miR-212-5p suppresses lipid accumulation by targeting FAS and SCD1

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Abstract

MicroRNAs, a class of small noncoding RNAs, are implicated in controlling a variety of biological processes. We have shown that leucine deprivation suppresses lipogenesis by inhibiting fatty acid synthase (FAS) expression in the liver previously; the aim of our current study is to investigate which kind of microRNA is involved in the regulation of FAS expression in response to leucine deprivation. Here, we indicated that microRNA-212-5p specifically binds to mouse FAS 3'UTR and inhibits its activity. Leucine deficiency significantly increased the mRNA levels of miR-212-5p in the livers of mice. Further studies proved that miR-212-5p also directly binds to the 3'UTR of stearoyl-CoA desaturase-1 (SCD1) to inhibit its activity. Overexpression of miR-212-5p decreases the protein levels of FAS and SCD1 *in vitro* and *in vivo*, and silencing of miR-212-5p has the opposite effects in mouse primary hepatocytes. Moreover, overexpression of miR-212-5p significantly decreases triglyceride (TG) accumulation in primary hepatocytes and in the livers of mice injected with adenovirus-mediated overexpressing of miR-212-5p (Ad-miR-212). Interestingly, inhibition of miR-212-5p reverses the suppressive effects of leucine deficiency on FAS and SCD1 expression, as well as TG accumulation in mouse primary hepatocytes. Finally, we demonstrate that leucine deficiency induces the expression of miR-212-5p in a GCN2/ATF4-dependent manner. Taken together, our results demonstrate a novel function of hepatic miR-212-5p in the regulation of lipid metabolism which represents a potential therapeutic target for the treatment of non-alcohol fatty liver diseases (NAFLD).

Key Words

- ▶ miR-212-5p
- ▶ FAS
- ▶ SCD1
- ▶ lipid accumulation
- ▶ NAFLD

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease worldwide and is characterized by hepatic lipid accumulation in the absence of heavy alcohol consumption (de Alwis & Day 2008). NAFLD is widely associated with obesity, type 2 diabetes, dyslipidemia and the metabolic syndrome

(Samuel *et al.* 2010, Samuel & Shulman 2012). In order to develop appropriate treatment, the knowledge of the molecular mechanisms underlying the development of NAFLD is essential. The largest contributor to liver steatosis is dysregulated hepatic *de novo* lipogenesis (DNL) (Fabbrini *et al.* 2010). DNL is a process in which

the cell makes fatty acids from nonfat materials and liver is the most important organ for DNL (Lawes & Gilbert 1877). Several transcription factors, including sterol regulatory element-binding proteins (SREBPs), liver X receptor (LXR) and carbohydrate response element-binding protein (ChREBP) regulate the expression of genes involved in DNL in the liver (Iizuka & Horikawa 2008, Ferre & Foulle 2010, Strable & Ntambi 2010, Ducheix *et al.* 2011). These transcriptional factors ultimately affect the expression of key enzymes for DNL, which include that fatty acid synthase (FAS) (Jensen-Ustad & Semenkovich 2012); and stearoyl-CoA desaturase 1 (SCD1) (Ntambi 1992). Given that $26 \pm 7\%$ of hepatic triglycerides are derived from DNL in humans with NAFLD (Donnelly *et al.* 2005), decreased DNL in liver is a potential and interesting target for the treatment of NAFLD. Therefore, a better knowledge of the regulation of the key enzymes that control DNL in liver, namely FAS and SCD1, may in the future help the development of potential therapeutic approaches for NAFLD.

MicroRNAs (miRNA) is a new class of small, noncoding RNAs that regulate gene expression at the posttranscriptional level by binding, in most cases, to the 3'-untranslated region (UTR) of target genes and inhibiting translation or causing mRNA cleavage (Ambros 2001, Bartel 2004, He & Hannon 2004, Wilfred *et al.* 2007). Previous work suggested that microRNAs are implicated in the process of NAFLD (Jin *et al.* 2009, Hoekstra *et al.* 2012, Feng *et al.* 2014). It is intriguing that circulating and liver miR-122 plays a role of physiological significance in the biology of NAFLD (Pirola *et al.* 2013, Miyaaki *et al.* 2014, Pirola *et al.* 2015). In addition, miR-185 significantly decreases the mRNA levels of *Fas* and sterol regulatory element-binding protein 1c (*Srebp1c*) and plays an important role in regulating fatty acid metabolism (Wang *et al.* 2014). Our previous study indicated that dietary deprivation of leucine suppresses lipid accumulation in the liver by downregulating the expression of FAS in a GCN2-dependent manner (Guo & Cavener 2007). The involvement of microRNA in this regulation, however, has not been reported. In the present study, we demonstrated that leucine deprivation inhibits FAS and SCD1 expression via GCN2/ATF4/miR-212-5p axis with results of decreased TG accumulation in hepatocytes. These findings suggested that nutritional or pharmacological modulation of miR-212-5p-FAS/SCD1 axis could be potential therapeutic approaches for treating NAFLD.

