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Leucine-Mediated *SLC7A5* Promotes Milk Protein and Milk Fat Synthesis through mTOR Signaling Pathway in Goat Mammary Epithelial Cells

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ABSTRACT: The *SLC7A5* gene encodes a Na^+ and pH-independent transporter protein that regulates cell growth by regulating the uptake of AA. This study, utilizing RNA-seq, aimed to explore the effect of *SLC7A5* on the synthesis of milk proteins and fats in goat mammary epithelial cells (GMECs) through gene interference and overexpression techniques. The results demonstrated that the overexpression of *SLC7A5* resulted in a significant increase in the expression of *CSN1S1*, *SCD*, *CEBPB*, *ACACA*, α_{S1}-casein, p-S6K, and p-S6. The levels of p-S6K and p-S6 gradually increased as the AA/Leu stimulation time lengthened. The overexpression of *SLC7A5* rescued the role of Torin1 in GMECs. In conclusion, *SLC7A5* plays a crucial role in promoting the synthesis of milk proteins and milk fats through the mTOR signaling pathway in GMECs, providing a theoretical foundation for improving the quality of goat milk.

KEYWORDS: *SLC7A5, milk protein and fat, mTOR, dairy goat, mammary epithelial cells*

1. INTRODUCTION

Goat milk is recognized as an essential component of human nutrition due to its superior composition. Notably, goat milk contains higher protein, fat, and mineral contents, with a nutritional profile that closely aligns with human milk.[1] Milk protein in the human diet is an important source of protein that is easily digested and absorbed and contains a wide range of essential amino acids (EAAs) and biologically active components required for the human body. Understanding the mechanism of milk protein synthesis in the mammary gland of dairy goats is of great significance for optimizing the quality and nutritional value of dairy products. The expression of milk proteins and fats in the mammalian mammary gland is regulated by the mTOR and JAK-STAT5 pathways.[2]

Branched-chain amino acids (BCAA), including leucine (Leu), isoleucine (Ile), and valine (Val), represent approximately 40% of the total essential amino acid (EAA) supply to the mammary glands of dairy cows.[3] The activity of mTOR is also regulated by BCAA, particularly Leu.[4] It is suggested that an increase in intracellular Leu enhances translation mainly through enhanced mTOR. The direct stimulatory effect of Leu and other AA on milk protein synthesis has been demonstrated in mouse and bovine mammary epithelial cells (BMECs), with stronger sensitivity observed in bovine.[5,6] Furthermore, the synthesis of milk proteins is highly correlated among various AA.[7] mTOR mediates AA regulation of milk proteins at the transcriptional and translational levels.[8] It is suggested that optimal AA regulation S6K1 and 4EBP1 phosphorylation by promoting mTOR phosphorylation, and thus promoting casein synthesis.[9,10] The availability of AA in the mammary gland is important not only for protein synthesis but also for the rate of AA transport. LAT1, first isolated by Kanai in 1998, encodes a novel Na^+-independent multipass transmembrane protein for neutral amino acids localized to the cell membrane.[11,12] The LAT1 protein encoded by the *SLC7A5* gene forms a disulfide bond with glycoprotein 4F2hc.[13] Functioning as an antiporter system, LAT1 and 4F2hc facilitate the cellular uptake of neutral BCAAs such as Leu, and the efflux of Gln, which is a critical pathway for EAA entry into the cell.[14,15] LAT1-4F2hc regulates intracellular AA flux, playing an essential role in mTOR regulation.[16] $LAT1^{KO}$ cells exhibit disruptions in AA homeostasis both in vitro and in vivo, leading to ATF4 induction, mTORC1 inhibition, and cessation of tumor growth. This clearly indicates that EAA transport is a key limiting step of LAT1 transport activity.[17] The expression of *SLC7A5* may be regulated by the mTORC1 signaling pathway, which eventually affects milk protein synthesis.[18]

Triglycerides (TG) are synthesized in ruminants mainly through the glycerol 3-phosphate process, in which *LPIN1* and *DGAT* play important roles.[19] Studies have shown that mTOR also regulates lipid metabolism.[20] Activation of mTORC1 promotes TG accumulation or inhibits TG catabolism by facilitating fatty acid (FA) elongation and by slowing down catabolic processes such as lipolysis and β-oxidation.[21] Research also suggests that the PI3K-AKT-mTOR signaling

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pathway regulates *SREBF1* and drives cellular lipid synthesis. *SREBF1*, as a transcription factor of lipid metabolism, senses various metabolic signals and can be activated by specific protein hydrolysis processing.[22] Following cleavage, mature *SREBF1* translocates to the nucleus and activates transcription. Target genes include those encoding key enzymes in the adipogenic pathway (such as *ACLY*, *ACACA*, *FASN*, and *SCD*) and genes mediating the gluconeogenesis and pentose phosphate pathways (such as *PKLR*, *PCK1*, *G6PC*, and *G6PD*).[23,24] Insulin has been shown to increase the expression of *SCD* in the liver by activating mTOR and *SREBF1* in chicken embryo hepatocytes and HepG2 cells.[25] It has also been demonstrated that mTORC1 regulates lipid synthesis in the liver by activating S6K1 and triggering the translocation of mature *SREBP1c* from the cytoplasm to the nucleus.[26]

In our previous study, RNA-seq was performed on mammary tissues of dairy goats with low (<3%) and high (>4.5%) milk fat percentage. The results hypothesized *SLC7A5* ($\log_2$[fold change] = 1.55, *P*-value = 1.13×10^{-05}) as a key candidate gene regulating the synthesis of milk components, such as milk fats and proteins, in dairy goats. Investigating amino acid transporters in the mammary gland is crucial for enhancing the content of protein, fat, and other nutrients in milk, thereby improving milk quality. However, limited research exists on the role of *SLC7A5* in milk protein and fat synthesis in dairy goats. Therefore, this study utilized overexpression and interference techniques to analyze the role and mechanism of *SLC7A5* in milk protein and fat synthesis in goat mammary epithelial cells (GMECs).

2. MATERIALS AND METHODS

2.1. Ethical Approval. All of the experimental procedures were performed under the approval of the Institutional Animals Care and Use Committee (IACUC) of Northwest A&F University, China (Approval No. DK2021054).

2.2. Vector Construction. Based on the predicted sequence of goat *SLC7A5* from NCBI (XM_005691915.3), specific cloning primers and enzyme digestion primers (Xho I, *Kpn*I) for *SLC7A5* were designed using Primer Premier 6.0 software and Primer-BLAST on NCBI. To construct the *SLC7A5* overexpression plasmids, pMD19-T-SLC7A5 was used as a plasmid template for polymerase chain reaction (PCR) amplification, and the coding sequence of *SLC7A5* was cloned into the pCMV-C-enhanced green fluorescent protein (EGFP) vector to produce fusion proteins with EGFP (Beyotime Biotechnology, China). Additionally, siRNA was designed based on the CDS sequence of *SLC7A5* using the open-source tool (http://www.detaibio.com/sms2/rest_summary.html). The most efficient siRNA from siRNA-1063 (named siRNA-SLC7A5) and siRNA-1243 was selected for subsequent experiments. The primer sequences are provided in Tables S1 and S2.

