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ORIGINAL ARTICLE

Downregulation of ubiquitous microRNA-320 in hepatocytes triggers RFX1-mediated FGF1 suppression to accelerate MASH progression



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Abstract Metabolic dysfunction-associated steatohepatitis (MASH), a severe type of metabolic dysfunction-associated steatotic liver disease (MASLD), is a leading etiology of end-stage liver disease worldwide, posing significant health and economic burdens. microRNA-320 (miR-320), a ubiquitously expressed and evolutionarily conserved miRNA, has been reported to regulate lipid metabolism; however, whether and how miR-320 affects MASH development remains unclear. By performing miR-320 *in situ* hybridization with RNAscope, we observed a notable downregulation of miR-320 in hepatocytes during MASH, correlating with disease severity. Most importantly, miR-320 downregulation in hepatocytes exacerbated MASH progression as demonstrated that hepatocyte-specific miR-320 deficient mice were more susceptible to high-fat, high-fructose, high-cholesterol diet (HFHC) or choline-deficient, amino acid-defined, high-fat diet (CDAHFD)-induced MASH compared with control littermates. Conversely, restoration of miR-320 in hepatocytes ameliorated MASH-related steatosis and fibrosis by injection of adeno-associated virus 8 (AAV8) carrying miR-320 in different types of diet-induced MASH models. Mechanistic studies revealed that miR-320 specifically regulated fibroblast growth factor 1 (FGF1) production in hepatocytes by inhibiting regulator factor X1 (RFX1) expression. Notably, knockdown of *Rfx1*

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in hepatocytes mitigated MASH by enhancing FGF1-mediated AMPK activation. Our findings underscore the therapeutic potential of hepatic miR-320 supplementation in MASH treatment by inhibiting RFX1-mediated FGF1 suppression.

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1. Introduction

Due to the heterogeneity and limitation of nonalcoholic fatty liver disease (NAFLD), metabolic dysfunction-associated steatotic liver disease (MASLD) has been officially proposed as a replacement for NAFLD and metabolic dysfunction-associated steatohepatitis (MASH) as a substitution for nonalcoholic steatohepatitis (NASH) in 2023¹. The change of nomenclature from NAFLD to MASLD highlights that MASLD is a chronic liver disease combined with obesity, insulin resistance, dyslipidemia, or hyperglycemia in the absence of excessive alcohol intake, which is characterized by systemic metabolic dysfunction resulting in hepatic excess lipid accumulation and encompasses metabolic dysfunction-associated steatotic liver (MASL) and MASH^{1,2}. Accumulating evidence indicates that approximately 99% of patients with NAFLD conform to MASLD criteria³. Compared to the phenotype and reversibility of MASL, MASH syndrome with more severe steatosis, ballooning, immune cell infiltration, and fibrogenesis may further develop into irreparable fibrosis, MASH-related cirrhosis, and hepatocellular carcinoma (HCC)⁴. In adults, approximately 20% of individuals with MASL progress to MASH, and 20%–50% of patients with MASH develop fibrosis or cirrhosis⁴. Similarly, in pediatric patients with MASLD diagnosed by biopsy, approximately 50% of patients develop MASH, and 16%–31% have advanced fibrosis^{5,6}. Unfortunately, effective FDA-approved drugs for the treatment of MASH remain scarce despite the growing global clinical and economic burden. Thus, there is an urgent need to elucidate the pathogenesis of MASLD and develop novel therapeutics specific to MASH.

The pathogenesis of MASH is complicated, and multiple factors simultaneously contribute to the development and progression of MASH. Initially, abnormal lipid metabolism in hepatocytes results in the storage of excess energy in the form of triglycerides in the liver and the generation of numerous toxic metabolic intermediates, which further sensitizes and predisposes hepatocytes to the following events. Subsequently, lipotoxicity, oxidative stress, immune response activation, and altered intestinal microbiome drive the progression of MASH^{7,8}. Metabolic dysfunction in hepatocytes is the root cause of MASLD development, although hepatic inflammation is believed to be the leading cause of MASL progression to MASH^{8–10}. Therefore, maintaining and regulating the balance of lipid metabolism in hepatocytes is critical to prevent the development of MASH. Referring to lipid metabolism in the liver, fatty acids are sourced in two ways: from dietary or blood intake and *de novo* lipogenesis (DNL), while their disposal fate is mitochondrial β -oxidation and storage as triglycerides. Although these processes are strictly regulated by many transcription factors and metabolic enzymes, such as sterol regulatory element binding protein-1c (SREBP-1c), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS), DNL is upregulated by 20%–30% in patients with MASLD compared to healthy individuals^{11–13}. To date, several candidate drugs targeting DNL and

hepatic lipid metabolism have shown promising results in treating MASLD in clinical trials including the ACC inhibitor Firsocostat, the fibroblast growth factor 21 (FGF21) receptor agonist Pegbelfermin (BMS986036), and the THR- β agonist Resmetirom (MGL3196)¹⁴. Recently, the FDA approved the first drug, MGL3196, for the treatment of MASLD¹⁵, suggesting that increasing liver-directed fat metabolism and reducing lipotoxicity are indispensable approaches for treating MASLD.

MicroRNAs (miRNA) are small noncoding RNAs with 20–24 nucleotides in length that function as degradation-targeted messenger RNA (mRNA) and/or repressors of their translation¹⁶. Different cell types¹⁷, such as hepatocyte-specific miR-122¹⁸, neutrophil-specific miR-223^{19,20}, and macrophage-specific miR-155²⁰, have unique miRNA profiles that play key roles in the pathogenesis of liver diseases. Unlike miRNAs that are specifically expressed or enriched in several cell types, miR-320 is an evolutionarily conserved and ubiquitously expressed miRNA^{21,22}, suggesting that miR-320 may be involved in fundamental cellular processes such as cell development, differentiation, and homeostasis.

In the present study, we demonstrated that hepatic levels of ubiquitous miR-320 are significantly downregulated in humans with MASH and in experimental mouse MASH models. More importantly, such downregulation may accelerate MAFLD progression as evidenced that hepatocyte-specific deletion of miR-320 exacerbates MASH, while restoration of miR-320 improves different types of diet-induced steatosis and fibrosis. Mechanistically, miR-320 limits hepatic lipogenesis by maintaining the production of anti-lipogenic fibroblast growth factor 1 (FGF1), which is controlled by the negative transcription factor regulatory factor of X-box 1 (RFX1) in hepatocytes. Silencing *Rfx1* in hepatocytes remarkably elevates FGF1 levels, thus ameliorating the development of MASH. Taken together, ubiquitous miR-320 in hepatocytes plays a protective role in the development of MASH, and supplementation with hepatic miR-320 may be a novel potential therapeutic strategy for the treatment of MASH.

2. Materials and methods

2.1. Human MAFLD datasets

The expression profiles of serum miR-320 in healthy volunteers and MASH patients were obtained from the public datasets GSE33857 and GSE89632. The correlations between miR-320 levels and hepatic gene expression, serum alanine aminotransferase (ALT), aspartate transaminase (AST), and severity score of MASH in 20 simple steatosis (MASL) patients, 19 MASH patients, and 24 healthy controls were analyzed from the public dataset GSE89632. Hepatic miR-320 expression in 5 patients with MASH-induced cirrhosis (MASH-CH) and 6 non-injured liver control samples from the public dataset GSE59492 was analyzed.

2.2. Mice

miR-320^{fllox/fllox} mice (Strain No. T007902) were purchased from GemPharmatech (Nanjing, China). Hepatocyte-specific miR-320 knockout mice (miR-320^{ΔHep}) were generated by several steps including crossing miR-320^{fllox/fllox} mice with albumin-Cre (Alb-Cre) mice. Alb-Cre mice were kindly provided by Prof. Cen Xie (SIMM, Chinese Academy of Sciences, Shanghai, China). For the miR-320 deletion experiment in adults, adeno-associated virus serotype 8 (AAV8)-TBG-Cre and its scrambled control (AAV8-scramble) were constructed by OBio (Shanghai, China). Eight-week-old miR-320^{fl/fl} male mice were injected intravenously with AAV8-TBG-Cre (2×10^{11} viral genomes) in a total volume of 150 μ L.

For the mouse models of MASH, mice were fed a high-fat, high-fructose, high-cholesterol diet (HFHC) containing 40% fat, 22% fructose, and 2% cholesterol (catalog No. D09100310, Research Diet, New Brunswick, USA) for 16 weeks, or choline-deficient, amino acid-defined, high-fat diet (CDAHFD, catalog no. A06071302, Research Diets, New Brunswick, USA) for 4 or 6 weeks.

For overexpression of miR-320 in the liver, AAV8-miR-320-ZsGreen (promoter U6, AAV8-miR-320) and its control viruses AAV8-null-ZsGreen (AAV8-scramble) were constructed by HanBio (Shanghai, China). Eight-week-old C57BL/6J male mice were purchased from Beijing Vital River Laboratory. After one week of adaptive feeding, the mice were fed an HFHC diet for 12 weeks and injected intravenously with AAV8-miR-320 and AAV8-scramble in a total of 1×10^{11} viral genomes per mouse, and then AAV8-miR-320 group and AAV8-scramble group mice were kept another 4-week HFHC diet. Similar to the experiment of overexpression of miR-320 in the liver, AAV8-*Rfx1* shRNA-EGFP (promoter U6, AAV8-sh*Rfx1*, HanBio) and its control virus AAV8-null-EGFP (AAV8-scramble, 1.5×10^{11} viral genomes per mouse) were intravenously injected into C57BL/6J male mice at 12 weeks after HFHC diet feeding to silence hepatic *Rfx1* expression. For the overexpression of miR-320 in low-density lipoprotein receptor (*Ldlr*) knockout mice (Strain No. 002207, The Jackson Laboratory, Bar Harbor, ME, USA), the mice were fed a high-fat diet (HFD, 60 kcal% fat; catalog no. D12492; Research Diets, New Brunswick, USA) for 8 weeks, and then injected with AAV8-miR-320 and its AAV8-scramble control (1×10^{11} viral genomes). After the AAV8 injection, all the above mice were fed an HFD or an HFHC for another 4 weeks.

All animal experiments were authorized by the Institutional Animal Care and Use Committee (IACUC) of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, and abided by the Institutional Ethical Guidelines on Animal Care. The IACUC approval no: 2021-09-HY-02, 2022-07-HY-03 and 2023-07-HY-06. All mice were housed in constant-temperature, specific pathogen-free rooms with a 12-h light/dark cycle and unrestricted access to food and water.

2.3. miRNA in situ hybridization

miRNA *in situ* hybridization was performed as previously reported²³. The probe for miR-320 and the miRNAscope™ HD Reagent Kit-red were purchased from Advanced Cell Diagnostics (Advanced Cell Diagnostics, catalog No. 324500, CA, USA) and the experiment was conducted following the manufacturer's instructions. In brief, liver-frozen sections were fixed with 4% PFA overnight and washed twice with PBS following dehydration with

different concentrations of ethanol. Then, the sections were pre-digested with protease for 30 min before hybridization with a preheated miR-320 probe in a hybridizer at 40 °C for 2 h. After that, the sections were hybridized with the detection reagents and stained with Fast Red reagents. Finally, after the termination of staining, the sections were counterstained with hematoxylin (Servicebio, catalog no. CR2301055, Wuhan, China) and mounted with neutral balsam (MACKLIN, catalog C16129365, Shanghai, China). Images were obtained using an Olympus BX43 microscope or an Olympus APEXVIEW APX100 microscope.

2.4. Bulk RNA sequencing (bulk RNA-seq)

RNA high-throughput sequencing was performed by Shanghai Majorbio Biopharm Biotechnology Co., Ltd. (Shanghai, China). Briefly, total RNA was extracted from the tissue using TRIzol® Reagent and reverse transcribed into double-stranded cDNA using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA libraries were constructed by Illumina® Stranded mRNA Prep, Ligation from Illumina (San Diego, CA, USA) using 1 μ g of total RNA. Libraries were size-selected for 300 bp cDNA target fragments using Phusion DNA polymerase. After quantification, the paired-end RNA-seq library was sequenced using a NovaSeq Xplus sequencer. The initial paired-end reads were shaped and quality controlled, and the resulting clean reads were aligned to the reference genome by HISAT2 software. Differentially expressed genes (DEGs) were identified according to the level of each transcript and gene abundance using the transcripts per million reads (TPM) method and RSEM. Furthermore, significantly differentially expressed genes are considered as the DESeq2 with $|\log_2FC| \geq 1$ and $FDR < 0.05$ (DESeq2). All data were analyzed on the Majorbio Cloud Platform (<https://cloud.majorbio.com/>).

2.5. Tissue processing and histological analysis

Liver tissues were fixed with 10% formaldehyde, embedded in paraffin, sectioned into 5- μ m-thick slices, and dewaxed with ethanol and xylene briefly. Hematoxylin and eosin (H&E) staining for histological analysis was performed by using H&E stain solution (catalog No. G1004, Servicebio, Wuhan, China) according to the manufacturer's instructions. For Sirius red staining, paraffin sections were stained with Picrosirius red solution (catalog No. PH1098, PhyGene, Fuzhou, China) for 2 h, and washed with 0.5% acetic acid three times for 5 min. Oil Red O staining was used to assess hepatic steatosis in OCT-embedded liver tissues. Briefly, frozen liver sections were stained with Oil Red O (catalog No. O0625, Sigma-Aldrich, St. Louis, MO, USA) solution in 60% isopropanol for 40 min, washed with 60% isopropanol three times for 30 s and counterstained with hematoxylin for 10 s. After washing and dehydration, the sections were covered with coverslips and imaged using an Olympus BX43 microscope (Olympus, Tokyo, Japan).

2.6. Immunohistochemistry (IHC) and immunofluorescence staining

Paraffin-embedded sections were subjected to antigen retrieval with citrate buffer (pH 6.0, catalog No. 005000, Thermo Fisher Scientific, Waltham, MA, USA). The slices were washed with PBS and treated with 3% hydrogen peroxide (H₂O₂) for 15 min to

remove endogenous peroxidase activity. After blocking with 3% normal goat serum buffer (NGS) (catalog No. abs933-50 ml, Absin), the sections were incubated with primary antibodies at 4 °C overnight. Afterward, the sections were washed with PBS and incubated with secondary antibodies (catalog No. 8814S or 8125S, Cell Signaling Technology [CST], Danvers, MA, USA) at room temperature for 40 min. After incubation, the sections were rewashed and incubated with DAB substrate (catalog No. SK-4105, Vector Laboratories, Burlingame, CA, USA) for visualization. The primary antibodies used were as follows: 4-hydroxynonenal (4-HNE, catalog No. MHN-100P, JaICA, Shi-zuoka, Japan), Malondialdehyde (MDA, catalog No. MMD-030n, JaICA), F4/80 (catalog No. 70076S, CST), Lymphocyte antigen 6 complex locus G6D (Ly6G, catalog No. 87048S, CST), alpha smooth muscle actin (α -SMA, catalog No. 19245S, CST), FGF1 (catalog No. PA5-79249, Invitrogen, CA, USA), and RFX1 (catalog No. A303-043A, Bethyl Laboratories, Hamburg, Germany). For FGF1 immunostaining in AML-12 cells, the cells were seeded on Nunc™ Lab-Tek™ Chamber slides and transfected with miR-320 mimics or *Rfx1* siRNA for 48 h. The slides were fixed with 4% paraformaldehyde for 2 h at room temperature and washed three times in PBS before incubation with an anti-FGF1 antibody at 4 °C overnight. Then, the cells were handled by the same step like hepatic FGF1 immunostaining, including washing, incubation with secondary antibodies, and DAB substrate staining. All Images were obtained using an Olympus BX43 microscope and quantified using Image J software. Due to the nuclear localization and ubiquitous expression of RFX1, a quantitation analysis of RFX1 immunostaining was performed using the Integrated option density (IOD). The quantification analysis was performed using Image J plus the “IHC profile” plug-in.

To detect FGF1 by immunofluorescence, paraffin-embedded sections were blocked with 3% NGS for 1 h and then incubated with anti-FGF1 (catalog No. PA5-79249, Invitrogen) and anti-hepatocyte nuclear factor 4 alpha (HNF4 α , hepatocyte marker, catalog No. MA1-199, Invitrogen) antibodies at 4 °C overnight. After washing, the slides were incubated with secondary antibodies (Alexa Fluor 488 conjugate goat anti-rabbit IgG [H + L], CST, catalog No. 4412S; Alexa Fluor 555 conjugate goat anti-mouse IgG [H + L], CST, catalog No. 4409S, Danvers, MA, USA) for 40 min. Nuclear staining was performed using 4',6'-diamino-2-phenylindole (DAPI) (Beyotime, catalog No. P0131, Shanghai, China). Images were captured with an Olympus APEXVIEW APX100 microscope (Olympus; Tokyo, Japan).

2.7. RNA isolation and quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from liver tissues or cell lysates using TRIzol reagent (catalog No. R401-01-AA, Vazyme, Nanjing, China) following the manufacturer's instructions. For mRNA quantitative real-time PCR, first-strand cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (catalog No. 4368813, Thermo Fisher Scientific) according to the manufacturer's instructions. Gene expression was detected by a ChamQ SYBR qPCR Master Mix kit (catalog No. Q311-02, Vazyme) in an Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific). The mRNA levels of 18S were used as an internal control. Each test was performed in triplicate and the $2^{-\Delta\Delta C_t}$ method was used to calculate the relative expression of mRNA.

For the miR-320 quantitative assay, reverse transcription reactions were performed with a miRNA 1st Strand cDNA Synthesis

Kit (by stem-loop) (catalog No. MR101-02, Vazyme). Then, the level of miR-320 was measured using a miRNA Universal SYBR qPCR Master Mix Kit (catalog No. MQ101-02, Vazyme) according to the manufacturer's instructions. snoRNA202 was used for the normalization of miRNA expression. The mRNA and miRNA primers used for RT-qPCR are listed in Supporting Information Tables S1 and S2.

2.8. Cell culture and mouse primary hepatic cell isolation

The mouse AML-12 cell line was purchased from the American Type Culture Collection (ATCC, US) and cultured with F12/DMEM (catalog No.10-092-CVRC, Corning, NY, USA) supplemented with 10% fetal bovine serum (catalog No. 10091-148, Gibco, NY, USA), 1% Insulin-Transferrin-Selenium (catalog No. 41400045, Gibco), 40 ng/mL dexamethasone (Cat: D4902, Sigma) and 1% penicillin–streptomycin (catalog No. 15140-122, Gibco) in a humidified incubator at 37 °C with 5% CO₂. For miRNA transfection assays, cells were seeded at 5×10^4 /mL in 24-well plates and transfected with 50 nmol/L miR-320 mimics (sense: AAAAGCUGGGUUGAGAGGGCGA, antisense: GCCCUCUC AACCCAGCUUUUUU, Sangon), *Rfx1* siRNA (sense: CC AGUUA AUGCCGCCUCUUTT, antisense: AAGAGGCGGC AUUAAACUGGTT; Sangon) or control siRNA/mimics for 48 h using Lipofectamine RNAiMAX transfection reagent (catalog No. 2487720, Invitrogen).

Mouse primary hepatocytes were performed as previously described²⁴. In brief, ethylene glycol tetraacetic acid (EGTA) buffer perfusion was used to remove blood cells and extracellular calcium. A total of 0.075% Collagenase Type I (catalog No. 41J21479, Worthington, CO, USA) in $1 \times$ HBSS buffer with 0.002% DNase I was perfused to digest the liver into single cells. Isolated hepatocytes were seeded at 5×10^5 /mL in 12-well plates and cultured in DMEM (catalog No. 10-013-CV, Corning) supplemented with 10% FBS and 1% penicillin–streptomycin. Mouse hepatic stellate cell (HSC) and Kupffer cell isolation were performed as previously described²⁵. After EGTA and Collagenase Type I perfusion and centrifugation, the cell fraction between GBSS and 11.5% opti-prep (catalog No. D1556, Merck, Darmstadt, Germany) was gently aspirated and washed again to obtain HSCs. The cell fraction between 20% opti-prep and 11.5% opti-prep was Kupffer cells, which were then collected and washed again.

2.9. Biochemical analysis of serum and liver samples

The ALT, AST, triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) levels were measured by the corresponding kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions and detected by a Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

For hepatic TG measurement, the liver tissues were weighed and homogenized using lysis buffer at a ratio of 1:9, and then the content of TG was measured using the Triglyceride assay kit (Nanjing Jiancheng Bioengineering Institute) by Multiskan FC microplate reader according to the manufacturer's instructions and the concentration of protein was quantified by BCA assay kit (Thermo Fisher Scientific) to normalize the hepatic TG content.

2.10. BODIPY staining

An indicator of neutral lipid dye, BODIPYTM 493/503 (catalog No. D3922, InvitrogenTM), was used to assess lipid accumulation in AML-12 cells *via* fluorescence staining and flow cytometry. AML-12 cells were seeded in NuncTM Lab-TekTM Chamber slides or 12-well plates and transfected with miR-320 mimics for 24 h before 0.15 mmol/L palmitic acid (PA) treatment for another 16 h. Then the cells were incubated with 2 μ mol/L BODIPY solution in the dark for 20 min at 37 °C and washed with PBS three times. For fluorescence staining, the cells were incubated with DAPI solution for 5 min, and the chamber slides were mounted after washing. Immunofluorescence images were obtained with an Olympus APEXVIEW APX100 microscope (Olympus; Tokyo, Japan). For flow cytometry, after incubation with BODIPY solution, the cells were digested and harvested from 12-well plates. Samples were acquired on a Cytoflex flow cytometer (Beckman Coulter, Brea, CA, USA), and the data were analyzed using CytExpert software.

2.11. Western blotting

Liver tissues and cell samples were homogenized or lysed in RIPA lysis buffer (catalog No. YH374135, Thermo Fisher Scientific) containing Halt Protease and Phosphatase Inhibitors (catalog No. 78447, Thermo Fisher Scientific). All samples were quantified and equally loaded on 10%–12% SDS-PAGE gels and transferred to the nitrocellulose membranes (catalog No. 0000208128, Merck). After blocking with 1% BSA, the membrane was incubated with primary antibodies at 4 °C overnight. Then the membrane was washed with Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated with anti-rabbit or anti-mouse IgG HRP-linked secondary antibodies (catalog No. 7074S or 7076S, CST). The protein bands were visualized with SuperSignal Maximum Sensitivity Substrate (catalog No. WG328673, Thermo Fisher Scientific). The following primary antibodies were used: FGF1 polyclonal antibody (dilution 1:1000, catalog No. PA5-79249, Invitrogen), RFX1 polyclonal antibody (dilution 1:3000, catalog No. A303-043A, Bethyl Laboratories), phospho-AMPK α (Thr172) antibody (dilution 1:1000, catalog No. 2535, CST), AMPK α antibody (dilution 1:1000, catalog No. 2532, CST) and β -Actin rabbit mAb (dilution 1:80,000, catalog no. AC026, ABclonal, Wuhan, China).

2.12. miRNA pull-down assay

The miRNA pull-down assay was performed to verify the direct binding of miR-320 and *Rfx1* using the miRNA pulldown Kit (catalog No. 202407, BersinBio, Guangzhou, China). Biotin-labeled probes of miR-320-3p (Bio-miR-320) and its control (Bio-miR-scr) were commercially synthesized by Gene Adv (Jiangsu, China). AML-12 cells were seeded at 10⁶/mL in a 10 cm dish overnight and transfected with Bio-miR-320 (experimental group) or Bio-miR-scr (negative control group) at a final concentration of 150 nmol/L for 48 h, respectively. AML-12 cells were harvested and lysed with lysis buffer for 20 min. Meanwhile, streptomycin magnetic beads were blocked with a blocking buffer and mildly rotated at 4 °C for 2 h. The lysed mixture was divided into the Input group at the ratio 1:10 and the other lysed mixture was mixed with blocked streptomycin magnetic beads as the RPD group and gently rotated at 4 °C for 4 h. Samples were separated by a magnetic stand and washed for 5 times. 100 μ L RNA Elution buffer was added and beads were boiled for 2 min to elute RNA, which was isolated by Trizol reagent. Finally, RNA was reverse

transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (catalog No. 4368813, Thermo Fisher Scientific). The levels of *Rfx1* using different primers were measured by RT-qPCR using a ChamQ SYBR qPCR Master Mix kit (catalog No. Q311-02, Vazyme).

