Aberrant miR199a-5p/caveolin1/PPARα axis in hepatic steatosis

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Abstract

The prevalence of non-alcoholic fatty liver disease (NAFLD), a condition characterized by an excessive accumulation of triglycerides (TGs) in hepatocytes, has dramatically increased globally during recent decades. MicroRNAs (miRs) have been suggested to play crucial roles in many complex diseases and lipid metabolism. Our results indicated that miR199a-5p was remarkably upregulated in free fatty acid (FA)-treated hepatocytes. To investigate the role of miR199a-5p in the pathogenesis of fatty liver and the potential mechanism by which miR199a-5p regulates NAFLD, we first transfected two hepatocyte cell lines, HepG2 and AML12 cells, with agomiR199a-5p or antagomiR199a-5p. Our results indicated that miR199a-5p overexpression exacerbated deposition of FA and inhibited ATP levels and mitochondrial DNA (mtDNA) contents. Consistently, suppression of miR199a-5p partially alleviated deposition of FA and increased ATP levels and mtDNA contents. Moreover, miR199a-5p suppressed the expression of mitochondrial FA β-oxidation-related genes through inhibition of caveolin1 (CAV1) and the related peroxisome proliferator-activated receptor alpha (PPARα) pathway. Furthermore, suppression of CAV1 gene expression by CAV1 siRNA inhibited the PPARα signalling pathway. Finally, we examined the expression of miR199a-5p in liver samples derived from mice fed a high-fat diet, db/db mice, ob/ob mice and NAFLD patients, and found that miR199a-5p was upregulated while CAV1 and PPARα were downregulated in these systems, which was strongly indicative of the essential role of miR199a-5p in NAFLD. In summary, miR199a-5p plays a vital role in lipid metabolism, mitochondrial activity and mitochondrial β-oxidation in liver. Upregulated miR199a-5p in hepatocytes may contribute to impaired FA β-oxidation in mitochondria and aberrant lipid deposits, probably via CAV1 and the PPARα pathway.

Key Words

• non-alcoholic fatty liver disease
• caveolin1
• PPARα
• miR199a-5p
• fatty acid β-oxidation

Introduction

Non-alcoholic fatty liver disease (NAFLD), characterised by excessive fat deposits in the liver, is a risk factor for a variety of metabolic diseases including obesity, dyslipidaemia and type 2 diabetes. It can also trigger a progressive cascade of lipid disorders, such as steatohepatitis, liver fibrosis, cirrhosis and hepatocellular carcinoma.
Hepatic steatosis, a hallmark of NAFLD, occurs when the rate of hepatic fatty acid (FA) uptake from plasma and de novo FA synthesis is more than the rate of FA oxidation and export. Obesity, which commonly originates from overnutrition, has been considered to be the most prevalent cause of the development of NAFLD (Fabbrini et al. 2010). It has been suggested that obesity-induced NAFLD in humans is associated with the suppression of several genes involved in the FA oxidation pathway (Wanless & Lentz 1990, Mitsuyoshi et al. 2009).

Intracellular hepatic FA metabolism is regulated mainly via the mitochondrial β-oxidation systems (Kurtz et al. 1998). Accordingly, decreased mitochondrial FA β-oxidation has been considered to be one of the major mechanisms underlying the disturbances in lipid metabolism in liver and steatosis (Ockner et al. 1993). Furthermore, results from studies of both animal models and humans showed that decreased mitochondrial FA β-oxidation is closely associated with mitochondrial dysfunction (Nassir & Ibdah 2014). Therefore, investigation of the molecular mechanism of mitochondrial dysfunction would help to identify unique targets for therapeutic intervention of hepatic steatosis.

MicroRNAs (miRs) are a class of small RNAs that negatively regulate gene expression via the repression of the corresponding target mRNAs. To date, several miRNAs have been suggested to play important roles in lipid metabolism, including miR-122, miR-24, miR-370, miR-378–378, miR-335, miR-125a-5p, and miR-33 (Fernandez-Hernando et al. 2011, Ceccarelli et al. 2013, Ng et al. 2014). It was previously reported that aberrant expression of miR199a-5p may contribute to lung and liver fibrotic processes (Lino Cardenas et al. 2013). NAFLD disease progression can include inflammation and fibrosis. Therefore, we tested the hypothesis that miR199a-5p is important in mitochondrial dysfunction and the pathogenesis of hepatic steatosis.

