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MicroRNA-205 ameliorates lipid accumulation in non-alcoholic fatty liver disease through targeting NEU1

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Abstract. – OBJECTIVE: The aim of this study was to explore the role of miR-205 in non-alcoholic fatty liver disease (NAFLD) and explore the underlying mechanism.

MATERIALS AND METHODS: High-fat diet (HFD) mice were used as an *in vitro* model of NAFLD. HepG2 and primary hepatocytes (PH) cells were treated with oleic acid (OA) and considered as *in vitro* models of NAFLD. qRT-PCR (quantitative real time polymerase chain reaction) and Western blot were respectively employed to investigate mRNA expression and protein expression level. Further analysis was then applied to analyze the underlying mechanisms. Livers were histologically examined using hematoxylin and eosin (H&E) and Oil Red O staining. TargetScan analysis and Luciferase assay were used to identify the target of miR-205.

RESULTS: MiR-205 was upregulated and NEU1 was downregulated in both HFD-fed mice and OA-treated HepG2 and PH cells. The overexpression of miR-205 caused the decreased weight of body and liver, downregulation of liver triglyceride, and resulted in the enhancement of glycerol concentration, and finally suppressed lipid accumulation. In addition, the TargetScan analysis and Luciferase assay identified neuraminidase 1 (NEU1) as a novel target of miR-205. *In vivo* study suggested that the knockdown of NEU1 ameliorated lipid accumulation. Finally, the *in vitro* investigation showed that the overexpression of miR-205 alleviated lipid accumulation in OA-induced HepG2 and PH cells by targeting NEU1.

CONCLUSIONS: Results revealed that miR-205 facilitated lipid accumulation by inhibiting NEU1 in NAFLD, suggesting that miR-205 might be a potential target for the therapeutic strategy for NAFLD.

Key Words:
MiR-205, NEU1, Non-alcoholic fatty liver disease, Lipid accumulation.

Introduction

Non-alcoholic fatty liver diseases (NAFLD) is a liver metabolic syndrome which is characterized by excessive lipid accumulation[1-3] and is associated with insulin resistance and hyperlipidemia[4]. NAFLD affects about 24% of the population worldwide and is emerged as one of the most common etiological factors leading to chronic liver disease[5,6]. It is well established that the pathogenesis of NAFLD is complicated and appears to be related to genes, gene products, and environmental factors[7-9]. Particularly, lipid accumulation is an important pathogenic factor for fatty liver diseases and may lead to hepatic steatosis[10,11]. Therefore, anti-glycemic drugs are considered as promising therapeutic approaches for NAFLD[12].

MicroRNAs (miRNAs) regulate the expression of the target genes[13], and have been recently regarded as novel biomarkers and potential therapeutic targets for NAFLD[14]. Aberrant expression of miRNAs was found to be associated with human or experimental NAFLD[15-18]. MiRNAs have the ability to modulate metabolic signal pathways, such as lipid and glucose metabolism, which thus appear to be involved in all stages of NAFLD[19]. For example, miR-34a was significantly upregulated in mice with high-fat diet (HFD) that could target Sirtuin-1 (SIRT1) and peroxisome proliferator-activated receptor-alpha (PPARα) to regulate the lipid metabolism that is associated with NAFLD[20]. MiR-185 modulates lipid metabolism and insulin sensitivity in mice fed with HFD and palmitate-induced HepG2 cells, and is considered as a potential therapeutic target for NAFLD[21]. Particularly, miR-205, an important miRNA in the regulation of tumorigenesis[22,23], has been recently reported to regulate lipid metabolism in

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hepatocellular carcinoma[24], and was identified as a modulator of insulin sensitivity[25]. These results indicated the potential role of miR-205 in the progression and development of NAFLD. However, the functional role of miR-205 in NAFLD was still unclear.

Neuraminidase (NEU) cleaves sialic acids from glycoconjugates and regulates the activity of the functional molecules[26]. Among them, NEU1 has been reported to modulate cell proliferation, differentiation, and inflammation[26], and the increased activity of NEU1 was associated with adipogenesis in 3T3-L1 preadipocytes[27]. Besides, in epididymal visceral adipose and livers of mice with obesity and diabetes, the activity of NEU1 was abnormal[28]. Further study revealed that the knockdown of NEU1 in 3T3-L1 adipocytes increased the glycerol concentration and the phosphorylation of perilipin 1 (Plin1), which has been reported to maintain lipid metabolism homeostasis in adipocytes[27,29]. Therefore, miR-205 might regulate lipid accumulation in NAFLD *via* regulating NEU1, but the detailed mechanism remains unclear.

In the present study, we firstly explored the fundamental effect of miR-205 on NAFLD; secondly, the downstream regulator of miR-205, NEU1, was identified. Lastly, we investigated the potential mechanisms underlying the biological role of miR-205/NEU1 axis in the progression of NAFLD. This study provides a basis and new target for the treatment of NAFLD.

Materials and Methods

Animal Models

This study was conducted in accordance with the guidelines for the Care and Use of Laboratory Animals by the National Institute of Health Guide. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Affiliated Hospital of the Guizhou Medical University. Healthy male C57BL/6 mice (6-8 weeks old) were housed separately with standard pellet diet and water before research. After two weeks, the mice were randomly divided into two groups. One group was fed with normal control diet (NCD) with 3.1 % fat, 16.1 % protein, 3.9 % fiber, and 5.1 % ash (minerals) for 6 weeks. Another group was fed with high-fat diet (HFD) with 21.4 % fat, 17.5 % protein, 50 % carbohydrate, 3.5 % fiber, and 4.1 % ash for 8 weeks. Mice were euthanized and livers were harvested. The body and liver weight of each mouse were recorded.

Hematoxylin & Eosin and Oil Red O staining

The livers were dissected and fixed with 4% paraformaldehyde solution, and then processed in a tissue-processing machine. After dehydration in graded ethanol, 5 μm sections were stained with hematoxylin and eosin (H&E). To visualize the lipids, the frozen sections were stained using Oil Red O. The stained sections were viewed under a bright field microscope (Nikon, Tokyo, Japan).