2.13. Statistical analysis

All the data were analyzed by GraphPad Prism Software (version 9, GraphPad, USA) and are presented as the mean $\pm$ standard error of mean (SEM). All *in vitro* data were collected from three independent experiments. Statistical analyses between two groups were performed with a two-tailed unpaired Student's *t*-test, while differences among multiple groups were compared by one-way analysis of variance (ANOVA) with Tukey's *post hoc* comparison test. *P* values less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. Hepatic miR-320 is significantly downregulated during MASH progression and negatively correlates with the severity of MASH

miR-320, a ubiquitously expressed and highly conserved miRNA, is widely expressed in multiple tissues of mice, such as the liver, heart, and adipose tissues²². As shown in Supporting Information Fig. S1A, miR-320 was indeed widely expressed in different organs. In addition, the miR-320 sequence is highly conserved in different mammalian species, especially, 100% conserved in humans and mice (Fig. S1B). To investigate the function of miR-320 in the development of MASH, we first analyzed the expression of miR-320 in MAFLD patients by utilizing GEO Datasets. As shown in Fig. 1A, serum and hepatic miR-320 levels were significantly downregulated in patients with MASH (GSE33857, GSE89632), or MASH-related cirrhosis compared with healthy control (GSE59492). Notably, the alteration of hepatic miR-320 was closely associated with the severity of MASH as demonstrated that miR-320 levels negatively correlated with the levels of serum ALT, AST, as well as NASH-Associated Score (NAS) and inflammation grade (Fig. 1B). Moreover, hepatic miR-320 levels had a negative correlation with some inflammatory and fibrotic genes, including *TGFB2*, *COL6A1*, *COL6A2*, *COL1A1*, *MMP3*, and *MMP13* (Fig. S1C). Consistent with findings in the human database, hepatic miR-320 was indeed downregulated in the livers of different diet-induced mouse MASH models (Fig. 1C). Finally, *in situ* hybridization with RNAscope analysis further demonstrated that miR-320 levels in the livers of MASH mice were significantly lower than those in the livers of chow diet-fed mice (Fig. 1D). Furthermore, to wonder why miR-320 levels were downregulated in hepatocytes during MASH development, the levels of miR-320 in AML-12 cells were measured after stimulation with some different factors involved in the development of MASH, including metabolic, inflammatory and oxidative stress inducers. As shown in Fig. S1D, miR-320 levels were downregulated after stimulation with high glucose or Lipopolysaccharides (LPS), as well as buthionine sulfoximine (BSO) treatment also contributed to the tendency of miR-320 downregulation, indicating that the downregulation of hepatic miR-320 was orchestrated with multiple factors. These results strongly indicated that the level of ubiquitous miR-320 is significantly

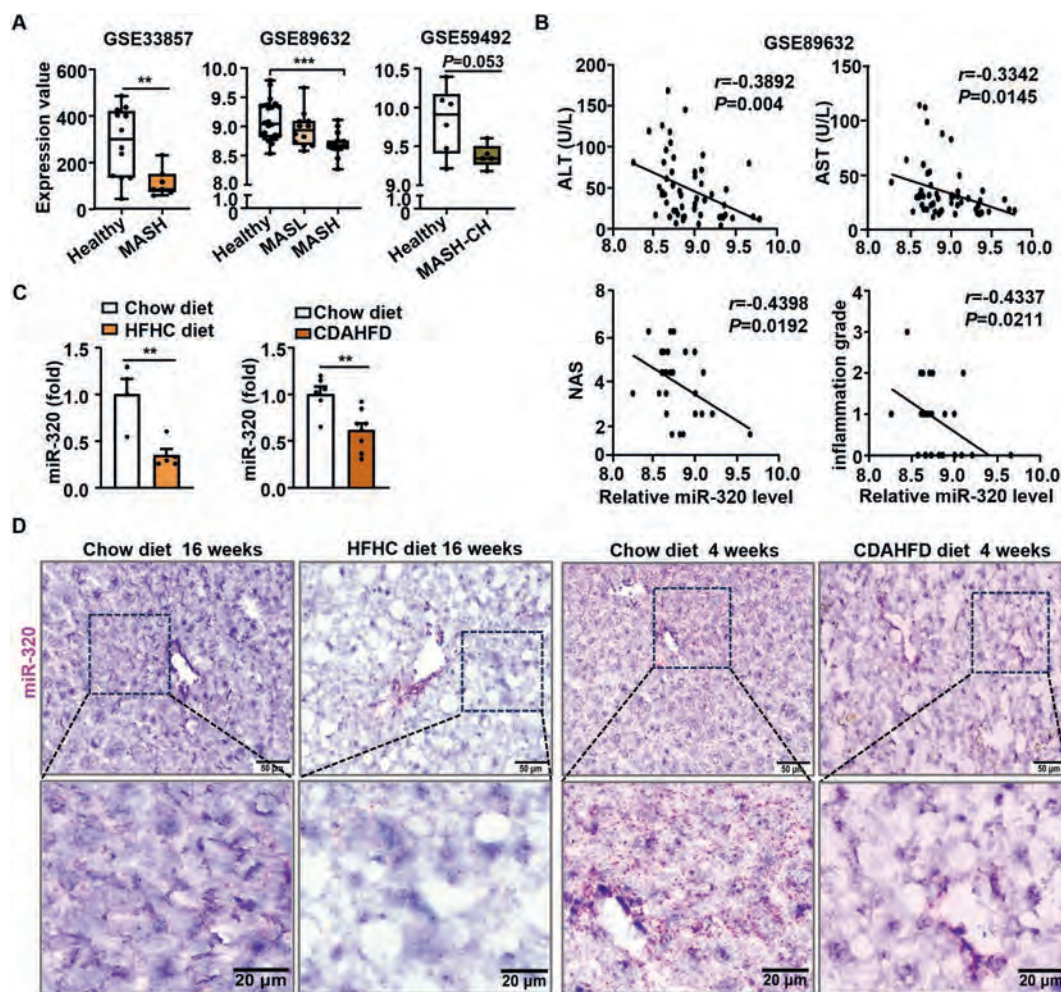


Figure 1 Hepatic miR-320 is downregulated during MASH progression and negatively correlates with the severity of MASH. (A) Serum miR-320 levels were analyzed from 7 MASH patients and 12 healthy volunteers in the GEO dataset GSE33857. Hepatic miR-320 levels were analyzed from the GSE89632 dataset containing 20 simple steatosis (MASL) patients, 19 MASH patients, and 24 healthy individuals, and the GSE59492 dataset including 5 MASH-related cirrhosis (MASH-CH) and 6 control liver samples (healthy). (B) The correlations between hepatic miR-320 levels and serum ALT, AST, NAS-Associated Score (NAS), and inflammation grade in MASLD patients were analyzed from the GSE89632 dataset. Analysis of the correlation between hepatic miR-320 levels and ALT/AST was performed among 39 patients and 24 healthy individuals. Analysis of the correlation between hepatic miR-320 levels and the NAS/inflammation grade was conducted in 39 MASLD patients. (C) Hepatic miR-320 expression was measured by RT-qPCR in HFHC and CDAHFD-induced mouse MASH models. $n = 4-8$. (D) Hepatic miR-320 expression was determined by *in situ* hybridization with RNAscope in HFHC and CDAHFD-fed mice. Values represent means $\pm$ SEM. ** $P < 0.01$, *** $P < 0.001$.

downregulated in the livers of patients with MASH or in mouse MASH models and is closely associated with the progression of MASH.

3.2. Hepatic miR-320 deficiency accelerates CDAHFD-induced MASH

It is well-established that the miR-320 family comprises the highly conserved miR-320-3p and poorly conserved miR-320-5p in mice. To further investigate whether the downregulation of hepatic miR-320 contributes to the progression of MASH, we generated hepatocyte-specific miR-320-3p deficient mice (miR-320^{ΔHep}). We examined the levels of miR-320-3p (3p) and miR-320-5p (5p) in the various tissues of hepatocyte-specific miR-320 deficient mice to evaluate the efficiency of miR-320 deletion.

As shown in Fig. 2A, compared with control littermates (miR-320^{f/f}), miR-320-3p levels were dramatically and specifically decreased in the livers of miR-320^{ΔHep} mice but not in other tissues. miR-320-5p levels in different tissues were comparable between miR-320^{f/f} and miR-320^{ΔHep} mice likely due to its very poorly conserved and low expression. Meanwhile, miR-320-3p expression was prominently lessened in hepatocytes isolated from miR-320^{ΔHep} mice compared with the hepatocytes from miR-320^{f/f} mice (Fig. 2A, right panel). Next, the CDAHFD-induced MASH model was used to evaluate the function of miR-320. After 2 or 4 weeks of CDAHFD feeding, the serum ALT and AST levels were comparable between the miR-320^{f/f} mice and the miR-320^{ΔHep} mice (Fig. 2B and Supporting Information Fig. S2A). Of note, compared to miR-320^{f/f} mice, miR-320^{ΔHep} mice fed with CDAHFD for 2 weeks had greater degree of

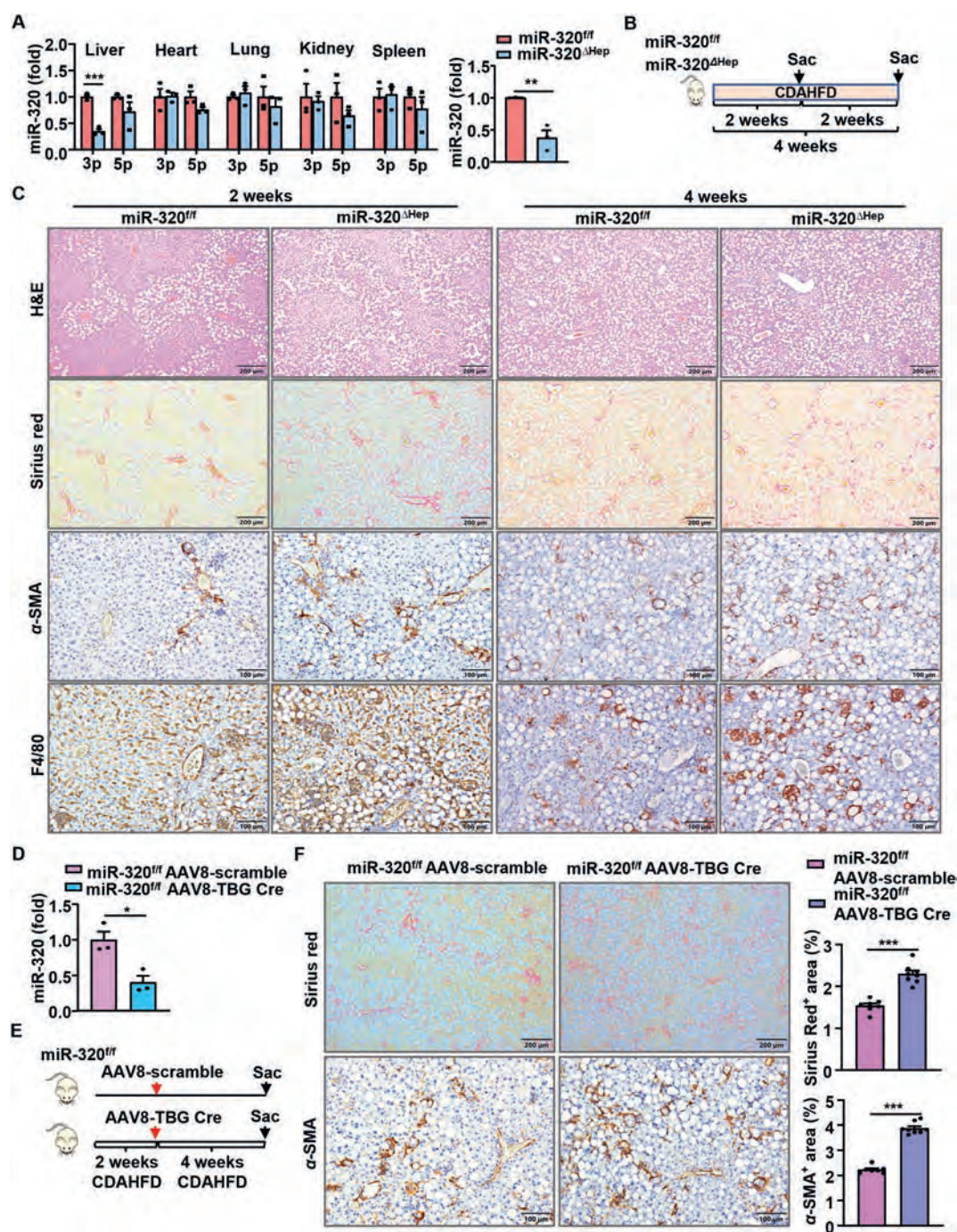


Figure 2 miR-320 deficiency in hepatocytes accelerates CDAHFD-induced MASH. (A) miR-320-3p or 5p levels in different tissues from miR-320^{ΔHep} and miR-320^{fl/fl} control littermate mice were measured by RT-qPCR (left panel). The levels of miR-320 in primary hepatocytes isolated from miR-320^{fl/fl} and miR-320^{ΔHep} mice were measured by RT-qPCR (right panel). $n = 3$ in each group. (B) Eight-week-old male miR-320^{ΔHep} and miR-320^{fl/fl} mice were fed the CDAHFD for 2 or 4 weeks. Serum and liver samples were collected. Schematic diagram of the CDAHFD-induced MASH model. $n = 5-10$. (C) Representative images of H&E (Scale bar: 200 μ m), Sirius red staining (Scale bar: 200 μ m), α -SMA (Scale bar: 100 μ m), and F4/80 (Scale bar: 100 μ m) immunostaining of liver tissue sections are shown. (D) The levels of miR-320 in primary hepatocytes isolated from AAV8-scramble or AAV8-TBG Cre-injected miR-320^{fl/fl} mice were measured by RT-qPCR. $n = 3$ in each group. (E) Schematic diagram of CDAHFD-fed AAV8-scramble/TBG Cre-injected miR-320^{fl/fl} mice. 8-week-old miR-320^{fl/fl} mice were subjected to CDAHFD for 2 weeks before AAV8-scramble or AAV8-TBG Cre injection by tail vein (2×10^{11} vg/mouse) and then fed with the CDAHFD for another 4 weeks. $n = 7$ or 8 per group. (F) Representative images of Sirius red (Scale bar: 200 μ m) and α -SMA (Scale bar: 100 μ m) immunostaining are shown. The fibrotic area per field was quantified. Values represent means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

steatosis while 4 week-CDAHFD feeding slightly promoted lipid accumulation in miR-320^{ΔHep} mice compared with miR-320^{fl/fl} mice as demonstrated by H&E staining and hepatic TG content (Fig. 2C and Fig. S2B). However, hepatic levels of lipid peroxidation markers including 4-HNE and MDA were remarkably enhanced in miR-320^{ΔHep} mice after CDAHFD feeding for 2 or 4 weeks (Fig. S2C). Notably, Sirius Red staining and α -SMA immunostaining displayed that CDAHFD-fed miR-320^{ΔHep} mice had more severe collagen deposition and fibrogenesis than miR-320^{fl/fl} mice (Fig. 2C and Fig. S2D). Besides, the number of F4/80 positive “crown-like structure” macrophages was markedly enlarged in CDAHFD-fed miR-320^{ΔHep} mice compared with their littermate miR-320^{fl/fl} mice (Fig. 2C and Fig. S2D). These results suggested that miR-320 deficiency in hepatocytes facilitated CDAHFD-induced steatosis in the early stage, but persistently aggravated oxidative stress, inflammation and fibrosis.

