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Keywords: Perilipin1, BMECs, milk fat, SREBP1

Posted Date: May 20th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-533321/v1

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Perilipin1 promotes the synthesis of milk fat by regulating the activity of SREBP1 in bovine mammary epithelial cells

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Abstract

Background: Milk fat content is an important index of milk product quality and one of the main traits of dairy cattle breeding. Perilipin1 is a predominant binding protein that mainly surrounds lipid droplets. Perilipin1 is important in the regulation of lipid metabolism. SREBP1 is a transcription factor that controls the expression of a variety of lipogenic genes and is the main regulator of milk fat synthesis. Here, we investigated the effect and mechanism of Perilipin1 on milk fat synthesis in bovine mammary epithelial cells (BMECs).

Results: We found that the number and volume of lipid droplets increased following periplipin1 overexpression, leading to increased triglyceride accumulation, increased relative expression of lipid synthesis-related genes, decreased expression of lipid lipolysis genes, and increased SREBP1 activity . On the contrary, periplipin1 silencing reduced the number of lipid droplets, inhibited the synthesis of triglycerides, decreased the relative expression of lipid synthesis-related genes, increased the expression of lipid lipolysis genes, and downregulated the activity of SREBPI.

Conclusions: Perilipin1 promotes the synthesis of milk fat via up-regulating the activity of SREBP1 in BMECs. These findings laid the foundation for Holstein dairy cows to increase their milk fat content in molecular breeding.

Key Words: Perilipin1, BMECs, milk fat, SREBP1

Background

Milk is rich in fat, protein, lactose, multiple vitamins, and mineral elements. It is widely referred to as "white blood" as it provides most of the nutrients required for human life. Fat is the main energy component in milk, determines many of the organoleptic qualities, manufacturing characteristics, and physical properties of milk and its products [1]. Therefore, milk fat is also considered to be the main target trait in dairy cattle breeding [2]. Milk fat is synthesized in breast epithelial cells (**BMECs**), and its synthesis process and regulation mechanism are extremely complex [3], it is coordinated and regulated by a variety of factors such as genetics, hormones, physiology, environment, and nutritional levels [3-5], while genetic factors are the core factors that determine milk fat synthesis in dairy animals [6]. A large number of genes related to milk fat metabolism have been identified, including LPL, FABP3, ACSL1, ACCα, FASN, SCD, FADS1, AGPAT6, LPIN1, PLIN2, Par, and SREBP1 [5, 7], however, our current understanding of the regulation on milk fat synthesis remains limited, it is necessary to further in-depth study and analysis.

As we all know, the PAT protein family is the most abundant protein in lipid droplets (**LDs**), with highly conserved similar sequences, it has an affinity for the surface of intracellular neutral lipid storage droplets and plays an important role in the biosynthesis and decomposition of LDs [8-10]. As a "molecular switch" for regulating lipid metabolism, perilipin1 (***PLIN1***) is a member of this family, has three subtypes, namely, *PLIN1*A, B, and C, they have a common N-terminal region but different C-terminal lengths; *PLIN1*A is the most abundant and is often referred to as *PLIN1* [11, 12]. The LD coating protein encoded by the *PLIN1* is the most abundant protein on the surface of LDs, it binds to lipid droplets in cells and promotes triglyceride (**TAG**) synthesis in fat cells, increasing the number and volume of LDs, resulting in large LDs [13-15]. TAG can be hydrolyzed into diglycerides (also referred to as diacylglycerol, **DAG**) by fatty triglyceride lipase (**ATGL**) and further hydrolyzed to glycerol by hormone-sensitive lipase (**HSL**) [16]. *PLIN1* inhibits the basic lipolysis of ATGL and HSL in adipocytes [11, 17, 18]. However, under starvation and catabolic conditions, *PLIN1* supports TAG breakdown by lipase [19, 20]. These results show that the *PLIN1* plays an important role in LD biosynthesis and lipid metabolism. Therefore, based on

the above findings, we used BMECs to explore the regulatory role of *PLIN1* in milk fat.

Sterol regulatory element-binding transcription protein 1 (SREBP1, gene name *SREBF1*) is a transcription factor that plays an important role in cholesterol biosynthesis and fatty acid metabolism, specifically fat biosynthesis. SREBP1 plays an important role in the regulation of milk fat in BMECs [21], and acts as a key positive regulator in the synthesis of milk fat [22]. Many studies have shown that SREBP1 is regulated by many genes [23-26], and a research has shown that *PLIN1* may activate SREBP1 in lipid metabolism [27]. Consequently, we hypothesized that *PLIN1* could activate SREBP1 and plays an important role in milk fat synthesis and metabolism in BMECs.

Materials methods

Cell culture and transfection

Mammary epithelial cells were extracted from the mammary gland parenchyma of 4 mid-lactating Holstein cows, as previously described [2, 3]. BMECs from the breast tissue were separated by collagenase digestion [3]. Cells were cultured in DMEM/F12 (Gibco, 12500062) complete medium containing 10% fetal bovine serum, 100 μg/mL streptomycin, 100 μg/mL penicillin culture BMEC in the medium. We incubated the cells in a humidified environment of 37°C, 95% air, and 5% CO2 for subsequent experiments. When the cells reach 80% confluence, we used 0.25% trypsin for digestion. Pure breast epithelial cells were isolated after 3-4 generations. Forty-eight hours before cell treatments, we replaced the complete medium with a milk production medium. The cells were seeded in a 6-well plate, and the cells were transfected when the cells reached 50-60% confluency, and the samples were collected after 48 hours for subsequent experiments. All experiments were processed in parallel in triplicate.

Total RNA extraction and real-time PCR

TRIzol reagent (Sigma, Louis) was used to extract total RNA of differently processed

BMECs according to the manufacturer's instructions after 48 h of transfection. The OD260/280 values of all RNA samples were between 1.8 and 2.0. The first-strand cDNA was synthesized with a reverse transcription kit (Takara), and quantitative reverse-transcription PCR (qRT-PCR) was performed with a Real-time PCR kit (Takara). Primer Premier 5.0 (Premier Biosoft) was used to design primers for qRT-PCR and synthesized by TSINGKE Biological Technology. The relative mRNA expression level was normalized with the housekeeping gene *UXT*. The obtained data were calculated using the $2^{-\Delta\Delta Ct}$ method.

Oil Red O staining

Treated BMECs (48 h) were washed three times with phosphate buffered saline (PBS, including KCl 0.02%, NaCl 0.8%, KH_2PO_4 0.02%, and Na_2HPO_4 0.29% in deionized distilled water). Cells were fixed with 4% tissue cell fixative for 40 min, washed three times with PBS, stained with oil red O working solution for 40 min, and then washed three times with PBS. Cells were observed and detected under a microscope.

Triacylglycerol assay

We determined the intracellular TAG content in BMECs after 48 h of transfection using the cell/tissue TAG analysis kit (Applygen Technologies). The total protein concentration was measured using the BCA kit (Takara), to calibrate the TAG content. The TAG values were expressed in micrograms per milligram of protein. All the above operations were performed per the agreement issued by the above manufacturer.