Materials and methods

Animals and treatment

Male 8- to 10-week-old C57BL/6J mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China), and fed a leucine-deficiency diet for 7 days. Mice were maintained on a 12h light/darkness cycle at 25°C and provided with free access to tap water. The Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences (Shanghai, China) approved all the animal-related experimental procedures in this study (permit number: INS09-1001).

Cell culture and treatments

Mouse primary hepatocytes were prepared from livers of male C57BL/6J mice as described previously (Wang *et al.* 2009). Mouse primary hepatocytes were maintained in Dulbecco's Modified Eagle's medium (DMEM) with 25 mmol/L glucose from Gibco, 10% FBS, 50 mg/mL penicillin and streptomycin at 37°C, and 5% CO₂-95% air. miRNA double-stranded mimics for miR-212-5p or miR-212-5p inhibitors were purchased from GenePharma (Shanghai, China) with the following sequences: miR-212-5p mimics (5'-accuuggcucuaagacugcuacu-3') and miR-212-5p inhibitors (5'-aguaagcagucuaagccaaggu-3', 2'Ome modification). Primary hepatocytes were transfected with miR-212-5p mimics, inhibitors or negative control for 48h.

Bioinformatic analysis

miRanda and miRBase Release 16.0 were used to identify the potential putative targets of miR-212-5p and miR-212-3p.

DNA constructs and mutagenesis

The DNA fragment encoding miR-212 pre-miRNA was amplified using mouse genomic DNA as the PCR template and inserted into the expressing vector named pShuttle-CMV. DNA fragments of 3'-untranslated regions (UTRs) from FAS and SCD1 containing potential miR-212-5p binding sites were amplified and cloned into Xba I site immediately downstream of the stop codon in the pGL3-promoter vector from Promega. Mutated FAS and SCD1 3'UTR reporter plasmids were constructed using the overlapping four-primer PCR to produce mutated 3'UTR pGL3 reporter plasmid.

Luciferase assay

Human embryonic kidney (HEK) 293 cells were cultured in 96-well plates and at ~90% confluence, 150 ng of plasmid containing either the mutated or the wild-type the 3'UTR sequence were transfected using Lipofectamine 2000 Transfection Reagent and Opti-MEM from Invitrogen. Renilla plasmid (5 ng) was cotransfected as the transfection control. The cells were cotransfected with either the negative control or the mimic (50 ng) and incubated for 24 h. Cells were then lysed using 1× Passive lysis buffer from Promega and luciferase activity was measured using a luminometer from Amersham Buchler (Braunschweig, Germany) according to the manufacturer's instructions. Firefly luciferase values were normalized to those of Renilla luciferase.

Generation and administration of recombinant adenoviruses

The recombinant adenoviruses used for miR-212 expression was generated using AdEasy Vector System from Qbiogene (CA, USA). Viruses were diluted in PBS and administered at a dose of 1×10^7 pfu/well in 12-well plate or 5×10^8 pfu/mice through tail vein injection.

RNA isolation and relative quantitative PCR

Total RNA was prepared from frozen tissues with TRIzol from Invitrogen reagent. For mRNA detection, Two micrograms of RNA were reversely transcribed with random primer and M-MLV reverse transcriptase from Takara. Quantitative amplification by PCR was carried out using SYBR Green I Master Mix reagent from Takara by ABI 7900 system. GAPDH was used as an internal control for each gene of interests. For miRNA detection, polyA tail was added to RNase-free DNase-digested total RNA using the *E. coli* polyA polymerase from NEB (Beverly, MA, USA). One microgram of the tailed total RNA was reverse transcribed with miR dTRT primer (5'-cgactcgatcca gtctcagggtcc gaggtattcgatc gactgcactt tttttttttv-3'). SYBR Green qRT-PCR was used to assay miRNA expression with the specific forward primers and the universal reverse primer complementary (Hi-rev: 5'-ccagtctcagggtccgaggtattc-3') to the anchor primer. U6 was used as internal control.