Intracellular Triglyceride and Glycerol Assay

Liver tissues or oleic acid (OA)-treated PH/HepG2 cells were homogenized in protein lysis buffer (Solarbio, Beijing, China). Intracellular triglyceride (TG) and glycerol content were determined by enzymatic kits (Nanjing Jiancheng Bioengineering, Nanjing, China).

Cell Culture

Human embryonic kidney 293T (HEK-293T) cells (Invitrogen, Carlsbad, CA, USA) were cultured in Dulbecco's Modified Eagle's Medium high glucose (H-DMEM; Gibco, Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher, Waltham, MA, USA). Mouse primary hepatocytes (PH) were isolated from the livers of male C57/BL6 mice. The cells were seeded into 6-well plates (6×10^5 cells per well) precoated with 400 μL 1 mg/mL collagen solution. Hepatocytes were cultured in William's medium E (WME, Pan Biotech GmbH, Aidenbach, Germany) containing 100 U/ml penicillin, 100 μg/mL streptomycin, 0.0000l% Insulin-Transferrin-Selenium (ITS), and 100 nM dexamethasone. Human hepatocellular carcinoma cell line HepG2 (ATCC; Manassas, VA, USA) was cultured in DMEM-based medium (Gibco, Thermo Fisher, Waltham, MA, USA) with 10% Fetal Bovine Serum (FBS), 100 μg/mL streptomycin and 100 U/ml penicillin, and maintained at 37¡C in a humidified incubator with 95% air/5% CO_2.

Cell Steatosis Model

PH and HepG2 cells (at 80% confluence) were cultured in FBS-free medium for 24 hours in 96-well culture plate. The cells were then treated with 200 μL of 0.5 mM OA solution for 24 hours. After removing the medium, 100 μL of the fixative solution was added and incubated at room temperature for 15 minutes. The control cells were treated with the OA-free medium containing albumin.

Construction and Transduction of Lentivirus and Adenovirus

The primary precursor sequence of miR-205 was amplified and cloned into lentiviral plasmid pLenti-DEST lentivector (Thermo Fisher Scientific, Waltham, MA, USA). For the transduction of lentivirus with miR control (Vehicle) and miR-205 (miR-205) into HEK-293T cells: 4 x 10^5 cells per well were seeded in 12-well plates and incubated overnight. We removed the culture medium and washed triple times with phosphate-buffered saline (PBS). The cells were transduced with Vehicle or miR-205 lentivirus vector in the presence of Vira Power[a] Packaging Mix (Thermo Fisher Scientific, Waltham, MA, USA) and 8 mg/mL polybrene. The viral supernatants were harvested after transduction for 3 days and the titer was analyzed at a multiplicity of infection (MOI) of 5.

The stable knockdown of NEU1 in HEK-293T cells was achieved using a BLOCK-iT Adenoviral RNAI Expression System (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, HEK-293T cells were transduced with virus expressing negative control (Ad-vector, 5'-CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG-3') or NEU1 shRNAs (Ad-1#NEU1, GTCAACCTTCAAGTGGAAGAT; Ad-2#NEU1, CCAGAAAGCTTGGCAGATAAT) with 8 mg/mL polybrene. The viral supernatants were harvested after transduction of adenovirus for 3 days and analyzed the titer at a multiplicity of infection (MOI) of 5. Moreover, HFD-fed mice were divided into six subgroups (six for each group): control group, HFD mice treated with Vehicle, HFD mice treated with miR-205, HFD mice treated with Ad-vector, HFD mice treated with Ad-1#NEU1, and HFD mice treated with Ad-1#NEU2. All treatments were executed *via* tail-vein injection. At 8th week, the mice were sacrificed, and the liver tissues were collected. The body weight and liver weight were recorded.

Plasmid Construction and Transfection

The miR-205 mimics and negative control (NC-mimics), inhibitor, and NC-inhibitor were synthesized by GenePharma (Shanghai, China). PCR was used to amplify NEU1. Expression plasmids pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) were then constructed and sequenced. The cells were transfected with miR-205 mimics/inhibitor (40 nM) or NC or pcDNA3.1-NEU1 *via* Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. 48 hours after transfection, the cells were harvested for RNA extraction.

Reporter Vectors Construction and Luciferase Assays

The sequences of wild-type or mutant of binding sites between miR-205 and NEU1 were amplified by PCR and subcloned into pmirGLO Dual-Luciferase miRNA target expression vectors (GenePharama, Shanghai, China). HEK-293 T cells were seeded in a 96-well plate and co-transfected with wild-type or mutant pmirGLO-NEU1 reporter plasmid and Vehicle or miR-205 overexpression, respectively. 48 hours later, the Luciferase activities were performed by Dual-Lucifer Reporter assay system (Promega, Madison, WI, USA) and detected by lumat LB 9501 luminator (EG&G Berthold, Bundoora, Victoria, Australia). Firefly Luciferase gene in the vector pGL3-control was used as a control.

RNA Preparation and qRT-PCR

RNAs from tissues or cells were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and miRNAs were extracted using miRcute miRNA isolation kit (Tiangen, Beijing, China). The cDNAs were synthesized using Reverse Transcription System Bestar qPCR RT Kit with ABI 7500 Real Time-PCR System (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal reference. The primer sequences were listed: miR-205, 5'-CTTGTCCTTCATTCCACCGGA-3' (forward) and 5'-TGCCGCCTGAACTTCACTCC-3′ (reverse); NEU1, 5'-TGTGACCTTCGACCCTGAGC-3' (forward) and 5'-TCGCAGGGTCAGGTTCACTC-3′ (reverse); GAPDH, 5'-TGTTCGTCATGGGTGTGAAC-3' (forward) and 5'-ATGGCATGGACTGTGGTCAT-3' (reverse).

