ORIGINAL ARTICLE

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Downregulation of GNAI3 Promotes the Pathogenesis of Methionine/Choline-Deficient Diet-Induced Nonalcoholic Fatty Liver Disease

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Background/Aims: The pathogenesis of nonalcoholic fatty liver disease (NAFLD) has not be fully elucidated, and the lack of therapeutic strategies for NAFLD is an urgent health problem. Guanine nucleotide binding protein, alpha inhibiting activity polypeptide 3 (GNAI3) participates in several biological processes, but its relationship with lipid metabolism and NAFLD has not yet been reported. We aimed to determine the function of GNAI3 in the development of NAFLD. **Methods:** Mice were fed a methionine and choline-deficient diet to induce NAFLD. An NAFLD model in HepG2 cells was induced by free fatty acid treatment. GNAI3 levels in HepG2 cells were downregulated by shRNA. Protein levels of related proteins were evaluated by Western blotting, and mRNA levels were determined by quantitative reverse transcription polymerase chain reaction. Hematoxylin and eosin and Oil Red O staining were used to observe histological changes in liver tissue. **Results:** The dysregulated hepatic lipid metabolism in the NAFLD mouse model was enhanced by GNAI3 knockout, which also provoked worse liver damage. In the NAFLD model in HepG2 cells, the downregulation of GNAI3 promoted cellular lipid accumulation and enhanced the changes in lipid metabolic enzyme levels. **Conclusions:** This study demonstrates that GNAI3 participates in the development of NAFLD in both cellular and mouse models. The data indicate that GNAI3 is a potential new target for the treatment of NAFLD in humans. **(Gut Liver 2020;14:492-499)**

Key Words: Non-alcoholic fatty liver disease; GNAI3; Free fatty acid

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease characterized by excessive lipid accumulation in hepatocytes without excessive alcohol consumption and other clear causes of liver damage. It is considered to be a group of acquired metabolic stress-related liver disease.[1] Nonalcoholic steatohepatitis (NASH) is developed from NAFLD and progresses to NASH cirrhosis and eventually hepatocellular cancer (HCC). It is also reported that metabolic syndrome and obesity both have strong correlation with NAFLD.[2] As the most common liver disease all over the world, the current prevalence of NAFLD is approximately 25%.[3,4] During the past 20 years, NAFLD in Asian countries has grown rapidly and has a trend of younger age. It is reported that up to 42% of adults in China are suffering from NAFLD.[5,6] Although NAFLD has become an important public health problem, its pathogenesis has not been fully understood. It is now confirmed that the development of hepatic fibrosis and hepatic fat in NAFLD are triggered by insulin resistance, type 2 diabetes mellitus, obesity, ferritin level elevation, and PNPLA3 I148M polymorphism.[7] The discovery of new regulator in the development of NAFLD may indicate us a new target for the therapy of NAFLD.

Guanine nucleotide–binding proteins play several different biological functions in cells, such as proliferation, differentiation, protein synthesis, and protein transport.[8] G-protein is composed of α, β, and γ subunits. Gα subunit can be divided into Gαs, Gαi, Gαq, and Gα12 family based on protein sequence and function.[9] One of the Gαi family protein GNAI3 has been proved to participate in the regulation of several cellular activities including invasion, apoptosis, migration, and proliferation.[10] It has been reported that the poor prognosis of hepatocellular carcinoma has correlation with depressed expression of GNAI3.[10] Because of its multiple functions in cellular activity and liver cancer, we hypothesized that GNAI3 might also play a role in the lipid metabolism and influence the pathogenesis of

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NAFLD.

In this study, we aim to explore whether GNAI3 has influence on the lipid metabolism and plays a regulatory function in the development of NAFLD *in vivo* and *in vitro*.

MATERIALS AND METHODS

1. Animal model

Wild-type (WT) male C57BL/6J mice served as controls. GNAI3 knockout mice were purchased from Shanghai Langata Biotech (Shanghai, China). Animal model of NAFLD prepared by methionine and choline-deficient (MCD) diet: 40 WT mice and 40 GNAI3 KO mice of 20 weeks' old were fed with chow diet (normal control) or MCD diet (NAFLD/NASH model mice) for 8 weeks (four groups in total, n=20 for each group). All animal studies were approved by the ethics commitment of Hangzhou First People's Hospital, the Affiliated Hospital of Medical School of Zhejiang University.

