The effects of cell death-inducing DNA fragmentation factor- α -like effector C (CIDEc) on milk lipid synthesis in mammary glands of dairy cows

Yang Yang,* Ye Lin,*† Xiaoyu Duan,* He Lv,* Weinan Xing,* Qingzhang Li,*† Xuejun Gao,*† and Xiaoming Hou*¹

*College of Life Science, Northeast Agricultural University, Harbin 150030, China

†Key Laboratory of Dairy Science of Education Ministry, Northeast Agricultural University, Harbin 150030, China

ABSTRACT

Adequate lipid synthesis by the mammary gland during lactation is essential for the survival of mammalian offspring. Cell death-inducing DNA fragmentation factor- α -like effector C (CIDEc) is a lipid droplet-associated protein and functions to promote lipid accumulation and inhibit lipolysis in mice and human adipocytes. However, the function of CIDEc in regulation of milk lipid synthesis in dairy cow mammary gland remains largely unknown. In this study, 6 multiparous Holstein cows (parity = 3) in early lactation were allocated to high-fat milk (milk yield 33.9 ± 2.1 kg/d, milk fat $>3.5\%$, $n = 3$) and low-fat milk (milk yield 33.7 ± 0.5 kg/d, milk fat $<3.5\%$, $n = 3$) groups according to their milk fat content. Lactating cows were slaughtered at 90 d in milk and mammary tissues were collected to detect CIDEc localization. Immunofluorescence staining of sections of lactating mammary glands with high- and low-fat milk showed that CIDEc was expressed in the cytoplasm of epithelial cells and localized to lipid droplets. Lipid droplets and CIDEc protein were also detected in isolated lactating mammary epithelial cells of dairy cows. Immunostaining of CIDEc in isolated mammary epithelial cells also confirmed its presence in the nucleus. The knockdown of *CIDEc* in cultured bovine mammary epithelial cells decreased milk lipid content and reduced expression of genes associated with mammary de novo fatty acid synthesis, short- and long-chain intracellular fatty acid activation, triacylglycerol synthesis, and transcription regulation. These genes included those for acetyl-CoA carboxylase (ACC, -60%), fatty acid synthase (FASN, -65%), acyl-CoA synthetase short-chain family member 2 (ACSS2, -50%), acyl-CoA synthetase long-chain family member 1 (ACSL1, -30%), diacylglycerol acyl-

transferase 1 (DGAT1, -60%), sterol regulatory element-binding protein 1 (SREBP1, -45%), and SREBP cleavage activating protein (SCAP, -66%). Conversely, in cells overexpressing *CIDEc*, triacylglycerol content was increased, and transcription of those genes involved in milk lipid synthesis was coordinately upregulated. These results suggest that CIDEc plays an important role in regulating milk lipid synthesis in dairy cow mammary gland via a mechanism involving gene expression, which provides further insight into the mechanisms regulating mammary lipogenesis in ruminants.

Key words: dairy cow, mammary gland, milk lipid, cell death-inducing DNA fragmentation factor- α -like effector C

INTRODUCTION

Lipids, primarily triacylglycerols (TAG), are major milk constituents of most mammals, providing a large percentage of the calories, essential fatty acids, and bioactive lipids required for neonatal growth and development (McManaman, 2009). Lactating mammary gland synthesizes large amounts of TAG from fatty acids (FA) derived from the blood and from de novo lipogenesis (Emery, 1973). In cows, about half of the milk FA are derived from de novo synthesis (Bauman and Griinari, 2003).

Milk lipid synthesis is a complicated process in vivo and is highly regulated by gene expression. Cell death-inducing DNA fragmentation factor- α -like effector C (CIDEc) is a lipid droplet-associated protein (Tan et al., 2014). It is highly expressed in both white and brown adipose tissues and is strikingly upregulated during adipogenesis in mice (Matsusue et al., 2008; Nishino et al., 2008; Toh et al., 2008). Cell death-inducing DNA fragmentation factor- α -like effector C has the ability to enhance neutral lipid accumulation not only in adipocytes but also in many nonlipogenic cells (Puri et al., 2007). In mice, *CIDEc* overexpression leads to promotion of the formation of intracellular lipid droplets through TAG accumulation in hepatocytes in vitro

Received June 2, 2016.

Accepted December 19, 2016.