To rule out the exacerbation of MASH in miR-320^{ΔHep} mice due to miR-320 deficiency in the embryonic period, we injected AAV8-TBG Cre into miR-320^{fl/fl} mice to deplete hepatic miR-320 in adulthood. The expression of miR-320 was distinctly decreased in isolated primary hepatocytes after miR-320^{fl/fl} mice were injected with AAV8-TBG Cre for 4 weeks (Fig. 2D). Eight-week-old miR-320^{fl/fl} mice were fed CDAHFD for 2 weeks and then were injected with AAV8-TBG Cre or scramble, and kept on CDAHFD for another 4 weeks (Fig. 2E). We found that miR-320 deletion in hepatocytes barely affected lipid accumulation and liver injury compared with control mice (Fig. S2E and S2F). Nevertheless, compared to AAV8-scramble-injected miR-320^{fl/fl} mice, hepatic 4-HNE and MDA levels were markedly increased in AAV8-TBG Cre-injected miR-320^{fl/fl} mice (Fig. S2F). More importantly, collagen deposition and fibrogenesis were dramatically greater in the livers of AAV8-TBG Cre-injected miR-320^{fl/fl} mice than in AAV8-scramble-injected miR-320^{fl/fl} mice after CDAHFD feeding (Fig. 2F). In addition, hepatic macrophage infiltration was also enhanced in AAV8-TBG Cre-injected miR-320^{fl/fl} mice as demonstrated by F4/80 immunostaining (Fig. S2F). Collectively, the data from miR-320^{ΔHep} mice and AAV8-TBG Cre-injected miR-320^{fl/fl} mice substantiate that miR-320 deficiency in hepatocytes accelerates CDAHFD-induced MASH progression.

3.3. Hepatocyte-specific miR-320 deletion exacerbates HFHC diet-induced MASH

To further evaluate the effect of hepatic miR-320 on obesity-related MASH, miR-320^{ΔHep} mice and their littermate miR-320^{fl/fl} mice were fed a HFHC diet for 16 weeks. First, we found that compared with miR-320^{fl/fl} mice, miR-320^{ΔHep} mice fed a chow diet for 16 weeks exhibited comparable liver/body weight ratio and serum ALT levels but a distinct rise of AST levels (Supporting Information Fig. S3A and S3B). As expected, chow diet-fed miR-320^{ΔHep} mice exhibited no distinct abnormalities in morphology, lipid accumulation, inflammation, and fibrogenesis as demonstrated by H&E, F4/80 immunostaining, and Sirius Red staining (Fig. S3C–S3E). Interestingly, the body weight of the miR-320^{ΔHep} mice had a tendency of weight gain than miR-320^{fl/fl} mice at the 9th week and reached a significant difference at the 13th week after HFHC feeding for 16 weeks, which may be partially due to the slight increase of food intake in HFHC-diet induced miR-320^{ΔHep} mice (Fig. 3A and Supporting Fig. S4A). The liver images of two group of mice showed the liver volume of the miR-320^{ΔHep} mice was greater than that of the miR-320^{fl/fl} mice. As expected, the liver weight and liver/body weight ratio of

miR-320^{ΔHep} mice were distinctly greater than those of miR-320^{fl/fl} mice (Fig. 3B and Fig. S4B). More importantly, miR-320^{ΔHep} mice had much higher levels of serum ALT and AST than miR-320^{fl/fl} mice (Fig. 3C). Although miR-320 deficiency in hepatocytes didn't affect serum levels of TG, total cholesterol (TCH) and low-density lipoprotein cholesterol (LDL-C) after HFHC feeding (Fig. S4C), H&E, Oil red O staining and hepatic TG measurement suggested that miR-320^{ΔHep} mice had more lipid accumulation and ballooning degeneration in the liver compared with miR-320^{fl/fl} mice (Fig. 3D, Fig. S4D). Furthermore, the contents of 4-HNE and MDA were remarkably augmented in the livers of HFHC diet-fed miR-320^{ΔHep} mice (Fig. 3D). Additionally, RT-qPCR analyses demonstrated that lipogenic genes including *Srebp-1c*, *Acc1*, and *Fasn* were upregulated, while β -oxidative genes including *Cpt1* and *Cpt2* were downregulated, and lipid peroxidation-related genes had an increased tendency in HFHC-diet-fed miR-320^{ΔHep} mice (Fig. 3E). Compared with miR-320^{fl/fl} mice, the populations of F4/80 positive “crown-like structure” macrophages and Ly6G-positive neutrophils were markedly augmented in miR-320^{ΔHep} mice (Fig. S4E). Furthermore, miR-320^{ΔHep} mice were more susceptible to liver fibrogenesis as demonstrated by Sirius Red staining and α -SMA immunostaining (Fig. 3F). Accordingly, inflammatory and fibrotic genes, such as *Adgre1*, *Ccl2*, *Col1a1*, *Col3a1*, and *Tgfb1*, were markedly upregulated in the livers of HFHC-diet-fed miR-320^{ΔHep} mice (Fig. S4F). Taken together, these results suggest that hepatocyte-specific miR-320 deletion exacerbates obesity-related MASH progression.

3.4. Restoration of hepatic miR-320 mitigates different diet-induced MASH

The above results suggest that MASH diet feeding results in hepatic miR-320 downregulation, which exacerbates MASH progression. Therefore, we wondered whether restoration of hepatic miR-320 could alleviate MASH progression (Fig. 4A). As shown in Fig. 4B and Supporting Information Fig. S5A, due to the ubiquitous and high expression of miR-320 in the liver, RT-qPCR and *in situ* hybridization with RNAscope analyses suggested that hepatic miR-320 levels were approximately 3-fold higher in AAV8-miR-320-injected mice than that in AAV8-scramble group after HFHC feeding. Of note, liver/body weight ratio but not body weight was lower in AAV8-miR-320-injected mice after HFHC feeding compared with AAV8-scramble-injected mice (Fig. S5B and S5C). Furthermore, although restoration of miR-320 didn't influence serum TG and HDL-C levels, serum ALT, AST, TCH, and LDL-C levels were distinctly reduced (Fig. 4C and Fig. S5D). H&E staining and Oil red O staining consistently revealed that AAV8-miR-320-injected mice exhibited lower steatosis in HFHC-fed mice, as well as consistent with hepatic TG content (Fig. 4C and D). Additionally, the contents of 4-HNE and MDA were also significantly decreased in the liver of AAV8-miR-320-injected mice (Supporting Fig. S5E). Most importantly, administration of AAV8-miR-320 obviously ameliorated HFHC-induced liver fibrosis as evidenced by lower levels of collagen deposition and α -SMA (Fig. 4D). Moreover, the numbers of F4/80-positive macrophages and Ly6G-positive neutrophils were reduced after restoration of miR-320 after HFHC feeding (Fig. S5F). Similarly, the expression of genes involved in liver lipogenesis and lipid peroxidation, such as *Srebp-1c*, *Acc1*, *Ncf2*, and *Cybb*, were markedly downregulated, while the expression of β -oxidative genes was upregulated in the liver of AAV8-miR-320-injected mice (Fig. 4E). Consistently, injection of AAV8-miR-320

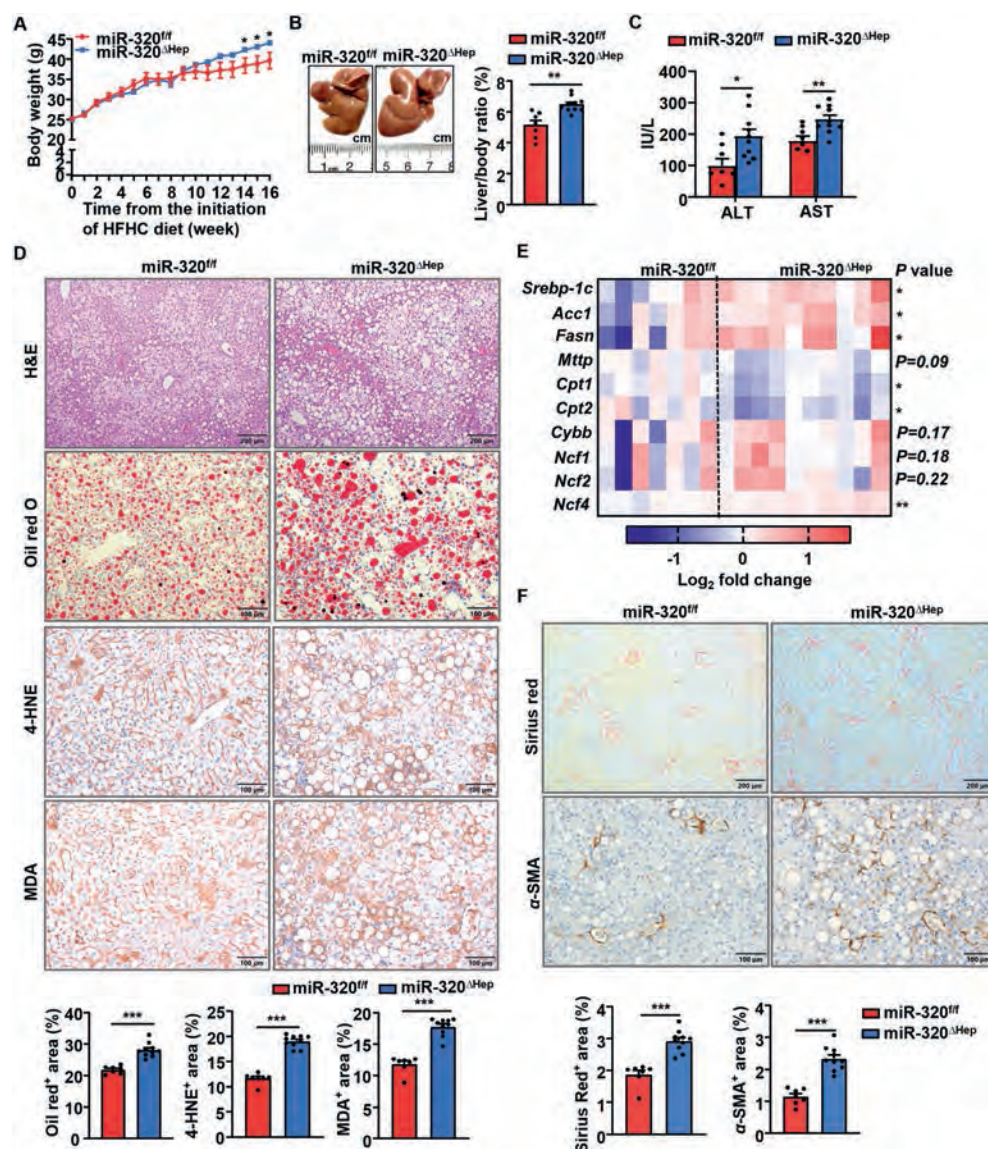


Figure 3 Hepatocyte-specific miR-320 deletion exacerbates HFHC-induced MASH. Eight-week-old male $\text{miR-320}^{\Delta\text{Hep}}$ mice and their littermate $\text{miR-320}^{\text{fl/fl}}$ mice were fed the HFHC diet for 16 weeks. Liver and serum samples were collected. $n = 7$ or 10 per group. (A) The body weight of $\text{miR-320}^{\text{fl/fl}}$ and $\text{miR-320}^{\Delta\text{Hep}}$ mice during HFHC diet feeding was measured. (B) Images of the livers of two groups of mice are shown. The ratio of liver/body weight in HFHC diet-fed $\text{miR-320}^{\text{fl/fl}}$ and $\text{miR-320}^{\Delta\text{Hep}}$ mice was determined. (C) Serum ALT and AST levels were measured. (D) Representative images of H&E staining (Scale bar: 200 μm), Oil Red O staining (Scale bar: 100 μm), 4-HNE and MDA staining (Scale bar: 100 μm) are shown. The positive areas of Oil Red O staining, 4-HNE and MDA staining per field were quantified. (E) A heatmap of genes involved in lipogenesis, β -oxidation, and oxidative stress was analyzed by RT-qPCR. (F) Representative images of Sirius red (Scale bar: 200 μm) and α -SMA (Scale bar: 100 μm) immunostaining are shown. The fibrotic area per field was quantified. Values represent means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. HFHC diet-fed $\text{miR-320}^{\text{fl/fl}}$ mice.

significantly downregulated several inflammatory and fibrotic gene levels after HFHC feeding (Fig. 4F). Finally, we evaluated the effect of AAV8-miR-320 on chow diet-fed mice. Injection of AAV8-miR-320 had minimal effects on liver weight, serum ALT and AST levels, liver steatosis, and inflammation (Supporting Information Fig. S6A–S6D). In addition, AAV8-miR-320 didn't alter the hepatic expression of some lipogenic, inflammatory, and fibrotic genes (Fig. S6E).

