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The serine protease 2 gene regulates lipid metabolism through the *LEP/ampkα1/SREBP1* pathway in bovine mammary epithelial cells

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ABSTRACT

Molecular breeding has brought about significant transformations in the milk market and production system during the twenty-first century. The primary economic characteristic of dairy production pertains to milk fat content. Our previous transcriptome analyses revealed that serine protease 2 (*PRSS2*) is a candidate gene that could impact milk fat synthesis in bovine mammary epithelial cells (BMECs) of Chinese Holstein dairy cows. To elucidate the function of the *PRSS2* gene in milk fat synthesis, we constructed vectors for *PRSS2* overexpression and interference and assessed intracellular triglycerides (TGs), cholesterol (CHOL), and nonesterified fatty acid (NEFA) contents in BMECs. Fatty acid varieties and components were also quantified using gas chromatography–mass spectrometry (GC–MS) technology. The regulatory pathway mediated by *PRSS2* was validated through qPCR, ELISA, and WB techniques. Based on our research findings, *PRSS2* emerges as a pivotal gene that regulates the expression of associated genes, thereby making a substantial contribution to lipid metabolism via the leptin (*LEP*)/Adenylate-activated protein kinase, alpha 1 catalytic subunit (*AMPKα1*)/sterol regulatory element binding protein 1(*SREBP1*) pathway by inhibiting TGs and CHOL accumulation while potentially promoting NEFA synthesis in BMECs. Furthermore, the *PRSS2* gene enhances intracellular medium- and long-chain fatty acid metabolism by modulating genes related to the *LEP/AMPKα1/SREBP1* pathway, leading to increased contents of unsaturated fatty acids C17:1N7 and C22:4N6. This study provides a robust theoretical framework for further investigation into the underlying molecular mechanisms through which *PRSS2* influences lipid metabolism in dairy cows.

1. Introduction

Milk is preferred by many consumers because of its rich nutritional value, health benefits, and good taste. Fat is the main energy substance in milk and is mainly composed of triglycerides (TGs), phospholipids, and sterols. Milk fat is mainly dispersed in milk in the form of milk fat globules (Fleming, Schenkel, Koeck, Malchiodi, Ali, Corredig, Mallard, Sargolzaei and Miglior [1]. TGs make up approximately 98 % of milk fat, making them the primary component of milk fat. As the main storage form of energy metabolism, triglycerides mainly combine with apolipoprotein plasma and are secreted in the form of chylous particles from mammary epithelial cells to the acinar cavity to form milk fat [2,3]. Cholesterol (CHOL), which is approximately 0.2–0.4 % of milk, is the most important steroid compound in the animal body and the precursor of physiologically active substances such as bile acids, steroid hormones and vitamin D3 synthesized by animals. Cholesterol metabolism mainly uses acetyl-CoA as a raw material and participates in the citric acid cycle [4]. Fatty acids (FA) account for approximately 0.1 % of milk fat and play an important role in organisms. It can form phospholipids and glycolipids, regulate the processes of cell and fat metabolism, ensure the precise release of free fatty acids, and regulate intracellular processes at several levels, such as transcriptional activation and protein post-transcriptional modification [5–7]. However, the biosynthesis of milk fat is a complex process regulated by multiple functional genes, and changes in functional gene expression directly affect differences in traits and phenotypes [8].

Adenylate-activated protein kinase (*AMPK*), a heterotrimeric complex that contains a catalytic α subunit and regulatory β and γ subunits, is crucial for controlling the balance of cellular energy. AMPK inhibits ATP-consuming biosynthetic activities such as gluconeogenesis, lipid

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synthesis, and protein synthesis [9,10]. Sterol regulatory element binding protein 1(*SREBP1*) is a member of the superfamily of fundamental helix-loop-helix leucine zipper transcription factors [11]. *AMPK* can attenuate the expression of the transcription factor *SREBP1* to induce fat oxidation, synthesis, and transcription in cells [12–14]. Leptin (*LEP*) is an endogenous protein hormone that is mainly synthesized and secreted by white adipocytes. It can also be synthesized and secreted in large quantities in breast epithelial cells and naturally exists in milk [15]. *LEP* plays a key role in regulating food intake, energy consumption and neuroendocrine function, which could also stimulate fatty acid oxidation and glucose uptake and prevent the accumulation of lipids in nonadipose tissues. Leptin promotes lipid oxidation [16] and TG storage [9,17] by regulating AMPK signalling in peripheral tissue, which suggests that there might be a connection between *AMPK* and *LEP* through direct or indirect pathways.

The bovine serine protease 2 (*PRSS2*) gene (ENSBTAT00000028731.5) is located on chromosome 4: 106, 071, 878–106, 075, 654 with one transcript. The DNA sequence of *PRSS2* contains a total of 3780 bases and has 5 exons and 4 introns. The *PRSS2* mRNA sequence contains a coding region of 808 bp, and the open reading frame encodes 247 amino acids. Although the *PRSS2* gene is conserved among different species, its expression levels vary in different tissues or cells. *PRSS2* is a multifunctional gene that participates in various metabolic processes involved in physiological and cellular activities, including substance absorption and digestion, blood solidification, cells and processes of humoral immunity, fibrin dissolution, fertilization, embryonic development, protein processing and tissue remodelling [18–20]. The study found that the ATPase H^+ transporting accessory protein 2 *(ATP6AP2)* and glucagon-like peptide 1 receptor (*GLP1R*) genes jointly regulate the glucagon-like peptide 1 (*GLP1*) gene to promote insulin secretion, leading to the expression of the *PRSS2* gene being significantly downregulated, suggesting that *PRSS2* may participate in glucose absorption and insulin secretion [21].

However, the function of *PRSS2* in regulating lipid metabolism has not been reported. Our previous transcriptome analysis showed that *PRSS2* is a gene that is differentially expressed in the bovine mammary epithelial cells (BMECs) of Chinese Holstein dairy cows that produce high-fat and low-fat milk [22,23]. This result suggested that *PRSS2* may be an important candidate gene involved in milk lipid metabolism. Thus, we aimed to explore the biological function of the *PRSS2* gene in the present study, which is possible and valuable. In particular, our experiment focused on the effects of *PRSS2* in BMECs lipid metabolism and its regulatory pathways to provide the basic theory for further study of related functional genes.

2. Materials and methods

2.1. Constructing the interference vector pb7SK-shPRSS2

According to the *PRSS2* gene sequence on the NCBI website, four (4) oligo primers were designed for the target gene (Table 1). The primers were annealed at 95 °C for 5 min and then ligated to the pb7SK/GFP/Neo vector (GenePharma, C02007, Shanghai, China). The 10 μL ligation system consisted of 1 μL of 350 U/μL T_4 ligase (Takara, Beijing, China), 1 μL of 10 × T_4 ligase buffer, 4 μL of 100 ng/μL annealing product, and 4 μL of 500 ng/μL pb7SK-GFP-Neo vector and was incubated at 16 °C for 5 h.

2.2. Constructing of the overexpression vector pBI-CMV3-PRSS2

PCR cloning was performed using the CDS region sequence of *PRSS2* in a 20 μL cloning system: 10 μL of 2 × GreenMix, 1 μL of 1 μg cDNA template, 0.4 μL of 10 μM forward primer (5′-CGGGATCCATGCATCCCCTGCTGAT-3′), 0.4 μL of 10 μM reverse primer (5′-CCCAAGCTTTCAGCTGTTGGCGGC-3′), and 8.2 μL of ddH_2O. The 744 bp PCR product was validated by DNA sequencing and ligated into the pBI-CMV3 plasmid (Clontech, 631,632, USA) to generate the pBI-CMV3-PRSS2 expression vector.

