New function of the *CD44* gene: Lipid metabolism regulation in bovine mammary epithelial cells

Ping Jiang,^{1*} Lixin Xia,^{2*} ZiKang Jin,¹ Shaokat Ali,¹ Mengyan Wang,² Xiaohui Li,² Runjun Yang,² Xibi Fang,^{2†} and Zhihui Zhao^{1†}

¹College of Agriculture, Guangdong Ocean University, Zhanjiang, 524088, P. R. China

²College of Animal Science, Jilin University, Changchun, 130062, P. R. China

ABSTRACT

The *CD44* gene encodes a cell-surface glycoprotein that participates in a variety of biological processes such as cell interactions, adhesion, hematopoiesis, and tumor metastasis. We compared the transcriptome in bovine mammary epithelial cells (bMEC) of Chinese Holstein dairy cows producing milk of high and low fat contents. Our results suggest that *CD44* might be a candidate gene affecting milk fat synthesis. In the present study, the overexpression of the *CD44* gene increased the contents of intracellular triglycerides (TG) and cholesterol (CHOL), whereas knockdown of the *CD44* gene decreased bMEC CHOL and TG contents. Gas chromatography analysis of fatty acid composition showed that the contents of α -linolenic acid, palmitic acid, and *cis*-8,11,14-eicosatrienoic acid were altered due to changes in the level of expression of the *CD44* gene. Additionally, elaidic acid, palmitoleic acid, tridecanoic acid, and oleic acid were markedly reduced in the *CD44* gene overexpression group compared with the control group. On the contrary, *cis*-5,8,11,14-eicosatetraenoic acid and stearic acid were markedly increased in the *CD44* knockdown group compared with the control group. And RT² Profiler PCR array (Qiagen, CLAB24070A Frankfurt, Germany) further suggested that overexpression or knockdown of the *CD44* gene altered expression levels of functional genes associated with lipid metabolism. The present data indicate that *CD44* plays a key regulatory role in lipid metabolism in bMEC.

Key words: *CD44*, triglycerides, cholesterol, fatty acids, bovine mammary epithelial cells

INTRODUCTION

The *CD44* gene (ENSBTAG00000011578) is located on bovine chromosome 15:66,454,331–66,541,790, with 3 alternatively spliced transcripts. The transcript of *CD44*-201 was selected. The *CD44* DNA sequence contains 87,460 base pairs with 9 exons and 8 introns. The *CD44* mRNA sequence contains a coding region of 1,107 bp, and the open reading frame encodes 368 amino acids. The *CD44* protein is a hyaluronan-binding surface receptor that is highly expressed in hematopoietic cells and immune cells (Schmits et al., 1997; Kang et al., 2013). High *CD44* expression is observed in early development of T- and B-cells, and upregulated expression of *CD44* was attributed to antigen receptor stimulation or mitogen activation (Camp et al., 1991). Fraser and colleagues observed that *CD44* was highly expressed in human adipogenic stem cells as a cell-surface marker (Fraser et al., 2006), which suggests that *CD44* may be related to the pluripotency and differentiation of adipocyte precursors. Furthermore, previous studies have observed that cells with high expression of *CD44*, but without the expression of *CD34* or *CD56*, have the capacity to differentiate into adipocytes (Khan et al., 2012). Previous study has also suggested that treatment with *CD44* monoclonal antibodies on mice with a high-fat diet inhibited high-fat diet-induced obesity, improved adipose tissue inflammation, and regulated the expression levels of lipid-related genes (Kodama et al., 2012). Furthermore, this effect of the antibody was superior to the effects of obesity drugs such as metformin (Kodama et al., 2012), suggesting that the *CD44* gene is closely associated with lipid metabolism in mice. Additionally, our previous transcriptome analysis also revealed that *CD44* is a differentially expressed gene in bovine mammary epithelial cells (bMEC) of Chinese Holstein dairy cows producing milk of high and low fat contents (Supplemental Table S1, <https://doi.org/10.3168/jds.2019-17415>). Thus, it is possible that *CD44* might play a key role in the regulation of lipid metabolism in bMEC.

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*These 2 authors contributed equally to this work.

†Corresponding authors: fangxibi@jlu.edu.cn and zhzhao@jlu.edu.cn

The main components of milk fat are triglycerides (**TG**), which constitute about 97 to 98% of the fat content, with fatty acids (**FA**) and cholesterol (**CHOL**) as the minor components that make up around 0.1% and 0.2 to 0.4% of the fat content, respectively (Jensen et al., 1991; Lubary et al., 2011).

Although the function of *CD44* in lipid metabolism has been examined in mice, its effects on milk fat synthesis in dairy cattle have not been studied, especially the effects on TG and CHOL metabolism in bMEC. In our previous studies, analysis of transcriptome results suggested that *CD44* might be involved in fat synthesis by regulating other milk fat metabolism-related genes. Therefore, the objective of the present study was to uncover the regulatory roles of *CD44* in bMEC on the contents of TG, CHOL, and FA, as well as on expression levels of genes involved in lipid and FA metabolism, using gene overexpression and RNA interference (**RNAi**) approaches. This study will be valuable for revealing the regulatory roles and molecular mechanisms of *CD44* in milk fat synthesis.

MATERIALS AND METHODS

Constructing Interference Vector *pb7SK-shCD44*

The target sequence of the *CD44* gene (GenBank: NM_174013.3) is GCGCAGATCGATCTGAATATA. The forward primer is 5'-AGAGGCGCAGATCGATCTGAATATATCTCTTGAATATATTCAGATCGATCTGCGCTTTTGTG-3', and the reverse primer is 5'-GATCCAAAAAGCGCAGATCGATCTGAATATATTCAGATATATTCAGATCGATCTGCGC-3'. Small interfering (si)RNA oligonucleotides were annealed and cloned into the *pb7SK/GFP/Neo* vector (C02007, GenePharma Corporation, Shanghai, China). The 20- μ L mixture contained 2 μ L of annealing buffer, 4 μ L of sense and antisense siRNA oligonucleotide mix (100 μ M), and 14 μ L of double-distilled water.