2. Cell culture

HepG2 cells were cultured in high sugar Dulbecco's Modified Eagle's Medium (DMEM; Life Technology, Pleasanton, CA, USA) medium containing 10% fetal bovine serum (Life Technology), penicillin and streptomycin. Cells were maintained at 37°C under 5% CO_2. Preparation of cell model of NAFLD: the control group was cultured in DMEM medium for 48 hours; the model group was cultured with 1 mM free fatty acid (FFA; Life Technology) for 48 hours.

3. Plasmids

The pLV-GFP, 8.91 and VSVG plasmids used to package lentiviruses were kept in our laboratory. G248-sh1-GNAI3-GFP interference plasmid, G248-sh2-PRAME-GFP, G248-sh3-GNAI3-GFP plasmid and interference control plasmid were synthesized and provided by Jikai Company (Shanghai, China). The pLV-GNAI3-GFP plasmid and the control plasmid were synthesized and supplied by GENEWIZ Gene Technology (Shanghai, China). The pCMV-GNAI3 plasmid using for the rescue of GNAI3 in HepG2 cells was synthesized using polymerase chain reaction (PCR) method.

4. qRT-PCR

The cells were fully lysed using Trizol at low temperature to extract total RNA. Reverse transcription reactions were performed on a PTC-type conventional PCR instrument as required by Takara-PrimeScript™ RT Master Mix kit instructions. The following primers were used: forward primer 5'-ATCGACCGCAACTTACGGG-3' and reverse primer 5'-AGTCAATCTTTAGCCGTCCCA-3' for GNAI3; forward primer 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse primer 5'-GGCTGTTGTCATACTTCTCATGG-3' for GAPDH. GAPDH was used as an internal control.

5. Western bolt

The following antibodies were used: anti-GNAI3 Antibody (PA5-27940; 1:1,000) was purchased from Invitrogen. Anti-ACOX2 antibody (SAB1406653; 1:500) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-ApoB antibody (20578-1-AP; 1:1,000) was purchased from Proteintech Group Inc. (Rosemont, IL, USA). Anti-ACCa antibody (4190; 1:1,000) and anti-GAPDH antibody (5174; 1:2,000) were purchased from Cell Signaling Technology (Danvers, MA, USA).

6. Histopathological analysis

Liver tissue samples were collected from mice and fixed in 4% paraformaldehyde over night at 4°C. Then the tissue samples were embedded with paraffin and sectioned. Hematoxylin and eosin the staining and Oil Red O staining were done based on the standard protocols.

7. Serum biochemical measurements

Free Fatty Acid Quantification Kit (Abcam, ab65341, Cambridge, MA, USA), Cholesterol Quantification kit (Abcam, ab65359), and Triglyceride Colorimetric Assay kit (Cayman Chemical Company: #10010303) were used for the measurement of lipids. Abcam mouse ELISA kit ab100718 and ab108785 were used for the detection of leptin and adiponectin.

8. Statistical analysis

All data were analyzed by SPSS 19.0 version software (IBM Corp., Armonk, NY, USA) and the results were shown as mean±standard deviation or mean±standard error of mean. Analysis was performed using the Student t-test. The $p<0.05$ indicates that the difference was statistically significant.

RESULTS

1. Metabolic syndrome-phenotype associated with NAFLD in mice is enhanced by GNAI3 knockout

We assessed the protein levels of GNAI3 in control and GNAI3KO mice with chow diet or MCD diet, and the results showed that there was no significant change between chow diet and MCD diet groups (Supplementary Fig. 1A and B). Forty WT mice and 40 GNAI3KO mice were fed chow diet and MCD diet for 8 weeks. MCD diet-fed mice had less body weight than chow diet-fed mice in both the WT and the GNAI3KO groups. The weight loss generated by MCD diet was promoted in GNAI3KO mice compared to WT mice (Fig. 1A). Beside the weight loss, MCD diet-fed mice were proved to be hypoleptinemic and hyperadiponectinemic. In GNAI3KO mice, the decrease in the level of leptin and the elevation in the level of adiponectin were both promoted when compared with the WT mice (Fig. 1B). The fasting serum glucose level and HOMA-IR index in GNAI3KO mice were both less than the WT mice after the induction of

