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Research paper

Thrombospondin 1 improves hepatic steatosis in diet-induced insulin-resistant mice and is associated with hepatic fat content in humans

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ABSTRACT

Background: Nonalcoholic fatty liver disease (NAFLD) is associated with altered production of secreted proteins. Increased understanding of secreted proteins could lead to improved prediction and treatment of NAFLD. Here, we aimed to discover novel secreted proteins in humans that are associated with hepatic fat content using unbiased proteomic profiling strategy, and how the identified Thbs1 modulates lipid metabolism and hepatic steatosis.

Method: NAFLD patients were enrolled and treated with lifestyle intervention. Patients who underwent liver biopsy were enrolled for analyzing the correlation between circulating Thbs1 and liver steatosis. Mice were fed on high-fat, high-sucrose diet and treated with recombinant Thbs1. Primary hepatocytes isolated from CD36 knockout (CD36-/-) mice and their wild-type littermates (controls) were treated with glucose plus insulin for 24 h together with or without recombinant Thbs1.

Finding: Serum Thbs1 levels are increased in participants with NAFLD and positively associated with liver steatosis grades. Improvement of liver steatosis after lifestyle intervention was accompanied with significant reduction of serum Thbs1 levels. Pharmacological administration of recombinant human Thbs1 attenuates hepatic steatosis in diet-induced obese mice. Treatment with Thbs1 protein or stably overexpression of Thbs1 causes a significant reduction of lipid accumulation in primary hepatocytes or HepG2 cells exposed to high glucose plus insulin, suggesting that Thbs1 regulates lipid metabolism in a hepatocyte-autonomous manner. Mechanistically, Thbs1 inhibits cleavage and processing of SREBP-1, leading to a reduction of target lipogenic gene expression and hepatic steatosis. Inhibitory effects of Thbs1 on lipogenesis and triglyceride accumulation are abrogated in CD36 deficient primary hepatocytes exposed to high glucose plus insulin. Interestingly, beneficial effects of Thbs1 on lipid accumulation are observed in primary hepatocytes treated with a Thbs1 nonapeptide mimetic ABT-526.

Interpretation: Thbs1 is a biomarker for NAFLD in humans, and pharmacological and genetic approaches for the modulation of Thbs1 activity may have the therapeutic potential for treating hepatic steatosis.

Fund: A full list of funding bodies that contributed to this study can be found in the Funding Sources section.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by lipid accumulation in liver, which is called hepatic steatosis [1]. Hepatic steatosis is associated with insulin resistance, impaired glucose and lipid metabolism, or cardiovascular disease. About 15−30% of the adult population in Asia suffer from NAFLD [2]. The

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Research in Context Section

Evidence before this study

Reduction of hepatic lipid content via life-style intervention causes improvement in metabolic homeostasis, which is associated with altered production of secreted proteins in humans. Increased understanding of secreted proteins could lead to improved prediction and treatment of NAFLD.

Added value of this study

We found that serum Thbs1 levels are increased in humans of NAFLD, and decreased after liver-fat-lowering intervention. Pharmacologically administration of Thbs1 improves hepatic steatosis in diet-induced obese mice. Mechanistically, hepatic CD36 is necessary for Thbs1 protein or peptide mimetic ABT-526 to inhibit SREBP-1 cleavage and target lipogenic gene expression. Therefore, Thbs1 may serve as a novel biomarker for NAFLD in humans.

Implication of all the available evidence

Circulating Thbs1 should be measured in subjects with metabolic dysfunction. Significant elevation of Thbs1 may indicate increases hepatic fat content and the risk of development of NASH. Importantly, Thbs1 plays a critical role in the pathogenesis of lipid metabolism and NAFLD and could be a novel therapeutic target for the treatment of this disease.

underlying mechanisms of NAFLD development have attracted extensive attentions with great interest in the intracellular signaling pathways. However, the characterization of the extracellular matrix and its contribution to the pathogenesis of NAFLD are not well understood.

Thrombospondins are a family of extracellular matrix proteins. Thrombospondin 1 (Thbs1 or Tsp-1) was first found in platelets when platelets were stimulated with thrombin [3]. It is produced and secreted into the extracellular space in many cell types, such as endothelial cells, smooth muscle cells, adipocytes, fibroblasts, hepatic stellate cells and keratinocytes [4,5]. One of the ligands of Thbs1 is CD36 molecule [6]. The anti-angiogenesis efficacy of Thbs1 is mediated though CD36 [7], suggesting an important role of Thbs1 in regulating tumor growth and metastasis. Interestingly, Thbs1 is able to regulate cellular adhesion, angiogenesis, cell migration and platelet accumulation via binding other receptors or proteins such as CD47, transforming growth factor-β, and integrins [8,9]. Recent studies showed that the expression levels of Thbs1 are increased in adipose tissue of high fat diet-fed mice [10] and obese and insulin-resistant individuals [11]· [12], and the expression levels of Thbs1 are positively correlated with inflammatory maker monocyte chemoattractant protein-1 (MCP-1) in adipose tissue of mice. Accordingly, Thbs1 was characterized as an adipokine that is enriched in adipose tissue [13]. However, it is not known whether circulating Thbs1 is associated with NAFLD in humans, and its implication in regulating lipid metabolism in the liver.

In this study, we sought to identify novel secreted proteins using the serum of NAFLD patients, and further characterize the newly identified Thbs1 that may act as regulator or potential therapeutic target for treating NAFLD during nutrient excess conditions. These in vivo and in vitro findings indicate that 1) serum Thbs1 levels are increased in humans of hepatic steatosis, and decreased after liver-fat-lowering intervention; 2) pharmacologically administration of Thbs1 improves hepatic steatosis in diet-induced obese mice; 3) hepatic CD36 is necessary for Thbs1 protein or peptide mimetic ABT-526 to inhibit SREBP-1 cleavage and target lipogenic gene expression; 4) Thbs1 may serve as a novel biomarker for NAFLD in humans.

2. Materials and methods

2.1. Study design

This study analyzed two groups of population, including a group of NAFLD patients selected from a lifestyle intervention of a previous randomized controlled trial (RCT) [14] and a liver biopsy cohort from Zhongshan Hospital, Fudan University.

For NAFLD patients selected from RCT, study designs were described previously [14]. Briefly, NAFLD patients with a more than 13% hepatic fat content were enrolled in the randomized, parallel controlled, open-label clinical trial in three medical centers, in which patients were treated with lifestyle intervention, lifestyle intervention plus berberine or lifestyle intervention plus pioglitazone for 16 weeks (NIH Registration number: NCT00633282). Patients with the following conditions were excluded: severe metabolic abnormalities and organ dysfunction; excessive alcohol consumption (>140 g per week for men and >70 g per week for women); positive for hepatitis B, hepatitis C or had other liver diseases; or use of hypoglycaemic or lipid-lowering agents, hepatic protectants, or hepatotoxic agents within 4 weeks before enrolment. For lifestyle intervention, patients were required to take the calorie limited-diet (30% of energy from fat, 20% from protein and 50% from carbohydrates) by subtracting 500 kcal from the mean daily calorie intake and achieve more than 150 min per week medium intensity aerobic exercise [15]. The lifestyle-intervention patients who finished the follow-up were subjected to serum proteomic profiling and ELISA validation before and after the intervention.