Western blotting

Mouse liver and cell extracts were lysed by RIPA lysis buffer (150 mmol/L Tris-HCl, 50 mmol/L NaCl, 1% NP-40, 0.1%

tween 20), and centrifuged at 15,000g for 20 min. Then, the supernate was mixed with isometric sodium dodecyl sulfate (SDS) buffer (125 mmol/L, Tris-HCl (pH 6.8), 10% mercaptoethanol (v/v), 4% SDS (w/v), 20% glycerol (v/v) and 0.002% bromophenol blue). The mixture was heated for 10 min at 100°C. Supernatants were subjected to 10% SDS-PAGE gels and were wet-blotted. The primary antibodies against FAS (BD Biosciences), SCD1 and SREBP1c (Santa Cruz Biotechnology), and ACTIN (Sigma) were incubated overnight at 4°C and visualized by ECL Plus from GE Healthcare. Band intensities were measured by Tanon-5500 Chemiluminescent Imaging System from Tanon Science & Technology (Shanghai, China).

Measurements of triglyceride (TG), cholesterol (TC) and free fatty acids (FFAs)

Hepatic and cellular lipids were extracted with chloroform-methanol (Folch *et al.* 1957) and TG, TC and FFAs were determined using TG kit, TC kit (Jiancheng, Nanjing, China) and FFAs kit (Wako Pure Chemical Industries), respectively, according to the manufacturer's instructions. The values obtained were normalized to the total protein content and are expressed as nmol/μg protein. Control and oleate sodium- (200 μmmol/L) treated cells and hepatic lipids were also evaluated for the levels of triglycerides in an identical manner. For hepatic lipid determination, the values obtained were normalized to the liver weight and are expressed as nmol/g liver.

Statistical analysis

All data are expressed as means ± S.E.M. Significant differences were assessed by two-tail Student's *t*-test or one-way ANOVA followed by the Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

Results

Leucine deficiency induces the expression of miR-212

Our previous study indicated that leucine deprivation inhibits lipogenesis in liver via downregulating FAS expression in the liver (Guo & Cavener 2007). To explore the possible role of miRNAs in this process, we used TargetScan, PicTar and miRanda to predict the potential miRNAs targeting FAS. These potential microRNAs included miR-27a, miR-27b, miR-33, miR-130a, miR-132, miR-191, miR-212 and miR-214. Further study showed that miR-27a, miR-27b, miR-132, miR-191, miR-212

and miR-214 can significantly suppress the activity of FAS 3'UTR (Fig. 1A). Among them, miR-212 is the most effective miRNAs for the regulation of FAS 3'UTR. To further investigate the possible role of miR-212 in the process of leucine deprivation-suppressed FAS expression and TG accumulation, we examined the expression of miR-212 in the liver of mice fed a control diet or leucine-deficiency diet. The results showed that both miR-212-3p and miR-212-5p were increased in the livers of mice fed a leucine-deficiency diet (Fig. 1B). However, it is miR-212-5p, not miR-212-3p, that significantly suppressed the activity of FAS 3'UTR (Fig. 1C).

miR-212-5p directly targets FAS and SCD1

Given that miR-212-5p, but not miR-212-3p, targets FAS, the effect of miR-212-5p on lipid metabolism was further investigated. FAS and SCD1, both key enzymes for lipogenesis in liver, are predicted to be direct targets of miR-212-5p (Fig. 2A). To verify this, we generated Renilla luciferase reporter plasmids containing miR-212-5p binding sites in the 3'UTRs of mouse FAS and SCD1. A reporter gene assay performed in HEK293 cells revealed miR-212-5p-dependent repression of luciferase activity from these constructs. Mutation of the miR-212-5p-target sites abrogated miR-212-mediated reduction in luciferase activity (Fig. 2B). To determine whether miR-212-5p

can target endogenous FAS and SCD1, primary mouse hepatocytes were isolated and infected with miR-212-5p mimics or miR-212-5p inhibitor or negative control. We found that miR-212-5p mimics significantly increased the expression of miR-212-5p in mouse primary hepatocytes (Fig. 2C). Overexpression of miR-212-5p did not influence the mRNA levels of *Fas* and *Scd1* (Fig. 2D), but inhibited the protein levels of FAS and SCD1 relative to negative control treatment while the protein level of SREBP1c had no difference (Fig. 2E). Conversely, miR-212-5p inhibitor significantly decreased the expression of miR-212-5p (Fig. 2F). Inhibition of miR-212-5p also did not influence the mRNA levels of *Fas* and *Scd1* (Fig. 2G), but significantly increased the protein levels of FAS and SCD1 while the protein level of SREBP1c had no difference (Fig. 2H).

miR-212-5p suppresses triglyceride accumulation in primary hepatocytes

Leucine deprivation suppresses the TG accumulation in liver, which is accompanied by elevated miR-212-5p. In the present study, the miR-212-5p mimics and adenovirus-mediated miR-212-5p overexpression were used to determine the effect of miR-212-5p on TG accumulation in mouse primary hepatocytes under basic or oleic acid-stimulated conditions. Results showed