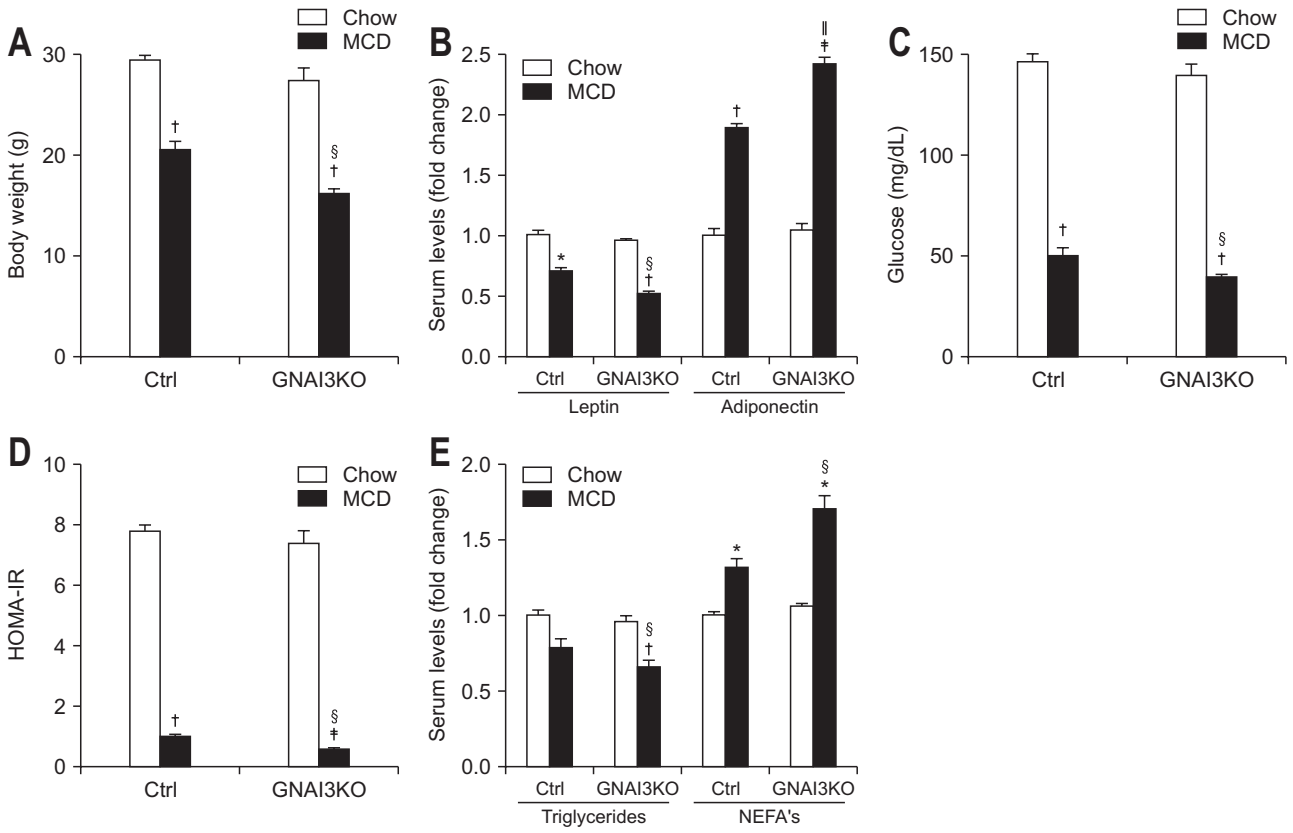


Fig. 1. Effects of GNAI3KO on the metabolic profile in nonalcoholic fatty liver disease (NAFLD) mice. Forty WT mice and 40 GNAI3KO mice were fed a chow diet or a methionine and choline-deficient (MCD) diet for 8 weeks (n=20 for each group); the mice were sacrificed at 20 weeks of age. (A) Body weight. (B) Adipokine levels (leptin and adiponectin). (C) Fasting serum glucose levels and (D) HOMA_IR index. (E) Serum triglycerides and NEFA's. The results were normalized to those in chow diet-fed mice, and the bar graphs show the mean±standard error of the mean.
Ctrl, control; HOMA-IR, homeostasis model assessment of insulin resistance; NEFA's, nonesterified fatty acids; WT, wild-type. *p<0.05, †p<0.01, and ‡p<0.001, MCD diet versus chow diet; §p<0.05 and ǁp<0.01 MCD diet-fed GNAI3KO mice versus MCD diet-fed WT mice.

