Repression of MicroRNA-124-3p Alleviates High-Fat Diet–Induced Hepatosteatosis by Targeting Pref-1

Guan Wang†, Haibo Zou†, Chunyou Lai, Xiaolun Huang, Yutong Yao* and Guangming Xiang*

Department of Hepatobiliary and Pancreatic Surgery, Cell Transplantation Center, Sichuan Academy of Medical Sciences, Sichuan Provincial People’s Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu, China

Nonalcoholic fatty liver disease (NAFLD) is the common disease in the liver, which is associated with metabolic syndrome and hepatocellular carcinoma. Accumulated evidence establishes that small non-coding microRNAs (miRNAs) contribute to the initiation and progression of NAFLD. However, the molecular repertoire of miRNA in NAFLD is still largely unknown. Here, using an integrative approach spanning bioinformatic analysis and functional approaches, we demonstrate that miR-124-3p participates in the development of NAFLD by directly targeting preadipocyte factor-1 (Pref-1). In response to high-fat diet (HFD), expression of miR-124-3p was increased in the liver. Inhibition of miR-124-3p expression led to a dramatic reduction of triglyceride contents in hepatocytes, in parallel with decreased inflammatory factors. Mechanistically, miR-124-3p directly controls the transcription of Pref-1, a secretory factor that has been proved to resist metabolic syndrome. Our work identifies a novel molecular axis in hepatosteatosis, and highlights miR-124-3p/Pref-1 as potential targets for clinical interventions of NAFLD.

Keywords: nonalcoholic fatty liver disease, high-fat diet, miR-124-3p, Pref-1, miRNA

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease, which is characterized by excessive hepatic fat deposition and a wide range of pathologies encompassing steatohepatitis, fibrosis, cirrhosis, liver failure, and hepatocellular carcinoma (1, 2). A number of molecular abnormalities that occur in hepatosteatosis confer to the initiation and progression of NAFLD. Evidence from experimental and clinical studies suggests a complicated interplay of multiple biological processes in the disease development, including obesity, dysbiosis of the intestinal microbiome, heightened intestinal barrier permeability, metabolic endotoxemia and inflammations (3–5). Despite the high prevalence and clinical importance, factors leading to NAFLD were still poorly understood and few efficacious therapies exist (6). Therefore, molecular understandings of the initiation and progression of NAFLD are urgently needed to provide a basis for therapeutic design.
MicroRNAs (miRNAs) are small non-coding RNAs with 18–24 nucleotides, which regulates gene expression by binding to mRNAs and impairs the process of translation (7). MiRNAs are emerging as new regulators of glucose and lipid metabolism during liver development and disease progression, including NAFLD (8, 9). For example, hepatic miR-122 affects gene expressions in cholesterol and lipid metabolism, and thereby maintains liver homeostasis (10, 11). miR-27a attenuated hepatic de novo lipogenesis and alleviated obesity-initiated NAFLD by inhibiting FASN and SCD1 in liver (12). miR-375 is up-regulated in NASH patients, and its inhibition ameliorates lipid accumulation and decreases inflammatory cytokines (13, 14). Despite these studies, however, the molecular repertoire of miRNA in NAFLD is still largely unknown.

In this study, we reveal a novel function of miR-124-3p in hepatic lipid metabolism. MiR-124-3p expression in the liver is increased under high-fat diet (HFD). Inhibition of MiR-124-3p reduces triglyceride contents as well as inflammatory factors in hepatocytes, and vice versa. Mechanistically, miR-124-3p directly controls the transcription of Pref-1, a secretory factor that resists metabolic syndrome. Our work establishes the novel function of miR-124-3p in maintaining liver homeostasis, and defines miR-124-3p as a potential target for clinical interventions of NAFLD.

MATERIALS AND METHODS

Animals and Cell Culture

C57BL/6J mice were obtained and raised in the animal center of Sichuan Academy of Medical Science. Experimental mice were kept in SPF conditions with standard housing conditions in a temperature-controlled environment with 12-h light/dark cycles and received chow diet (CD) or high-fat diet (HFD, from 8-week age) and water ad libitum for 12 weeks (15). Animals studies were conducted in accordance with institutional guidelines. For primary hepatocyte culture, we conducted a two-step collagenase perfusion method as described (16). Isolated hepatocytes were cultured in Williams E medium (Sigma) including HepExtend™ Supplement (Gibco), plus penicillin/streptomycin and gentamycin in 37°C under 5% CO2.

Oil Red O Staining

For oil red O staining to validate the hepatosteatosis in mice with HFD, fresh liver samples were fixed in 4% paraformaldehyde (PFA) solution, and embedded with OCT for subsequent staining with Oil Red O, according to standard protocols (17).