2.3. Cell Culture and AA/Leu Treatment. The GMECs used in this study were obtained from our previous research.[27] Briefly, the GMECs were cultured in cell culture dishes with growth medium at 37 °C and 5% CO_2, with the medium being changed every 24 h. The growth medium contained DMEM/F12 (Gibco) and was supplemented with 10% fetal bovine serum (FBS, Gibco), 10 ng/mL epidermal growth factor (EGF, Invitrogen), 5 μg/mL insulin (Sigma-Aldrich, Germany), 5 mg/L hydrocortisone (Sigma-Aldrich, Germany), and 1% penicillin−streptomycin (Pricella, China). Three hours before treatment, the GMECs were switched to serum-free medium, where FBS was replaced with fatty acid-free bovine serum albumin (BSA, 1 g/L, Solarbio, China). The pEGFPC1-SLC7A5 plasmids were used at 50 nmol/L for transient transfection. Overexpression and interference experiments were conducted using Lipofectamine 2000 (Invitrogen) and Lipofectamine RNAiMAX Reagent (Invitrogen), respectively. The GMECs were cotreated with an AA mix and siRNA-SLC7A5 or with Leu (0.8 mmol/L) and siRNA-SLC7A5 for 0, 5, 10, and 15 min, respectively.[28] The AA premix was prepared at the following ratios: arginine 0.60 mmol/L; cystine 0.10 mmol/L; histidine 0.20 mmol/L; isoleucine 0.40 mmol/L; leucine 0.40 mmol/L; lysine 0.40 mmol/L; methionine 0.10 mmol/L; phenylalanine 0.20 mmol/L; threonine 0.40 mmol/L; tryptophan 0.05 mmol/L; tyrosine 0.20 mmol/L; valine 0.40 mmol/L.

2.4. Rescue Assay. For the overexpression assays, GMECs were transfected with pEGFPC1-NC or pEGFPC1-SLC7A5, followed by treatment with dimethyl sulfoxide (DMSO) or Torin1 (2.5 μmol/L) for 48 h, respectively. For the *SLC7A5* interference assay, GMECs were transfected with siRNA-NC or siRNA-SLC7A5 and subsequently treated with DMSO or MHY1485 (10 μmol/L) for 48 h, respectively.

2.5. Western Blot. The total protein from GMECs was lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (PC101, Beyotime, China) supplemented with phenylmethanesulfonyl fluoride (Epizyme Biotech, China) and a phosphatase inhibitor cocktail (Epizyme Biotech, China). The protein concentration was determined using a BCA Kit (Epizyme Biotech, China). A 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel was used to separate proteins, followed by transfer to a polyvinylidene difluoride membrane. The membrane was then blocked with 5% BSA for 1 h at room temperature and subsequently incubated with primary antibodies at 4 °C overnight. The primary and secondary antibodies used in this study are listed in Table S3. Afterward, the membrane was incubated with a secondary antibody for 1 h at room temperature. Blots were visualized using the Omni-ECL Femto Light Chemiluminescence Kit (Epizyme Biotech, China) on a QuickChemi 5200 chemiluminescence imaging system (Monad Biotech, China). All protein levels were analyzed with ImageJ and normalized to β-actin.

2.6. Quantitative Real-Time PCR (RT-qPCR). The total RNA from GMECs was extracted using TRIzol reagent (Invitrogen, CA) according to the manufacturer's instructions. The concentration and integrity of the total RNA were measured using a NanoDrop One spectrophotometer (Thermo Scientific) and agarose gel electrophoresis. First-strand cDNA was synthesized using a PrimeScript RT Kit (Takara, Japan). RT-qPCR assays were performed using an SYBR Premix Ex TaqTM II Kit (Takara, Japan) to determine mRNA expression of genes on a QuantStudio 5 (Applied Biosystems), following the previously published protocols.[29] Gene-specific primers (Table S4) were designed and synthesized by Sangon Biotech (China) and were optimized before initial screening and quantitative analysis.

2.7. BODIPY Staining. BODIPY staining was performed according to previous research.[28] Briefly, cells were fixed for 30 min at 4 °C using 4% paraformaldehyde. After the paraformaldehyde was discarded, 300 μL of BODIPY staining solution (Invitrogen Corporation) was added to each well under light avoidance conditions for an additional 30 min. Subsequently, 200 μL of 4′,6-diamidino-2-phenylindole (DAPI) nuclear staining solution (Gibco) was added.

2.8. Triglyceride Assay. The TG content in GMECs was determined using assay kits from Nanjing Jiancheng Bioengineering Institute according to the manufacturer's procedures (A110-1-1, Jiancheng, China). The results were detected using a microplate reader (Synergy LX, BioTek).

2.9. Statistical Analysis. All data are presented as the mean ± standard error of the mean (SEM) of at least three independent assays. Statistical significance was determined with two-tailed Student's *t* tests or analysis of variance (ANOVA), as indicated in the figure legends, using Statistical Package for the Social Sciences (SPSS) and Prism 9 (GraphPad) software. BODIPY staining was measured three times per sample. Relative gene expression levels were normalized to the endogenous RNA control *RPS9* and *UXT* with the $2^{-\Delta\Delta Cq}$ method. Densitometric quantification of Western blot bands was performed using ImageJ (National Institutes of Health). *P*-values were considered significant as follows: $^*P < 0.05$, $^{**}P < 0.01$, and