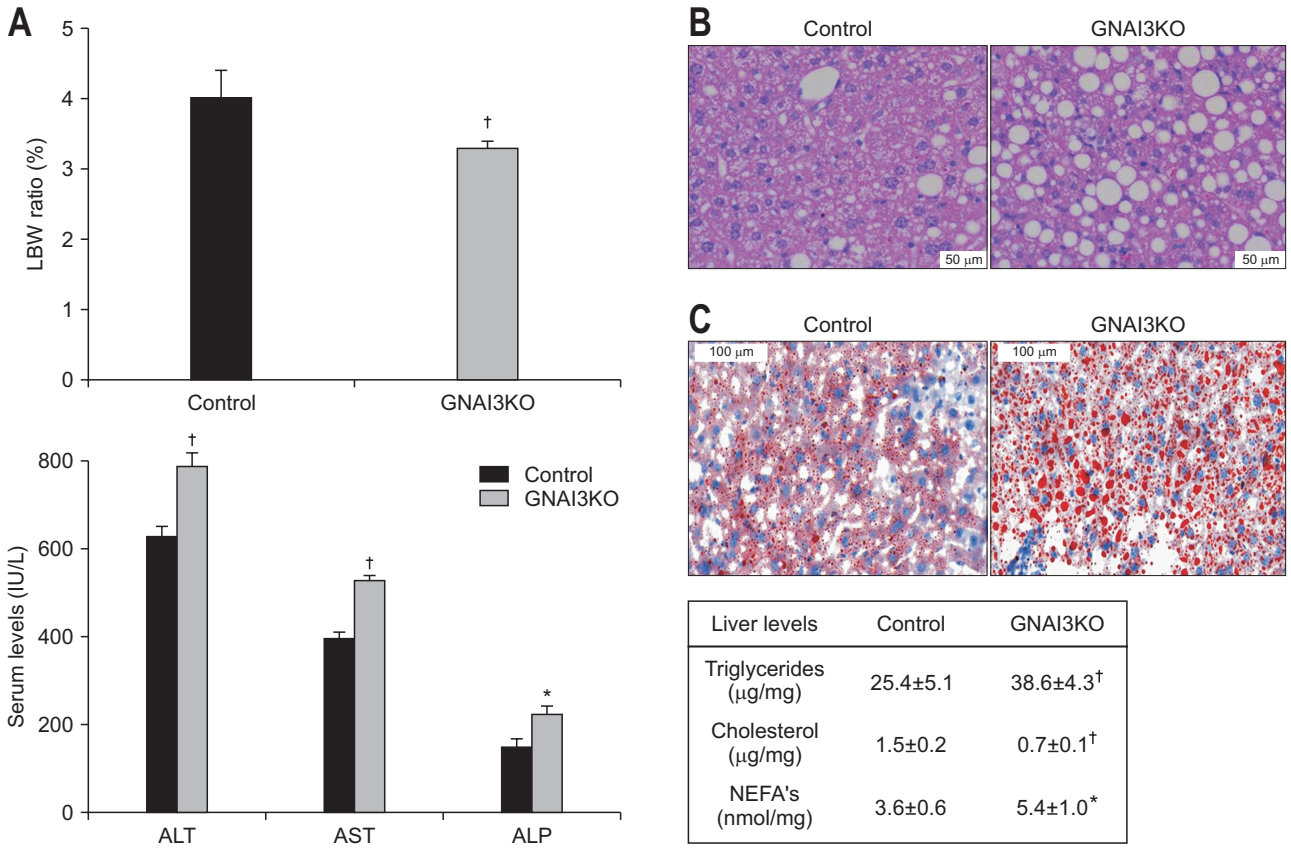
NAFLD (Fig. 1C and D). The dyslipidemia generated by NAFLD was demonstrated by the changes in the levels of triglycerides and nonesterified fatty acids (NEFA's) in serum. Fig. 1E shown that compared to MCD diet-fed WT mice, the changes in the level of triglycerides and NEFA's were both promoted by GNAI3 knockout in MCD diet-fed mice. Based on these data, metabolic syndrome-phenotype associated with NAFLD in mice is promoted by GNAI3 knockout.

2. Liver damage generated by NAFLD in mice is enhanced by GNAI3 knockout

To further investigate the influence of GNAI3 on the pathogenesis of NAFLD in mice, the liver damage was detected. The loss of liver mass caused by MCD diet generated a lower liver-to-body weight ratio than chow diet-fed mice. The lower liver-to-body weight ratio in MCD diet GNAI3KO mice indicated that the loss of GNAI3 generated a smaller liver than MCD diet WT mice (Fig. 2A). Serum aminotransferases (alanine aminotransferase and aspartate aminotransferase) and alkaline phosphatase levels in serum were also increased by GNAI3 knockout (Fig. 2A). Beside the increased liver enzyme levels in serum, it is demonstrated by hematoxylin and eosin staining that histologic liver inflammation in MCD diet-fed mice was also increased by the loss of GNAI3 (Fig. 2B). The more impressive hepatic steatosis in GNAI3KO mice than WT mice was also proved by Oil Red O staining (Fig. 2C). Fig. 2C also demonstrated that the changes in the levels of liver triglyceride, cholesterol, and NEFA's generated by NAFLD were all improved by GNAI3 knockout. All these results proved that GNAI3 knockout provokes worse liver damage in NAFLD mice model.