¹Corresponding author: xiaominghou@126.com

and in vivo, whereas knockdown of *CIDEA* results in loss of hepatic lipids (Matsusue et al., 2008). In 3T3-L1 adipocytes, *CIDEA* depletion significantly stimulates lipolysis and reduces the size of lipid droplets (Keller et al., 2008). The mammary gland is one of the major lipid-synthesizing organs in dairy cows. However, little is known about the effect of *CIDEA* on milk lipid synthesis in the mammary gland of dairy cows.

Increased milking frequency in dairy cows results in greater milk production (Hillerton et al., 1990). Microarray analysis of the bovine mammary gland has shown that *CIDEA* expression is upregulated by increased milking frequency (Connor et al., 2008). This result suggests that *CIDEA* may participate in milk lipid synthesis. In mice and human adipocytes, *CIDEA* localizes to lipid droplets (Puri et al., 2007). However, no study directly investigating *CIDEA* expression in mammary gland of lactating cows has been reported. Peroxisome proliferator-activated receptor- γ (PPAR γ) belongs to the nuclear receptor superfamily and is expressed in numerous types of cells, including adipocytes, macrophages, and mammary epithelial cells (Kang et al., 2015). In bovine and dairy goat mammary glands, PPAR γ is considered to be a central regulator of lipid metabolism (Bionaz and Loo, 2008b; Kang et al., 2015). Gene network analysis in bovine mammary tissue shows that expression of PPAR γ and its putative target genes is upregulated during lactation, suggesting a role for this nuclear receptor in the regulation of milk lipid synthesis (Bionaz and Loo, 2008b). The *CIDEA* has been identified as a direct target gene of PPAR γ , and its upregulation can elevate TAG levels in mouse hepatocyte (Matsusue et al., 2008). Whether *CIDEA* participates in regulating milk lipid synthesis in lactating dairy cow mammary gland has not been well established.

The objective of this study was to reveal the specific role of *CIDEA* in regulating milk lipid synthesis in the mammary gland of dairy cows. To meet this objective, 6 lactating Holstein cows in the third parity were allocated to high-fat milk (milk yield 33.9 ± 2.1 kg/d, milk fat $>3.5\%$, $n = 3$) and low-fat milk (milk yield 33.7 ± 0.5 kg/d, milk fat $<3.5\%$, $n = 3$) groups. Lactating cows were slaughtered at 90 DIM and mammary tissues were collected to detect *CIDEA* expression. To evaluate the

role of *CIDEA* in mediating milk lipid synthesis in the dairy cow mammary gland, mammary epithelial cells were isolated from lactating mammary tissues of dairy cows with high-fat milk. The expression of genes encoding enzymes and transcription factors involved in milk lipid synthesis were examined in cultured mammary epithelial cells by quantitative real-time PCR (qPCR) when *CIDEA* was knocked down or overexpressed.

MATERIALS AND METHODS

Animals and Tissue Collection

The Northeast Agricultural University Animal Care and Use Committee (Harbin, China) approved all procedures involving dairy cows. Six multiparous Holstein cows (in the third parity, calving at 52 to 54 mo of age) in early lactation were used in this study. Amplification of the polymorphism *DGAT1* K232A by tetra-primer amplification refractory mutation system PCR (Steinberg et al., 2009) showed that all cows carried the AA genotype of *DGAT1* (Supplemental Figure S1; <https://doi.org/10.3168/jds.2016-11549>). All animals were offered grass silage ad libitum supplemented with concentrates. Animals had access to a constant supply of fresh water and were milked twice daily at 0800 and 1530 h.

Lactating cows were allocated to 2 groups (3 cows per group), the high-fat milk (milk fat $>3.5\%$) and low-fat milk (milk fat $<3.5\%$, milk fat decreased likely due to diet-induced milk fat depression) groups, according to their milk fat content (Table 1). Milk yield of lactating cows with high-fat milk was 33.9 ± 2.1 kg/d. Milk yield of lactating cows with low-fat milk was 33.7 ± 0.5 kg/d. Somatic cell count was $<50,000$ cells/mL for all cows. The 6 lactating cows were slaughtered at 90 DIM. Immediately after exsanguination, several parenchyma samples were aseptically removed from the core of the mammary gland. Tissue samples for quantitation of protein and RNA were frozen immediately in liquid nitrogen and stored at -80°C until assay. Mammary tissues for cell culture were collected in 50-mL conical centrifuge tubes with Hanks' Balanced Salt Solution (14170, Life Technologies Corporation, Grand Island, NY), transported, and preserved at 4°C until cell isolation and culture.

