Lnc027912 Reduces Lipid Accumulation Through Akt/MTOR/SREBP1C Axis in Hepatic Cells

Kaifei Chu
Wenzhou Medical University
Niannian Zhao
Wenzhou Medical University
Rong Feng
Wenzhou Medical University
Li Zhang
Wenzhou Medical University
Xudong Hu
Wenzhou Medical University
Li Liu
Wenzhou Medical University
Wei Li
Wenzhou Medical University
Lin Liu (liulin0829@wmu.edu.cn)
Wenzhou Medical University

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Abstract

Background: Various metabolism diseases are closely related to lipid metabolism disorder, but long noncoding-RNAs (lncRNA) involve in regulating function of lipid was limited elucidated. Previous our work have found that lnc027912 involve in cholesterol metabolism. Here, we further explore the role of lipid metabolism-associated lncRNA-lnc027912 in oleic acid- (OA) and palmitic acid (PA)-induced hepatic cells.

Methods: The overexpression of lnc027912 cell model was constructed by using virus particles transfection, and the level of lnc027912 in AML12 cells were detected by RT-qPCR. High fat cell model was established by treating AML12 cells with OA and PA, and the level of lipid drops was detected by Oil red O staining and triglyceride analyze Kit. The lipid metabolism related-genes, such as SREBP1C, FAS, PPARγ, MTTP, ApoE and ApoC3 level, was detected using RT-qPCR and Western blot. The role of SREBP1C in lipid metabolism was further analyzed using double luciferase reporter gene assay and Immunofluorescence. The Akt/mTOR signal pathway related genes was detected by Western blot.

Results: We found that TG level was inhibited in overexpression of lnc027912 cell. Upregulated lnc027912 of AML12 cells treated with OA and PA showed a significant decrease in lipid accumulation and TG levels. Furthermore, overexpression of lnc027912, the lipid biosynthesis genes of SREBP1C, FAS and PPARγ was significantly decreased and a significant increase in expression of MTTP and ApoE. Interestingly, lnc027912 inhibited Akt/mTOR signaling axis and decreased SREBP1C transit into nucleus and the promoter activity of SREBP1C and regulated expression of its targets.

Conclusions: Our study revealed a new insights into the molecular function of lnc027912 in lipid metabolism by Akt/mTOR/SREBP1C signaling axis and highlights the potential of lnc027912 as a new therapeutic target for lipid disorder diseases (such as, NAFLD).

Background

Lipid metabolism is a dynamic biological process that includes lipid biosynthesis, accumulation, distribution and secretion [1]. Lipids synthesized by the liver is in an equilibrium state in terms of synthesis and catabolism [2]. The synthesis of lipids is mainly regulated by transcription factor, such as cholesterol regulating element binding protein (SREBP), which control the levels of the key enzyme, such as fatty acid synthase (FAS) , stearoyl-CoA desaturase (SCD) and acetyl-CoA carboxylase (ACC) [3]. Recent studies have demonstrated that SREBP-1C is central to dietary regulation of most hepatic lipogenic genes [4,5], and SREBP-1C transit into the nuclei and activate the expression of theses lipogenic genes. Furthermore, SREBP-1C facilitates fatty acid (FA) synthesis and high SREBP-1C cause hepatic TG accumulation and other lipid disorders [6,7]. Therefore, the regulation of lipid homeostasis is crucial for treating for lipid disorder diseases, such as NAFLD, obesity, type 2 diabetes, dyslipidemia, and arteriosclerotic cardiovascular disease.

Long nocoding-RNAs (lncRNAs) are non-coding transcripts more than 200 nucleotides in length with no protein-coding capacity [8]. As the function of modulated by lncRNAs continues to grow, it has become more important that they are key regulators of a wide range of biological processes, in particular lipids homeostasis. A large number of studies have indicated that lncRNAs involved in lipid metabolism various processes, such as synthesis, transport and secretion [9]. LncHULC (highly upregulated in liver cancer), is the first identified liver-specific lncRNA, which was acted on PPARA to regulate the transcriptional factor of long-chain acy-CoA synthetase 1(ACSL1) involved in cellular fatty acid metabolism [10]. Moreover, lncRNAs, including MALAT-1[10], HOTAIR[11], H19[12], HR1[13], LeXis [14] and Gm16551[15] were found to be related with lipid metabolic disorder. Recent studies have demonstrated that lncH19 suppressed lipid metabolism which may act as therapeutic target of atherosclerosis [16]. These results suggested that lncRNAs played a vital role in remaining lipid homeostasis. Previous our work have found that lncRNA, NONMUG027912 (after abbreviate it to lnc027912), is implicated in the process of cholesterol biosynthesis [17]. Depending on cholesterol levels, metabolic pathways are activated resulting in fatty acid or cholesterol synthesis. However, the function of lnc027912 in regulating lipid metabolism have not be investigated.