3. GNAI3 knock-down accelerated the cellular lipid accumulation in HepG2 cell NAFLD model

We first assessed the protein levels of GNAI3 in control and FFA-treated HepG2 cells, and the results showed that there was no significant change between the two groups (Supplementary Fig. 1C). To further investigate GNAI3 function in the development of NAFLD, we also used HepG2 cell NAFLD model. To knock-down the mRNA expression of GNAI3, HepG2 cells were infected with lentivirus carrying GNAI3-targeted shRNA. It is shown by the quantitative reverse transcription PCR (qRT-PCR) that G248-sh2-GNAI3-GFP had the highest knock-down



Liver levels	Control	GNAI3KO
Triglycerides (μg/mg)	25.4±5.1	38.6±4.3†
Cholesterol (μg/mg)	1.5±0.2	0.7±0.1†
NEFA's (nmol/mg)	3.6±0.6	5.4±1.0*

Fig. 2. Effects of GNAI3KO on hepatic steatosis in nonalcoholic fatty liver disease mice. (A) Liver-to-body weight (LBW) ratio, alkaline phosphatase (ALP), and serum aminotransferase (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) levels in WT (n=20) and GNAI3KO mice (n=20) on the methionine and choline-deficient diet. (B) Liver sections from mice (H&E). (C) Oil Red O staining of representative mouse liver sections and liver lipid levels. The results are presented as the mean±standard error of the mean.
NEFA's, nonesterified fatty acids; WT, wild-type. *p<0.05 and †p<0.01, GNAI3KO mice versus WT mice.

efficiency, so we use GNAI3 shRNA2 to construct the GNAI3 KD HepG2 cell line (Fig. 3A). The decline in the protein level of GNAI3 in GNAI3 KD HepG2 cell line was demonstrated by Western blot (Fig. 3B). Intracellular lipid accumulation in HepG2 cell NAFLD model can be revealed by Oil Red O staining. When compared with control group, the lipid accumulation caused by NAFLD in HepG2 cells was significantly promoted by the knock-down of GNAI3, and the change were rescued by subsequent transfection of GNAI3-expressing vectors (Fig. 3C). These data demonstrated that GNAI3 also participate in the development of NAFLD in HepG2 cells.

4. The downregulation of GNAI3 promoted NAFLD development through influence on *de novo* lipogenesis and fatty acid esterification

Since GNAI3 can regulate the pathogenesis of NAFLD, we wanted to investigate which signaling pathway is regulated by GNAI3 in the lipid metabolism process. Firstly, the mRNA levels of genes encoding lipid metabolic enzymes involved in fatty acid esterification, *de novo* lipogenesis and very low-density lipoprotein (VLDL) secretion in GNAI3KO and WT mice liver were detected by qRT-PCR. Fig. 4A illustrated that the mRNA levels of ACOX (fatty acid esterification), ACCa (*de novo* lipogenesis), and ApoB (VLDL secretion) were significantly altered in GNAI3KO mice when compared with WT mice. In GNAI3 KD HepG2 cell line, qRT-PCR data also shown the same tendency in the mRNA levels of ACOX, ACCa, and ApoB (Fig. 4B). The downregulated ACOX expression and upregulated ACCa and ApoB expression on the protein level in GNAI3 KD HepG2 cells was detected by Western blot (Fig. 4C). GNAI3 participated in the regulation of fatty acid esterification and *de novo* lipogenesis through the regulation of ACOX and ACCa expression in the NAFLD liver.

DISCUSSION

During recent years, a number of studies has focused on the discovery of NAFLD progression mechanisms and several different mechanisms has been proposed.[11,12] Although several important molecular signaling pathways have been reported to be critical in NAFLD, the details in the pathogenesis of NAFLD are still waiting to be discovered. We aimed to figure out a new