Materials and methods

Mice experiments and human liver tissues

Eight to ten weeks old, male C57BL/6 lean mice, db/db mice and ob/ob mice (Coleman & Hummel 1973, Chen et al. 1996) that originated from the JAX Lab (Bar Harbor, ME, USA) were purchased from the Model Animal Research Center of Nanjing University. The mice were put on a high fat diet (HFD) and the Chow diet as described previously (Lu et al. 2014). The mice were housed in standard cages and conditions (at 20–24 °C, relative humidity 50–60%, with a 12 h light:12 h darkness cycle). Serum free FA (FFA) was measured using the Non-esterified Fatty Acids (NEFA) Commercial Kit (Wako, Osaka, Japan). The animal protocol was reviewed and approved by the Animal Care Committee of Shanghai Jiao Tong University School of Medicine. The human liver tissues were gifts from Dr Lu who purchased them from Alena Bio. Company (Xi’an, China) as described previously (Lu et al. 2014). Those samples were screened for non-alcoholic steatohepatitis (NASH) or fibrosis by H&E staining, only liver samples with simple hepatosteatosis were selected for our experiments. The protocol was reviewed and approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine.

Extraction of tissue and cell lipid and measurement of triglyceride

 Cultured cells were harvested using a cell scraper and homogenised by sonication. For determination of lipid contents, extracts were obtained from cell homogenates or liver tissues using a methanol–chloroform mixture by the Folch method (Folch et al. 1957). After evaporation of the organic solvent, the triglyceride (TG) content of each sample was measured using the TG measurement reagent (Sigma–Aldrich and BioVision (Milpitas, CA, USA)) according to the manufacturer’s instructions.

Cell culture and miR199a-5p transfection

The HepG2 cells were grown in DMEM supplemented with 10% fetal bovine serum, 1% l-glutamine and 1% penicillin–streptomycin (Invitrogen). Mouse liver 12 (AML12) hepatocytes were cultured in a 1:1 mixture of DMEM:F12 (Invitrogen) with 10% fetal bovine serum, 1% l-glutamine, 1% penicillin–streptomycin, 0.005 mg/ml insulin, 0.005 mg/ml transferrin and 40 ng/ml dexamethasone as previously described, with minor adjustment (Tateya et al. 2013). To overexpress miR-199a-5p, 20 nM AGOmiR199a-5p (sense, 5'-CCAGUGUUCAGACCCAGUG-3' and anti-sense, 5’-GAAAGUUCAGACCCAGUG-3`) or the negative control (sense, 5’-UCUACCUUCUGAGUGUGUGA-3' and anti-sense, 5’-UCUACCUUCUGAGUGUGUGA-3`) (Biomics, Jiangsu, China) was transfected into HepG2 and AML12.
cells using Lipofectamine 2000 (Invitrogen). To suppress miR199a-5p, 100 nM ANTmiR199a-5p (sense, 5'-GAACAGGUAGUCUGAAGACUGGG-3') or its negative control (sense, 5'-UCUACUCUUUUCUGAGGGUGUA-3') (Biomics) was transfected into HepG2 and AML12 cells. Eight hours after transfection, the medium was replaced with DMEM or DMEM/F12 containing 1 mM BSA-conjugated oleate acid/palmitate acid (OA/PA) for another 24 h.

Quantitative PCR and western blotting

Total RNA and protein extracts were isolated from the hepatocytes lysates or livers for gene expression analysis. The primer sequences are listed in Supplementary Table 1, see section on supplementary data given at the end of this article. To quantify the expression level of miR199a-5p, RT-PCR was carried out with M-MLV (Promega) followed by the EzOmics SYBR qRT-PCR (Biomics). miR199a-5p primer and U6 primer were purchased from Biomics. U6 and β-actin served as internal controls. The primary antibodies used in western blotting analysis included anti-peroxisome proliferator-activated receptor alpha (PPARα; Millipore, Billerica, MA, USA), anti-caveolin1 (CAV1; Proteintech, Chicago, IL, USA), anti-carnitine palmitoyltransferase 1a (CPT1a), anti-β-actin (Cell Signaling, Danvers, MA, USA), anti-medium-chain acyl-coenzyme A dehydrogenase (MCAD) and anti-lamin B (Santa Cruz Biotechnology).

Detection of cellular ATP levels

Cellular ATP levels were measured using a firefly luciferase-based ATP assay kit (Beyotime, Jiangsu, China). Briefly, HepG2 hepatocytes in triplicate were lysed and centrifuged at 12 000 g for 5 min, and 100 μl supernatant was mixed with 100 μl ATP detection dilution. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and GloMax 20/20 Single TubeLuminometer (Promega), and total ATP levels were expressed as nmol/mg protein.

Cotransfection with AGOmIR, ANTMIR and pGL3 plasmid constructs

Luciferase vectors in pGL3 backbone (Promega) were generated as described previously (Lino Cardenas et al. 2013). Briefly, the annealed oligonucleotides derived from the CAV1 3' UTR or the oligonucleotides mutated at the miR199a-5p-binding site were cloned into pGL3 vector between the Xhol and HindIII sites. HepG2 cells were plated into a 24-well plate and co-transfected using Lipofectamine 2000 (Invitrogen) with 100 nM of pGL3 plasmid construct and AGOmIR199a-5p, ANTMIR199a-5p or control miRs. 48 h after transfection, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and GloMax 20/20 Single TubeLuminometer (Promega).

Quantification of mitochondrial DNA content by real-time PCR

To quantify mitochondrial DNA (mtDNA) content, total genomic DNA was extracted from HepG2 cells using the NucleoSpin Kit (Macherey–Nagel, Bethlehem, PA, USA), and real-time PCR was carried out to detect two mtDNA-specific sequences, mitochondrially encoded cytochrome b (MT-CYB) and 12S rRNA with β-actin as nuclear DNA control. The primer sequences were listed in Supplementary Table 1.

CAV1 silencing

siRNAs were synthesised according to the method described in a previous report (Collett et al. 2010). HepG2 cells were transfected with 10 nM CAV1 siRNA or non-silencing control siRNA control using RNAiMAX Lipofectamine reagent (Invitrogen). Twenty-four hours after transfection, the medium was replaced with a complete growth medium for an additional 24 h before harvest.

Statistical analyses

All data are presented as mean ± S.E.M. Student’s t-test was used to compare two groups; the ANOVA test was used to compare differences among multiple groups, and P < 0.05 was considered significant.

Results

miR199a-5p regulates hepatic steatosis in HepG2 and AML12 hepatocytes

To test the role of miR199a-5p in hepatic steatosis, HepG2 cells and AML12 hepatocytes were exposed to OA and PA, two abundant FAs in the serum of obese mice (Escande et al. 2010), or the no OA/PA control. We found that miR199a-5p was upregulated in both HepG2 and AML12 cells treated with OA/PA (Fig. 1A and B).

To further explore the role of miR199a-5p, HepG2 cells and AML12 hepatocytes were transfected with AGOmIR199a-5p, a synthetic small RNA used to over-express miR199a-5p, or its negative control AGOmIR NC.
miR199a-5p regulates hepatic steatosis in HepG2 and AML12 hepatocytes. (A) HepG2 cells and AML12 cells (B) were stimulated with 1 mM OA/PA or BSA control, the expression levels of miR199a-5p were examined. Results shown are the average of data from three experiments. **P<0.01 and ***P<0.001, t-test. HepG2 cells (C) and AML12 cells (D) were transfected with AGOmiR199a-5p or AGOmiR NC and stimulated with 1 mM OA/PA or BSA control, lipid accumulation was examined. Results shown are the average of data from three experiments. *P<0.05, **P<0.01 and ***P<0.001, t-test. ### P<0.001, four groups ANOVA test. HepG2 cells (E) and AML12 cells (F) were transfected with ANTmiR199a-5p or ANTMir NC and stimulated with 1 mM OA/PA or BSA control, lipid accumulation level was also examined. Results shown are the average of data from three experiments. *P<0.05, **P<0.01 and ***P<0.001, t-test. ### P<0.001, four groups ANOVA test.