In this study, we explore the role of lnc027912 in regulating lipid metabolism. Up-regulated lnc027912 significantly reduced lipid accumulation in AML12 cells. Mechanistically, lnc027912 negatively modulated the expression of SPEBP-1C, PPARγ and FAS, meanwhile, positively regulated the expression of MTTP and ApoE, thereby reducing lipid accumulation induced by oleic acid (OA) and palmitic acid (PA). Furthermore, our results further confirmed that lnc027912 inhibited the SREBP1C promoter activity and reduced SREBP1C transit into nucleus through restraining Akt/mTOR signaling axis. Together, our study provides new insights into the molecular function of lnc027912 in lipid metabolism and highlights the potential of lnc027912 as a new therapeutic target for lipid disorder diseases (such as, NAFLD).

Methods

Cell cultured and transfection

AML12, Hepa1-6 and HEK-293T cells were obtained from the Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). AML12 cells were cultured in DMEM/F-12 medium (Gibco) with 10% (v/v) fetal bovine serum (FBS) (Gibco), 1% (v/v) ITS liquid media supplement (Sigma), and 40 ng/ ml dexamethasone (Sigma). HEK-293T and Hepa1-6 cells were cultured in DMEM medium (Gibco) and RPMI-1640 medium (Gibco), respectively, with 10% (v/v) fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin. All cells were cultured under standard culture conditions of 37°C in a humidified atmosphere containing 5% CO_2.

Hepa1-6 cells were transfected with 50 nM siRNA (RiboBio) and Lipofectamine™ RNAiMAX (Invitrogen). The negative control siRNA has no significant sequence similarity with mouse gene sequences. Si-lnc027912 sequence: GUGUAUUGCUUUGAGUUCATTUGAACUCAAAGCAAUCCACTT; si-control sequence: UUCUCCGAACGUGUCACGUTTAC GUGACACGUUCGGAGAATT.

Construction of overexpression cell line

After mixing the plasmids (pCDH-GFP-lnc027912 and pCDH-GFP) and pPACKH1-plasmid Mix, they were transfected into HEK-293T cells, the culture medium was collected, and the virus particles were precipitated with PEG-it. Puromycin was added to AML12 cells to screen out the cell line stably expressing lnc027912, and the concentration of puromycin in AML12-GFP or AML12- lnc027912 cells was 0.8 μg/ml.

Oleic acid (OA) and palmitic acid (PA) treatment, and Oil red O staining

The AML12-GFP and AML12-lnc027912 cells were seeded in 12 wells at the density of 5×10^4 cells per well in DMEM/F-12 medium. After for 12 h, the cells were washed with 1×PBS for one time, then 200 μM OA (Sigma) or PA (Sigma) treatment for 24 h. All samples were fixed with 4% (wt/vol) paraformaldehyde in PBS, and refrigerated at 4°C overnight. Then, cells were stained with Oil red O, as previous described [18].

Measurement of triglyceride (TG) in hepatic cell

The AML12-GFP and AML12-lnc027912 cells were treated with OA (200 μM) and PA (200 μM) for 24 h, then samples were lysed with cell lysis buffer and obtained the liquid supernatant. The TG was measured according to the TG manufacturer's protocols (Jiancheng Bioengineering Institute, Nanjing, China).

Quantitative RT-PCR

Total RNA was isolated from cells using the TRIzol reagent (Invitrogen). Remove genomic DNA with 4×gDNA wiper Mix, and then add 5×HiScript qRT SuperMix (Vazyme;) to synthesize cDNA. Quantitative RT-PCR was performed on the CFX96TM real-time PCR system (Applied Biosystems). The PCR reagent mix was obtained from Vazyme. The data was analyzed using the ΔΔCT method. GAPDH expression was an control to normalize the data. All primers sequences used are listed in the Table S1.

Western blot analysis

Cellular protein was extracted with cell lysis buffer and measured using an BCA Protein Assay Kit (Beyotime). Then, the proteins were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was performed using select primary antibodies, followed by probing with the recommended secondary antibodies, GAPDH was used as a control. Finally, the signals were detected using a ChemiDoc^{TM} Imaging System (Bio-Rad).The antibodies used are listed in the Table S2.

Immunofluorescence

AML12-GFP and AML12-lnc027912 cells were treated with OA and PA. Then, samples were fixed with 4% paraformaldehyde and further permeabilized with 0.5% Triton X-100. The specially primary (FAS and

SREBP1C) and secondary (Alexa Fluor 555-labeled Donkey Anti-Mouse IgG(H+L), (Beyotime) and Cy3-labeled Goat Anti-Mouse IgG (H+L) , (Beyotime)) antibodies were incubated with the cells. The nuclei were stained with 4',6-Diamidino-2-phenylindole (DAPI, Beijing Solarbio Science & Technology Co., Ltd. Beijing, China) . The images were captured under a laser confocal microscope (Nikon, Tokyo, Japan), and these pictures were further quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

Luciferase reporter assays

The SREBP1C promoter containing the far-upstream element sequence (about-2000-0 bp) was sub-cloned and inserted into the pGL3-basic vector (Promega, Madison, WI, USA). The recombinant plasmid (pGL3-SREBP1C-promoter vector) was obtained by MiaoLingBio. HEK-293T cells were seeded onto 48-well plate and transfected with pCDH-lnc027912, pGL3-SREBP1C, and phRL-TK vectors. After incubation for 36 h, the cells were treated with OA (200 μM) and PA (200 μM) for 24 h. Then, the cells were lysed in 1×passive lysis. A Dual-luciferase reporter assay system (Promega) was performed according the manufacturer's protocols, with Renilla luciferase serving as a transfection control.

Statistical analysis

All experiments were repeated at least three times, and the results were presented as mean ± SD. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA). The *P* values were calculated using a one-way ANOVA. A *P* value of <0.05 was considered statistically significant.

Results

Up-regulated lnc027912 reduced the total triglyceride level in hepatic cells

Our previous studies have demonstrated that lnc027912 played a key role in regulating of cholesterol homeostasis [17]. Cholesterol level is closely related to lipid metabolism, inducing lipid biosynthesis, transport or secretion, etc [19]. To further explore the function of lnc027912 in regulating lipid metabolism, we used virus particles (obtaining pCDH-lnc027912-GFP and pCDH-GFP vectors) to infect AML12 cells, as shown in Fig. 1a, above 95% cells showing green fluorescence. Compared with the control group, the CT value of lnc027912 was decreased by 10 (Fig. 1b). These results suggest that over-expression of lnc027912 cell line was successfully constructed. At the same time, the total triglyceride (TG) level were analyzed. As showing in Fig.1c, overexpression of lnc027912, the TG level was significantly inhibited in AML12 cells. To further analyze the mechanism of lnc027912 in regulating lipid metabolism, these lipid metabolism related genes (SREBP1C, PPARγ, FAS, MTTP, ApoE, ApoC3) mRNA expression were measured. As showing in Fig.S1, the key lipogenesis genes of SPEBP1C and FAS were obviously decreased, simultaneously, the gene of MTTP was increased in AML12-lnc027912 cells. These results further suggested lnc027912 involved in lipid metabolism.

Lnc027912 could alleviate OA and PA induced lipid accumulation

To further demonstrate the effect of lnc027912 on lipid level, cells were treatment with OA and PA, then the total triglyceride (TG) level was assessed by oil red O staining and TG kit. Firstly, oil red O staining was performed to analyses the cellular lipid level. As shown in Fig. 2a, compared with control group, upregulation of lnc027912 in cells resulted in a decrease in oil red O positive lipids. Importantly, overexpression of lnc027912 could significantly reduce lipid accumulation induced by OA and PA. Then, the number of lipid drops was measured using image Pro plus 6.0, showing that lipid drops significantly reduced in upregulated of lnc027912 groups (Fig. 2b). Furthermore, we used TG kit to measure the TG level, results showing that the TG decreased by 32.7% (OA-treated, Fig. 2c) and 32.3% (PA-treated, Fig. 2d), respectively, than the control group. To further explore the role of lnc027912, silencing lnc027912 was performed in Hepa1-6 cells, results showing that lnc027912 level decreased by 54% (Fig. S1a). While, we noticed that the TG level was no change (Fig. S1b). Together, these results strongly suggested that lnc027912 inhibited lipid accumulation in cells.

