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Original Article

MicroRNA-199a-3p attenuates hepatic lipogenesis by targeting Sp1

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Abstract: Emerging studies have demonstrated that microRNAs (miRs) are profoundly involved in non-alcoholic fatty liver disease (NAFLD) and related metabolic diseases. Previously, we revealed a repertoire of miRs dysregulated in NAFLD by high-throughput sequencing. Here, we showed that microRNA-199a-3p was down-regulated in the livers of C57BL/6J mice fed a high-fat-diet (HFD) and oleic acid/palmitic acid-induced Hepa1-6 cells. Gain-of-function and loss-of-function studies demonstrated that microRNA-199a-3p exhibited a suppressive role in hepatic lipogenesis. Adenoviral mediated microRNA-199a-3p expression in C57BL/6J mice largely attenuated triglyceride (TG) accumulation and expression of lipogenic genes. Furthermore, we identified Specificity Protein 1 (Sp1) as the functional target of miR-124. Restoration of Sp1 expression largely compromised the effect of microRNA-199a-3p on hepatic TG metabolism. Taken together, our findings uncover a novel function of microRNA-199a-3p/Sp1 axis in NAFLD and provide a mechanism underlying perturbations of hepatic TG homeostasis.

Keywords: microRNA-199a-3p, metabolic disease, high-fat-diet, triglyceride metabolism, NAFLD

Introduction

Nonalcoholic fatty liver disease (NAFLD) is initially described as the pathological accumulation of lipids in hepatocytes in the absence of alcohol consumption, viral or toxic causes of liver diseases. NAFLD refers a spectrum of diseases, including isolated hepatic steatosis, nonalcoholic steatohepatitis (NASH) and cryptogenic fibrosis/cirrhosis [1, 2]. And NASH can progress to irreversible liver damage, such as cirrhosis and hepatocellular carcinoma [3]. Apart from liver pathology, NAFLD is also an important part of metabolic syndrome and increases the risk of type 2 diabetes and cardiovascular disease [4]. With the expanding epidemic of obesity, the incidence of NAFLD is increasing, especially in developed societies; in the adult population, the prevalence of NAFLD in obesity is more than 90% [5]. Therefore, it is imperative to gain a better understanding of the exact molecular mechanisms underlying the pathogenesis of NAFLD.

MicroRNAs (miRNAs), which are short noncoding RNA molecules composed of 18-25 nucleotides, regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of target messenger RNAs (mRNAs) [6]. Ample studies have demonstrated that miRNAs are involved in a wide spectrum of biological processes and metabolic homeostasis [7]. Recently, miRNAs have emerged as novel biomarkers and potential therapeutic targets in the management of NAFLD [8]. And many miRNAs are critically implicated in the pathogenesis of NAFLD. For instance, miR-122, the well-studied miRNAs in NAFLD, has been shown to regulate cholesterol and fatty acid metabolism [9]. And aberrant miR-34a regulates steatosis by targeting peroxisome proliferator-activated receptor-alpha [10]. Additionally, miRNAs are also involved other aspects of NAFLD, such as insulin resistance and obesity [11], oxidative stress [12], and mitochondrial dysfunction [13].

Previously, we employed the high-throughput sequencing to screen the expression profiles of miRNA in high-fat diet (HFD)-induced fatty liver tissue [14]. And a series of differentially expressed miRNAs between the normal group and HFD group was identified. Among these

miRNAs, we demonstrated that up-regulated miRNA-421 induces hepatic mitochondrial dysfunction by inhibiting sirtuin 3 in NAFLD mice. In this study, we further found that miR-199a-3p was mostly down-regulated both in NAFLD animal and cell models. Moreover, by *in vitro* and *in vivo* studies, we identified a critical role of miR-199a-3p in the regulation of lipogenic genes expression and hepatic homeostasis by inhibiting Sp1.

Materials and methods

Subjects and NAFLD model

Male C57BL/6J mice (20 to 25 g body weight) at age of 8-12 weeks were purchased from the Shanghai Center of Laboratory Animals. All surgical and care procedures administered to the animals were in accordance with the institutional guide lines of Shanghai Center of Laboratory Animals. All mice were housed at 21 ± 1°C with a humidity of 55 ± 10% and a 12-hr light/12-hr dark cycle with free access to food and water. Once they had adapted to the environment, C57BL/6J mice in the normal control group were fed with standard chow diet (SCD), while the others were fed with HFD (approximately 1.67 kcal/g), which was composed with 79.5% corn powder, 0.5% cholesterol and 20% lard. Each group has at least 5 mice. Additionally, C57BL/6J mice fed a HFD diet were injected intravenously through the tailvein with the adenovirus expressing a miR-199a-3p mimic or mouse Sp1 and negative control adenovirus vector. Viruses were diluted with PBS and each mouse received 2×10^9 plaque-forming units. Two weeks after adenovirus injection, the mice were anesthetized, and livers were harvested for further analysis.