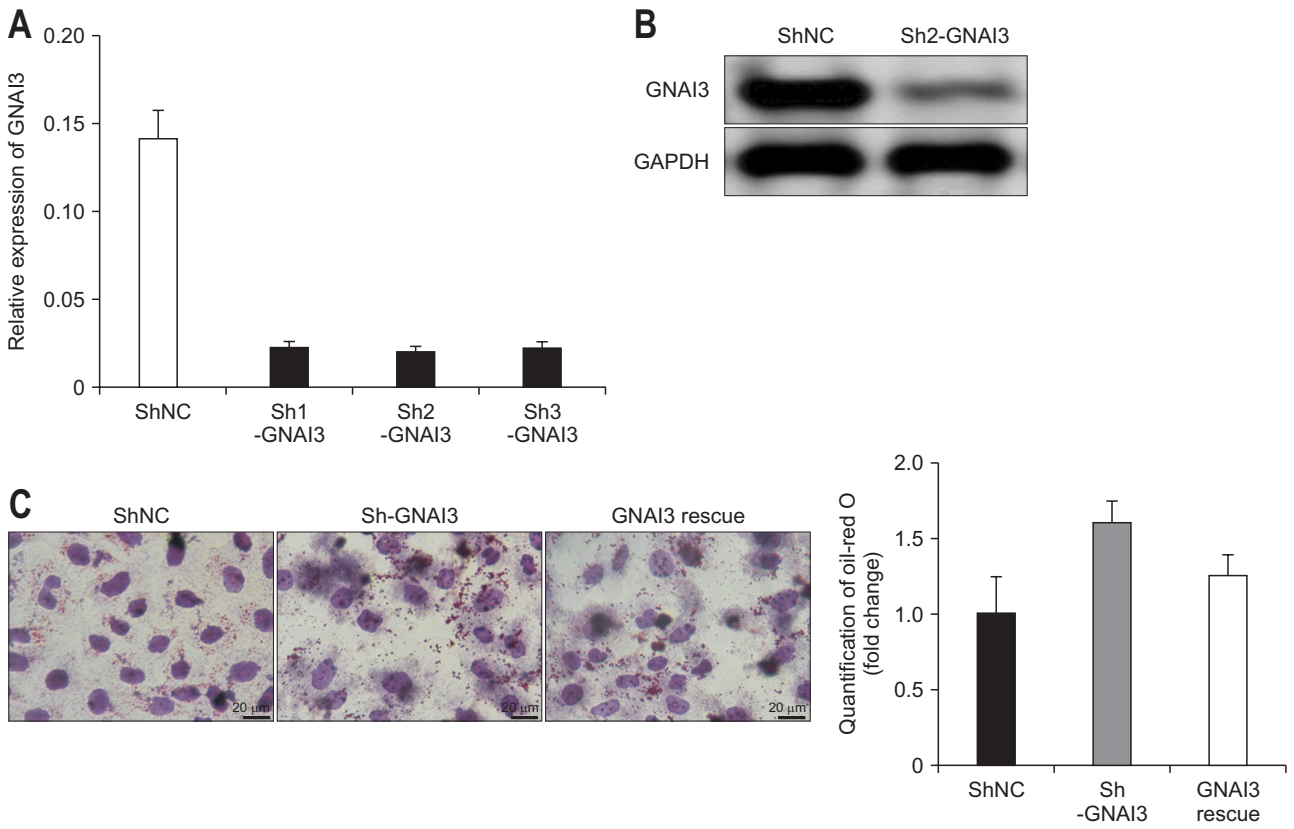


Fig. 3. Construction of GNAI3 KD HepG2 cells and the effect of GNAI3 KD on cellular lipid accumulation. (A) Quantitative reverse transcription polymerase chain reaction analysis of GNAI3 mRNA expression in HepG2 cells transfected with lentiviral vectors carrying GNAI3 shRNA. (B) Western blotting analysis of GNAI3 protein expression levels in GNAI3 KD and control HepG2 cells. (C) Oil Red O staining of control, GNAI3 KD and rescued HepG2 cells after free fatty acid treatment. Positive staining for Oil Red O indicates intracellular lipid accumulation. Quantification of Oil Red O staining is shown in the right panel. The bar graphs show the mean±standard error of the mean. $p<0.01$, $p<0.001$, GNAI3 KD (sh-GNAI3) versus control; $p<0.05$, GNAI3 rescue versus GNAI3 KD (sh-GNAI3).

molecule which participates in the regulation of NAFLD pathogenesis. The discovery of new NAFLD relative molecule may help us understand the mechanism of NAFLD development and provide us a potential new target for the therapy of NAFLD.

GNAI3 is a multifunction protein and has been illustrated to participate in the regulation of cell invasion, migration, proliferation, and apoptosis.[13-16] Recently, the significantly declined GNAI3 expression level was proved to have a strong correlation with the induction of poor prognosis in HCC.[10] During the NAFLD pathogenesis, NAFL (nonalcoholic fatty liver) evolved into NASH (non-alcoholic steatohepatitis). NASH caused liver cirrhosis and ultimately generated HCC.[17] These evidences indicated that GNAI3 may also be involved in the pathogenesis of NAFLD.