2.3. Transfection of plasmids into BMECs

BMECs were provided by our laboratory [24,25]. Cells were cultured in 10 cm culture plates (Falcon, 353,003, Franklin, Lake, NJ, USA) in DMEM/F12 (Corning, New York, USA) supplemented with 10 % foetal bovine serum (Tian Hang, 11,011–6123, Zhejiang, China), 25 μL hydrocortisone (25 μg/mL, ab141250, Abcam, Cambridge, UK) and 25 μL insulin (0.25 EU/mg, I0305000, Sigma, USA) with 1 % antibiotics (HyClone, SV30010, Logan, USA), which were inoculated into 6-well culture plates (Falcon, 353,003, Franklin, Lake, NJ, USA) with a density of 1 × 10^5 cells in an incubator. When the cell density reached 70 % ~80 % confluence, 3 μg of plasmid DNA, 6 μL of transfection reagent, and 200 μL of DMEM/F12 were mixed for 15 min and transfected into the cells following the manufacturer's protocol (ViaFectTM Transfection Reagent). The tests were repeated three times by transfecting an equal number of cells in each well using identical vectors.

2.4. RNA extraction and RT-qPCR

The total RNA from the cells in the six-well plates was extracted with the TaKaRa MiniBEST Universal RNA Extraction Kit (Takara, Beijing, China) following the manufacturer's protocol. One microgram of total RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Beijing, China). According to TB Green® Premix Ex Taq™II (Tli RNaseH Plus) (Takara, Beijing, China), the fluorescence quantification of each sample cell was performed on a PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Beijing, China). The relative expression levels of the genes were calculated using the $2^{-\Delta\Delta Ct}$ method [25–27]. The list of primers for RT-qPCR used is shown in Table 2.

2.5. Protein extraction and western blotting

After the cells were transfected for 48 h, they were washed with prechilled phosphate-buffered saline (PBS, Corning, 21-040-CVR, China) 2–3 times, collected in a centrifuge tube, and incubated for 15–20 min on ice using lysis buffer containing protease and phosphatase inhibitors (Dalian Meilun Biotech, Dalian, China). The samples were centrifuged at 4 °C and 12,000 rpm for 15 min, and the supernatants

Table 1
The target sequence and annealed DNA oligo sequence of *PRSS2* shRNA.

Name	Target sequence (5′-3′)	DNA oligo sequence (5′-3′)
pb7SK-PRSS2-shRNA1	GCGGAGAATTCTGTCCCTTAC	AGAGGCGGAGAATTCTGTCCCTTACTCAAGAGGTAAGGGACAGAATTCTCCGCTTTTTTG GATCCAAAAAAGCGGAGAATTCTGTCCCTTACCTCTTGAGTAAGGGACAGAATTCTCCGC
pb7SK-PRSS2-shRNA2	GGCTGGGAGAATACAACATTG	AGAGGGCTGGGAGAATACAACATTGTCAAGAGCAATGTTGTATTCTCCCAGCCTTTTTTG GATCCAAAAAAGGCTGGGAGAATACAACATTGCTCTTGACAATGTTGTATTCTCCCAGCC
pb7SK-PRSS2-shRNA3	GCTGGGAGAATACAACATTGA	AGAGGCTGGGAGAATACAACATTGATCAAGAGTCAATGTTGTATTCTCCCAGCTTTTTTG GATCCAAAAAAGCTGGGAGAATACAACATTGACTCTTGATCAATGTTGTATTCTCCCAGC
pb7SK-PRSS2-shRNA4	GGGAGAATACAACATTGATGT	AGAGGGGAGAATACAACATTGATGTTCAAGAGACATCAATGTTGTATTCTCCCTTTTTTG GATCCAAAAAAGGGAGAATACAACATTGATGTCTCTTGAACATCAATGTTGTATTCTCCC

Table 2
List of primers for RT-qPCR used in this study.

Gene symbol	mRNA sequence number	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product size (bp)
PRSS2	ENSBTAT00000028731.5	CTGAGCCACGCCGACTGT	TTCTGGGCACAGCCGTAG	173
HSL	NM_001080220.1	GATGAGAGGGTAATTGCCG	GGATGGCAGGTGTGAACT	100
SCD1	NM_173959.4	ATTTATCCGACCTAAGAGCCG	CGTTTCATCCCACAGATACCAT	122
ACSS2	NM_001105339.1	GCTCCACAGGCAAACCCAA	CACATCCTCCGCATGAAAGTC	104
GSK3B	NM_001101310.1	GCTACCATCCTTATTCCTCCTCA	TTATTGGTCTGTCCACGGTCTC	110
Cpt1A	NM_001304989.2	CGAGAGGGCAGAACGGAGA	CCACGTAGAGGCAGAAGAGGTG	196
leptin	NM_173928.2	CTCATCAAGACAATTGTCACCAGG	CAGCTGCCGCAACATGTCCTG	383
AMPKα1	NM_001109802.2	CCCGTATTATTTGCGTGTTCG	TCTGTGGCGTAGCAGTCCCT	161
SREBP1	NM_001113302.1	CTGTCCACAAAAGCAAATCGC	ACTTCCACCGCTGCTACTGC	191
β-actin	NM_173979.3	AGAGCAAGAGAGGCATCC	TCGTTGTAGAAGGTGTGGT	102

Note: *PRSS2*, serine protease 2; *HSL*, hormone-sensitive lipase; *SCD1*, stearoyl-coenzyme A desaturase 1; *ACSS2*, acyl-CoA synthetase short chain family member 2; *GSK3B*, glycogen synthase kinase 3 beta; *CPT1A*, carnitine palmitoyltransferase 1 A; *LBP*, leptin; *AMPKα1*, AMP-activated catalytic subunit alpha 1; *SREBP1*, sterol regulatory element binding protein 1; *Actb*, β-actin.

were obtained as the protein samples for Western blot analysis. The BCA Protein Assay Kit (Takara, Beijing, China) was used to determine the protein content in the samples. PRSS2 antibody (Bioss Biotechnology, Beijing, China), SREBP1 antibody (Bioss Biotechnology, Beijing, China), AMPKα1 antibody (Bioss Biotechnology, Beijing, China), *P*-AMPKα1 (Ser496) antibody (Bioss Biotechnology, Beijing, China), and β-actin antibody (Bioss Biotechnology, Beijing, China) were incubated with the PVDF membrane. After performing a secondary antibody reaction, the specific signal of the horseradish peroxidase (HRP) substrate (Bioworld Technology, Minnesota, USA) coupled to the secondary antibody was detected by Western fluorescence detection reagent BeyoECL Star (Beyotime Biotechnology, Shanghai, China).

2.6. Measurements of LEP expression levels by ELISA

After overexpression or interference with the PRSS2 gene, the expression of the LEP gene was determined by a bovine LEP ELISA kit (Jiangsu Meimian Industrial, Jiangsu, China). After culturing for 24 h, the total protein in the cells was extracted by lysis buffer containing protease and phosphatase (Dalian Meilun Biotech, Dalian, China), and then the protein concentration of the samples was detected by the BCA Protein Assay Kit (Takara, Beijing, China). The concentration of LEP was detected according to the operation steps of the ELISA kit.

2.7. Analysis of the triglycerides, cholesterol and non-esterified fatty acid contents in BMECs

The TG and CHOL contents were detected with a tissue and/or cell triglyceride/cholesterol kit (Applygen Technology, Beijing, China). The NEFA content in the cells was detected with a nonesterified fatty acid kit (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China). The absorbance was measured with a microplate reader (YongChuang SM600, Shanghai, China).