Western Blot Analysis

Liver tissues or cells were homogenized in the protein lysis buffer (Solarbio, Beijing, China). The extracted proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane. The membranes were blocked overnight in 5% skim milk solution. After washing with Tris-Buffered Saline containing 0.05% Tween 20 (TBST) for three times, the membranes were incubated with primary antibodies (NEU1, 1:1000; β-actin and GAPDH, 1:2000, Cell Signaling Technology (Danvers, MA, USA) for 2 hours. Washed with TBST for 10 minutes, the membranes were then incubated with secondary antibodies (1:3000, anti-rabbit IgG antibodies, Sigma-Aldrich, St. Lou-

is, MO, USA) at room temperature for 1 hour. Immunoreactivity was determined using enhanced chemiluminescence (Millipore, Billerica, MA, USA), and analyzed by Image J program. β-actin and GAPDH were used as controls.

Statistical Analysis

Data were presented as mean ± SEM (standard error of the means). All experiments were performed at least in three independent times. The statistical significance between the two groups was analyzed by One-way analysis of variance (ANOVA) followed by Duncan's multiple-comparison test using SPSS 19.0 (IBM Corp., Armonk, NY, USA). $p<0.05$, $p<0.01$ or $p<0.001$ was considered as statistically significant.

Results

MiR-205 Was Upregulated and NEU1 Was Downregulated in Livers of HFD-Fed Mice and OA-Treated PH and HepG2 Cells

In order to explore the potential role of miR-205 and NEU1 in the progression of NAFLD, we initially used HFD (high-fat diet)-fed mice and examined hepatic expression of miR-205 or NEU1. The establishment of NAFLD model was firstly verified using H&E and Oil Red O staining in HFD-fed mice. As depicted in Figure 1A, the histological examination of liver tissues showed structural abnormalities and fatty degeneration in HFD-fed mice compared to that in NCD (normal control diet) group. Oil Red O staining showed more lipid accumulation, characterized by the enhancement of triglyceride (TG) in HFD as compared to that in the NCD group (Figure 1A). These results confirmed the successful construction of the NAFLD mice model.

MiR-205 expression was significantly increased in steatotic livers from HFD-fed mice as compared to that from NCD mice ($p<0.001$) (Figure 1B). By contrast, the mRNA (Figure 1B) and protein (Figure 1C) expressions of NEU1 were decreased in steatotic livers from HFD-fed mice as compared to that from NCD mice, suggesting the potential role of miR-205 and NEU1 in the progress of NAFLD.

The expression of miR-205 and NEU1 was also detected in the *in vitro* experiment. Consistently, miR-205 was also rapidly increased after OA stimulation in both HepG2 ($p<0.001$) and PH

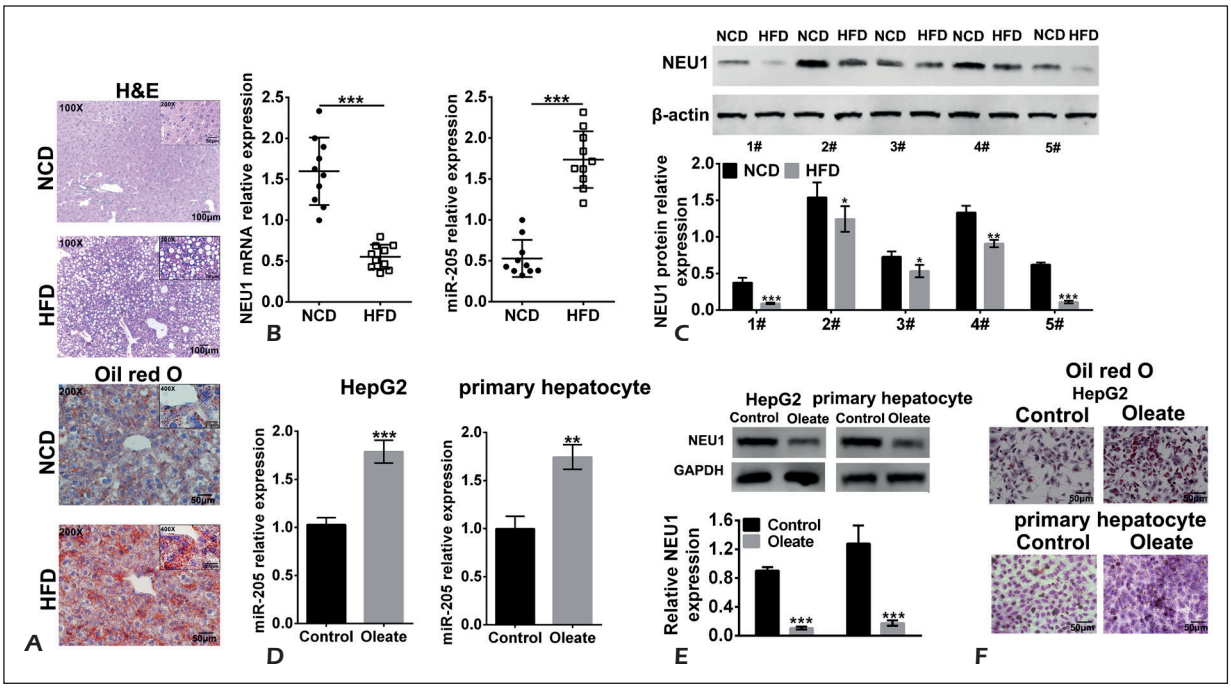

Figure 1. MiR-205 was upregulated and NEU1 was downregulated in livers of HFD-fed mice and OA-treated PH and HepG2 cells. **A**, H&E and Oil Red O staining of liver tissues of HFD and NCD-fed mice. Magnifications, 400x. **B**, qRT-PCR analysis of miR-205 and NEU1 in the livers of HFD-fed *vs.* NCD-fed mice. *** HFD *vs.* NCD $p<0.001$. **C**, Western blot analysis of NEU1 in the livers of HFD-fed *vs.* NCD-fed mice. *, **, *** HFD *vs.* NCD $p<0.05$, $p<0.01$, $p<0.001$. **D**, qRT-PCR analysis of miR-205 in OA-treated and control PH and HepG2 cells. **, *** OA *vs.* control $p<0.01$, $p<0.001$. **E**, Western blot analysis of NEU1 in OA-treated and control PH and HepG2 cells. *** OA *vs.* control $p<0.001$. **F**, Oil Red O staining of OA-treated and control PH and HepG2 cells. Magnifications, 400x.