Glycerol assay

The content of intracellular glycerol in BMECs after 48 h of transfection was measured with a tissue cell glycerase assay kit (Applygen Technologies). The total protein concentration measured by the BCA kit (Takara) was used to calibrate the glycerol content. The glycerol values were expressed in micrograms per milligram of protein. All operations were carried out per the freehand notice issued by the manufacturer.

Western blotting

The total protein in BMECs was collected after 48 h of transfection. The cells were digested from the 6-well plate with 0.25% trypsin (Solarbio), and then the digestion was terminated with DMEM/F12 (Gibco, 12500062) complete medium. We discarded the supernatant after centrifugation. Cell pellets were washed with PBS and supplemented with 200 μL of RIPA buffer containing 1% PMSF (Solarbio) and 10% phosphatase inhibitor cocktail (Roche). They were then placed on ice for lysis for 15 min. We collected 180 μL of supernatant, to be used for western blotting. The protein concentration was determined using the BCA method (Takara). The protein samples were diluted with 5× SDS-PAGE (BioSharp) and boiled in a metal bath at 100°C for 10 minutes to denature it. Samples were separated on 12% SDS-PAGE gel and transferred to a membrane. The membranes were blocked with Quickblock™ blocking buffer (Beyotime) for 30 minutes and incubated with the primary antibody overnight at 4°C. Membranes were incubated with the secondary antibody for 1 hour at room temperature and protected from light. The chemiluminescent HRP substrate was used to capture western blot images on the Bio-Rad molecular imager. The images were analyzed using Image Lab software (Bio-Rad Laboratories). The antibodies used in this experiment were as follows: anti-GAPDH antibody (1:8000, Abcam), anti-perilipinA antibody (1:6000, AB10200, Sigma-Aldrich), SREBF1 Polyclonal antibody (1:500, 14088-1-AP, Proteintech), rabbit anti-SREBF1 (Phospho-Ser439) polyclonal antibody (1:600, D151451-0025, Sangon Biotech).

Statistical analysis

All experiments were processed in parallel, in triplicate. All the experimental data obtained were sorted by Office Excel and then analyzed by statistical software SPSS17.0. Data were expressed as means ± standard deviation (means ± SD). The independent sample t-test was used for comparative analysis. The P-value (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) was used to indicate the significant differences. Figures were generated using the GraphPad Prism 6.01 software.

Results

PLIN1 promotes milk fat synthesis in BMECs

We designed *PLIN1* overexpression (op-*PLIN1*; Figure 1A) and *PLIN1* interference (si-*PLIN1*; Figure 1B) vectors. We found that compared with the control group, op-*PLIN1* significantly promoted LD formation (Figure 1C) and resulted in an increase in TAG synthesis (Figure 1E). Additionally, si-*PLIN1* inhibited LD formation (Figure 1F) and decreased TAG synthesis (Figure 1D) compared with the control group. The data above showed that *PLIN1* promoted milk fat synthesis in BMECs.

PLIN1 promotes the expression of lipid synthesis-related genes in BMECs

In the op-*PLIN1* group, the relative mRNA expression of lipid synthesis-related genes increased (Figure 2A). In the si-*PLIN1* group, the relative mRNA expression of lipid synthesis-related genes decreased (Figure 2B). These data suggest that *PLIN1* promoted the expression of lipid synthesis-related genes in BMECs.

PLIN1 inhibits lipid lipolysis in BMECs

Compared with the control group, op-*PLIN1* reduced the mRNA expression of HSL and ATGL (Figure 3A) and caused a significant decrease in the glycerol content of BMECs (Figure 3C). Additionally, compared with the control group, si-*PLIN1* promoted the mRNA expression of HSL and ATGL (Figure 3B) and led to a decrease in glycerol content in BMECs (Figure 3D). Based on these results, we concluded that *PLIN1* inhibited lipid lipolysis in BMECs.

PLIN1 improves SREBP1 activity in BMECs

To further clarify the regulation mechanism of *PLIN1* in milk fat synthesis in BMECs, we tested the relative mRNA and protein expressions of *SREBP1*. We found that *PLIN1*

did not affect SREBP1 mRNA levels (Figure 4A, B). WB results showed that the *PLIN1* did not affect the total protein of SREBP1, however, had a significant effect on phosphorylated SREBP1. The expression of phosphorylated SREBP1 in the op-*PLIN1* group increased significantly compared with the control group (Figure 4C). The expression of phosphorylation in the si-*PLIN1* group was significantly decreased (Figure 4D). The above experimental results suggested that *PLIN1* increased SREBP1 activity in BMECs.

PLIN1 promotes milk fat synthesis in BMECs by increasing SREBP1 activity

To verify the role of SREBP1 in the regulation of milk fat synthesis by *PLIN1*, we co-transfected op-*PLIN1* and si-SREBP1 (op-*PLIN1*&si-SREBP1) in BMECs. We found that the relative expression of *PLIN1* increased in the op-*PLIN1*&si-SREBP1 group in BMECs, and *SREBP1* decreased (Figure 5A). Staining with oil red O found that in the op-*PLIN1* group, the number of lipid droplets was significantly increased compared with the corresponding control group. The LD number was significantly reduced in the si-*PLIN1* group, and in the op-*PLIN1*&si-SREBP1 group, the number was roughly the same as the number of the control group in the first two groups (Figure 5C). We further verified this trend by using the TAG enzymatic assay technology (Figure 5B). The WB results further showed that in the op-*PLIN1*&si-SREBP1 groups, the expression level of phosphorylated SREBP1 was comparable to that of the control group (Figure 5D). These results verified that *PLIN1* regulated milk fat synthesis by regulating SREBP1 activity, in BMECs.

Discussion

Milk fat content is an important indicator of milk quality and dairy cattle breeding, with TAG as its main component. It is well known that *PLIN1* is highly expressed in white fat, can promote the accumulation of TAG in adipocytes, and plays an important role in lipid biosynthesis and lipid metabolism of adipocytes [3, 19]. However, the effect of

PLIN1 on milk fat synthesis and its regulatory mechanism remains unclear in BMECs. Therefore, we mainly explored the adipogenic effect and potential mechanism of *PLIN1* in BMECs.

PLIN1 is the most abundant protein associated with the LDs of adipocytes and surrounds them [28, 29]**.** *PLIN1* prevents basal lipolysis, increases LD formation and TAG accumulation under basal conditions [30]. *PLIN1* knockout increases basal lipolysis, reduces the size of LD in adipocytes, and inhibits the accumulation of TAG [31]. We found that *PLIN1* promotes the accumulation of TAG in BMECs, consistent with previous results in fat cells of mice and cattle [19, 20, 28, 30].