2.8. Analysis of the FA content in BMECs by gas chromatography-mass spectrometry (GC-MS)

The contents of medium- and long-chain saturated and unsaturated fatty acids in cells were detected by GC–MS at Shanghai Applied Protein Technology Co. Ltd. To further verify the biological function of the *PRSS2* gene. A mixture of 40 kinds of fatty acid methyl esters was used to make standard curve solutions. Then, 500 μL of the mixed standard fatty acids and 25 μL of methyl hexadecanoate at a concentration of 500 ppm were mixed in the injection bottles. After transfecting mammary epithelial cells with *PRSS2* gene overexpression or interference vectors for 48 h, 1×10^7 cells were washed twice with prechilled PBS, digested with trypsin solution, and centrifuged at 1000 rpm for 5 min at 4 °C to obtain the cell precipitate. The cells were collected in 2 mL glass centrifuge tubes, 1 mL of chloroform-methanol solution was added, and the mixture was sonicated for 45 min with an ultrasonic crusher. Then, 2 mL of 1 % sulfuric acid-methanol solution was added to the supernatant, and the mixture was placed in an 80 °C water bath for 45 min. One millilitre of hexane was added, and then 5 mL of ddH_2O was used for washing after methyl esterification; 25 μL of methyl hexanoate was added as the reference standard to 500 μL of supernatant, and the solution was mixed in glass centrifuge tubes. The initial column temperature was set at 50 °C for 3 min, which was then heated to 220 °C at a rate of 10 °C/min for 5 min. The velocity of the carrier helium gas was 1.0 mL/min. In the sample queue, a QC sample was used to measure and assess the stability and repeatability of the system. Finally, an Agilent 7890/5975C GC–MS was used for mass analysis. The detailed operating conditions were as follows: The temperatures of the injection port, ion source, and transmission line were 280 °C, 230 °C, and 250 °C, respectively. The injection volume was 1 μL, the split ratio was 10:1, and shunt injection was used. Cleaning was conducted after every four samples until all samples were tested.

2.9. Statistical analysis

The comparative Ct method ($2^{-\Delta\Delta Ct}$) [26] was used to analyse the relative qRT–PCR gene expression. The chromatographic peak area and retention time were extracted by MSD ChemStation software. The TGs, CHOL, FFA, ELISA and Western blotting data were analysed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, California, USA) with a two-tailed (unpaired) *t*-test. Experimental data are shown as the mean ± SEM. Statistically significant differences were defined as $P < 0.05$ (P value).

3. Result

3.1. Construction of the PRSS2 expression vector

Four interference vectors of the *PRSS2* gene were successfully constructed and transfected into BMECs. The interference efficiency of pb7SK-PRSS2-shRNA4 had the highest significance compared with other interfering vectors ($P < 0.0001$, Fig. 1A–E). The overexpression vector of pBI-CMV3-PRSS2 was detected in the BMECs by determining the expression of green fluorescent protein (Figure F–G).

3.2. Analysis of PRSS2 mRNA expression

PRSS2 gene expression was upregulated in the pBI-CMV3-PRSS2 overexpression vector group compared with that in the pBI-CMV3 vector group ($P < 0.0005$, Fig. 2B). In addition, the mRNA expression in the pb7SK-PRSS2-shRNA4 vector-transfected BMECs group was decreased compared with that in the pb7SK/GFP/Neo negative control group ($P < 0.0001$, Fig. 2A).

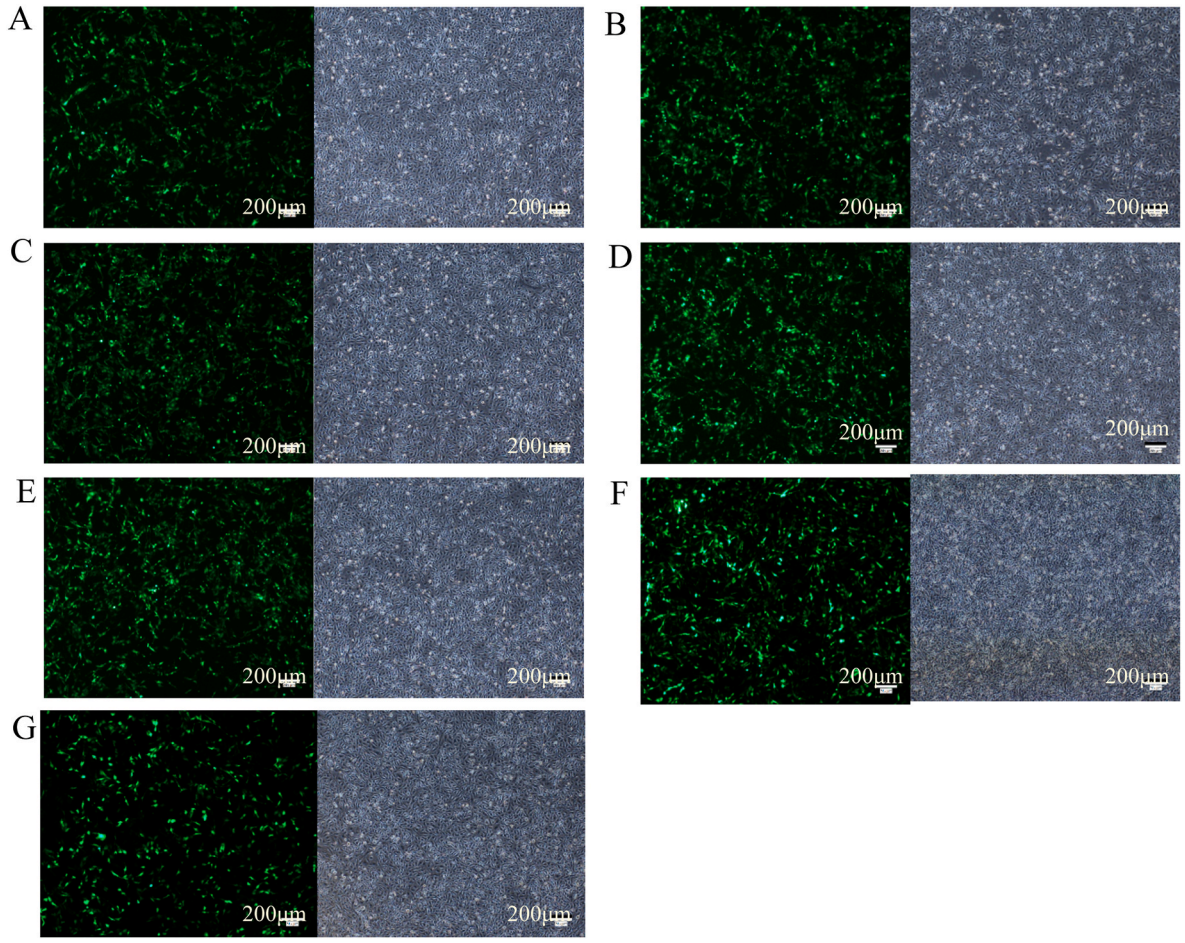

Fig. 1. Transfection of the PRSS2 gene overexpression vector and the interference vector. (A) Transfection of pb7sk-PRSS2-shRNA1 vector into BMECs; (B) Transfection of pb7sk-PRSS2-shRNA2 vector into BMECs; (C) Transfection of pb7sk-PRSS2-shRNA3 vector into BMECs; (D) Transfection of pb7sk-PRSS2-shRNA4 vector into BMECs; (E) Transfection of pb7sk-GFP-Neo vector into BMECs; (F) Transfection of pBI-CMV3-PRSS2 vector into BMECs; (G) Transfection of pBI-CMV3 vector into BMECs.