Table 1. Milk components of lactating Holstein dairy cows

Milk component	Dairy cows with high-fat milk	Dairy cows with low-fat milk
Milk protein (%)	3.27 ± 0.04	2.89 ± 0.02
Milk fat (%)	4.17 ± 0.01	3.20 ± 0.06
Lactose (%)	4.84 ± 0.03	4.52 ± 0.09
DM (%)	11.93 ± 0.01	10.89 ± 0.03

Mammary Epithelial Cell Culture

Primary cell culture from lactating mammary parenchymal tissues of 3 dairy cows with high-fat milk was performed as described (Hou et al., 2016). Briefly, the tissue samples were minced using surgical scissors. Minced samples were digested with collagenase III (C8490, Solarbio, Beijing, China) for 2 h at 37°C. The digest was filtered through a nylon mesh and the filtrate centrifuged at $150 \times g$ for 10 min at room temperature. The cell inoculum was plated in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (12400, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (04-001-1ACS, BI Biological Industries, Kibbutz Beit-Haemek, Israel), 100 U/mL of penicillin, 100 µg/mL of streptomycin and lactating hormones (5 µg/mL insulin, 1 µg/mL of prolactin, and 1 µg/mL of hydrocortisone; Sigma-Aldrich, St. Louis, MO) in cell culture flasks coated with 0.5% rat tail collagen (Sigma-Aldrich) and incubated in 5% CO₂ at 37°C. The primary cells were trypsinized at ~80% confluency and passaged. Pure mammary epithelial cells were obtained after 3 passages. Immunofluorescence was used to detect cytokeratin 18 expression in the cultured cells to confirm that the isolated cells were purified mammary epithelial cells (Supplemental Figure 2A; <https://doi.org/10.3168/jds.2016-11549>). All experiments were conducted using cells within passage 10. Lipid droplets and β-casein expression were detected by immunofluorescence before experiments to ensure that the cells we used were capable of synthesizing milk components (Supplemental Figures 2B and 2C; <https://doi.org/10.3168/jds.2016-11549>).

Plasmid Construction

Total RNA from lactating mammary tissue samples was extracted using TRIzol reagent (15596, Life Technologies). The RNA quantity and purity were determined by a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE). The RNA integrity was analyzed by denaturing agarose gel electrophoresis with the 28S rRNA band appearing approximately twice as intense as the 18S rRNA band. Total RNA was reverse transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase (C28025, Life Technologies) with oligo dT at 37°C for 50 min. To construct the *CIDE*C expression plasmid, full-length bovine *CIDE*C was amplified from mammary tissue cDNA using PCR and subcloned into the *Bam*H I/*Hind* III sites of the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA). The following PCR primer pair was used: forward = 5'-CCCAAGCTTATGGAATACGC-

CATGAAGTCCCTCAGC-3'; reverse = 5'-CGCG-GATCCTCACTGCAGCATCTTCAGACTG-GTTG-3'. The integrity of plasmid DNA was verified by DNA sequencing.

To construct the short hairpin RNA (shRNA) plasmid for targeting *CIDE*C, a pair of oligonucleotides specific for bovine *CIDE*C sequence and a pair of nontargeting oligonucleotides (scramble) were chemically synthesized and subcloned into the *Bam*HI/*Hind*III sites of pSilencer 3.1-H1 (Ambion, Carlsbad, CA). The shRNA sequences used in this study were as follows: *CIDE*C, forward = 5'-GATC-CGCACATACTCCGTCTCCTATTCAAGAGATAG-GAGACGGAGTATGTGCTTTTTTGGAAA-3'; reverse = 5'-AGCTTTTCCAAAAAAGCACATACTC-CGTCTCCTATCTCTTGAATAGGAGACGGAG-TATGTGCG-3'. Scramble, forward = 5'-GATCCG-CACAAGCTGGAGTACAACCTTCAAGAGAGTTG-TACTCCAGCTTGTGCTTTTTTGGAAA-3'; reverse = 5'-AGCTTTTCCAAAAAAGCACAAAGCTGGAG-TACAACCTCTCTTGAAGTTGTACTCCAGCTTGT-GCG-3'.

Transfection

Transient transfection was performed using Lipofectamine 2000 (11668-019, Life Technologies) according to the manufacturer's instructions (Hou et al., 2016). Briefly, mammary epithelial cells were plated in 6-well plates at 1×10^6 cells/well. For *CIDE*C knockdown, ~80% confluent cells were transfected with either 2.5 µg of *CIDE*C-specific shRNA or nontargeting shRNA as a negative control. For *CIDE*C overexpression, cells were transfected with either 2.5 µg of pcDNA3.1-*CIDE*C or pcDNA3.1(+) as a negative control. All experiments were performed in triplicate. After 24 h of transfection, cells were harvested for qPCR assay. After 48 h of transfection, cells were harvested for lipid droplet immunostaining and TAG content assays.