For patients who underwent liver biopsy diagnosis, a total of 200 patients were initially enrolled from 2012 to 2018 in Zhongshan Hospital, Fudan University. Hepatic steatosis was evaluated by liver biopsy steatosis grade and ^{1}H-MRS scanning. Of those, 98 patients with the following conditions were excluded: advanced NASH or cirrhosis [16], known acute or chronic disease with the exception of obesity or T2DM, excessive alcohol consumption, or use of hepatic protectants or hepatotoxic agents in recent years. Of the 102 patients, 21 with normal-liver-fat, 7 with simple steatosis, and 74 with mild NASH [16], which were divided into 4 groups: grade 0 (<5% hepatocytes with steatosis), grade 1 (5–33% hepatocytes with steatosis), grade 2 (34–66% hepatocytes with steatosis), and grade 3 (>66% hepatocytes with steatosis) according to biopsy steatosis grade [17]. Normal-liver-fat specimens were obtained for exclusion of liver malignancy during surgery. All patients gave their written consent for their samples to be collected. The study was approved by the Ethics Committee of Zhongshan Hospital, Fudan University, and was conducted in accordance with the 1975 Declaration of Helsinki.

2.2. Measurement of human hepatic fat content via ^{1}H-MRS scanning

A 1.5T magnetic resonance (MR) scanner (Siemens Avanto, Erlangen, Germany) equipped for proton spectroscopy acquisitions was used for hepatic fat content detection. Sagittal, coronal, and axial slices covering the whole liver were acquired, and a single voxel of 8 cm^3 within the right lobe avoiding major vascular structures and subcutaneous fat tissues was chosen. The proton spectrum was calculated using the body coil after shimming over the volume of chosen voxel via a point-resolved spectroscopy sequence (PRESS) with the following parameters: repetition time=1500 milliseconds, echo time=135 milliseconds. There are two peaks on the proton spectrum curve: signal intensities of water peak (SW) at 4.8 ppm, and signal intensities of lipid peak (SL) at 1.4 ppm. Hepatic

fat content of chosen voxel was calculated with the formula 100% × SL/(SL+SW) [18,19].

2.3. Human serum proteomics

Whole blood of 5 chosen patients before and after intervention was collected by venipuncture. Blood was allowed to clot at room temperature in plain collection tubes for 30 min. Serum was collected after centrifugation at 2000 g for 10 min. An aliquot (100 μL) was removed for the proteomics. After pretreatment for the removal of lipids and high abundance proteins, serum was further processed for reduction with dithiothreitol (DTT), alkylation with Iodoacetic acid (IAA) and trypsin digestion. Finally, 2D-LTQ mass spectrum analysis was performed to identify proteins in serum [20].

2.4. Animal model and diets

Male C57BL/6 mice at 8 weeks of age were purchased from Shanghai Laboratory Animal Co. Ltd, China. Mice were fed on high-fat, high-sucrose diet (D12327, Research Diets, New Brunswick, NJ, USA) for 13 weeks, followed by treatment without or with recombinant human thrombospondin 1 protein (0.5 mg/kg/day, Novoprotein Scientific Inc., China) or vehicle (PBS) via subcutaneous injection every day for 10 days. CD36 knockout mice were purchased from Shanghai Model Organisms Center, Inc. While CD36 KO strains were first designed by Dr. Maria Febbraio at Weill Medical College (Cornell University) [21]. All mice were housed under a 12:12-h light/dark cycle at controlled temperature in the research animal facility at Shanghai Institutes for Biological Sciences. Mice were sacrificed under isoflurane anesthesia. Tissues were rapidly excised, freshly frozen in liquid nitrogen and stored at −80 °C, while some were fixed for histological analysis. All animal experimental protocols were approved by Institutional Animal Care and Use Committee at Shanghai Institute of Nutrition and Health, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

2.5. Liver histological analysis

Livers were fixed in 10% phosphate-buffered formalin acetate at 4 °C overnight and embedded in paraffin wax. Paraffin sections (5 μm) were cut and mounted on glass slides for hematoxylin and eosin (H&E) staining as previously described [22]. Livers embedded in optimum cutting temperature compound (Tissue-Tek, Laborimpex, Vorst, Belgium) were used for Oil Red O staining for the assessment of hepatic steatosis according to the manufacturer's instructions (American MasterTech, Lodi, CA, USA).

2.6. Generation of plasmids encoding Thbs1

The expression plasmids encoding Thbs1 were constructed by PCR-mediated amplification of the regions corresponding to amino acids of human Thbs1, followed by subcloning into a shuttle vector (pCDH-CMV).

2.7. Thbs1 peptide mimetic ABT-526

The Thbs1-drived peptide N-Ac-Sar-Gly-Val-D-Ile-Thr-Nva-Ile-Arg-Pro-NHEt (ABT-526) was synthesized by China Peptides (Shanghai).

2.8. In vitro lipid accumulation

Lipid accumulation model in hepatocytes was performed as described previously [23]. The Thbs1 or vehicle stably overexpressed HepG2 hepatocytes were starved in serum-free DMEM (with 1% penicillin/streptomycin) for 24 h and treated for an additional 24 h in DMEM containing 30 mM glucose and 100 nM insulin. Similarly, primary hepatocytes extracted from wild-type mice or CD36 knockout mice were starved in serum-free DMEM (with 1% penicillin/streptomycin) for 24 h and treated with Thbs1 or ABT-526 for additional 24 h in DMEM containing 30 mM glucose and 100 nM insulin. The accumulation of lipid droplets was visualized by Oil Red O staining or quantified by colorimetric enzymatic assay (Applygen Technologies Inc. Beijing, China).

2.9. Statistical analysis

Normally distributed data are expressed as mean±SD or SEM, whereas non-normally distributed data are expressed as median with interquartile range. For NAFLD patients from the RCT, serum Thbs1 levels and metabolic parameters before and after lifestyle intervention were compared using paired Student's *t*-test or Wilcoxon signed-rank test. For liver biopsy cohort, serum Thbs1 levels and metabolic parameters were compared with steatosis grade using one-way ANOVA model, except for the non-normally distributed variables, such as serum liver enzymes, which were compared using Kruskal-Wallis test. The correlation between serum Thbs1 and hepatic fat content was evaluated using Pearson correlation analysis. For animal and in vitro studies, statistical significance was evaluated using the unpaired Student's *t*-test and among more than two groups by analysis of one-way ANOVA. Differences were considered significant at a P value<0.05. Data were analyzed with GraphPad Prism software, version 8.0 (GraphPad Software Ins., La Jolla, CA, USA).

2.10. Data deposition and materials sharing

Serum proteomics data have been deposited in the National Omics Data Encyclopedia (http://www.biosino.org/node) under the accession number OEP000881.

3. Results

3.1. Serum Thbs1 in NAFLD patients is decreased following liver-fat-lowering intervention

To identify candidate regulators for NAFLD, 2D-LTQ mass spectrometry was performed to identify secreted proteins in serums of NAFLD patients who underwent lifestyle intervention to reduce liver fat content (NIH Registration number: NCT00633282) [14]. Of 53 patients who completed lifestyle intervention follow-up, 5 patients with significant reduction of hepatic fat content as measured by proton magnetic resonance spectroscopy (^{1}H-MRS) were chosen for serum proteomic analysis (Fig. S1). As shown in Fig. 1a and 1b, lifestyle intervention caused a significant reduction of hepatic fat content in these patients, which is consistent with previous observations ([15], [24]).