Figure 1 miR-212-5p but not miR-212-3p inhibits the activity of FAS 3'UTR. (A) Luciferase activity assay for FAS 3'UTR by miR-27a, miR-27b, miR-33, miR-130a, miR-132, miR-191, miR-212 and miR-214 in 293T cells ($n=3$). (B) Expression of miR-212-3p and miR-212-5p in the livers of mice fed a leucine-deficiency diet ((-) leu) or control diet (con) for 7 days ($n=6$). (C) Effect of miR-212-5p or miR-212-3p on activity of FAS-3'UTR in 293T cell ($n=3$). Means $\pm$ s.e.m.s shown are representative of at least two independent *in vitro* experiments, with the number of each group in each experiment indicated. Statistical significance was determined by two-tailed Student's *t*-test for the effect of miR-212-5p mimics or miR-212-3p mimics vs control (* $P<0.05$).

Figure 2

miR-212-5p directly targets FAS and SCD1 in mouse primary hepatocytes. (A) miR-212-5p binding site on the mouse FAS 3'UTR and mouse SCD1 3'UTR is shown, and the mutant of FAS 3'UTR (FAS-3'UTR-Mut) and the mutant of SCD1 3'UTR (SCD1-3'UTR-Mut) are shown. (B) *In vitro* luciferase reporter assay in HEK293 cells. miR-212-5p mimics or negative control was cotransfected with the pGL3-3'UTR reporter plasmids containing the FAS-3'UTR, SCD1-3'UTR, FAS-3'UTR-Mut or SCD1-3'UTR-Mut ($n=3$). (C, D and E) Mouse primary hepatocytes were transfected with miR-212-5p mimics for 48 h ($n=6$). (F, G and H) Mouse primary hepatocytes were transfected with miR-212-5p inhibitor for 48 h ($n=6$). (C and F) Expression of miR-212-5p. (D and G) *Fas* and *Scd1* mRNA levels. (E and H) *FAS*, *SCD1* and *SREBP1c* protein levels (left, Western blot; right, quantitative measurements of *FAS*, *SCD1* and *SREBP1c* protein relative to *ACTIN*). Means $\pm$ S.E.M.s shown are representative of at least two independent *in vitro* experiments, with the number of each group in each experiment indicated. Statistical significance was determined by two-tailed Student's *t*-test for the effect of miR-212-5p mimics or miR-212-5p inhibitor vs control ($*P<0.05$).

that miR-212-5p mimics significantly decreased the TG accumulation in primary hepatocytes under both basic and oleatic sodium-stimulated conditions (Fig. 3A). For the convenience in investigating the role of miR-212-5p in

lipid metabolism *in vivo*, we constructed the adenovirus-mediated miR-212-5p overexpression (Ad-miR-212) system. Overexpression of miR-212-5p in primary hepatocytes is about 4-fold with Ad-miR-212 (Fig. 3B)

Figure 3

miR-212-5p significantly inhibits TG accumulation in mouse primary hepatocytes. (A) Assay of the TG content of mouse primary hepatocytes transfected with miR-212-5p mimics or control for 48 h with or without the 200 μ M sodium oleate treatment ($n=4$). (B) Expression of miR-212-5p in mouse primary hepatocytes infected with Ad-miR-212 for 48 h ($n=6$). (C) Assay of the TG content of mouse primary hepatocytes transfected with Ad-miR-212 or Ad-GFP for 48 h with or without the 200 μ M sodium oleate treatment ($n=4$). Means $\pm$ S.E.M.s shown are representative of at least two independent *in vitro* experiments, with the number of each group in each experiment indicated. Statistical significance was determined by two-tailed Student's *t*-test for the effect of miR-212-5p mimics or Ad-miR-212 vs control (* $P<0.05$).

and also significantly inhibited the TG accumulation in primary hepatocytes under both conditions (Fig. 3C).

Hepatic overexpression of miR-212-5p suppresses TG accumulation in liver via inhibiting lipogenesis

Based on the above results, we speculated that miR-212-5p might also be involved in the regulation of hepatic lipid metabolism *in vivo*. To test this possibility, mice were injected with Ad-miR-212 or Ad-GFP. Actually, miR-212-5p mRNA levels were significantly increased about 3-fold in the livers of mice injected with Ad-miR-212 compared with control mice (Fig. 4A) while there were no difference in the expression of miR-212 in other tissues such as adipose tissue and brown adipose tissue (Supplementary Fig. 1A and B, see section on supplementary data given at the end of this article). Injection of Ad-miR-212 for 7 days did not influence body weight and food intake of mice (Fig. 4B and C). However, the liver to body weight ratio was significantly decreased in the mice injected with Ad-miR-212 compared with that in control mice (Fig. 4D). Furthermore, the TG content and the level

of FFAs in the livers of mice injected with Ad-miR-212 were significantly decreased compared with those of Ad-GFP mice while the cholesterol (TC) content has no change (Fig. 4E). Moreover, the serum TG content and TC content were significantly decreased in the livers of mice injected with Ad-miR-212 compared with those of control mice although the serum level of FFAs has no difference (Fig. 4F). Consistent with these changes, lipid accumulation was significantly decreased in the livers of Ad-miR-212 mice, as demonstrated by Oil Red O staining and H&E staining (Fig. 4G). Hepatic lipogenesis, β -oxidation, and uptake and secretion of fatty acids contribute to the total TG content in the liver. For this reason, we examined the mRNA levels of genes related to these processes. These included acetyl CoA carboxylase 1 (ACC1) and FAS, which together catalyze the rate-limiting step in the production of palmitate 16:0; stearoyl-CoA desaturase (SCD), which catalyzes the synthesis of oleic acid (18:1, *n*-9); ATP-citrate lyase (ACL), which catalyzes the conversion of citrate to CoA and oxaloacetate; and sterol receptor element-binding protein 1c (SREBP1c) and peroxisome proliferator-activated receptor γ (PPAR γ), which are key regulators of lipogenic genes including FAS and SCD1. We found that the Fas and Scd1 mRNA levels were significantly decreased by Ad-miR-212 in the livers of C57BJ/6L mice, while there were no differences in Srebp1c, Ppar γ , Acc1 and Acl mRNA levels (Fig. 4H). Otherwise, impaired β -oxidation of fatty acids is another contributor of liver steatosis (Kersten *et al.* 1999, Leone *et al.* 1999). Peroxisome proliferator-activated receptor- α (PPAR α), a key regulator of β -fatty acid oxidation, peroxisome proliferator-activated receptor c coactivator 1 α (PGC1 α), a cofactor of PPAR α , and CPT1, a direct target of PPAR α , were not changed in the livers of mice injected with Ad-miR-212 (Fig. 4I). In addition to triglyceride synthesis and β -oxidation, misregulation of triglyceride uptake and secretion could also contribute to fatty liver (Bradbury 2006). We therefore examined the genes related to the secretion of fatty acids, including apolipoprotein B (ApoB), apolipoprotein E (ApoE) and apolipoprotein H (ApoH) and the genes related to the uptake of fatty acids, including fatty acid transport protein (FATP), fatty acid-binding protein (FABP) and fatty acid translocase (CD36). The results showed that only Cd36 was significantly increased by Ad-miR-212 (Fig. 4J), which contributes to increased TG accumulation in livers. By considering the suppressive effect of Ad-miR-212 on TG accumulation, it is assumed that the suppressed lipogenesis is the main contributor in this process. As expected, the FAS and

Figure 4

miR-212-5p significantly suppresses the expression of FAS and SCD1 and inhibits the lipid accumulation in the livers of mice. (A, B, C, D, E, F, G, H, I, J and K) eight- to ten-week-old male mice were injected with Ad-miR-212 (+Ad-miR-212) or Ad-GFP (–Ad-miR-212) for 7 days ($n=6$). (A) miR-212-5p and miR-212-3p mRNA levels. (B) Body weight. (C) Food intake. (D) Ratio of liver weight to body weight (%). (E) Liver TG, TC and FFA content. (F) Serum TG, TC and FFA content. (G) Representative photomicrographs of liver stained with Oil Red O (top) or Hematoxylin-Eosin (H&E) (bottom). (H) *Fas*, *Srebp1c*, *Ppar γ* , *Scd1*, *Acc1* and *Acl* mRNA levels. (I) *Pgc1 α* , *Ppara* and *Cpt1* mRNA levels. (J) *ApoB*, *ApoE*, *ApoH*, *Fatp*, *Fabp* and *Cd36* mRNA levels. (K) FAS, SCD1 and SREBP1c protein levels (left, Western blot; right, quantitative measurements of FAS, SCD1 and SREBP1c protein relative to ACTIN). Means $\pm$ S.E.M.s shown are representative of two independent *in vivo* experiments, with the number of each group indicated. Statistical significance was determined by two-tailed Student's *t*-test for the effect of Ad-miR-212 vs control (* $P<0.05$).

