) in livers of Loxl1fl/flGfapcre and their control mice fed with CSAA or CDAA diet for 16 weeks. (E) Quantification of liver TG (right panel) and NEFA (left panel) levels in Loxl1fl/flGfapcre and their control mice fed with CDAA diet for 16 weeks. (F) Serum TG levels in Loxl1fl/flGfapcre and control mice fed with CDAA diet for 16 weeks. (G) qPCR analysis of lipogenesis related genes in the livers of Loxl1fl/flGfapcre and their control mice fed with CSAA or CDAA diet for 16 weeks. Data in (A, B, D, E, F,

G) are presented as the mean ± SEM. Statistical analysis was performed by unpaired two-tailed Student's t test and one-way ANOVA followed by Tukey post hoc test (*p < 0.05).

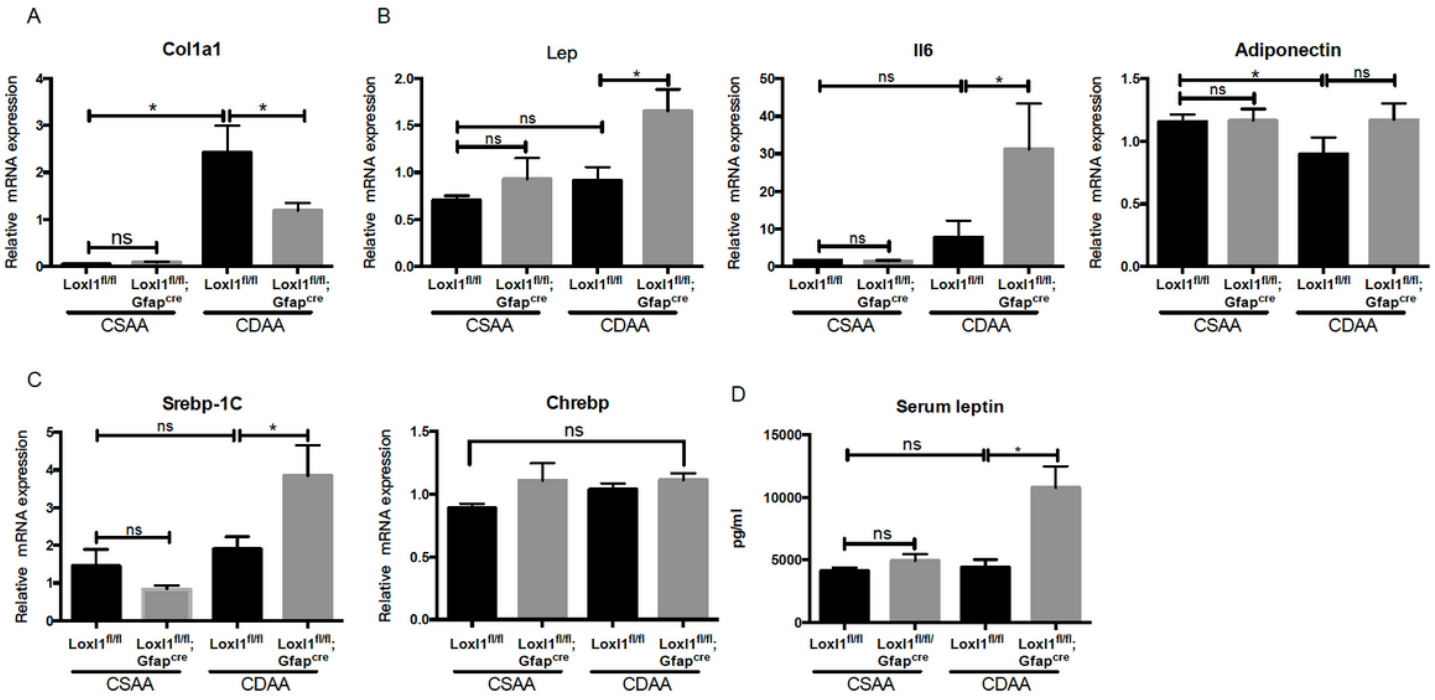

Figure 6

HSCs-specific Loxl1 deletion improved adipose tissue function in murine NASH. (A-C) qPCR analysis for Col1a1 (A), adipikine-related (B) and lipogenic genes (C) in adipose tissues from Loxl1fl/flGfapcre and their control mice fed with CSAA or CDAA diet for 16 weeks. (B) Elisa assay measurement of serum leptin levels in Loxl1fl/flGfapcre+ and their control mice fed with CSAA or CDAA diet for 16 weeks. Data in (A, B C) are presented as the mean ± SEM. Statistical analysis was performed by unpaired two-tailed Student's t test and one-way ANOVA followed by Tukey post hoc test (*p < 0.05).