MicroRNA Assay and Analysis

To detect the profile of miRNAs in mouse livers under HFD and CD, a total of miRNAs were extracted using mirNeasy mini kit (QIAGEN). The microarray hybridization of miRNAs was performed using GeneChip miRNA 3.0 Array (Affymetrix). Microarray datasets were presented in volcano plot and GO biological process classification, and miRNAs with 2-fold or greater fold change with P-value < 0.05 were considered differentially expressed.

Plasmid Construct and Luciferase Reporter Assays

To confirm that miR-124-3p directly targets Pref-1, a luciferase reporter assay was performed. Briefly, the pmirGL-control luciferase vector, pmirGLO dual-Luciferase miRNA target expression vector and luciferase reporter assay system were purchased from Toubia (China). The pmirGLO-control vector was used to construct pGL-pref-1, which contained the 3’UTR of mouse pref-1 (Genbank accession NM0003836). DNA fragment of Pref-1 was constructed into vector (18). For luciferase reporter assay, control vector or miR-124-3p plasmid and reporter plasmids were co-transfected into cells. Luciferase activities were measured at 48 h post-transfection by using the dual-luciferase assay system (Turner BioSystem, USA).

Protein Lysis and Western Blots

For western blots, cultured cells were lysed in 2% SDS buffer plus protease and phosphatase inhibitors (Thermo Scientific, USA). The lysates of equivalent total proteins were separated on SDSPAGE. Then proteins were transferred into PVDF membrane (Millipore) for incubation with primary/secondary antibodies. The primary antibodies used were as follows, Pref-1 (Cell Signaling Technology, Cat#2069) and GAPDH (Cell Signaling Technology, Cat#2118).

RNA Extraction and qPCR

Total RNA was extracted by Animal miRNA Isolation Kit (Foregene, China) and Cell Total RNA Isolation Kit (Foregene, China) followed by standard procedure. The PrimeScriptTM RT reagent Kit (Takara) was used to reverse transcript to cDNA, and SYBR Premix Ex TaqTM II (Takara) was used to qPCR assay in Bio-rad system. The qPCR primers were list as follows: miR-124-3p, F: 5’-TAAGGACACGCGTGTAATGCC-3’, R: 5’-GATTGAATCGGACACCGATGTAC-3’; TNFα, F: 5’-CAGGGCTTGCTCTGCTCT-3’, R: 5’-GGATCTCCCCCAGGTTTGATT-3’; Pref-1, F: 5’-TTCGGCCACAGCACCTATG-3’, R: 5’-GGGAGCTGTACACCTTGTCAT-3’.

RNA Interference

For Pref-1 knockdown, targeted siRNA were transfected into cells using Lipofectamine RNAiMAX reagent (Invitrogen, USA) in Opti-MEM (Invitrogen, USA) over 72 h. The siRNA sequences were as follows, siRNA negative-control: sense-UCC GGAACUGUUACGUGAA; antisense-UUCACGUAAACAG UUCCGGA. siRNA Pref-1: sense: UCCUGAAGGUGUCC AUGGA; antisense: UUCAUGGACCCUUCCAGGA.

Statistical Analysis

Statistical analysis was performed in GraphPad Prism. Data were expressed as mean ± SD at least three independent experiments. Statistical differences between two groups were analyzed by Student’s t test, and multi-group comparisons by one-way ANOVA followed by Tukey post hoc tests. P<0.05 was considered statistically significant; **indicates p<0.01, and *** p<0.001.
RESULTS

MiR-124-3p Expression Is Increased by HFD-Induced Hepatosteatosis

To investigate the miRNAs involved in hepatosteatosis, we assessed the hepatic miRNA profile in C57BL/6J mice fed with high fat diet (HFD) for 12 weeks, in comparison to littermate controls fed with chow diet (CD). Then we extracted the total miRNA from mouse livers and performed microarray hybridization using GeneChip miRNA 3.0 Array (Affymetrix). Histochemical results confirmed the lipid accumulation by Oil Red O staining in the liver of HFD mice (Figure 1A). By miRNA screening, we found that altered miRNAs between HFD and CD mice were enriched in metabolic process by GO (Gene Ontology) biological process classification (Figure 1B). As shown in the volcano plot, we differentiated the increased and decreased miRNAs. Particularly, it was found that miR-124-3p was dramatically increased in the liver of HFD mice (Figure 1C). And this increasing was further validated by quantitative evaluation from more paired HFD/CD mice (Figure 1D). All these data suggest that increased miR-124-3p may play a vital role in HFD-induced hepatosteatosis.