Cell culture

The mouse hepatocyte cell line Hepa1-6 and NCTC1469 was obtained from China Cell Culture Center (Shanghai, China). Hepa1-6 cell were cultured in routine DMEM supplemented with 10% fetal bovine serum (Gibco BRL, Carlsband, CA,USA). The NCTC1469 cells were cultured in low-glucose DMEM (Invitrogen). All cells were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator at 37°C with 5% CO_2. Oleate (OA) and palmitate (PA; Sigma, St Louis, MO, USA) were used to induce the fat overloading cells. Briefly, stock solutions of 5 mM oleate and 5 mM palmitate were prepared in the culture medium containing 1% bovine serum albumin (BSA). The Hepa1-6 cells at 80% confluency were exposed to a long train mixture of OA and PA (HFFA) at a final ratio of 2:1 and final concentration of 1 mM for 24 hr. In order to achieve the maximal fat accumulation with minimal cytotoxic effects of HFFA, the induction parameters should be optimized in a preliminary test.

Cell transfection

The miR-199a-3p mimics and inhibitors as well as the negative control (NC) were purchased from GenePharma (Shanghai). The miR-199a-3p mimic is chemically modified double-stranded RNAs that mimic endogenous miR-199a-3p, and the miR-199a-3p inhibitor is a LNA/OMe modified antisenseoligonucleotide designed to specifically bind to and inhibit endogenous miR-199a-3p. DharmaFect1 (Dharmacon, Lafayette, CO) was used for transfection of miRs.

Quantitative real-time PCR

Total RNA were extracted from cells or tissues using standard TRIzol method according to the manufacturers' instructions (Invitrogen, Shanghai). Real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Japan) on Light Cycler480 (Roche, Switzerland) in a 20 μL reaction system. Specially, for the detection of miRNA, total RNA was reverse-transcribed using miScript II RT Kit (QIGEN, Shanghai). The relative expression level of a miRNA or mRNA was determined by normalizing to an internal invariant control, U6 or β-actin. Each reaction was performed in triplicate. The primers used in this study were shown as follows: FASN forward, 5'-ACAGCGGGGAATGGGTACT-3', FASN reverse: 5'-GACTGGTACAACGAGCGGAT-3'; SREBP1 forward, 5'-ACAGTGACTTCCCTGGCCTAT-3', SREBP1 reverse: 5'-GCATGGACGGGTACATCTTCAA-3'.

Western blot analysis

Protein from indicated cells were prepared with radioimmunoprecipitation (RIPA) buffer containing 50 mMTris-HCl (pH 8.0), 150 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM NaF, 1% NP_40 and 0.1% SDS, supplemented with protease and phosphatase inhibitors (Millipore, USA). Protein concentration was quantified by the BCA method. Cell lysates were loaded onto 10% SDS-PAGE for separation, transferred to