cells (p<0.01) (Figure 1D). The protein expression of NEU1 was also downregulated in *in vitro* cell models (Figure 1E). Oil Red O staining revealed that OA treatment led to a significant enhancement of intracellular lipids in HepG2 and PH cells (Figure 1F). In conclusion, the expression of miR-205 was upregulated and NEU1 was downregulated in both *in vivo* and *in vitro* NAFLD models.

Overexpression of Mir-205 Ameliorated Lipid Accumulation in HFD-Fed Mice

To detect the functional role of miR-205 in the lipid accumulation of NAFLD, miR-205 overexpressing plasmid was transduced into HEK-293 T cells by lentivirus system. The supernatant of cell culture was then injected into HFD-fed mice, and the transduction efficiency was confirmed (Figure 2A). H&E and Oil Red O staining revealed that the overexpression of miR-205 could ameliorate the structural abnormalities and lipid accumulation in HFD-fed mice (Figure 2B). The increased weight of body (Figure 2C) and liver (Figure 2D) in HFD-fed mice were reversed after the overexpression of miR-205. Based on the fact that highly elevated TG levels derived from glycerol may cause fatty liver disease and are strongly associated with NAFLD, the levels of liver TG and glycerol were thus determined. The results showed that liver TG was elevated in HFD-fed mice, while the overexpression of miR-205 decreased the liver TG level (Figure 2E). Besides, the overexpression of miR-205 also increased the downregulation of glycerol level in HFD-fed mice (Figure 2F). In general, elevated expression of miR-205 could effectively ameliorate lipid accumulation in HFD-fed mice.

Knock Down of NEU1 Ameliorated Lipid Accumulation in HFD-Fed Mice

To detect the functional role of NEU1 in the lipid accumulation of NAFLD, we firstly transduced NEU1 shRNA into HEK-293 T cells by adenovirus system (Ad-1#NEU1 or Ad-2#NEU1), and the su-

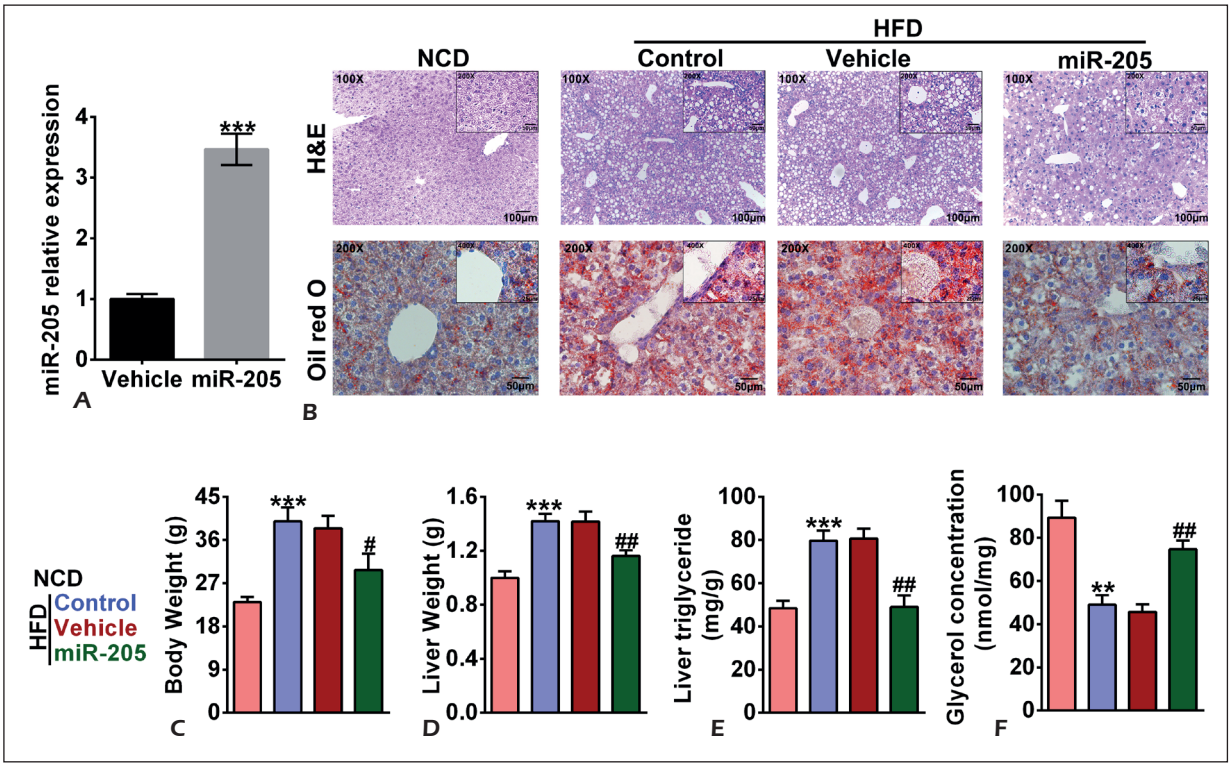

Figure 2. The overexpression of miR-205 ameliorated lipid accumulation in HFD-fed mice. **A**, qRT-PCR analysis of miR-205 in HFD-fed mice injected with supernatant in cell culture of miR-205 over-expression (miR-205) and negative control (Vehicle). *** miR-205 *vs.* Vehicle p<0.001. **B**, H&E and Oil Red O staining analysis on the effect of miR-205 on tissue morphology and lipid accumulation in HFD-fed mice. Magnifications, 400x. **C**, The effect of miR-205 on body weight in HFD-fed mice. *** HFD *vs.* NCD p<0.001. # miR-205 *vs.* Vehicle p<0.05. **D**, The effect of miR-205 on liver weight in HFD-fed mice. *** HFD *vs.* NCD p<0.001. ## miR-205 *vs.* Vehicle p<0.01. **E**, The effect of miR-205 on liver triglyceride in HFD-fed mice. *** HFD *vs.* NCD p<0.001. ## miR-205 *vs.* Vehicle p<0.01. **F**, The effect of miR-205 on liver glycerol in HFD-fed mice. ** HFD *vs.* NCD p<0.01. ## miR-205 *vs.* Vehicle p<0.01.