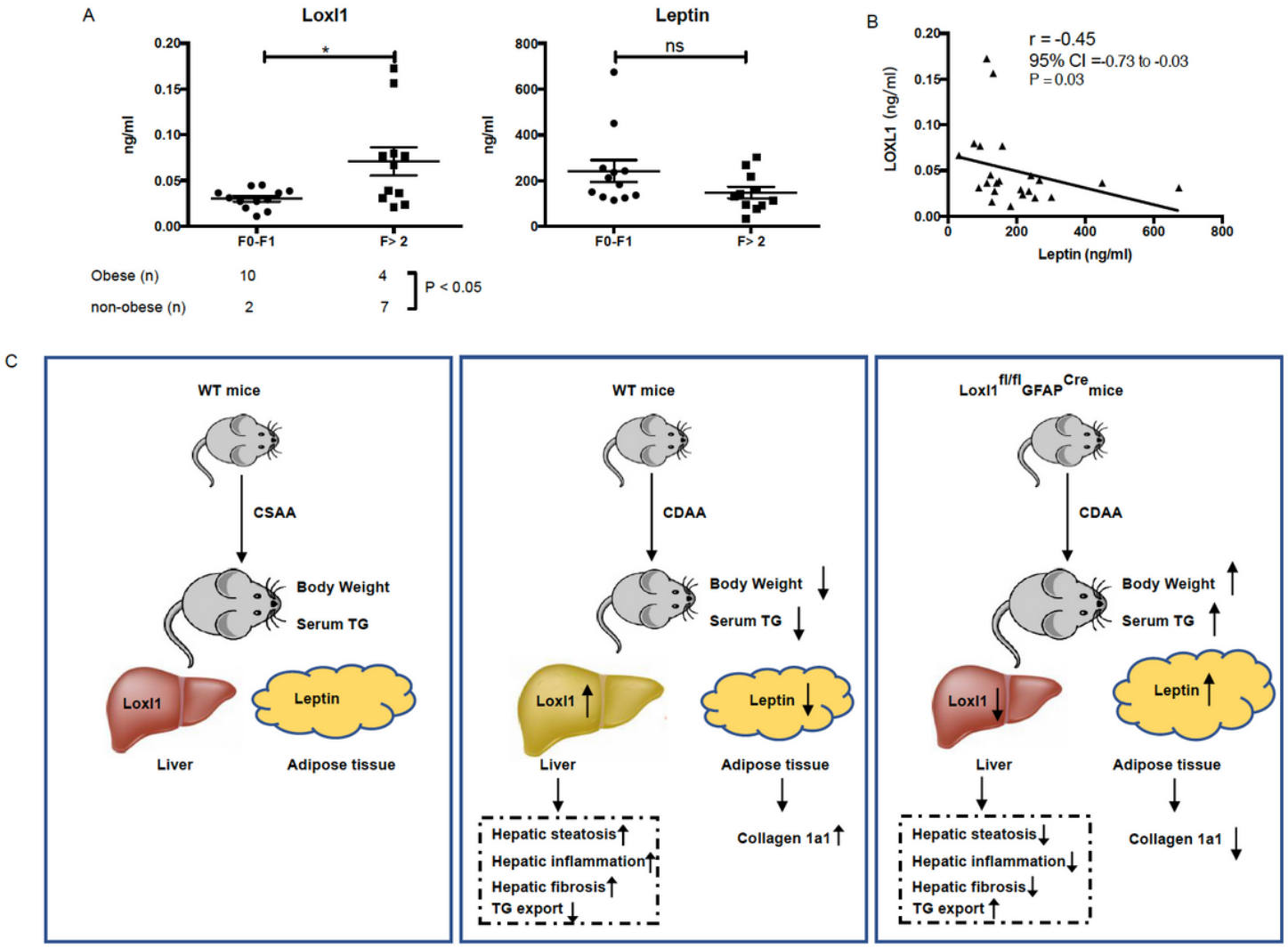

Figure 7

Inverse correlation between the serum LOXL1 levels and the serum leptin levels in NAFLD patients. (A) Levels of serum of LOXL1 between the different stages of fibrosis (F = 0/1 vs. F ≥ 2) in NAFLD patients. (B) Levels of serum of leptin between the different stages of fibrosis (F = 0/1 vs. F ≥ 2) in NAFLD patients. (C) Correlation of serum LOXL1 with serum leptin (Spearman rank correlation test). (D) Proposed mechanisms. Data in (A, B) are presented as the Median (25% Percentile, 75% Percentile). *p < 0.05.

Supplementary Files

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- SupplementaryMaterialandMethodsforhepatologyint.docx