SCD1 protein levels were significantly decreased in the livers of Ad-miR-212-treated mice compared with those in the control mice, while the SREBP1c protein level has no difference (Fig. 4K).

Above data suggested that miR-212 regulate lipid metabolism in WT mice, based on this, we speculated that miR-212 might be involved in the regulation of liver steatosis in diet-induced NAFLD. To test this possibility, 4-week-old male mice were fed with HFD for 13 weeks and then injected with Ad-miR-212 or Ad-GFP. Consistent with WT mice, we found that overexpressed miR-212 dramatically decreased lipid accumulation in livers of HFD mice, which was also mediated by inhibiting the expression of FAS and SCD1 (Supplementary Fig. 2).

miR-212-5p inhibitor reverses leucine deprivation-suppressed TG accumulation in primary hepatocytes

Our previous study showed that leucine deprivation decreased TG accumulation via suppressing lipogenesis in the liver (Guo & Cawener 2007). We further explore the possibility of miR-212-5p-mediated effect of leucine deprivation on TG accumulation in primary hepatocytes.

Above data indicated that leucine deprivation induced the expression of miR-212-5p, which plays a key role in the regulation of the expression of FAS and SCD1, as well as TG accumulation. We speculated that increased miR-212-5p expression may contribute to the suppressed lipogenesis and TG accumulation under the condition of leucine deprivation. To test this hypothesis, we knocked down miR-212-5p expression using miR-212-5p inhibitor in primary hepatocytes (Fig. 5A). miR-212-5p knockdown effectively reversed the effect of leucine deprivation on the expression of FAS and SCD1 in primary hepatocytes (Fig. 5B and C). Furthermore, the TG content was also significantly reversed in the group of miR-212-5p inhibitor with leucine deprivation compared with the group of leucine deprivation (Fig. 5D).

miR-212-5p is induced by leucine deprivation via GCN2/ATF4 pathway

Although miR-212-5p is involved in leucine deprivation-induced decreased TG accumulation and suppressed lipogenesis, the mechanisms underlying leucine deprivation regulating the expression of miR-212-5p

Figure 5

Inhibition of miR-212-5p can reverse the effect of leucine deprivation on expression of FAS and SCD1, as well as TG accumulation. (A, B, C and D) Mouse primary hepatocytes were transfected with miR-212-5p inhibitor with or without leucine deprivation treatment ($n=4$). (A) miR-212-5p mRNA level. (B) *Fas* and *Scd1* mRNA levels. (C) FAS and SCD1 protein levels (Upper, Western blot; lower, quantitative measurements of FAS and SCD1 protein relative to ACTIN). (D) TG content. Means $\pm$ S.E.M.s shown are representative of two independent *in vitro* experiments, with the number of each group indicated. Statistical significance was determined by two-tailed Student's *t*-test or one-way ANOVA followed by the SNK test for the effect of leucine deprivation vs control ($*P<0.05$), or the effect of miR-212-5p inhibitor vs negative control under the condition of leucine deprivation ($#P<0.05$).

are largely unclear. GCN2 is a serine protein kinase that functions as a sensor for amino acid deprivation (Hinnebusch 1994), suggesting the possible involvement of GCN2 in the regulation of miR-212-5p expression during leucine deprivation. To test this possibility, we examined miR-212-5p expression in primary hepatocytes of GCN2 knockdown maintained on a control or leucine deprivation medium for 48 h. As predicted, the expression of GCN2 was decreased in shGCN2-treated cells, as well as ATF4, a downstream target gene of GCN2 (Fig. 6A). Leucine deprivation-induced miR-212-5p expression was largely blocked in primary hepatocytes with GCN2 knockdown (Fig. 6B). GCN2/ATF4 pathway is critical to cell survival in response to amino acid deprivation (Ye *et al.* 2010, Averous *et al.* 2011, Wang *et al.* 2013), suggesting that the leucine deprivation-induced GCN2/ATF4 activity might be involved in the regulation of miR-212-5p expression. To test this possibility, we examined whether the knockdown of ATF4 decreases leucine deprivation-induced miR-212-5p expression. As predicted, leucine

deprivation-induced miR-212-5p expression was significantly blocked in primary hepatocytes with ATF4 knockdown under the condition of leucine deprivation (Fig. 6C and D).