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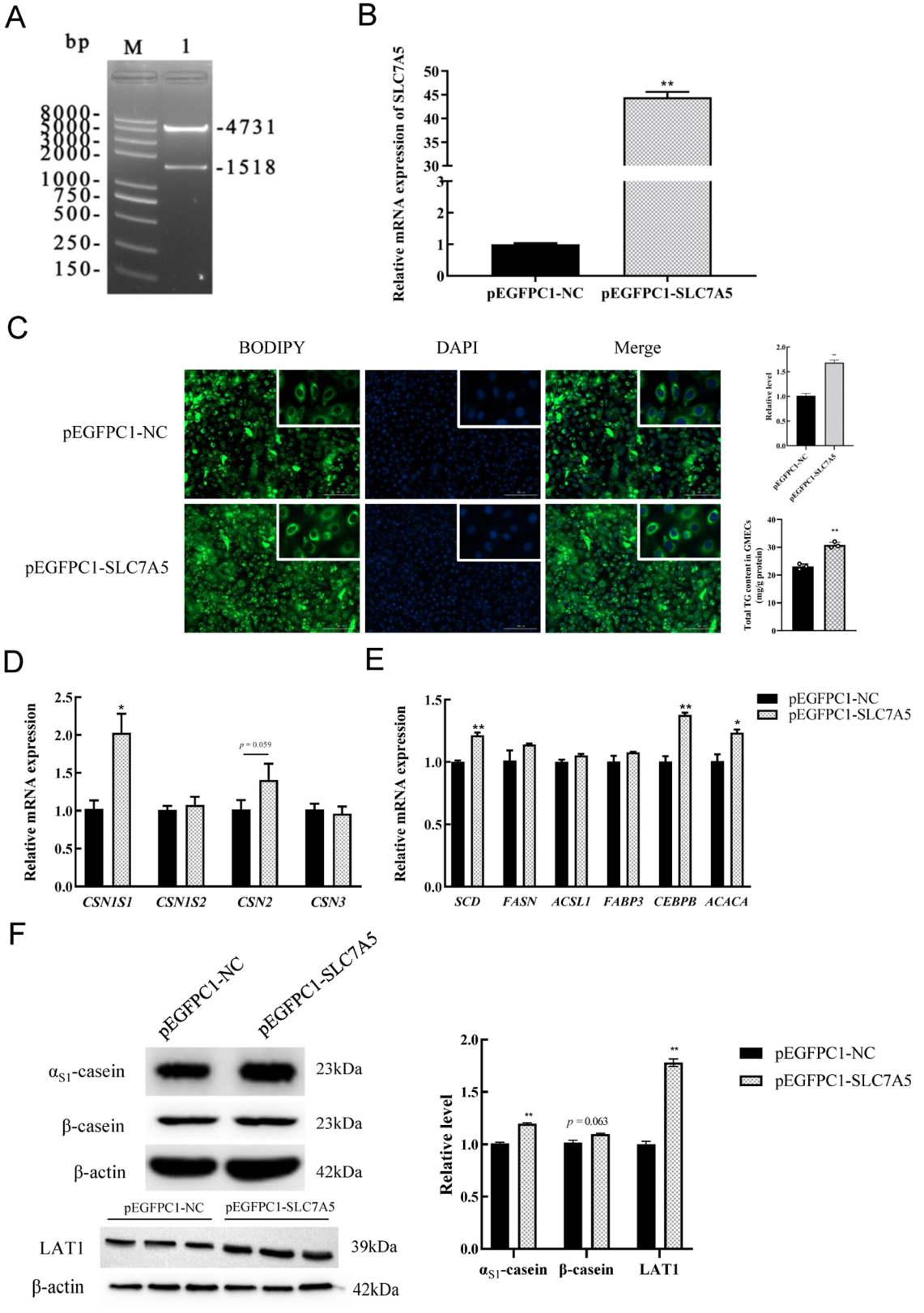

Figure 1. Effect of *SLC7A5* overexpression on milk protein- and fat-related genes in GMECs. (A) Identification of plasmids of pEGFPC1-SLC7A5. Line 1: pEGFPC1-SLC7A5 plasmids digested; M represents marker. (B) Overexpression efficiency of pEGFPC1-SLC7A5 plasmid. (C) Effects of the overexpression of *SLC7A5* on lipid droplet accumulation in GMECs. (D, F) Effect of overexpression with *SLC7A5* on the mRNA expression and protein level of milk protein synthesis genes in GMECs. (E) Effect of *SLC7A5* overexpression on milk fat-related genes in GMECs. The relative mRNA expression was normalized to *RPS9* and *UXT*. The relative protein levels were normalized to *β*-actin and measured thrice per sample. The data are presented as mean ± SEM of three independent experiments (n = 3); *P < 0.05, **P < 0.01, and ***P < 0.001.

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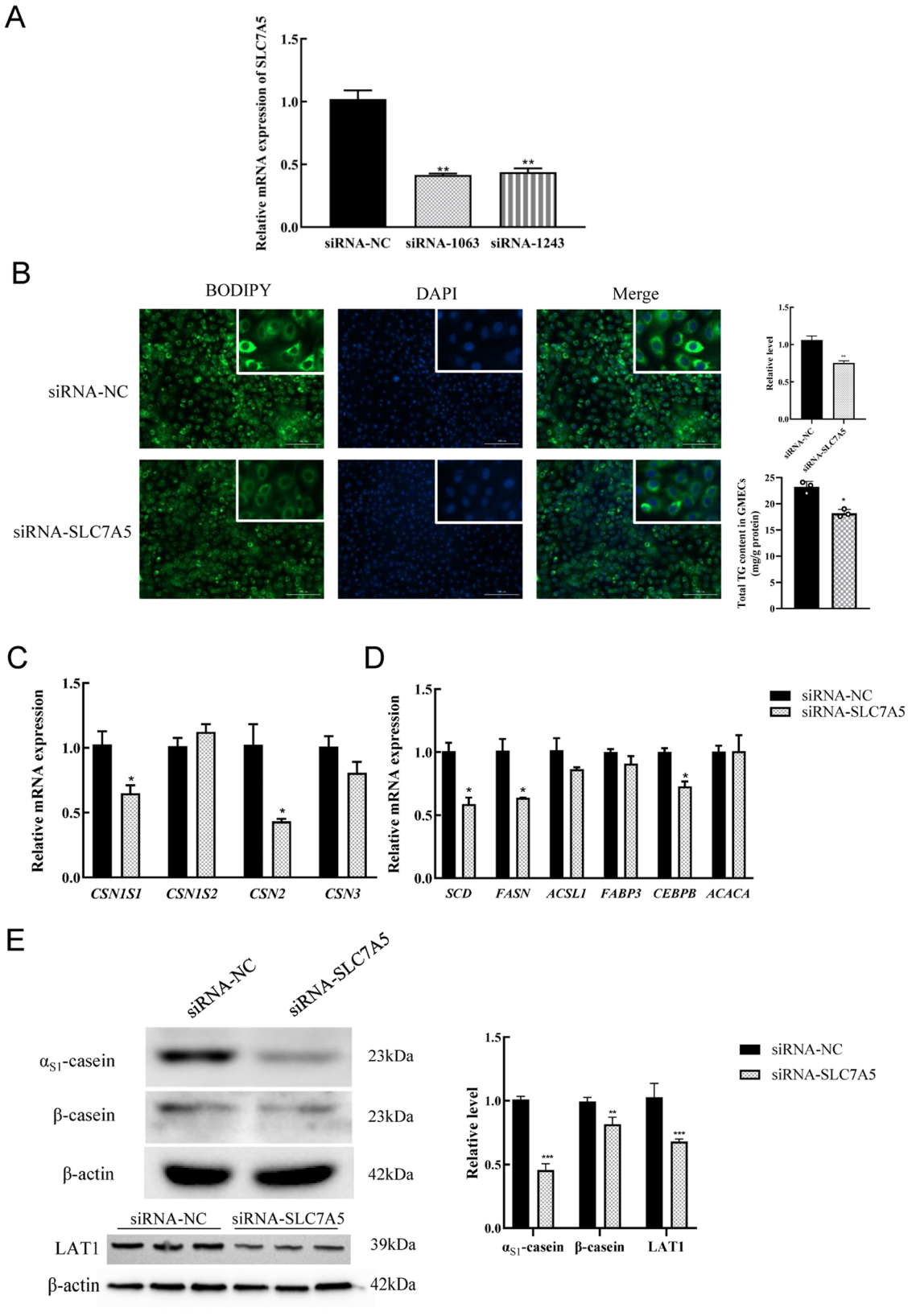

