Gm12664-201, A Pseudogene-Transcribed RNA Promotes triglyceride Accumulation Via Activating Endoplasmic Reticulum Stress Pathway in Hepatocytes

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Research Article

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Abstract

Background: Typical pseudogenes expressions have been reported to be associated with liver diseases. However, whether pseudogenes contribute to non-alcoholic fatty liver disease (NAFLD) and triglyceride (TG) accumulation and the underlying molecular mechanisms are poorly understood. We aimed to elucidate the role of Gm12664-201 in TG accumulation and the potential mechanism.

Method and Results: We identified Gm12664-01 in high-fat diet (HFD) fed mice using microarray and confirmed its expression in both vivo and vitro high fat models with quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Then changed the level of Gm12664-201 in vitro to determine the role of it in TG accumulation, which evaluated by Oil red O staining and enzymatic method. The underlying mechanisms of Gm12664-01 in TG accumulation were investigated by detecting signaling markers BiP, PERK, ATF6, CHOP, eIF2 α , XBP1 and ATF4 with qRT-PCR and western blotting. We found that Gm12664-201 was markedly increased in liver tissue of HFD mice and steric acid (SA) incubated AML-12 cells. Gm12664-201 overexpression rendered AML-12 cells susceptible to triglyceride (TG) accumulation, and silencing pseudogene Gm12664-201 expression efficiently alleviated SA-induced TG accumulation. Further studies revealed that Gm12664-201 regulates endoplasmic reticulum stress signaling markers BiP, PERK, ATF6, CHOP, eIF2 α , XBP1 and ATF4 expression, which may be responsible for increased TG accumulation in hepatocytes.

Conclusions: Upregulation of pseudogene Gm12664-201 induced by HFD promotes triglyceride accumulation and activates ER stress pathway in hepatocytes.

Introduction

The prevalence of non-alcoholic fatty liver disease (NAFLD) has been on a rise, accompanying a worldwide increase in diabetes and metabolic syndrome [1]. NAFLD includes a continuum of liver abnormalities from hepatic simple steatosis to nonalcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and hepatocellular carcinoma [2]. The majority of NAFLD patients are obese or even morbidly obese with insulin resistance [3]. Accumulation of hepatic fat predominantly induced by elevated peripheral fatty acids and hepatic lipogenesis contributes to the NAFLD [3]. The major physiologic mechanism responsible for the accumulation of fat and maintenance of steatosis in NAFLD is to increase the supply of free fatty acid and triglyceride (TG) production in the liver, with an inability of the liver to adequately increase TG export [4]. Although the underlying mechanism of NAFLD is still not completely understood, endoplasmic reticulum stress (ERs) and mitochondrial dysfunction, as well as systemic inflammatory mediators exert pivotal roles in the process of NAFLD[3, 5].

The ER is a cellular organelle for the synthesis and folding of the protein, calcium homeostasis, lipid biogenesis, and posttranslational modification and trafficking [6]. In response to environmental, physiological, and pathological insults, an adaptive signaling pathway referred to as the unfolded protein response (UPR) is triggered initially to maintain ER homeostasis [7]. The ERs responses play an essential

role in hepatic steatosis, since most of lipid synthesis takes place in the smooth ER [7]. Chronic ERs regulates lipid metabolism in live specially by inducing *de novo* lipogenesis[5, 8, 9], the alteration of TG export as VLDL[10, 11] and triggering insulin resistance [12]. The major ERs signaling pathway responsible to perturbation of hepatic lipid metabolism involves the activation of three principal transmembrane sensors inositol-requiring enzyme 1 (IRE1), protein kinase RNA-like ER kinase (PERK) and activating transcription factor (ATF6)[7, 11].