Discussion

NAFLD usually begins with an aberrant fat accumulation in the hepatocytes due to metabolic imbalances such as increased DNL (Postic & Girard 2008). Our previous study indicated that leucine deprivation suppresses hepatic DNL (Guo & Cavener 2007). Previous work has suggested that microRNAs play a key role in the regulation of lipid accumulation in liver (Soh *et al.* 2013, Dai *et al.* 2016). We have been interested in the role of microRNAs in regulating lipogenesis, especially in the process of leucine deprivation-suppressed lipogenesis, in liver. In our current study, we concentrated on investigating the function of miR-212 on lipid metabolism. miR-212 was involved in tumorigenesis (Incoronato *et al.* 2011, Wei *et al.* 2014,

Figure 6

Leucine deprivation induces the expression of miR-212-5p in a GCN2/ATF4-dependent pathway. (A and B) Mouse primary hepatocytes were transfected with shGCN2 or negative control with or without leucine deprivation treatment ($n=4$). (C and D) Mouse primary hepatocytes were transfected with siATF4 or negative control with or without leucine deprivation treatment ($n=4$). (A) GCN2 and ATF4 protein levels (Upper, Western blot; lower, quantitative measurements of GCN2 and ATF4 protein relative to ACTIN). (B and D) miR-212-5p mRNA level. (C) ATF4 protein level (Upper, Western blot; lower, quantitative measurements of ATF4 protein relative to ACTIN). (E) Working model. Means $\pm$ s.e.m.s shown are representative of two independent *in vitro* experiments, with the number of each group indicated. Statistical significance was determined by two-tailed Student's *t*-test or one-way ANOVA followed by the SNK test for the effect of leucine deprivation vs control ($*P<0.05$), or the effect of shGCN2 or siATF4 vs control under the condition of leucine deprivation ($\#P<0.05$).

Liang *et al.* 2013, Jiang *et al.* 2014, Ma *et al.* 2014) and mouse mammary gland development (Ucar *et al.* 2010). Though miR-212 can target Cyclin D1 (Jiang *et al.* 2014), which is involved in the regulation hepatic lipogenesis (Hanse *et al.* 2012), a direct effect of miR-212 on hepatic lipogenesis has not been described previously. Our work showed that miR-212 is a potential microRNA which could target 3'UTR of FAS and SCD1. The expression of miR-212-5p and miR-212-3p was increased in the livers of mice fed a leucine-deficiency diet compared with those of control mice, which suggested that miR-212 might be involved in leucine-deficiency-regulated hepatic lipogenesis. Further study showed that miR-212-5p, but not miR-212-3p, targets FAS, implying that miR-212-5p might be involved in hepatic lipogenesis. Furthermore, miR-212-5p directly target FAS and SCD1 to suppress TG accumulation *in vitro* and *in vivo*. What is more, our study indicated that the increased expression of miR-212-5p was induced by leucine deprivation-dependent GCN2/ATF4 signaling.

microRNA.org (<http://www.microrna.org>) is a comprehensive resource of microRNA target predictions, which are based on the development of a miRanda algorithm (Betel *et al.* 2008). Using FAS 3'UTR as target, the predicted microRNAs include miR-27a, miR-27b, miR-33, miR-130a, miR-132, miR-191, miR-212 and miR-214. Furthermore, miR-212 is most effective microRNA which significantly suppresses the activity of FAS 3'UTR. miR-212-5p (accession no. MIMAT0017053) and miR-212-3p (accession no. MIMAT0000659) are two isoforms from the same gene in mouse with different sequences. Though both miR-212-5p and miR-212-3p are predicted microRNAs-targeted FAS, only miR-212-5p, but not miR-212-3p, significantly inhibits the activity of FAS 3'UTR. On the other hand, leucine deprivation induced the expression of miR-212-5p and miR-212-3p. The results implied that miR-212-5p is the main isoform suppressing the expression of FAS in response to leucine deprivation while whether miR-212-3p plays a role in this process needs further investigation in the future.

FAS is a large multi-subunit protein that synthesizes the sixteen-carbon saturated fatty acid palmitate through the sequential addition of two carbon units at a time, with the carbons donated by malonyl-CoA. SCD is a delta-9 desaturase that catalyzes the conversion of saturated fatty acids to their monounsaturated fatty acid (MUFA) counterparts. Both FAS and SCD1 are key enzymes for DNL in liver (Strable & Ntambi 2010). Using bioinformatics tools, we found that both FAS and SCD1

are the target genes of miR-212-5p. The luciferase assay indicated that miR-212-5p directly binds the conserved sequence GCCAA located in the 3'UTR of FAS and SCD1 to suppress the activity of FAS 3'UTR or SCD1 3'UTR. Though miR-212-5p significantly inhibits the protein levels of FAS and SCD1, the mRNA levels of *Fas* and *Scd1* cannot be influenced, which suggested that miR-212-5p regulates the expression of FAS and SCD1 on the translational level.