miR199a-5p is associated with ATP activity and mtDNA copy

After HepG2 hepatocytes were exposed to OA/PA, the cellular ATP contents were notably decreased (Fig. 2A and D), indicating that mitochondrial function is crucial in FA metabolism. Thus, we tested whether the effect of miR199a-5p on lipid accumulation was related to mitochondrial function. Indeed, overexpression of miR199a-5p in HepG2 cells resulted in less ATP content under both basal and OA/PA stimulation conditions (Fig. 2A and D). To determine whether the AGOmiR199a-5p-inhibited ATP activity was associated with mitochondrial biogenesis, the copy number of two mtDNA genes, cytochrome b (MT-CYB) and 12S rRNA (Kocher et al. 1989), was measured by real-time PCR. As shown in Fig. 2B and C, overexpressed miR199a-5p in HepG2 cells also led to reduced copy numbers of MT-CYB (Fig. 2B) and 12S rRNA (Fig. 2C).
miR199a-5p is associated with ATP activity and mitochondrial DNA copy number. (A, B and C) After HepG2 cells were transfected with AGOmiR199a-5p or AGOmiR NC and stimulated with OA/PA or BSA control, cellular ATP levels were measured (A). In addition, the copy numbers of MT-CYB (B) and 12S rRNA (C) were assayed by real-time PCR. Results shown are the average of data from three experiments. *P<0.05, **P<0.01 and ***P<0.001, four groups ANOVA test. (D, E and F) After HepG2 cells were transfected with ANTmiR199a-5p or ANTmiR and stimulated with OA/PA or BSA control, cellular ATP levels (D) and the copy numbers of MT-CYB (E) and 12S rRNA (F) were determined. Results shown are the average of data from three experiments. *P<0.05 and **P<0.01, t-test. **P<0.01 and ***P<0.001, four groups ANOVA test.

Consistently, overexpression of miR199a-5p in AML12 cells also resulted in less ATP content and lower copy numbers of MT-CYB and 12S rRNA (Supplementary Figure 2A, B and C, see section on supplementary data given at the end of this article). Moreover, the suppression of miR199a-5p expression in HepG2 cells resulted in significantly increased ATP content (Fig. 2D), and higher copy numbers of MT-CYB (Fig. 2E and F) and 12S rRNA under both basal and OA/PA exposure conditions.

miR199a-5p regulates CAV1 expression and the PPARα pathway in HepG2 hepatocytes

A previous study demonstrated that CAV1 is a direct bona fide target of miR199a-5p (Lino Cardenas et al. 2013). Using an in silico approach, they revealed the position of a miR-199a-5p target site in the CAV1 3’ UTR, and validated it using luciferase reporter assays in HEK293 cells. Herein, a dual luciferase reporter gene assay was performed to further examine whether CAV1 is a direct target of miR199a-5p in HepG2 cells. In HepG2 cells transfected with luciferase reporter plasmid, driven by WT CAV1 3’ UTR, the AGOmiR199a-5p inhibited, whereas the ANTmiR199a-5p increased luciferase reporter activity (Supplementary Figure 2A). However, this effect was not observed in cells transfected with luciferase reporter plasmid driven by CAV1 3’ UTR containing mutated miR199a-5p recognition site (Supplementary Figure 2A). This result confirms that CAV1 is a direct target of miR199a-5p in hepatocytes. To further examine this effect in hepatocytes, AGOmiR199a-5p or ANTmiR199a-5p was transfected into HepG2 cells, and the expression level of CAV1 was examined by real-time PCR and western blotting analysis. AGOmiR199a-5p significantly downregulated CAV1 mRNA and CAV1 protein levels in HepG2 cells (Fig. 3A), while ANTmiR199a-5p significantly upregulated CAV1 mRNA and CAV1 protein levels in HepG2 cells (Fig. 4A).

CAV1 knockout mice have altered mitochondrial FA β-oxidation, lipid metabolism and amino acid metabolism in the liver (Fernandez-Rojo et al. 2013); all processes which are under the control of the nuclear receptor PPARα (Mandard et al. 2004). To examine the effect of miR199a-5p on the PPARα pathway, AGOmiR199a-5p or ANTmiR199a-5p was transfected into HepG2 cells. Then, cells and the expression of hepatic PPARα and its three mitochondrial FA β-oxidation-related target genes, namely CPT1a, CPT1b and MCAD, were measured by real-time PCR and western blotting analysis (no western blotting data for CPT1b due to the lack of good antibody
for CPT1b). The overexpression of miR199a-5p in HepG2 cells led to significantly decreased expression of PPARα (Fig. 3B) and its target genes (Fig. 3C, D and E) under both basal and OA/PA exposure conditions. Similar results were found in AML12 cells overexpressing miR199a-5p (Supplementary Figures 3 and 4A, B, C, D and E, see section on supplementary data given at the end of this article). Consistently, suppressed miR199a-5p expression was correlated with elevated level of PPARα (Fig. 4B) and its target genes (Fig. 4C, D and E).