The different types of non-coding RNA (ncRNA) transcripts, once termed as 'junk' DNA, have recently been found to be transcriptionally active [13]. Of note, pseudogene, a type of ncRNAs, is now known to have crucial biological roles. Pseudogene is defined as defunct genomic loci with sequence similarity to functional genes but lacking the function of encoding for coding functional proteins [14]. Increasing evidence indicates that certain pseudogenes are critical parts of modulating gene expression, specifically as modulators of miRNAs, and maintaining physiological function[15, 16]. This was recently described by Kong *et al.*, where pseudogene PDIA3P1 was discovered to promote cell proliferation, migration and invasion, and suppress hepatic apoptosis in hepatocellular carcinoma via inhibiting p53 pathway [17]. To investigate new mechanisms by which pseudogene act as a regulator in triglyceride accumulation, we studied the long-noncoding RNAs profile expression in mice fed HFD in comparison with the normal diet (ND) mice, using microarray analysis technique. We acquired a set of RNAs up- or down-regulated in HFD mice compared with ND mice. Among them, we focused our attention on pseudogene Gm12664-201, the most overexpressed RNA. Here, we demonstrate that upregulation of pseudogene Gm12664-201 induced by HFD promotes triglyceride accumulation and activates ER stress pathway in hepatocytes.

Materials And Methods

2.1. Chemicals

Palmitic acid (PA) and stearic acid (SA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of PA and SA were prepared as described previously. The Gm12664-201 small interfering RNA (siRNA sequence: 5'-CCCTCTTCTTTGAATTCAA-3') and the corresponding negative control were synthesized from RiboBio (Guangzhou, China).. For the Gm12664-201 overexpression assay, a pcDNA3.1 vector was designed to specifically express the open reading frame of Gm12664-201 containing full-length 3'-UTR and purchased from GENERAY Biotechnology (Shanghai, China).

2.2. Animals

8-weeks male C57BL/6 mice were purchased from Vital River Laboratories (Beijing, China). After 1-week adaptation, the mice were randomly divided into two groups and supplied with either a normal diet (ND, 15% fat) or a high fat diet (HFD, 36% fat) for 10 weeks. Food intake and body weight were recorded daily and weekly, respectively. At the end of the experiment, all mice were sacrificed and blood samples and livers were collected and stored at -80 °C for further use. The study was approved by the Harbin Medical

University Institutional Animal Care Committee and performed in accordance with the Harbin Medical University guidelines for the care and use of laboratory animals.

2.3. Cell culture, treatments and transfection

Alpha mouse liver-12 hepatocytes (AML-12, ATCC Cat# CRL-2254, RRID: CVCL_0140) were purchased from the ATCC, and cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12, SH30023.02, HyClone Laboratories, USA) with 0.005 mg/ml insulin (A189560, Gibco, USA), 40 ng/ml dexamethasone (D4902-25MG, Sigma-Aldrich) and 10% fetal bovine serum (G11-70500, Genial Biological Inc., USA), at 37°C in a humidified 5% CO₂ incubator. Cells were treated with PA, or SA at different times and doses to determine the most appropriate intervention conditions.

AML-12 cells were transfected with 100 nM Gm12664-201 siRNA /negative control siRNA or 2.5 mg per well (6 wells plate) Gm12664-201 overexpression plasmid using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. After 24-hours transfection, the cells were harvested for further assays or treated with SA or PA for further 24 hours.

2.4. RNA isolation and qRT-PCR analysis

The total RNA was isolated from liver tissues or cultured cells using TRIzol reagent (15596026, Invitrogen, USA), then reversed (High Capacity cDNA Reverse Transcription Kits, Applied Biosystems, USA) and quantified by qRT-PCR (7500 Real-Time PCR System, Applied Biosystems, USA). For Gm12664-201 and mRNA detection were performed using SYBR Green PCR Master Mix (Applied Biosystems, USA). The quantification data were normalized to β -actin expression. The primers were purchased from Sangon (Shanghai, China) and corresponding sequences were listed in table 1.