DNL is critical for lipid accumulation in the liver (Postic & Girard 2008). Given that miR-212-5p significantly suppressed the expression of FAS and SCD1, the effect of miR-212-5p on lipid accumulation in mouse primary hepatocytes and in the liver was determined. As expected, overexpression of miR-212-5p significantly inhibited the lipid accumulation *in vitro* and *in vivo*. Moreover, we found that miR-212-5p inhibitor can reverse the suppressive effect of leucine deprivation on the expression of FAS and SCD1, as well as lipid accumulation in mouse primary hepatocytes. These results indicated that miR-212-5p plays a key role in leucine deprivation-suppressed DNL. Based on the finding that miR-212 specifically suppressed the mRNA levels of *Fas* and *Scd1* and increased the mRNA level of *Cd36*, we speculated that miR-212 suppressed lipid accumulation mainly due to the suppressed DNL. Otherwise, miR-212-5p could not influence the mRNA levels of *Fas* and *Scd1* in mouse primary hepatocytes, which is different to the effect of miR-212 in mice. We surmised that two reasons might be responsible to this difference. One is the potential function of Ad-miR-212 which also expresses the isoform of miR-212-3p, besides of miR-212-5p. The other reason is the possible result of Ad-miR-212 acting on the complex environment *in vivo*. It is worth noting that miR-212 decreased the protein levels of FAS and SCD1 both *in vitro* and *in vivo*.

Previous work indicated that insulin (Shukla *et al.* 2013) and ethanol (Tang *et al.* 2008) induces the expression of miR-212. Our work suggests that leucine deprivation also induces the expression of miR-212. These results implied that miR-212 might be regulated by nutrition states, which need further investigation in the future. The GCN2/ATF4 pathway is critical for cell survival in response to leucine deprivation (Ye *et al.* 2010, Wang *et al.* 2013). Our study showed that leucine deprivation stimulated the GCN2/ATF4 pathway, and blocking the GCN2/ATF4 pathway via shGCN2 or siATF4 can reverse the higher expression of miR-212-5p induced by leucine deprivation in mouse primary hepatocytes. These results suggest that GCN2/ATF4 pathway mediates the expression of miR-212 induced

by leucine deficiency, although the mechanisms need further investigation. It has been reported that FAS expression in liver is not suppressed by leucine-deficient diet in GCN2 knockout mice; as a sensor of amino acid deficiency, GCN2 was reported to be involved in lipid metabolism via regulating genes related to the synthesis of fatty acids such as SREBP1c and its target FAS (Guo & Cavener 2007). In our current study, we found that GCN2 mediates the inhibition of FAS expression by miR-212. Consistently, previous studies have suggested that GCN2 may have a relationship with body TG levels. It is shown that the GCN2/mTOR/S6K1 and AMPK pathways play important roles in regulating hepatic insulin sensitivity by leucine deprivation (Xiao *et al.* 2011); many studies have demonstrated that insulin resistance is accompanied by liver steatosis (Savage *et al.* 2007, Kumashiro *et al.* 2011, Birkenfeld & Shulman 2014), which suggests that GCN2 may be involved in regulating hepatic TG levels. On the other hand, GCN2 may regulate other transcription factors like C/EBP β and LXR α (Guo & Cavener 2007), which may be involved in regulating fatty liver (Rahman *et al.* 2007, Schroeder-Gloeckler *et al.* 2007, Hijmans *et al.* 2015, Hsieh *et al.* 2016).

In this study, we also explored the possible role of miR-212 in regulating lipid metabolism following treatment with a HFD and found that miR-212 significantly ameliorated liver steatosis of mice induced by HFD. Various strategies have been proposed to treat NAFLD, including lifestyle modifications and pharmacologic interventions (Suzuki *et al.* 2005, Musso *et al.* 2010); however, our work suggests that miR-212 was involved in the regulation of nutrient-induced fatty liver, which may provide potential therapeutic approaches to NAFLD.

In summary, our study showed that leucine deprivation induces the expression of miR-212-5p in a GCN2/ATF4-dependent manner. miR-212-5p suppresses lipid accumulation in liver by targeting FAS and SCD1 under both normal diet and high-fat diet conditions. These findings proved a novel function of miR-212-5p in sensing leucine deprivation and regulating lipid metabolism in the livers of mice, which suggested that nutritional or pharmacological modulation of miR-212-5p-FAS/SCD1 axis could be potential therapeutic approaches for treating NAFLD.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-16-0179>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Y Guo and C Wang researched data and wrote, reviewed and edited the manuscript. J Yu, K Li, B Liu, Y Du, F Xiao and S Chen provided research material. F Guo directed the project, contributed to discussion, and wrote, reviewed, and edited the manuscript.

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