pernatant of cell culture was collected and injected into HFD-fed mice. By using qRT-PCR (Figure 3A) and Western blotting (Figure 3B), NEU1 was significantly downregulated in HFD-fed mice after treatment of Ad-1#NEU1 or Ad-2#NEU1 compared to that of Ad-Vector ($p < 0.001$). H&E and Oil Red O staining also revealed that the structural abnormalities and lipid accumulation in Ad-Vector and NC (negative control) were ameliorated by knockdown of NEU1 (Figure 3C). The knockdown of NEU1 also decreased the body weight (Figure 3D), liver weight (Figure 3E), and liver TG level (Figure 3F) in HFD-fed mice as compared to that in the Ad-Vector and NC groups. The knockdown of NEU1 also increased the glycerol level in HFD-fed mice (Figure 3G). Therefore, these results suggested that the knockdown of NEU1 could alleviate lipid accumulation in HFD-fed mice.

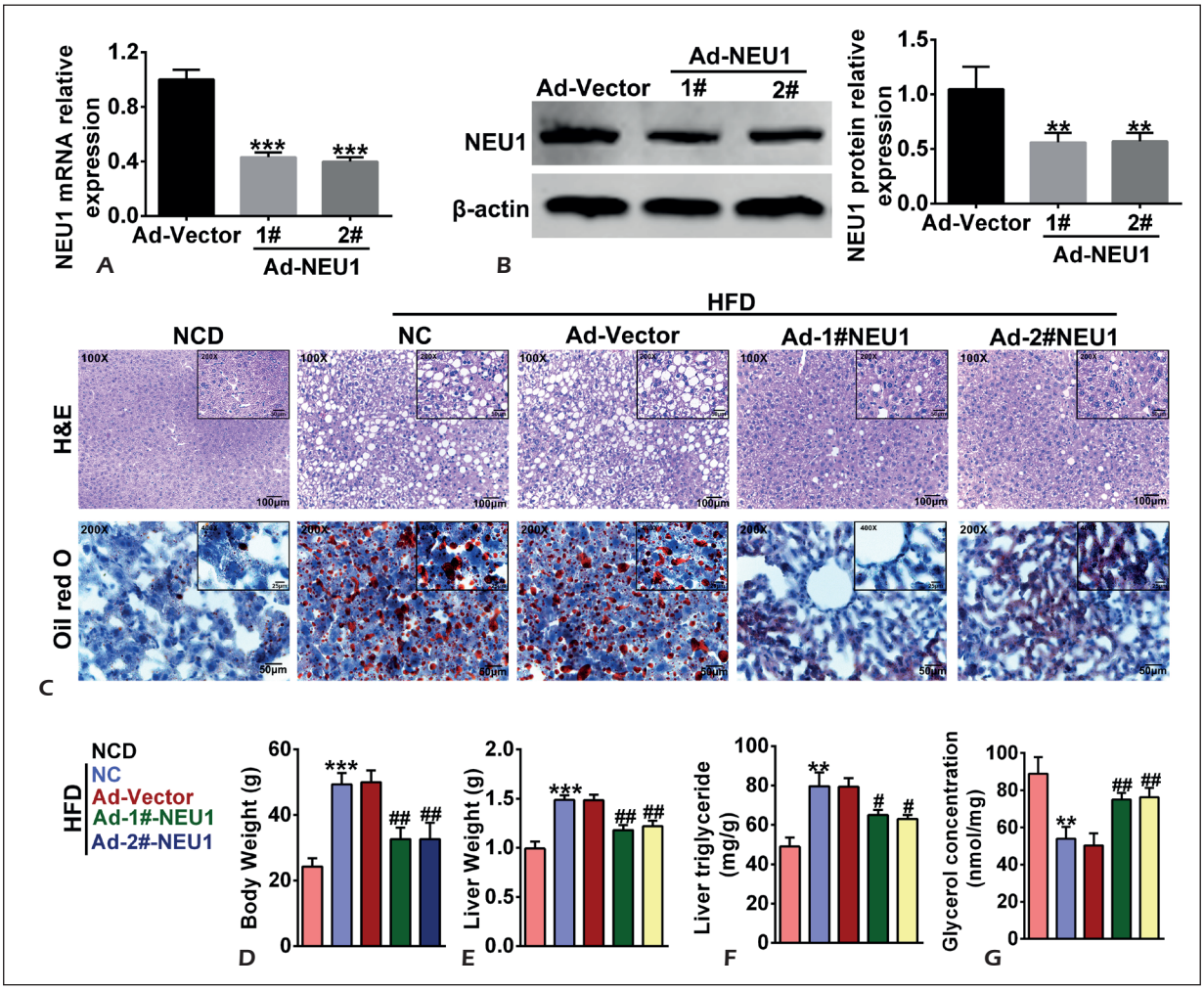

Figure 3. Knockdown of NEU1 ameliorated lipid accumulation in HFD-fed mice. **A**, qRT-PCR analysis of NEU1 in HFD-fed mice injected with supernatant in cell culture of NEU1 knockdown (Ad-1#NEU1, Ad-2#NEU1) and negative control (Ad-Vector). *** Ad-1#NEU1, Ad-2#NEU1 *vs.* Ad-Vector $p<0.001$. **B**, Western blot analysis of NEU1 in HFD-fed mice injected with Ad-1#NEU1, Ad-2#NEU1, and Ad-Vector. ** Ad-1#NEU1, Ad-2#NEU1 *vs.* Ad-Vector $p < 0.01$. **C**, H&E and Oil Red O staining analysis on the effect of Ad-1#NEU1, Ad-2#NEU1 on tissue morphology and lipid accumulation in HFD-fed mice. Magnifications, 400x. **D**, The effect of Ad-1#NEU1, Ad-2#NEU1 on body weight in HFD-fed mice. *** HFD *vs.* NCD $p<0.001$. ## Ad-1#NEU1, Ad-2#NEU1 *vs.* Vehicle $p<0.01$. **E**, The effect of Ad-1#NEU1, Ad-2#NEU1 on liver weight in HFD-fed mice. *** HFD *vs.* NCD $p<0.001$. ## Ad-1#NEU1, Ad-2#NEU1 *vs.* Vehicle $p<0.01$. **F**, The effect of Ad-1#NEU1, Ad-2#NEU1 on liver triglyceride in HFD-fed mice. ** HFD *vs.* NCD $p<0.01$. # Ad-1#NEU1, Ad-2#NEU1 *vs.* Vehicle $p<0.05$. **G**, The effect of Ad-1#NEU1, Ad-2#NEU1 on liver glycerol in HFD-fed mice. ** HFD *vs.* NCD $p<0.01$. ## Ad-1#NEU1, Ad-2#NEU1 *vs.* Vehicle $p<0.01$.

NEU1 Was a Target of MiR-205

TargatScan analysis predicted that miR-205 could target 3'UTR of NEU1 (Figure 4A). Luciferase assays were then performed to verify it, and the result suggested that miR-205 mimics dramatically decreased the Luciferase activity of cells that transfected with wild-type NEU1 3'UTR in comparison to that transfected with Vehicle (p<0.001) (Figure 4B). These results indicated that NEU1 was a direct target of miR-205. The transfection efficiency of miR-205 mimics and inhibitor were respectively determined (Figure 4C). Western blot (Figure 4D) revealed that miR-205 mimics significantly inhibited NEU1 expression in HepG2 and PH cells. By contrast, miR-205 inhibitor led to an upregulation of the protein expression of NEU1 (Figure 4D). These results revealed that miR-205 directly targeted and negatively regulated NEU1 expression.

MiR-205 Regulated Lipid Accumulation in PH and HepG2 Cells Via Inhibition of NEU1

The mRNA (Figure 5A) and protein (Figure 5B) expression levels of NEU1 in OA-treated PH and HepG2 cells that transfected with pcDNA-3.1-NEU1 were increased. MiR-205 mimics decreased NEU1 expression in both OA-treated PH and HepG2 cells (Figure 5C), while the addition of pcDNA-3.1-NEU1 resulted in a significant reduction of NEU1 expression (Figure 5C). Furthermore, consistent with the *in vivo* results, miR-205 mimics prevented lipid accumulation (Figure 5D), while NEU1 overexpression resulted in a dramatical induction of lipid accumulation (Figure 5D). The levels of TG and glycerol were also respectively decreased and increased by miR-205 mimics in HepG2 (Figure 5E) and PH cells (Figure 5F). Therefore, these investiga-