In serum proteomics, a total of 435 proteins were identified via mass spectrum in 5 pairs of blood samples from patients before- and after-lifestyle intervention (Fig. S2a). Spearman correlations between every two samples and principal component analysis (Fig. S2b and S2c) showed great sample consistency and significant group difference. Notably, as shown in Fig. 1c, 11 proteins were identified with profound changes after intervention, including immunoglobins, complements, as well as thrombospondin 1 (Thbs1). Furthermore, 35 pairs blood samples of patients who received lifestyle intervention were subjected to further verification of Thbs1's concentration. As shown in Fig. 1d and 1e, a significant reduction of circulating Thbs1 is further validated in these 35 patients. Strikingly, the reduction ratio of circulating Thbs1 is positively correlated with the reduction ratio of hepatic fat content (Fig. 1f). Additionally, partial improvement of

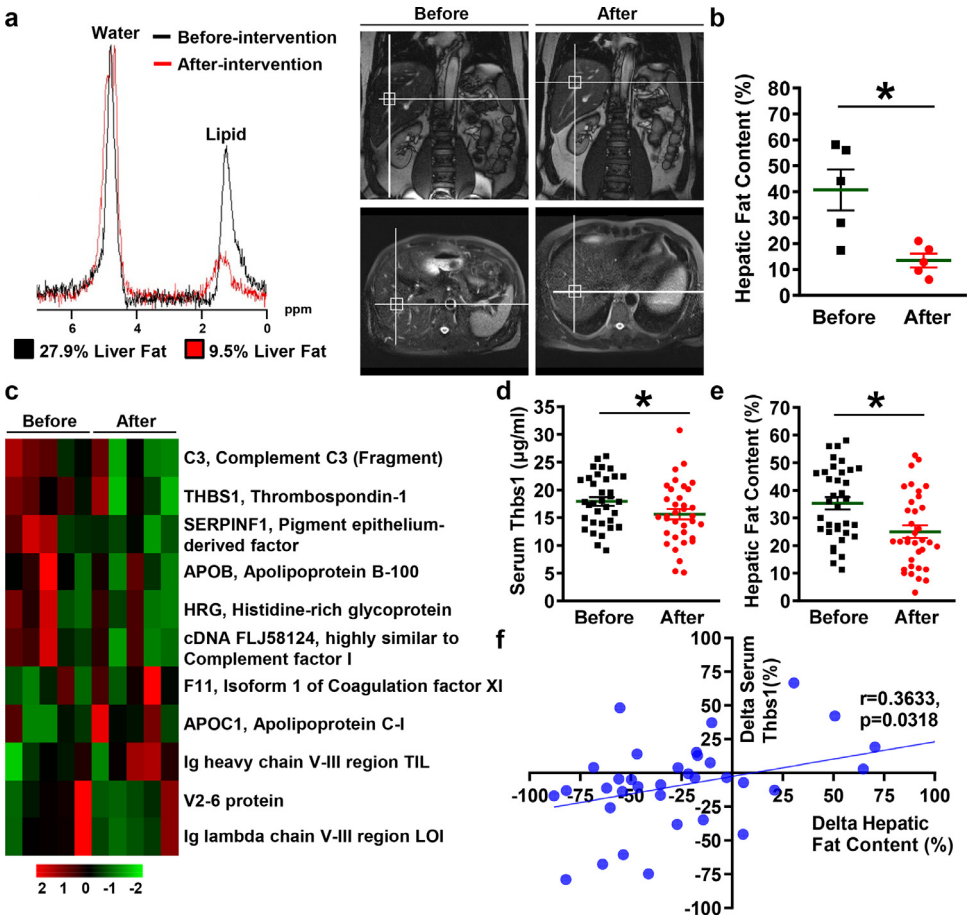

Fig. 1. Circulating levels of Thbs1 are reduced after liver-fat-lowering intervention in NAFLD patients. Total 184 NAFLD patients with a more than 13% hepatic fat content were officially enrolled in the randomized, parallel controlled, open-label clinical trial, in which 62 patients treated with lifestyle intervention were chosen for the study. Notably, total 53 patients of lifestyle intervention completed the follow-up, of which 5 patients with significant reduction of hepatic fat content were chosen for serum proteomic analysis. (a) Hepatic fat content was detected by a proton magnetic resonance spectroscopy (^{1}H-MRS) before and after intervention. Hepatic fat content=100% × area under the curve (AUC) of lipid peak/(AUC of water peak + AUC of lipid peak). (b) Hepatic fat content of 5 patients subjected to proteomic analysis is shown. (c) Clustering analysis of serum secreted proteins. Statistical significance was assessed by paired Student's *t*-test. $n = 5$, $^*p<0.05$, vs. before lifestyle intervention. (d–f) Validation of the reduction of Thbs1 levels is shown. Serum Thbs1 levels were measured in 35 randomly selected patients before and after lifestyle intervention by ELISA analysis. Serum Thbs1 levels (d) and hepatic fat content (e) are shown. (f) Thbs1 reduced ratio (Delta Serum Thbs1) is positively correlated with the difference of hepatic fat content after and before liver-fat-lowering treatment (Delta Hepatic Fat Contents) in patients. Statistical significance was assessed by paired Student's *t*-test. $n = 35$, $^*p<0.05$, vs. before lifestyle intervention.

glucose intolerance and obesity were observed in these patients (Fig. S3 and Table S1). Taken together, these results suggest that reduction of Thbs1 may serve as a proteomic biomarker for amelioration of NAFLD in humans.

3.2. Serum Thbs1 levels are positively correlated with hepatic steatosis

To further demonstrate the role of Thbs1 as a biomarker for hepatic steatosis, serum levels of Thbs1 were measured in another group of patients who underwent liver biopsy diagnosis. As shown in Fig. 2a and Table 1, total 102 subjects were divided into 4 groups according to biopsy steatosis grade: grade 0 (<5% hepatocytes with steatosis), grade 1 (5–33% hepatocytes with steatosis), grade 2 (34–66% hepatocytes with steatosis), and grade 3 (>66% hepatocytes with steatosis). Strikingly, serum levels of Thbs1 are positively correlated with increased steatosis grade (Fig. 2b). Furthermore, positive correlation between serum Thbs1 levels and hepatic fat content measured by 1H-MRS scanning was also observed (Fig. 2c). Notably, serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and triglyceride (TG) were significantly increased in patients of the higher steatosis grade (Fig. 2d and 2e). Together with the above observation in patients who received lifestyle intervention, these data strongly demonstrate that Thbs1 indeed serves as a biomarker of NAFLD in humans.