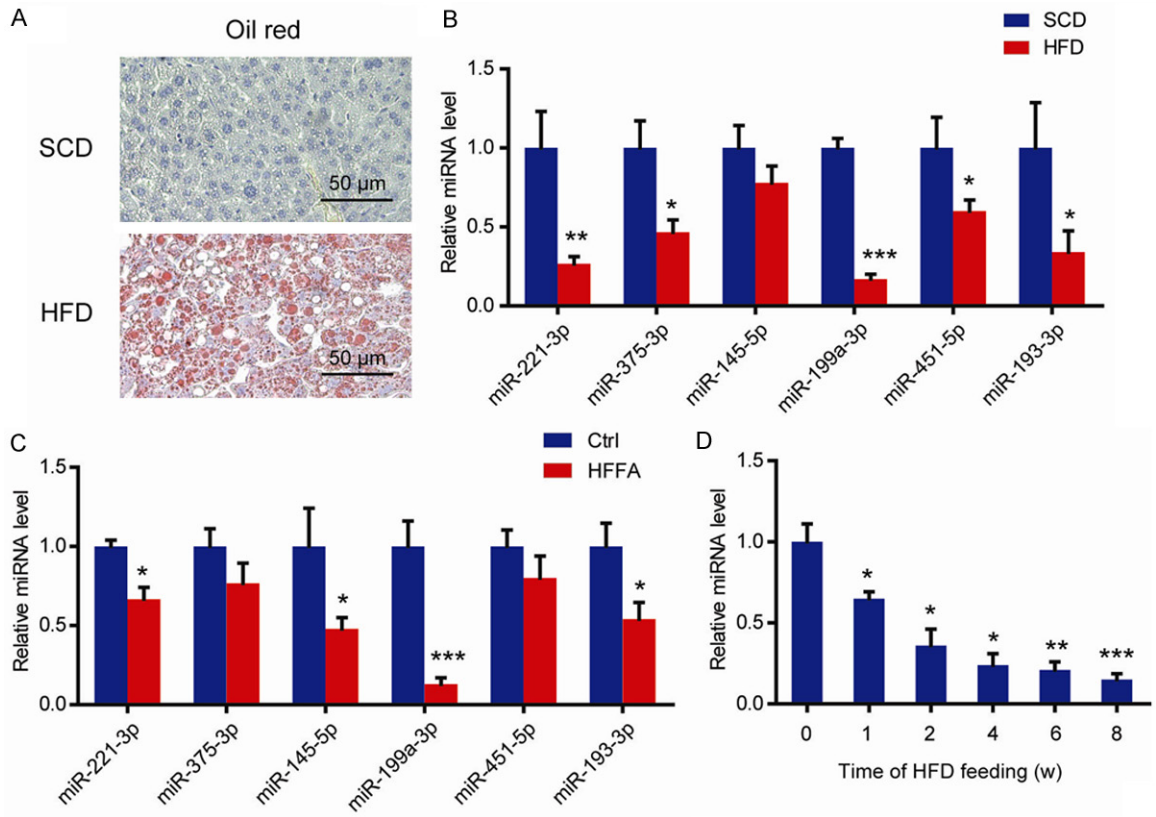

Figure 1. miR-199a-3p is down-regulated in the HFD-fed Mice and model cells. A. Oil red O staining of liver frozen sections from SCD and HFD mice. B. RT-qPCR certified a list of indicated miRNAs in the liver tissue from SCD and HFD mice. C. Relative expression of indicated miRNAs in Hepa1-6 cells with or without Oleate and palmitate (HFFA) treatment. D. miR-199a-3p expression was validated by qPCR in HFD mice challenged with treatment at different weeks. Bars are represented from three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001.

PVDF membrane, blocked with 5% defatted milk powder, and incubated with a primary antibody against Sp1 (1:4000, Abcam, ab13370) or β-actin (1:2000, Abcam, ab8227) at 4°C overnight. After washing in TBST for three times, secondary antibody (1:1000, Shanghai biotech, China) was applied for 1 hr incubation at room temperature. Then the blot was incubated with HRP-conjugated anti-IgG membrane and the immunoreactivity was developed by ECL method (Millipore).

Oilred O staining

Liver tissues were fixed in 4% paraformaldehyde for 1 h at 37°C, then embedded in optimum cutting temperature compound (OCT) on dry ice, and cryosectioned into 5 μm sections. To test lipiddroplet accumulation, slides were incubated with pre-warmed Oil Red O (Solarbio, Beijing, China) for 30 min at 37°C and washed with 60% 1,2-propanediol. Finally, tissues stained with Oil Red O were visualized under a microscope (CK Microscope, Olympus, Tokyo, Japan).

Hepatic and cellular TG measurement

Harvested liver tissues were homogenized in chloroform/methanol (2:1 v/v) using a Polytron tissue grinder (Kinematica AG, Luzern, Switzerland) and lipid extracts were prepared by the classical Folch method. Extracts were dried and dissolved in isopropanol. Intracellular triglyceride (TG) content was quantified in cell extracts by using the commercial chemicalcolorimetric kit (JianchengBioeng. Com., Nanjing, China).