To ensure that the ameliorative effect of miR-320 restoration on MASH was robust, we administered AAV8-miR-320 to CDAHFD-fed mice. Eight-week-old male C57BL/6J mice were

intravenously injected with AAV8-scramble or AAV8-miR-320. After 1 week, the mice were fed a CDAHFD for another 3 weeks (Fig. 5A). As expected, restoration of hepatic miR-320 obviously reduced serum ALT and AST levels compared with those in AAV8-scramble-injected mice (Fig. 5B). The hepatic TG content and H&E staining demonstrated that administration of AAV8-miR-320 improved liver steatosis after CDAHFD feeding, especially in the central vein region (Fig. 5C). Furthermore, lipid peroxidation was markedly suppressed in the livers of AAV8-miR-320-treated mice as demonstrated by 4-HNE and MDA immunostaining (Fig. 5D). Consistently, hepatic genes related to

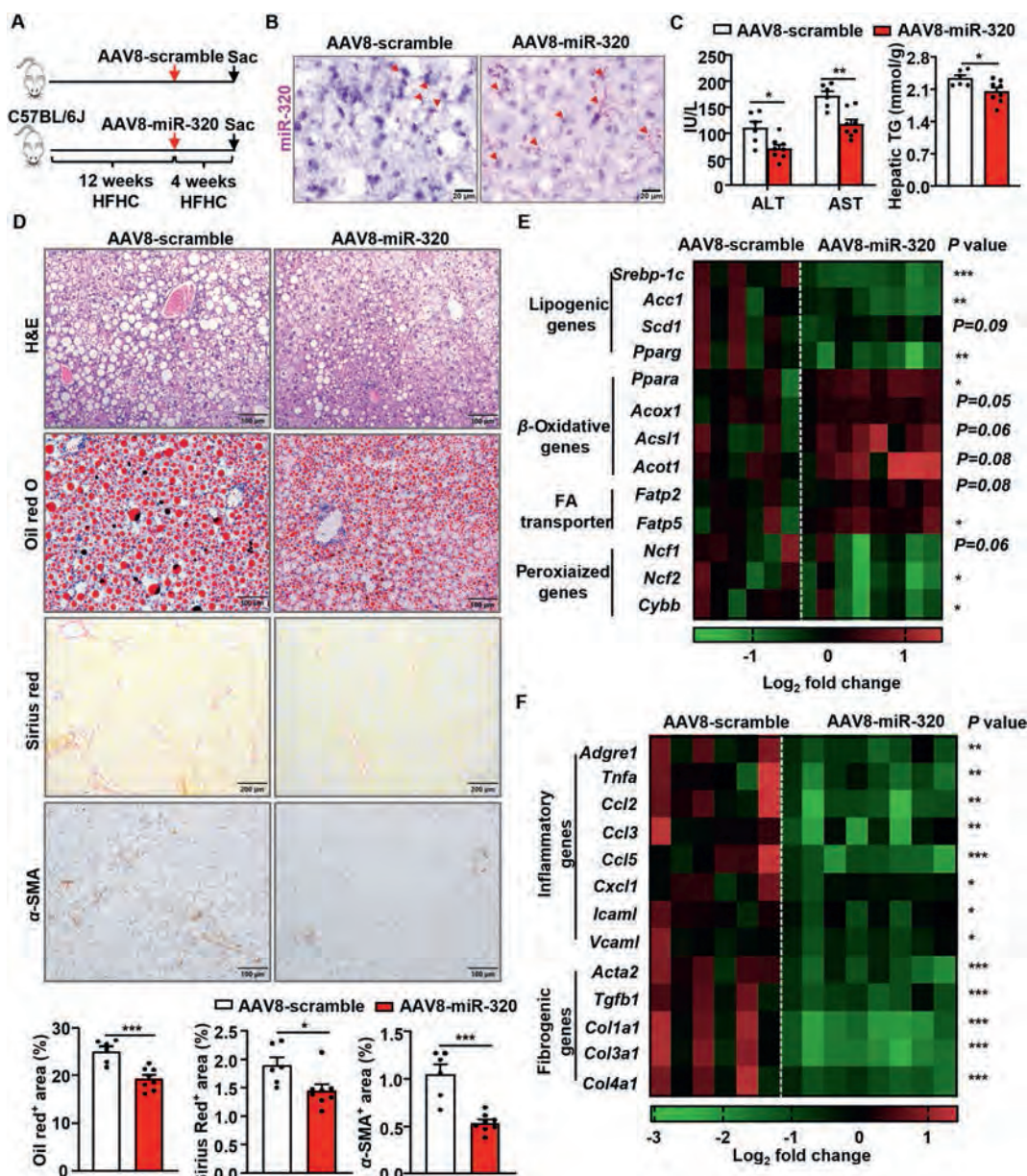


Figure 4 Restoration of hepatic miR-320 mitigates HFHC-induced MASH. (A) Schematic diagram of miR-320 restoration in the HFHC-induced MASH model. Eight-week-old male C57BL/6J mice were fed the HFHC diet for 12 weeks prior to intravenously injecting AAV8-scramble or AAV8-miR-320 (1×10^{11} vg/mouse) and then continued HFHC feeding for another 4 weeks. Liver and blood samples were collected. $n = 6$ or 8 per group. (B) miR-320 expression was analyzed by performing *in situ* hybridization with RNAscope in the liver of HFHC-fed AAV8-scramble or AAV8-miR-320-injected mice. The red arrows indicate miR-320 expression. (C) Serum ALT and AST levels, and hepatic TG content were measured. (D) Representative images of H&E staining (Scale bar: 100 μ m), Oil red O staining (Scale bar: 100 μ m), Sirius red (Scale bar: 200 μ m) and α -SMA (Scale bar: 100 μ m) staining are shown. The positive area per field of Oil Red O, 4-HNE and MDA staining were quantified. (E) The hepatic expression of genes involved in lipogenesis, β -oxidation, and oxidative stress was analyzed by RT-qPCR. (F) RT-qPCR analyses of hepatic inflammatory and fibrogenic gene expression. Values represent means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. HFHC diet-fed AAV8-scramble mice.

lipogenesis and lipid peroxidation were distinctly downregulated, while the expression of β -oxidative genes had an increasing tendency in the liver of mice supplemented with miR-320 (Fig. 5E). CDAHFD-induced collagen deposition, fibrogenesis and inflammatory cell infiltration were dramatically attenuated in the livers of mice administrated AAV8-miR-320 (Fig. 5F). In agreement, the expression of inflammatory and fibrogenic genes was significantly downregulated in the livers of AAV8-miR-320 group mice

(Fig. 5G). The above study demonstrated the preventive effect of miR-320 in the development of CDAHFD-induced MASH.

Next, we administrated AAV8-miR-320 in the early period of CDAHFD feeding to assess the therapeutic effect of miR-320 overexpression. Different from the above experiment, AAV8-miR-320 and its control virus (AAV8-scramble) were intravenously injected into C57BL/6J mice after CDAHFD feeding for 1 week and then the mice were kept CDAHFD feeding for another 3

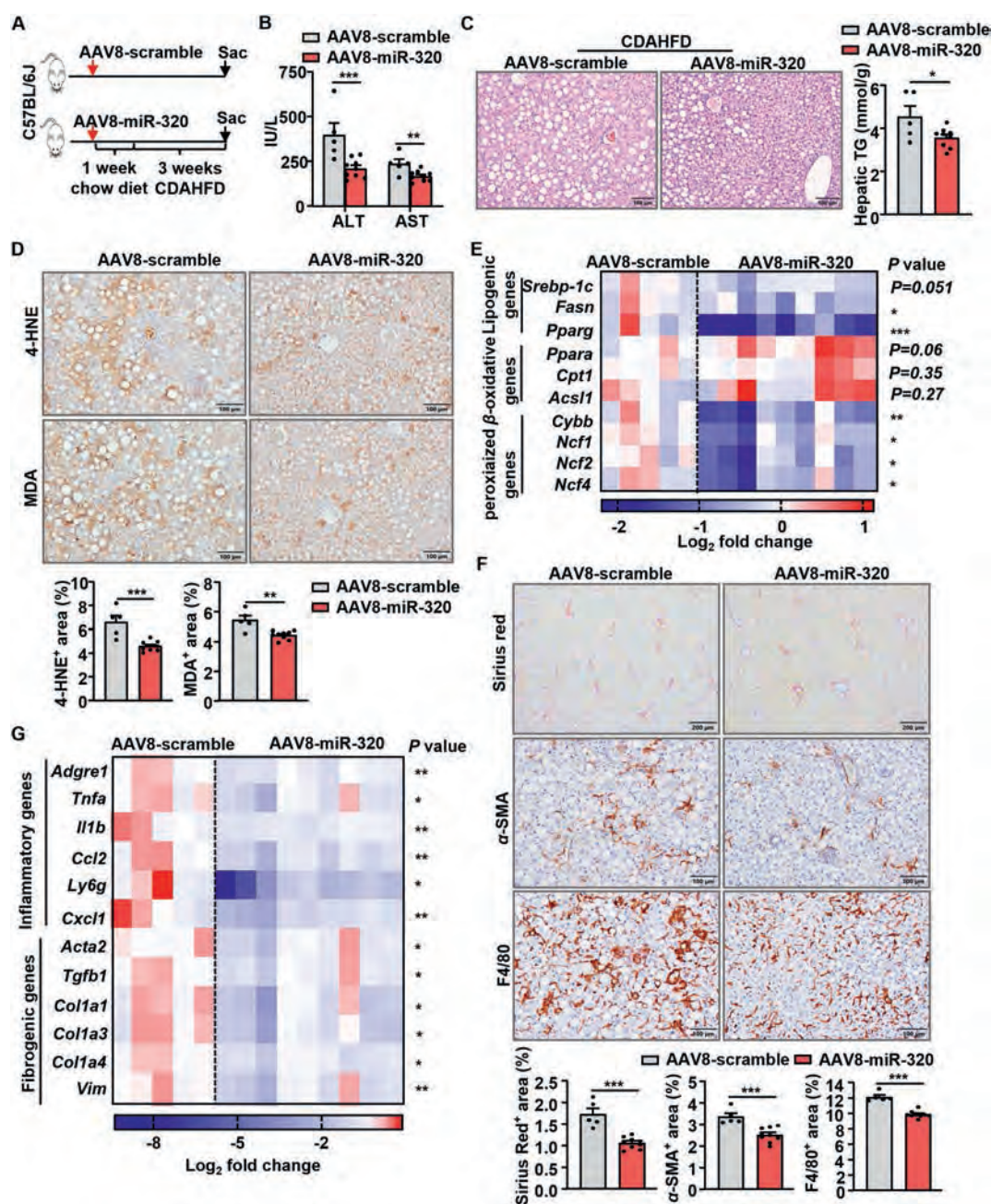


Figure 5 Restoration of hepatic miR-320 prevents mice from CDAHFD-induced MASH. (A) Schematic diagram of the CDAHFD-induced MASH model. After adaptive feeding, 8-week-old male C57BL/6J mice were intravenously injected with AAV8-scramble or AAV8-miR-320 (1×10^{11} vg/mouse). After 1 week, the mice were fed the CDAHFD for another 3 weeks. Liver and blood samples were collected. $n = 5$ or 9 per group. (B) Serum ALT and AST levels were measured. (C) Representative images of H&E staining are shown. Hepatic TG content was measured in two groups. (D) Representative images of 4-HNE and MDA immunostaining are shown. Scale bar: 100 μ m. The positive areas of 4-HNE and MDA staining per field were quantified. (E) The expression of genes involved in lipogenesis, β -oxidation, and oxidative stress were analyzed by RT-qPCR. (F) Representative images of Sirius red (Scale bar: 200 μ m) staining, α -SMA (Scale bar: 100 μ m) and F4/80 (Scale bar: 100 μ m) immunostaining were shown. The fibrotic area and F4/80 positive area per field were quantified. (G) Hepatic inflammatory and fibrotic gene expression was analyzed by RT-qPCR. Values represent means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. CDAHFD-fed AAV8-scramble mice.

weeks (Supporting Information Fig. S7A). Consistently, compared with CDAHFD-fed AAV8-scramble group mice, restoration of hepatic miR-320 markedly reduced serum ALT and AST levels (Fig. S7B), inhibited hepatic lipid accumulation and peroxidation as shown in H&E staining, 4-HNE and MDA immunostaining

(Fig. S7C). Importantly, administration of AAV8-miR-320 effectively suppressed fibrogenesis and inflammatory cell infiltration in the livers of CDAHFD-fed mice (Fig. S7D). Taken together, restoration of hepatic miR-320 not only prevents CDAHFD-induced MASH but also has therapeutic effects.