3.3. Inhibition of hepatic lipogenic gene expression and hepatic steatosis by administration of human recombinant Thbs1 in HFHS diet-fed mice

Given that circulating levels of Thbs1 is dynamically changed according to the hepatic fat content, we hypothesize that Thbs1 may regulate lipid metabolism in the liver. To test this hypothesis, administration of recombinant Thbs1 was performed in mice fed with a diet composed of high-fat, high-sucrose (HFHS) [25] as shown in Fig. 3a. Interestingly, treatment with 0.5 mg/kg/day recombinant Thbs1, a dose that was used in previous studies [26,27], or vehicle via subcutaneous injection for 10 days showed alleviated hepatic steatosis, which is evidenced by gross liver morphology, H&E and Oil Red O staining, indicating improved hepatic lipid metabolism (Fig. 3b-3c). Consistently, liver and plasma triglyceride levels decreased significantly in HFHS diet-fed mice after Thbs1 administration, suggesting that Thbs1 treatment has beneficial effects on lipid metabolism (Fig. 3d-3e). As shown in Fig. S4a-4c, plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were not obviously changed by administration of Thbs1 in mice fed with HFHS diet, a hepatic steatotic and insulin resistant diet [22,28]. Future

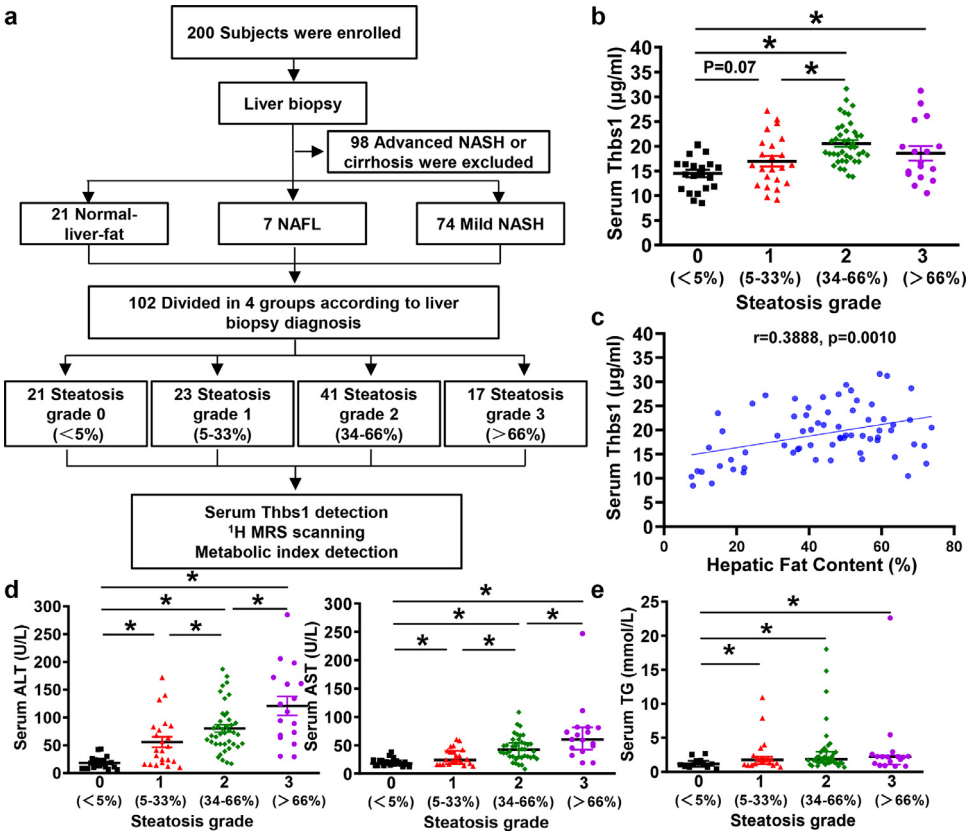

Fig. 2. Correlation between serum levels of Thbs1 and hepatic fat content in 102 patients with liver biopsy. A total of 200 patients were initially enrolled. Of those, 98 patients were excluded. Of the 102 patients included into analysis, 21 had normal-liver-fat, 7 had simple steatosis, and 74 had mild NASH, and they were divided into 4 groups: grade 0 (<5% hepatocytes with steatosis), grade 1 (5–33% hepatocytes with steatosis), grade 2 (34–66% hepatocytes with steatosis), and grade 3 (>66% hepatocytes with steatosis) according to steatosis grade in NAFLD activity score (NAS). (a) Enrollment and outcomes. (b) Correlation between serum Thbs1 and biopsy steatosis grade. (c) Correlation between serum Thbs1 levels and hepatic fat content measured by ^{1}H-MRS, $n = 68$. (d–e) Serum ALT and AST levels (d) and TG (e) levels are shown. The data are represented as scatter dot with mean±SEM or median with interquartile range. Statistical significance was assessed by one-way ANOVA or Kruskal–Wallis test. $n = 102$. *$p<0.05$.

studies are needed to investigate the effects of pharmacological Thbs1 on NASH phenotypes using NASH diets, such as high-fat, fructose and cholesterol (HFC) diet [29-31]. A mild reduction of liver weight to body weight ratio was observed in mice treated with Thbs1. Notably, compared with vehicle, no significant changes of food intake by Thbs1 administration were observed.

Next, the mechanisms underlying Thbs1-attenuated hepatic steatosis were investigated. As shown in Fig. 3f-3g, administration of Thbs1 caused a significant reduction of nuclear forms of SREBP-1 and its downstream lipogenic enzymes involved in fatty acid and triglyceride synthesis, including acyl-CoA desaturase 1 (SCD1) and fatty acid synthase (FAS). Real-time PCR was performed to measure the mRNA expression levels of key enzymes participating in de novo lipogenesis pathway. As shown in Fig. 3h, de novo lipogenic genes, such as SREBP-1c, ACC1, FAS, and SCD1 were downregulated in livers of HFHS diet-fed mice injected with Thbs1, indicating decreased lipid biosynthesis. Moreover, as shown in Fig. S4d-4e, no obvious changes of Thbs1 on VLDL secretion were observed as evidenced by the expression levels of VLDL assembly-related genes, such as microsomal TG transfer protein (MTTP) that is a rate limiting molecule in VLDL assembly and secretion; diacylglycerol acyltransferase 2 (DGAT2) that is involved in converting fatty acids into triglyceride; cell death-inducing like-effector type B (CIDEB) that is involved in lipidation of particles; and small GTP-binding protein a (SAR1a) that facilitates the movements of VLDL particles toward the Golgi apparatus [32-34].

As shown in Fig. S5a-5b, administration of Thbs1 attenuated glucose intolerance and insulin resistance in mice fed with HFHS diet, suggesting an improvement of glucose metabolism. Moreover, treatment with Thbs1 caused a mild reduction of plasma glucose and insulin levels, as well as calculated HOMA-IR (Fig. S5c-5e). Notably, a mild reduction of body weight, although not significant, may account for reduced lipogenesis and lipid accumulation in livers of HFHS diet-fed mice treated with Thbs1 (Fig. S5f). These results further support the salutary effects of Thbs1 activators on lipid metabolic disorders and insulin resistance. As shown in Fig. S6a-6b, administration of pharmacological Thbs1 causes reduction of HFHS diet-induced expression of endogenous levels of Thbs1 and CD36 in the liver of mice fed with HFHS diet, which is correlated with the improvement of hepatic steatosis. Together, these results suggest that administration of Thbs1 ameliorates HFHS diet-induced hepatic steatosis, possibly through inhibition of SREBP-1 cleavage and activation, and downregulation of hepatic de novo lipogenesis in mice.

3.4. Inhibition of hepatic steatosis by treatment with Thbs1 protein or peptide mimetics in hepatocytes exposed to high glucose plus insulin

To investigate whether Thbs1 has the ability to improve hepatic steatosis in vitro, Thbs1 stably overexpressed HepG2 cells were treated with high glucose plus insulin to induce intracellular lipid accumulation, which has been proved able to mimic hepatic steatosis [35]. Strikingly, consistent with in vivo results above, overexpression of Thbs1 suppressed high glucose plus insulin-induced lipid accumulation, as demonstrated by a reduction of lipid staining using Oil Red O staining (Fig. 4a-4b). Moreover, high glucose plus insulin-induced lipid accumulation in primary hepatocyte could also be suppressed by exogenous Thbs1 or its peptide mimetic ABT-526, demonstrated by a reduction of lipid staining via Oil Red O staining (Fig. 4a-4b) and

Table 1
Characteristics of 102 subjects with liver biopsy.

	Steatosis grade				*P* value
	0 (5%)	1 (5–33%)	2 (34–66%)	3 (>66%)	
Sex (M/F)	6/15	10/13	27/14	9/8	0.039
Age (year)	52.19 ± 15.96	50.91 ± 12.28	38.66 ± 13.02	32.88 ± 10.40	<0.001
Wight (kg)	557.95 ± 9.68	73.49 ± 15.96	80.62 ± 17.33	84.84 ± 18.94	<0.001
Waist (cm)	80.43 ± 7.35	93.35 ± 12.90	96.54 ± 10.32	99.00 ± 12.20	0.004
Hip (cm)	90.71 ± 6.78	99.82 ± 9.51	101.81 ± 9.31	102.55 ± 9.99	0.033
Waist to hip ratio	0.89 ± 0.05	0.93 ± 0.06	0.94 ± 0.06	0.96 ± 0.06	0.037
BMI (kg/m2)	22.45 ± 3.24	26.57 ± 4.50	27.93 ± 3.97	30.08 ± 4.91	<0.001
SBP (mmHg)	130.29 ± 17.46	126.91 ± 14.56	133.28 ± 14.54	131.53 ± 14.17	0.454
DBP (mmHg)	80.57 ± 6.98	78.65 ± 10.37	85.03 ± 9.54	86.00 ± 9.16	0.02
Hepatic fat content (%)	11.40 ± 3.98	29.85 ± 13.40	48.28 ± 11.87	60.39 ± 8.42	<0.001
Lipid profile					
TC (mmol/L)	4.50 ± 0.99	4.67 ± 1.09	4.93 ± 1.60	4.89 ± 1.22	0.742
TG (mmol/L)	0.75 (1.17–1.64)	1.76 (1.16–2.23)	1.85 (1.36–2.98)	2.19 (1.10–2.43)	0.039
HDL-c (mmol/L)	1.33 ± 0.35	0.98 ± 0.26	1.11 ± 0.53	1.15 ± 0.24	0.115
LDL-c (mmol/L)	2.58 ± 0.84	2.60 ± 0.78	2.66 ± 1.07	2.56 ± 1.04	0.981
APO-A (g/L)	1.52 ± 0.23	1.23 ± 0.21	1.22 ± 0.21	1.31 ± 0.25	<0.001
APO-B (g/L)	0.77 ± 0.23	0.90 ± 0.22	0.92 ± 0.27	0.96 ± 0.27	0.241
APO-E (mg/L)	49 (38.25–53.25)	47 (35–66)	50.5 (39.75–58.25)	47 (41.5–66.75)	0.789
Lpa (mg/L)	66.5 (28.5–249.35)	97 (49.5–378.5)	52 (37–166.5)	56 (29.75–172)	0.28
Liver enzyme					
ALT (U/L)	18.14 ± 10.71	55.74 ± 44.94	80.22 ± 42.70	120.53 ± 69.68	<0.001
AST (U/L)	18 (14.5–23)	24 (17–40)	42 (29.5–54)	60 (42–81.5)	<0.001
γ-GT (U/L)	18 (14.5–29.5)	42 (29–77)	58 (41–91)	56 (43.5–75.5)	<0.001
ALP (U/L)	63.38 ± 19.73	72.61 ± 25.85	77.27 ± 24.44	72.12 ± 23.36	0.197

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglycerides; HDL-c, high-density lipoprotein; LDL-c, low-density lipoprotein; APO-A, apolipoprotein-A; APO-B, apolipoprotein-B; APO-E, apolipoprotein-E; LPa, Lipoprotein a; ALT, alanine amino transaminase; AST, aspartate amino transaminase; γ-GT, γ-glutamyl transferase; ALP, alkaline phosphatase. Normally distributed data are presented as mean±SD, whereas non-normally distributed data as median with interquartile range.

decreased triglyceride levels measured by quantitative assay kit (Fig. 4c). Overexpression of Thbs1 is evidenced by immunoblots showing overexpressed FLAG-tagged Thbs1 in stable transfected HepG2 cells or transient transfected HEK293A cells (Fig. 4d-4e), and efficiency of FLAG-Thbs1 plasmids is confirmed by a Thbs1 ELISA kit which was used to detect Thbs1 levels in supernatant of FLAG-Thbs1 transiently transfected HEK293A cell line (Fig. 4f). Together, these observations indicate that Thbs1 is able to inhibit lipid accumulation in hepatocytes in vitro.

3.5. CD36 is required for the lipid lowering effects of Thbs1 in hepatocytes

To explore whether Thbs1's inhibition effect in de novo lipogenesis is mediated by CD36 receptor, primary hepatocytes were extracted from wild-type littermates or CD36 knockout mice (Fig. 5a) and then treated with glucose plus insulin for 24 h together with or without recombinant Thbs1 protein. Exogenous Thbs1 inhibits cleavage of SREBP-1 and protein levels of FAS and SCD1 in WT primary hepatocytes treated with high glucose plus insulin, but not in CD36 KO primary hepatocytes (Fig. 5b). Consistently, mRNA levels of lipogenic genes, such as FAS, SCD1, ACC1 and ACLY, were downregulated in WT but not CD36 KO primary hepatocytes treated with Thbs1 (Fig. 5c). Decreased triglycerides accumulation after Thbs1 treatment in WT hepatocytes or unchanged triglycerides accumulation after Thbs1 treatment in CD36 KO hepatocytes was reflected by a colorimetric enzymatic assay (Fig. 5d), suggesting that CD36 may mediate Thbs1's inhibition of lipogenesis. ABT-526 can also inhibit cleavage of SREBP-1 and protein levels of FAS and SCD1 in WT primary hepatocytes treated with high glucose plus insulin in a dose-dependent manner (Fig. 5e). Significantly decreased triglycerides accumulation after peptide mimetics ABT-526 treatment in WT hepatocytes or slightly decreased triglycerides accumulation after ABT-526 treatment in CD36 KO hepatocytes was reflected by a colorimetric enzymatic assay (Fig. 5f). These results reveal that the inhibitory effect of Thbs1 or its peptide mimetics on hepatic de novo lipogenesis is likely mediated by CD36.

4. Discussion

This study demonstrates that an extracellular protein Thbs1 may serve as a biomarker for hepatic steatosis in humans, and pharmacological administration of Thbs1 is sufficient to attenuate hepatic steatosis in HFHS diet-induced obese mice. Thbs1-CD36 axis-mediated inhibition of lipogenesis may represent a mechanism by which pharmacological and genetic activation of Thbs1 attenuate hepatic steatosis (Fig. 6). These findings support the potential clinical applications of Thbs1 and its peptide mimetics for treating NAFLD and related metabolic diseases.

4.1. Thbs1 serves as a biomarker for NAFLD in humans

Although thrombospondins belong to extracellular matrix proteins, which were previously believed to play structure roles, recent studies demonstrate that kinds of extracellular matrix proteins are not only existed in extracellular space, but also able to be secreted into circulation. One of the most important findings of this report is that Thbs1 serves as a biomarker for NAFLD in humans. First, unbiased proteomic profiling strategy reveals Thbs1 as a secreted protein that is dynamically changed in humans of NAFLD. Second, serum Thbs1 levels are increased in NAFLD humans, and are positively correlated with hepatic steatosis grade that is measured by liver biopsy diagnosis. Importantly, serum Thbs1 levels are reduced in NAFLD patients after lifestyle intervention to reduce liver fat. The findings that circulating levels of Thbs1 are positively correlated with hepatic fat contents in NAFLD patients are consistent with previous observations showing Thbs1 as a marker of obesity [12] as well as adipose inflammation [13]. Interestingly, consistent with simple steatosis and mild NASH, serum levels of Thbs1 are elevated in advanced NASH subjects compared with normal control, whereas no further elevation were observed (unpublished data). These results

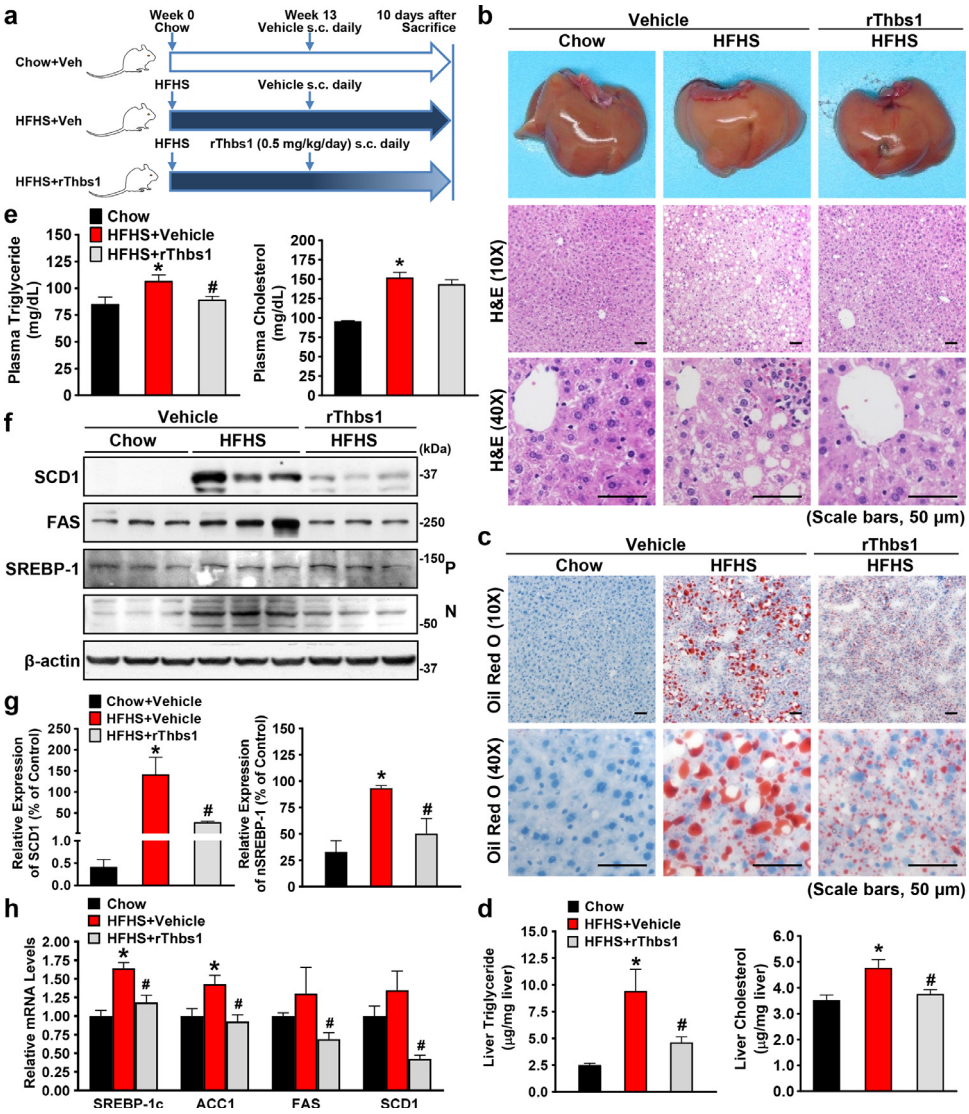

Fig. 3. Administration of human recombinant Thbs1 inhibits hepatic lipogenic gene expression and attenuates hepatic steatosis in HFHS diet-fed mice. Eight-week-old male C57BL/6 mice were fed on a chow diet for 13 weeks or a HFHS diet for 13 weeks, followed by treatment with 0.5 mg/kg/day recombinant human thrombospondin 1 (rThbs1) or vehicle (PBS) via subcutaneous injection for 10 days. (a) Schematic illustration showing the strategy for the in vivo experiment. (b–c) Representative gross morphology, H&E and Oil Red O staining are shown (scale bars: 50 μm). (d) Liver triglyceride and cholesterol levels were assessed. (e) Plasma triglyceride and cholesterol levels in mice. (f–g) Injection of Thbs1 inhibits cleavage of SREBP-1 and protein levels of SCD1 in livers of HFHS diet-fed mice. Representative immunoblots and densitometric quantification for expression of SREBP-1-N (N represents nucleus) and SCD1 in livers. Relative expression levels were normalized to β-actin. (h) Hepatic expression of lipogenic genes, including SREBP-1c, ACC1, FAS and SCD1, were determined by real-time PCR. The data are represented as the mean±SEM. Statistical significance was assessed by unpaired Student's *t*-test or one-way ANOVA. $n = 4–6$. $^*p<0.05$, vs. chow and vehicle; $\#p<0.05$, vs. HFHS and vehicle.

indicate that Thbs1 shows better performance as a biomarker for the early stage than the full spectrum of NAFLD. The effects of Thbs1 on advanced NASH which is characterized by pathological changes including inflammation, necrosis and fibrosis ([36], [37]) are currently under investigation. Given that Thbs1 is produced and secreted in many cell types, such as endothelial cells, smooth muscle cells, adipocytes, fibroblasts, hepatic stellate cells and keratinocytes ([4], [5]), the relative contribution of these cell types to the induction of circulating Thbs1 in NAFLD mice and humans requires further investigation. Taken together, these results demonstrate Thbs1 as a proteomic biomarker for NAFLD in humans, which may represent a noninvasive approach for the diagnosis of hepatic steatosis and quantification of hepatic fat content.

4.2. Administration of Thbs1 improves aberrant lipid metabolism under hepatic steatosis conditions

There are several lines of evidence support the salutary effects of Thbs1 on lowering lipogenesis and improving hepatic steatosis. First, administration of recombinant Thbs1 attenuates hepatic steatosis in diet-induced hepatic steatosis mice. Second, human HepG2 cells stably transfected with Thbs1 plasmid or treated with recombinant Thbs1 protein show reduced triglyceride levels. Third, given that high protein molecular weight and multiple structure domains restrict druggability of Thbs1[6], several small peptide mimetics of Thbs1 that are based on CD36 binding domain have been developed [38]. Strikingly, treatment with ABT-526, one of Thbs1 peptide mimetics that has been used in dogs and humans in phase 1 and 2 clinical trials [39], is sufficient to reduce triglycerides accumulation in hepatocytes. However, the expected beneficial effects of endogenous Thbs1 to lower hepatic steatosis is absent in NAFLD condition. It is likely that high endogenous or physiological levels of Thbs1 appear to be ineffective or insufficiency due to Thbs1 resistance, which may account for compensatory overproduction of Thbs1 in the circulation. The findings that administration of recombinant Thbs1 causes a reduction of hepatic steatosis and endogenous expression of Thbs1 in diet-induced insulin resistant mice further support the notion of compensatory overexpression of Thbs1.