We also found that *PLIN1* significantly increased the expression level of lipid-related genes, such as PPARγ, C/EBPa, C/EBPβ, FABP4, and FASN. PPARγ causes lipid production and adipocyte differentiation in adipocytes [32, 33], C/EBPα and C/EBPβ are the main adipogenic factors [34-36]. As a lipid chaperone protein, FABP4 marks the production of lipids [37]. Previous studies have shown that *PLIN1* promotes the mRNA expression of ACC, FASN, PPARγ, DGAT2, FABP4, and LPL genes in bovine adipocytes [19, 20], and knockout of *PLIN1* affects the ability of some transcription factors (such as E2F1, PLAG1, C/EBPβ, SMAD3) to regulate lipid metabolism in bovine adipocytes [38-40]. From this, we can conclude that *PLIN1* regulates milk fat synthesis via affecting the expression of lipid synthesis-related genes in BMECs. Additionally, we found that *PLIN1* inhibited the expression of HSL and ATGL. As the two most abundant enzymes in adipocytes, HSL and ATGL, have significant hydrolytic activities on different stages of TAG [19, 20]. Once *PLIN1* is phosphorylated, it actively promotes the action of lipase, predominantly by transporting HSL to the surface of LDs. As a result, *PLIN1* knockouts increase basal lipolysis and reduce LDS size in adipocytes [32], and studies have shown that lack of ATGL leads to lipid accumulation [33, 34]. Therefore, we concluded that *PLIN1* promoted the synthesis of milk fat by promoting and inhibiting the expression of lipid synthesis-related and lipolysis genes, respectively, in BMECs.

Interestingly, we found that *PLIN1* has no affect the expression of SREBP1, however, it can promote its activity. In mice, the fat marker gene *SREBP1* remains unchanged;

however, its activity was significantly reduced following the knockout of *PLIN1* [27, 41, 42]. In mouse mammary glands, SREBP was shown to be a key molecule in the process of milk fat synthesis [43]. SREBP1 promotes milk fat synthesis and increases glucose transport by upregulating GLUT1 [44]. SREBP1 regulates lipid synthesis by regulating the transcription of SCD1, FABP3, FASN, and other coding genes in BMECs [45]. Collectively, we found that *PLIN1* regulated the transcription of lipid genes by promoting SREBP1 activity, thereby affecting the synthesis of milk fat in BMECs.

Conclusions

Our findings showed that *PLIN1* regulates the synthesis of milk fat by affecting the activity of SREBP1, which in turn affects the transcription of lipid-related genes in BMECs. These results provide a novel direction or ways to improve milk fat content in dairy cows through molecular breeding.

Abbreviations

BMECs：bovine mammary epithelial cells; LDs: lipid droplets; PLIN1:Perilipin; TAG:triglyceride; DAG: diglyceride; ATGL: fatty triglyceride lipase; HSL: hormone-sensitive lipase; SREBP1: element-binding transcription protein 1; qRT-PCR: quantitative reverse-transcription PCR.

Acknowledgements

We acknowledge financial support by the Natural Science Foundation of Shaanxi Province (2021JM-100) and the Postdoctoral Scientific Foundation of China (2019M653775).

Conflict of interest

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

Authors' contributions

WM conceived and designed the experiments, WYJ and WXH extract RNA and perform data analysis. JHR, YCQ and ZL performed part of the data analysis and helped explain the data. ZLS and YWC interpreted the data and helped write the manuscript. All authors have read and approved the final version of the manuscript.

Founding

Research supported by the Natural Science Foundation of Shaanxi Province (2021JM-100) and the Postdoctoral Scientific Foundation of China (2019M653775).

Availability of data and materials

All data generated or analyzed during this study available from the corresponding authors on reasonable request.

Ethics statement

All operations in this study are in full compliance with the "Regulations on the Management of Laboratory Animals (Chinese Ministry of Science and Technology, revised in 2004). All animal experiment procedures were approved by the Experimental Animal Management Committee (EAMC) of Northwest A&F University. And, as far as possible to ensure the welfare of animals, reduce the number of uses.

Competing interests

The authors declare that they have no competing interests.

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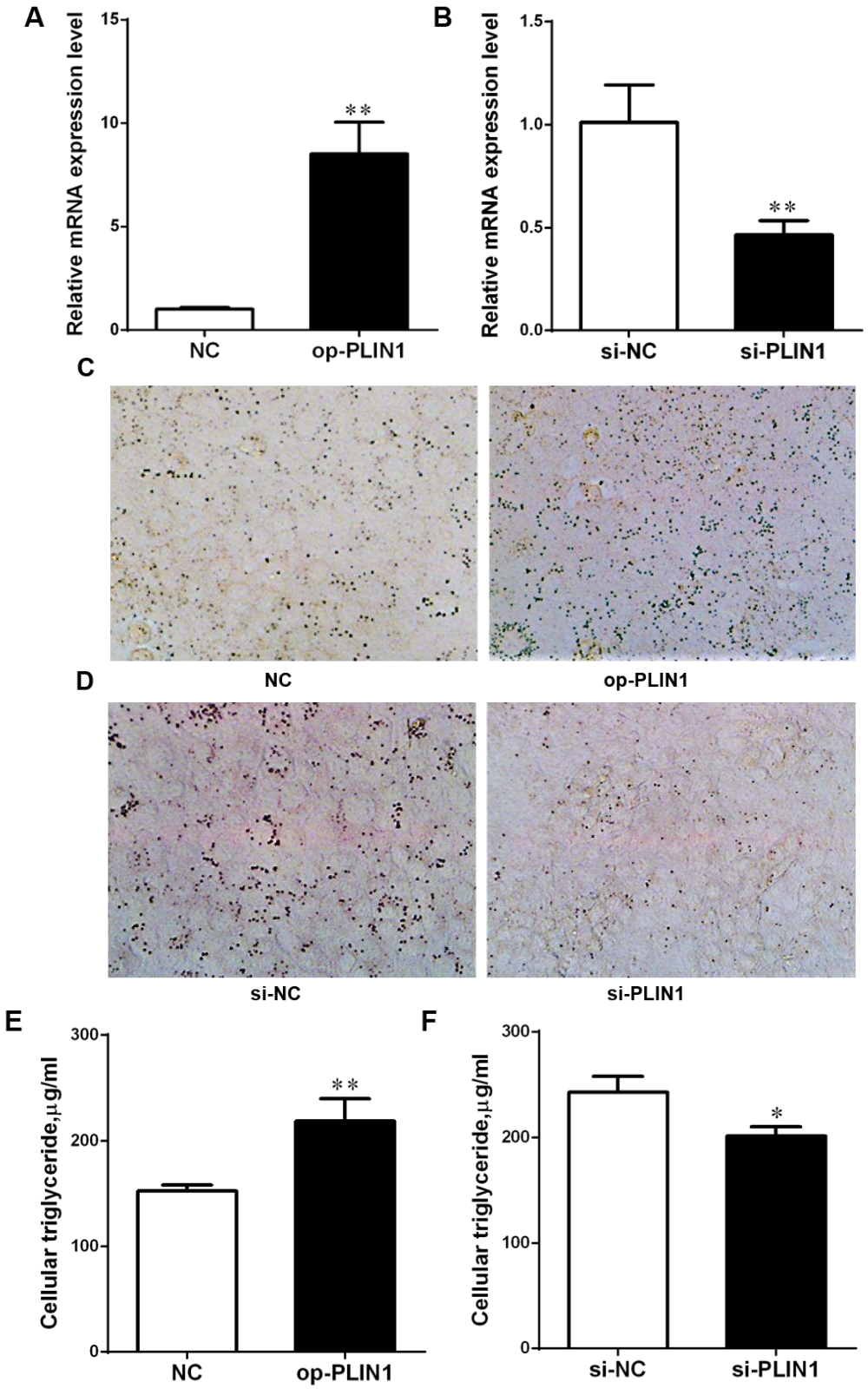


Figure 1. Effect of *PLIN1* on milk fat synthesis in BMECs. (A, B) The mRNA expression levels of *PLIN1* in BMECs after 48 h; (C, D) Representative images of Oil Red O–stained cells; (E, F) TAG levels in BMECs. Values are expressed as mean ± SEM, "*" represents $P < 0.05$; "**" represents $P < 0.01$. NC = negative control, TAG = intracellular triglyceride, BMECs = bovine mammary epithelial cells.