Constructing Overexpression Vector *pBI-CMV3-CD44*

The coding region of *CD44* in cow was amplified by PCR with forward primer 5'-ACGCGTATGGA-CACGTTTTGGTGGC-3' (the underlined sequence indicates a *Mlu*I site) and reverse primer 5'-GCGGC-CGCTTACACCCCAATCTTCATATCCAC-3' (the underlined sequence indicates a *Not*I site). The 1,107-bp PCR product (*Mlu*I to *Not*I) was validated through DNA sequencing and ligated into the *pBI-CMV3* plasmid (#631632, Clontech Laboratories, Mountain

View, CA) to generate the *pBI-CMV3-CD44* expression vector.

Transfection of Plasmids into bMEC

The bMEC in the study were purified and cultured from a healthy dairy cow according to previous work performed in our laboratory using the method of tissue nubble culture (Lu et al., 2012). Animal experiments were performed in strict accordance with the guidelines for the care and use of laboratory animals by Jilin University (Animal Care and Use Committee permit number: SY201901007). The bMEC were cultured in 10-cm culture plates (353003, Falcon, Franklin Lakes, NJ) in Dulbecco's modified Eagle medium with nutrient mixture F-12 (12-719Q, HyClone, Logan, UT) supplemented with 10% fetal bovine serum (11011-6123, Tian Hang, Zhejiang, China), 25 μ L of hydrocortisone (25 μ g/mL, ab141250, Abcam, Cambridge, UK), and 25 μ L of insulin (0.25 EU/mg, I0305000, Sigma-Aldrich, St. Louis, MO) with 1% antibiotics (SV30010, HyClone). To investigate the regulatory role of *CD44* on bMEC, cells were seeded into 6-well culture plates (353090, Falcon) at a concentration of 2×10^5 cells/well and cultured at 37°C and 5% CO₂ in a CO₂ incubator (Thermo Fisher Scientific, Waltham, MA). When cells reached 80% confluence, the cell culture medium was changed to a medium without antibiotics for transfection (Jiang et al., 2018). The various vectors were transfected into cells using a FuGENE HD Transfection Reagent according to the manufacturer's protocol (PRE2311, Promega, Madison, WI). Briefly, the mixture of 3 μ g of plasmid DNA and 7.5 μ L of FuGENE HD in 150 μ L of Opti-MEM (Thermo Fisher Scientific) was incubated at room temperature for 15 min and then added into the culture medium in each well of bMEC. After 24 h, the expression of green fluorescent protein (**GFP**) in cells was observed under a fluorescence microscope (TE2000, Nikon, Tokyo, Japan) to determine the transfection efficiency. Three replicates of the experiments were carried out by transfecting equal numbers of cells using the same vectors in the various wells.

Analysis of *CD44* Expression by Quantitative Real-Time PCR

Cells transfected with *CD44* RNAi or overexpression vector were harvested at 24 h post-transfection. Then, 1 μ g of total RNA was extracted with an RNA extraction kit (Analytik Jena, Jena, Germany), and the concentration and purity of the RNA were determined using a spectrophotometer, further confirmed via 1% agarose gel electrophoresis. The cDNA was obtained using a

cDNA synthesis kit (RR047A, TaKaRa Biotechnology, Dalian, China). Then, quantitative real-time (qRT)-PCR was performed with SYBR Premix Ex Taq (RR420L, TaKaRa Biotechnology) with forward primer 5'-CCTCGGATACCAGAGACTACG-3' and reverse primer 5'-CACACCTTCTCCTACTGTTGAC-3'. We performed PCR amplification in 10- μ L reaction volume under the following conditions: an initial denaturing step of 95°C for 30 s, followed by 40 cycles of 95°C for 10 s, and then 60°C for 45 s. All samples were adjusted to the same concentration. The mRNA expression was measured via qRT-PCR with β -actin as the reference gene for internal control. All qRT-PCR were repeated at least 3 times.

Determination of CD44 Protein Expression by Western Blotting

Cells were collected at 48 h after transfection and were washed 2 to 3 times with PBS (21-040-CVR, Corning Inc., Corning, NY). Cells were then resuspended in radio immunoprecipitation assay buffer (AR0105, Boster Bio, Pleasanton, CA) containing protease and phosphatase inhibitors. Cell lysates were centrifuged at 10,000 $\times g$ for 10 min at 4°C, and the supernatants that contained the target protein were collected. The total protein concentration was measured with the Enhanced BCA Protein Quantitation Assay Kit (KGP902, Keygen Biotech, Jiangsu, China). The 3 repeated experiments on the same sample were performed as technical replicates, and protein concentration was measured in triplicate. A negative control treatment was included, in which double-distilled water was added instead of the protein. Averages were calculated to analyze the contents. Polyvinylidene fluoride membrane was then incubated with rabbit polyclonal anti-*CD44* antibody (1:750 dilution in 1 $\times$ Tris-buffered saline, bs-4916, Bioss Antibodies, Beijing, China) and β -actin (1:10,000 dilution in 1 $\times$ Tris-buffered saline, ab8227, Abcam, Shanghai, China) for 14 h at 4°C. The procedure was performed according to a previously published protocol (Jiang et al., 2018). The relative expressions of the target proteins in different samples were calculated with a gray value analyzer (5200, Tanon Science and Technology Co. Ltd., Shanghai, China).

Analysis of the Triglyceride and Cholesterol Contents in bMEC

Triglyceride content was measured using a TG detection kit, and CHOL content was determined using a tissue and cell total CHOL assay kit (E1015-105 and E1013, Applygen Technologies, Beijing, China). Cells

were seeded at 2×10^5 cells per well in 6-well culture plates and transfected with different vectors, with triplicates for each vector (353090, Falcon). Cells were collected 48 h after transfection. A blank control group was set as the reference group using double-distilled water. The cellular contents of TG and CHOL were adjusted by each microgram of protein. The concentrations were measured using a microplate reader (SM600, Shanghai YongChuang Medical Instrument Co. Ltd., Shanghai, China).

FA Extraction and Content Analysis in bMEC by GC

Fatty acids were extracted from bMEC 48 h after transfection. Cells were trypsinized after washing 3 times with PBS. Cell pellets were collected through centrifugation. Folch's solution (CHCl₃:CH₃OH = 2:1, vol/vol), an internal reference FA (ginkgolic acid C13:0, 49962, Sigma-Aldrich) was added to cells, and a standard FA (Supelco 37, 18919-1AMP, Sigma-Aldrich) was selected as the standard solution. The tubes with cell pellets were then filled with high-purity nitrogen and shaken vigorously. After evaporation of chloroform, 1 mL of methyl esterified mixed solvent composed of 35% BF₃ methanol (14%) (33040-U, Sigma-Aldrich), 45% methanol, 20% hexane was added to the glass tubes. Finally, 1 mL of hexane and 0.4 mL of NaCl (0.88%) were added, and the supernatant was transferred to clean glass sample bottles with caps via a Pasteur pipette with a long straw for GC analysis (GC7980, ALS7020, Techcomp, Hong Kong, China). Detailed operating conditions were as follows: the column used was an HP-FFAP elastic quartz capillary column (100 m $\times$ 0.25 mm, 0.2- μ m film thickness), and the initial column temperature was set at 70°C. The injection and detector temperatures were 250°C and 255°C, respectively. The split ratio was 1:1, and the carrier gas was nitrogen. The injection volume was 1.0 μ L under operating conditions. The flow rate of hydrogen, nitrogen, and air gases were 25, 20, and 150 mL/min at the exit port, respectively. Relative retention time was 119.5 min. Cleaning was conducted after every 4 samples. The contents of FA components were calculated by peak area normalization. The 37 standard FA were measured, and then the emergent times of the 37 FA could be shown. The concentration and the added volumes of the internal reference FA were known, and the contents of the tested FA could be calculated according to the quantity of the internal reference FA and that FA's proportion of total FA, and the proportion of tested FA in total FA. The experiment was repeated 3 times, providing 3 biological replicates, and each replicate was measured 3 times.

Analysis of Lipid and FA Metabolism by RT² Profiler PCR Array

One microgram of total RNA was extracted from bMEC with an RNeasy Mini Kit (74134, Qiagen). Then cDNA was synthesized with an RT² First Strand Kit (330404, Qiagen) according to the manufacturer's protocol. The qRT-PCR was carried out with the Mx3005p system (Stratagene, Agilent, Santa Clara, CA). Transcript levels of related genes in lipid and FA metabolism pathways were determined using an RT² Profiler PCR Array (CLAB24070A, Qiagen). Five different housekeeping genes were chosen as the reference controls: β -actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), tyrosine 3-monooxygenase (*YWHAZ*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and TATA-box binding protein (*TBP*). Threshold count (Ct) values were normalized based on the selection of reference genes. The expression fold change or regulation was calculated through a data analysis web portal using the $\Delta\Delta C_t$ method, in which ΔC_t is calculated between the gene of interest and an average of reference genes, followed by $\Delta\Delta C_t$ calculations [$\Delta C_t(\text{test group}) - \Delta C_t(\text{control group})$]. Fold change was then calculated using the $2^{-\Delta\Delta C_t}$ formula.

Statistical Analysis

Experimental data are shown as mean $\pm$ standard error of the mean. Statistically significant differences were defined as $P < 0.05$. Relative gene expression data from qRT-PCR were analyzed using the comparative Ct method ($2^{-\Delta\Delta C_t}$). GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA) with a 2-tailed (unpaired) *t*-test was used for the data analysis. Relative gene expression data were analyzed using the online RT² Profiler PCR Array data analysis software (<https://www.qiagen.com/cn/shop-old/data-interpretation-systems/bioinformatics/geneglobe-data-analysis-center/>). The negative control was used as the control group. We present RNAi or overexpression of *CD44* as relative changes compared with the control.

RESULTS

Construction of the *CD44* Expression Vector

The RNAi vector pb7SK-sh*CD44* and overexpression vector pBI-CMV3-*CD44* were constructed to examine the function of *CD44* on the contents of TG and CHOL (Figure 1A). Plasmid vectors were transfected into bMEC (Figure 1B). The expression of green fluorescence protein was observed in the transfected group,

but not in the untransfected group, 24 h after transfection (Figure 1C).

Analysis of *CD44* mRNA Expression

Expression of *CD44* was increased in the pBI-CMV3-*CD44* overexpression vector group compared with the pBI-CMV3 vector group ($P < 0.05$, Figure 2A). In addition, the mRNA expression in the pb7SK-sh*CD44* vector-transfected bMEC group was lower compared with the pb7SK/GFP/Neo negative control group ($P < 0.05$, Figure 2B).

Analysis of *CD44* Protein Expression

Western blotting results showed that *CD44* protein expression in pBI-CMV3-*CD44* group was upregulated relative to that in the pBI-CMV3 group (Figure 2C), and the protein expression in the pb7SK-sh*CD44* group was downregulated relative to that in pb7SK/GFP/Neo negative control group (Figure 2D). The *CD44* protein level was expressed as the ratio of *CD44* protein to β -actin.

Effect of *CD44* on TG Content

Levels of TG were higher in bMEC transfected with pBI-CMV3-*CD44* than in the control bMEC, which were transfected with the pBI-CMV3 empty vector ($P < 0.05$, Figure 3A). In addition, TG content was lower in bMEC transfected with pb7SK-sh*CD44* than in the control bMEC, which were transfected only with pb7SK/GFP/Neo empty vector ($P < 0.05$, Figure 3B).