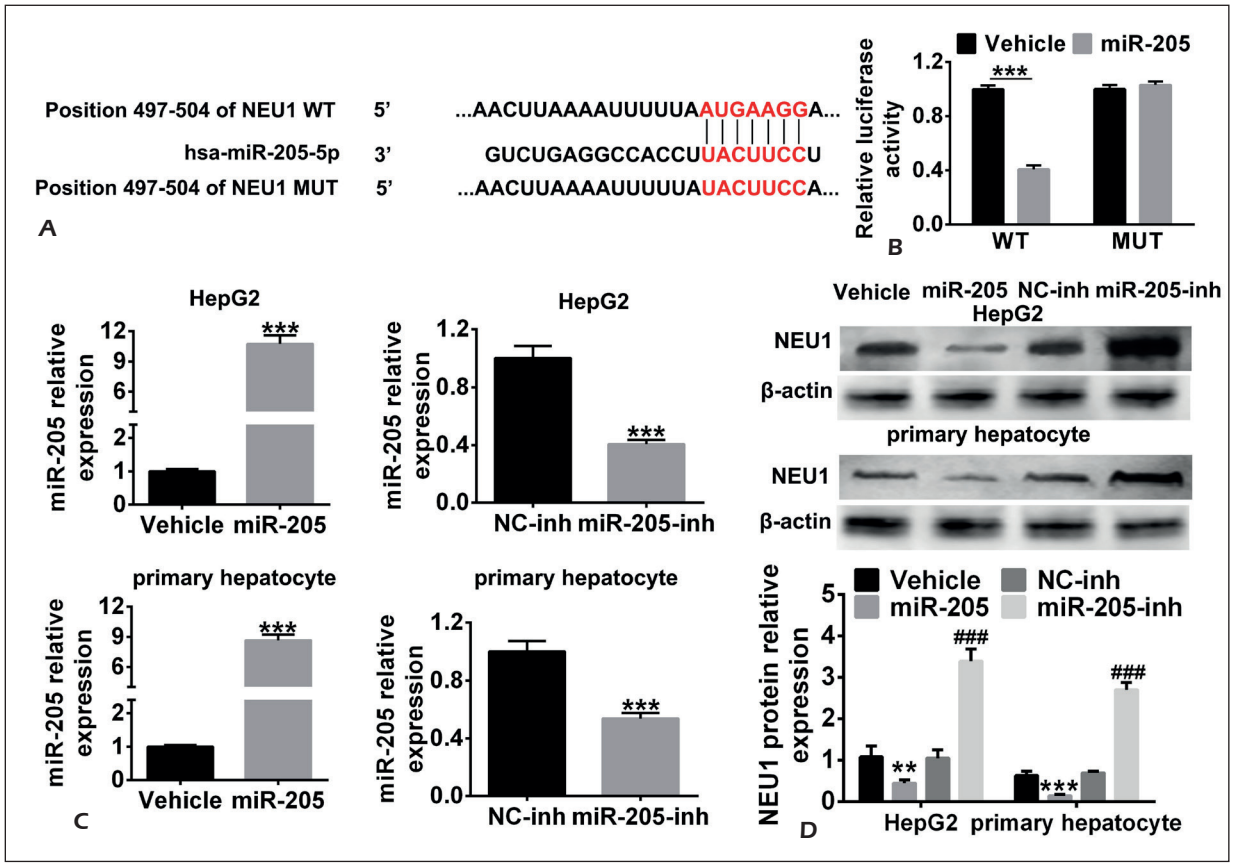

Figure 4. NEU1 was a target for miR-205. **A**, The predicted target sites between the 3'UTR of NEU1 and miR-205, as well as the mutant sequences. **B**, Luciferase assays of miR-205 mimics (miR-205) and Vehicle with wild-type or mutant NEU1 3'UTR. *** miR-205 *vs.* Vehicle p<0.001. **C**, qRT-PCR analysis on the effect of miR-205 or miR-205-inhibitors (miR-205-inh) on the expression of miR-205 in PH and HepG2 cells. *** miR-205 *vs.* Vehicle p<0.001, ### miR-205-inh *vs.* NC-inh p<0.001. **D**, Western blot analysis on the effect of miR-205 or miR-205-inhibitors (miR-205-inh) on the expression of NEU1 in PH and HepG2 cells. **, *** miR-205 *vs.* Vehicle p<0.01, p<0.001, ### miR-205-inh *vs.* NC-inh p<0.001.