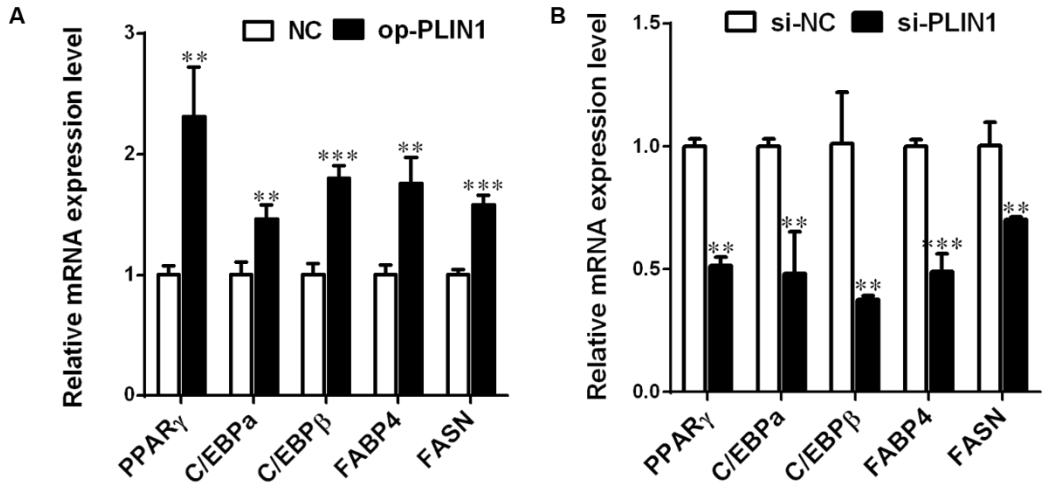


Figure 2. (A, B) Effect of *PLIN1* on lipid synthesis-related genes on mRNA in BMECs. Values are expressed as mean ± SEM, "*" represents $P < 0.05$; "**" represents $P < 0.01$; "***" represents $P < 0.001$. NC = negative control, BMECs = bovine mammary epithelial cells.

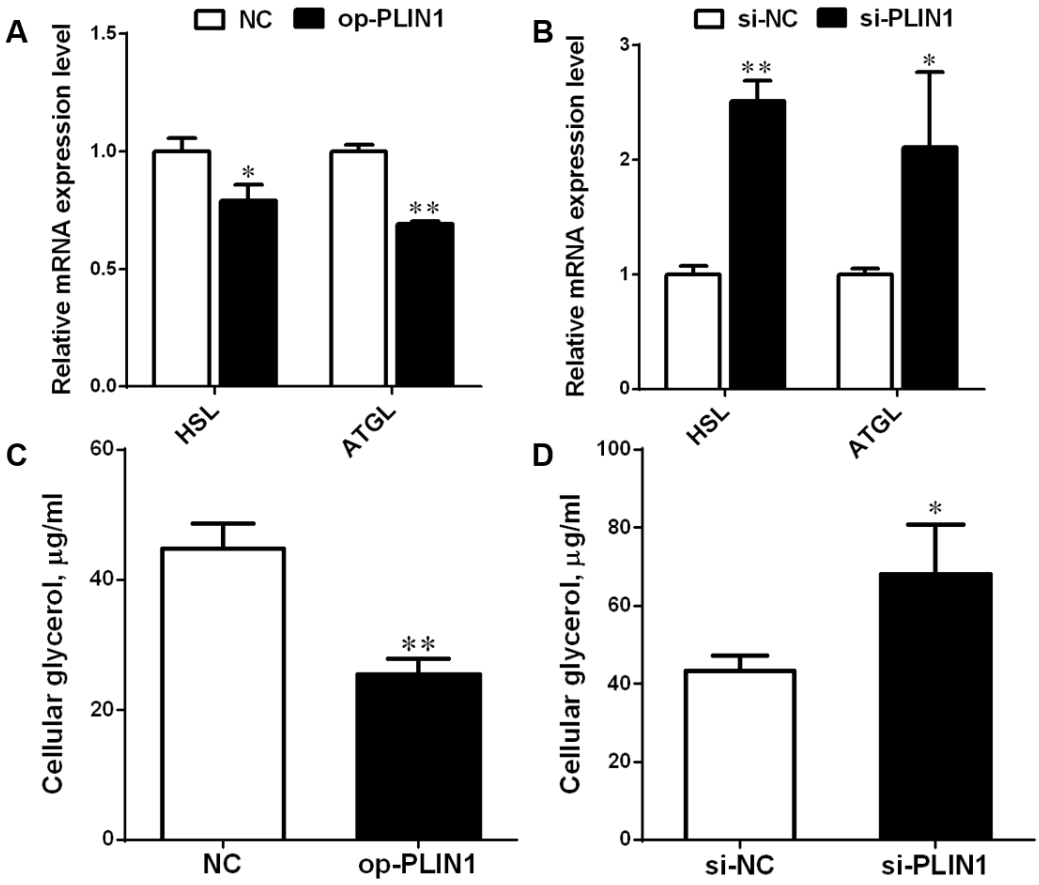


Figure 3. Effect of *PLIN1* on lipid lipolysis in BMECs. (A, B) The mRNA changes in fat metabolism-related genes in BMECs. (C, D) The glycerol levels in BMECs. Values are expressed as mean ± SEM, "*" represents $P < 0.05$; "**" represents $P < 0.01$. NC = negative control, BMECs = bovine mammary epithelial cells.