2.5. Oil red O staining

After the AML-12 cells were transfected with plasmids or siRNA and further cultured with SA for 24 hours, remove and discard culture medium, rinse the cell layer with PBS to remove remain medium. The cells were fixed with 4% paraformaldehyde for 20 minutes, followed by briefly rinsed with 60% isopropyl alcohol and stained with Oil Red O (Sigma-Aldrich) for 30 minutes, and counterstained with Mayers hematoxylin (Sigma-Aldrich) for 1 minute. The digital images were captured using a Nikon EclipseTi-S microscope (Nikon, Tokyo, Japan).

2.6. Triglyceride detection

Triglyceride (TG) deposits in the serum were measured using a Triglyceride test Kit (ApplyGen, Beijing, China, E1013) according to the manufacturer's instructions. Intracellular TG contents were detected using kits obtained from Solarbo Technologies Inc (Beijing, China).

2.7. Western blot analysis

Western blot was done as described previously. All the primary antibodies (Cell Signaling Technology Cat# 3177, RRID: AB_2119845; Cell Signaling Technology Cat# 4970, RRID: AB_2223172) were diluted by 1:1000 according to the western blotting protocol. Alkaline phosphatase (AP) enzyme conjugated secondary antibody (Promega Cat# S3731, RRID: AB_430872) was applied with a working dilution of 1:7,500 as suggested.

2.8. Statistical analysis

All data are expressed as the mean $\pm$ standard deviation (SD) of at least three independent experiments. Comparisons between groups were analyzed using Student's t-test. The statistical analyses were performed using the GraphPad Prism (GraphPad Prism, RRID: SCR_002798). A two-sided *P* value < 0.05 was considered statistically significant.

Results

3.1. Upregulation of Gm12664-201 in vivo and vitro high fat models

After a 10-week HFD feeding period, the higher body weights were seen in HFD mice compared with ND mice (Fig. 1 a). The serum TG contents of the HFD mice were significantly higher than that of the ND mice (Fig. 1 b). In our previous study [18], microarray analysis revealed that Gm12664-201 was significantly upregulated in liver tissues of HFD mice compared with ND mice (Fig. 1 c). Upregulation of Gm12664-201 in liver of HFD mice was verified by qRT-PCR (Fig. 1 c). Next, we assessed whether free fatty acid, such as PA and SA can regulate Gm12664-201 expression in AML-12 cells. Similarly, we found that treatment with PA or SA significantly up-regulated Gm12664-201 expression (Fig. 1 d).

3.2. Gm12664-201 promotes TG accumulation in AML-12 cells

To determine the role of Gm12664-201 in TG accumulation, we first increased Gm12664-201 expression by transfecting Gm12664-201 overexpression plasmid into AML-12 cells. Transfection efficacy was determined by qRT-PCR (Fig. 2 a). Our results showed that enhanced the expression of Gm12664-201 markedly increased TG content in AML-12 cells (Fig. 2 c). Similar results were seen in oil red O staining (Fig. 2 d). We then investigated whether knockdown of Gm12664-201 expression could reverse the SA-induced TG accumulation. To this end, we silenced Gm12664-201 by transfecting Gm12664-201 siRNA to AML-12 cells (Fig. 2 b). We found that silencing Gm12664-201 expression efficiently rescued SA-increased triglyceride accumulation (Fig. 2 c and d). These results suggest that up-regulated pseudogene Gm12664-201 promotes the intracellular triglyceride accumulation.

3.3. Gm12664-201 regulates endoplasmic reticulum stress in AML-12 cells

Previous study reported ERs mediate TG accumulation and drives lipogenesis and steatohepatitis [5]. We observed that the levels of BiP protein were significantly increased in HFD mice liver tissues (Fig. 3 a). And in vitro, our results revealed that SA increased BiP protein levels in a dose-dependent manner (Fig. 3 b). To further identify the mechanism by which Gm12664-201 augments TG accumulation, we analyzed BiP and other ER stress signaling markers using qRT-PCR and western blot analysis (Fig. 3 c and e). On the other hand, silencing pseudogene Gm12664-201 expression significantly attenuated higher levels of BiP which were induced by SA (Fig. 3 d and e). In addition, ER stress signaling markers PERK, ATF6, CHOP, eIF2 α , XBP1 and ATF4 mRNA levels were also increased by enhancing Gm12664-201 expression, and SA-upregulated mRNA levels of those gene were efficiently attenuated by silencing pseudogene Gm12664-201 expression (Fig. 4).

