



Original Research Article

Sodium acetate promotes fat synthesis by suppressing TATA element modulatory factor 1 in bovine mammary epithelial cells

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ABSTRACT

Short-chain fatty acids are important nutrients that regulate milk fat synthesis. They regulate milk synthesis via the sterol regulatory element binding protein 1 (SREBP1) pathway; however, the details are still unknown. Here, the regulation and mechanism of sodium acetate (SA) in milk fat synthesis in bovine mammary epithelial cells (BMECs) were assessed. BMECs were treated with SA supplementation (SA+) or without SA supplementation (SA-), and milk fat synthesis and activation of the SREBP1 pathway were increased ($P = 0.0045$; $P = 0.0042$) by SA+ and decreased ($P = 0.0068$; $P = 0.0031$) by SA-, respectively. Overexpression or inhibition of SREBP1 demonstrated that SA promoted milk fat synthesis ($P = 0.0045$) via the SREBP1 pathway. Overexpression or inhibition of TATA element modulatory factor 1 (TMF1) demonstrated that TMF1 suppressed activation of the SREBP1 pathway ($P = 0.0001$) and milk fat synthesis ($P = 0.0022$) activated by SA+. Overexpression or inhibition of TMF1 and SREBP1 showed that TMF1 suppressed milk fat synthesis ($P = 0.0073$) through the SREBP1 pathway. Coimmunoprecipitation analysis revealed that TMF1 interacted with SREBP1 in the cytoplasm and suppressed the nuclear localization of SREBP1 ($P = 0.0066$). The absence or presence of SA demonstrated that SA inhibited the expression of TMF1 ($P = 0.0002$) and the interaction between TMF1 and SREBP1 ($P = 0.0001$). Collectively, our research suggested that TMF1 was a new negative regulator of milk fat synthesis. In BMECs, SA promoted the SREBP1 pathway and milk fat synthesis by suppressing TMF1. This study enhances the current understanding of the regulation of milk fat synthesis and provides new scientific data for the regulation of milk fat synthesis.

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1. Introduction

Milk fat is one of the important nutrient components of milk (Osorio et al., 2016). In bovine mammary glands, the synthesis of milk fat is controlled by genetics, hormones, nutrition and the

environment (Bionaz et al., 2008; Li et al., 2018; Luo et al., 2018c; Li et al., 2019). Short-chain fatty acids, such as sodium acetate (SA), are important nutrients that regulate the synthesis of milk fat (Sun et al., 2013; Yan et al., 2019). They not only provide basic material for milk fat synthesis as an important precursor but also regulate milk synthesis by activating pathways (Bedford et al., 2018; Vargas-Bello-Pérez et al., 2019).

The sterol regulatory element binding protein 1 (SREBP1) pathway is one of the most important pathways that regulates milk fat synthesis in bovine mammary epithelial cells (BMEC) (Che et al., 2019; Li et al., 2018). It is also one of the main pathways through which short-chain fatty acids regulate the synthesis of milk fat (Urrutia et al., 2017a, 2017b). In BMECs, SREBP1 responds to short-chain fatty acids, enters the nucleus, and then promotes the expression of its target genes, such as acetyl-CoA carboxylase (ACC),

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fatty acid synthesis (FAS), stearoyl-CoA desaturase (SCD) and fatty acid binding protein 3 (FABP3), subsequently leading to milk fat synthesis (Laaksonen et al., 2006; Liang et al., 2014). However, the upstream pathway of SREBP1, i.e., the way in which SREBP1 is activated by short-chain fatty acids, is poorly understood and requires further clarification.

TATA element modulatory factor 1 (TMF1), also named ARA160, is a Golgi-associated protein (Lerer-Goldshstein et al., 2010). Studies have shown that the structure of TMF1 contains a coiled-coil forming domain, and this domain mediates the interaction of TMF1 with other cellular factors. TMF1 is involved in various cellular physiological functions (Bel et al., 2012; Hsiao et al., 1999; Miller et al., 2013). Our previous study suggests that TMF1 can interact with SREBP1 and regulate SA-dependent milk fat synthesis by participating in the regulation of the upstream pathway of SREBP1 in BMECs (Li et al., 2014). However, the pathway and mechanism of this regulation are unknown.

In this study, the pathway of SA regulates fat synthesis through SREBP, and in particular, SA activates SREBP1, and its mechanisms in BMECs were explored. We found that TMF1 is an important inhibitor of SA mediated activation of SREBP1 and fat synthesis. Sodium acetate activated the SREBP1 pathway and subsequently stimulated fat synthesis by suppressing TMF1. This study enriches our understanding of the regulatory pathway and mechanism of fat synthesis, provides scientific data for basic research on the regulation of fat synthesis and is important for the development of the dairy industry.

2. Materials and methods

2.1. Animal ethics statement

All the experimental procedures applied in this study were conducted according to the principles of Jiamusi University (JMSU-243, Jiamusi, China) and Southern Medical University (L2019010, Guangzhou, China) Animal Care and Use Committee, which approved the study protocols.

2.2. Cell culture and treatment

BMECs were obtained as previously reported (Yu et al., 2019) and cultured with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (11,330,032, Thermo Fisher Scientific Co., Ltd, California, USA) containing 10% fetal bovine serum (FBS; 26140079, Thermo Fisher Scientific Co., Ltd) and penicillin-streptomycin solution (100 U/mL each of penicillin and streptomycin) (C0222, Beyotime Biotechnology Co., Ltd, Shanghai, China).

For SA treatment experiments, BMECs were plated in 6 well plates and cultured with DMEM/F12 containing 10% FBS. After 12 h, the cells were starved of FBS for 12 h, cultured with FBS free DMEM/F12 and treated with SA. For the SA concentration optimization experiment, BMECs were treated with 0, 3, 6, 9, 12, 15 and 18 mmol/L SA for 24 h, and the 0 mmol/L SA group was used as a control. For the treatment time optimization experiment, BMECs were treated with 12 mmol/L SA for 0, 6, 12, 18, 24, 30, 36, 42 and 48 h, and the

0 h group was used as a control. The medium of each group was collected to test triglyceride (TG) secretion. The cells were collected for Western blotting (WB) or co-immunoprecipitation (co-IP). For the gene function evaluation experiments, BMECs were first treated with gene overexpression or silencing and then treated with SA (SA+) or no SA (SA-).

2.3. Western blotting (WB)

WB was completed following a normal procedure (Luo et al., 2020). The total protein concentration of each group sample was measured by a BCA Protein Assay Kit (P0012S, Beyotime Biotechnology Co., Ltd). Approximately 30 µg of protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene fluoride (PVDF) membrane (FFP24, Beyotime Biotechnology Co., Ltd). The PVDF membrane was blocked with TBSTw (ST671, Beyotime Biotechnology Co., Ltd) containing 5% bovine serum albumin (ST025, Beyotime Biotechnology Co., Ltd) and then incubated with primary antibody (diluted with blocking solution). The membrane was washed three times with TBSTw and then incubated with HRP-conjugated secondary antibody (diluted with blocking solution). Then, the membrane was washed 3 times with TBSTw and visualized with Super ECL Plus (P0018M, Beyotime Biotechnology Co., Ltd). The primary antibodies used in this experiment were as follows: anti-β-actin (1:1,000, # 4970, Cell Signaling Technology, Massachusetts, USA), anti-SREBP1 (1:1,000, ab191857, Abcam, Cambridgeshire, UK), anti-ARA160 (TMF1) (1:500; sc-398411, Santa Cruz Biotechnology, California, USA), anti-ACC (1:1,000, # 3662S, Cell Signaling Technology, USA), anti-FAS (1:1,000, ab22759, Abcam, UK), anti-SCD (1:1,000, ab23331, Abcam, UK), anti-FABP3 (1:1,000, ab45966, Abcam, UK), anti-Lamin B1 (1:1,000, ab16048, Abcam, UK), anti-β-Tubulin (1:500; sc-5274, Santa Cruz Biotechnology, USA), and anti-Myc (1:1,000, AM933, Beyotime Biotechnology Co., Ltd), anti-Flag (1:1,000, AF519, Beyotime Biotechnology Co., Ltd).