Western Blot Analysis

Cultured mammary epithelial cells were lysed with RIPA buffer (P0013C, Beyotime Biotechnology, Shanghai, China) containing 0.5 mM PMSF (ST506, Beyotime Biotechnology), 5 µg/mL of aprotinin (A1153, Sigma-Aldrich), and 5 µg/mL of leupeptin (L2884, Sigma-Aldrich), and quantitated by a BCA protein assay kit (23227, Thermo Fisher Scientific Inc., Waltham, MA). Equal amounts of protein (30 µg) were separated on a 10% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. The membrane was blocked for 1 h with 5% skim milk in Tris-buffered saline with

0.1% (vol/vol) Tween-20 and incubated overnight at 4°C with antibody specific to CIDEA (1:500 dilution, ab198204, Abcam, Cambridge, MA) or β -actin (1:1,000 dilution, #4970, Cell Signaling Technology, Beverly, MA). The blot was then treated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000 dilution, ZB-2301, ZSGB-BIO, Beijing, China) for 1 h. Proteins were visualized using enhanced chemiluminescence detection reagent (6683, Cell Signaling Technology). Densitometry analysis was performed with Image-Pro Plus 6.0 (Media Cybernetics Inc., Warrendale, PA) and target protein expression was normalized to β -actin expression.

Immunostaining and Confocal Microscopy

For determination of CIDEA localization in mammary tissues and cultured mammary epithelial cells, 8 μ m-thick cryosections or cultured mammary epithelial cells were fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.05% Triton X-100 (vol/vol) in PBS, blocked with 10% goat serum in PBS for 1 h at 37°C, and treated overnight at 4°C with anti-CIDEA (1:100 dilution, ab198204, Abcam). Sections or cells were washed 3 times with PBS and incubated with tetraethyl rhodamine isothiocyanate-conjugated goat anti-rabbit IgG (1:200 dilution, ZF-0316, ZSGB-BIO) for 30 min at 37°C. Following 3 washes, sections or cells were stained with Bodipy 493/503 (1:1,000 dilution, D3922, stock concentration 1 mg/mL, Invitrogen) for lipid droplet staining, and nuclei were stained with 4',6-diamidino-2-phenylindole. After extensive washing, sections or cells were mounted in 1,4-diazabicyclooctane triethylenediamine. Fluorescence was observed by confocal microscopy (TCS SP2, Leica Microsystems GmbH, Wetzlar, Germany).

For detection of lipid droplets in CIDEA knockdown or overexpressed cells, mammary epithelial cells plated on coverslips were washed twice with PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. After 3 washes with PBS, cells were stained for 20 min at 37°C with Bodipy 493/503 (1:1,000 dilution, D3922, Invitrogen) for lipid droplets, following by incubation for 10 min with 4',6-diamidino-2-phenylindole for nuclei staining. Cells were washed 3 times with PBS and mounted in 1,4-diazabicyclooctane triethylenediamine. Fluorescence was observed by confocal microscopy (TCS SP2, Leica Microsystems GmbH).

Measurement of TAG Content

Mammary epithelial cells were washed 3 times with pre-warmed PBS and then incubated in serum-free me-

dium containing 1% fatty acid-free BSA (A8806, Sigma-Aldrich) for 24 h before TAG content assay. For cellular TAG content determination, lipids were extracted from cultured mammary epithelial cells with hexane/isopropanol (3:2, vol/vol). The lipids were dried with nitrogen and dissolved in ethanol. The TAG levels were detected by a TAG assay kit (E1013, Applygen Technologies Inc., Beijing, China). Protein concentrations in the samples were measured by a BCA protein assay kit (23227, Thermo Fisher Scientific Inc.), and TAG levels were normalized to the protein concentration of each sample. Experiments were performed in triplicate.

qPCR

Total RNA was isolated from cultured mammary epithelial cells with TRIzol reagent (15596, Life Technologies) according to the manufacturer's instructions (Hou et al., 2016). The RNA concentrations were measured on a NanoDrop 2000c spectrophotometer (Thermo Scientific). Total RNA was reverse-transcribed to cDNA with M-MLV reverse transcriptase (28025, Life Technologies). Oligo dT was used as the primer for reverse transcription. Expression levels of selected genes involved in milk lipid synthesis were determined by qPCR analysis using gene-specific primer sets (Table 2). Primers were designed using Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA). Real-time qPCR was performed using the SYBR Premix Ex Taq (RR420A, TaKaRa Biotechnology Co., Ltd., Dalian, China) in the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Grand Island, NY). Thermal cycling conditions were: 30 s of predenaturalization at 95°C, followed by 40 cycles of 5 s denaturation at 95°C and 34 s annealing and extension at 60°C. The PCR product specificity was verified by postamplification melting curve analysis and by running products on an agarose gel. Each gene was amplified in a separate reaction and each reaction was performed in triplicate. The abundance of β -actin was not significantly different between treatments; therefore, the relative expression of target genes was normalized to β -actin and calculated by the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008).