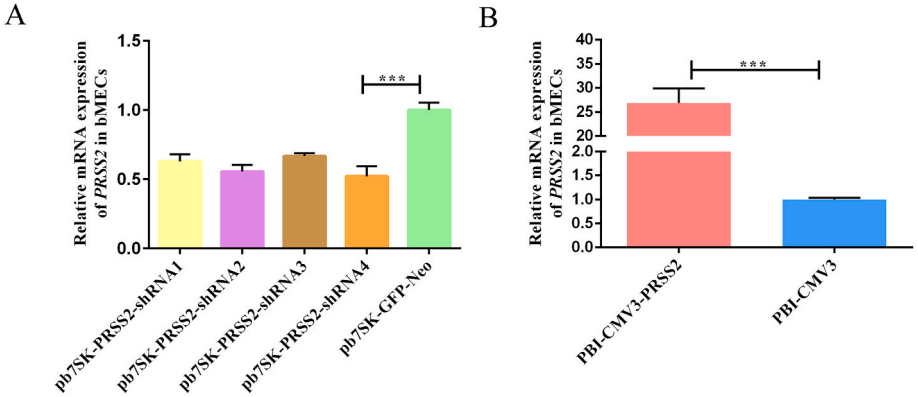

Fig. 2. The mRNA levels of the *PRSS2* gene in BMECs. (A) The mRNA levels of the *PRSS2* gene in BMECs transfected with the interference vector. (B) The mRNA levels of the *PRSS2* gene in BMECs transfected with the overexpression vector. Experimental data are shown as the mean ± SEM. GFP, green fluorescent protein. ***$P < 0.0005$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Analysis of PRSS2 protein expression

Compared with that in the pBI-CMV3 vector group, the PRSS2 protein level in the pBI-CMV3-PRSS2 group was increased ($P < 0.05$, Fig. 3A and B). Additionally, the PRSS2 protein level in the pb7SK-PRSS2-shRNA4 group was lower than that in the pb7SK-GFP-Neo group ($P < 0.001$, Fig. 3A and B). The PRSS2 protein level was expressed as the ratio of PRSS2 protein to β-actin.

3.4. Effect of PRSS2 on TGs content

The level of TGs in the pBI-CMV3-PRSS2 group was 41 % lower than that in the pBI-CMV3 group ($P < 0.005$, Fig. 4A). In addition, the TG content in the pb7SK-PRSS2-shRNA4 group was 36 % higher than that in

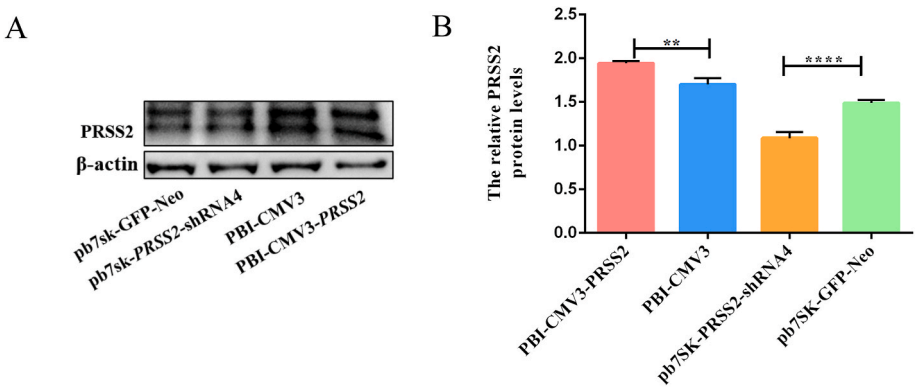

Fig. 3. The protein levels of the *PRSS2* gene in BMECs. (A) Western blot results of the *PRSS2* gene in BMECs transfected with the interference vector and the overexpression vector. (B) The protein levels of the *PRSS2* gene in BMECs transfected with the interference vector and the overexpression vector. Experimental data are shown as the mean ± SEM. GFP, green fluorescent protein. $^{**}P < 0.01$, $^{****}P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

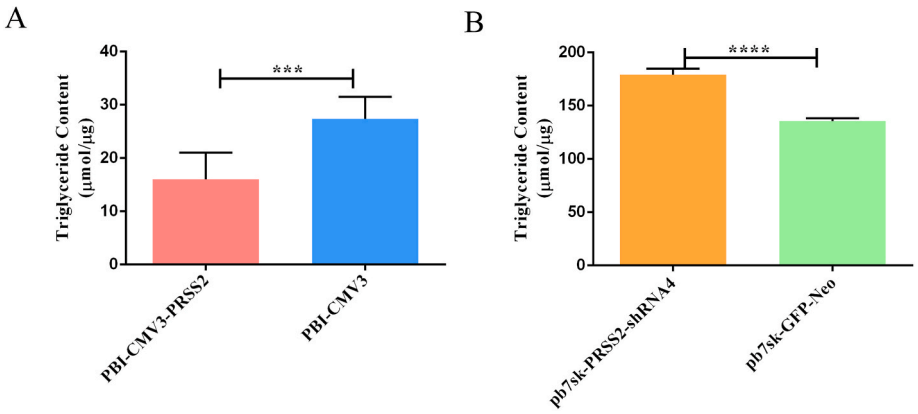

Fig. 4. Effects of *PRSS2* on the triglycerides (TGs) contents in BMECs. (A) TGs content after transfection with the pBI-CMV3-PRSS2 overexpression vector. (B) TGs content after transfection with the pb7SK-PRSS2-shRNA4 vector. Experimental data are shown as the mean ± SEM. GFP, green fluorescent protein. $^{***}P < 0.005$; $^{****}P < 0.0005$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the pb7SK-GFP-Neo group ($P < 0.0005$, Fig. 4B).

3.5. Effect of PRSS2 on CHOL content

The CHOL level was measured using a CHOL kit to examine whether the *PRSS2* gene is involved in the CHOL metabolic pathway. The results showed that the CHOL content in BMECs transfected with pBI-CMV3-PRSS2 was 28 % lower than that in the control group ($P < 0.005$, Fig. 5A). In addition, the CHOL content in BMECs transfected with pb7SK-PRSS2-shRNA4 was 19 % higher than that in the control group ($P < 0.05$, Fig. 5B).

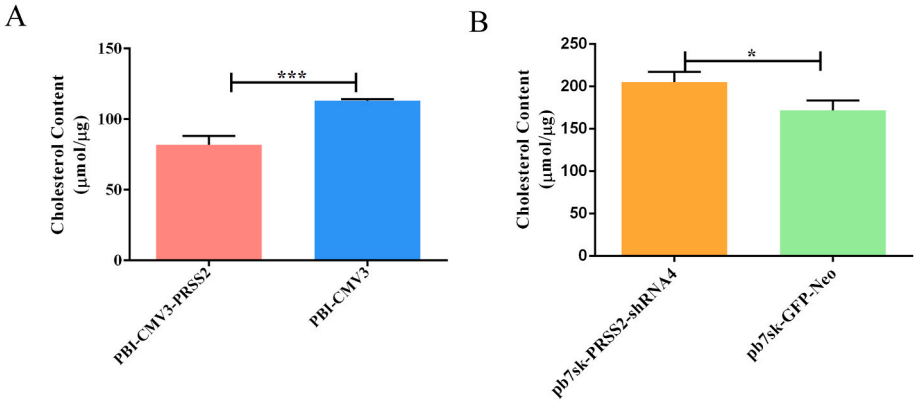

Fig. 5. Effects of *PRSS2* on the cholesterol (CHOL) content in BMECs. (A) CHOL content after transfection with the pBI-CMV3-PRSS2 overexpression vector. (B) CHOL content after transfection with the pb7SK-PRSS2-shRNA4 vector. Experimental data are shown as the mean ± SEM. GFP, green fluorescent protein. $^{*}P < 0.05$, $^{***}P < 0.005$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.6. Effect of PRSS2 on NEFA content

The level of NEFAs was detected with a NEFA kit, and whether the *PRSS2* gene is involved in the NEFA metabolic pathway was assessed. The findings revealed that the NEFA levels in the PBI-CMV3-PRSS2 group exhibited an approximate 1.9-fold increase compared to those observed in the PBI-CMV3 group ($P < 0.01$, Fig. 6A). In addition, the level of NEFAs in the BMECs transfected with pb7SK-PRSS2-shRNA4 decreased approximately 0.43 times compared with that in the control group ($P < 0.005$, Fig. 6B).