2.4. Triglyceride secretion

The secretion of TG was evaluated by measuring the concentration of TG in the collected medium. The TG content in the medium was tested using a triglyceride test kit (BC0625, Solarbio Science & Technology Co., Ltd, Beijing, China) according to the manufacturer's instructions.

2.5. Immunofluorescence (IF)

The IF experiment was completed as previously reported (Luo et al., 2019). BMECs were plated on cover slips in 6 well plates and cultured with DMEM/F12. After 12 h, the cells were treated with SA+ and/or subjected to gene overexpression or silencing. Cell crawling was evaluated as previously reported (Luo et al., 2018b). The primary antibodies used in this study were TMF1 and SREBP1 antibodies. The secondary antibodies were goat - anti - mouse Alexa Fluor 488-conjugated IgG (1:500, bs-0296G-AF488, bioss,

Table 1

Primer sequences used for plasmid construction.

Gene	Primer sequence (5'–3') ¹	
	Forward primer	Reverse primer
TMF1	TCC <u>CCCGGG</u> ATGAGCTGGTTCACGCCTCCAG (the <i>Sma</i> I site is underlined)	GCTCTAGAACTGAGCCTTTGTCTTAGAAGTTC (the <i>Xba</i> I site is underlined)
SREBP1	TCC <u>CCCGGG</u> ATGGACGAGCCACCTTCAACG (the <i>Sma</i> I site is underlined)	GCTCTAGAGCTGGAGGTACAGTGGTCCAC (the <i>Xba</i> I site is underlined)

TMF1 = TATA element modulatory factor 1; SREBP1 = sterol regulatory element-binding protein 1.

¹ The primers were designed using Primer Expression software Primer Premier 5.

Table 2
siRNA sequences.

Gene	siRNA sequence (5'–3')	
TMF1	Sense	GCCAGAAGUCUAUUGACATT
	Antisense	UGUCAAUAGACUUCUGGGCTT
SREBP1	Sense	CCUAUUUGACCCACCCUAUTT
	Antisense	AUAGGGUGGGUCAAAUAGGTT
Negative control	Sense	UUCUCCGAACGUGUCACGUTT
	Antisense	ACGUGACACGUUCGGAGAATT

TMF1 = TATA element modulatory factor 1; SREBP1 = sterol regulatory element-binding protein 1.

Beijing, China) and goat-anti-rabbit Alexa Fluor 647-conjugated IgG (1:500, bs-0295G-AF647, bioss, China). The nucleus was stained using 4',6-diamidino-2-phenylindole (DAPI; C1002, Beyotime Biotechnology Co., Ltd) or propidium iodide (PI; P3566, Invitrogen,

California, USA). Fluorescence was observed with laser scanning confocal microscopy (LEICA, Germany).

2.6. Plasmid construction and cell transfection

The TMF1-Myc or SREBP1-Flag overexpression plasmids were constructed as previously reported (Luo et al., 2013). The specific primers used in this research are shown in Table 1. The eukaryotic expression plasmids used in this study were pCMV-C-Flag (D2632, Beyotime Biotechnology Co., Ltd) and pCMV-C-Myc (D2672, Beyotime Biotechnology Co., Ltd).

For the gene overexpression experiment, BMECs were plated into 6 well plates and cultured with DMEM/F12. After 12 h, the medium was changed to OPTI-MEM medium (11058021, Thermo Fisher Scientific Co., Ltd, USA), and the overexpression plasmids (SREBP1-Flag or TMF1-Myc) were transfected with Lipo6000

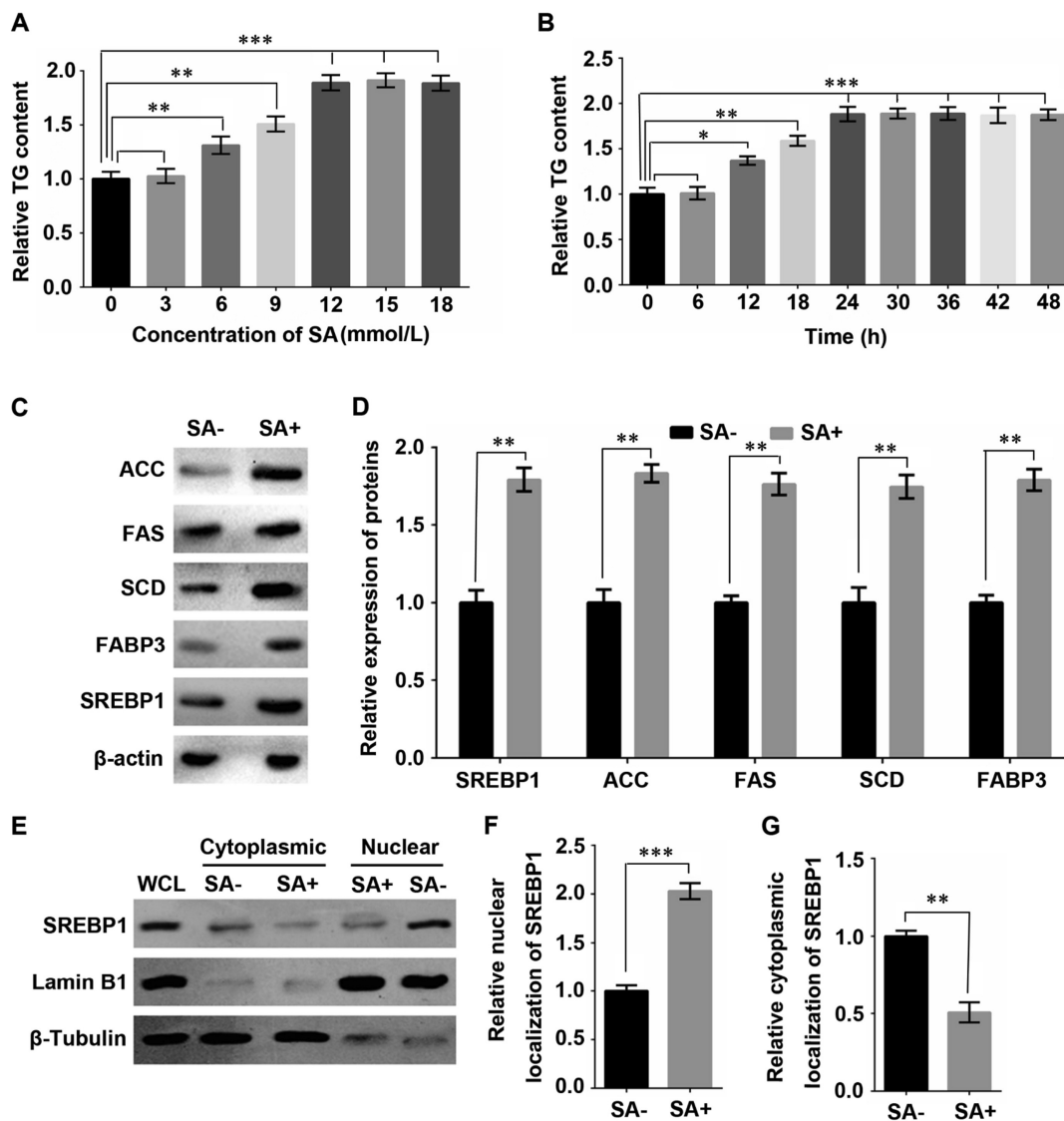


Fig. 1. Sodium acetate (SA) promotes fat synthesis and the SREBP1 pathway in bovine mammary epithelial cells (BMECs). (A and B) Secretion of triglyceride (TG). (C) Expression of SREBP1, ACC, FAS, SCD and FABP3 was tested by western blotting. (D) Quantitation of (C). (E) Nuclear localization and cytoplasmic localization of SREBP1 was tested by western blotting. (F) Quantitation of nuclear localization of SREBP1. (G) Quantitation of cytoplasmic localization of SREBP1. BMECs were cultured with DMEM/F12 medium +0, 3, 6, 9, 12 and 18 mmol/L SA for 24 h (A), or + 12 mmol/L SA for 0, 6, 12, 18, 24, 30, 36, 42 and 48 h (B), or with DMEM/F12 medium (SA-), or DMEM/F12 medium + 12 mmol/L SA (SA+) for 24 h (C–F). Data are expressed as the mean values and standard deviations ($n = 3$); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ACC = acetyl CoA carboxylase; FAS = fatty acid synthesis; SCD = stearoyl-CoA desaturase; FABP3 = fatty acid binding protein 3; SREBP1 = sterol regulatory element-binding protein 1; SA+ = SA supplementation; SA- = no SA supplementation.