MiR-124-3p Positively Regulates Lipid Contents in Hepatocytes

Based on the finding that miR-124-3p was induced by HFD in mouse liver, we asked whether induction of miR-124-3p has a causal effect on lipid accumulation. Therefore, we examined the lipid contents in hepatocytes after miR-124-3p inhibition. As a first step, we knocked down miR-124-3p expression by transfection of its inhibitors in primary hepatocytes, and the transfection efficiency was confirmed by RT-qPCR (Figure 2A). Next, we inactivated miR-124-3p expression in palmitic acid (PA) treated hepatocytes (Figure 2B). Although PA could increase triglycerides in control hepatocytes (19), however, miR-124-3p inhibitors dramatically reduced triglyceride contents by PA treatment (Figure 2C). Excessive lipid accumulation often coincides with increased inflammation (20). We found that miR-124-3p inhibitors could also decrease the level of TNFα by PA treatment, indicating that miR-124-3p inactivation...
con reporter plasmid with a luciferase reporter assay. Data showed that the activity of the examined whether overexpression of Pref-1 in Hepatocytes.

Pref-1 is a transmembrane protein that could be cleaved at the extracellular domain to generate a soluble form that reduces hepatosteatosis and hyperglycemia (21, 22). To study whether miR-124-3p regulates hepatic lipid metabolism through Pref-1, we examined the effect of Pref-1 after PA treatment in miR-124-3p inactivated hepatocytes. Western blot results showed that miR-124-3p inhibitors could induce expression of full and cleaved Pref-1 with or without PA (Figures 4A–C). It’s noted that Pref-1 expression was slightly increased by PA, however, dramatically increased by miR-124-3p inhibitors (Figures 4A–C), suggesting that miR-124-3p inactivation was necessary for Pref-1 expression under lipid stress conditions.

To strengthen the point that miR-124-3p regulates hepatosteatosis through Pref-1, we examined the effect of Pref-1 knockdown on the lipid metabolism and inflammation in hepatocytes (Figures 4D–F). Results showed that Pref-1 knockdown did not dramatically alter triglyceride levels or TNFα expression under basal condition. However, in the presence of PA, Pref-1 knockdown caused a more dramatic increasing of triglyceride levels or TNFα expression, indicating that Pref-1 is required for the resistance of triglyceride accumulation under lipid stress (Figures 4G–H). Thus, we conclude that miR-124-3p regulated Pref-1 expression is critical for the maintenance of lipid homeostasis in hepatocytes.

ameliorsates liver inflammation (Figure 2D). Reciprocally, we examined whether overexpression of miR-124-3p by its mimics is sufficient to increase lipid contents and inflamations in hepatocytes. Results showed that miR-124-3p overexpression increased triglyceride levels and inflammation in hepatocytes (Figures 2E–H). Taken together, these results suggest that miR-124-3p positively regulates lipid contents and inflammation in hepatocytes.

Pref-1 has been proved to resist HFD-induced hepatosteatosis and obesity (23, 24). To study whether miR-124-3p regulates hepatic lipid metabolism through Pref-1, we assessed the changes of Pref-1 after PA treatment in miR-124-3p inactivated hepatocytes. Western blot results showed that miR-124-3p inhibitors could induce expression of full and cleaved Pref-1 with or without PA (Figures 4A–C). It’s noted that Pref-1 expression was slightly increased by PA, however, dramatically increased by miR-124-3p inhibitors (Figures 4A–C), suggesting that miR-124-3p inactivation was necessary for Pref-1 expression under lipid stress conditions.

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**MiR-124-3p Directly Targets Pref-1 in Hepatocytes**

To clarify how miR-124-3p participates in the regulation of lipid metabolism, we next examined the potential targets of miR-124-3p. By sequence analysis, we found that Preadipocyte factor 1 (Pref-1) was a potential candidate of miR-124-3p in the liver (Figure 3A). Pref-1 is a transmembrane protein that could be cleaved at the extracellular domain to generate a soluble form that reduces hepatosteatosis and hyperglycemia (21, 22). To confirm that miR-124-3p directly targets Pref-1, we performed a luciferase reporter assay. Data showed that the activity of the reporter plasmid with miR-124-3p mimic was decreased, indicating that miR-124-3p binds to the 3′-UTR of Pref-1 and thus regulates Pref-1 expression (Figure 3B). Results of qPCR showed that the mRNA level of Pref-1 was decreased by miR-124-3p overexpression and increased by its knockdown (Figure 3C). Consistently, the protein levels of both full and cleaved Pref-1 were both negatively regulated by miR-124-3p (Figures 3D–F).

Therefore, we propose that miR-124-3p directly targets Pref-1 to regulate its expression.