Figure 2. Effects of interference with *SLC7A5* on milk protein- and fat-related genes in GMECs. (A) Detection of the interference efficiency of siRNA on *SLC7A5*. (B) Effects of the interference of *SLC7A5* on lipid droplet accumulation in GMECs. (C, E) Effect of interference with *SLC7A5* on the relative mRNA expression and protein level of milk protein synthesis genes in GMECs. Measured thrice per sample. (D) Effect of *SLC7A5* interference on milk fat-related genes in GMECs. The relative mRNA expression was normalized to *RPS9* and *UXT*. The relative protein levels were normalized to *β*-actin and measured thrice per sample. The data are presented as mean ± SEM of three independent experiments ($n = 3$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

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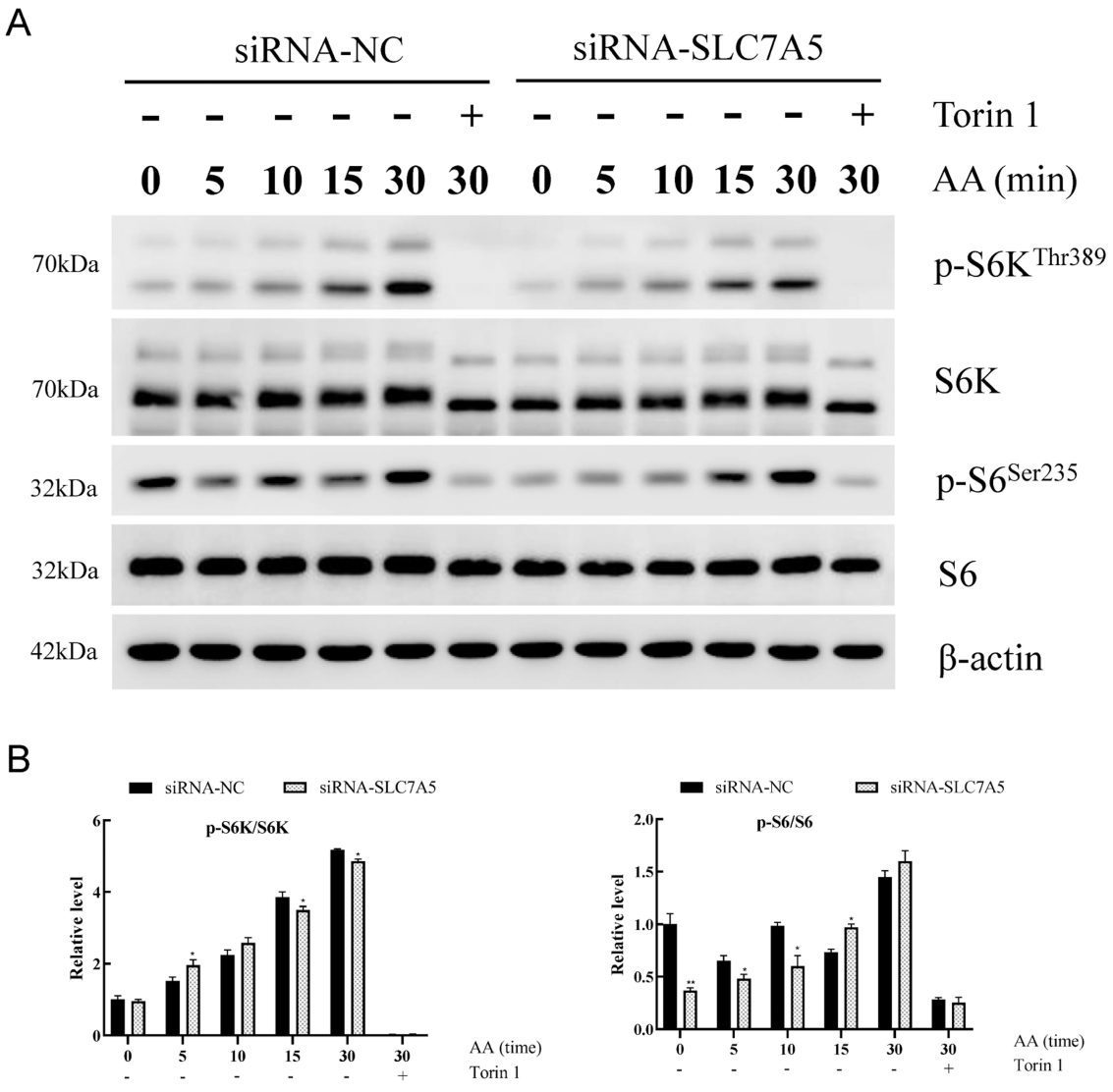

Figure 3. Effects of interference with *SLC7A5* on AA-stimulated mTOR in GMECs. (A) Detection of AA-stimulated mTOR by interference with *SLC7A5*. (B) Visualization analysis of Western blot. The relative protein levels were normalized to β-actin and measured thrice per sample. The data are presented as the mean ± SEM of three independent experiments (n = 3); *P < 0.05, **P < 0.01, and ***P < 0.001.

***P < 0.001. All statistical analyses considered a trend at 0.05 < P < 0.10.

3. RESULTS

3.1. *SLC7A5* Promotes the Expression of Key Genes Involved in Milk Protein and Fat Synthesis in GMECs. To construct the overexpression plasmid for *SLC7A5*, pEGFPC1-SLC7A5 was identified by double enzyme digestion, resulting in band sizes of 4731 bp (pEGFPC1) and 1518 bp (*SLC7A5*), respectively (Figure 1A). To explore the regulatory mechanism of *SLC7A5* on milk protein and fat synthesis, pEGFPC1-NC and pEGFPC1-SLC7A5 plasmids were transferred into GMECs for 24 h. The relative level of *SLC7A5* expression increased significantly (Figure 1B,F). BODIPY staining and the TG content assay ensured consistent cell density and revealed that overexpression of *SLC7A5* significantly increased the accumulation of intracellular lipid droplets (Figure 1C). By detecting the effect of *SLC7A5* on genes related to milk protein and fat synthesis in GMECs, the results exhibited a significant increase in the relative mRNA expression of *CSN1S1*, *SCD*, *CEBPB*, and *ACACA* (P < 0.05), and a trend of increase in *CSN2* (P = 0.059) (Figure 1D,E). Overexpression of *SLC7A5* in GMECs significantly increased the expression of α_{S1}-casein (P < 0.05), and β-casein tended to increase (P = 0.063) (Figure 1F).

3.2. Interference with *SLC7A5* Inhibits the Expression of Key Genes for Milk Protein and Fat Synthesis in GMECs. To explore the regulatory mechanism of *SLC7A5* on milk protein and fat synthesis, two siRNAs were designed and individually transfected into GMECs for 24 h. The siRNA-1063 (named as siRNA-SLC7A5) was selected as the most efficient for subsequent experiments based on the results (Figure 2A). BODIPY staining and TG content assay ensured consistent cell density and revealed that interference with *SLC7A5* significantly decreased the level of accumulation of intracellular lipid droplets (Figure 2B). By detecting the effect of *SLC7A5* on genes related to lipid metabolism in GMECs, we observed a significant decrease in the relative mRNA expression of *CSN1S1*, *CSN2*, *SCD*, *FASN*, and *CEBPB* (P < 0.05) (Figure 2C,D). Interference with *SLC7A5* in GMECs significantly decreased the expression of α_{S1}-casein, β-casein, and LAT1 (P < 0.05) (Figure 2E).

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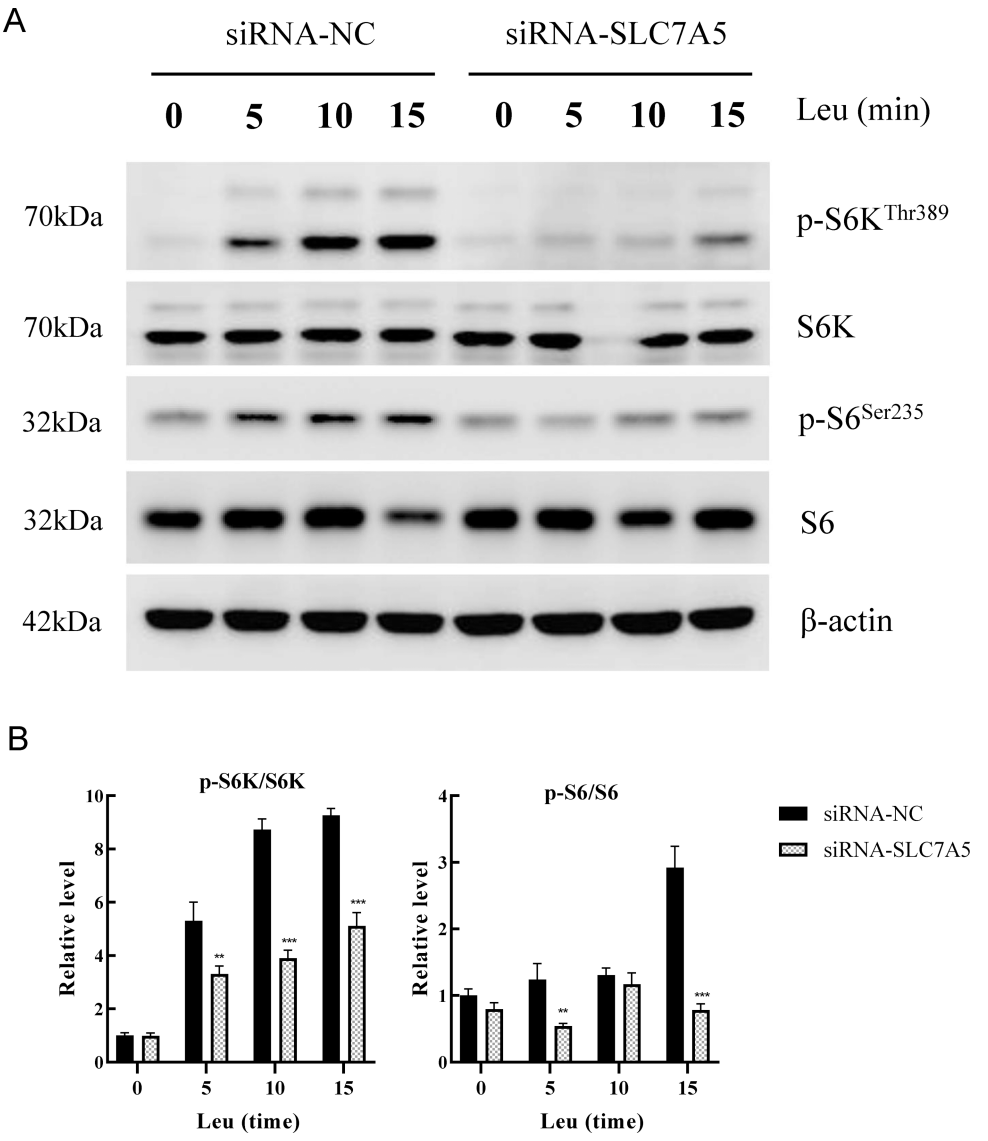

Figure 4. Effects of interference with *SLC7A5* on Leu-stimulated mTOR in GMECs. (A) Detection of Leu-stimulated mTOR by interference with *SLC7A5*. (B) Visualization analysis of Western blot. The relative mRNA expression was normalized to *RPS9* and *UXT*. The relative protein levels were normalized to *β*-actin and measured thrice per sample. The data are presented as the mean ± SEM of three independent experiments ($n = 3$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

3.3. Leucine Promotes Protein Synthesis through *SLC7A5* Activation of mTOR in GMECs. After siRNA-NC or siRNA-SLC7A5 was transferred into GMECs for 24 h, the GMECs were starved with AA-free Dulbecco's modified Eagle's medium (DMEM) for 3 h, followed by the addition of AA for 0, 5, 10, 15, and 30 min, respectively.

Results as shown in Figure 3A,B, compared to the NC and AA cotreated GMECs, the relative level of p-S6K/S6K significantly decreased at 15 and 30 min with siRNA-SLC7A5 and AA cotreatment, and the relative level of p-S6/S6 significantly decreased at 0, 5, and 10 min with the siRNA-SLC7A5 and AA cotreatment ($P < 0.05$). Moreover, the relative level of p-S6K/S6K gradually increased with the prolongation of AA stimulation. The relative level of p-S6K/S6K significantly decreased in the siRNA-SLC7A5 group compared to the siRNA-NC group at the same time with AA treatment. Compared to AA treatment of GMECs at 30 min, when Torin1 (the mTOR pathway inhibitor) was simultaneously added to the AA, the levels of p-S6K/S6K and p-S6/S6 significantly decreased ($P < 0.05$).