Taken together, these data indicate that pseudogene Gm12664-201 regulates ER stress signaling, which may be responsible for increased TG accumulation in hepatocytes.

Discussion And Conclusions

NAFLD is characterized by ectopic fat storage in the liver, which is closely linked to with metabolic syndrome and insulin resistance [19]. On a cellular level, hepatic steatosis results from the imbalance of excess fatty acid uptake, lipid synthesis, lipid β -oxidation, and VLDL-TG secretion [20]. A number of pseudogenes have been identified as regulators in the pathology of tumor, insulin resistance and type 2 diabetes[21, 22]. For example, POU5F1B promotes hepatocellular carcinoma (HCC) proliferation by activating AKT pathway [23]. To acquire a more profound understanding of genetic markers contributing to hepatic steatosis, we applied long noncoding RNA microarray analysis and determined a set of differentially expressed gene response to HFD feeding. Gm12664-201 has emerged as novel potential candidate gene based on the most prominent alterations in the expression which showed elevation in HFD mice.

In the present study, we have discovered that enhancing pseudogene Gm12664-201 expressions markedly increased TG accumulation, and silencing pseudogene Gm12664-201 expression significantly attenuated elevated levels of TG which were induced by SA in AML-12 cells. Of note, pseudogene Gm12664-201 overexpression increased BiP expression in the protein and mRNA levels and activated endoplasmic reticulum stress signaling markers, PERK, ATF6, CHOP, eIF2 α , XBP1 and ATF4, which may contribute to intracellular TG accumulation in hepatocytes.

To date, there have been no reports regarding the function of Gm12664-201. Pseudogenes have long been considered as nonfunctional genomic sequences, evolutionary remnants from past genetic duplication events [24]. However, recent increasing evidence suggests that a large number of pseudogenes exhibit transcriptional activity commonly in a tissue specific manner, which suggests

pseudogenes play crucial regulatory roles[14, 25, 26]. Owing to their high sequence homology to their parent gene, an actively transcribed pseudogene will share miRNA target sites that may compete for miRNA binding [27]. PTENP1, the first reported pseudogene that is homologous to the tumor suppressor PTEN regulates PTEN transcript levels by preventing miRNAs from targeting the PTEN transcript in cancer cells [28]. Cristina *et al.* indicate that transcribed pseudogene *RPSAP52*, that runs antisense to the oncofetal gene HMGA2, influences post-transcriptional regulation in the cytoplasm through the interaction with the RNA binding protein IGF2BP2 [29]. Toward this end, further study is necessary to determine the mechanism by which Gm12664-201 impacts TG accumulation in hepatocytes.

While our results suggested Gm12664-201 as a novel modulator of ER stress, whether the hepatic TG accumulation is attributable specifically to regulating of ER stress signaling pathway remained unclear. Thus, the relationship between the hepatic TG accumulation and ER stress needs to be further elucidated.

In conclusion, we have discovered that pseudogene Gm12664-201 expression markedly increased in HFD mice liver. Mechanistic investigations revealed that enhancing pseudogene Gm12664-201 expressions markedly increased TG accumulation and this phenomenon was accompanied by activation of ER stress signaling in AML-12 cells.

Declarations

Funding

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Conflicts of interest/Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Authors' contributions

Lixin Na and Changhao Sun conceived and designed the project, Yanhe Zhao, Yubing Jia and acquired the data, Yanhe Zhao analysed and interpreted the data, Zhen Tian and Yanhe Zhao wrote the paper, Dan

Shi and Xinyue Wang polished the article.

Ethics approval

The study was approved by the Institutional Animal Care Committee from Harbin Medical University and conducted in accordance with the University guidelines for the care and use of laboratory animals (approval number:12– 0003).