3.7. Effect of PRSS2 on FA content

In this experiment, thirty-seven (37) different fatty acids in the cells were detected by GC–MS. The concentrations of SFAs, MUFAs, PUFAs, and n-3 and n-6 PUFAs in the PRSS2 overexpression group were found to be lower than those in the negative control group, although the differences were not statistically significant ($P > 0.05$, Table 3). Conversely, the concentrations of these fatty acids were observed to be higher in the PRSS2 interference group than in the negative control group; however, this difference was also not statistically significant ($P > 0.05$, Table 4). Furthermore, the C17:1N7, C18:3N6, C22:2N6 and C22:4N6 concentrations were significantly lower, while the C8:0, C20:5N3 and C23:0 concentrations were significantly higher in the PRSS2 overexpression group than in the negative control group ($P < 0.05$). The concentration of C24:1N9 was found to be significantly lower ($P < 0.05$) whereas other fatty acids including C8:0, C12:0, C14: 0, C23: 0, C24: 0, C14: 1 N^5, C16: 1 N^7, C17: 1 N^7, C18: 1 N^9, C18: 2 TTN6, C20: 1 N^9, and C22:4 N^6 were observed to be significantly higher in the *PRSS2* interference group than those in the negative control group ($P < 0.05$).

3.8. Expression of genes involved in the lipid metabolism pathway

To elucidate the underlying mechanism through which *PRSS2* regulates lipid metabolism, we further determined the impact of *PRSS2* overexpression and silencing on the mRNA transcription levels of signalling molecules associated with lipid metabolism. In this experiment, the gene analysis results revealed that PRSS2 overexpression in BMECs upregulated the mRNA levels of the Hormone-sensitive lipase (*HSL*), Stearoyl-Coenzyme A desaturase 1(*SCD1*), Acyl-CoA synthetase short chain family member 2 (*ACSS2*), Glycogen synthase kinase 3 beta (*GSK3B*), Carnitine palmitoyltransferase 1 A (*CPT1A*), *LEP*, and *AMPKα1* genes but downregulated the mRNA level of the *SREBP1* gene (Fig. 7A). However, the mRNA levels of the *HSL*, *SCD1*, *ACSS2*, *GSK3B*, *CPT1A*, *LEP*, and *AMPKα1* genes were decreased, but the mRNA level of the *SREBP1* gene in the pb7SK-PRSS2-shRNA group was decreased

Table 3
The concentrations of various fatty acids in bovine mammary epithelial cells transfected with pBI-CMV3-PRSS2 and pBI-CMV3.

Fatty acids component	pBI-CMV3-PRSS2	pBI-CMV3	P value
8:0	0.0004 ± 0.0183	0.0003 ± 0.0174	0.036
10:0	0.0003 ± 0.0507	0.0002 ± 0.0183	0.99
11:0	0.0003 ± 0.0428	0.0002 ± 0.0197	0.34
12:0	0.0195 ± 0.0004	0.0181 ± 0.0017	0.46
13:0	0.0056 ± 0.0003	0.00570 ± 0.0004	0.80
C14:0	2.9900 ± 0.1140	2.9800 ± 0.0503	0.97
14:1N5	0.1080 ± 0.0059	0.0924 ± 0.0092	0.23
15:0	0.4400 ± 0.0132	0.5070 ± 0.0212	0.056
15:1N5	0.0848 ± 0.0046	0.0628 ± 0.0101	0.39
16:0	27.3000 ± 1.1300	25.8000 ± 0.440	0.10
16:1N7	5.0900 ± 0.6990	4.8200 ± 0.3170	0.75
17:0	0.3590 ± 0.0110	0.4010 ± 0.0169	0.11
17:1N7	0.8440 ± 0.0321	0.9960 ± 0.0262	0.013
18:0	11.2000 ± 0.4030	10.6000 ± 0.1760	0.22
18:1N9	9.0600 ± 0.4700	9.9700 ± 0.3530	0.20
18:1TN9	50.4000 ± 2.5600	34.9000 ± 17.5000	0.39
18:2N6	6.5600 ± 0.0987	7.2000 ± 0.2180	0.056
18:2TTN6	0.2720 ± 0.0121	0.2590 ± 0.0110	0.49
18:3N6	0.0602 ± 0.0015	0.0718 ± 0.0006	0.0018
18:3N3	0.4370 ± 0.0307	0.5690 ± 0.4040	0.76
20:0	0.6630 ± 0.0272	0.5710 ± 0.0298	0.086
20:1N9	1.6700 ± 0.1330	1.6800 ± 0.0433	0.94
20:2N6	0.4600 ± 0.0395	0.5380 ± 0.0149	0.14
20:3N6	2.4600 ± 0.1420	2.6700 ± 0.2190	0.47
20:4N6	11.8000 ± 0.6640	12.3000 ± 1.1000	0.73
20:3N3	9.7100 ± 0.2640	7.0900 ± 2.7400	0.66
20:5N3	0.2050 ± 0.0110	0.1730 ± 0.0016	0.046
21:0	0.0310 ± 0.0008	0.0289 ± 0.0021	0.48
22:0	0.6200 ± 0.0128	0.5850 ± 0.0775	0.68
22:1N9	0.5100 ± 0.0284	0.5140 ± 0.0055	0.88
22:2N6	0.0476 ± 0.0020	0.0633 ± 0.0042	0.0065
22:4N6	1.6200 ± 0.0344	1.9200 ± 0.0527	0.0086
22:5N3	1.6700 ± 0.0387	1.7100 ± 0.0553	0.57
22:5N6	0.3750 ± 0.0024	0.3940 ± 0.0075	0.065
23:0	0.0236 ± 0.0009	0.0192 ± 0.0003	0.0079
24:0	0.0434 ± 0.0010	0.0422 ± 0.0005	0.37
24:1	2.3300 ± 0.0530	2.2100 ± 0.0507	0.19
n-3	12.0000 ± 0.2960	12.3000 ± 0.2120	0.44
n-6	23.6000 ± 0.9280	25.4000 ± 1.2600	0.33
SFA	43.7000 ± 1.6800	41.5000 ± 0.5800	0.29
MUFA	70.1000 ± 3.8900	73.2000 ± 1.1700	0.48
PUFA	35.6000 ± 1.2200	37.7000 ± 1.2000	0.30

Note: Experimental data are shown as the mean ± SEM. Unit: μg/10^7.