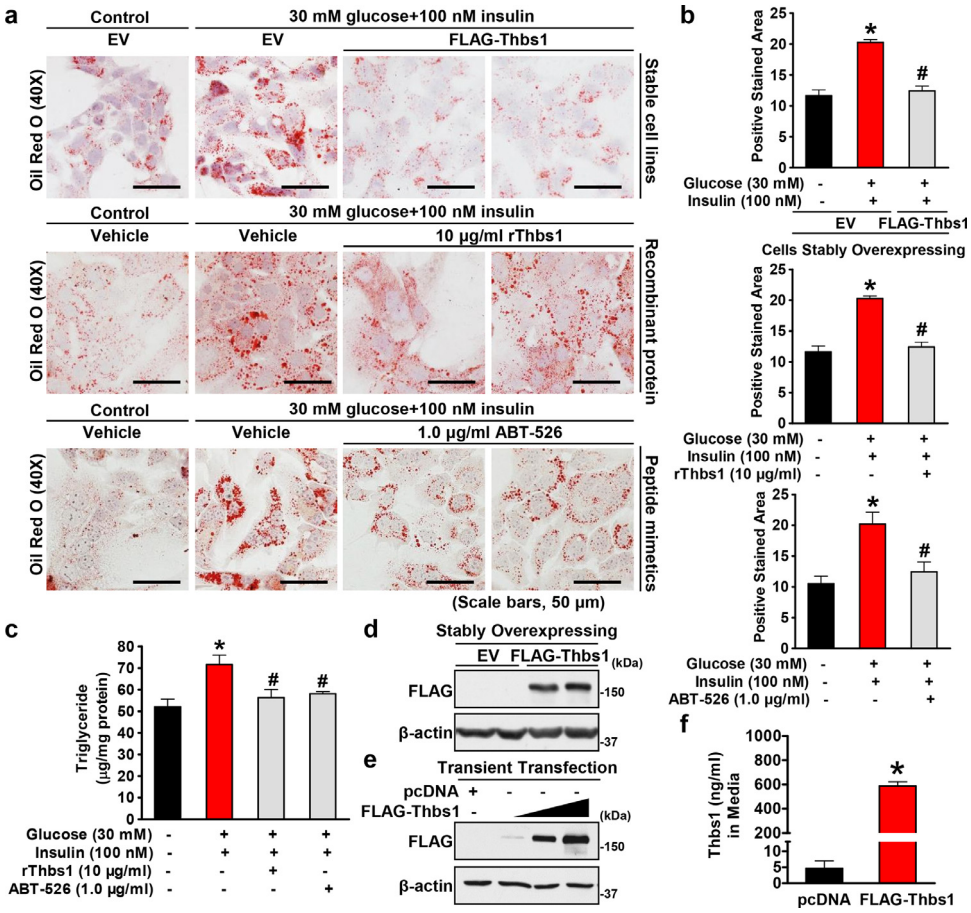

Fig. 4. Administration of Thbs1 protein or peptide mimetics inhibits hepatic steatosis in hepatocytes exposed to high glucose plus insulin. (a) HepG2 cells stably transfected with expressing plasmid encoding Thbs1 protein were fasted for 24 h, followed by treatment without or with 30 mM glucose plus 100 nM insulin for 24 h. And primary hepatocytes were also fasted for 24 h, followed by treatment without or with 30 mM glucose plus 100 nM insulin for 24 h, during which time, 10 μg/ml recombinant Thbs1 or 1.0 μg/ml peptide mimetics (ABT-526) were added. Decreased lipid accumulation was shown by Oil Red O staining in stably overexpressing Thbs1 HepG2 cells and recombinant Thbs1 protein or its peptide mimetic treated primary hepatocytes. (b) Representative Oil Red O staining quantification in each group is shown (scale bars: 50 μm). (c) Decreased triglycerides accumulation in primary hepatocyte was confirmed by a colorimetric enzymatic assay. (d–e) Immunoblots of FLAG-Thbs1 protein shows successful transient or stable transfect of Thbs1 overexpression plasmids in both HEK293A and HepG2 cells. (f) HEK293A cells were transfected with expressing plasmid encoding Thbs1 protein. Overexpression of Thbs1 protein in supernatant was detected by means of Thbs1 ELISA assay kit (*p<0.05, vs. cells transfected with pcDNA). The data are represented as the mean±SEM. Statistical significance was assessed by unpaired Student's t-test or one-way ANOVA. n = 3–5. *p<0.05, vs. vehicle control; #p<0.05, vs. high glucose plus insulin.

The data that pharmacological administration of Thbs1 attenuates hepatic steatosis in diet-induced insulin resistant mice are consistent with the observation that administration of recombinant Thbs1 or its analogue ameliorated lipid accumulation in endothelial and vascular smooth muscle cells [40], [41]. Although these data appear to contradict the previous studies showing improved weight gain and insulin resistance in Thbs1 whole-body knockout mice [10], [42], tissue-specific effects of systemic pharmacological Thbs1 in NAFLD mice and net effects of whole-body Thbs1 deficiency may account for this difference. The biology of Thbs1 and its mechanism of actions require further investigation using tissue-specific knockout mice of Thbs1 and its receptor. Taken together, this study characterizes Thbs1 as a biomarker for NAFLD in humans, and demonstrates salutary effects of pharmacological administration of Thbs1 on hepatic steatosis.

4.3. CD36 mediates effects of Thbs1 on inhibiting lipogenesis and attenuating hepatic steatosis

This report demonstrates that CD36 is necessary for Thbs1's effects on inhibiting lipogenesis and excessive triglyceride storage in the hepatocytes, suggesting that CD36 is required for the pharmacological actions of Thbs1 on inhibiting hepatic lipogenesis. Although these results appear to contradict the previous studies showing decreased expression of lipogenic genes in CD36 deficient mice [43], it is likely that CD36 signaling is differentially activated to either increase or decrease hepatic lipogenesis in response to differential cellular ligands or environmental stimuli. It is conceivable that the hepatic fat content is likely controlled by the precise pathophysiological context, which may be determined by net effects of CD36 on lipogenesis in hepatocytes. Future studies are needed to evaluate the relative contribution of CD36 to Thbs1's effects on inhibiting hepatic lipogenesis using liver-specific CD36 knockout mice. In addition to its action as a membrane protein for fatty acid translocase function [44], our study demonstrated that CD36 is required for regulating hepatic lipogenesis in response to Thbs1 stimulation. Further studies are required to investigate the mechanisms of CD36-mediated inhibition of cleavage and processing of SREBP-1, and the potential effects of Thbs1 on CD36-mediated lipid uptake in hepatocytes using fatty acid-induced hepatic steatotic model.