**miR-124-3p Regulates Lipid Contents via Pref-1 in Hepatocytes**

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overexpression of miR-124-3p decreased PA-induced triglyceride levels, and particularly, in hepatocytes. Our data showed that inhibition of expression may have a causal effect on the lipid accumulation expression is dynamically regulated by lipid stress hepatocytes. Therefore, we conclude that miR-124-3p triglyceride level. Moreover, we revealed that increased PA treatment. Thus, miR-124-3p treatment could induce This increasing was consistent with our finding that PA knockdown alone failed to produce a similar effect like miR-124-3p overexpression (Figure 4G), suggesting that miR-124-3p may regulate lipid metabolism partly through Pref-1 independent mechanisms. Interestingly, after PA treatment, Pref-1 knockdown phenocopies the effect of miR-124-3p overexpression, indicating that miR-124-3p-controlled lipid accumulation is largely

DISCUSSION

Altered miRNA expression in response to genetic/epigenetic factors or environmental conditions confers to the onset of NAFLD (25). However, the molecular repertoire of miRNA in the initiation and progression of NAFLD is largely unknown. The present study demonstrates that miR-124-3p participates in hepatosteatosis in response to lipid stress. Inhibition of miR-124-3p in hepatocytes promotes Pref-1 expression and thus ameliorates lipid accumulation (Figure 4I).

In decades, numerous miRNAs were identified to regulate a wide spectrum of metabolic processes, including lipid homeostasis, glucose catabolism, and inflammation, which were known to be epigenetically deregulated in NAFLD (26). Altered hepatic miRNA profile has been described in both in humans and animal models of NAFLD (27). A recent study assessed circulating miRNAs in NASH patients and found that among 84 circulating miRNAs (13). For example, miR-122 and miR-192 were significantly upregulated and others were downregulated. Comparing to these studies, we revealed that miR-124-3p was particularly increased in the liver of HFD mice. This increasing was consistent with our finding that PA treatment could induce miR-124-3p expression in primary hepatocytes. Therefore, we conclude that miR-124-3p expression is dynamically regulated by lipid stress in vitro and in vivo. Moreover, we revealed that increased miR-124-3p expression may have a causal effect on the lipid accumulation in hepatocytes. Our data showed that inhibition of miR-124-3p decreased PA-induced triglyceride levels, and particularly, overexpression of miR-124-3p is sufficient to increase triglyceride level (Figure 2G), which is more pronounced after PA treatment. Thus, miR-124-3p is a critical regulator in lipid homeostasis of the liver. The impact of miR-124-3p on lipid metabolism indicates its potential therapeutic implication of NAFLD.

MiR-124-3p is a widely expressed miRNA in mammalian cells, and previous studies reveal its biological function in tumorigenesis and neural diseases. For example, miR-124-3p regulates aerobic glycolysis and induces chemoresistance in glioma cells through AMPK pathway (28). miR-124-3p also represses the migration and invasion of bladder cancer cells via ROCK1 (29). In the liver, miR-124-3p modulates autophagy through Beclin and LC3 and thus participates in hepatic impact injury (30). However, the role of miR-124-3p in metabolic diseases is not yet well understood. A recent study showed that miR-124 represses genes associated with fatty acid and triglyceride breakdown, and thus promotes triglyceride accumulation in hepatoma cells (31). Consistent with this finding, our study demonstrate that miR-124-3p has a direct effect in the liver under lipid stress conditions. We further revealed that miR-124-3p directly targets Pref-1 that resists hepatic lipid accumulation. Pref-1 belongs to the NOTCH family of epidermal growth factor-like repeat-containing proteins, which can inhibit adipogenesis and resist to high fat diet-induced obesity in mice (21, 32). In this study, we identified that miR-124-3p negatively regulates the expression of Pref-1 and its soluble fragment. It’s noticed that miR-124-3p overexpression could induce a slight but reproducible triglyceride increasing in hepatocytes under basal condition (Figure 2G). However, Pref-1 knockdown alone failed to produce a similar effect like miR-124-3p overexpression (Figure 4G), suggesting that miR-124-3p may regulate lipid metabolism partly through Pref-1 independent mechanisms. Interestingly, after PA treatment, Pref-1 knockdown phenocopies the effect of miR-124-3p overexpression, indicating that miR-124-3p-controlled lipid accumulation is largely
dependent on Pref-1 under lipid stress condition. Nevertheless, our study at least reveals that miR-124-3p/Pref-1 is a novel molecular axis in hepatosteatosis. Future studies would be directed at the assessment of miR-124-3p/Pref-1 axis in resistance to hepatosteatosis in animal models and clinical trials.

CONCLUSION

In summary, our study identifies a novel function of miR-124-3p in the liver. Hepatocytes with miR-124-3p knockdown have decreased triglyceride contents and inflammatory cytokines. This work provides a therapeutic target for potential interventions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Sichuan Provincial People’s Hospital.

AUTHOR CONTRIBUTIONS

GW, YY, and GX designed the study and wrote the manuscript. GW, HZ, CL, XH, YY, and GX performed the experiments and analyzed the data. All authors contributed to the article and approved the submitted version.

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