Luciferase reporter assay

Cells were seeded at 3×10^5 cells/wells in 24-cell plates. The complete 3'-UTR of murine Sp1 containing either the wild type (WT) or mutated miR-199a-3p binding sites was cloned and inserted into pGL3-null vector (Promega,

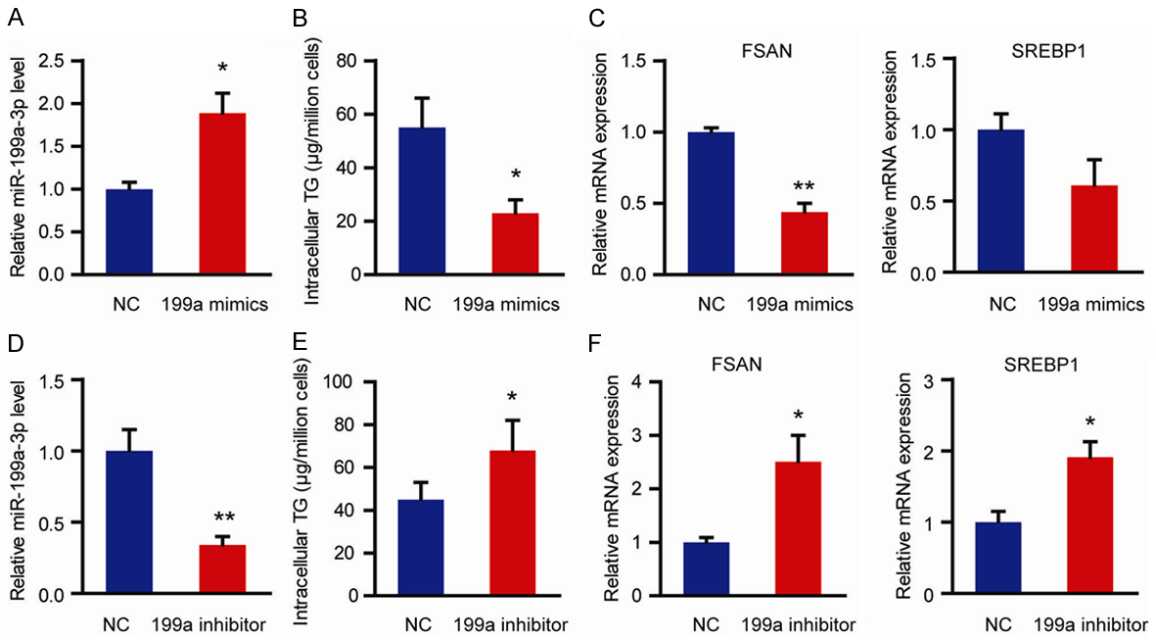

Figure 2. miR-199a-3p regulates hepatic lipogenesis *in vitro*. A. The miR-199a-3p expression level after transfection of miR-199a-3p mimics. B. Influence of miR-199a-3p mimics on intracellular TG content in Hepa1-6 cells. C. Influence of miR-199a-3p mimics on FASN and SREBP1 expression in Hepa1-6 cells. D. The miR-199a-3p expression level after transfection of miR-199a-3p inhibitors. E. Influence of miR-199a-3p inhibitors on intracellular TG content in Hepa1-6 cells. F. Influence of miR-199a-3p inhibitors on FASN and SREBP1 expression in Hepa1-6 cells. Results were from three independent experiments. *P < 0.05 and **P < 0.01.

USA). For luciferase assays, cells were co-transfected with miR-199a-3p mimic or scramble and pGL3-Sp1-3'-UTR, pGL3-Sp1-3'-UTR Mut plasmid and phRL-SV40 control vector (Shanghai Biotech, China) by using the Lipofectamine2000 (Invitrogen, CA, USA). The luciferase activities were measured consecutively by using the Dual Luciferase Reporter Assay System (Promega, USA).

Statistical analysis

Data were shown as the means ± SD. The calculations were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software Inc., San Diego, CA). The two-tailed unpaired Student's t-tests were used for comparisons of two groups. The ANOVA multiple comparison test was used for comparisons of two more groups. Statistical significance is displayed as *P < 0.05, **P < 0.01 and ***P < 0.001.

Results

miR-199a-3p is down-regulated in the HFD Mice and model cells

Previously, we performed genome-wide miRNA expression profiles in the liver tissues of HFD and control mice, and found several significantly changed miRNAs [14]. In this study, we firstly tested six down-regulated miRNAs (miR-221-3p, miR-375-3p, miR-145-5p, miR-199a-3p, miR-451-5p, and miR-193-3p) in SCD and HFD mice (**Figure 1A**). RT-qPCR showed that all the miRs detected were significantly down-regulated in HFD mice compared to SCD mice except for miR-145-5p (**Figure 1B**). Next, we determined the expression of these miRs in HFFA-induced Hepa1-6 cells. The result showed that miR-221-3p, miR-145-5p, miR-199a-3p and miR-193-3p were markedly decreased in HFFA-induced Hepa1-6 cells compared with the controls (**Figure 1C**). Notably, miR-199a-3p had the remarkable fold change. Interestingly, miR-199a-3p expression was gradually decreased when the HFD feed time was extended (**Figure 1D**).

miR-199a-3p regulates hepatic lipogenesis in vitro

To evaluate the potential role of miR-199a-3p in lipid metabolism, gain-of-function and loss-of-function assays were carried out. As shown in **Figure 2A**, transfection with miR-199a-3p mimics in HFFA-induced Hepa1-6 resulted in an

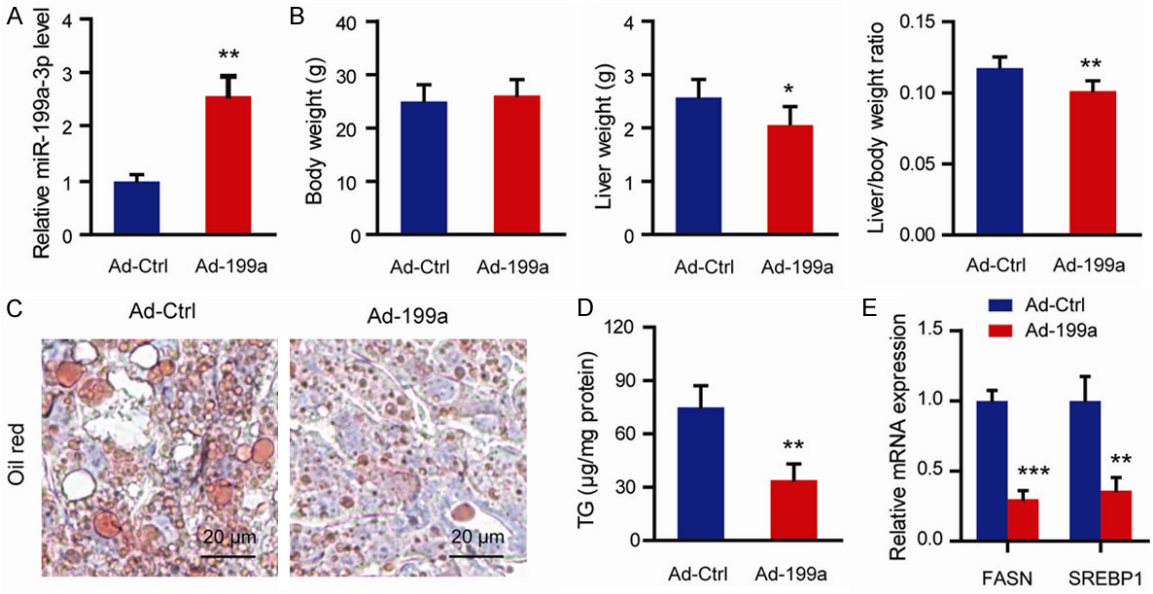

Figure 3. miR-199a-3p inhibits hepatic lipogenesis *in vivo*. A. The miR-199a-3p expression level in liver tissues after injection of Ad-miR-199a-3p. B. Quantification of bodyweight, liver weight, and liver weight/body weight ratio in Ad-199a and Ad-Ctrl HFD mice. C. Oil red O staining of liver frozen sections from Ad-199a and Ad-Ctrl mice. D. Relative hepatic TG content in Ad-199a and Ad-Ctrl mice. E. Relative FASN and SREBP1 expression in liver tissues from Ad-199a and Ad-Ctrl mice. *P < 0.05, **P < 0.01 and ***P < 0.001.