Transfection Reagent (C0526, Beyotime Biotechnology Co., Ltd) according to the manufacturer's instructions. The Lipo6000 Transfection Reagent (5 μ L per well) and the overexpression plasmids (2.5 μ g per well) were diluted into 125 μ L OPTI-MEM medium, and then incubated for 5 min at room temperature. The diluted overexpression plasmids and Lipo 6000 Transfection Reagent were then mixed and incubated at room temperature for 15 min. The mixture was added to 6 well plates containing cells. After 6 h, the medium was changed to DMEM/F12 containing FBS and penicillin - streptomycin and the cells were cultured for 12 h. Then, the cells were starved with FBS for 12 h and treated with SA for 24 h. The cells that were not transfected were blank controls (B), and the cells transfected with empty plasmids pCMV-C-Flag or pCMV-C-Myc were empty plasmid controls (EV).

2.7. Small interfering RNA (siRNA) transfection

The specific siRNAs targeting the *TMF1* gene, *SREBP1* gene and negative control used in this study were synthesized by GenePharma Co., Ltd. (GenePharma, Shanghai, China). The siRNA sequences used in this research are shown in Table 2. The siRNAs (*TMF1* gene, *SREBP1* gene or negative control) were transfected using Lipo 6000 Transfection Reagent. The transfection process was the same as that performed for gene overexpression. The amounts of siRNA and Lipo6000 Transfection Reagent applied were 100 pmol and 5 μ L per well, respectively. The untransfected cells were blank controls (B), and the cells transfected with negative control siRNA were negative controls. The experimental process was the same as that performed for overexpression plasmid transfection.

2.8. Nuclear and cytoplasmic protein extraction

Treated BMECs were harvested, and nuclear and cytoplasmic proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (P0027, Beyotime Biotechnology Co., Ltd) according to the manufacturer's instructions. Lamin B1 and β -tubulin were used as nuclear and cytoplasmic markers, respectively.

2.9. Co-immunoprecipitation

BMECs were either not transfected or transfected with *TMF1*-Myc and/or *SREBP1*-Flag. Then, cell lysates from each group were collected with cell lysis buffer for Western blotting and IP (P0013, Beyotime Biotechnology Co., Ltd) containing 1 mmol/L phenylmethanesulfonyl fluoride (PMSF) (ST506, Beyotime Biotechnology Co., Ltd). The co-IP experiment was completed using Protein A + G Agarose (P2012, Beyotime Biotechnology Co., Ltd) according to the manufacturer's instructions (Luo et al., 2018a). The primary antibodies used for co-IP in this research were TMF1 and Myc antibodies, and the antibodies used to test the immunoprecipitate were TMF1, Myc, Flag and SREBP1 antibodies.

2.10. Statistical analysis

The data were analyzed using GraphPad Prism 6 software and reported as the mean \pm standard deviation ($n = 3$). Statistical significance between groups was analyzed by one-way ANOVA. $P \geq 0.05$ was considered not a significant difference, $0.01 \leq P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered

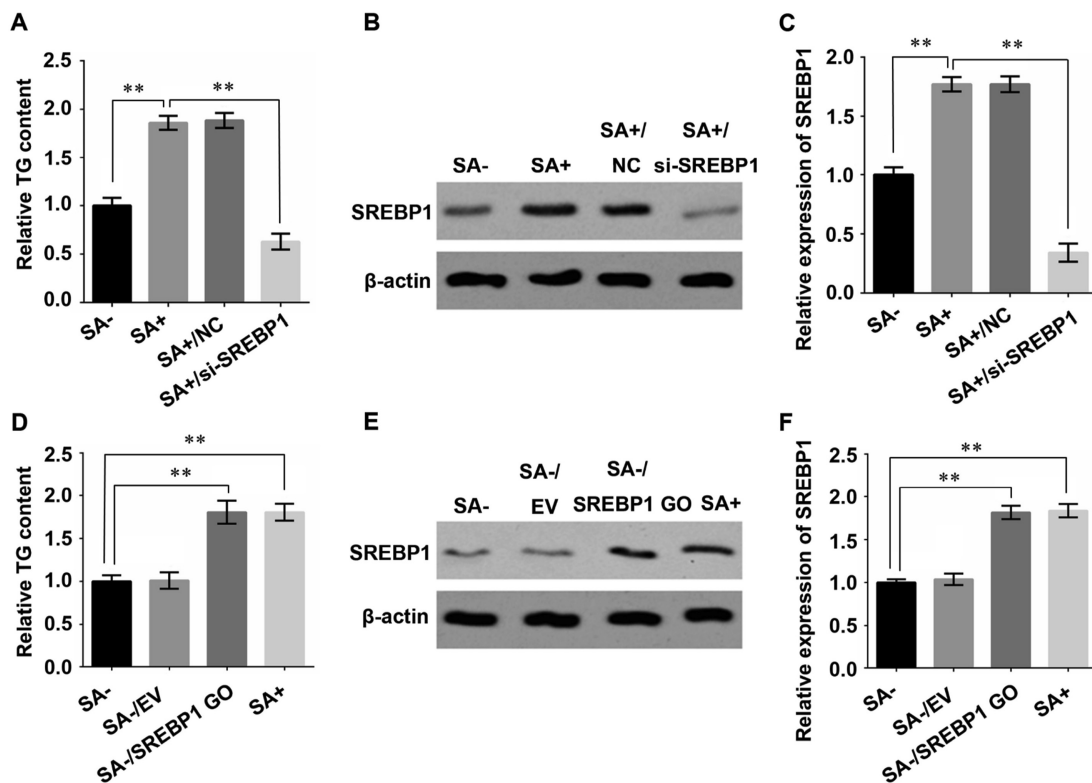


Fig. 2. Sodium acetate (SA) promotes fat synthesis through the SREBP1 pathway. (A) Secretion of triglyceride (TG). (B) Expression of SREBP1 was tested by Western blotting. (C) Quantitation of (B). Bovine mammary epithelial cells were cultured with DMEM/F12 medium (SA-), DMEM/F12 medium + 12 mmol/L SA (SA+), SA+ and transfected with negative control siRNA (SA+/NC) or SA+ and transfected with *SREBP1* siRNA (SA+/si-SREBP1). (D) Secretion of TG. (E) Expression of SREBP1 was tested by Western blotting. (F) Quantitation of (E). Bovine mammary epithelial cells were cultured with DMEM/F12 medium (SA-), SA- and transfected with empty vector (SA-/EV), SA- and transfected with *SREBP1* expression vector (SA-/SREBP1 GO) or DMEM/F12 medium + 12 mmol/L SA (SA+). Data are expressed as the mean values and standard deviations ($n = 3$); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. SREBP1 = sterol regulatory element-binding protein 1; SA+ = SA supplementation; SA- = no SA supplementation.

highly significantly different. Grayscale scans of the WB bands and the colocalization of IF were analyzed with ImageJ 2X software. All of the data in this study were averaged from 3 independent experiments.

3. Results

3.1. Sodium acetate promotes fat synthesis and the SREBP1 pathway

To explore the effect of SA on fat synthesis in BMECs, cells were treated with different concentrations (0, 3, 6, 9, 12, 15 and 18 mmol/L) of SA for 24 h. The secretion of TG was tested. The results showed that the secretion of TG ($P = 0.3680$; $P = 0.0066$; $P = 0.0023$; $P = 0.0002$; $P = 0.0001$; $P = 0.0001$) was increased by SA (≥ 6 mmol/L) stimulation, and this increase was dose-dependent when the concentration of SA was less than 12 mmol/L (Fig. 1A). BMECs were treated with 12 mol/L SA for 0, 6, 12, 18, 24, 30, 36, 42 and 48 h. The results showed that the secretion of TG ($P = 0.4260$; $P = 0.0159$; $P = 0.0075$; $P = 0.0002$; $P = 0.0007$; $P = 0.0002$; $P = 0.0001$;

$P = 0.0008$) was increased by SA stimulation (≥ 12 h), and this increase was time-dependent in less than 24 h (Fig. 1B).

The SREBP1 pathway is one of the crucial pathways regulating fat synthesis. To investigate the effect of SA on the SREBP1 pathway, BMECs were treated with 12 mmol/L SA for 24 h. The expression levels of the SREBP1 pathway-associated proteins SREBP1, ACC, FAS, SCD and FABP3 and the nuclear localization of SREBP1 were tested. The results showed that the expression levels of SREBP1 ($P = 0.0021$), ACC ($P = 0.0045$), FAS ($P = 0.0037$), SCD ($P = 0.0058$) and FABP3 ($P = 0.0012$) (Fig. 1C and D) and the nuclear localization of SREBP1 ($P = 0.0027$) (Fig. 1E and F) were increased by SA stimulation and that the cytoplasmic localization of SREBP1 ($P = 0.0061$) (Fig. 1E and G) was decreased by SA stimulation.

To avoid the influence of FBS on the experimental results, BMECs were starved with FBS for different lengths of time. The secretion of TG and cell growth were tested. The result showed that the effect of FBS on the secretion of TG (Figs. S1 and S2) and cell growth (Fig. S3) did not affect our experimental results.

These results suggested that SA promoted fat synthesis and activated the SREBP1 pathway in BMECs.

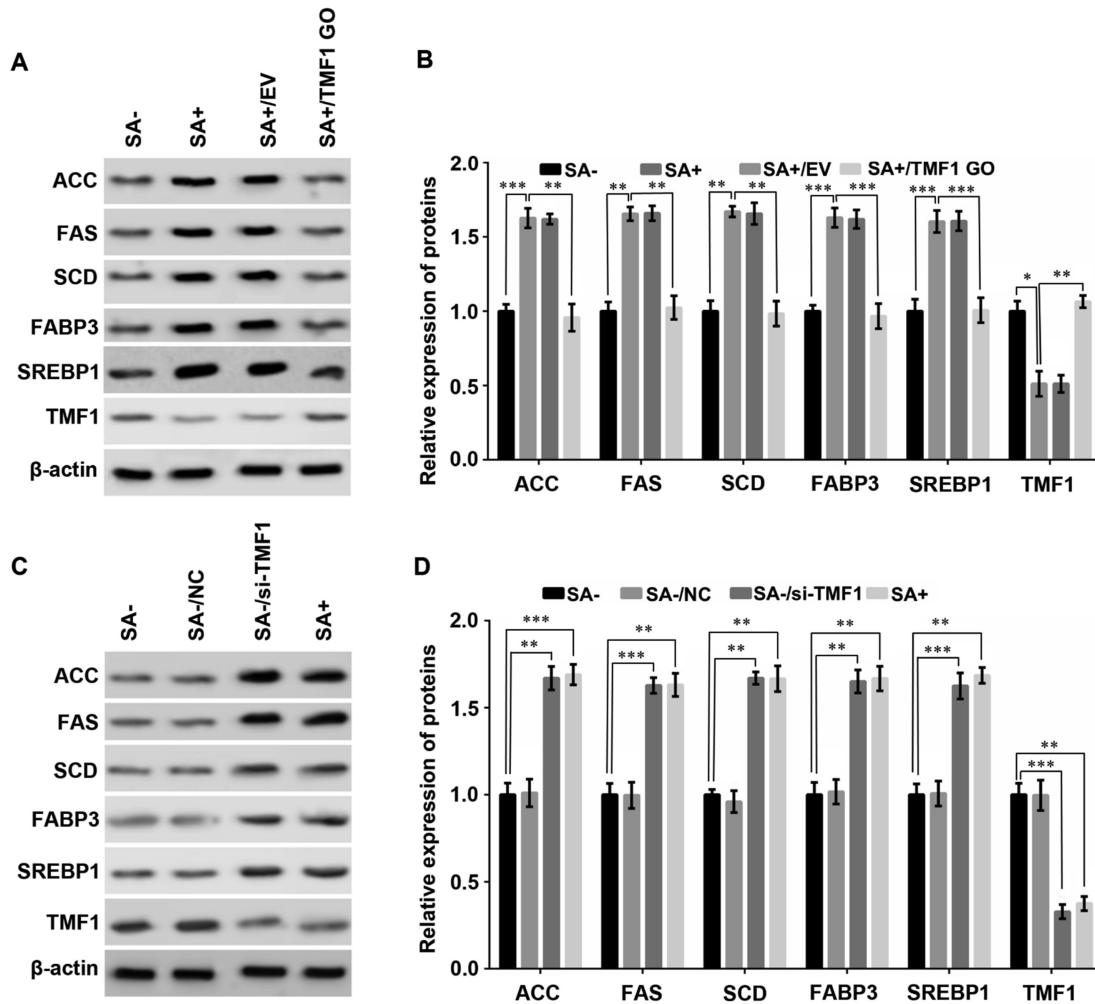


Fig. 3. TATA element modulatory factor 1 (TMF1) is an important inhibitor of sodium acetate (SA) mediated activation of the sterol regulatory element binding protein 1 (SREBP1) pathway. (A) Expression of SREBP1, ACC, FAS, SCD and FABP3 was tested by Western blotting. (B) Quantitation of (A). Bovine mammary epithelial cells were cultured with DMEM/F12 medium (SA-), DMEM/F12 medium + 12 mmol/L SA (SA+), SA+ and transfected with empty vector (SA+/EV) or SA+ and transfected with *TMF1* expression vector (SA+/TMF1 GO). (C) Expression of SREBP1, ACC, FAS, SCD and FABP3 was tested by Western blotting. (D) Quantitation of (C). Bovine mammary epithelial cells were cultured with DMEM/F12 medium (SA-), SA- and transfected with negative control siRNA (SA-/NC), SA- and transfected with *TMF1* siRNA (SA-/si-TMF1) or DMEM/F12 medium + 12 mmol/L SA (SA+). Data are expressed as the mean values and standard deviations ($n = 3$); $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. ACC = acetyl CoA carboxylase; FAS = fatty acid synthesis; SCD = stearoyl-CoA desaturase; FABP3 = fatty acid binding protein 3; SA+ = SA supplementation; SA- = no SA supplementation.

3.2. SA promotes fat synthesis through the SREBP1 pathway

To explore whether the SREBP1 pathway was involved in the regulation of fat synthesis mediated by SA, BMECs were treated with SREBP1 overexpression or silencing, and then with SA+ or SA-. The secretion of TG and the expression of SREBP1 were then measured. The results showed that the secretion of TG ($P = 0.0045$) (Fig. 2A) and the expression of SREBP1 ($P = 0.0042$) (Fig. 2B and C) were increased by SA+, but these increases were blocked ($P = 0.0021$; $P = 0.0015$) by SREBP1 silencing. Conversely, the secretion of TG ($P = 0.0068$) (Fig. 2D) and the expression of SREBP1 ($P = 0.0031$) (Fig. 2E and F) were decreased in cells in the control group (no SA was added, SA-) compared with cells in the SA+ group, but these decreases were restored ($P = 0.0057$; $P = 0.0033$) by SREBP1 overexpression.

These results suggested that the SREBP1 pathway was involved in the regulation of fat synthesis mediated by SA and that SA promoted fat synthesis through the SREBP1 pathway.

3.3. TMF1 is an important inhibitor of SA mediated activation of the SREBP1 pathway

Our previous report showed that TMF1 was a potential regulator of the SREBP1 pathway and fat synthesis in BMECs (Li et al., 2014). To investigate whether TMF1 was involved in SA mediated activation of the SREBP pathway, BMECs were treated with TMF1 overexpression or silencing, and then with SA+ or SA-. The expression levels of the SREBP1 pathway-associated proteins ACC, FAS, SCD and FABP3 in BMECs were then assessed. The results showed that the expression levels of SREBP1 ($P = 0.0003$), ACC ($P = 0.0003$), FAS ($P = 0.0044$), SCD ($P = 0.0036$) and FABP3 ($P = 0.0003$) were

increased in cells treated with SA+, but these increases were blocked ($P = 0.0001$; $P = 0.0061$; $P = 0.0011$; $P = 0.0033$; $P = 0.0002$) by TMF1 overexpression (Fig. 3A and B). Conversely, the expression levels of SREBP1 ($P = 0.0040$), ACC ($P = 0.0009$), FAS ($P = 0.0069$), SCD ($P = 0.0039$) and FABP3 ($P = 0.0019$) were decreased in cells in the SA-group compared with cells in the SA+ group, but these decreases were restored ($P = 0.0001$; $P = 0.0061$; $P = 0.0002$; $P = 0.0014$; $P = 0.0072$) by TMF1 silencing (Fig. 3C and D). The efficiency of TMF1 overexpression and silencing is shown in Fig. S4, S5 and S6.