Lnc027912 regulate lipid metabolism by inhibiting lipid biosynthesis and improving lipid transport

To determine the mechanism by which lnc027912 influences lipid level, we analyzed the expression of lipid metabolism related-genes by qPCR and Western Blot. Overexpression of lnc027912, the lipogenesis genes of SREBPC1 and FAS was significantly inhibited, concurrently, VLDL metabolism genes of MTTP was induced by RT-qPCR (Fig. S2). Importantly, we found that lnc027912 could inhibit lipid biosynthesis and increase lipid transport, when cells was treated with OA (Fig.3a, b and Fig.S2). These results suggested that lnc027912 could alleviate OA induced lipid accumulation. Additionally, the lipid level was analyzed under PA treatment. The decreased of SREBPC1 and FAS, and increased of MTTP was found by RT-qPCR (Fig. S2) and Western blot (Fig. 3c, d). These results sufficiently confirmed that lnc027912 could inhibit OA- or PA-induced lipid accumulation in hepatic cell. To further confirm the lnc027912 role of lipid metabolism, the FAS level was detected by immunofluorescence (Fig. 3e). We found that the FAS, which the lipid biosynthesis key gene, was significantly inhibited in overexpressed of lnc027912 cells. Concurrently, we found that lnc027912 can effectively reduce the elevation of FAS caused by OA or PA (Fig. 3e, f). Together, these findings suggest that lnc027912 could inhibit lipid accumulation by reducing lipid biosynthesis and inducing lipid transport.

Overexpression of lnc027912 could inhibit SREBP1C activity

SREBP1C is a key transcription regulator of fatty acid synthesis, a process known as de novo lipogenesis [20]. Therefore, we reasoned that lnc027912 may involve in regulating of SREBP1C activity to modulate the lipid metabolism. Firstly, to investigate the effect of lnc027912 on promoter activity of SREBP1C, the dual-luciferase reporter assay was performed. As shown in Fig. 4a, the SREBP1C promoter activity was significantly inhibited when lnc027912 was upregulated. Interestingly, SREBP1C promoter activity was activated under the treatment with OA and PA, while, overexpression of lnc027912 can significantly reduce the activation of SREBP1C promoter induced by OA and PA. These results suggested that lnc027912 may affect lipid deposition by inhibiting SREBP1C activity. Additionally, we also found that the

fluorescence density of SREBP1C was significantly inhibited in unregulated of lnc027912 cells (Fig. 4b and Fig. S3). Furthermore, we found that overexpression of lnc027912 reduced the expression of SREBP1C in nuclei (Fig. 4c), which may reduce the expression of lipid-related genes. To further explore the mechanism of lnc027912 regulation of SREBP1C, the Akt and mTOR levels were detected. As shown in Fig. 4d, the p-mTOR (Ser2448) and p-Akt (Ser473 and Thr308) level were inhibited after overexpression of lnc027912, while, the levels of mTOR and Akt remained unchanged. These results suggested that lnc027912 inhibited the expression lipid synthesis genes and lipid accumulation through Akt/mTOR/SREBP1C signaling axis.

In summary, our findings investigated that lnc027912 inhibited SREBP1C transferred into the nuclei through reducing phosphorylation of Akt and mTOR, then decreased of the activity of SREBP1C promoter, resulted in decreased of lipid synthesis and increased of the transport of lipid, and alleviated to lipid accumulation (Fig. 5).

Discussion

Excessive lipids in the liver could disrupt lipid synthesis and transport balance, and lipid metabolism can be perturbed [21,22]. Lipid homeostasis disorders not only causes NAFLD, but is also a component of metabolic syndrome with obesity, type 2 diabetes, dyslipidemia, and arteriosclerotic cardiovascular disease [23,24]. Recent studies have suggested that lncRNAs played a key role in regulating lipid metabolism, such as lncHC, lncLSTR, lncHULC and lncMALAT1 [25]. LncRNAs often target several transcription factors that play essential roles in the regulation of lipid metabolism, such as sterol regulatory element binding proteins (SREBPs). Previous our work have found that lnc027912 involved in regulation of cholesterol metabolism via modulating the expression of farnesyl-diphosphate farnesyltransferase 1(Fdft1), which is key enzyme for cholesterol biosynthesis [17]. While, lnc027912 involving in lipid metabolic diseases has not been elucidated yet.