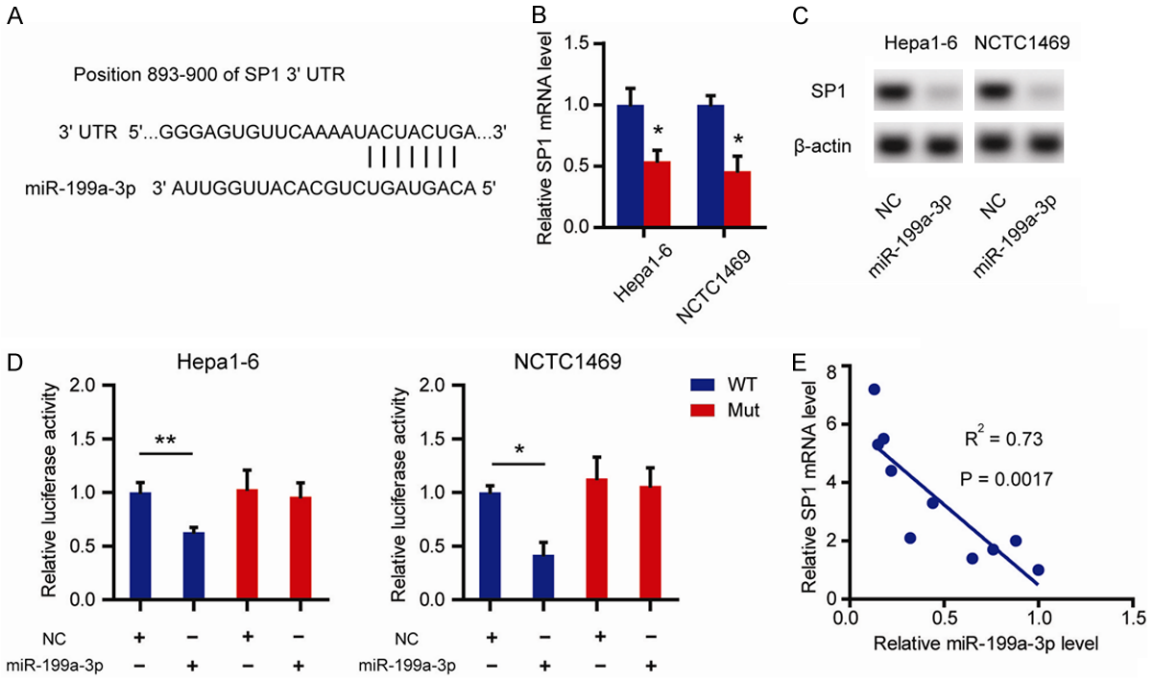

Figure 4. Sp1 is a direct target of miR-199a-3p. A. Sequence alignment of the 3'-UTR of Sp1 and miR-199a-3p. B. Effect of miR-199a-3p mimics on Sp1 mRNA expression in Hepa1-6 and NCTC1469 cells. C. Effect of miR-199a-3p mimics on Sp1 protein expression in Hepa1-6 and NCTC1469 cells. D. Relative luciferase activity of the firefly reporter containing the 3'-UTR of Sp1 was detected in Hepa1-6 and NCTC1469 cells co-transfected with miR-199a-3p mimics. E. Correlation between miR-199a-3p and Sp1 in liver tissues of HFD mice. *P < 0.05 and **P < 0.01.

approximately two-fold up-regulation of miR-199a-3p expression. Hepa1-6 cells transfected with miR-199a-3p mimics had a significant decrease in intracellular TG contents compared