CAV1 deficiency impairs the PPARα signalling pathway in hepatocytes

To further explore the role of CAV1 in the PPARα pathway, HepG2 cells were transfected with CAV1 siRNA or negative control siRNA. The cells transfected with CAV1 siRNA displayed significantly downregulated CAV1 mRNA levels (Fig. 5A) and CAV1 protein levels (Fig. 5B). Consistently, decreased expression of PPARα (Fig. 5C and D) and its target genes was observed (Fig. 5E, F, G and H) after CAV1 expression was inhibited, indicating that the expression of these genes was regulated by CAV1.

The miR199a-5p/CAV1/PPARα axis in NAFLD mice

To further investigate the role of miR199a-5p in the development of NAFLD, we performed miR199a-5p expression analysis using livers obtained from three obese mice models, including HFD mice, db/db mice and ob/ob mice. As expected, hepatic TG contents and serum FFA levels among these models were elevated in both models (Fig. 6A and B). Interestingly, real-time PCR results indicated that miR199a-5p expression levels were upregulated (Fig. 6C, D and E) while Cav1 and Ppara were downregulated (Fig. 6F and G) in the livers of these models, indicating the potential correlation between the miR199a-5p/CAV1/PPARα axis and NAFLD in mouse models.
The miR199a-5p/CAV1/PPARα axis in NAFLD patients

To explore the clinical relevance of miR199a-5p, we further performed miR199a-5p expression analysis in liver samples obtained from NAFLD patients. As expected, hepatic TG contents were elevated in livers of patients (Fig. 7A). Intriguingly, the level of expression of miR199a-5p was also upregulated (Fig. 7B) while CAV1 and PPARα were downregulated (Fig. 7C and D) in the liver samples from patients, indicating a strong correlation between miR199a-5p/CAV1/PPARα axis and NAFLD in patients.

Discussion

Results from previous studies indicated that the aberrant expression of miR199a-5p was associated with the progression of liver and lung fibrosis (Murakami et al. 2011, Lino Cardenas et al. 2013). We first showed that miR199a-5p was increased in OA/PA-induced steatosis in cultured HepG2 and AML12 hepatocytes as well as in the livers from HFD-fed NAFLD mice, in which we found that serum FFA levels were simultaneously increased. Secondly, hepatic steatosis and elevated serum FFA levels with increased liver miR199a-5p expression were observed in db/db mice and ob/ob mice, two obese and diabetic animal models with deficiencies in leptin signalling (Coleman & Hummel 1973, Chen et al. 1996). More importantly, we found that miR199a-5p was increased in NAFLD patients. Our results indicated that miR199a-5p was comprehensively upregulated in hepatic steatosis, and could be regulated by FFA. Notably we found that OA/PA could further decrease miR199a-5p-suppressed CAV1/PPARα expression and ATP activity, indicating that FFA may
The expression profile of miR199a-5p in livers of NAFLD mice. (A) The hepatic TG contents in livers of HFD, db/db and ob/ob mice. Results are presented as mean ± S.E.M., **P < 0.01, t-test, n=4. (B) The serum free fatty acid (FFA) levels in livers of HFD, db/db and ob/ob mice. Results are presented as mean ± S.E.M., *P < 0.05, **P < 0.01, t-test, n=4. (C, D and E) The expression of miR199a-5p in livers from HFD mice, db/db mice (D) and ob/ob mice (E). U6 were used as internal controls. Results are presented as mean ± S.E.M., *P < 0.05, t-test, n=4. (F and G) The expression of Cav1 and Ppara in livers from HFD mice, db/db mice and ob/ob mice. β-actin was used as an internal control. Results are presented as mean ± S.E.M., *P < 0.05, **P < 0.01 and ***P < 0.001, t-test, n=4.
Nevertheless, further investigation is needed to determine the detailed mechanism.