SREBP1C is major transcription factors control fatty acid synthesis and play key role in the process of lipid metabolism. Some works have demonstrated that lncRNAs could affect lipid synthesis via regulating the level of SREBP1C. LncMALAT1 could inhibit PA-induced upregulate of SREBP1C and hepatic lipid accumulation through inhibition of FAS, which is the principle target of SREBP1C [26]. Overexpression of lncHR1 could decrease triglyceride (TG) through inhibiting activation of SREBP1C and FAS in oleic acid (OA)-induced hepatic cells [13]. These results indicated that lncRNAs played an important role in inhibiting lipid accumulation in OA- and PA-treated cells. In our study, we found that lnc027912 could abrogates PA- or OA-induced lipid accumulation through a mechanism of action that increase SREBP1C protein. Further the mechanism exploration, we found that lncRNA027912 could inhibit promoter activity of SREBP1C. Additionally, studied have revealed that the SREBP1C transited into cell nuclei and involved in regulating lipid biosynthesis [27]. However, lncRNAs induced SREBP1C transferred into the nucleus have been rarely reported. Interestingly, in this study, our findings showing that, lnc027912 reduced SREBP1C transit into the nucleus. Furthermore, Akt activation appears to be both necessary for the induction of hepatic SREBP1C [28], and recently studies have shown Akt activated

mTOR was through the phosphorylation [29].Our findings were consistent with previous studies showing lnc027912 inhibited Akt and mTOR of phosphorylation, then reduced lipid accumulation.

Furthermore, PPARγ plays an important role in regulation of lipid metabolism.-PPARγ as well as SREBP1 and FAS are important transcription factors that modulate genes involved in fatty acid metabolism and TG synthesis [30]. In this study, lnc027912 inhibited PPARγ protein level and reduced hepatic lipid accumulation. In addition, lipid transport is also vital process in lipid metabolism. Some research groups have confirmed that lncRNAs modulate lipid homeostasis via regulating the VLDL metabolism key genes expression of APOA4, APOC3, MTTP, and APOE [31]. Down regulation of lncAPOA4-AS inhibited APOA4 expression, resulting in decreased triglyceride (TG) concentrations, indicating that lncAPOA4-AS may be a potential target for fatty liver disease [32]. LncMTTP-AS affected the MTTP stability and translation, which may be involve in lipid transport [33]. These results demonstrated that lncRNAs play a vital for lipid metabolism efflux. Interestingly, in this study, we found that lnc027912 promoted the expression of MTTP and ApoE, resulting in decreased lipid accumulation in hepatic cells.

Conclusions

In this study, we found that lnc027912 inhibited SREBP1C activity through Akt/mTOR signal pathway and involved in lipid metabolism balance. Lnc027912 can not only reduce lipid synthesis, but also promote lipid transport, thereby reducing intracellular lipid accumulation. Therefore, lnc027912 played a dual regulatory role in the regulation of lipid metabolism homeostasis and may be an effective target for the regulation of lipid metabolism diseases, such as lipid disorder diseases (NAFLD).

Abbreviations

LncRNA: long noncoding RNA; OA: oleic acid; PA: palmitic acid; NAFLD: Non-alcoholic fatty liver disease; SREBP: cholesterol regulating element binding protein; FAS: fatty acid synthase; SCD: stearoyl-CoA desaturase; ACC: acetyl-CoA carboxylase; FA:fatty acid; ACSL1: acy-CoA synthetase 1; FBS: fetal bovine serum; TG: triglyceride; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; PPARγ: peroxisome proliferator-activated receptor γ ; LXRs: liver X receptors; Fdft1: farnesyl-diphosphate farnesyltransferase 1; MTTP: microsomal triglyceride transfer protein

Declarations

Acknowledgements

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Authors' contributions

LL (Lin Liu) and WL guided and revised manuscript. KFC participated in experiment. NNZ, RF, LZ, XDH and LL(Li Liu) helped to perform experiments and the statistical analysis and figures. All authors

reviewed the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

All study data are contained within the manuscript and additional materials. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interest

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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Figures