Statistical Analysis

Results are presented as the mean $\pm$ SEM. Data from 3 independent experiments were subjected to statistical analysis using Prism 6 software (GraphPad, La Jolla, CA). The effect of CIDEA knockdown or overexpression on transcript abundance of genes encoding enzymes and transcription factors involved in milk lipid synthesis in bovine mammary epithelial cells was

Table 2. Primer sequences used for quantitative real-time PCR¹

Gene	GenBank accession number	Primer sequence (5' to 3')	Product size (bp)
<i>ACC</i>	NM_174224.2	F: AGACAAACAGGGACCATT R: AGGGACTGCCGAAACAT	141
<i>FASN</i>	NM_001012669.1	F: CCACGGCTGTCGGTAAT R: CGCTCCCACTCATCCTG	163
<i>ACSS2</i>	NM_001105339.1	F: GGCGAATGCCTCTACTGCTT R: GGCCAATCTTTTCTCTAATCTGCTT	100
<i>CD36</i>	NM_001278621.1	F: AAGAACTGGTGACACTCCCTC R: GCTCAAGCCACATCTGTAGAA	168
<i>FABP3</i>	NM_174313.2	F: GAACTCGACTCCCAGCTTGAA R: AAGCCTACCACAATCATCGAAG	102
<i>ACSL1</i>	NM_001076085.1	F: GTGGGCTCCTTTGAAGAAGTGT R: ATAGATGCCTTTGACCTGTTCAAAT	120
<i>SCD1</i>	NM_173959.4	F: CGACCTAAGAGCCGAGAAGC R: CCAAGGGCATAACGGAATAA	162
<i>AGPAT6</i>	NM_001083669.1	F: TCCAGTTTGCCAATAGGGT R: TGGTTGCCGACAATCATCT	151
<i>DGAT1</i>	NM_174593.2	F: CCACTGGGACCTGAGGTGTC R: GCATCACCACACACCAATTCA	101
<i>SREBP1</i>	NM_001113302.1	F: GACACCACCAGCATCAACCA R: GCCAAGGAGAAGAGCACCAG	137
<i>SCAP</i>	NM_001101889.1	F: TGTGAAATCTTCCGTGTCTCC R: CGTGGTTCCGAATCTCCTC	107
<i>INSIG1</i>	NM_001077909.1	F: GGAGGCAGTGAGTGGGAGGT R: GTTTGGGCCAAGACGGAGA	128
<i>PPARγ</i>	NM_181024.2	F: TCAAAGTCCAGCCTGTATC R: CATAGTGGAACCCGTGACG	138
<i>PPARGC1A</i>	NM_177945.3	F: GTACCAGCACGAAAGGCTCAA R: ATCACACGGCGCTCTTCAA	120
β -Actin	NM_173979	F: TGTTAGCTGCGTTACACCTT R: CTGTCACCTTACCGTTCC	163

¹*ACC* = acetyl-CoA carboxylase; *FASN* = fatty acid synthase; *ACSS2* = acyl-CoA synthetase short-chain family member 2; *CD36* = cluster of differentiation 36 (fatty acid translocase); *FABP3* = fatty acid binding protein 3; *ACSL1* = acyl-CoA synthetase long-chain family member 1; *SCD1* = stearoyl-CoA desaturase 1; *AGPAT6* = 1-acylglycerol-3-phosphate-O-acyltransferase 6; *DGAT1* = diacylglycerol acyltransferase 1; *SREBP1* = sterol regulatory element-binding protein 1; *SCAP* = SREBP cleavage activating protein; *INSIG1* = insulin-induced gene 1; *PPAR γ* = peroxisome proliferator-activated receptor- γ ; *PPARGC1A* = peroxisome proliferator-activated receptor gamma coactivator-1 α . F = forward; R = reverse.

assessed using a mixed model ANOVA. Fixed effect in the model was treatment, whereas the random effect was replicate. