Rapid communication: lipid metabolic gene expression and triacylglycerol accumulation in goat mammary epithelial cells are decreased by inhibition of *SREBP-1*

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ABSTRACT: In mammals, sterol regulatory element binding protein-1 (*SREBP-1*) is the master regulator of fatty acid and triacylglycerol synthesis. Recent gene silencing studies in mammary cells indicate that *SREBP-1* has a central role in milk fat synthesis. However, *SREBP-1* knockdown studies in goat mammary cells have not been performed; hence, its direct role in controlling mRNA expression of lipid metabolism genes and triacylglycerol synthesis remains unknown. Inhibition of *SREBP-1* in goat mammary epithelial cells (**GMEC**) by small interference RNA (**siRNA**) markedly reduced the content of cellular triacylglycerol (~50% decrease, $P < 0.05$) and was partly related to downregulation of *AGPAT6*, *LPIN1*, and *DGAT2* (−23%, −28% and −19%, respectively. $P < 0.05$), which are key enzymes involved in triacylglycerol synthesis, cellular triacylglycerol content and lipid droplet accumulation all decreased by *SREBP-1* inhibition. The expression of lipid droplet formation and secretion genes was not altered in response to treatment. Although the lack of effect on expression of *ACACA* and *FASN* (rate-limiting enzymes for de novo fatty acid synthesis) with *SREBP-1* knockdown was unexpected ($P > 0.05$), the downregulation of genes related to synthesis of acetyl-CoA and acetate activation (*ACLY*, *ACSS2,* and *IDH1*, $P < 0.05$) suggests that lipogenesis was inhibited. *SREBP-1* knockdown also resulted in decreased expression of genes associated with fatty acid desaturation and elongation (*SCD1* and *ELOVL6*, $P < 0.05$), long-chain fatty acid (LCFA) activation and transport (*ACSL1*, *FABP3,* and *SLC27A6*, $P < 0.05$). The results underscored the essential role of *SREBP-1* not only in fatty acid synthesis but also in desaturation, elongation, and esterification in GMEC. Clearly, the lack of effect on *ACACA* and *FASN*, both of which are considered the key lipogenic enzymes, implies that there may be different regulatory mechanisms in goat compared with bovine mammary cells.

Key words: dairy goat, gene expression, lactation, mammary cell

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INTRODUCTION

The high content of MUFA in goat milk confers it a high nutritional value (Park et al., 2007). By virtue of the high content of short- and medium-chain fatty acids and smaller fat globules, goat milk is considered to be the best substitute for

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human milk (Chilliard et al., 2003; Haenlein, 2004). Since milk fat has a high nutritional value (Bernard et al., 2005) and is composed of 98% triacylglycerol (**TAG**; Dils, 1984), investigation of the regulatory mechanism of fatty acid and TAG synthesis is of biological and practical importance.

Sterol regulatory element binding proteins (***SREBPs***) are members of a basic-loop-helix leucine zipper (**bHLH-Zip**) transcription factor superfamily (Chieko Yokoyama, 1993) and are known to be master regulators of lipid and cholesterol metabolism (Horton et al., 2002; Espenshade, 2006; Raghow et al., 2008). The sterol regulatory element binding protein-1 (***SREBP-1***) isoform (gene name ***SREBF1***) participates mainly in fatty acid and TAG synthesis. It was reported that the two isoforms of *SREBP-1* (*SREBP-1a* and *SREBP-1c*) differ only in their first exon in humans and mice (Horton, 2002; Horton et al., 2002). However, the exact sequence difference between these two isoforms has not been distinguished in ruminants (Lengi and Corl, 2010); SREBP-2 (gene name ***SREBF-2***) is mainly involved in the regulation of cholesterol metabolism (Eberle et al., 2004; Espenshade, 2006).

Studies in mice have documented the developmental changes in mRNA expression of *SREBP-1c* and fatty acid composition in milk (Rudolph et al., 2010), suggesting that this isoform plays a key role in de novo fatty acid synthesis. In rodents, the key enzymes involved in de novo fatty acid synthesis, e.g., acyl-coenzyme A synthase short-chain family member 2 (***ACSS2***), acetyl-CoA carboxylase alpha (***ACACA***), and fatty acid synthase (***FASN***) are transcriptionally regulated by *SREBP-1* (Luong et al., 2000; Harvatine et al., 2009; Li et al., 2015), and these genes are all correlated with fat yield in buffalo (Yadav et al., 2015) and bovine (Bionaz and Loor, 2008). Furthermore, the inhibitory effect of PUFA on fatty acid synthesis in nonruminants appears to be mediated by *SREBP-1* (Takeuchi et al., 2010; Nakakuki et al., 2014). Despite all these available data, the exact mechanism of how *SREBP-1* regulates fatty acid (especially TAG) synthesis and milk-fat formation is still largely unknown.

The hypothesis of this study was that alteration of *SREBP-1* expression in goat mammary epithelial cells (**GMEC**) would cause changes in mRNA expression of fatty acid synthesis-related genes and also TAG synthesis. The specific objective of the present study was to knockdown the expression of *SREBP-1* using small interference RNA (**siRNA**) and measure the effects on expression of genes associated with de novo synthesis, desaturation, elongation, and esterification along with TAG content, lipid droplet formation and accumulation.

MATERIALS AND METHODS

All experimental procedures involving dairy goats were conducted under the approval of the Animal Care and Use Committee of Northwest A&F University of Agriculture and Technology (Yangling, Shaanxi, China). GMEC were isolated, purified, and cultured as previously described (Lin et al., 2013; Shi et al., 2015; Xu et al., 2016). Briefly, cells were incubated at 37 °C in 5% CO_2 and air. The basal culture medium consisted of DMEM/F12 medium (Hyclone Laboratories, Beijing, P. R. China) supplemented with 10% fetal bovine (Hyclone), bovine insulin (5 μg/mL; Sigma, St. Louis. MO), hydrocortisone (5 mg/liter; Sigma), penicillin/streptomycin (10 kU/liter; Harbin Pharmaceutical Group, Harbin, P. R. China), and epidermal growth factor (10 ng/mL; Sigma). The cells used in the experiments were isolated and pooled from at least three different individual goats, and each treatment was performed with three biological replicates on three different cell culture plates. Before treatment, GMEC were seeded in 60-cm^2 cell culture plates (Corning Inc, Corning, NY) and switched from the basal culture medium to a lactogenic medium to induce lactogenesis. The lactogenic medium contained BSA (1 mg/mL; Sigma) instead of fetal bovine serum and was supplemented with prolactin (2.0 μg/mL; Sigma) without antibiotics. After 12 h incubation (approximately 70%–80% confluence), the chemosynthetic siRNA-Cy3 (Ribobio, Guangzhou, China) that did not target specific genes (Scrambled siRNA, siSCR) was diluted with diethyl pyrocarbonate water and then transfected into GMEC at different concentrations (10, 20, 30, 50, or 100 nM) using Lipofectamin™ RNAiMAX (Invitrogen, USA) following a forward transfection protocol. Six hours later, the red fluorescence for each treatment was captured under a Leica DMI4000B inverted fluorescence microscope (Leica Geosystems, Co., Ltd, Germany) to determine the best transfection efficiency.

With the same method, the siRNA targeting the *SREBP-1* gene (***siSREBP-1***, sense: 5′-GCUCCUCACUUGAAGGCUUTT-3′; antisense: 5′-AAGCCUUCAAGUGAGGA GCTT-3′) was transfected into GMEC with the selected concentration, using siSCR as a negative control. After 48 h incubation, GMEC were harvested. For RT-qPCR, total RNA was extracted using an RNAprep pure cell/bacteria kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's instructions. The mRNA expression of target genes was evaluated using the SYBR Premix Ex Taq II (Takara Bio Inc, Otsu, Japan) on a CFX96

Real-time PCR Detection System (Bio-Rad, Hercules, CA). Mitochondrial ribosomal protein L39 (***MRPL39***), ribosomal protein S9 (***RPS9***), and ubiquitously expressed transcript (***UXT***) were used as internal control genes for normalization of target genes. Gene-specific primers used for RT-PCR are listed in Supplementary Table S1.

Total protein was extracted with RIPA buffer (Solarbio Tech Co. Ltd., Beijing, China) supplemented with PMSF (1 mM; Pierce, Rockford, IL). Western blots were performed with the primary antibody β-actin (CW0096, 1:1,000; CW Biotech, Beijing, China) and *SREBP-1* (ab3259, 1:500; Abcam, Cambridge, United Kingdom) as described previously (Xu et al., 2016). Lipid droplet accumulation of GMEC was evaluated by staining with Oil Red O (Shi et al., 2015), followed by visualization in a Leica DMI4000B inverted fluorescence microscope (Leica Geosystems, Co., Ltd). After 48 h transfection with siRNA, GMEC were stained with Oil Red O to measure the lipid droplet accumulation by a method described previously (Mehlem et al., 2013). Briefly, GMEC were washed three times with cold PBS and then fixed with 10% pre-cold paraformaldehyde for at least 1 h at 4 °C. Cells were then washed twice with cold PBS and stained with 0.5% Oil red O (m/v, 0.05 g Oil red O dissolved in 10 mL 70% ethanol) for 30 min at room temperature. Lastly, GMEC were washed three times with cold PBS and observed under a Leica DMI4000B inverted fluorescence microscope (Leica Geosystems, Co., Ltd., Wetzlar, Germany).

Total cellular TAG and cholesterol content was measured using a tissue/cell triacylglycerol assay kit (E1013-105) and tissue/cell total cholesterol assay kit (E1015) according to the manufacturer's instructions (Applygen Technologies Inc, Beijing, China). The inter- and intra-assay coefficients of variation for assays were between 1.8% and 5.3%, and 1.5% and 6%, respectively. Total cellular protein was quantified by using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA) and used to normalize total TAG and cholesterol content.

Data are presented as means ± SEM of three independent biological replicates. The results of RT-qPCR were analyzed using the $2^{-\Delta\Delta Ct}$ method relative to the negative control group (Ct is the cycle threshold). A one-way ANOVA was performed with significant differences declared at $P < 0.05$.

RESULTS AND DISCUSSION

In the present study, as shown in Figure 1, the best transfection efficiency (with the most red fluorescence) was observed at 100 nM siRNA transfection, and we also noticed that the transfection efficiency of siRNA increased in a concentration-dependent manner. Compared with the siSCR-transfected group, 100 nM *siSREBP-1* transfection led to more than a 60% decrease in expression of both *SREBF1* and *SREBF-1a* (Figure 2A, $P < 0.01$). Western blot analysis revealed that the abundance of the precursor (located in cytoplasm) form of *SREBP-1* did not change, while the mature form of *SREBP-1* protein (located in cell nuclei) decreased in the siSREBP1-transfected group compared with the siSCR control group (Figure 2B).

Although *ACACA* and *FASN* were not affected (Figure 3B), the inhibition of *SREBP-1* by siRNA significantly decreased mRNA expression of other genes associated with de novo fatty acid synthesis, including ATP citrate lyase (***ACLY***) by 15%, *ACSS2* by 30%, isocitrate dehydrogenases 1 (NADP+) soluble (***IDH1***) by 35% (Figure 3B). This treatment also decreased the expression of key enzymes for fatty acid desaturation and elongation (stearoyl-Coenzyme A desaturase 1, ***SCD1*** by 35% and elongase of very LCFAs, ***ELOVL6*** by 24%, Figure 3A). *SREBP-1* inhibition also caused a significant decrease of expression of LCFA activation- and transport-related genes (Figure 3C): fatty acid binding protein 3 (***FABP3***) by 53%, acyl-CoA synthase long-chain family member 1 (***ACSL1***) by 13%, solute carrier family 27 member 6 (***SLC27A6***) by 31%. Expression of the mitochondrial oxidation of LCFAs [(carnitine palmitoyltransferase 1A, ***CPT1A***) gene also decreased by 31% ($P < 0.01$)]. In addition, *SREBP-1* inhibition was associated with downregulation of the lipid metabolism regulators insulin-induced gene 1 (***INSIG1***), SREBP cleavage-activating protein (***SCAP***), and peroxisome proliferator-activated receptor gamma (***PPARG***), which decreased by 15%, 16%, and 14%, respectively (Figure 3D, $P < 0.05$). However, opposite to the decrease in the expression of lipogenic genes, the expression of peroxisome proliferator-activated receptor alpha (***PPARA***), xanthine dehydrogenase (***XDH***), and acyl-coenzyme A oxidase (***ACOX***) increased by 1.15-fold, 1.17-fold, and 1.30-fold, respectively (Figure 3D and F, $P < 0.05$).

The mRNA expression of the TAG synthesis-related genes 1-acylglycerol-3-phosphate O-acyltransferase 6 (***AGPAT6***), lipin 1 (***LPIN1***), and diacylglycerol O-acyltransferase 1 (***DGAT2***; except for glycerol-3-phosphate acyltransferase, ***GPAM***, and *DGAT1*) were all downregulated by *SREBP-1* knockdown (Figure 4A). Total content of cellular TAG also decreased by approximately 50%

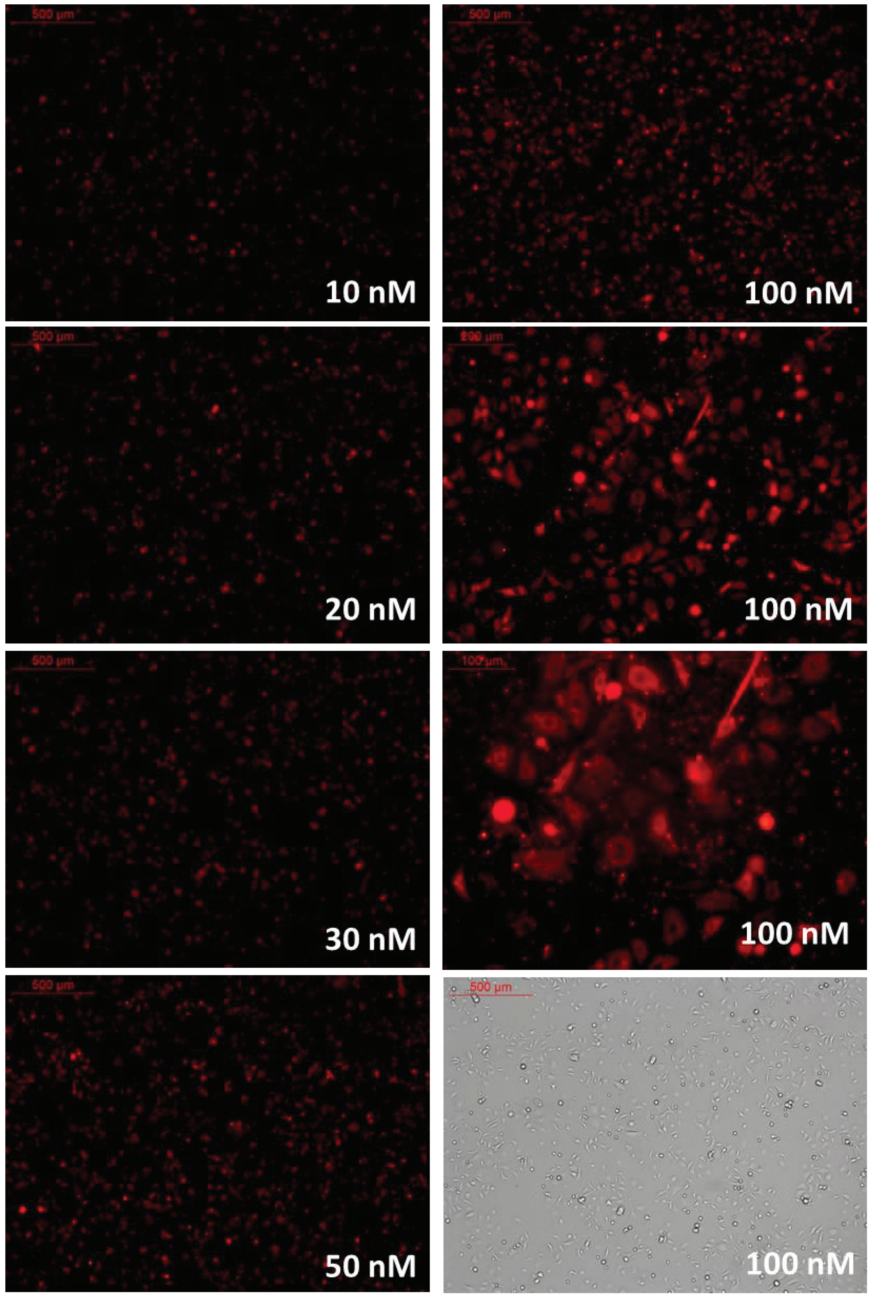

Figure 1. Comparison of transfection efficiency with different concentrations of siSCR in GMEC. GMEC were transfected with 0 nM, 10 nM, 20 nM, 30 nM, 50 nM, and 100 nM siSCR, 6 h later, red fluorescence of different treatment were captured.

in the *siSREBP-1* transfected group (Figure 4B, $P < 0.01$), and this decrease was further supported and validated by the decrease of lipid droplet accumulation in GMEC (Figure 4C). The content of cellular cholesterol did not differ after *SREBP-1* knockdown (Figure 4D, $P > 0.05$).

The role of *SREBP-1* on de novo fatty acid synthesis and TAG synthesis has been well established in mouse and bovine mammary epithelial cells (Rudolph et al., 2010; Ma and Corl, 2012; Li et al., 2014). Studies also identified that *ACSS2*, a gene encoding a protein that activates acetate to acetyl-CoA, is a target gene of *SREBP-1* (Luong et al., 2000), and our previous studies revealed that *SREBP-1* overexpression induced a more than 40-fold increase of *ACSS2* expression in GMEC (Xu et al., 2016). In accordance with results observed in bovine (Ma and Corl, 2012), the significant decrease of *ACSS2* by *SREBP-1* knockdown further supports that it is a target gene of *SREBP-1*.

Although there are no reports about direct regulation of *ACLY* and *IDH1* by *SREBP-1*, we uncovered a positive correlation of the mRNA expression of these two genes and *SREBP-1* (Figure 3B) as in bovine mammary cells (Ma and Corl, 2012). The rate-limiting enzymes for de novo

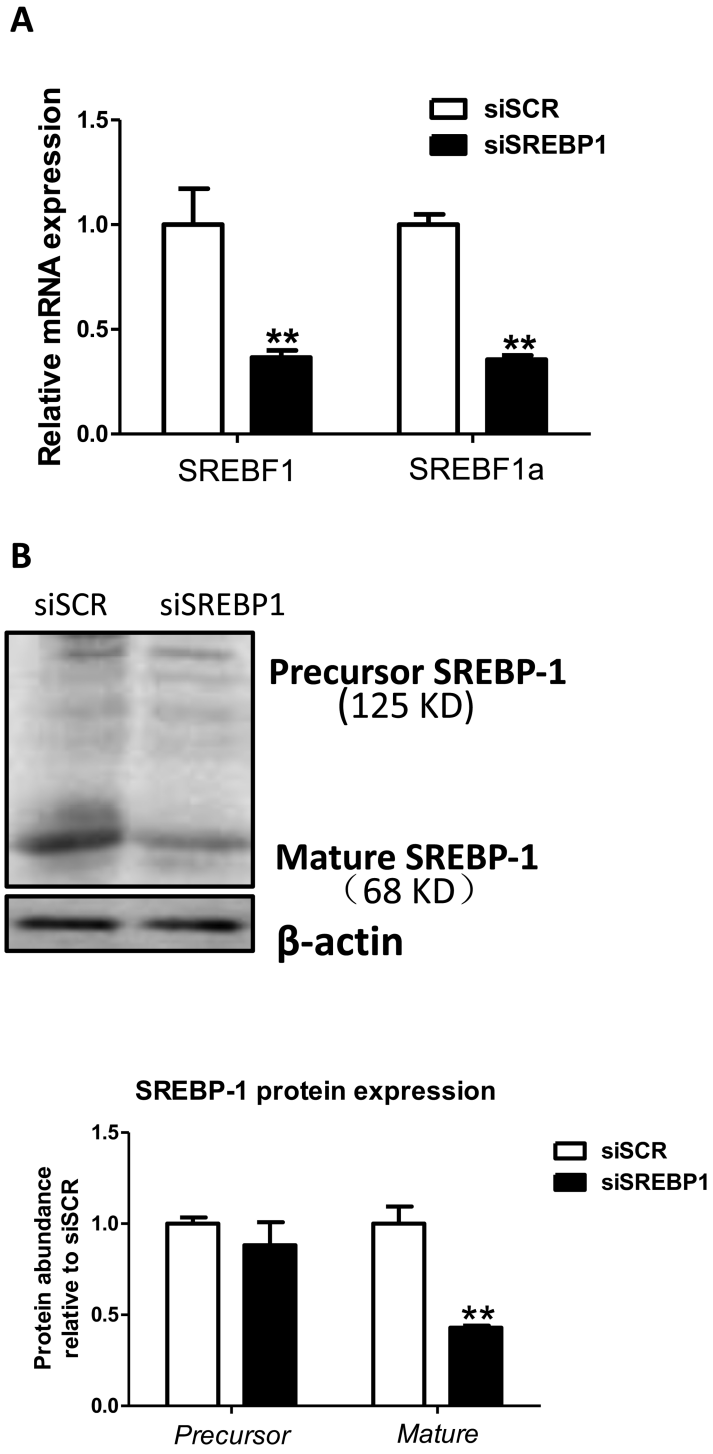

Figure 2. siRNA-mediated inhibition of *SREBP1* in GMEC. (A) Relative mRNA expression of *SREBF1* and *SREBF1a* after 48 h transfection with siSREBP1. Values are presented as LSM ± SE for three replicates; $^{**}P < 0.01$. (B) western blot analysis of precursor (125 kDa) and mature (68 kDa) SREBP1 protein levels after 48 h transfection with siSREBP1. Protein abundance was normalized to β-actin. The blots are representative of three independent experiments.

fatty acid synthesis, *FASN* and *ACACA*, that catalyze the condensation of acetyl-CoA and malonyl-CoA (Mashima et al., 2009; Currie et al., 2013), are regulated directly by *SREBP-1* (Lopez et al., 1996; Oh et al., 2003; Li et al., 2015). The lack of change in expression of *ACACA* and *FASN* in the present study after *SREBP-1* inhibition indicates that these lipogenic genes are regulated by other transcription factors in goat mammary cells. Thus, this represents a novel finding in the context of milk fat synthesis regulation in that mechanisms seem to differ in goat compared with bovine.

The expression of *SCD1*, encoding the desaturase of C16:0 and C18:0 (Bionaz and Loor, 2008),

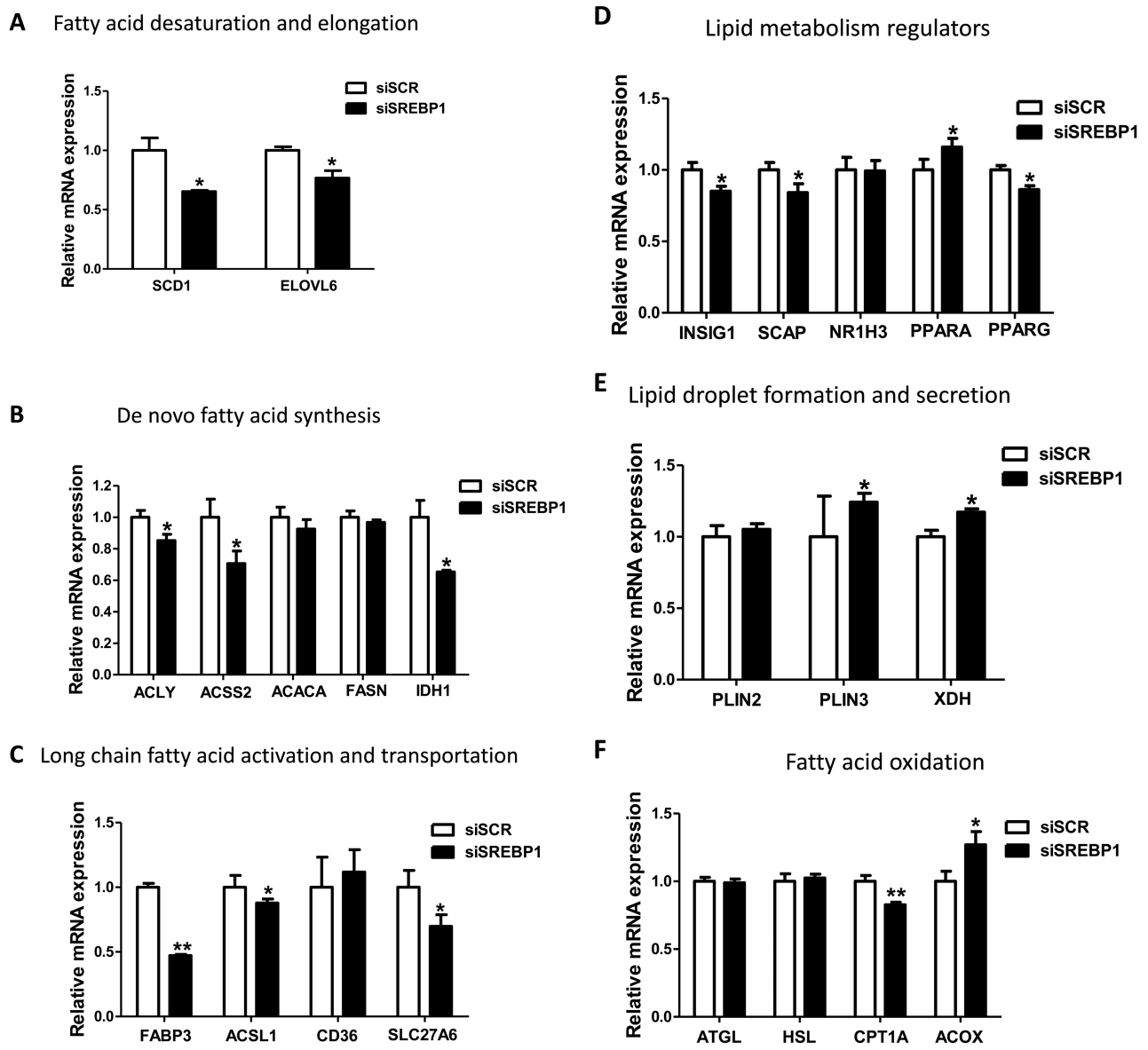

Figure 3. Effects of *SREBP-1* inhibition on the mRNA abundance of genes related to (A) fatty acid desaturation and elongation (*SCD1* and *ELOVL6*); (B) de novo fatty acid synthesis (*ACLY*, *ACSS2*, *ACACA*, *FASN,* and *IDH1*); (C) long-chain fatty acid activation and transportation (*FABP3*, *ACSL1*, *CD36,* and *SLC27A6*); (D) lipid metabolism regulators (*INSIG1*, *SCAP*, *NR1H3*, *PPARA,* and *PPARG*); (E) lipid droplet formation and secretion (*PLIN2*, *PLIN3,* and *XDH*); (F) fatty acid oxidation (*ATGL*, *HSL*, *CPT1A,* and *ACOX*). RT-qPCR measurements of gene expression are expressed as fold change compared with siSCR (negative control) transfected group. Values are presented as LSM ± SE for three replicates; *$P < 0.05$; **$P < 0.01$.

appears to be exclusively regulated by *SREBP-1* (Liang et al., 2002; Oppi-Williams et al., 2013). Consistent with previous studies, the 35% decrease of *SCD1* expression after *SREBP-1* inhibition suggested a direct regulation via *SREBP-1* also in GMEC (Ma and Corl, 2012; Yao et al., 2016). Although in nonruminants, it is well established that ELOVL6 is a key enzyme involved in the elongation of C16:0 to C18:0 (Jakobsson et al., 2006), little is known about ELOVL6 gene function and regulation in ruminants. In the mouse, *ELOVL6* is a target gene of *SREBP-1* (Kumadaki et al., 2008). Overexpression and knockdown studies of *SREBP-1* revealed that expression of *ELOVL6* was positively correlated with *SREBP-1* (Xu et al., 2016). Further studies in nonruminants underscored that the coordinated induction of FA elongase and desaturase activity is required for the balance of MUFA synthesis (*n*-7 vs. *n*-9 species), and ELOVL6 is the major elongase resulting in C18:1n-9 production. The elongation and desaturation pathways in mammary gland epithelial cells are important in the context of cellular membrane composition (Green et al., 2010; Mida et al., 2012).

Previous research in nonruminants demonstrated that *SREBP-1* activation increases TAG synthesis (Ishimoto et al., 2009; Li et al., 2014; Xu et al., 2016), which we also observed upon

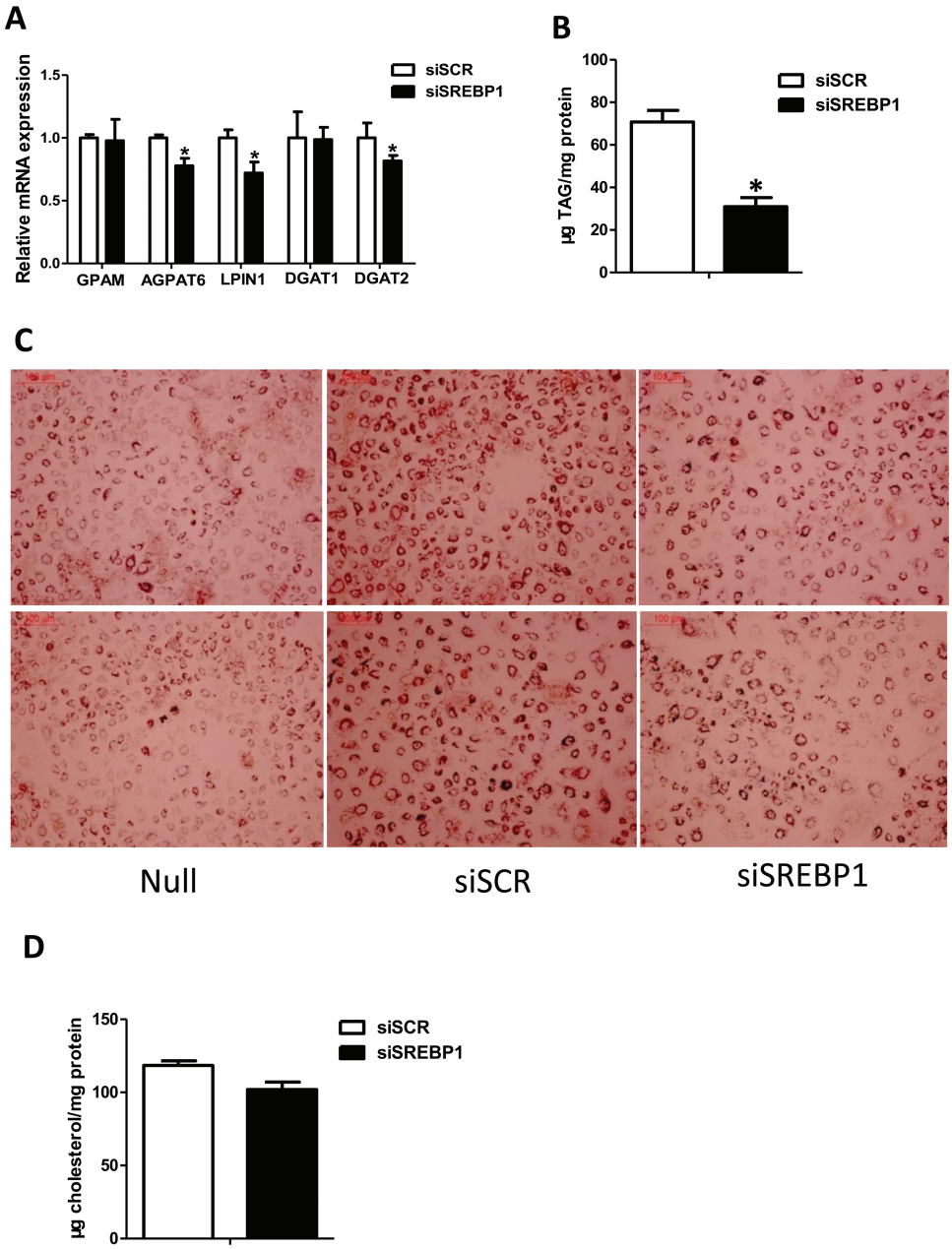

Figure 4. Effects of *SREBP-1* inhibition on TAG and cholesterol synthesis. GMEC was transfected with siSREBP1, 48 h later, (A) mRNA expression of TAG synthesis-related genes (*GPAM*, *AGPAT6*, *LPIN1*, *DGAT1,* and *DGAT2*) decreased; (B) cellular TAG content decreased significantly; while (C) lipid droplet accumulation was obviously decreased in siSREBP1 transfected group (Scale bar = 100 μm); (D) cellular cholesterol had no significant change. Values are presented as LSM ± SEM for three replicates. *$P < 0.05$.

SREBP-1 knockdown in GMEC (Figure 4). We speculate that most of this response was due to *SREBP-1* inhibition bringing about a significant decrease of *AGPAT6*, *LPIN1*, and *DGAT2*, all of which are key enzymes for TAG synthesis, and some are known targets of *SREBP-1* in the mouse (Beigneux et al., 2006; Ishimoto et al., 2009). The TAG data are further supported by the decrease of lipid droplet accumulation in siSREBP1-transfected GMEC, despite the fact that genes responsible for lipid droplet formation and secretion (*PLIN2*, *PLIN3,* and *XDH*) were not decreased by *SREBP-1* knockdown. These data seem to indicate that decreased expression of *AGPAT6, LPIN1,* and *DGAT2* mainly contribute to the suppression of cellular lipid accumulation. The remarkable decrease of *FABP3*, *ACSL1,* and *SLC27A6* after *SREBP-1* knockdown likely contributed to the decrease of TAG synthesis and lipid droplet accumulation because these genes play crucial roles in fatty acid uptake, activation, an intracellular transport to the esterification pathway. Despite the strong effect of *SREBP-1* knockdown on TAG synthesis, the lack of effect on cholesterol synthesis agrees with its function in rodents (Figure 4D).

A previous study from our laboratory detected a large increase of *SREBP-1* from pregnancy to lactation in goat mammary gland (Xu et al., 2016). Furthermore, overexpression of *SREBP-1* increased the expression of genes related to milk fat synthesis, cellular TAG content, and the amounts of C16:0 and C18:1 (Xu et al., 2016). The results from the present study provide further confirmation of the role of *SREBP-1* in coordinating these metabolic pathways in GMEC. Considering that *SREBP-1* is a master regulator of lipid metabolism and is central in the regulatory network of milk fat synthesis, there might be some compensatory mechanisms to counteract the effects of *SREBP-1* inhibition, hence, partly explaining the modest inhibition of target gene expression. Clearly, more experiments including promoter analysis, western blot, and EMSA or ChIP assays are needed to clarify additional mechanisms controlling milk fat synthesis-related genes in GMEC.

Taken together, the inhibition of *SREBP-1* induced a modest but widespread downregulation of genes related to de novo fatty acid synthesis, LCFA activation and transport, and TAG synthesis. Understanding the regulatory effects of *SREBP-1* on lipid metabolism may contribute to efforts in defining better mechanisms responsible for milk fat synthesis regulation.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

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