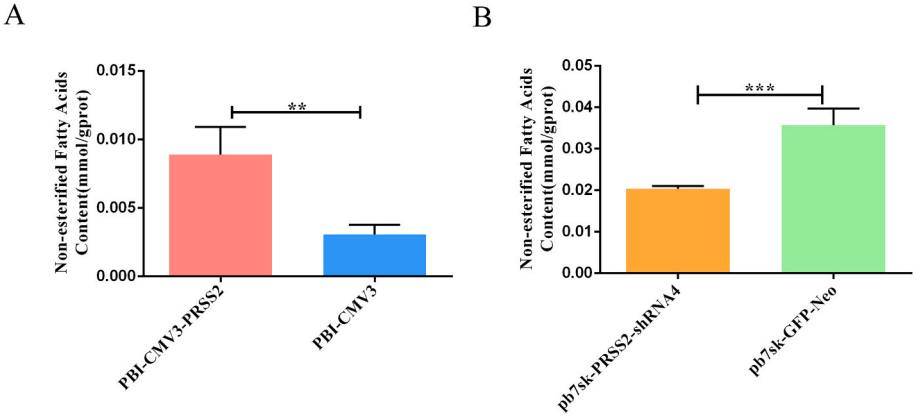

Fig. 6. Effects of *PRSS2* on the nonesterified fatty acid (NEFA) contents in BMECs. (A) NEFA content after transfection with the pBI-CMV3-PRSS2 overexpression vector. (B) NEFA content after transfection with the pb7SK-PRSS2-shRNA4 vector. Experimental data are shown as the mean ± SEM. GFP, green fluorescent protein. $^{**}P < 0.01$; $^{***}P < 0.005$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4
The concentrations of various fatty acids in bovine mammary epithelial cells transfected with the pb7SK-PRSS2-shRNA4 vector and the negative control.

Fatty acid component	pb7SK-PRSS2-shRNA4	pb7SK-GFP-Neo	P value
8:0	0.0006 ± 0.0177	0.0004 ± 0.0107	0.0063
10:0	0.0003 ± 0.0139	0.0002 ± 0.0146	0.23
11:0	0.0003 ± 0.0449	0.0003 ± 0.0247	0.43
12:0	0.0142 ± 0.0004	0.0109 ± 0.0002	0.0015
13:0	0.00480 ± 0.0002	0.00430 ± 0.0218	0.093
14:0	2.6600 ± 0.0683	2.3700 ± 0.0306	0.018
14:1N5	0.0700 ± 0.0026	0.0573 ± 0.0006	0.0089
15:0	0.4150 ± 0.0118	0.4220 ± 0.0088	0.66
15:1N5	0.0619 ± 0.0031	0.0588 ± 0.0016	0.43
16:0	23.1000 ± 0.7040	17.4000 ± 4.1700	0.010
16:1N7	4.6300 ± 0.1380	3.8100 ± 0.0474	0.0050
17:0	0.3250 ± 0.0077	0.3310 ± 0.0048	0.55
17:1N7	0.9010 ± 0.0190	0.5610 ± 0.2520	0.012
18:0	9.2500 ± 0.3540	9.2800 ± 0.0374	0.92
18:1N9	9.1600 ± 0.2710	7.9400 ± 0.2680	0.033
18:1TN9	47.1000 ± 1.410	43.6000 ± 0.5340	0.078
18:2N6	6.4100 ± 0.1840	6.0500 ± 0.1550	0.20
18:2TTN6	0.2610 ± 0.0022	0.1420 ± 0.0708	0.00010
18:3N6	0.0675 ± 0.0014	0.0656 ± 0.0019	0.46
18:3N3	0.1690 ± 0.0155	2.1600 ± 1.9200	0.21
20:0	0.5280 ± 0.0286	0.5050 ± 0.0050	0.47
20:1N9	1.5200 ± 0.0539	3.6200 ± 2.2800	0.049
20:2N6	0.4570 ± 0.0206	0.4610 ± 0.0093	0.86
20:3N6	2.4400 ± 0.08120	2.3500 ± 0.0213	0.35
20:4N6	11.6000 ± 0.3120	11.0000 ± 0.0430	0.11
20:3N3	9.5500 ± 0.2250	9.0500 ± 0.0361	0.093
20:5N3	0.1590 ± 0.0042	0.1510 ± 0.0018	0.19
21:0	0.0251 ± 0.0013	0.0177 ± 0.0088	0.25
22:0	0.4240 ± 0.0126	0.4410 ± 0.0163	0.46
22:1N9	0.4650 ± 0.0218	0.4420 ± 0.0074	0.37
22:2N6	0.0527 ± 0.0011	0.0507 ± 0.0011	0.27
22:4N6	1.8500 ± 0.0231	1.7500 ± 0.0184	0.031
22:5N3	1.6100 ± 0.0404	1.5400 ± 0.0495	0.35
22:5N6	0.4030 ± 0.0073	0.3800 ± 0.0207	0.59
23:0	0.0206 ± 0.0005	0.0161 ± 0.0004	0.0023
24:0	0.0420 ± 0.0004	0.0374 ± 0.0010	0.013
24:1N9	2.1900 ± 0.0427	2.7000 ± 0.0422	0.0010
n-3	11.5000 ± 0.2830	11.0000 ± 0.1230	0.17
n-6	23.6000 ± 0.6120	22.3000 ± 0.2460	0.13
SFA	36.8000 ± 1.1700	34.9000 ± 0.3250	0.20
MUFA	66.1000 ± 1.9300	60.8000 ± 0.3420	0.053
PUFA	35.1000 ± 0.8950	33.3000 ± 0.3680	0.14

Note: Experimental data are shown as the mean ± SEM. Unit: μg/10^7.

compared with that in the pb7SK-GFP-Neo group (Fig. 7B).

3.9. *PRSS2* regulates the signalling pathways associated with lipid metabolism

To further explore the pathway by which *PRSS2* regulates lipid metabolism, we determined the effects of *PRSS2* overexpression and silencing on the signalling pathway associated with milk fat synthesis at the translation level. We determined whether *PRSS2* regulates the *LEP/AMPKα1/SREBP1* pathway in BMECs. The results revealed that overexpression of *PRSS2* negatively regulated *SREBP1* gene expression but positively regulated *LEP* and *AMPKα1* gene expression (Fig. 8A–C). Silencing the *PRSS2* gene significantly increased *SREBP1* gene expression but decreased *LEP* and *AMPKα1* gene expression (Fig. 8A–C). However, we also found that silencing or overexpressing the *PRSS2* gene had no effect on *AMPKα1* phosphorylation (Fig. 8B–D).

4. Discussion

The main physiological function of dairy cow mammary tissue is to synthesize and secrete milk, and BMECs are the target and vital cells for studying the mechanism of lactation in mammary tissue in vitro. Importantly, BMECs serve as a valuable experimental model for investigating functions involved in milk fat component synthesis and identifying associated regulatory metabolic pathways. The successful isolation and culture of BMECs has been widely used in the functional verification of exogenous genes. BMECs can accumulate TGs in lipid droplets, however, excessive accumulation of TGs in the body can alter lipid metabolism [28]. CHOL, a pivotal lipid constituent of cellular membranes, plays a crucial role in maintaining membrane stability and regulating flexibility [29]. However, excessive CHOL intake can cause diseases such as myocardial ischaemia, coronary atherosclerosis, and coronary heart disease [30]. Therefore, it is necessary to obtain milk with a low cholesterol content and to provide it to consumers for dairy consumption. NEFAs are found in milk and are an important component of the lipid structure of cell membranes. NEFAs play a pivotal role in the pathogenesis of obesity by modulating lipid metabolism [13]. However, the synthesis and decomposition of these three types of lipids, namely, FAs, TGs and CHOL, are mainly regulated by functional genes. Recent studies have demonstrated that the *CD44* gene exerts regulatory control over lipid metabolism-related genes in BMECs, thereby promoting the synthesis of FAs, TGs, and CHOL [25]. The activation of *AMPKα* inhibits de novo synthesis of lipids and lactose in BMECs [31]. These results both indicated that manipulation of these genes has the potential to modulate cellular lipid metabolism. Importantly, BMECs serve as a valuable experimental model for investigating functions involved in milk fat component synthesis and identifying associated regulatory metabolic pathways.