These results suggested that TMF1 was a negative regulator of SA-dependent activation of the SREBP1 pathway and that SA activated the SREBP1 pathway by suppressing TMF1.

3.4. TMF1 negatively regulates SA activated fat synthesis

To investigate whether TMF1 was involved in the regulation of fat synthesis mediated by SA, BMECs were treated with TMF1 overexpression or silencing and then with SA+ or SA- and the secretion of TG was assessed. The results showed that the expression of TMF1 ($P = 0.0033$) (Fig. 4A and B) was decreased, but the secretion of TG ($P = 0.0001$) (Fig. 4C) was increased in cells treated with SA+; however, this decrease ($P = 0.0006$) and increase ($P = 0.0022$) were restored and blocked by TMF1 overexpression, respectively. Conversely, the expression of TMF1 ($P = 0.0076$) (Fig. 4D and E) was increased, but the secretion of TG ($P = 0.0048$) (Fig. 4F) was decreased in cells treated with SA-; however, this increase ($P = 0.0045$) and decrease ($P = 0.0058$) were restored and blocked by TMF1 silencing, respectively.

These results indicated that TMF1 was a negative regulator of fat synthesis and suppressed SA-dependent fat synthesis in BMECs.

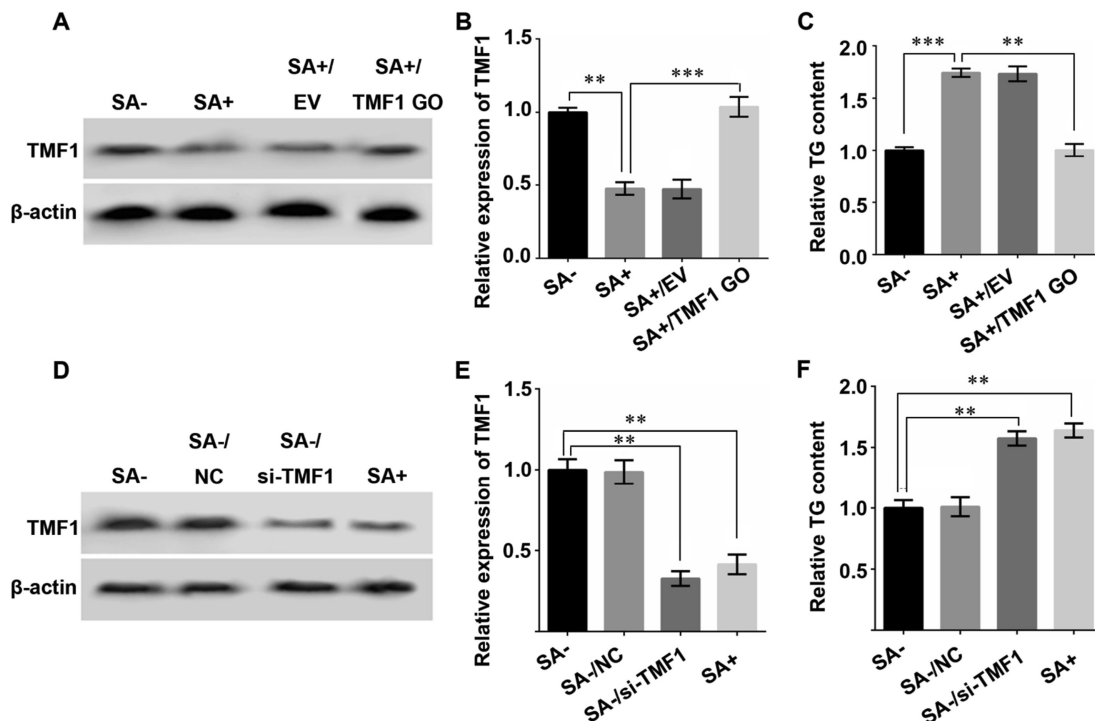


Fig. 4. TATA element modulatory factor 1 (TMF1) negatively regulates sodium acetate (SA) activated fat synthesis. (A) Expression of TMF1 was tested by Western blotting. (B) Quantitation of (A). (C) Secretion of triglyceride (TG). Bovine mammary epithelial cells were cultured with DMEM/F12 medium (SA-), DMEM/F12 medium + 12 mM SA (SA+), SA+ and transfected with empty vector (SA+/EV), or SA+ and transfected with TMF1 expression vector (SA+/TMF1 GO). (D) Expression of TMF1 was tested by Western blotting. (E) Quantitation of (D). (F) Secretion of TG. Bovine mammary epithelial cells were cultured with DMEM/F12 medium (SA-), SA- and transfected with negative control siRNA (SA-/NC), SA- and transfected with TMF1 siRNA (SA-/si-TMF1) or DMEM/F12 medium + 12 mM SA (SA+). Data are expressed as the mean values and standard deviations ($n = 3$); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. SA+ = SA supplementation; SA- = no SA supplementation.

3.5. TMF1 negatively regulates fat synthesis through the SREBP1 pathway

To investigate whether the SREBP1 pathway was involved in the regulation of fat synthesis by TMF1, BMECs were cotreated with TMF1 and SREBP1 overexpression or silencing. SREBP1 expression and TG secretion by BMECs were assessed. The results showed that the expression of SREBP1 ($P = 0.0107$) (Fig. 5A and B) and the secretion of TG ($P = 0.0073$) (Fig. 5C) were decreased in cells overexpressing TMF1, but these decreases were restored ($P = 0.0016$; $P = 0.0115$) by SREBP1 overexpression. Conversely, the expression of SREBP1 ($P = 0.0173$) (Fig. 5D and E) and the secretion

of TG ($P = 0.0021$) (Fig. 5F) were increased in cells treated with TMF1 silencing, but these increases were blocked ($P = 0.0067$; $P = 0.0083$) by SREBP1 silencing (Fig. 5D and F).

These results indicated that the SREBP1 pathway was involved in the regulation of fat synthesis by TMF1 and that TMF1 negatively regulated fat synthesis through the SREBP1 pathway.

3.6. TMF1 directly interacts with SREBP1

To explore the mechanism by which TMF1 inhibits the SREBP1 pathway and fat synthesis, the interaction between TMF1 and SREBP1 was identified. The results showed that TMF1 directly

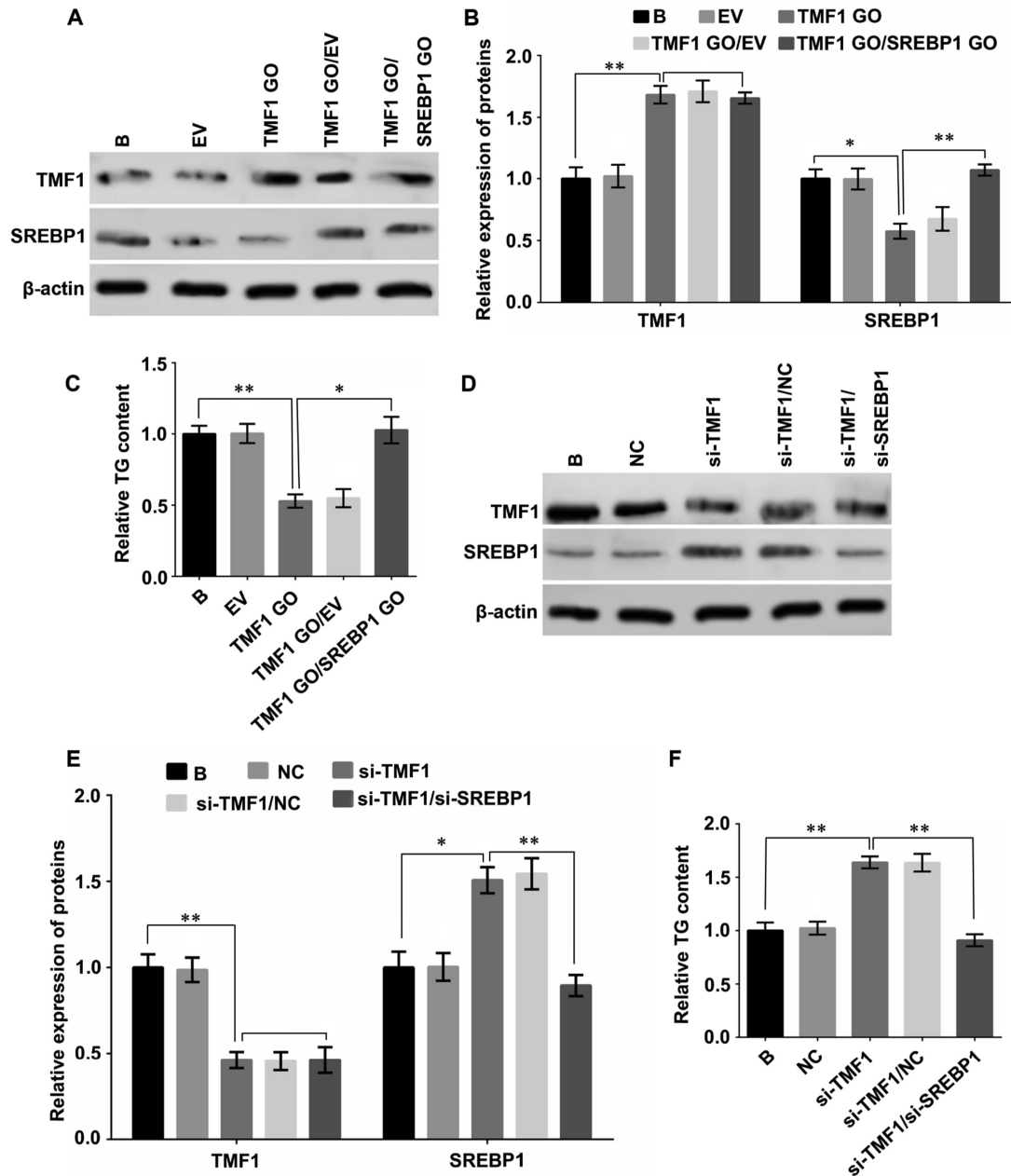


Fig. 5. TATA element modulatory factor 1 (TMF1) negatively regulates fat synthesis through the sterol regulatory element binding protein 1 (SREBP1) pathway. (A) Expression of TMF1 and SREBP1 was tested by Western blotting. (B) Quantitation of (A). (C) Secretion of triglyceride (TG). Bovine mammary epithelial cells were cultured with no transfection (B), transfection with empty vector (EV), TMF1 expression vector (TMF1 GO), TMF1 expression vector + empty vector (TMF1 GO/EV) or TMF1 expression vector + SREBP1 expression vector (TMF1 GO/SREBP1 GO). (D) Expression of TMF1 and SREBP1 was tested by Western blotting. (E) Quantitation of (D). (F) Secretion of TG. Bovine mammary epithelial cells were cultured with no transfection (B), transfection with negative control siRNA (NC), TMF1 si-RNA (si-TMF1), TMF1 si-RNA + negative control siRNA (si-TMF1/NC) or TMF1 si-RNA + SREBP1 si-RNA (si-TMF1/si-SREBP1). Data are expressed as the mean values and standard deviations ($n = 3$); $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

interacted with SREBP1 (Fig. 6A). To confirm the specificity of this interaction, BMECs were transfected with *TMF1*-Myc and/or *SREBP1*-Flag. The interaction between *TMF1* and *SREBP1* was tested by co-IP with Myc and Flag antibodies. The results showed that *SREBP1*-Flag was detected only in cells that were cotransfected with *TMF1*-Myc and *SREBP1*-Flag, and *SREBP1*-Flag was detected with Flag antibody or *SREBP1* antibody (Fig. 6B and C). In addition, the colocalization of *TMF1* and *SREBP1* was identified; the results showed that *TMF1* and *SREBP1* were colocalized and their colocalization existed only in the cytoplasm (Fig. 6D).

These results suggested that *TMF1* specifically interacted with *SREBP1* in BMECs.

3.7. *TMF1* suppresses the nuclear localization of *SREBP1*

To explore whether the nuclear localization of *SREBP1* was regulated by *TMF1*, BMECs were treated with *TMF1* overexpression or silencing, and the nuclear localization of *SREBP1* was tested. The results showed that the nuclear localization of *SREBP1* (Fig. 7C: $P = 0.0083$; Fig. 7E: $P = 0.0101$) was decreased and that the cytoplasmic localization of *SREBP1* ($P = 0.0049$) was increased in cells overexpressing *TMF1* (Fig. 7A, B, C, D and E). Conversely, the nuclear localization of *SREBP1* (Fig. 7H: $P = 0.0066$; Fig. 7J: $P = 0.0042$) was increased, and the cytoplasmic localization of *SREBP1* ($P = 0.0073$) was decreased in cells treated with *TMF1* silencing (Fig. 7F, G, H, I and J).

These results indicated that *TMF1* suppresses the nuclear localization of *SREBP1*.

3.8. Sodium acetate inhibits the expression of *TMF1* and the interaction between *TMF1* and *SREBP1*

To investigate whether the expression of *TMF1* and the interaction between *TMF1* and *SREBP1* were affected by SA, BMECs were treated with SA+ or SA-. The expression of *TMF1* and the interaction between *TMF1* and *SREBP1* were assessed. The results showed that the expression of *TMF1* ($P = 0.0002$) was decreased in cells treated with SA (Fig. 8A and B). The interaction between *TMF1* and *SREBP1* ($P = 0.0001$) (Fig. 8C and D) and the colocalization of *TMF1* and *SREBP1* ($P = 0.0135$) (Fig. 8E and F) were decreased in cells treated with SA.

These results indicated that SA suppressed the expression of *TMF1* and the interaction between *TMF1* and *SREBP1*.

4. Discussion

SA is one of the most important precursors of fatty acid synthesis in cells (X Li et al., 2019; Urrutia et al., 2019; Yan et al., 2019). Studies have shown that milk fat synthesis in mammary gland cells in vitro can be promoted by the addition of SA to the medium (Sun et al., 2013; Urrutia et al., 2017a; 2017b). In this study, the secretion of TG was increased in BMECs treated with SA supplementation. This result is consistent with the conclusions of previous research (Sun et al., 2013; Urrutia et al., 2017a, 2017b).

TMF1 is a DNA-binding factor that preferentially binds to the TATA element in human immunodeficiency virus 1 (Garcia et al., 1992; Lerer-Goldshtein et al., 2010). In eukaryotic cells, *TMF1* is

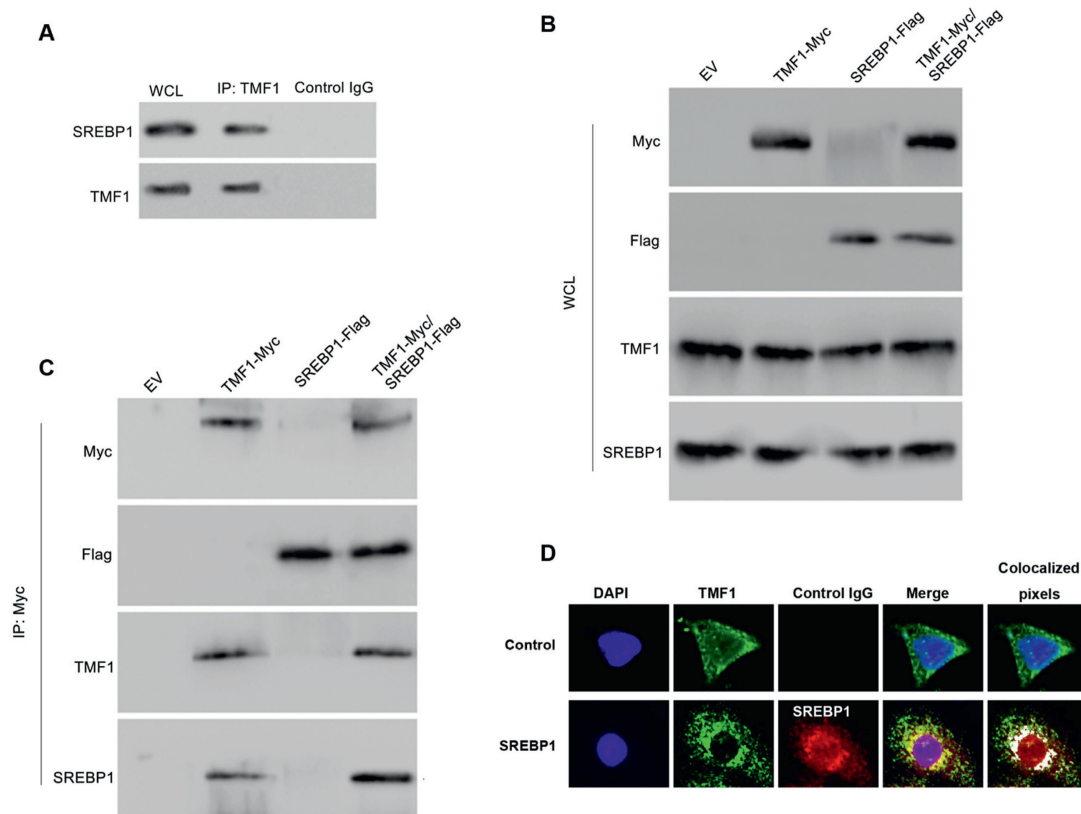


Fig. 6. TATA element modulatory factor 1 (*TMF1*) directly interacts with sterol regulatory element binding protein 1 (*SREBP1*). (A) Co-immunoprecipitation (co-IP) of *TMF1*. IgG was used as a control. (B–C) Co-IP of Myc vector (Myc). (D) Colocalization of *TMF1* and *SREBP1*. In (A) and (D), bovine mammary epithelial cells (BMECs) were cultured with DMEM/12 medium. In (B) and (C), BMECs were cultured with DMEM/12 medium, and then transfected with empty vector (EV), *TMF1*-Myc, *SREBP1*-Flag vector (*SREBP1*-Flag), and *TMF1*-Myc vector + *SREBP1*-Flag vector (*TMF1*-Myc/*SREBP1*-Flag). WCL = whole cell lysate.

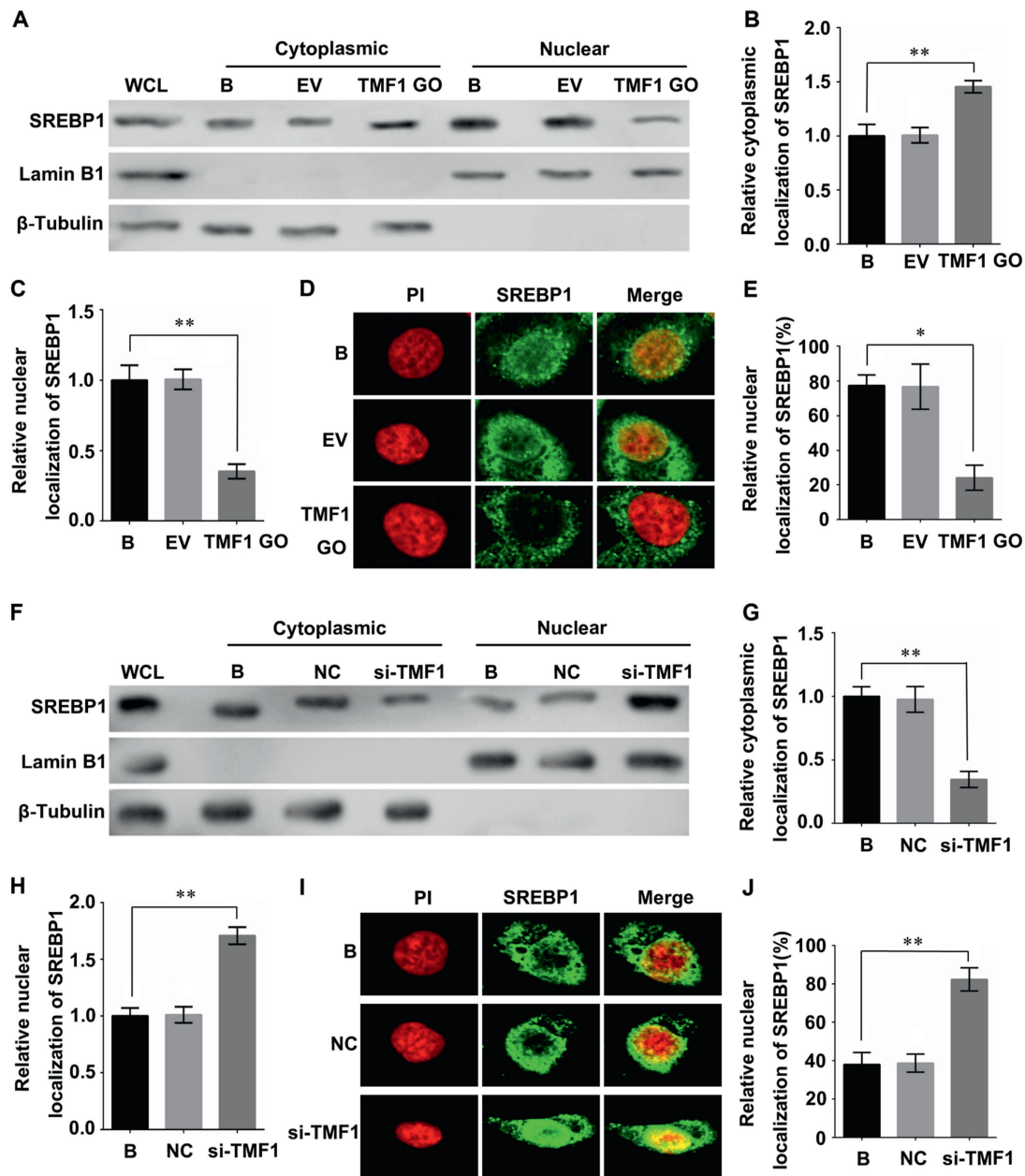


Fig. 7. TATA element modulatory factor 1 (TMF1) suppresses the nuclear localization of sterol regulatory element binding protein 1 (SREBP1). (A) Cytoplasmic localization and nuclear localization of SREBP1 was tested by Western blotting. (B) Quantitation of cytoplasmic localization of SREBP1. (C) Quantitation of nuclear localization of SREBP1. (D) Nuclear localization of SREBP1 was tested by immunofluorescence. (E) Quantitation of (D). Bovine mammary epithelial cells were cultured with DMEM/F12 medium, and then treated with no transfection (B) transfection with empty vector (EV) or TMF1 expression vector (TMF1 GO). (F) Cytoplasmic localization and nuclear localization of SREBP1 was tested by Western blotting. (G) Quantitation of cytoplasmic localization of SREBP1. (H) Quantitation of nuclear localization of SREBP1. (I) Nuclear localization of SREBP1 was tested by immunofluorescence. (J) Quantitation of (I). Bovine mammary epithelial cells were cultured with DMEM/F12 medium, and then treated with no transfection (B), negative control siRNA (NC) or TMF1 siRNA (si-TMF1). Data are expressed as the mean values and standard deviations ($n = 3$); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

widely involved in various cell signaling pathways and physiological processes. Studies have shown that TMF1 is dispersed in the cytoplasm of stressed cells and exerts E3 ubiquitin ligase activity (Perry et al., 2004). TMF1 is also a key regulator of the morphogenesis of mature sperm (Elkis et al., 2015) and mediates the degradation of Stat3 (Perry et al., 2004). Moreover, TMF1 down-regulates proangiogenic genes, attenuates the progression of PC3 xenografts (Abraham et al., 2009), and mediates the sorting and transport of proteins in the Golgi apparatus (Fridmann-Sirkis et al., 2004). In our previous study, we found that TMF1 could be involved in the regulation of fat synthesis in BMECs. The current study demonstrated that TMF1 negatively regulated fat synthesis in

response to SA supplementation. Cotreatment with SA and silencing or overexpression of TMF1 in BMECs revealed that SA activated the SREBP1 pathway and promoted fat synthesis by suppressing TMF1.

The SREBP1 pathway is one of most important pathways involved in milk fat synthesis. It responds to short- or long-chain fatty acids and then promotes fat synthesis (Liu et al., 2019). In this study, SA application promoted the expression of SREBP1 and its target genes related to milk fat synthesis, such as *ACC*, *FAS*, *SCD* and *FABP3*. In addition, silencing or overexpression of TMF1 in BMECs demonstrated that TMF1 negatively regulated the expression of SREBP1 and its target gene controlled by SA. These results

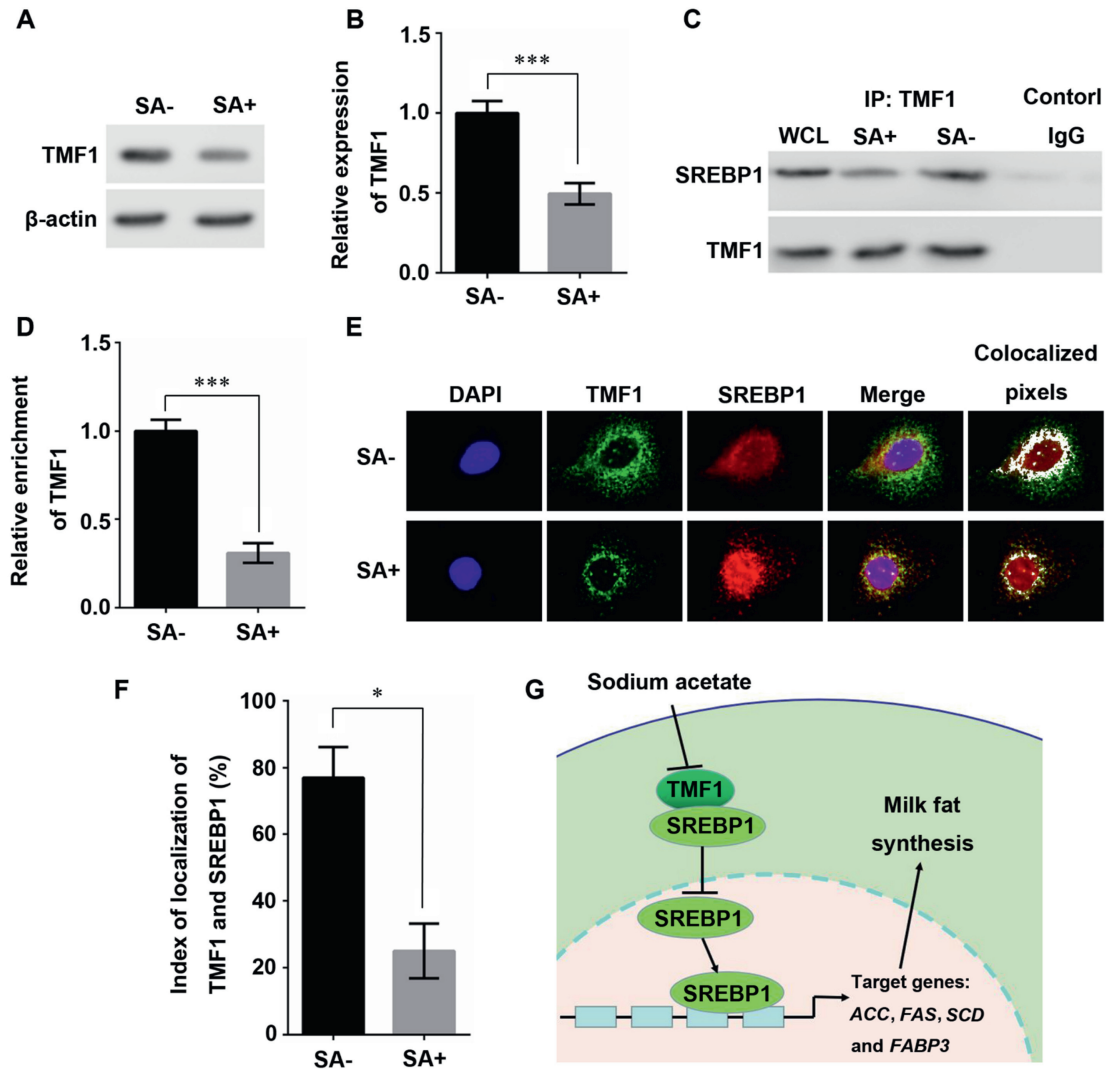


Fig. 8. Sodium acetate (SA) inhibits the expression of TATA element modulatory factor 1 (TMF1) and the interaction between TMF1 and sterol regulatory element binding protein 1 (SREBP1). (A) Expression of TMF1 was tested by Western blotting. (B) Quantitation of (A). (C) Interaction between TMF1 and SREBP1. (D) Quantitation of (C). (E) Colocalization of TMF1 and SREBP1. (F) Quantitation of (E). (G) Diagram of the pathway by which SA regulates fat synthesis. Bovine mammary epithelial cells were cultured with DMEM/F12 medium (SA-) and DMEM/F12 medium + 12 mmol/L SA (SA+) for 24 h. Data are expressed as the mean values and standard deviations ($n = 3$); * $P < 0.05$, *** $P < 0.001$. SA+ = SA supplementation; SA- = no SA supplementation.

suggested that TMF1 was involved in SA-dependent regulation of the SREBP1 pathway and fat synthesis and is an important negative regulator in this regulatory process in BMECs.

As a main regulator of fat synthesis, SREBP1 responds to fatty acids and then transfers into the cell nucleus and promotes the expression of its target gene and subsequent milk fat synthesis (Harvatine et al., 2006). In our study, the regulatory effect of TMF1 on the nuclear localization of SREBP1 was tested, and the results showed that the nuclear localization of SREBP1 was blocked by the overexpression of TMF1.

Because its central and C-terminal parts contain coiled-coil forming domains, TMF1 can interact with many other proteins, such as Golgi and acrosome-associated variants of the tyrosine kinase Fer-FerT (Bel et al., 2012; Kierszenbaum et al., 2008), Rab6 GTP-binding protein (Yamane et al., 2007), and ATPase subunits of the human chromatin remodeling complex SNF/SWI-hbrm/hSNF2a and BRG-1/hSNF2b (Hsiao et al., 1999; Miller et al., 2013; Mori et al., 2002). This combination enables it to participate in a wide range of cellular physiological processes and functions. In the current study, co-IP experiments indicated that TMF1 can interact with SREBP1, IF

colocalization observations demonstrated that TMF1 and SREBP1 interact in the cytoplasm rather than in the nucleus. Combined with the previous results in this study, we suggest that TMF1 interacts with SREBP1 directly in the cytoplasm and then suppresses the nuclear localization of SREBP1, the expression of SREBP1 target genes (*ACC*, *FAS*, *SCD* and *FABP3*) and subsequent fat synthesis in BMECs.

In addition, the effect of SA on the expression of TMF1 and the interaction between TMF1 and SREBP1 were investigated in this study, and the results indicated that SA supply suppressed the expression of TMF1 and the interaction between TMF1 and SREBP1. This finding suggests that TMF1 is an important negative regulator of the SA-dependent SREBP1 pathway and fat synthesis in BMECs.

5. Conclusions

In summary, the current study found that SA is an important positive regulator of fat synthesis and that it promotes the SREBP1 pathway and fat synthesis by suppressing TMF1. In BMECs, TMF1 is an inhibitor of fat synthesis, and it prevents the nuclear localization of SREBP1 by interacting with SREBP1 in the cytoplasm. Sodium

acetate suppresses the expression of TMF1, prevents the interaction between TMF1 and SREBP1, and then activates the SREBP1 pathway and subsequent fat synthesis. This study enhances the current understanding of the regulation of fat synthesis and provides new scientific data for the artificial regulation of fat synthesis.

Author contributions

Chaochao Luo: Co-developed the conception and design of the study, co-collected and analyzed the data, wrote the original manuscript and supervised the project. **Nan Li:** Co-developed the conception and design of the study, co-collected and analyzed the data and wrote the original manuscript. **Qingzhu Wang:** Provided the BMECs, and reviewed and edited the manuscript. **Chunjiang Li:** Suggested the research idea, supervised the project, and reviewed and edited the manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aninu.2023.01.002>.

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