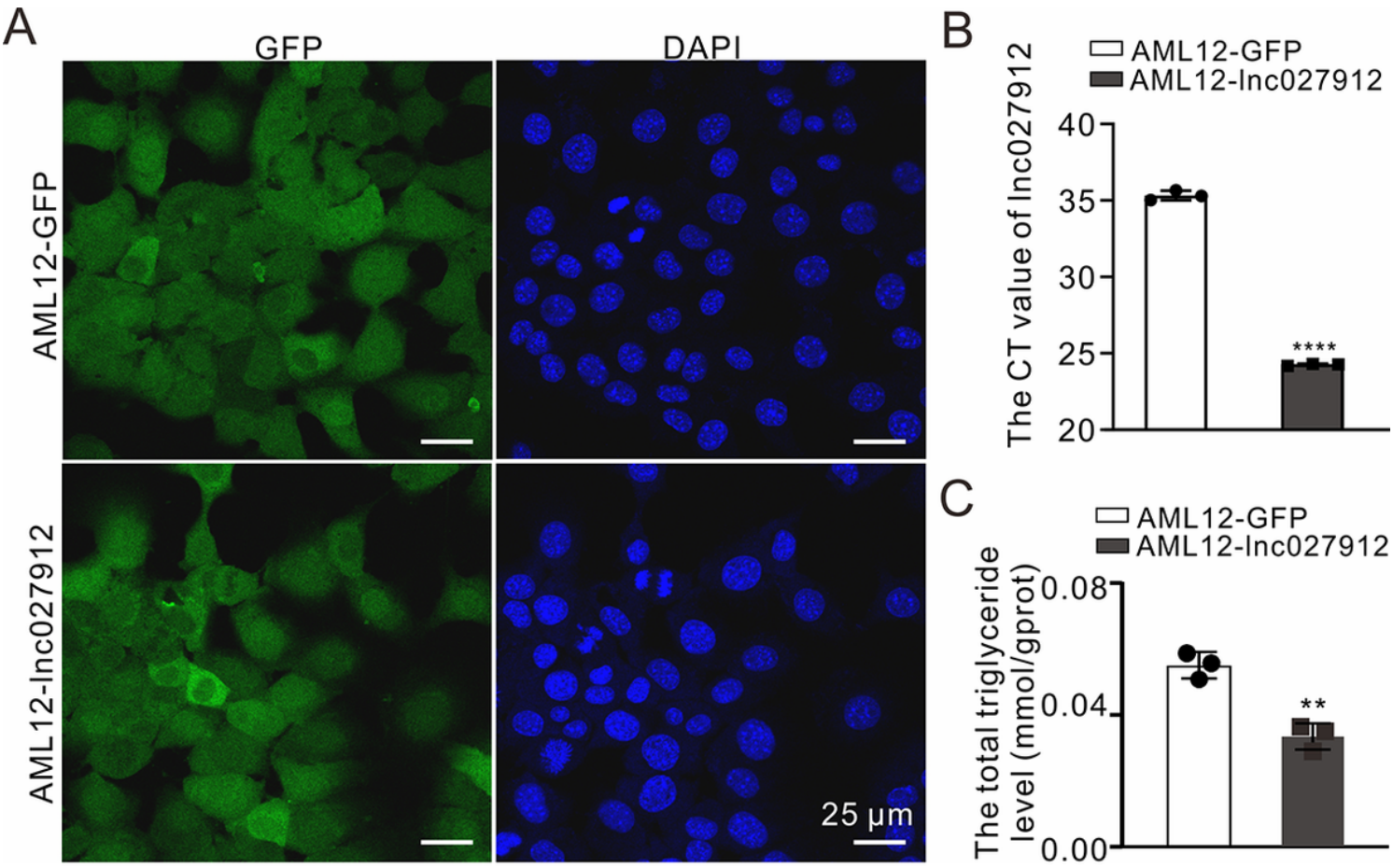

Figure 1

Construct of pCDH-GFP and pCDH-GFP-lnc027912 AML12 cell lines. **a** AML12 cells were transfected with pCDH-GFP or pCDH-GFP-lnc027912 virus particles. After 48 h, the puromycin (0.8mg/ml) was added to the complete medium. After 7 days, the image was obtained using laser confocal microscopy. **b** RT-qPCR analysis the level of lnc027912 in pCDH-GFP- lnc027912 AML12 cells. **c** The TG kit analysis the level of TG according to manufacturer's protocols. ** P<0.01, *** P<0.001