To explore the effect of leucine on protein synthesis, GMECs were treated with leucine for 0, 5, 10, and 15 min, respectively, as described above. The results are shown in Figure 4A,B. Compared to the siRNA-NC and Leu cotreated GMECs, the relative level of p-S6K/S6K were significantly decreased at 5, 10, and 15 min with siRNA-SLC7A5 and Leu cotreatment, and the relative level of p-S6/S6 were significantly decreased at 5 and 15 min with siRNA-SLC7A5 and Leu cotreatment ($P < 0.05$). Furthermore, with the prolongation of Leu treatment, the relative levels of p-S6K/S6K and p-S6/S6 gradually increased.

3.4. *SLC7A5* Promotes Casein Synthesis through mTOR in GMECs. A rescue assay was carried out to investigate the mTOR-dependent regulation of milk proteins by *SLC7A5*. As shown in Figure 5A, compared to control, the relative mRNA expression of *CSN2* was significantly decreased when Torin1 was used to treat the GMECs ($P < 0.05$), and

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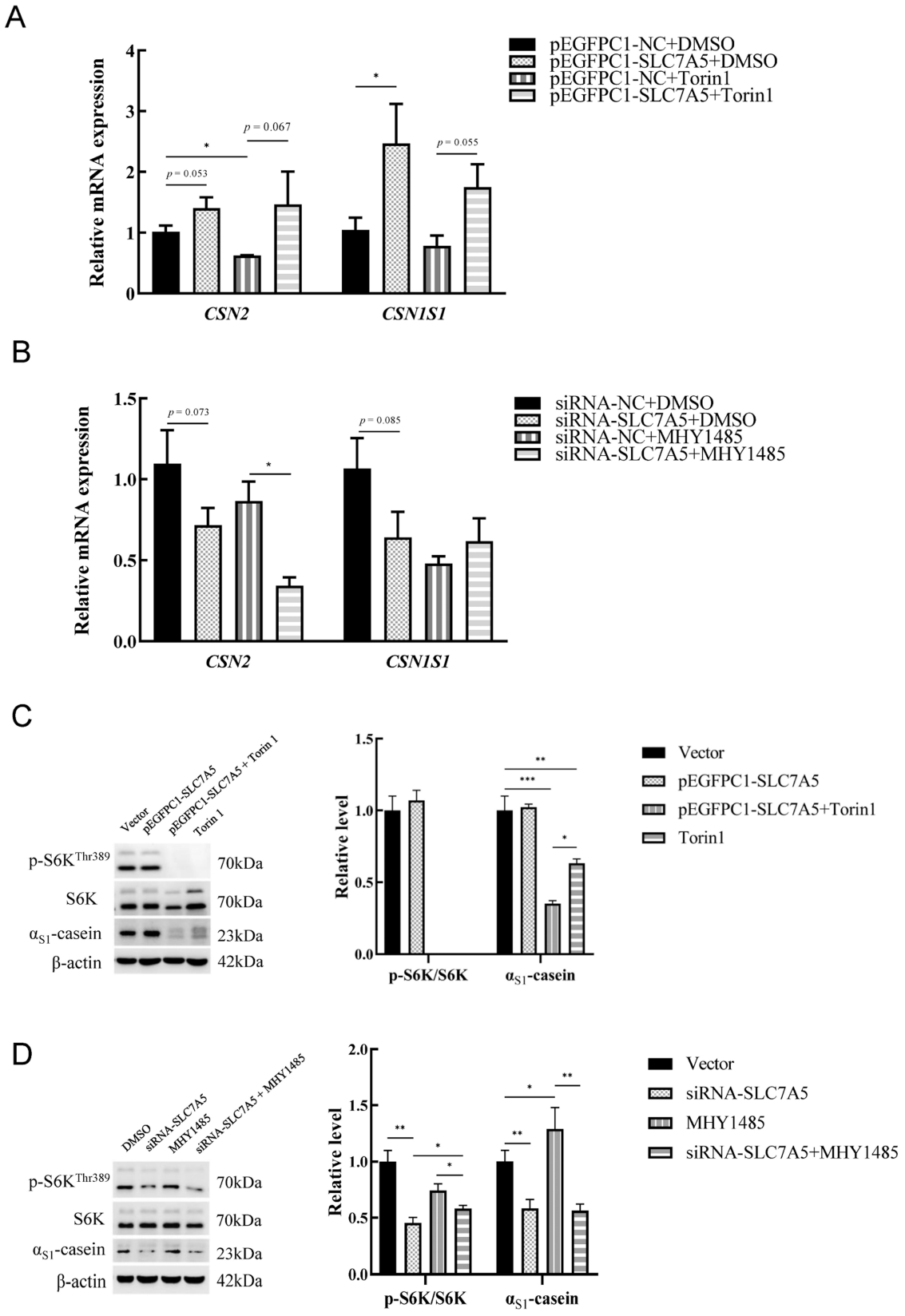