Mitochondria play a central role in the generation of energy from nutrient oxidation. It has been reported that mitochondria have a role in FA metabolism, and impaired mitochondrial function is thought to contribute to NAFLD (Garcia-Ruiz et al. 2013). In the current study, FFA treatment significantly decreased ATP levels in HepG2 cells, and miR199a-5p overexpression further decreased ATP levels. On the other hand, miR199a-5p suppression partially rescued FFA-induced mitochondrial damage. It is known that the copy number of mtDNA serves as a parameter for evaluating mitochondrial function (Nassir & Ibldah 2014). In line with this, we found that miR199a-5p influenced mtDNA copy number, indicating that miR199a-5p is closely associated with mitochondrial dysfunction in the HepG2 and AML12 cultured liver cell lines.

A recent report has described how miR199a-5p is upregulated during fibrogenic response by targeting CAV1 (Lino Cardenas et al. 2013). Consistently, we provided direct evidence that CAV1 is a bona fide miR199a-5p target in HepG2 hepatocytes. As CAV1 is an integral membrane protein and serves as the main structural protein of caveolae in non-muscle cells, it has been linked with several cellular functions (Fernandez-Rojo et al. 2013), including the maintenance of hepatic lipid homeostasis (Fernandez et al. 2006, Martin & Parton 2006, Parton & Simons 2007, Fernandez-Rojo et al. 2012) and mitochondrial regulation (Bosch et al. 2011, Asterholm et al. 2012). CAV1 was shown to be necessary for hepatic PPARα-dependent FA oxidation and ketogenesis (Fernandez-Rojo et al. 2013), indicating the important link between CAV1 and PPARα signalling in lipid metabolism. On the basis of results obtained using Cav1−/− mice, the mRNA expression of hepatic PPARα and its target genes, Cpt1a, Cpt1b and Mcad and the level of PPARα protein were significantly reduced in comparison with Cav1+/+ mice.

Our results reveal dysregulation of Cav1 and Ppara in HFD, db/db and ob/ob mice steatosis. Furthermore, we showed that miR199a-5p regulated the expression of CAV1, PPARα and its target genes in OA/PA-induced steatosis in hepatocytes. In addition, silencing of CAV1 resulted in decreased expression of PPARα and its target genes, indicating that CAV1 regulates the expression of genes in PPARα pathway.

We have demonstrated that miR199a-5p expression is upregulated in hepatocyte cell cultures and in livers from mice and patients with NAFLD. Furthermore, we demonstrated that AGOmiR-induced overexpression or ANTmiR199a-5p-induced suppression of miR199a-5p increased or decreased cellular TG in OA/PA-induced steatosis in HepG2 hepatocytes, respectively. A previous report described how the expression of miR199a-5p was upregulated in CCL4 and bile-duct-ligation-induced liver steatosis. The expression of miR199a-5p, CAV1 and PPARα in livers of patients. U6 and β-actin were used as internal controls. Results are presented as mean ± S.E.M., *P < 0.05, t-test, n = 7. (B, C and D)
fibrosis, but downregulated during fibrosis regression (Lino Cardenas et al. 2013), results from other studies also indicated that NASH could be present in one-third of NAFLD cases, for those with NASH, are prone to the risk of fibrosis (Farrell & Larter 2006, Zhang et al. 2014), indicating that miR199a-5p might have a role in the progression of NAFLD–NASH–fibrosis. However, we have not obtained direct evidence to support miR199a-5p's role in disease progression, which is the limitation of our study and will be an objective of our further studies.

In summary, our findings provide strong evidence of miR199a-5p's role in the regulation of hepatic steatosis in vivo and in vitro, and that FFA is a potential stimulator of miR199a-5p and acts synergistically in the development of NAFLD. Furthermore, we uncovered a novel mechanism by which in NAFLD the dysregulated axis of miR199a-5p/CAV1/PPARz leads to aberrant mitochondrial FA oxidation and hepatic steatosis (Fig. 8). As a transcriptional mediator of PPARz signalling, our study also demonstrated that CAV1 may serve as a potential therapeutic target for NAFLD and other fatty liver diseases.

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