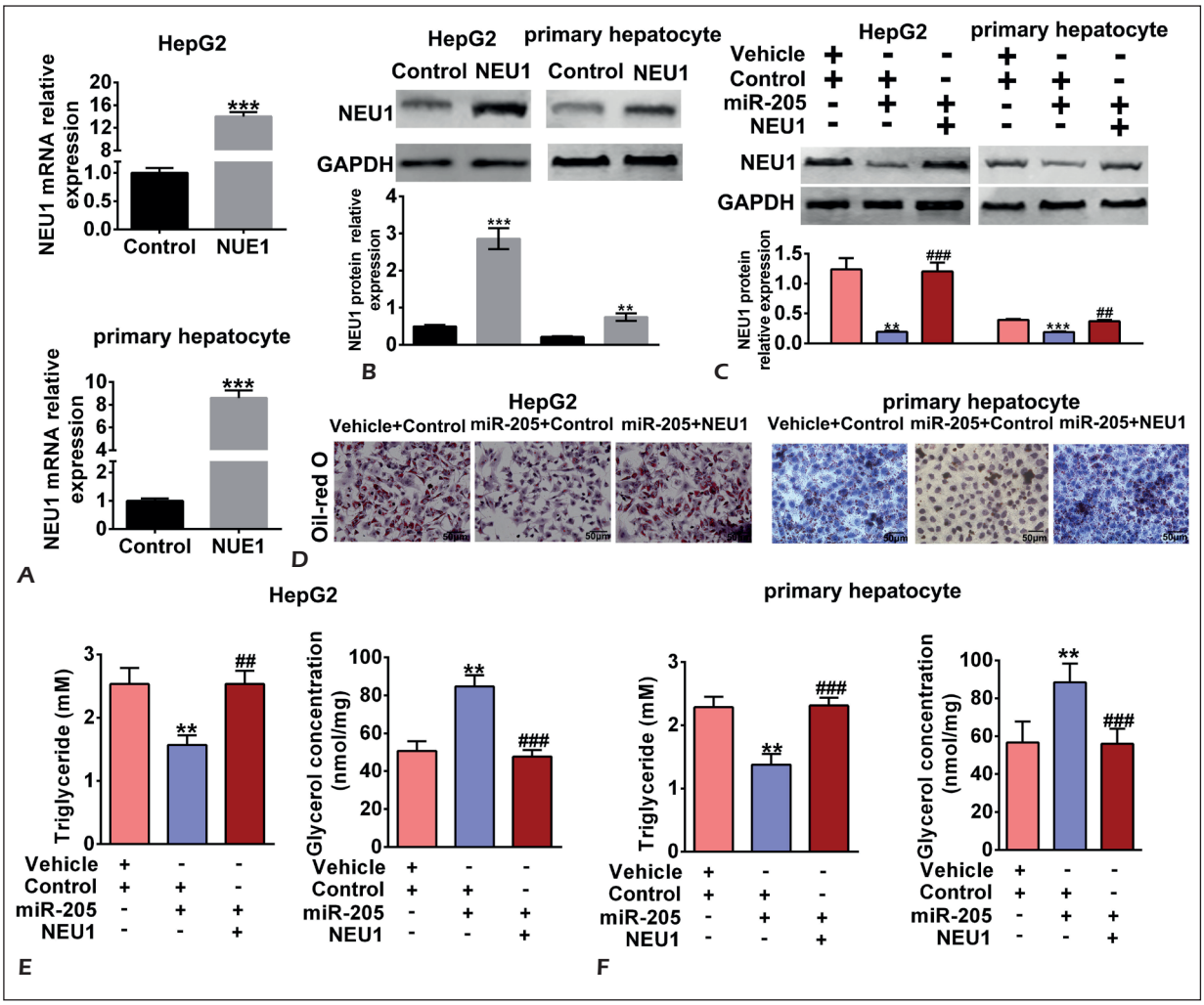

Figure 5. MiR-205 regulated lipid accumulation in PH and HepG2 Cells via inhibition of NEU1. **A**, qRT-PCR analysis on the effects of NEU1 over-expression (NEU1) on HepG2 and PH cells. *** NEU1 *vs.* Control $p<0.001$. **B**, Western blot analysis of NEU1 in HepG2 and PH cells. **, *** NEU1 *vs.* Control $p<0.01$, $p<0.001$. **C**, Western blot analysis on the effect of miR-205 and NEU1 on the protein expression of NEU1 in HepG2 and PH cells. **, *** miR-205 *vs.* control $p<0.01$, $p<0.001$, ##, ### miR-205+NEU1 *vs.* control $p<0.01$, $p<0.001$. **D**, Oil Red O staining analysis on the effect of miR-205 and NEU1 on lipid accumulation in HepG2 and PH cells. Magnifications, 400x. **E**, The effect of miR-205 and NEU1 on triglyceride in HepG2 cells. ** miR-205 *vs.* control $p<0.01$, ##, ### miR-205+NEU1 *vs.* control $p<0.01$, $p<0.001$. **F**, The effect of miR-205 and NEU1 on triglyceride in PH cells. ** miR-205 *vs.* control $p<0.01$, ### miR-205+NEU1 *vs.* control $p<0.001$.

tions demonstrated that miR-205 could suppress lipid accumulation in OA-induced PH and HepG2 Cells *via* inhibition of NEU1.

Discussion

Due to the unhealthy diet and modern lifestyle of people, NAFLD is gradually emerged as a worldwide metabolic syndrome that could ultimately contribute to liver fibrosis, cirrhosis, or hepatocellular carcinoma[30-32]. Hepatocellular steatosis with over 5% fat presented in hepatocytes is the hallmark and histological diagnosis criterion of NAFLD[33]. Researchers[34,35] have shown that fat accumulation in liver caused oxidative stress or the release of inflammatory cytokines, thus leading to steatohepatitis, fibrosis, and even NAFLD. Thus, it is urgent to explore the effective therapeutic schedule that can inhibit lipid accumulation in NAFLD. In the present study, we indicated that miR-205 overexpression attenuated NAFLD by suppressing lipid accumulation, representing a new hope for cure of this disease.

Previous investigations[36,37] have shown that excessive production of free fatty acids (FFAs) may

induce NAFLD by several mechanisms beyond direct cytotoxicity. OA is the most abundant unsaturated fatty acids in livers of patients with NAFLD[38]. Therefore, HFD-fed mice and OA-treated PH and HepG2 cells were respectively used as *in vivo* and *in vitro* NAFLD models. MiRNAs function as post-transcriptional gene regulators and that play vital roles controlling lipid metabolism[39]. Our study firstly showed the overexpression of miR-205 both *in vivo* and *in vitro*, suggesting its potential role in the development of NAFLD. Consistently, miR-205 deregulates lipid metabolism in hepatocellular carcinoma by targeting acylCoA synthetase long-chain family member 1 (ACSL1) mRNA, which is an important and abundant lipid metabolism enzyme in liver[40]. Tao et al[39] indicated that miR-205 is an important miRNA related to adipogenesis and lipid metabolism, and negatively regulates hepatic acetyl-CoA carboxylase β (ACAC β) mRNA in lipid metabolism of *Oreochromis niloticus*. These investigations suggested the important role of miR-205 in lipid metabolism.