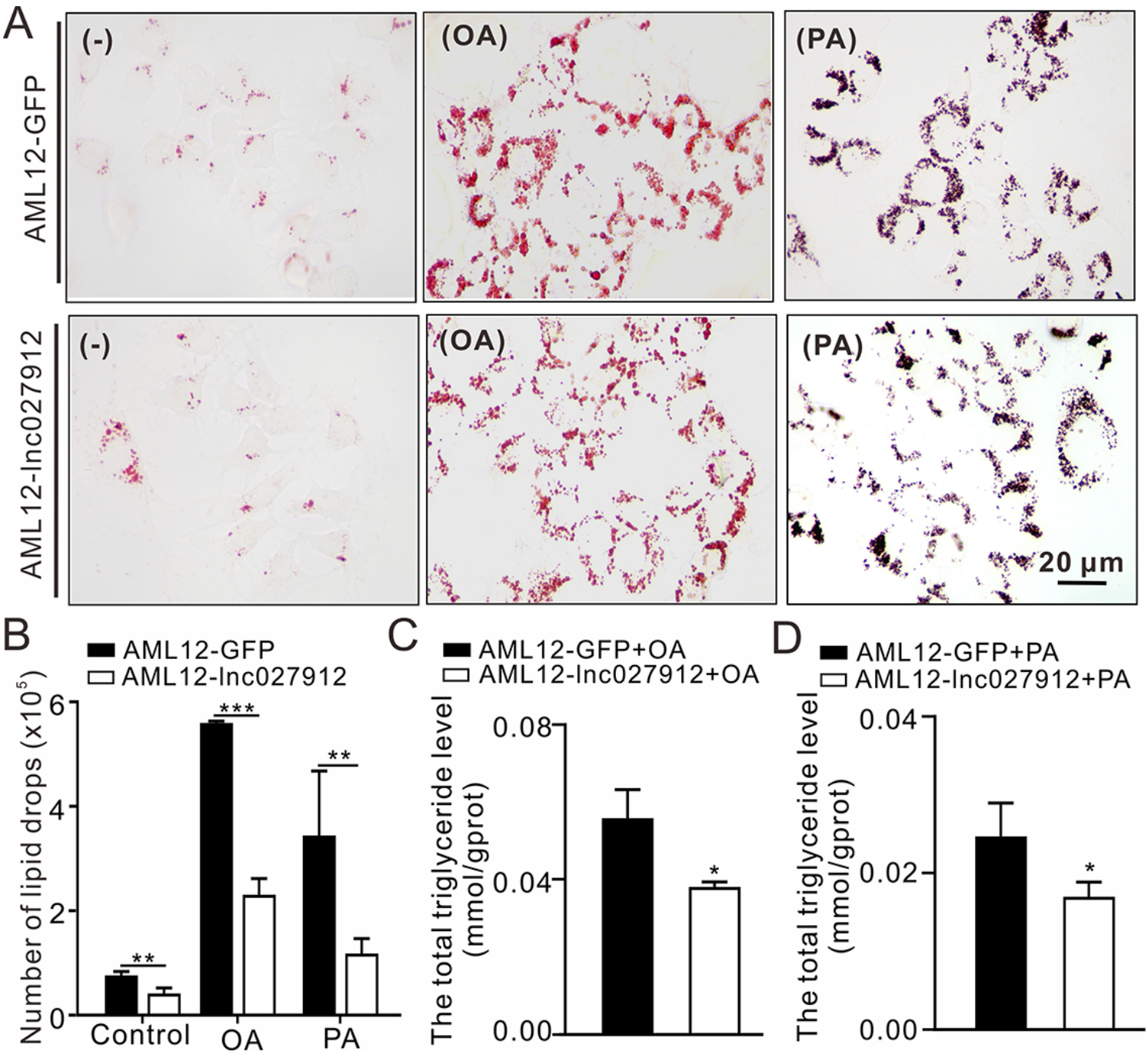

Figure 2

Lnc027912 inhibits fat accumulation in AML12 cells. **a** Oil red O staining analyzes lipid levels in cells under OA and PA treatment. (**a**). **b** The number of lipid drops was assessed using image Pro plus 6.0. The cellular total triglyceride level was analyzed under OA (**c**) and PA (**d**) treatment. (n=3, * $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Figure 3

Lnc027912 regulates the related genes expression of lipogenesis and VLDL metabolism involved in lipid metabolism. (**a-d**) Changes of key genes related to intracellular adipogenesis and VLDL metabolism were analyzed by Western Blot, respectively, after OA (**a, b**) and PA (**c, d**) treatments. The immunofluorescence of FAS was detected by special primary antibody (**e**). The fluorescence intensity of FAS was analyzed using Image J. Data are presented as the mean ± SD. (n≥3, * *P*<0.05, ** *P*<0.01, ****P*<0.001 green bar: 5 μm, white bar: 25 μm).