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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References

1. Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ (2018) Mechanisms of NAFLD development and therapeutic strategies. *Nat Med* 24:908–922. <https://doi.org/10.1038/s41591-018-0104-9>
2. Kim Y-R, Lee E-J, Shin K-O et al (2019) Hepatic triglyceride accumulation via endoplasmic reticulum stress-induced SREBP-1 activation is regulated by ceramide synthases. *Exp Mol Med* 51:1–16. <https://doi.org/10.1038/s12276-019-0340-1>
3. Tilg H, Moschen AR (2010) Evolution of inflammation in nonalcoholic fatty liver disease: The multiple parallel hits hypothesis. *Hepatology* 52:1836–1846. <https://doi.org/10.1002/hep.24001>
4. Fabbrini E, Mohammed BS, Magkos F et al (2008) Alterations in Adipose Tissue and Hepatic Lipid Kinetics in Obese Men and Women With Nonalcoholic Fatty Liver Disease. *Gastroenterology* 134:424–431. <https://doi.org/10.1053/j.gastro.2007.11.038>
5. Kim JY, Garcia-Carbonell R, Yamachika S et al (2018) ER Stress Drives Lipogenesis and Steatohepatitis via Caspase-2 Activation of S1P. *Cell* 175:133–145.e15.

<https://doi.org/10.1016/j.cell.2018.08.020>

6. Han J, Kaufman RJ (2016) The role of ER stress in lipid metabolism and lipotoxicity. *J Lipid Res* 57:1329–1338. <https://doi.org/10.1194/jlr.R067595>
7. Lebeaupin C, Vallée D, Hazari Y et al (2018) Endoplasmic reticulum stress signalling and the pathogenesis of non-alcoholic fatty liver disease. *J Hepatol* 69:927–947. <https://doi.org/10.1016/j.jhep.2018.06.008>
8. Xiao G, Zhang T, Yu S et al (2013) ATF4 Protein Deficiency Protects against High Fructose-induced Hypertriglyceridemia in Mice. *J Biol Chem* 288:25350–25361. <https://doi.org/10.1074/jbc.M113.470526>
9. Forouhi NG, Koulman A, Sharp SJ et al (2014) Differences in the prospective association between individual plasma phospholipid saturated fatty acids and incident type 2 diabetes: the EPIC-InterAct case-cohort study. *The Lancet Diabetes Endocrinology* 2:810–818. [https://doi.org/10.1016/S2213-8587\(14\)70146-9](https://doi.org/10.1016/S2213-8587(14)70146-9)
10. Irshad Z, Chmel N, Adya R, Zammit VA (2019) Hepatic VLDL secretion: DGAT1 determines particle size but not particle number, which can be supported entirely by DGAT2. *J Lipid Res* 60:111–120. <https://doi.org/10.1194/jlr.M089300>
11. Song MJ, Malhi H (2019) The unfolded protein response and hepatic lipid metabolism in non alcoholic fatty liver disease. *Pharmacol Ther* 203:107401. <https://doi.org/10.1016/j.pharmthera.2019.107401>
12. Hummasti S, Hotamisligil GS (2010) Endoplasmic Reticulum Stress and Inflammation in Obesity and Diabetes. *Circ Res* 107:579–591. <https://doi.org/10.1161/CIRCRESAHA.110.225698>
13. Rutnam ZJ, Du WW, Yang W et al (2014) The pseudogene TUSC2P promotes TUSC2 function by binding multiple microRNAs. *Nat Commun* 5:2914. <https://doi.org/10.1038/ncomms3914>
14. Pei B, Sisu C, Frankish A et al (2012) The GENCODE pseudogene resource. *Genome Biol* 13:R51. <https://doi.org/10.1186/gb-2012-13-9-r51>
15. De Martino M, Palma G, Azzariti A et al (2017) The HMGA1 Pseudogene 7 Induces miR-483 and miR-675 Upregulation by Activating Egr1 through a ceRNA Mechanism. *Genes* 8:330. <https://doi.org/10.3390/genes8110330>
16. Wen Y-Z, Zheng L-L, Liao J-Y et al (2011) Pseudogene-derived small interference RNAs regulate gene expression in African Trypanosoma brucei. *Proceedings of the National Academy of Sciences* 108:8345–8350. <https://doi.org/10.1073/pnas.1103894108>
17. Kong Y, Zhang L, Huang Y et al (2017) Pseudogene PDIA3P1 promotes cell proliferation, migration and invasion, and suppresses apoptosis in hepatocellular carcinoma by regulating the p53 pathway. *Cancer Lett* 407:76–83. <https://doi.org/10.1016/j.canlet.2017.07.031>
18. Wang L, Xu F, Song Z et al (2020) A high fat diet with a high C18:0/C16:0 ratio induced worse metabolic and transcriptomic profiles in C57BL/6 mice. *Lipids in Health Disease* 19:1–13. <https://doi.org/10.1186/s12944-020-01346-z>