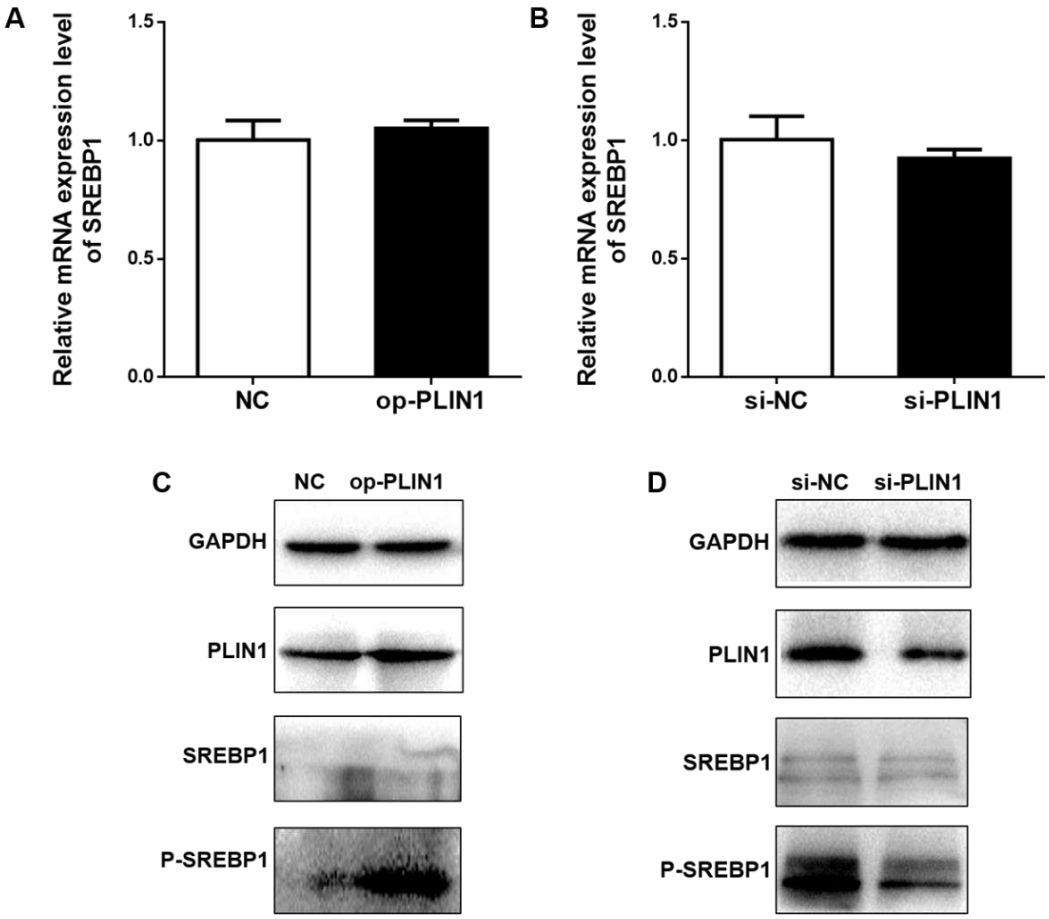
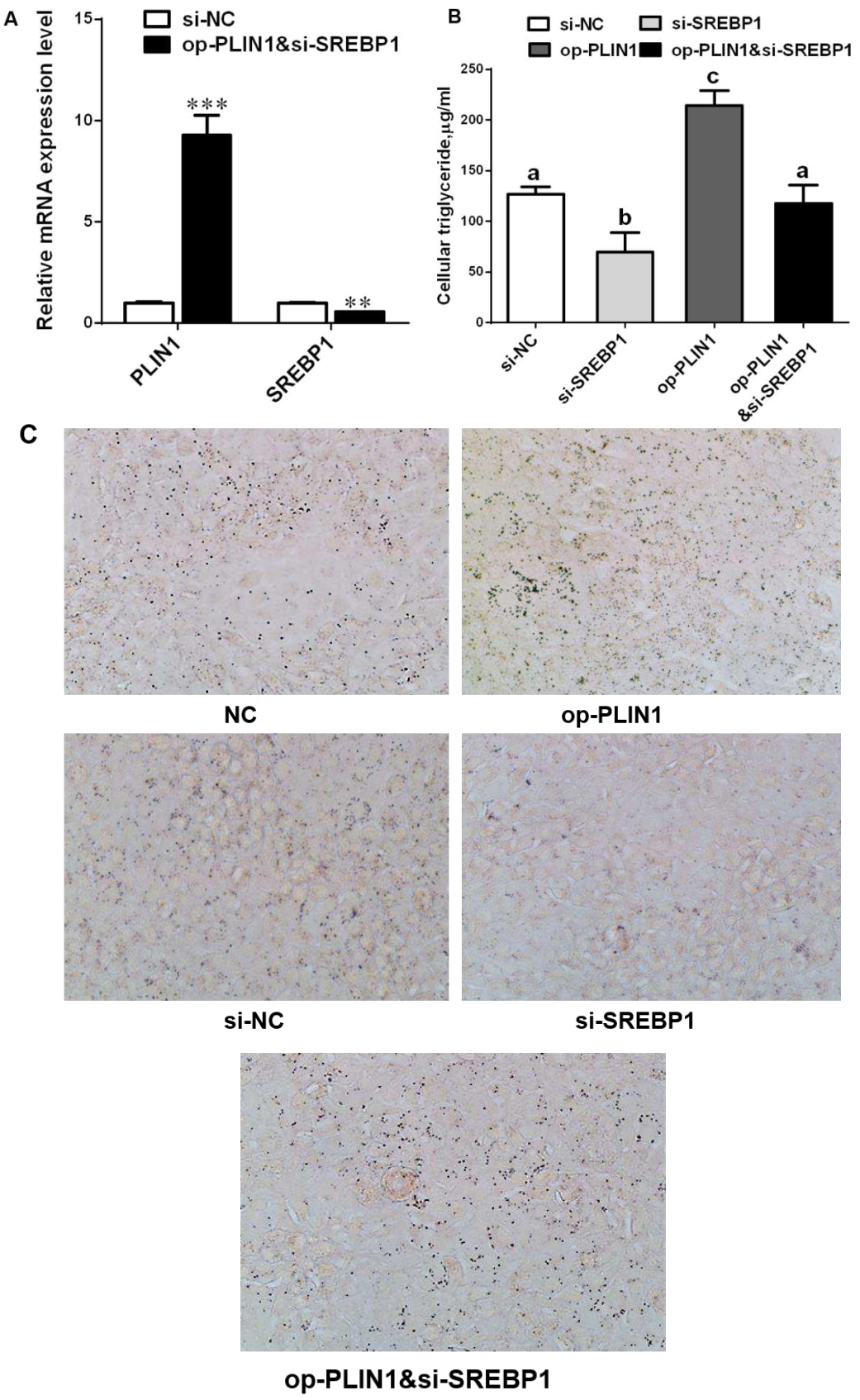


Figure 4. (A, B) Effect of *PLIN1* on SREBP1 mRNA in BMECs; (C, D) The effect of *PLIN1* on SREBP1 protein expression level in BMECs. Values are expressed as mean ± SEM, "*" represents $P < 0.05$; "**" represents $P < 0.01$. NC = negative control, BMECs = bovine mammary epithelial cells.


A
Relative mRNA expression level
15
10
5
0
si-NC
op-PLIN1&si-SREBP1
***
**
PLIN1
SREBP1
B
si-NC
si-SREBP1
op-PLIN1
op-PLIN1&si-SREBP1
Cellular triglyceride,μg/ml
250
200
150
100
50
0
a
b
c
a
si-NC
si-SREBP1
op-PLIN1
op-PLIN1 &si-SREBP1
C
NC
op-PLIN1
si-NC
si-SREBP1
op-PLIN1&si-SREBP1