Effect of *CD44* on CHOL Content

Levels of CHOL were detected with a CHOL kit, to examine whether the *CD44* gene participated in the CHOL metabolism pathway. The results showed that CHOL content was higher in bMEC transfected with pBI-CMV3-*CD44* than in the control group ($P < 0.05$, Figure 3C). Furthermore, CHOL content was lower in bMEC transfected with pb7SK-sh*CD44* than in the control group, which were transfected with only pb7SK/GFP/Neo empty vector ($P < 0.05$, Figure 3D).

Effect of *CD44* on FA Content

The FAME mix of 37 components were measured via GC. The contents of various FA are described in Tables 1 and 2. Concentrations of various FA, such as tridecanoic acid, palmitoleic acid, elaidic acid, stearic acid, linoleic acid, and oleic acid, were lower in the *CD44*

overexpression group compared with the negative control group, whereas the concentrations of α -linolenic acid; *cis*-8,11,14-eicosatrienoic acid, *cis*-5,8,11,14-eico-

satetraenoic acid, palmitic acid, and myristic acid were increased. The concentrations of diverse FA, such as tridecanoic acid, palmitic acid, palmitoleic acid, elaidic

Figure 1. Construction of the *CD44* gene overexpression vector and the interference vector. (A) The *CD44* gene chromosomal location and structural features in bovine. (B) Vector construction for overexpression and knockdown of the *CD44* gene. (i) Construction of the *CD44* gene interference vector (pb7SK-shCD44). (ii) The construction of the *CD44* gene overexpression vector (pBICMV3-CD44). (C) Overexpression and interference vectors transfected into bovine mammary epithelial cells (bMEC). GFP = green fluorescent protein.

acid, linoleic acid, α -linolenic acid, and *cis*-8,11,14-eicosatrienoic acid were decreased in the *CD44* knockdown group. Moreover, the concentrations of stearic acid and *cis*-5,8,11,14-eicosatetraenoic acid were increased in this group compared with the control group. In addition, oleic acid was not detected in the *CD44* knockdown group, which might have resulted from the silencing of *CD44* expression. However, myristic acid was detected

in the overexpression group, suggesting that its content may be regulated by the *CD44* gene.

Expression of Genes Involved in Lipid Metabolism Pathway

The results indicated that RNAi of *CD44* in bMEC led to upregulation of 4 genes, and the other 4 genes

Figure 2. Protein and mRNA expression levels of the *CD44* gene in bovine mammary epithelial cells (bMEC). (A) Relative expression levels of *CD44* in bMEC transfected with the pBI-CMV3-CD44 vector. (B) Relative expression levels of *CD44* in bMEC transfected with the pb7SK-shCD44 vector. (C and D) Protein expression levels of CD44 in bMEC transfected with the pBI-CMV3-CD44 and pb7SK-shCD44 vectors. Gray value ratios were calculated as target protein content or β -actin content. Experimental data are shown as mean $\pm$ SEM. GFP = green fluorescent protein.

Table 1. The concentrations of various fatty acids in bovine mammary epithelial cells transfected with pBI-CMV3-CD44 and pBI-CMV3; experimental data are shown as mean $\pm$ SEM

Fatty acid component	pBI-CMV3-CD44	pBI-CMV3	P-value
Tridecanoic acid	0.1471 $\pm$ 0.01279	0.3923 $\pm$ 0.0266	0.0011
Myristic acid	0.0767 $\pm$ 0.0000	0	
Palmitoleic acid	0.1471 $\pm$ 0.0128	0.2394 $\pm$ 0.0115	0.0058
Stearic acid	0.1279 $\pm$ 0.0128	0.1396 $\pm$ 0.0000	0.4102
Elaidic acid	2.5703 $\pm$ 0.1535	3.3245 $\pm$ 0.0931	0.0137
Oleic acid	0.2813 $\pm$ 0.0231	0.4056 $\pm$ 0.0290	0.0284
Linoleic acid	0.3772 $\pm$ 0.0169	0.3923 $\pm$ 0.0266	0.6580
α -Linolenic acid	0.0320 $\pm$ 0.0064	0.01995 $\pm$ 0.000	0.1332
<i>cis</i> -8,11,14-eicosatrienoic acid	0.3005 $\pm$ 0.0279	0.2074 $\pm$ 0.0925	0.3898
<i>cis</i> -5,8,11,14-eicosatetraenoic acid	0.1023 $\pm$ 0.0064	0	
Palmitic acid	6.2404 $\pm$ 0.5427	5.8311 $\pm$ 0.3595	0.5636

Figure 3. Effects of *CD44* on triglyceride (TG) and cholesterol (CHOL) content in bovine mammary epithelial cells (bMEC). (A) TG content in bMEC transfected with the pBI-CMV3-CD44 overexpression vector. (B) TG content in bMEC transfected with the pb7SK-shCD44 vector. (C) CHOL content in bMEC transfected with the pBI-CMV3-CD44 overexpression vector. (D) CHOL content in bMEC transfected with the pb7SK-shCD44 vector. Experimental data are shown as mean $\pm$ SEM. GFP = green fluorescent protein. * $P < 0.05$; *** $P < 0.0001$.

Table 2. The concentrations of various fatty acids are described in bovine mammary epithelial cells transfected with the pb7SK-shCD44 vector and negative control; experimental data are shown as mean $\pm$ SEM

Fatty acid component	pb7SK-shCD44	Negative control	P-value
Tridecanoic acid	0.1661 $\pm$ 0.0315	0.1887 $\pm$ 0.0360	0.6615
Palmitic acid	5.8566 $\pm$ 0.4477	6.7060 $\pm$ 0.6347	0.3356
Palmitoleic acid	0.1180 $\pm$ 0.0076	0.1769 $\pm$ 0.0068	0.0044
Stearic acid	0.2010 $\pm$ 0.0087	0.1494 $\pm$ 0.0079	0.0117
Elaidic acid	1.5822 $\pm$ 0.7045	2.4135 $\pm$ 0.2039	0.3203
Linoleic acid	0.2622 $\pm$ 0.0303	0.3695 $\pm$ 0.0283	0.0609
α -Linolenic acid	0.6381 $\pm$ 0.0350	0.7626 $\pm$ 0.0283	0.0506
<i>cis</i> -8,11,14-eicosatrienoic acid	0.1923 $\pm$ 0.0231	0.2987 $\pm$ 0.0208	0.0267
<i>cis</i> -5,8,11,14-eicosatetraenoic acid	0.2360 $\pm$ 0.0262	0.1336 $\pm$ 0.0079	0.0201
Oleic acid	0	1.1557 $\pm$ 0.7904	

were downregulated. Overexpression of *CD44* in bMEC upregulated 21 genes and downregulated 23 genes (Tables 3 and 4). All the differentially expressed genes involved in lipid and FA metabolism pathways are presented in Tables 3 and 4.

DISCUSSION

The main physiological function of dairy cow mammary tissue is to synthesize and secrete milk. Changes in lipid metabolism can affect numerous cellular processes, including cell growth and differentiation. The biosynthesis and storage of TG are an important cellular process. Cells such as bMEC can accumulate TG in lipid droplets. The successful isolation and culture of bMEC has been widely used in the functional verification of exogenous genes, the mechanism of study of dairy cow mastitis and udder development. Recent studies have showed that different micro (mi)RNA, such as miR-21-3p targets *Elovl5* and regulates triglyceride production in bMEC (Li et al., 2019); miR-152 regulates triglyceride production in bMEC by targeting the *ACAA2* and *HSD17B12* genes, which are associated with lipid metabolism (Yang et al., 2018). Altering the expression of these genes could regulate the TG content in bMEC. In addition, the *GRK2* gene can negatively regulate milk protein synthesis by activating

the mitogen-activated protein kinase pathway in bMEC (Hou et al., 2016). Therefore, bMEC can be used as an experimental model to study the function of genes that regulate the synthesis of milk fat components and identify candidate genes related to milk fat synthesis. Triglycerides, CHOL, and FA are the predominant components of milk fat and are recognized as important sources of nutrients in organisms, building blocks for synthesis of cellular components, and energy sources (Lock and Bauman, 2004).

Our previous transcriptome analysis revealed that *CD44* is a differentially expressed gene that might be associated with milk fat synthesis. There are certain limitations to this previous model for studying TG synthesis in the mammary gland (Hosseini et al., 2013). Previous studies have shown that bMEC could be used as a cell model to study target genes associated with milk component synthesis (Hou et al., 2016), so we designed our experiment with bMEC to provide information to gain insight into the regulation mechanisms of the *CD44* gene, contributing to milk fat synthesis.

The *CD44* gene could regulate the CHOL content of milk. The ATP-binding cassette (ABC) subfamily E member 1 (*ABCE1*) is a key member of the superfamily of ABC transporters, which is regulated by *CD44* and predicted to be associated with *CD44* via IID UniProtKB STRING (https://version11.string-db.org/newstring.cgi/show_edge_details.pl?identifiers=9606.ENSP00000398632%0D9606.ENSP00000296577) in the present study. At the same time, the ABC family proteins could utilize ATP as a source of energy for transporting lipids and cholesterol across membranes (Dean et al., 2001), which indicates that *CD44* might regulate CHOL by altering the expression of ABC-family proteins.

Fatty acids have different turnover rates in vivo and are either broken down by a series of mitochondrial β -oxidation processes into acetyl-CoA to contribute to ATP generation through catabolic metabolism or incorporated into triacylglycerol and CHOL esters through

Table 3. Fold changes in the expression of genes involved in lipid metabolism that are regulated by *CD44* silencing in bovine mammary epithelial cells

Gene symbol	Fold change	Reference sequence
<i>ACAD11</i>	4.1125	NM_001075893
<i>ACADS</i>	0.2207	NM_001034401
<i>ACADSB</i>	0.6507	NM_001017933
<i>ACOT9</i>	0.0579	NM_001034560
<i>ACOX2</i>	1.7171	NM_001102015
<i>ACSBG1</i>	2.8481	NM_001024548
<i>ACSBG2</i>	0.6285	NM_174290
<i>ELOVL3</i>	1.5801	NM_001192306

Table 4. Fold changes in expression of genes involved in lipid and fatty acid metabolism that are regulated by *CD44* overexpression in bovine mammary epithelial cells