Figure 5. *SLC7A5* regulates the relative mRNA expression and protein level of casein synthesis through mTOR. (A) pEGFPC1-SLC7A5 and Torin1 were cotreated to detect the mRNA expression levels of *CSN2* and *CSN1S1*. (B) siRNA-SLC7A5 and MHY1485 were cotreated to detect the relative mRNA expression of *CSN2* and *CSN1S1*. (C) pEGFPC1-SLC7A5 and Torin1 were cotreated to detect the levels of S6K, p-S6K, and α_{S1}-casein. (D) siRNA-SLC7A5 and MHY1485 were cotreated to detect the levels of S6K, p-S6K and α_{S1}-casein. The relative mRNA expression was normalized to *RPS9* and *UXT*. The relative protein levels were normalized to β-actin and measured thrice per sample. The data are presented as the mean ± SEM of three independent experiments (n = 3); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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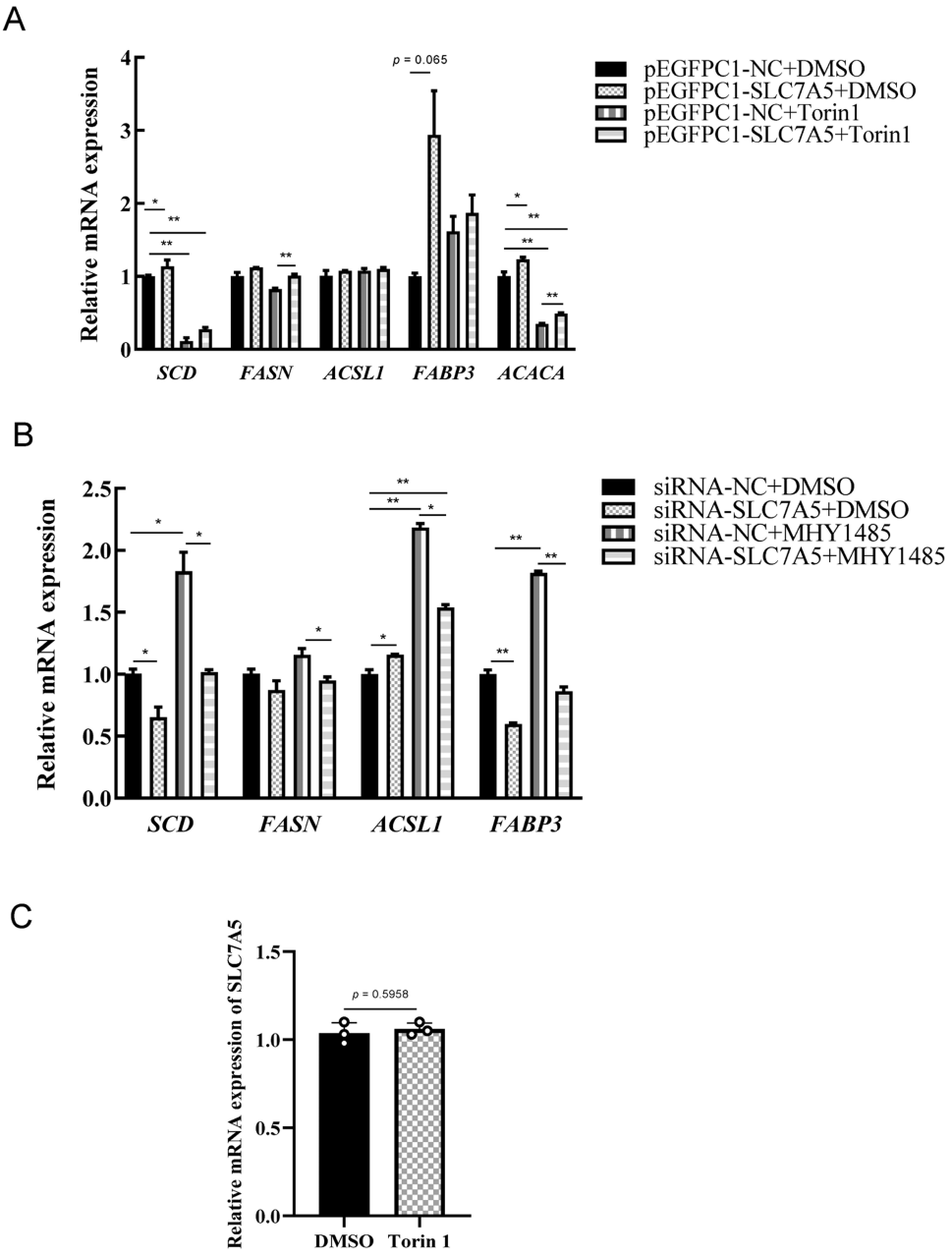

Figure 6. *SLC7A5* regulates the expression of milk fat-related genes through mTOR. (A) pEGFPC1-SLC7A5 and Torin1 were cotreated to detect the relative mRNA expression. (B) siRNA-SLC7A5 and MHY1485 were cotreated to detect the relative mRNA expression. (C) Torin1 did not significantly affect the relative mRNA expression of *SLC7A5*. The relative mRNA expression was normalized to *RPS9* and *UXT*. The data are presented as the mean ± SEM of three independent experiments (n = 3); *P < 0.05, **P < 0.01, and ***P < 0.001.

when the GMECs were cotreated with pEGFPC1-SLC7A5 and Torin1, the relative expression of *CSN2* returned to the control level. The trend in the relative expression of *CSN1S1* was similar to that of *CSN2*. As shown in Figure 5B, interference with *SLC7A5* in GMECs tended to decrease the relative mRNA expression of *CSN2* (P = 0.073). When MHY1485 (the mTOR pathway activator) was used to treat GMECs, interference with *SLC7A5* resulted in a decrease in *CSN2* and did not restore it to normal. In addition, variation in the relative expression of *CSN1S1* was insensitive to MHY1485.

Western blot demonstrated that the promotion of α_{S1}-casein synthesis by *SLC7A5* in GMECs cotreated with pEGFPC1-SLC7A5 and Torin1 was decreased with the relative level of p-S6K and p-S6 (Figure 5C). As shown in Figure 5D, interference with the *SLC7A5* significantly decreased the relative level of α_{S1}-casein (P < 0.05). However, when GMECs were cotreated with MHY1485 and siRNA-SLC7A5, the relative level of p-S6K significantly increased, while the relative level of α_{S1}-casein did not.

3.5. *SLC7A5* Regulates Milk Fat Synthesis through mTOR in GMECs. A rescue assay was carried out to investigate the mTOR-dependent regulation of milk fats by *SLC7A5*. As shown in Figure 6A, the relative expression of *SCD* and *ACACA* significantly decreased when GMECs were treated with Torin1 (P < 0.05). When pEGFPC1-SLC7A5 and Torin1 were used to cotreated GMECs, the inhibitory effect of Torin1 on the relative expression of *SCD*, *ACACA*, and *FASN* was attenuated. As shown in Figure 6B, interference with

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SLC7A5 in GMECs significantly decreased the relative expression of *SCD* and *FABP3*. When GMECs were treated with pEGFPC1-SLC7A5 and MHY1485, the relative expression of *SCD* and *FABP3* was slightly restored.

4. DISCUSSION

The results of this study suggest that overexpression of *SLC7A5* significantly increases the relative mRNA expression of *CSN1S1* and the levels of α_{S1}-casein. Conversely, interference with *SLC7A5* led to a decrease in the relative mRNA expression of *CSN1S1* and the levels of α_{S1}-casein. Elevated expression of *SLC7A5* facilitated the translocation of sufficient AA within cells, promoting milk protein synthesis and secretion. The positive regulation of milk proteins by *SLC7A5* aligns with the findings of Lin et al. in lactating Holstein cows.[30] Previous research has demonstrated a significant increase in β-casein expression in BMECs overexpressing LAT1, another key player in AA transport.[30] In the present study, overexpression of *SLC7A5* in GMECs significantly increased the level of α_{S1}-casein, with a noticeable trend toward an increase in β-casein ($P = 0.063$).

Studies on *SLC7A5* have predominantly focused on milk proteins with fewer studies examining milk fats. The present study revealed that overexpression of *SLC7A5* significantly increased the relative mRNA expression of *SCD*, *CEBPB*, and *ACACA*, and the accumulation of lipid droplets. In contrast, interference with *SLC7A5* significantly decreased the relative mRNA expression of *SCD*, *FASN*, and *CEBPB*, and the accumulation of lipid droplets. Consequently, *SLC7A5* appears to positively regulate milk fats. The expression of *SREBF1* target genes, such as *FASN*, *ACACA*, and *SCD*, was decreased by the inhibition of mTORC1, indicating the essential role of mTORC1 in fatty acid biosynthesis.[31] Studies have revealed that activation of the AKT/mTOR pathway, particularly *MIEF2*, enhances de novo fatty acid synthesis by upregulating the expression of *SREBF1* and its transcriptional target lipogenic genes *ACC1*, *FASN*, and *SCD1*.[32] Therefore, this study aimed to explore the possibility that *SLC7A5* may affect protein and lipid synthesis in GMECs through the mTOR pathway.