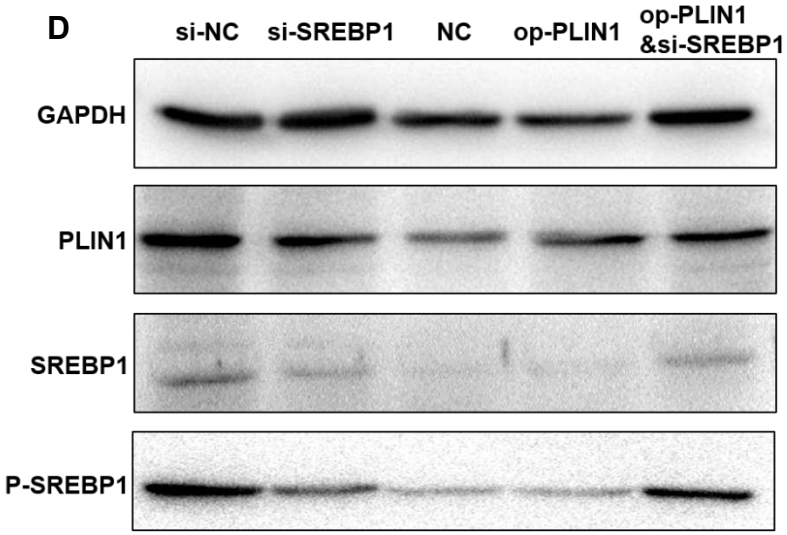


Figure 5. Effect of *PLIN1* on SREBP1 activity. (A) The relative mRNA expression of *PLIN1* and *SREBP1* in BMECs. (B) TAG measurement results; (C) Image of oil red O stained cells; (D) changes in total and phosphorylated protein levels. Values are expressed as mean ± SEM, "*" represents significant differences, $P < 0.05$; "**" represents significant differences, $P < 0.01$; (a-c) The same letter indicates no significant difference ($P > 0.05$), whereas different letters indicate a significant difference ($P < 0.05$). NC = negative control, BMECs = bovine mammary epithelial cells.

Figures

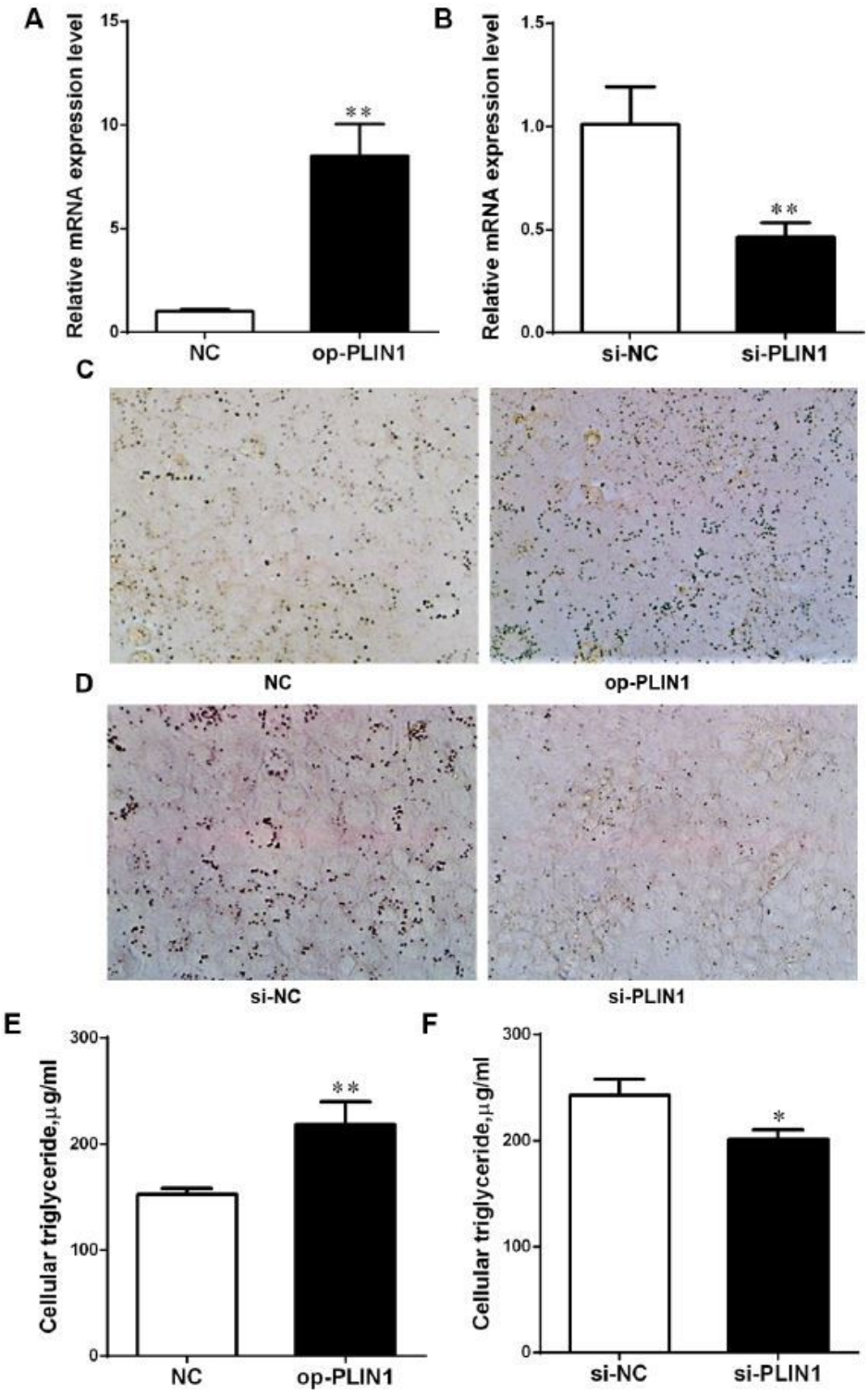


Figure 1

Effect of PLIN1 on milk fat synthesis in BMECs. (A, B) The mRNA expression levels of PLIN1 in BMECs after 48 h; (C, D) Representative images of Oil Red O–stained cells; (E, F) TAG levels in BMECs. Values are

expressed as mean ± SEM, “*” represents P < 0.05; “**” represents P < 0.01. NC = negative control, TAG = intracellular triglyceride, BMECs = bovine mammary epithelial cells.