Gene name	Fold change	Reference sequence	Gene name	Fold change	Reference sequence	Gene name	Fold change	Reference sequence
<i>ACAA1</i>	7.6529	NM_001034319	<i>ACSBG2</i>	0.0453	NM_174290	<i>FABP2</i>	0.3222	NM_001025332
<i>ACAA2</i>	0.4078	NM_001035342	<i>ACSL1</i>	0.2216	NM_001076085	<i>FABP3</i>	0.0072	NM_174313
<i>ACAD10</i>	0.6012	NM_001192292	<i>ACSL5</i>	0.197	NM_001075650	<i>FABP4</i>	0.0121	NM_174314
<i>ACAD11</i>	0.5889	NM_001075893	<i>ACSL6</i>	8.4914	NM_001038042	<i>FABP5</i>	0.0003	NM_174315
<i>ACADS</i>	1.5115	NM_001034401	<i>ACSM4</i>	0.4494	NM_002698016	<i>FABP7</i>	0.0072	NM_001078162
<i>ACADSB</i>	0.0746	NM_001017933	<i>ALDH2</i>	0.0264	NM_001075367	<i>FADS1</i>	0.0054	NM_002699285
<i>ACADVL</i>	2.1977	NM_174494	<i>BDH1</i>	7.3922	NM_001034600	<i>FASN</i>	3.0441	NM_001012669
<i>ACAT1</i>	0.3753	NM_001046075	<i>CPT1A</i>	2.9201	NM_001789518	<i>GK</i>	4.8099	NM_001075236
<i>ACAT2</i>	0.0913	NM_001075549	<i>CPT1C</i>	0.5344	NM_002695120	<i>GPD1</i>	12.5186	NM_001035354
<i>ACOT2</i>	0.6859	NM_001101938	<i>CPT2</i>	0.4918	NM_001045889	<i>HADHA</i>	0.6579	NM_174335
<i>ACOT7</i>	3.0652	NM_001075682	<i>CRAT</i>	2.4217	NM_001075587	<i>HMGCL</i>	2.7625	NM_001075132
<i>ACOT8</i>	13.3244	NM_001075157	<i>CROT</i>	0.0641	NM_177494	<i>HMGCS1</i>	0.0673	NM_001206578
<i>ACOX1</i>	0.4652	NM_001035289	<i>DECRL2</i>	1.5115	NM_001078122	<i>HMGCS2</i>	0.2824	NM_001045883
<i>ACOX2</i>	6.1305	NM_001102015	<i>ECHS1</i>	0.5021	NM_001025206	<i>LIPE</i>	1.7851	NM_001080220
<i>LPL</i>	0.1578	NM_001075120	<i>ECI2</i>	0.3069	NM_001034242	<i>SLC27A3</i>	1.9265	NM_001790632
<i>MCEE</i>	0.5091	NM_001045995	<i>EHHADH</i>	1.5326	NM_001075780	<i>SLC27A6</i>	5.3000	NM_001101169
<i>ME1</i>	0.0014	NM_001144853	<i>PRKACB</i>	0.0499	NM_174585	<i>PRKAB1</i>	2.5597	NM_001024558
<i>PECR</i>	0.4717	NM_002685568	<i>SLC27A1</i>	9.5533	NM_001033625	<i>PRKAB2</i>	0.475	NM_001192328

anabolic metabolism (Tang et al., 2018). Overexpression of *CD44* in bMEC increased the contents of TG and CHOL, and the FA concentrations of α -linolenic acid, *cis*-8,11,14-eicosatrienoic acid, and palmitic acid were also increased. Furthermore, the inhibition of *CD44* expression in bMEC suppressed TG and CHOL contents, and the concentrations of α -linolenic acid, *cis*-8,11,14-eicosatrienoic acid, and palmitic acid were also reduced relative to the negative control group. Remarkably, α -linolenic acid, palmitic acid, and *cis*-8,11,14-eicosatrienoic acid were regulated by the expression of *CD44*. *Cis*-8,11,14-eicosatrienoic acid, palmitic acid, and elaidic acid are essential for lipid metabolism (Strum-Odin et al., 1987; Schirmer and Phinney, 2007; Ishibashi et al., 2016; Tran et al., 2016; Yang et al., 2016; Montakhab-Yeganeh et al., 2018). Unexpectedly, palmitoleic acid in the *CD44* overexpression group was significantly decreased compared with the negative control group, and also decreased in the *CD44* knockdown group. We speculated that the palmitoleic acid content in cells might be regulated by other genes or pathways, and the mechanism for palmitoleic acid content decreasing by altered *CD44* expression requires further study.

Many protease receptors and transporters are involved in the process of lipid metabolism. These receptors and transporters are regulated by signal transduction pathways to form a complex and delicate regulatory network that maintains the lipid metabolism balance of cells as well as of the whole organism. Previous studies have also indicated that *CD44* is a key gene involved in lipogenesis. The present study showed that *CD44* is a crucial gene affecting the contents of CHOL, FA, and TG in bMEC. The expression of *CD44* significantly regulates genes related to lipid metabolism, such as *ACAD11*, an important member of the acyl-CoA dehydrogenase family that catalyzes the α , β -dehydrogenation of acyl-CoA esters using the electron transfer flavoprotein as the physiologic electron acceptor in mitochondria (He et al., 2011), and the expression of *ACAD11* was significantly increased by knockdown of the *CD44* gene, whereas the expression of *ACAD11* was significantly reduced by overexpression of *CD44*, indicating that *ACAD11* is negatively regulated by *CD44* gene expression. Acyl-CoA dehydrogenase short chain (*ACADS*) is another member of the acyl-CoA dehydrogenase family and catalyzes the first step in mitochondrial β -oxidation of FA (He et al., 2011). Interestingly, the expression of *ACADS* was significantly reduced by silencing the *CD44* gene. Correspondingly, the expression of *ACADS* was significantly increased by the overexpression of *CD44*. This data revealed that *ACADS* expression is positively regulated by *CD44*. The *CD44* gene inhibited the expression of *ACADSB* and *ACSBG2*, while promoting the expression of *ACOX2*.

The expression level of *ACADSB* was positively correlated with cellular TG content and was also actively involved in FA metabolism pathway in bMEC and in milk fat synthesis in dairy cattle (Jiang et al., 2018). These results indicate that *CD44* may regulate FA metabolism in bMEC by regulating the expression levels of related genes such as the *ACADS* gene.

Carnitine palmitoyltransferase 1A (*CPT1A*) catalyzes the transfer of acyl of long-chain FA-CoA conjugates onto carnitine, which is a crucial step for mitochondrial uptake of long-chain FA and their subsequent β -oxidation in mitochondria (Skotte et al., 2017). Carnitine palmitoyltransferase 1C (*CPT1C*) regulates the β -oxidation and transport of long-chain FA into mitochondria and play an important role in the regulation of feeding behavior and energy homeostasis. The *CPT1C* related pathways include AMP-activated protein kinase (*AMPK*) signaling (Wang et al., 2017). Carnitine palmitoyltransferase 2 (*CPT2*) oxidizes long-chain FA in the mitochondria together with carnitine palmitoyltransferase I and is involved in the PPAR- α pathway and metabolism (Lee et al., 2016). Moreover, long-chain acyl-CoA synthetases (*ACSL*) activate long-chain FA (12 to 20 carbons), which are important in FA metabolism (Ehehalt and Füllekrug, 2010; Fuencisla et al., 2011). These results indicate that the expression of *CD44* could affect transport and metabolism of FA by up- or down-regulating transcription of related genes and thereby affect the concentration of FA.