19. Wang J-J, Zhang Y-T, Tseng YJ, Zhang J (2019) miR-222 targets ACOX1, promotes triglyceride accumulation in hepatocytes. *Hepatobiliary Pancreatic Diseases International* 18:360–365. <https://doi.org/10.1016/j.hbpd.2019.05.002>
20. Ress C (2016) Mechanisms of intrahepatic triglyceride accumulation. *World J Gastroenterol* 22:1664. <https://doi.org/10.3748/wjg.v22.i4.1664>
21. Lou W, Ding B, Zhong G et al (2019) Dysregulation of pseudogene/lncRNA-hsa-miR-363-3p-SPOCK2 pathway fuels stage progression of ovarian cancer. *Aging* 11:11416–11439. <https://doi.org/10.18632/aging.102538>
22. Chiefari E, Iritano S, Paonessa F et al (2010) Pseudogene-mediated posttranscriptional silencing of HMGA1 can result in insulin resistance and type 2 diabetes. *Nat Commun* 1:40. <https://doi.org/10.1038/ncomms1040>
23. Pan Y, Zhan L, Chen L et al (2018) POU5F1B promotes hepatocellular carcinoma proliferation by activating AKT. *Biomed Pharmacother* 100:374–380. <https://doi.org/10.1016/j.biopha.2018.02.023>
24. Kim K, Theusch E, Kuang Y-L et al (2018) ZNF542P is a pseudogene associated with LDL response to simvastatin treatment. *Sci Rep* 8:12443. <https://doi.org/10.1038/s41598-018-30859-y>
25. Piehler AP, Hellum M, Wenzel JJ et al (2008) The human ABC transporter pseudogene family: Evidence for transcription and gene-pseudogene interference. *BMC Genom* 9:165. <https://doi.org/10.1186/1471-2164-9-165>
26. Muro EM, Mah N, Andrade-Navarro MA (2011) Functional evidence of post-transcriptional regulation by pseudogenes. *Biochimie* 93:1916–1921. <https://doi.org/10.1016/j.biochi.2011.07.024>
27. Thomson DW, Dinger ME (2016) Endogenous microRNA sponges: evidence and controversy. *Nat Rev Genet* 17:272–283. <https://doi.org/10.1038/nrg.2016.20>
28. Poliseno L, Salmena L, Zhang J et al (2010) A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature* 465:1033–1038. <https://doi.org/10.1038/nature09144>
29. Oliveira-Mateos C, Sánchez-Castillo A, Soler M et al (2019) The transcribed pseudogene RPSAP52 enhances the oncofetal HMGA2-IGF2BP2-RAS axis through LIN28B-dependent and independent let-7 inhibition. *Nat Commun* 10:3979. <https://doi.org/10.1038/s41467-019-11910-6>

Tables

Table 1. Primer sequences of mRNAs for qRT-PCR.

Gene	Primer (5' to 3')
ATF4-F ¹	CCTTCGACCAGTCGGGTTTG
ATF4-R ²	CTGTCCCGGAAAAGGCATCCA
ATF6-R	TGCCCAGAAAGTAGAGGT
ATF6-R	TGCCCAGAAAGTAGAGGT
CHOP-F	GCACCTATATCTCATCCCCAG
CHOP-R	TGCGTGTGACCTCTGTTG
eIF2a-F	TCCAGGAAGTGACAAGCCATT
eIF2a-R	ACAGGAGTAGGAGCCGCATC
Gm12664-201-F	ACAGTCCACACAGTCCCAAG
Gm12664-201-R	CCACCTCCTTCATCTTCTTCA
GAPDH-F	TGGCAAAGTGGAGATTGTTGCC
GAPDH-R	AAGATGGTGATGGGCTTCCCG
BiP-F	GGAAAGAAGGTTACCCATGC
BiP-R	AGAAGAGACACATCGAAGGT
IRE1-F	TTCTGAGGTTCTTAGCCA
IRE1-R	CATGCATTACAAACATGA
Neat1-F	TTGGGACAGTGGACGTGTGG
Neat1-R	TCAAGTGCCAGCAGACAGCA
PERK-F	ATTTATGTCGGTAGTGTCA
PERK-R	CTTGAAAGAAGTCATAATAGTT
XBP1s-F	CTGAGTCCGAATCAGGTGCAG
XBP1s-R	GTCCATGGGAAGATGTTCTGG
β-Actin-F	TTGAACATGGCATTGTTACCAA
β-Actin-R	TGGCATAGAGGTCTTTACGGA

¹F, forward primer; ²R, reverse primer.

Figures

Figure 1

Upregulation of Gm12664-201 expression in liver of HFD mice and AML-12 cells Legend: a, body weights of HFD and ND mice; b, serum TG contents of HFD and ND mice; c, the expression of Gm12664-201 in liver tissues of HFD and compared to the expression of Gm12664-201 in control mice, n=3 per group; d, AML-12 cells were cultured in 300 μ M PA or SA for 24 hours and harvested for qRT-PCR of Gm12664-201. Data show mean $\pm$ SD; *, $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2

Effects of Gm12664-201 on triglyceride accumulation in AML-12 cells Legend: AML-12 cells were transfected with Gm12664-201 overexpression plasmid (OE-G) or Gm12664-201 siRNA (si-G). a, transfection efficacy of Gm12664-201 overexpression plasmid was verified by qRT-PCR; b, transfection efficacy of Gm12664-201 siRNA was verified by qRT-PCR; c, the contents of TG were tested; d, oil red O

staining. Each in vitro test was performed at least three times. All values are expressed as the mean $\pm$ SD. *, $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3

Gm12664-201 regulates BiP expression Legend: AML-12 cells were treated with the 300 μ M SA or transfected with Gm12664-201 overexpression plasmid (OE-G) or Gm12664-201 siRNA (si-G) (or treated with the indicated dose of stearic acid) for 24 hours. a, western blot analysis of BiP in liver tissue of HFD

and ND mice, n = 5; b, western blot analysis of BiP protein expression in AML-12 cells; c, western blot analysis of BiP protein expression in AML-12 cells; d, western blot analysis of BiP protein expression in AML-12 cells; e, qRT-PCR analysis of BiP in AML-12 cells. Each in vitro test was performed at least three times. All values are expressed as the mean $\pm$ SD. *, P < 0.05, **P < 0.01, ***, P < 0.001.

Figure 4

Gm12664-201 regulates ER stress signaling markers expression Legend: Relative ER stress signaling markers PERK, ATF6, CHOP, eIF2 α , XBP1 and ATF4 mRNA levels were measured by qPCR in control, Gm12664-201 overexpression plasmid (OE-G) or Gm12664-201 siRNA (si-G)-transfected AML-12 cells with/without SA exposure. The levels of PERK, ATF6, CHOP, eIF2 α , XBP1 and ATF4 mRNA. Each in vitro test was performed at least three times. All values are expressed as the mean $\pm$ SD. *, P < 0.05, **P < 0.01, ***, P < 0.001.