The activation of mTOR requires AA. Deprivation of AA completely abolishes mTORC1 activity, while the addition of AA activates mTOR, recruiting it to the surface of the lysosomes. The lysosomal surface mTORC1 activator, Rheb, binds to and activates mTORC1. Consequently, phosphorylation of mTORC1 facilitates downstream phosphorylation of S6K1 and S6, initiating translation.[9,33] The exchange of intracellular Gln with extracellular Leu triggers activation of mTORC1. The efficiency of the LAT1-4F2hc heterodimer in transporting EAA is a limiting factor for mTORC1 activation by EAA and LAT1 acts upstream of the mTOR pathway.[34,35] In mammalian cells, mTORC1 plays an essential role in the phosphorylation of S6K1 and S6.[36] Previous studies have shown increased expression of *SLC7A5* in rodent milk protein synthesis.[37] Research on dairy cows has revealed an upregulation of the level of *SLC7A5* expression during lactation. However, no significant differences were found in the mTOR-independent AA transporter protein (including *SLC1A1* and *SLC7A1*). Thus, *SLC7A5*, regulated by mTORC1, is involved in milk protein synthesis.[18]

In the present study, interference with *SLC7A5* resulted in a significant inhibition of mTOR activation and a decrease in the levels of p-S6K and p-S6 during AA stimulation of GMECs under the same conditions. As the duration of AA stimulation increased, both groups showed a gradual increase in p-S6K and p-S6 levels, but the stimulated intensity in the interference with *SLC7A5* was weaker than that in the control group. This phenomenon could be attributed to the reduced LAT1 levels, leading to a decrease in the AA update per unit of time, subsequently reducing the phosphorylation of mTOR and its downstream factors. In the rescue assay, the inhibition of mTOR activity using Torin1 weakened the promotion of milk protein synthesis by *SLC7A5*, whereas the activation of mTOR using MHY1485 failed to rescue the inhibitory effect of interference with milk protein synthesis by *SLC7A5*. In addition, probably due to the off-target effects, the role of MHY1485 did not meet expectations.[38] Indeed, studies suggest that *SLC7A5* and mTOR can form a feedback loop that *SLC7A5* is a transporter mediating the import of leucine, which activates the AKT/mTOR pathway; on the other hand, *IGF2BP2*-mediated upregulation of *SLC7A5* expression through AKT/mTOR pathway activation.[39] This observation suggests that the lack of AA limits the synthesis of milk protein. Therefore, this study speculates that the upregulation of the *SLC7A5* gene in GMECs enhances the expression of α_{S1}-casein and β-casein by regulating the uptake of AA, especially leucine, and subsequently activating mTOR.

Lipid metabolism includes the biosynthesis and degradation of lipids, including fatty acids, TG, and total cholesterol (TC). In ruminants, fatty acids (FA) serve as the basic substances for TG synthesis. FA in milk is derived from the blood and synthesized in the epithelial cells of the mammary gland, primarily through acetic and butyric acids.[2,40] Acetate, a major short-chain fatty acid due to rumen fermentation, stimulates milk fat synthesis in lactating dairy cows by increasing de novo FA synthesis in a dose-dependent manner.[41] It has been suggested that mTORC1 stimulates FA synthesis in breast cancer cell lines through activation of *SREBF1*.[42] Acetate, which regulates milk fat synthesis through the mTOR/eIF4E pathway, can be effectively inhibited by rapamycin, emphasizing the essential role of mTOR in fat synthesis.[43] In the current study, inhibition of mTOR activity using Torin1 inhibited the promotion of *SCD* and *FASN* by *SLC7A5*, whereas activation of mTOR using MHY1485 rescued the interference of *SLC7A5*, alleviating its inhibitory effect on *SCD* and *FASN*. However, the inconsistent effects of *SLC7A5* on *ACACA* and *FASN* may be explained by the different sensitivities of the two enzymes to intracellular energy metabolism. The overexpression of *SLC7A5* promotes the entry of a large number of BCAAs into cells, and leucine can be converted into acetyl-CoA. This not only affects lipid metabolism but also promotes the tricarboxylic acid cycle, which promotes the mRNA expression of *ACACA*. Although *FASN* is positioned downstream in fatty acid synthesis, its expression increase is not significant. Interference with *SLC7A5* affects the entry of BCAAs into GMECs and the activation of mTOR, and therefore affects fatty acid elongation in the first place. In addition, studies have shown that rapamycin-induced inhibition of mTOR downregulates the relative mRNA expression of *SREBF1*, *ACACA*, *FASN*, and *SCD*, as well as PPARγ activity in various cell types in mice and humans.[44,45] Therefore, *SLC7A5* likely regulates the relative mRNA expression of *SCD* and *FASN* through activation of mTOR, thereby influencing the synthesis of milk fats. In summary, our findings indicate that *SLC7A5* promotes the synthesis of milk proteins and milk fats in GMECs by positively regulating

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mTOR and affecting the relative mRNA expression of *CSN1S1*, *CSN2*, *SCD*, and *FASN*.

In conclusion, *SLC7A5* acts as a positive regulator of milk protein and fat synthesis in GMECs. The overexpression of *SLC7A5* upregulates the expression of genes and proteins associated with casein and lipid synthesis. Moreover, *SLC7A5* activates mTOR in response to AA stimulation, particularly Leu, thereby promoting the synthesis of milk proteins and fats in GMECs. The present study lays a theoretical foundation for improving the quality of goat milk.

■ ASSOCIATED CONTENT

Data Availability Statement

The data sets used or analyzed during the present study are available from the corresponding author upon reasonable request.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c02087.

Design of SLC7A5 restriction primers (Table S1); design of SLC7A5-specific siRNA sequences online (Table S2); antibodies information (Table S3); and primer pairs for RT-qPCR analysis (Table S4) (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AA, amino acid; Leu, leucine; GMECs, goat mammary epithelial cells; BCAA, branched-chain amino acid; BMECs, bovine mammary epithelial cells; LAT1, large neutral amino acid transporter small subunit 1; 4F2hc, 4F2 cell surface antigen heavy chain; RT-qPCR, quantitative real-time PCR; DMSO, dimethyl sulfoxide; siRNA, small interfering RNA; TG, triglyceride; S6K, ribosomal protein S6 kinase B1; S6, ribosomal protein S6; *SLC7A5*, solute carrier family 7 member 5; *CSN1S1*, casein α s1; *CSN2*, casein β; *FABP3*, fatty acid binding protein 3; *ACACA*, acetyl-CoA carboxylase α; *SCD1*, stearoyl-CoA desaturase; *ACSL1*, acyl-CoA synthetase long-chain family member 1; *CEBPB*, CCAAT enhancer binding protein β; *RPS9*, ribosomal protein S9; *UXT*, ubiquitously expressed prefoldin like chaperone; FA, fatty acid; TC, total cholesterol

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