In summary, our results showed that *CD44* participates in regulating the metabolism of long-chain FA by regulating the expression of related genes involved in FA metabolism pathway. The results demonstrated that *CD44* is a key gene that plays a major role in lipid metabolism by regulating the expression of related genes such as *ACADS* and *ACAD11*. The *CD44* gene could promote the synthesis of TG and CHOL from FA. This study revealed the function of the *CD44* gene on the metabolism of lipid components in vitro in bMEC and identified several key players in the regulatory network of the *CD44* gene in FA metabolism, which lays the foundation for further research to unravel the molecular mechanism through which *CD44* influences the synthesis of milk fat components in vivo.

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REFERENCES

- Camp, R. L., T. A. Kraus, M. L. Birkeland, and E. Puré. 1991. High levels of CD44 expression distinguish virgin from antigen-primed B cells. *J. Exp. Med.* 173:763–766. <https://doi.org/10.1084/jem.173.3.763>.
- Dean, M., Y. Hamon, and G. Chimini. 2001. The human ATP-binding cassette (ABC) transporter superfamily. *J. Lipid Res.* 42:1007–1017. <https://doi.org/10.1101/gr.GR-1649R>.
- Ehehalt, R., and J. Füllekrug. 2010. CD36, ACSLs and lipid rafts, determinants for cellular fatty acid uptake. *Chem. Phys. Lipids* 163:S12. <https://doi.org/10.1016/j.chemphyslip.2010.05.038>.
- Fraser, J. K., I. Wulur, Z. Alfonso, and M. H. Hedrick. 2006. Fat tissue: An underappreciated source of stem cells for biotechnology. *Trends Biotechnol.* 24:150–154. <https://doi.org/10.1016/j.tibtech.2006.01.010>.
- Fuencisla, M. D. B., M. Fedetz, D. Ndagire, A. Alcina Madueño, and M. Sabio, inventors. 2011. Long chain acyl coenzyme-a synthetases (ACSLs) as cyto-serological biomarkers in inflammatory or autoimmune diseases. WO2011/135133. Assignee: Consejo Superior De Investigaciones Científicas (CSIC), Fundación Fibao, Fundación Para La Investigación Biosanitaria De Andalucía Oriental-Alejandro Otero, M. Del Barrio, M. Fedetz, D. Ndagire, A. Alcina Madueño, and M. Sabio.
- He, M., Z. Pei, A. W. Mohsen, P. Watkins, G. Murdoch, P. P. Van Veldhoven, R. Ensenauer, and J. Vockley. 2011. Identification and characterization of new long chain Acyl-CoA dehydrogenases. *Mol. Genet. Metab.* 102:418–429. <https://doi.org/10.1016/j.ymgme.2010.12.005>.
- Hosseini, A., R. Sharma, M. Bionaz, and J. J. Loo. 2013. Transcriptomics comparisons of Mac-T cells versus mammary tissue during late pregnancy and peak lactation. *J. Adv. Dairy Res.* 1:103.
- Hou, X., H. Hu, Y. Lin, B. Qu, X. Gao, and Q. Li. 2016. The effect of G protein-coupled receptor kinase 2 (GRK2) on lactation and on proliferation of mammary epithelial cells from dairy cows. *J. Dairy Sci.* 99:5828–5836. <https://doi.org/10.3168/jds.2015-10560>.
- Ishibashi, K., K. Nehashi, T. Oshima, N. Ohkura, and G.-I. Atsumi. 2016. Differentiation with elaidate tends to impair insulin-dependent glucose uptake and GLUT4 translocation in 3T3-L1 adipocytes. *Int. J. Food Sci. Nutr.* 67:99–110. <https://doi.org/10.3109/09637486.2016.1144721>.
- Jensen, R. G., A. M. Ferris, and C. J. Lammi-Keefe. 1991. Symposium: Milk fat—Composition, function, and potential for change: Composition of milk fat. *J. Dairy Sci.* 74:3228–3243. [https://doi.org/10.3168/jds.S0022-0302\(91\)78509-3](https://doi.org/10.3168/jds.S0022-0302(91)78509-3).
- Jiang, P., X. Fang, Z. Zhao, X. Yu, B. Sun, H. Yu, and R. Yang. 2018. The effect of short/branched chain acyl-coenzyme A dehydrogenase gene on triglyceride synthesis of bovine mammary epithelial cells. *Arch. Tierzucht* 61:115–122. <https://doi.org/10.5194/aab-61-115-2018>.
- Kang, H. S., G. Liao, L. M. Degraff, K. Gerrish, C. D. Bortner, S. Garantziotis, and A. M. Jetten. 2013. CD44 plays a critical role in regulating diet-induced adipose inflammation, hepatic steatosis, and insulin resistance. *PLoS One* 8:e58417. <https://doi.org/10.1371/journal.pone.0058417>.
- Khan, W. S., A. B. Adesida, S. R. Tew, U. G. Longo, and T. E. Hardingham. 2012. Fat pad-derived mesenchymal stem cells as a potential source for cell-based adipose tissue repair strategies. *Cell Prolif.* 45:111–120. <https://doi.org/10.1111/j.1365-2184.2011.00804.x>.
- Kodama, K., M. Horikoshi, K. Toda, S. Yamada, K. Hara, J. Irie, M. Sirota, A. A. Morgan, R. Chen, H. Ohtsu, S. Maeda, T. Kadowaki, and A. J. Butte. 2012. Expression-based genome-wide association