To investigate the function of GNAI3 in the development of NAFLD, we used both *in vitro* and *in vivo* models of NAFLD in this study. MCD diet was provided to induce NAFLD in mice and chow diet was provided as control. NAFLD model in HepG2 cell was induced by FFA treatment. It is reported that the hypermetabolism caused by NAFLD in MCD diet-fed mice causes body weight loss when compared with chow diet-fed mice.[10,18] In GNAI3KO mice, the loss of GNAI3 protein promoted the weight loss caused by MCD diet. It is reported that MCD diet does not mimic the NAFLD/NASH impacted systemic metabolic risks in mice. Based on previous studies, mice fed with MCD-diet were hypoleptinemic (decrease in leptin level) and hyperadiponectinemic (increase in adiponectin level) simultaneously.[19] Based on our result, the decline of leptin level and the elevation of adiponectin level were both promoted by GNAI3 knockout in MCD diet-fed mice. The more severe hypoglycemic after fasting and liver insulin resistance were proved to exist in the MCD diet-fed mice when compared with chow diet-fed mice.[20,21] The changes in fasting serum glucose levels and HOMA-IR index both confirmed that the phenotypes generated by MCD diet are enhanced by GNAI3 knockout in mice. So, the metabolic syndrome-phenotypes in MCD diet-fed mice were enhanced by the down-regulation of GNAI3 in mice. All these data demonstrated that the inhibition of GNAI3 expression did not influence the effect of MCD diet on the induction of NAFLD in mice.

The MCD diet provoked liver damage is shown by the decreased liver-to-body weight ratio caused by liver mass loss and the increased serum liver enzymes (alkaline phosphatase,