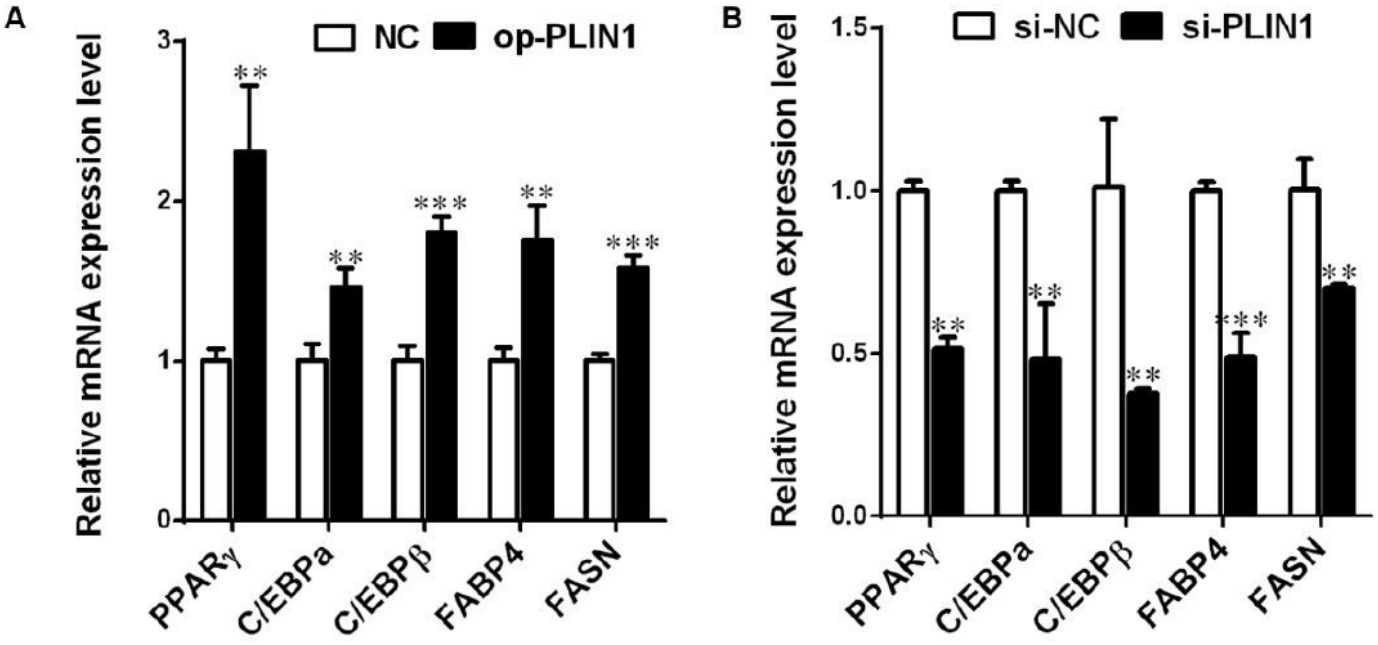


Figure 2

(A, B) Effect of PLIN1 on lipid synthesis-related genes on mRNA in BMECs. Values are expressed as mean ± SEM, “*” represents P < 0.05; “**” represents P < 0.01; “***” represents P < 0.001. NC = negative control, BMECs = bovine mammary epithelial cells.

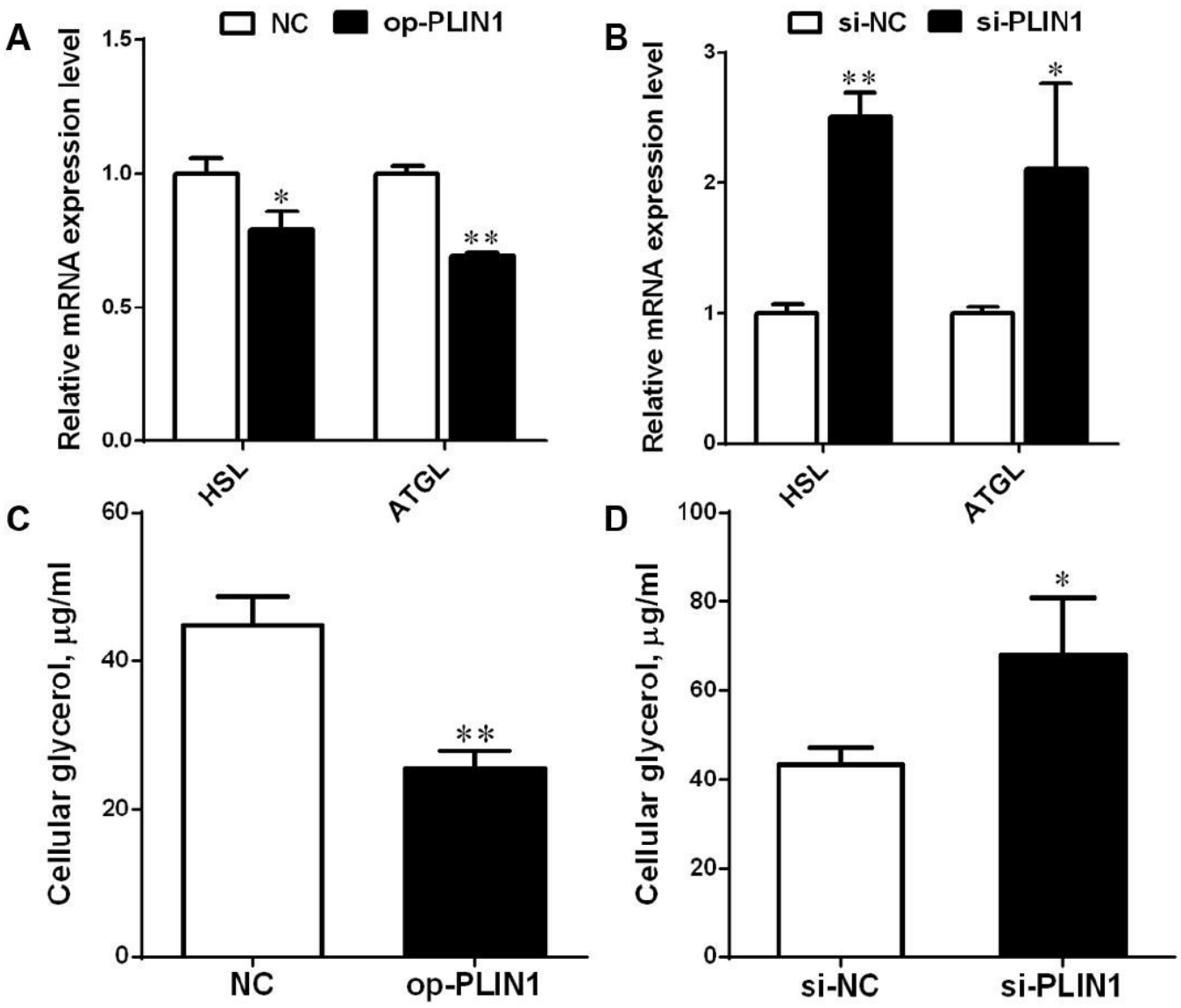


Figure 3

Effect of PLIN1 on lipid lipolysis in BMECs. (A, B) The mRNA changes in fat metabolism-related genes in BMECs. (C, D) The glycerol levels in BMECs. Values are expressed as mean ± SEM, "*" represents $P < 0.05$; "**" represents $P < 0.01$. NC = negative control, BMECs = bovine mammary epithelial cells.