Lipid accumulation in livers may promote hepatocellular damage[41], and induced a release of Òdanger signalsÓ, such as various cytokines, thus facilitating the progression of NAFLD[42,43]. The observation that miR-205 overexpression in HFD-fed mice ameliorated lipid accumulation clearly revealed a vital role of miR-205 in the progress of NAFLD. Fundamentally, miR-205 overexpression was not restricted to decrease body and liver weight of HFD-fed mice, but also was beneficial for the hallmark of NAFLD, characterized by decreased TG accumulation and increased glycerol level, which were also found in other studies[44,45]. Consistently, previous researches have shown that miR-205 inhibition increased cell proliferation and lipid accumulation in 3T3-L1 preadipocytes[46]. Cui et al[47] showed that miR-205 mediated cu-induced lipid accumulation in yellow catfish Pelteobagrus fulvidraco. Therefore, we concluded that miR-205 may be beneficial for NAFLD by inhibiting lipid accumulation in livers and over-expression of miR-205 may be used as a potential approach for NAFLD treatment.

Further analysis identified NEU1 as a direct target of miR-205. Gain-of-function for NEU1 caused increased content of TG and decreased glycerol level in PH and HepG2 cells, and the loss-of function resulted in decreased TG level and increased glycerol level in mouse livers, further suggesting the vital role of NEU1 in NAFLD. Consistently, Natori et al[27] revealed that the knockdown of NEU1 in adipocytes increased the glycerol concentration and the phosphorylation of Plin1, which has been reported to maintain lipid metabolism homeostasis in adipocytes. Moreover, the overexpression of miR-205 could suppress NEU1 expression, indicating that miR-205/NEU1 axis could be used for NAFLD treatment. Differently from previous conclusion[18,48] indicating that inhibition of specific miRNAs in HFD-fed mice prevented lipid accumulation and hyperlipidemia in NAFLD, miR-205 showed converse effect, because its overexpression relieved side effects caused by NAFLD, which was partially consistent with previous study[49]. Indeed, different targets for miR-205 were also reported previously[24], and the exact targets for miR-205 and the detailed mechanism in patients with NAFLD need further investigation.

Conclusions

This research revealed that miR-205 could be a physiological factor for the pathogenesis of NAFLD, and act as an important anti-lipotoxic factor through the downregulation of lipid accumulation. Understanding the modulatory mechanism of miR-205 in NAFLD could be helpful for exploring useful clinical biomarkers or indicators for NAFLD. Moreover, our study suggested that miR-205/NEU1 might be a potential therapeutic target for treatment of NAFLD. The lack of clinic trial studies to confirm the therapeutic potential of miR-205/NEU1 is one of the limitations of this study.

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Conflict of Interest

The authors declare that they have no conflict of interests, and all authors confirm its accuracy.

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