In conclusion, we have shown that pharmacological administration of Thbs1 is sufficient to mitigate excessive lipid deposition in the liver. Mechanistically, CD36 mediates Thbs1's salutary effects on inhibiting lipogenic gene expression and alleviating hepatic steatosis. These findings demonstrate that Thbs1 may be a novel biomarker for NAFLD in humans, and therapeutic approaches targeting Thbs1-CD36 axis such as Thbs1 recombinant protein or peptide mimetic may

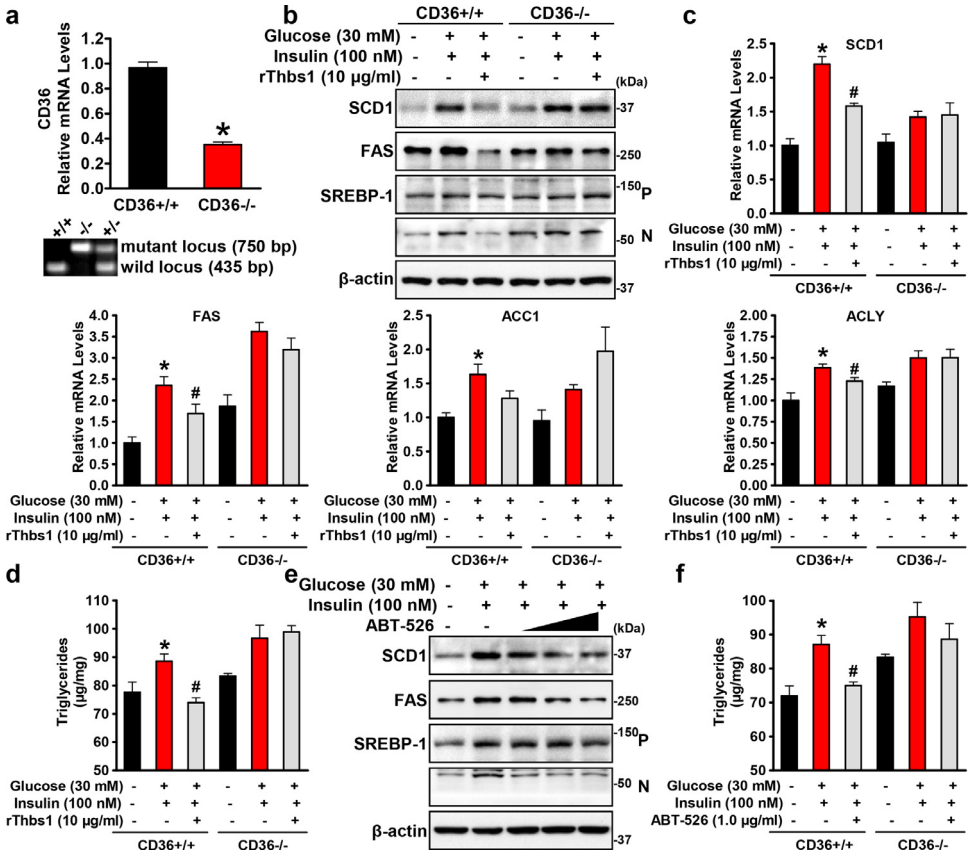

Fig. 5. The lipid lowering effects of Thbs1 are compromised by CD36 deficiency in hepatocytes. Primary hepatocytes from wild-type littermates or CD36 knockout mice were fasted and then treated with 30 mM glucose plus 100 nM insulin for 24 h with or without 10 μg/ml recombinant Thbs1 protein. (a) Expression of CD36 genes were determined by real-time PCR, and genotyping of wild-type littermates or CD36 knockout mice was made. (b) Exogenous Thbs1 inhibits cleavage of SREBP-1 and protein levels of FAS and SCD1 in WT primary hepatocytes treated with high glucose plus insulin, but not in CD36 KO primary hepatocytes. (c) Expression of lipogenic genes —- FAS, SCD1, ACC1 and ACLY were determined by real-time PCR in WT or CD36 KO primary hepatocytes treated with Thbs1. (d) Decreased triglycerides accumulation after Thbs1 treatment in WT hepatocytes or unchanged triglycerides accumulation after Thbs1 treatment in CD36 KO hepatocytes was reflected by a colorimetric enzymatic assay. (e) Peptide mimetics of Thbs1 inhibits cleavage of SREBP-1 and protein levels of FAS and SCD1 in WT primary hepatocytes treated with high glucose plus insulin in a dose-dependent manner. (f) Significant decreased triglycerides accumulation after peptide mimetics ABT-526 treatment in WT hepatocytes or slightly decreased triglycerides accumulation after peptide mimetics ABT-526 treatment in CD36 KO hepatocytes was reflected by a colorimetric enzymatic assay. The data are represented as the mean±SEM. Statistical significance was assessed by unpaired Student's *t*-test or one-way ANOVA. $n = 4-6$. *$p<0.05$, vs. vehicle control; #$p<0.05$, vs. high glucose plus insulin.

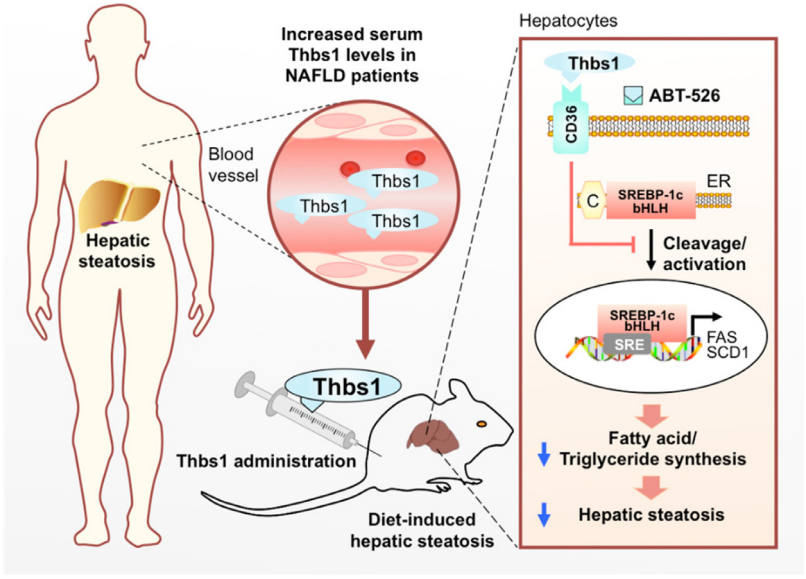

Fig. 6. The proposed model for Thbs1 as a novel regulator for lipid metabolism in the liver. Serum Thbs1 levels are increased in humans of NAFLD, and decreased after liver-fat-lowering intervention. Pharmacologically administration of Thbs1 improves hepatic steatosis in diet-induced obese mice. The Thbs1-CD36 axis may represent a novel molecular mechanism by which extracellular glycoprotein regulates intracellular metabolic pathway. These findings suggest that Thbs1 acts as a novel metabolic regulator with the therapeutic potential for treating hepatic steatosis via inhibiting hepatic de novo lipogenesis.

provide effective approaches for treating hepatic steatosis and related metabolic diseases.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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Authors' contributions

This work was carried out in collaboration among all authors. J.B., Y.Li. and X.G. contributed to experiment design; M.X., Y.X., F.M., A.C., Y.S., Y.H., X.X., F.Z., Z.H., Z.L., Y.Liu., J.G., G.C., W.S., and X.H. contributed to the acquisition and analysis of data; M.X., H.Y., and X.C. provided reagents and material support; X.S., H.W., H.B., and P.X. reviewed the manuscript; X.G., Y.Li. and J.G. obtained the funding; J. B., Y.Li. and X.G. wrote the manuscript. All authors have read and approved the final manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.102849.

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