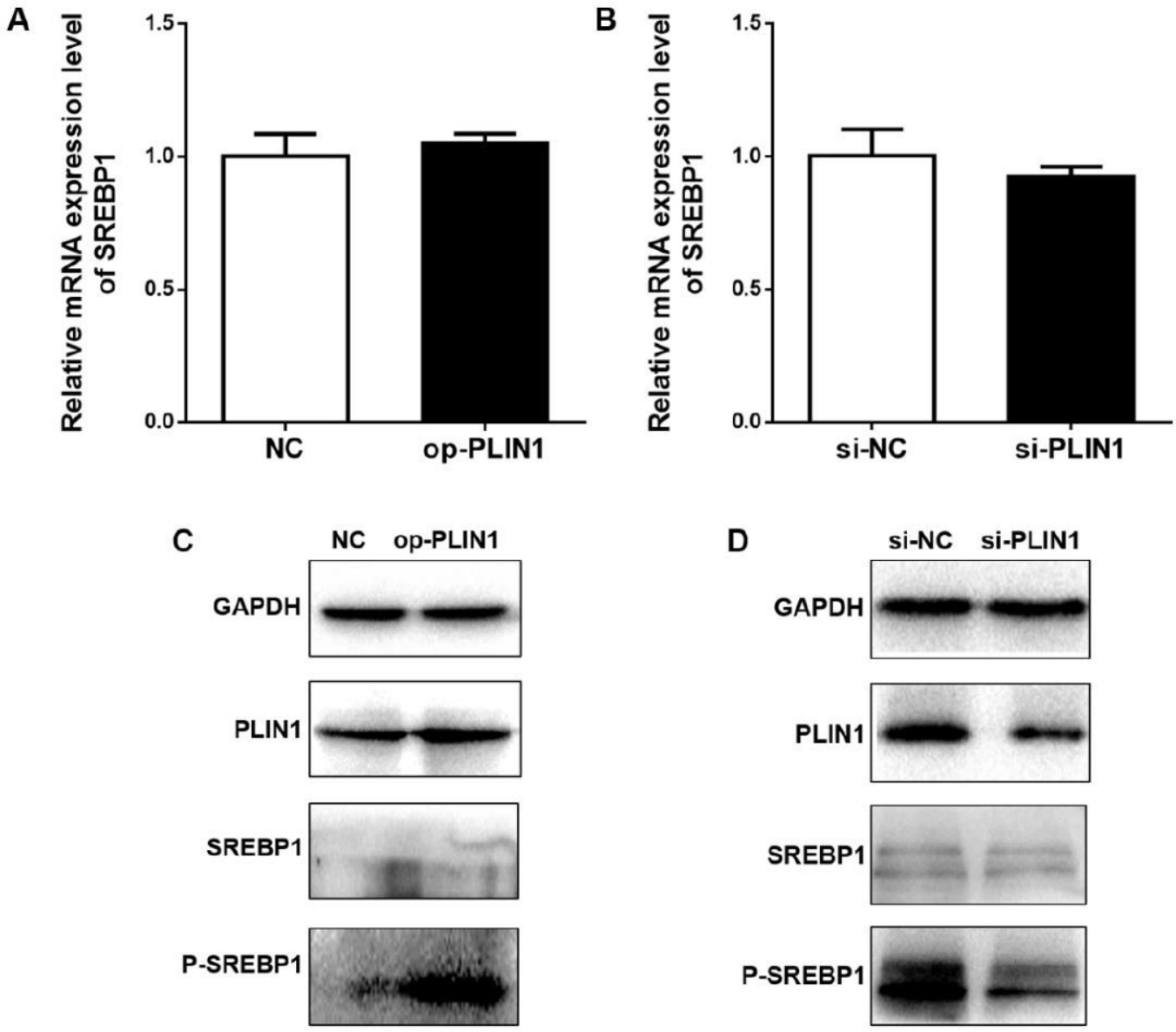


Figure 4

(A, B) Effect of PLIN1 on SREBP1 mRNA in BMECs; (C, D) The effect of PLIN1 on SREBP1 protein expression level in BMECs. Values are expressed as mean ± SEM, "*" represents $P < 0.05$; "**" represents $P < 0.01$. NC = negative control, BMECs = bovine mammary epithelial cells.

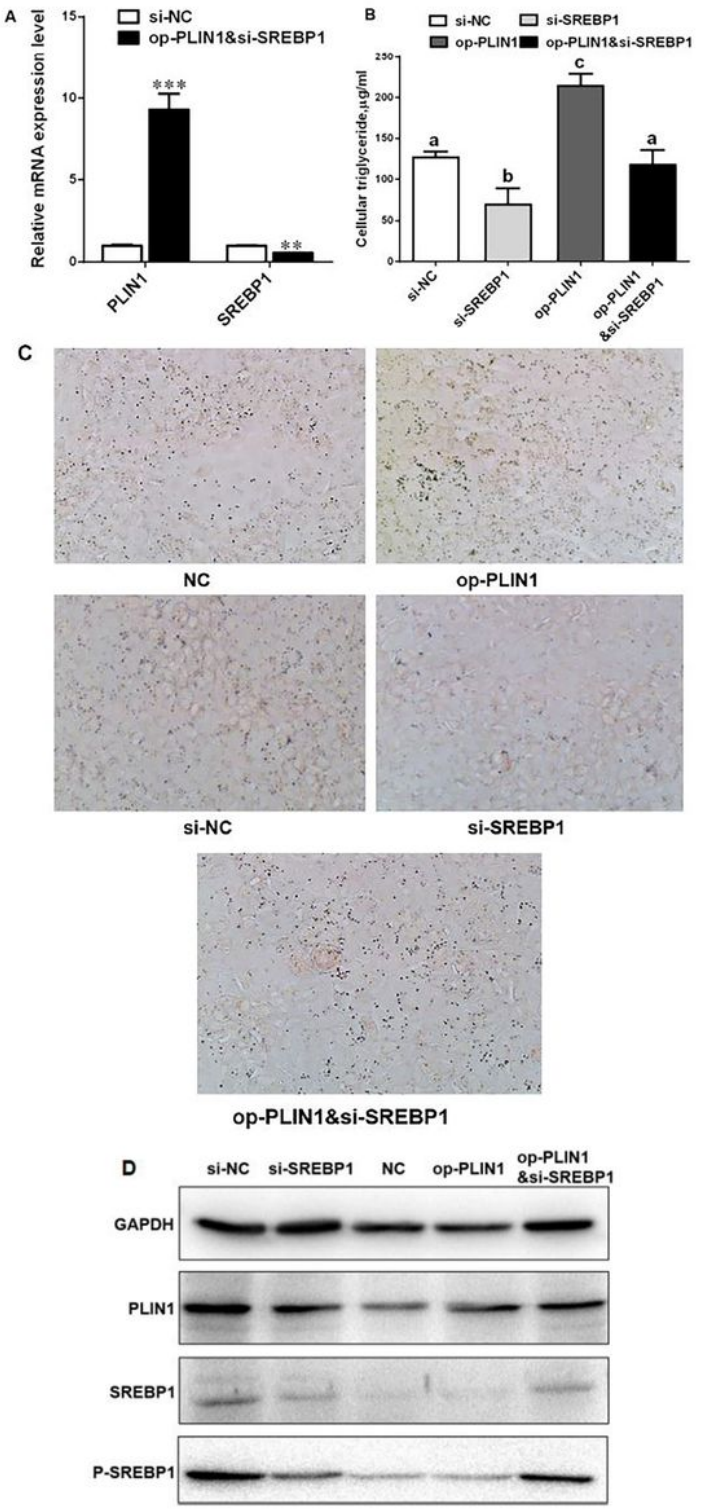


Figure 5

Effect of PLIN1 on SREBP1 activity. (A) The relative mRNA expression of PLIN1 and SREBP1 in BMECs. (B) TAG measurement results; (C) Image of oil red O stained cells; (D) changes in total and phosphorylated protein levels. Values are expressed as mean ± SEM, “*” represents significant differences, $P < 0.05$; “**” represents significant differences, $P < 0.01$; (a-c) The same letter indicates no

significant difference ($P > 0.05$), whereas different letters indicate a significant difference ($P < 0.05$). NC = negative control, BMECs = bovine mammary epithelial cells.