- study links the receptor *CD44* in adipose tissue with type 2 diabetes. *Proc. Natl. Acad. Sci. USA* 109:7049–7054. <https://doi.org/10.1073/pnas.1114513109>.
- Lee, J., J. Choi, S. Aja, S. Scafidi, and M. J. Wolfgang. 2016. Loss of adipose fatty acid oxidation does not potentiate obesity at thermoneutrality. *Cell Rep.* 14:1308–1316. <https://doi.org/10.1016/j.celrep.2016.01.029>.
- Li, X., P. Jiang, H. Yu, Y. Yang, L. Xia, R. Yang, X. Fang, and Z. Zhao. 2019. miR-21-3p targets *Elovl5* and regulates triglyceride production in mammary epithelial cells of cow. *DNA Cell Biol.* 38:352–357. <https://doi.org/10.1089/dna.2018.4409>.
- Lock, A. L., and D. E. Bauman. 2004. Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. *Lipids* 39:1197–1206. <https://doi.org/10.1007/s11745-004-1348-6>.
- Lu, C., R. Yang, B. Liu, Z. Li, B. Shen, S. Yan, Y. Zhang, L. Zhang, and Z. Zhao. 2012. Establishment of two types of mammary epithelial cell lines from Chinese Holstein dairy cow. *J. Anim. Vet. Adv.* 11:1166–1172. <https://doi.org/10.3923/javaa.2012.1166.1172>.
- Lubary, M., G. W. Hofland, and J. H. Ter Horst. 2011. The potential of milk fat for the synthesis of valuable derivatives. *Eur. Food Res. Technol.* 232:1–8. <https://doi.org/10.1007/s00217-010-1387-3>.
- Montakhab-Yeganeh, H., H. Babaahmadi-Rezaei, and M. Doosti. 2018. Effect of elaidic acid on ABCA1 expression in raw 264.7 cells. Is it through PPAR-gamma? *EXCLI J.* 17:864–870.
- Schirmer, M. A., and S. D. Phinney. 2007. γ -Linolenate reduces weight regain in formerly obese humans. *J. Nutr.* 137:1430–1435. <https://doi.org/10.1093/jn/137.6.1430>.
- Schmits, R., J. Filmus, N. Gerwin, G. Senaldi, F. Kiefer, T. Kundig, A. Wakeham, A. Shahinian, C. Catzavelos, J. Rak, C. Furlonger, A. Zakarian, J. J. L. Simard, P. S. Ohashi, C. J. Paige, J. C. Gutierrez-Ramos, and T. W. Mak. 1997. *CD44* regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. *Blood* 90:2217–2233. <https://doi.org/10.1182/blood.V90.6.2217>.
- Skotte, L., A. Koch, V. Yakimov, S. Zhou, B. Søborg, M. Andersson, S. W. Michelsen, J. E. Navne, J. M. Mistry, P. A. Dion, M. L. Pedersen, M. L. Børresen, G. A. Rouleau, F. Geller, M. Melbye, and B. Feenstra. 2017. *CPT1A* missense mutation associated with fatty acid metabolism and reduced height in Greenlanders. *Circ. Cardiovasc. Genet.* 10:e001618. <https://doi.org/10.1161/CIRCGENETICS.116.001618>.
- Strum-Odin, R., B. Adkins-Finke, W. L. Blake, S. D. Phinney, and S. D. Clarke. 1987. Modification of fatty acid composition of membrane phospholipid in hepatocyte monolayer with *n*-3, *n*-6 and *n*-9 fatty acids and its relationship to triacylglycerol production. *Biochim. Biophys. Acta* 921:378–391. [https://doi.org/10.1016/0005-2760\(87\)90040-3](https://doi.org/10.1016/0005-2760(87)90040-3).
- Tang, Y., J. Zhou, S. C. Hooi, Y. M. Jiang, and G. D. Lu. 2018. Fatty acid activation in carcinogenesis and cancer development: Essential roles of long-chain acyl-CoA synthetases. *Oncol. Lett.* 16:1390. <https://doi.org/10.3892/ol.2018.8843>.
- Tran, D. Q., E. H. Ramos, and D. D. Belsham. 2016. Induction of *Gnrh* mRNA expression by the ω -3 polyunsaturated fatty acid docosahexaenoic acid and the saturated fatty acid palmitate in a GnRH-synthesizing neuronal cell model, mHypoA-GnRH/GFP. *Mol. Cell. Endocrinol.* 426:125–135. <https://doi.org/10.1016/j.mce.2016.02.019>.
- Wang, R., Y. Cheng, D. Su, B. Gong, X. He, X. Zhou, Z. Pang, L. Cheng, Y. Chen, and Z. Yao. 2017. *Cpt1c* regulated by AMPK promotes papillary thyroid carcinomas cells survival under metabolic stress conditions. *J. Cancer* 8:3675–3681. <https://doi.org/10.7150/jca.21148>.
- Yang, W.-M., K.-H. Min, and W. Lee. 2016. MiR-1271 upregulated by saturated fatty acid palmitate provokes impaired insulin signaling by repressing *INSR* and *IRS-1* expression in HepG2 cells. *Biochem. Biophys. Res. Commun.* 478:1786–1791. <https://doi.org/10.1016/j.bbrc.2016.09.029>.
- Yang, Y., X. Fang, R. Yang, H. Yu, P. Jiang, B. Sun, and Z. Zhao. 2018. MiR-152 regulates apoptosis and triglyceride production in MECs via targeting *ACAA2* and *HSD17B12* genes. *Sci. Rep.* 8:417. <https://doi.org/10.1038/s41598-017-18804-x>.

ORCIDS

- Ping Jiang <https://orcid.org/0000-0001-7341-9070>
 Lixin Xia <https://orcid.org/0000-0001-7712-4851>
 Mengyan Wang <https://orcid.org/0000-0002-1157-0047>
 Runjun Yang <https://orcid.org/0000-0003-1401-4318>
 Xibi Fang <https://orcid.org/0000-0003-1134-0003>
 Zhihui Zhao <https://orcid.org/0000-0001-5907-0669>