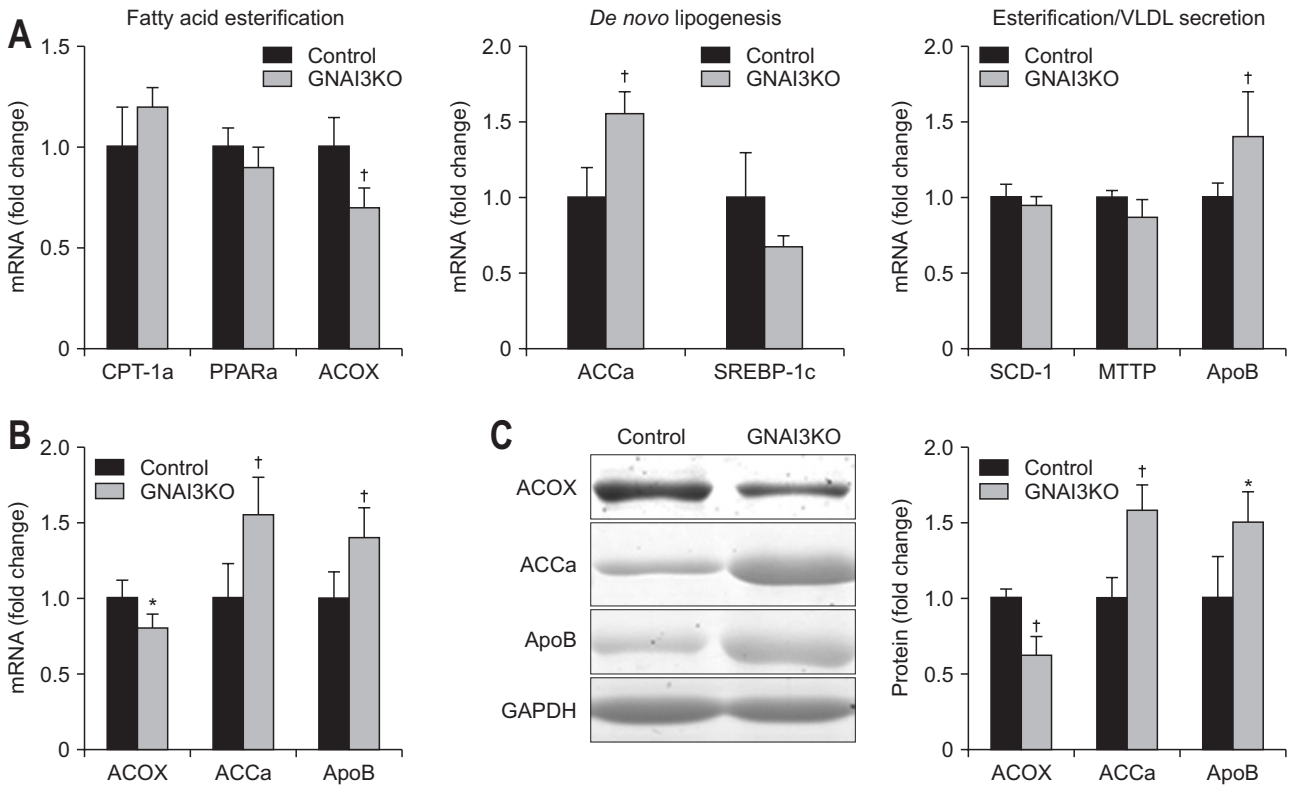


Fig. 4. Effects of downregulating GNAI3 expression on lipid metabolic enzymes. (A) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of genes encoding lipid metabolic enzymes involved in fatty acid esterification, *de novo* lipogenesis and very low-density lipoprotein (VLDL) secretion in the livers of GNAI3KO and wild-type mice. (B) qRT-PCR analysis of ACOX, ACCa and ApoB mRNA expression levels in GNAI3 KD and control HepG2 cells. (C) Western blotting analysis of ACOX, ACCa and ApoB protein levels in GNAI3 KD and control HepG2 cells. Bar graphs show the mean±standard error of the mean (A, B) or mean±SD (C). *p<0.05 and †p<0.01, GNAI3KO/KD versus control.

alanine aminotransferase, and aspartate aminotransferase) levels. Based on our results, the loss of GNAI3 generated a smaller liver mass and higher serum liver enzymes levels than WT mice in NAFLD mice model. We also demonstrated the influence of GNAI3 on NAFLD liver tissue by histopathological analysis. Hematoxylin and eosin staining data illustrated the promoted fibrosis and Oil Red O staining proved the promoted liver fat accumulation in GNAI3KO mice NAFLD model when compared with the WT mice. And the Oil Red O staining in HepG2 cell NAFLD model also confirmed that the lack of GNAI3 can accelerate the liver fat accumulation. GNAI3 knockout provoked a worse liver damage in both NAFLD mice model and NAFLD cell model.

Although we have proved that GNAI3 participates in the regulation of NAFLD pathogenesis, the molecular mechanism of GNAI3 function in NAFLD is still not clear. So, we analyzed the effects of downregulation of GNAI3 expression on the expression levels of lipid metabolic enzymes involved in fatty acid esterification, *de novo* lipogenesis and VLDL. Based on data from previous study, MCD diet caused the upregulation of *de novo* lipogenesis gene ACCa and the downregulation of fatty acid esterification gene ACOX.[22] In both mice liver and HepG2 cells, the lack of GNAI3 upregulated the ACCa expression and downregulated the ACOX expression in NAFLD model. So GNAI3 regulated the pathogenesis of NAFLD by influence the *de novo* lipogenesis and fatty acid esterification through ACCa and ACOX transcription regulation. In WT NAFLD mice model, the expression of VLDL relative gene ApoB is not influenced. When the GNAI3 expression is downregulated, the expression of ApoB was significantly upregulated in both *in vitro* and *in vivo* models of NAFLD. GNAI3 participates in several signaling cascades as a G protein-coupled receptors downstream transducer. It has been reported that the activity of receptor-regulated K+ channels is regulated by GNAI3.[23] Meanwhile, GNAI3 is suggested to be a direct target of miR-222 and downregulated by miR-222 in HCC.[24] Based on its interaction with microRNA molecules, GNAI3 may influence the expression of other genes through sponging microRNA. These data indicated that GNAI3 may not only regulates the expression of NAFLD related genes but also participates in the regulation of other genes in liver.

Although we have proved the function of GNAI3 in the pathogenesis of NAFLD and demonstrated the molecular mechanism, the details in the mechanism of GNAI3 remain to be understand. Bioinformatics methods should be used to predict the interactive proteins that may be combined with GNAI3. At the same time, epigenetic research should be used to predict up-

stream miRNAs that may regulate the expression of GNAI3. The understanding of the regulation mechanism upstream or downstream of GNAI3 will give us a new sight in the pathogenesis of NAFLD.

In this study, we demonstrated that GNAI3 participates in the development of NAFLD in both cell and mice model by influence the *de novo* lipogenesis and fatty acid esterification through ACCa and ACOX transcription regulation. GNAI3 can be considered as a new target for the therapy of NAFLD in human.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

Study concept and design: H.Z. Data acquisition: K.G. Data analysis and interpretation: J.L. Drafting of the manuscript; critical revision of the manuscript for important intellectual content: C.J. Statistical analysis: H.Z. Obtained funding: C.J. Administrative, technical, or material support; study supervision: C.J.

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