Our previous transcriptome analysis revealed that *PRSS2* is a differentially expressed gene that might be associated with milk fat component synthesis in BMECs. Thus, this study focused on verifying whether *PRSS2* could regulate TGs, CHOL, and NEFAs in BMECs. The study of the *PRSS2* gene is mostly related to human pancreatitis [12,32]. *PRSS2* is also one of the most abundantly secreted proteins in pancreatic

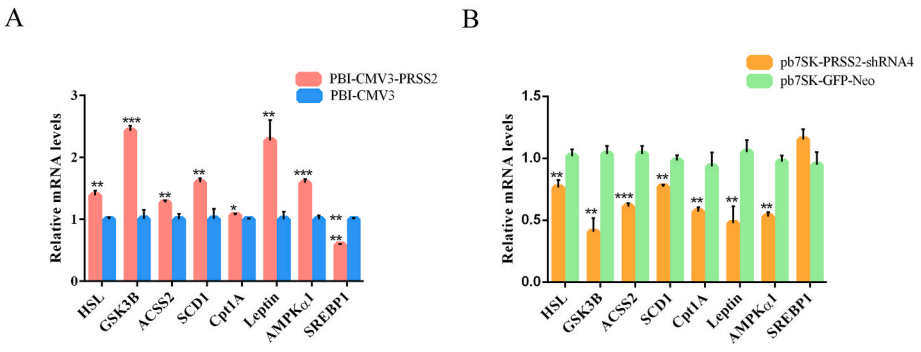

Fig. 7. Gene expression analysis in BMECs with *PRSS2* gene overexpression and interference vectors. (A) mRNA levels of lipid metabolism pathway-related genes in BMECs transfected with the pBI-CMV3-PRSS2 overexpression vector. (B) The mRNA levels of lipid metabolism pathway-related genes in BMECs transfected with the pb7SK-PRSS2-shRNA4 vector. Experimental data are shown as the mean ± SEM. GFP, green fluorescent protein. *$P < 0.05$; **$P < 0.005$ ***$P < 0.0005$; ****$P < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

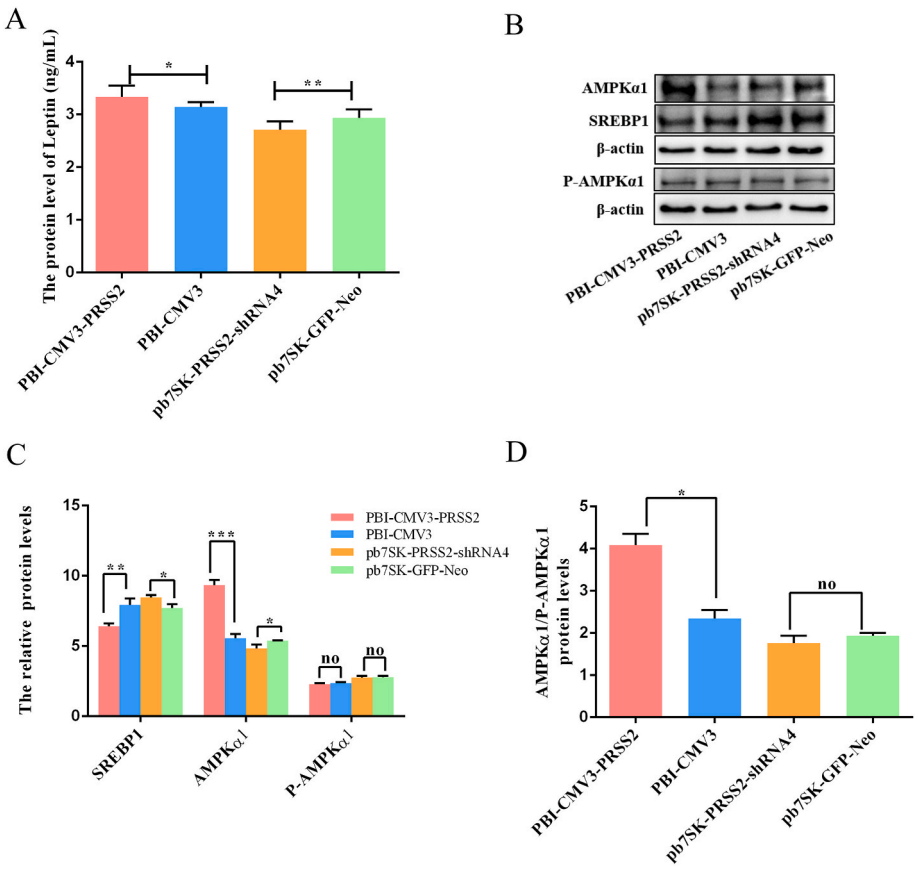

Fig. 8. Gene and protein expression analysis in BMECs with *PRSS2* gene overexpression and interference vectors. (A–C) The protein levels of the *LEP/AMPKα1/SREBP1* pathway in BMECs. (D) The AMPKα1/P-AMPKα1 ratio in BMECs. (E) Simulation of the mechanism by which the *PRSS2* gene affects lipid metabolism through the *LEP/AMPKα1/SREBP1* pathway in BMECs. Experimental data are shown as the mean ± SEM. GFP, green fluorescent protein. $^{*}P < 0.05$; $^{**}P < 0.005$ $^{***}P < 0.0005$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cancer and was first detected in pancreatic cance [33,34]. Trypsin 2 is encoded by the *PRSS2* gene. By converting the antibacterial compound pro-defensin into human α-defensin 5, trypsin 2 plays a pivotal role in modulating the innate immune response of the small intestine [35]. Khushdeep Bandesh found that *PRSS2* was overexpressed in pancreatic tissue when they studied genetic variations that caused type 2 diabetes and the development of a congenital immune disease [36]. This suggests that *PRSS2* might be involved in glycolipid metabolism. Nevertheless, there have not been any reports about the impact of the *PRSS2* gene on lipid metabolism. Thus, in this study, overexpression and interference vectors of the *PRSS2* gene were successfully constructed, and the mRNA and protein expression levels of the *PRSS2* gene were revealed. The effects of *PRSS2* on TGs, CHOL, NEFA and lipid metabolism-related genes in BMECs were analysed. Inhibiting the expression of the *PRSS2* gene can increase TGs and CHOL contents but can decrease NEFA content. It is suggested that *PRSS2* can inhibit cellular TGs and CHOL synthesis but can promote the synthesis of free fatty acids, which may be due to the decrease in triglyceride synthesis.