Finally, another genetic alteration-induced MASH model was also utilized. It's well known that low-density lipoprotein receptor (*Ldlr*) knockout (KO) mice are more susceptible to MASH phenotypes after HFD feeding for 3 months²⁰. After *Ldlr* KO mice were fed an HFD for 2 months, the mice were intravenously injected with AAV8-scramble or AAV8-miR-320 and then continued HFD feeding for another 4 weeks (Supporting Information Fig. S8A). Overall, hepatic miR-320 was elevated in the livers of HFD-fed *Ldlr* KO mice after injection of AAV8-miR-320 (Fig. S8B). Administration of AAV8-miR-320 didn't affect the liver/body ratio (Fig. S8C). Interestingly, compared to AAV8-scramble group, AAV8-miR-320-treated *Ldlr* KO mice exhibited remission of MASH phenotypes, as evidenced by lower levels of serum ALT and AST (Fig. S8D), less lipid accumulation and peroxidation in the liver (Fig. S8E and S8F), and fewer inflammatory cell infiltration and fibrogenesis as shown by immunostaining and RT-qPCR analyses (Fig. S8G–S8I). Taken together, the data from three different mouse MASH models strongly suggest that restoration of hepatic miR-320 mitigates the progression of MASH.

3.5. miR-320 suppresses lipid biosynthesis by upregulating fibroblast growth factor 1, thus activating the AMPK α signaling pathway in hepatocytes

To clarify how miR-320 affects the development and progression of MASH, liver samples from HFHC-fed miR-320^{fl} mice and miR-320^{ΔHep} mice were subjected to transcriptomic analysis by performing bulk RNA-sequencing (RNA-seq). We identified 938 genes that were differentially expressed in the livers of miR-320^{ΔHep} mice, of which 507 genes were upregulated and 431 were downregulated (Supporting Information Fig. S9A and S9B). Among these aberrant genes, the Reactome enrichment analysis indicated that these differentially expressed genes (DEGs) were mainly enriched in metabolism, biological oxidation, and lipid metabolism (Fig. 6A). Gene Set Enrichment Analysis (GSEA) analysis suggested that the gene set involved in metabolism, biological oxidations, lipid metabolism, and fatty acid metabolism were dramatically enhanced in the livers of miR-320^{ΔHep} mice compared with miR-320^{fl} mice (Fig. 6B and Fig. S9C). Furthermore, GO enrichment analysis demonstrated that these DEGs were enriched in lipid biosynthetic and metabolic processes, such as regulation of lipid biosynthetic process, fatty acid biosynthetic process, neutral lipid metabolic process and cholesterol homeostasis, suggesting that miR-320 may be involved in lipid biosynthetic process in hepatocytes (Fig. 6C). To address whether miR-320 inhibited lipid accumulation, BODIPYTM 493/503 dye, an indicator of neutral lipid, was utilized to detect lipid accumulation in the PA-induced hepatocyte cell line AML-12 cells with or without miR-320 mimics. As shown in Fig. S9D, fluorescence staining showed that overexpression of miR-320 in AML-12 cells had no effect on lipogenesis without PA treatment; however, overexpression of miR-320 remarkably weakened PA-induced lipid accumulation. Accordingly, flow cytometry analysis further confirmed that the intensity of BODIPY fluorescence was distinctly reduced in the miR-320 mimics-transfected hepatocytes after PA treatment compared with the negative control (NC) group (Fig. S9E). These results suggest that miR-320 could be identified as a negative regulator of the lipid biosynthetic process.

To wonder whether miR-320 has a protective effect on hepatocyte damage, the hepatocytes were treated with a high concentration of PA or H₂O₂ *in vitro*. Overexpression of miR-320 in

AML-12 cells mitigated PA or H₂O₂-induced hepatocyte damage by performing cell activity and AST measurement (Fig. S10A and S10B). Furthermore, to assess the impact of miR-320 on inflammation and fibrosis, we examined the expression of miR-320 in primary hepatic cells and the direct effect of miR-320 on HSCs and macrophages. The expression of miR-320 in HSCs and Kupffer cells (KCs) was lower than that in primary hepatocytes (PHs) isolated from C57BL/6J mice (Fig. S10C). Overexpression of miR-320 in human HSC line LX2 cells partially inhibited TGF β -induced fibrotic gene upregulation, including *COL1A1* and *COL3A1* (Fig. S10D). Similarly, overexpression of miR-320 in mouse macrophage cell line RAW264.7 markedly reduced LPS-induced pro-inflammatory gene expression including *Il6* and *Il1b* (Fig. S10E). These findings suggest that miR-320 not only plays a role in lipid biosynthesis in hepatocytes but also exerts anti-inflammatory and anti-fibrotic effects in HSCs and Kupffer cells.

To further elucidate the underlying mechanism by which miR-320 inhibits lipid accumulation, we performed an intersection analysis of five GO enrichment genes, yielding 6 genes including *Fgf1*, StAR related lipid transfer domain containing 4 (*Stard4*), cytochrome P450 family 7 subfamily a member 1 (*Cyp7a1*), apolipoprotein a4 (*Apoa4*), *Ldlr*, and farnesyl pyrophosphate synthase (*Fdps*), of which three genes were downregulated and other three genes were upregulated (Fig. 6D). Although *Apoa4*, *Ldlr*, and *Fdps* are not the potential direct targets of miR-320 predicted by TargetScan 7.0 database, we detected their mRNA levels in miR-320 mimics-transfected AML-12 cells and found that the expression of *Ldlr* and *Fdps* were inhibited in AML-12 cells after overexpression of miR-320 (Supporting Information Fig. S11A). However, based on the literature research, we paid more attention to the downregulated gene *Fgf1* in the liver of HFHC-fed miR-320^{ΔHep} mice since FGF1 is a fibroblast growth factor family member that is well-known to prevent oxidative damage and lipid accumulation by activating the AMPK signaling pathway^{26,27}. First, we verified that FGF1 located in the cytoplasm of hepatocytes in C57BL/6J mice by double immunofluorescence staining for FGF1 and hepatocyte marker HNF-4 α , which is consistent with the finding of *Fgf1* mRNA expression in hepatocytes according to single-cell and spatial multiomics database (MERSCOPE) analysis (Fig. S11B and S11C). FGF1 protein levels were upregulated in the livers of mice with MASH (Fig. S11D). As shown in Fig. 6E and Fig. S11E, hepatic *Fgf1* mRNA and protein expression were suppressed in miR-320^{ΔHep} mice, subsequently leading to decreased AMPK α activation (phosphorylated AMPK α , *p*-AMPK α). In contrast, restoration of hepatic miR-320 in HFHC-fed mice enhanced the levels of *Fgf1* mRNA and protein without affecting the expression of other hepatokines (Fig. 6F, Fig. S11F and S11G). Immunostaining further confirmed that FGF1 expression and the levels of *p*-AMPK α were dramatically suppressed in the livers of HFHC diet-fed miR-320^{ΔHep} mice but markedly amplified in those of HFHC diet-fed AAV8-miR-320-injected mice (Fig. 6G and H). Finally, hepatic FGF1 expression was evidently reduced in miR-320^{ΔHep} mice after CDAHFD feeding but enhanced in CDAHFD-fed mice or HFD-fed *Ldlr* KO mice after administration of AAV-8-miR-320 (Supporting Information Fig. S12A and S12B). However, serum FGF1 levels were comparable in each group after HFHC or CDAHFD feeding, suggesting that miR-320 doesn't influence extrahepatic FGF1 levels (Fig. S12C and S12D). Taken together, miR-320 may suppress lipid biosynthesis to ameliorate MASH through upregulating intrahepatic FGF1 expression, thus activating the AMPK α signaling pathway in hepatocytes.

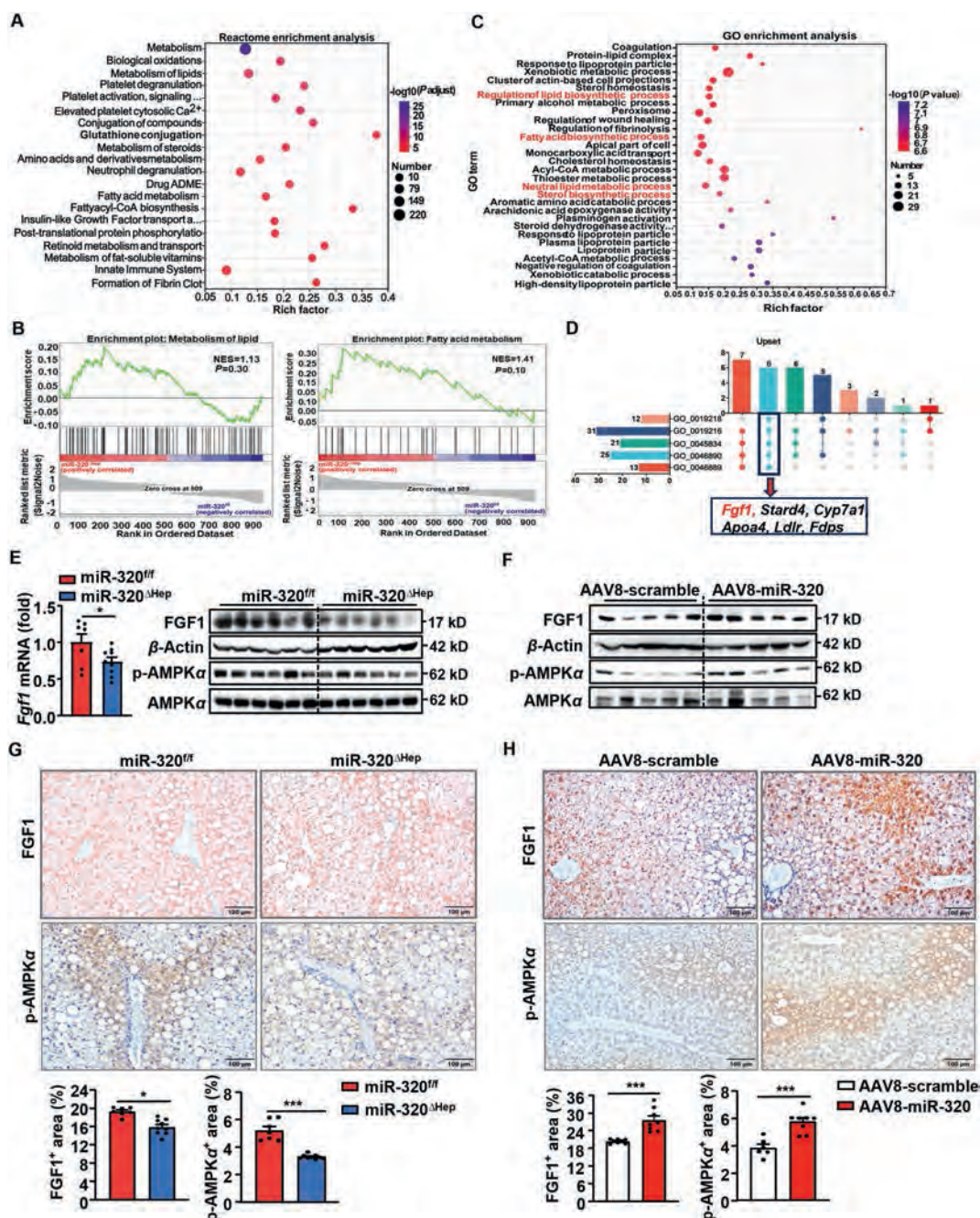


Figure 6 miR-320 suppresses lipid biosynthesis by upregulating fibroblast growth factor 1, thus activating the AMPK α signaling pathway in hepatocytes. Eight-week-old male miR-320^{ΔHep} mice and their littermate miR-320^{fl/fl} mice were fed the HFHC diet for 16 weeks. Liver samples were subjected to transcriptomic analysis *via* bulk RNA sequencing. $n = 4$ per group. (A) Reatome enrichment analysis of the differentially expressed genes from the RNA-sequencing dataset shows the Top20 Reatome pathways. (B) GSEA analysis of “metabolism of lipid” and “fatty acid metabolism” clusters from Reatome enrichment analysis. NES, normalized enrichment score, indicates the differences in gene set size and correlation between gene sets and expression matrices. The P value, a statistical analysis of the ES, indicates the confidence of the enrichment results. (C) GO enrichment analysis of the differentially expressed genes from the RNA-sequencing dataset. The GO terms with a red font refer to the lipid metabolism-related GO category. (D) Intersection analysis of Biological Process (BP) which related to lipid metabolism. GO_0019218: positive regulation of lipid biosynthetic process; GO_0019216: regulation of lipid metabolic process; GO_0045834: positive regulation of lipid metabolic process; GO_0046889: regulation of steroid metabolic process; GO_0046890: regulation of lipid biosynthetic process. The genes in the frame collectively emerged in the above five GO terms. (E) HFHC diet-fed miR-320^{fl/fl} and miR-320^{ΔHep} mice as described in Fig. 3. Hepatic *Fgf1* mRNA, FGF1, and phosphorylated AMPK α protein levels were respectively analyzed by RT-qPCR and Western blot. (F) HFHC diet-fed AAV8-scramble and AAV8-miR-320 as described in Fig. 4A. Hepatic FGF1 and phosphorylated AMPK α protein levels were analyzed by Western blot. (G, H) Representative images of FGF1 and p-AMPK α immunostaining are shown. Scale bar: 100 μ m. FGF1 positive area and p-AMPK α positive area per field were quantified. Values represent means $\pm$ SEM. $*P < 0.05$, $***P < 0.001$.