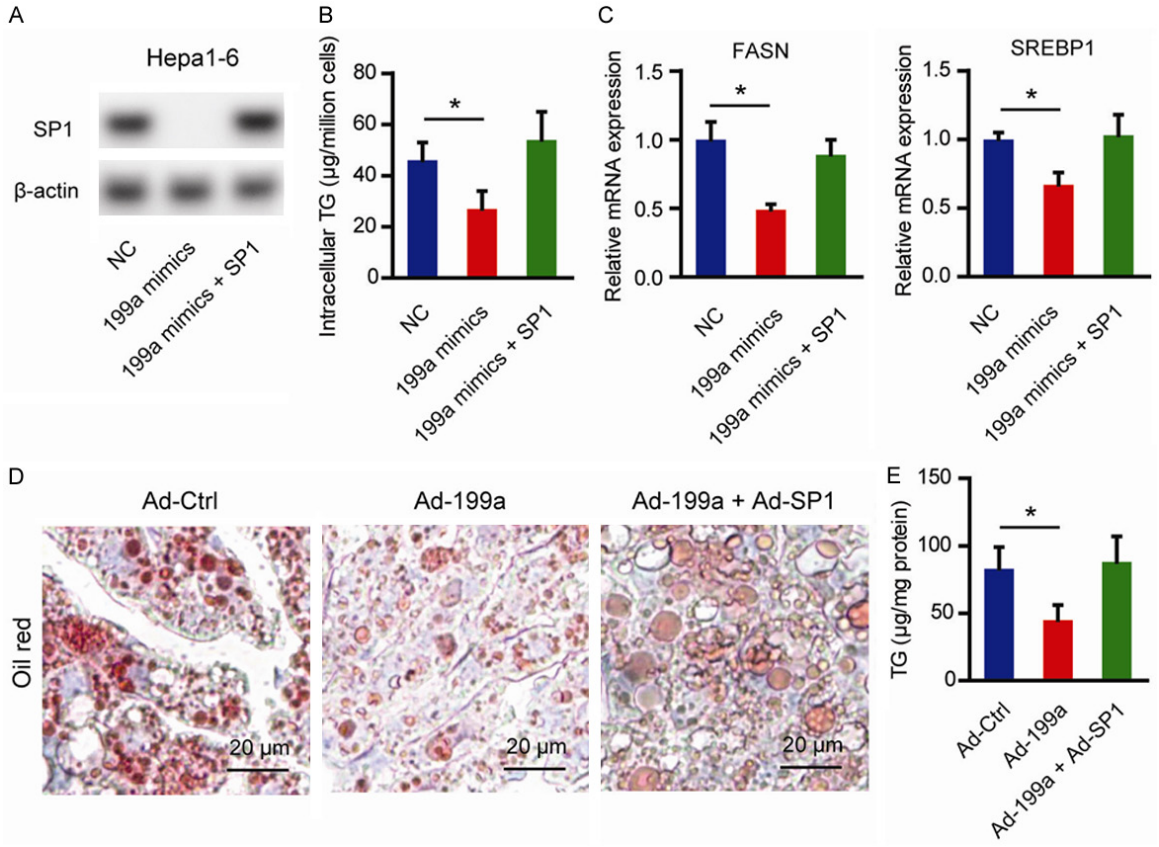

Figure 5. Restoration of Sp1 abolishes the effect of miR-199a-3p. (A) Western blotting analysis of the Sp1 protein level in Hepa1-6 cells upon transfection of miR-199a-3p mimics or both miR-199a-3p mimics and pcDNA3.1-Sp1. Liver TG content (B) and FASN and SREBP1 expression (C) in Hepa1-6 cells upon transfection of miR-199a-3p mimics or both miR-199a-3p mimicsand PCDNA3.1-Sp1. (D) Oil red O staining of liver frozen sections from Ad-199a or both Ad-199a and Ad-Sp1 mice. (E) Liver TG content in mice infected Ad-199a or both Ad-199a and Ad-Sp1. *P < 0.05.

with NC (**Figure 2B**). Meanwhile, we also found that lipogenic gene SREBP1 and its downstream target FASN were decreased by miR-199a-3p mimics (**Figure 2C**). And expectedly, inhibition of miR-199a-3p expression by transfection of miR-199a-3p inhibitors significantly increased intracellular TG contents and expression of lipogenic genes (**Figure 2D-F**).

miR-199a-3p inhibits hepatic lipogenesis in vivo

To further determine the effect of miR-199a-3p on HFD-induced hepatic lipogenesis in vivo, the adenovirus system was used deliver the miR-199a-3p to mouse hepatocytes. To rule out the effects of adenovirus vectors on inflammation, adenovirus vectors were used as control (Ad-Ctrl) to compare with adenovirus vectors expressing miR-199a-3p (Ad-199a). As depicted in **Figure 3A**, Ad-199a resulted in about 2.5-fold increase in hepatic miR-199a-3p expression. And this up-regulation was associated with a significant reduction in liver weight and liver/body weight ratios (**Figure 3B**). Oil Red O staining showed that the miR-199a-3p reduced the steatosis degree in the liver tissues (**Figure 3C**). Consistent with morphological findings, hepatic TG (**Figure 3D**) and lipogenic genes (**Figure 3E**) were also reduced in Ad-199a infected mice compared to Ad-Ctrl infected mice.

Sp1 is a direct target of miR-199a-3p

To identify the potential target of miR-199a-3p-mediated lipid accumulation, bioinformatics approach was employed and several putative mouse miR-199a-3p target genes, among which the gene encoding Sp1 harbored a miR-

199a-3p binding site (**Figure 4A**). Sp1 is a transcription factor than can regulate the expression of a large number of genes involved in a variety of processes such as cell growth, differentiation, immune responses and lipogenesis. Transfection of miR-199a-3p mimics in Hepa1-6 and NCTC1469 cells led to a significant decrease in Sp1 expression, at both mRNA (**Figure 4B**) and protein level (**Figure 4C**). To further confirm whether Sp1 is a direct target of miR-199a-3p, a luciferase reporter containing Sp1 3'-UTR was constructed in Hepa1-6 and NCTC1469 cells. Excitingly, miR-199a-3p markedly inhibited the luciferase reporter activity of WT Sp1 3'-UTR in both cells. However, mutation of Sp1 3'-UTR abrogated the repressive ability of miR-199a-3p, suggesting that the specificity of miR-199a-3p target sequence (**Figure 4D**). And consistently, mRNA level of Sp1 was negatively correlated with the expression of miR-199a-3p (**Figure 4E**).