To further explore the effect of the *PRSS2* gene on fatty acid metabolism, we determined the type and composition of intracellular fatty acids. Fatty acids are an important component of lipids in organisms. Different fatty acids in the chemical structure are very important for cellular energy storage, cell membrane structure, cell migration, and signal transduction [37]. Fatty acids can be decomposed into acetyl-CoA through a series of β-oxidation processes in the mitochondria, and they can also be synthesized into TGs and CHOL esters by anabolism. There are two forms of fatty acids in milk: esterified fatty acids (EFA) and free fatty acids (FFA). Most of the fatty acids are bound fatty acids, and less than 0.5 % are FFAs. Generally, fatty acids in bovine milk include 70 % SFAs, 25 % MUFAs, and 5 % PUFAs [38–40]. The results generally show that the content of approximately 14 fatty acids in milk is more than 1 %. Among them, C16:0 or C18:1-c9 are the most abundant fatty acids in milk, accounting for 15 % and 30 % of the total fatty acid content, respectively [41,42]. In our study, the results demonstrated that overexpression of the *PRSS2* gene could reduce the concentrations of the total n-3 family, n-6 family, MUFAs, and PUFAs, while inhibition of *PRSS2* expression could increase the concentrations of the total n-3 family, n-6 family, MUFAs, and PUFAs. *CD36* is almost completely responsible for AMPK-mediated stimulation of long-chain fatty acid (LCFA) uptake by cardiomyocytes, indicating that *CD36* plays a key role in mediating changes in cardiac LCFA flow [43]. The lack of the *HSL* gene decreased the contents of the n-6 PUFA LNA and n-3 PUFA ALA fatty acids in mouse testes [44]. Stearoyl-CoA desaturase (*SCD*), a microsomal protease found in lactating mammary glands and adipose tissue and belonging to the dehydrogenase family, is the rate-limiting enzyme for fatty acid desaturation and catalyses the desaturation of saturated fatty acyl-CoA Δ9-cis to generate MUFAs [45]. The prevention of obesity and insulin resistance in *SCD1* knockout mice is observed upon exposure to a high-fat diet [46,47], but limiting the intake of MUFAs can lead to severe weight loss and hypoglycaemia. Unexpectedly, C17:1N7 and C22:4N6 in the *PRSS2* overexpression group showed a significant reduction compared to the negative control group and were increased in the *PRSS2* inhibition group. Typically, specific fatty acids such as pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), and *trans*-palmitoleic acid (t-C16:1N7) are commonly utilized as indicators of dairy fat consumption in milk [48–50]. Studies have found that C17:1 in Ginkgo biloba extract may induce cell apoptosis by activating caspase-3, upregulating Bax expression, and inhibiting the migration of

SMMC7721 cells [51]. In cells, adrenic acid (22:4N6, ADA) is an endogenous PUFA formed by the extension of arachidonic acid (20:4N6, AA) by an extension enzyme called ultralong-chain fatty acid protein (ELOVL) 2 or 5 [52]. In summary, the *PRSS2* gene can change the content of long-chain fatty acids in cells, such as C17:1N7 and C22:4N6, suggesting that the *PRSS2* gene may modulate fatty acid synthesis in cells or milk.

Numerous protease receptors and transcription factors participate in the intricate process of metabolism, which is tightly regulated by signal transduction pathways to establish a complex and finely tuned regulatory network that maintains cellular and organismal lipid homeostasis. To confirm the molecular pathways involved, we constructed overexpression and interference vectors of *PRSS2* to affect fatty acid metabolism-related genes and the AMPK signalling pathway. A novel time-dependent effect of 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (*AICAR)* was to induce *AMPK* activation of lipolysis, which involves antagonistic modulation of *HSL* and patatin like phospholipase domain containing 2 (*ATGL)* [53]. *SCD1* knockout in mice can activate *AMPK* in the liver [54] and skeletal muscle [55] to enhance fatty acid oxidation, thereby reducing lipid biosynthesis and increasing thermogenesis [56]. A study demonstrated the effective regulation of lipid metabolism by aleurain-like protease (*ALP*), which reduces the risk of atherosclerosis in type 2 diabetic rats through modulation of the *SREBP1/SCD1* axis [57]. The mRNA of *ACSS2* in the mammary gland increased significantly during lactation, and its expression pattern was negatively correlated with the amount of milk fat during the whole lactation period [8,58]. Thus, we speculated that *PRSS2* may be involved in regulating lipid metabolism by affecting the expression of the *HSL*, *SCD1*, *ACSS2*, *GSK3B*, and *CPT1A* genes. *LEP* is present in bovine milk and is secreted by bovine mammary epithelial cells, and its SNPs are associated with milk production [59,60]. The administration of *LEP* resulted in an augmentation of lipolysis in white adipocytes [61], thereby facilitating the breakdown of TGs in skeletal muscle and enhancing the oxidation of fatty acids in both skeletal muscle and liver [62–64]. *LEP* could also promote lipid oxidation [16] and TG storage [9,65] by increasing AMPK signalling in peripheral tissue. The activation of *AMPK* is regulated by the transcription factors *SREBP1* and peroxisome proliferator activated receptor alpha (*PPARα*) to induce lipid metabolism [66,67]. The transcriptional activation of *SREBP1* is consistent with the synthesis of a large amount of milk fat in the early stage of lactation and throughout the lactation process of dairy cows, and it also regulates the fat synthesis of BMECs by regulating the transcription of *ACSS2* genes [68,69]. Transcription factors such as *SREBP1* induce the expression of adipogenesis-related genes and promote the activity of fatty acids. Fatty acid synthesis increased in mice that overexpressed the *SREBP1* gene [70]. In cow hepatocytes, *AMPKα* suppressed the expression level and transcriptional activity of the *SREBP1* protein, which decreased the expression of adipogenic genes, resulting in a decrease in lipid synthesis [71]. Furthermore, protein phosphorylation is a process in which the phosphate group of ATP is transferred to the receptor protein by a protein kinase. The phosphorylated form of *AMPK* is the activated form. The regulation of lipid metabolism by activated *AMPK* occurs mainly by directly acting on metabolic enzymes related to lipid metabolism, promoting fat catabolism and inhibiting its synthesis [72]. *AMPK* can be activated by the upstream kinases hepatokinase B1 (*LKB1*) and Ca^{2+}/calmodulin kinase β (*CaMKKβ*), AMPK activators (*AICAR*) and cytokines (leptin, insulin, and adiponectin). However, protein kinases and phosphatases are similar to a pair of "switches". Phosphorylation activates protein activity, and dephosphorylation inhibits protein activity accordingly [73,74]. However, in this experiment, the *PRSS2* gene regulated lipid metabolism by increasing dephosphorylated *AMPKα1*. Moreover, it may activate *AMPKα1* by increasing *LEP* while decreasing *SREBP1* to promote fat catabolism, inhibit fat synthesis, and promote fatty acid oxidation. These results suggest that the *PRSS2* gene can modulate the synthesis of TGs and fatty acids in BMECs by mediating the *LEP/AMPKα1/SREBP1* pathway.

5. Conclusions

The findings demonstrated that *PRSS2* serves as a pivotal gene in lipid metabolism through its regulation of associated genes such as *HSL*, *SCD1*, *ACSS2*, *GSK3B*, and *CPT1A* through the *LEP/AMPKα1/SREBP1* pathway to inhibit the accumulation of TGs and CHOL but may promote the synthesis of NEFAs in BMECs. Moreover, the *PRSS2* gene upregulated the expression of genes associated with the *LEP/AMPKα1/SREBP1* pathway to modulate intracellular medium- and long-chain fatty acid metabolism, leading to an increase in unsaturated fatty acids 17:1N7 and C22:4N6. This study establishes a foundation for further investigation into the molecular mechanism underlying the influence of *PRSS2* on lipid metabolism synthesis in dairy cows.

Institutional review board statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of GUANGDONG OCEAN UNIVER-SITY (protocol code University Science and Technology (2019) No. 1 and October 7, 2023).

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CRediT authorship contribution statement

Huixian Lu: Writing – original draft, Methodology, Data curation. **Zhihui Zhao:** Writing – original draft, Supervision, Funding acquisition, Data curation. **Haibin Yu:** Investigation, Data curation. **Ambreen Iqbal:** Investigation. **Ping Jiang:** Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors report there are no competing interests to declare.

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