3.6. miR-320 inhibits the negative transcription factor RFX1 expression to elevate FGF1

To determine whether miR-320 upregulates the expression of FGF1 *in vitro*, we overexpressed miR-320 *in vitro* in hepatocytes. As shown in Fig. 7A–C and Supporting Information Fig. S13A, overexpression of miR-320 remarkably elevated FGF1 expression and led to AMPK activation, suggesting that miR-320 upregulated FGF1 expression in hepatocytes. Additionally, *Fgf1* mRNA levels were markedly lower in primary hepatocytes from miR-320^{ΔHep} mice than in hepatocytes isolated from miR-320^{fl} mice (Fig. S13B). It's well-known that miRNAs inhibit the expression of target genes. Given that miR-320 positively regulates FGF1 expression, FGF1 is not a direct target of miR-320 in hepatocytes. According to the manner of miRNA-mRNA function and the database, RFX1 is a negative transcriptional regulator of FGF1^{28,29}. Indeed, we demonstrated that both the mRNA and protein levels of FGF1 were significantly elevated after *Rfx1* knockdown in AML-12 cells (Fig. 7D–F), and such elevation of FGF1 activated the phosphorylation of AMPK α (Fig. 7F and Fig. S13C). Notably, RFX1 may be a predicted potential target of miR-320 in hepatocytes (Fig. S13D). Transfection of miR-320 mimics in hepatocytes could remarkably suppress RFX1 expression, simultaneously strengthening FGF1 levels (Fig. 7G and Fig. S13E). Noteworthy, hepatocyte-specific deletion of miR-320 upregulated, while restoration of hepatic miR-320 limited RFX1 expression in hepatocytes after HFHC feeding as demonstrated by RFX1 immunostaining (Fig. 7H). As well as similar RFX1 expression was identified in the livers of miR-320^{ΔHep} mice and AAV8-miR-320-injected mice after CDAHFD feeding (Fig. S13F). To confirm whether RFX1 is a direct target of miR-320 in hepatocytes, we performed an miRNA pull-down assay (Fig. 7I and J). As shown in Fig. 7K, miR-320 indeed binds to different 3'-UTR regions of *Rfx1* mRNA, suggesting that miR-320 directly targets RFX1. Most importantly, silencing of *Fgf1* in hepatocytes partially abolished *Rfx1* knockdown-induced AMPK α activation in hepatocytes (Fig. S13G). Overall, miR-320 suppresses the expression of RFX1 *in vitro* and *in vivo*, which negatively regulates FGF1 production in hepatocytes.

3.7. Silencing of *Rfx1* in hepatocytes alleviates MASH by enhancing FGF1-mediated AMPK α activation

To further illustrate the function of RFX1 in the development of MASH, we first measured RFX1 expression in the MASH model. As demonstrated in Supporting Information Fig. S14A, hepatic RFX1 was significantly enhanced after HFHC feeding. Next, knockdown of *Rfx1* by injection of AAV8-*Rfx1* shRNA (AAV8-sh*Rfx1*) or AAV8-scramble was performed in HFHC diet-fed mice. Western blot analysis showed that RFX1 protein levels had a decreasing tendency in the livers of the AAV8-sh*Rfx1* group (Fig. S14B), possibly because RFX1 is broadly expressed in liver cells. Thus, we performed immunostaining to confirm RFX1 knockdown in AAV8-sh*Rfx1* injected mice. As shown in Fig. 8A—as expected, RFX1-positive hepatocytes were obviously less abundant in the livers of AAV8-sh*Rfx1*-injected mice than in those of AAV8-scramble-injected mice. Interestingly, silencing *Rfx1* in hepatocytes upregulated FGF1 protein and mRNA expression after HFHC feeding (Fig. 8B and Fig. S14B). Moreover, the phosphorylation of AMPK was aggrandized in the livers of AAV8-sh*Rfx1* group mice, which was consistent with the supplement of miR-320 in the liver of HFHC-fed mice (Fig. 8C).

Finally, we demonstrated that hepatic knockdown of *Rfx1* led to a distinct reduction in serum ALT levels after HFHC feeding without influencing the liver/body ratio (Fig. 8D and Fig. S14C). Compared to the AAV8-scramble group, the AAV8-sh*Rfx1*-treated mice had milder steatosis and lipid peroxidation, fewer macrophage infiltration and less fibrosis as shown by hepatic TG content, H&E staining, 4-HNE, MDA, F4/80 and α -SMA immunostaining (Fig. 8D–F, Fig. S14D and S14E), suggesting that knockdown of hepatic *Rfx1* ameliorates HFHC-induced MASH by enhancing FGF1 production.

4. Discussion

A ubiquitous miRNA is a type of miRNA that is expressed in a wide range of tissues and cell types, playing essential roles in maintaining fundamental cellular biological functions. In the present study, we demonstrated that hepatic ubiquitous miR-320 levels were downregulated during MASH and correlated with MASH severity. Our five lines of *in vivo* evidence from hepatocyte-specific miR-320 deletion mice and AAV8-mediated miR-320 supplemented mice substantiated that miR-320 deficiency in hepatocytes accelerated MASH development, while restoration of hepatic miR-320 served as a promising therapeutic strategy for MASH treatment by inhibiting RFX1-mediated FGF1 suppression (Fig. 8G).

miRNAs are well-known to exhibit tissue-specific expression; however, miR-320 is an evolutionarily conserved and ubiquitously expressed miRNA, which is directly encoded upstream of POLR3D, a conserved subunit of RNA polymerase III²¹. Indeed, our data revealed that compared to liver-specific miR-122, the CT value of miR-320 was basically equivalent in different mouse tissues. Despite its high and conserved expression in the liver, the loss of miR-320 in hepatocytes barely affects liver function and does not result in spontaneous steatosis and inflammation during chow diet feeding, suggesting that miR-320 is involved in this pathological process. A previous study showed that plasma miR-320 was upregulated in the early stages of drug-induced liver damage and presented as a steatotic biomarker for liver damage³⁰. Another study reported that hepatic global miR-320 levels were unchanged because the nuclear miR-320 levels were increased and miR-320 levels in the cytosol were decreased in HFD or streptozotocin (STZ)-induced mouse models and leptin receptor-deficient *db/db* mice³¹. In contrast, our study revealed that the serum and hepatic miR-320 levels were remarkably decreased in patients with MASH, as shown in GEO datasets and different diet-induced experimental mouse MASH models. The lower reduction in hepatic miR-320 in the CDAHFD model than in the HFHC model was probably due to greater immune cell infiltration in the CDAHFD-induced MASH. More importantly, although miR-320 deficiency in hepatocytes didn't affect liver injury in CDAHFD-induced MASH, hepatocyte-specific miR-320 deficient mice were more susceptible to CDAHFD or HFHC-induced liver steatosis, inflammation, and fibrosis. Besides, hepatocyte-specific miR-320 deficiency increased the body weight of mice after HFHC feeding, not only liver weight but also adipose tissue weight (Data not shown). The reason for the weight gain of miR-320^{ΔHep} mice was attributed to the function of miR-320 in lipid biosynthesis and metabolism, as well as partially due to the slight increase in food intake. Interestingly, the increased steatosis in hepatocyte-specific miR-320 deficient mice was continuous in the HFHC MASH model. However, miR-320 deficiency in

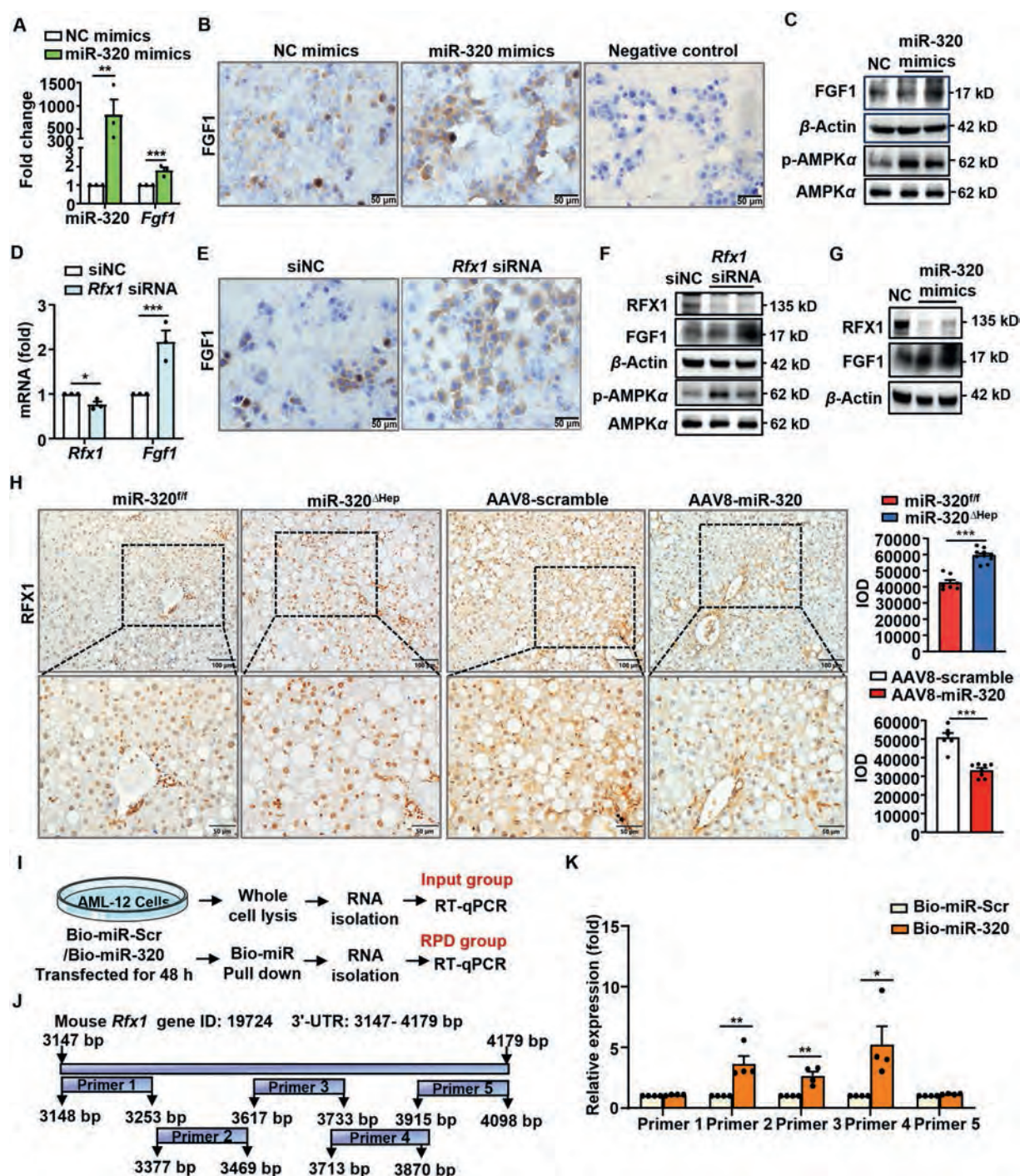


Figure 7 miR-320 suppresses the negative transcription factor RFX1 expression to elevate FGF1 production in hepatocytes. (A) AML-12 cells were transfected with Normal Control mimics (NC mimics) or miR-320 mimics for 48 h. The expression of miR-320 and *Fgf1* was measured by RT-qPCR. (B) Representative images of FGF1 immunostaining in AML-12 cells after transfection with NC or miR-320 mimics are shown. The negative control didn't incubate with the FGF1 primary antibody. Scale bar: 50 μ m. (C) The levels of FGF1 protein and phosphorylated AMPK α in NC or miR-320 mimics-transfected AML-12 cells were determined. (D) The expression of *Rfx1* and *Fgf1* in AML-12 cells after silencing *Rfx1* by transfecting with siRNA for 48 h was analyzed by RT-qPCR. (E) Representative images of FGF1 immunostaining in AML-12 cells after silencing *Rfx1* are shown. Scale bar: 50 μ m. (F) The levels of RFX1, FGF1 protein, and phosphorylated AMPK α in AML-12 cells after silencing *Rfx1*. (G) AML-12 cells were transfected with NC mimics or miR-320 mimics. RFX1 and FGF1 protein levels were analyzed. (H) The mice were subjected to HFHC feeding as described in Figs. 3 and 4. Representative images of RFX1 immunostaining in different liver sections are shown. Scale bar: 100 or 50 μ m. The integrated optical density (IOD) of RFX1 positive hepatocytes per field was quantified. (I) Schematic diagram of miRNA pull-down assay. (J) Different primers targeting different regions of 3'-UTR of *Rfx1* mRNA were designed. (K) Relative expression of *Rfx1* after pull down by Bio-miR-320 for different regions was measured. The data are from three independent *in vitro* experiments. Values represent means $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

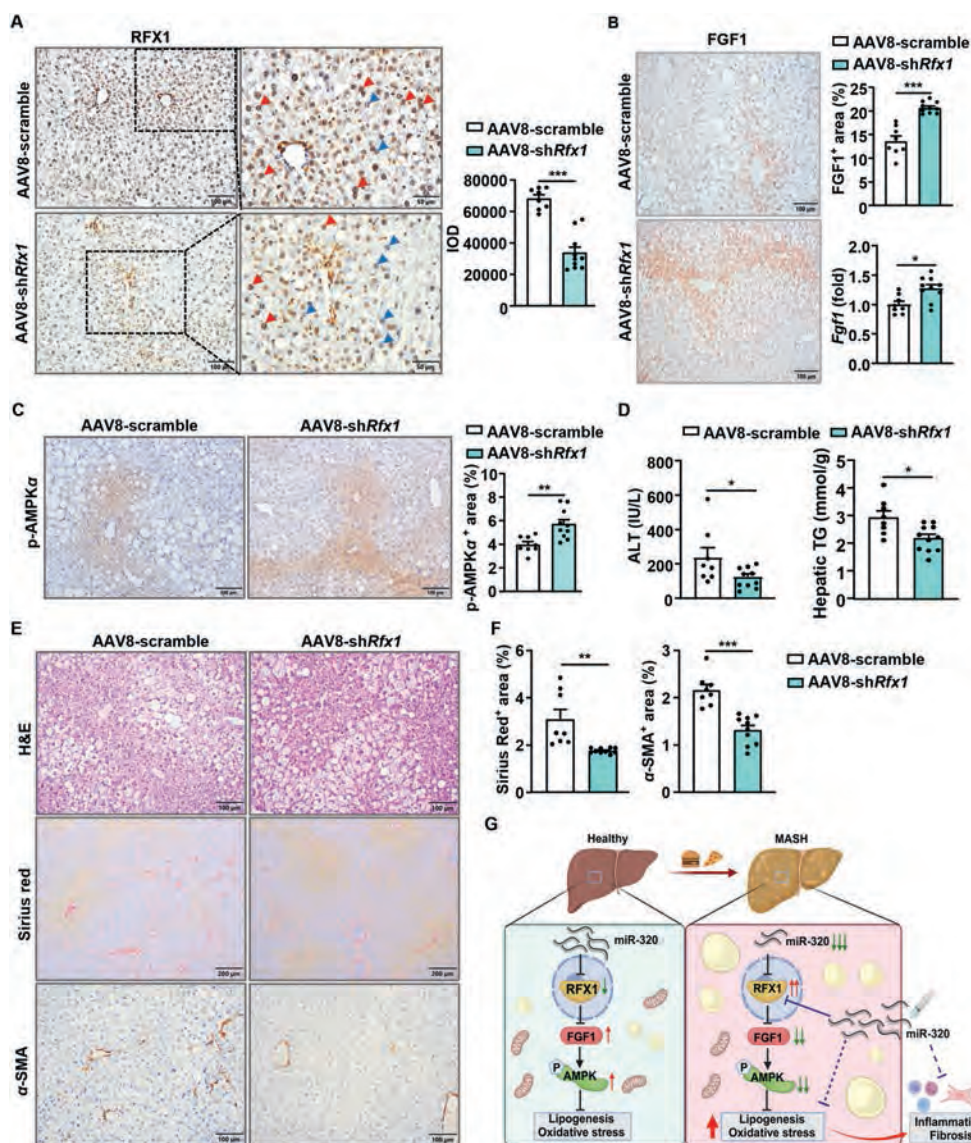


Figure 8 Silencing of *Rfx1* in hepatocytes alleviates MASH by activating the FGF1–AMPK α signaling pathway. Eight-week-old male C57BL/6J mice were fed the HFHC diet for 12 weeks prior to intravenous injection of AAV8-scramble or AAV8-*Rfx1* shRNA (AAV8-Sh*Rfx1*, 1.5×10^{11} vg/mouse) and continued HFHC feeding for another 4 weeks. $n = 8$ or 10 per group. (A) Representative images of RFX1 immunostaining are shown. The red arrows indicate RFX1-positive hepatocytes. The blue arrows indicate RFX1-negative hepatocytes. Scale bar: 100 or 50 μ m. The IOD of RFX1-positive hepatocytes per field was quantified. (B) Representative images of FGF1 immunostaining are shown. Scale bar: 100 μ m. The FGF1 positive area (top panel) and the expression of *Fgf1* mRNA (bottom panel) were quantified. (C) Representative images of p-AMPK α immunostaining are shown. Scale bar: 100 μ m. The p-AMPK α positive area per field was quantified. (D) Serum ALT levels and Hepatic TG content were measured. (E) Representative images of H&E staining (Scale bar: 100 μ m), Sirius red (Scale bar: 200 μ m) and α -SMA staining (Scale bar: 100 μ m) are shown. (F) The fibrotic area per field was quantified. Values represent means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. HFHC diet-fed AAV8-scramble mice. (G) A schematic model depicting diet or obesity-induced miR-320 downregulation triggers RFX1-mediated FGF1 suppression to accelerate MASH progression. Downregulation of miR-320 elevates the negative transcription factor RFX1 expression to suppress FGF1 production. Restoration of miR-320 ameliorates MASH development and progression by suppressing RFX1 expression, finally upregulating anti-lipogenic FGF1 production and AMPK activation in hepatocytes. Created in <https://BioRender.com>.

hepatocytes exhibited a moderate pro-lipogenesis effect in the early stage of the CDAHFD model but disappeared in the chronic CDAHFD feeding. The difference of miR-320 deficiency in steatosis between the HFHC and CDAHFD models may be due to mechanistic differences in the two dietary models, HFHC diet-induced steatosis is driven by lipid overload including the process of lipogenesis, β -oxidation, and lipid intake, while

CDAHFD-induced steatosis is largely driven by choline deficiency-mediated impairment of lipid exporting, oxidative stress, and cellular damage³². In addition, the transcriptomic analysis of HFHC-fed miR-320^{ΔHep} mice showed that DEGs were mainly enriched in lipid metabolism and biosynthesis, indicating the critical role of miR-320 deficiency in promoting steatosis. Moreover, restoration of hepatic miR-320 in three different types

of diet-induced MASH models mitigated the phenotypes of MASH. Considering the extensive and uncertain mRNA targets of miRNAs, developing miRNA-based drugs poses significant challenges, including the potential emergence of side effects. However, the ubiquitously expressed miR-320 presents a promising avenue for overcoming these obstacles. By restoring miR-320 levels in hepatocytes, there is potential to address these remaining challenges, particularly in the treatment of MASH. Nevertheless, how hepatic miR-320 levels are downregulated during MASH development remains unknown. Our findings suggested that high-glucose and oxidative stress but not free fatty acids decreased miR-320 expression in hepatocytes, which may explain why a high glucose diet is closely associated with the incidence of MAFLD³³.

One interesting finding from the current study is that miR-320 specifically controls FGF1 production without affecting the expression of other hepatokines. FGF1, a member of the FGF family, functions as an autocrine/paracrine hormone and is ubiquitously expressed in various tissues including the brain, adipose tissue, liver, skeletal muscle, and heart³⁴. FGF1 occupies a unique niche within the FGF family because it is the sole member capable of binding to all FGF receptors (FGFR1-4) and their subtypes and is well-recognized as a molecule with potent glucose-lowering ability except insulin^{34,35}. Numerous studies have demonstrated that central or peripheral recombinant FGF1 (rFGF1) administration markedly lowers blood glucose and enhances insulin sensitivity in various obese and diabetic mouse models by restraining the hypothalamus–pituitary–adrenal axis^{36–38}. FGF1 can promote glucose uptake by upregulating the expression of glucose transporters 1 and 4 (GLUT1 and GLUT4) in skeletal muscle and adipose tissues^{39,40}. In addition, FGF1 remodels adipose tissues and inhibits lipolysis *via* FGFR1-mediated activation of phosphodiesterase 4D (PDE4D)^{41,42}. Inspiringly, the administration of rFGF1 or an engineered FGF1 partial agonist with 3 substitutions (FGF1^{ΔHBS}) effectively suppressed hepatic steatosis and inflammation not only in *db/db* and *ob/ob* mice but also in multiple diet-induced MASLD models including HFD-induced mice, choline-deficient, L-amino acid-defined (CDAA)-fed mice, high-fat/high-sucrose (HFHS)-fed mice and HFHC diet-fed apolipoprotein E knockout mice^{26,27}. Coincidentally, supplementation with miR-320 intensified FGF1 expression in the liver and protected mice from CDAHFD and HFHC-induced MASH, which is in line with the ameliorated phenotypes induced by rFGF1 treatment in MASLD models. It has been demonstrated that HFD feeding induces FGF1 expression in adipose tissue, which is regulated by at least two independent mechanisms, such as direct regulation of FGF1 transcription *via* peroxisome proliferator-activated receptor γ (PPAR γ) (nutrient sensing) and mechanical stress-induced release of FGF1 due to adipocyte hypertrophy^{42,43}. Consistently, the GEO dataset and our results supported that FGF1 levels were elevated in the livers of HFHC diet-fed mice, which may be the compensatory response of organisms to pathological conditions. However, the exact mechanisms of FGF1 secretion in the liver remain unclear, and FGF1 expression is not totally regulated by miR-320, other mechanisms may be involved in the regulation of FGF1. In our study, the enhancement of FGF1 by miR-320 overexpression seems to further raise yield for sensitizing the FGF1 signaling. Activating AMPK α signaling is a major cascade of FGF1–FGFRs and it has been reported that the effect of FGF1 on inhibiting lipogenesis and antioxidants in the liver is mediated by the activation of AMPK α *via* hepatocyte FGFR4^{27,44}. As expected, miR-320 upregulated FGF1 expression and

simultaneously activated the phosphorylation of AMPK α *in vivo* and *in vitro*, while miR-320 deficiency suppressed FGF1 levels and AMPK α phosphorylation. In addition, it has been demonstrated that the anti-inflammatory effect of rFGF1 could be independent of its antisteatotic effects probably due to other pathways, such as reducing endothelial vascular cell adhesion molecule 1 (VCAM-1) expression that is implicated in leukocyte recruitment²⁷. Therefore, it is reasonable to speculate that the restoration of miR-320 mitigates MASH by elevating FGF1 and its downstream AMPK activation.

Previous studies have demonstrated that RFX1 can bind to the promoter of FGF1, leading to the suppression of FGF1 transcription and subsequent inhibition of human glioblastoma cell proliferation^{28,29}. Notably, bioinformatics analysis identified RFX1 as a potential target of miR-320. However, Western blot analysis suggested that miR-320 deficiency or overexpression didn't significantly affect hepatic RFX1 protein expression, possibly due to the broad expression of *Rfx1* gene in liver cells. Therefore, immunohistochemical staining may offer a more effective approach for assessing RFX1 expression in hepatocytes. Indeed, immunohistochemical staining revealed increased RFX1 expression in the hepatocytes of HFHC-fed miR-320^{ΔHep} mice but decreased RFX1 expression in those of HFHC-fed AAV8-miR-320 mice. Furthermore, *in vitro* experiments verified that overexpression of miR-320 in AML12 cells suppressed RFX1 expression, leading to upregulated FGF1 production. Conversely, silencing *Rfx1* in AML-12 cells elevated the expression of FGF1 and the phosphorylation of AMPK. Importantly, miRNA pull-down assay confirmed that RFX1 is a direct target of miR-320. These findings strongly support the role of miR-320 in regulating FGF1 expression in hepatocytes through targeting RFX1. RFX1 has been reported to be an essential regulator of major histocompatibility complex type II (MHC II) gene expression, for example, RFX1 negatively regulates the expression of Toll-like receptor 4 (TLR4), transforming growth factor beta 2 (TGF β 2) and type I collagen fibers^{45–47}. Although RFX1 expression is associated with poor prognosis and high recurrence in HCC patients and is involved in doxorubicin-induced HBV reactivation^{48,49}, the functional role of RFX1 during MASH development remains unclear. Our data suggested that silencing *Rfx1* in hepatocytes alleviates HFHC-induced MASH, which may be due to FGF1 upregulation. Recently, it has been reported that RFX1 induces macrophage M1 polarization by promoting DNA demethylation in autoimmune inflammation, and RFX1 has also been identified as a neutrophil fate decision factor to drive the transdifferentiation of mouse fibroblasts to neutrophils and regulates foam cell formation and atherosclerosis by mediating CD36 expression^{50–52}. These findings suggest that RFX1 may be involved in other possible mechanisms involved in improving diet-induced MASH models, and it is necessary to determine the roles of RFX1 and miR-320 in other cell types.

5. Conclusions

In conclusion, hepatic miR-320 levels are downregulated during MASH progression, which correlates with the severity of MASH. Such downregulation aggravates diet-induced MASH, while restoration of hepatic miR-320 expression protects mice against MASH by inhibiting RFX1-mediated FGF1 suppression. Our study not only highlights the functional role of miR-320 in the progression of MASH but also provides strong evidence that miR-320 is a promising target for the treatment of MASH.

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Author contributions

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Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apsb.2025.06.007>.

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