Restoration of Sp1 abolishes the effect of miR-199a-3p

To further test whether Sp1 mediates the functional effects of miR-199a-3p in hepatic steatosis, we restored Sp1 expression in the HFD mouse model and Hepa1-6 cell model. Transfection of pcDNA3.1-Sp1 plasmid restored the inhibitory effect of miR-199a-3p on Sp1 protein expression (**Figure 5A**), intracellular TG contents (**Figure 5B**) and expression of lipogenic genes (**Figure 5C**). Moreover, adenoviral-mediated restoration of Sp1 compromised the reduced hepatic steatosis induced by Ad-199a as demonstrated by Oil red O staining (**Figure 5D**) and hepatic TG contents (**Figure 5E**). Collectively, these data above suggest that miR-199a-3p mediated down-regulation of Sp1 is critically involved in hepatic lipogenesis and steatosis.

Discussion

In this study, we for the first time identified miR-199a-3p as a modulator in hepatic TG homeostasis. We demonstrated that miR-199a-3p can regulate the degree of steatosis and expression of lipogenic genes by directly targeting Sp1. Therefore, this newly identified miR-199a-3p/Sp1 pathway might provide an alternative approach for amelioration of hepatic TG accumulation and become a therapeutic target for the treatment of NAFLD.

The expression profile and cellular functions of miR-199a-3p have been intensively studied in cancers. However, it was unknown how miR-199a-3p contributes functionally to the pathogenesis of NAFLD. In the current study, using miRNA analysis of the livers of HFD-fed mice and Hepa1-6 cells treated with an oleic acid/palmitic acid mixture, we showed that miR-199a-3p was down-regulated both in the liver tissues of HFD mice and HFFA-induced cells. In human adipocytes, miR-199a-3p level can be significantly induced by fatty acid, TNF-α, IL-6 and leptin, and represents a factor in the modulation of obesity-associated insulin resistance and inflammatory responses [15]. In contrast to this observation, our data suggest that reduced expression of miR-199a-3p in hepatic steatosis might be a mechanism aimed to promote disease progression. Subsequent loss-of-function and gain-of-function studies further confirmed this notion that miR-199a-3p acts a suppressor in regulating lipogenesis as demonstrated by measurement of TG contents and lipogenic genes expression.

MicroRNAs negatively regulate gene expression post transcriptionally by binding to the 3'-UTR of their target mRNAs. Based on informatics analysis, several potential targets of miR-199a-3p with high match score were revealed and we finally focused on Sp1 because it regulates de novo lipogenesis. We then confirmed the regulation of the predicted miR-199a-3p targeted SP1 mRNAs by measuring both its mRNA and protein levels in two cell models, Hepa1-6 and NCTC1469. And by luciferase reporter assay, we revealed the post-transcriptional regulation of miR-199a-3p on Sp1. In cancer cells, Sp1 promotes lipogenesis by up-regulating sterol regulatory element-binding protein-1c (SREBP-1c) and FASN expression [16]. Consistent with this finding, restoration of Sp1 largely abolished the inhibitory effects of miR-199a-3p on TG accumulation and expression of lipogenic genes, including SREBP1 and FASN, suggesting miR-199a-3p/Sp1 axis might facilitate the development of NAFLD mainly by regulating lipogenesis.

Accumulating studies have indicated that miR-199a-3p is involved in multiple cellular processes by repressing various target genes. For example, miR-199a-3p suppresses the expansion and tumorigenic capabilities of prostate

cancer stem cells through directly or indirectly targeting several additional mitogenic molecules, including c-MYC, cyclin D1 and EGFR [17]. In prostate cancer, miR-199a-3p inhibits aurora kinase A and attenuates xenograft tumor growth [18]. Notably, miR-199a-3p inhibits cell proliferation and induces apoptosis by targeting YAP1 in hepatocellular carcinoma (HCC) [19]. Meanwhile, several other targets of miR-199a-3p have been identified, such as CD44 [20], AXL [21], ZHX1 [22], NLK [23] and Sp1 [24]. Therefore, we cannot fully exclude other factors except for Sp1 in the regulatory functions of miR-199a-3p in NAFLD. Notably, circulating miR-199a-3p can be served as a potential biomarker for the diagnosis of HCC, colorectal cancer and glioma [25-27], whether it is possible to be a biomarker for NAFLD warrants further investigation.

In conclusion, we reveal that miR-199a-3p is critically involved in the regulation of hepatic TG accumulation and is an inhibitor of Sp1. And our data offer novel insights into the knowledge of mechanisms underlying the pathogenesis of NAFLD.

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Disclosure of conflict of interest

None.

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