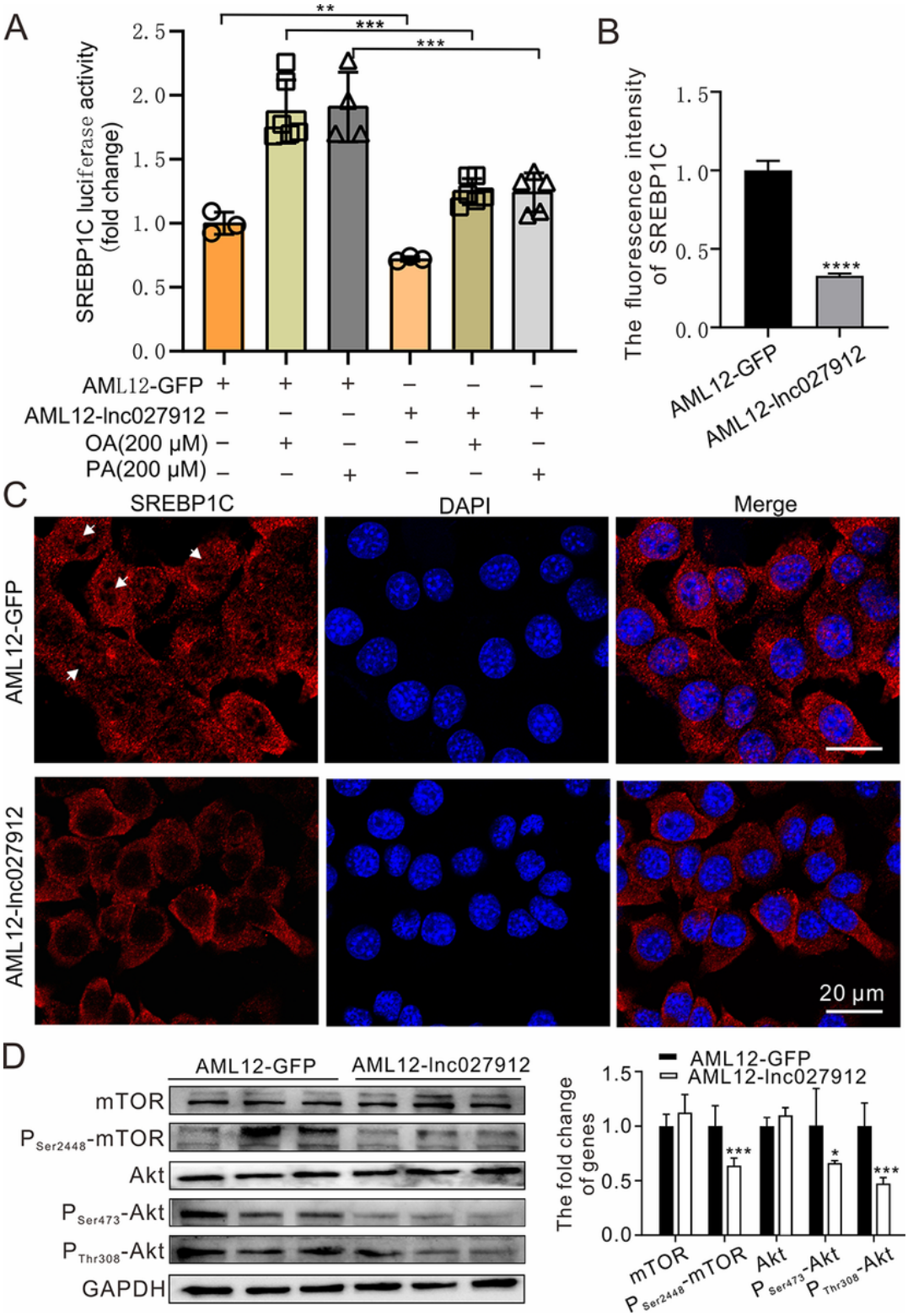

Figure 4

Lnc027912 regulate SREBP1C activity. **a** Changes of SREBP1C promoter activity was detected by dual-luciferase reporter assay. **b** The SREBP1C fluorescence intensity was analyzed in AML12-GFP and AML12-lnc027912 cells. **c** The SREBP1C location was detected by immunofluorescence (Red: SREBP1C antibody staining; Blue: DAPI stained cell nucleus, white arrow indicate SREBP1C). (n≥3). **d** Western blot analyzed the mTOR, $P_{Ser2448}$-mTOR, P_{Ser473}-AKT and P_{Thr308}-AKT expression level. (n=3). Data are presented as the mean ± SD, * P<0.01, ** P<0.01, *** P<0.001, **** P<0.0001.

Figure 5

Diagram for the role of lnc027912 in regulating lipid metabolism. Lnc027912 could inhibit SREBP1C transfer into the nucleus through decreased of p-Akt and p-mTOR, then inhibited SREBP1C promoter activity and. further regulated lipid metabolism-related genes (FAS, et.al) and reduced lipid accumulation in hepatocytes cells.

Supplementary Files

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- SupplementaryMaterials.docx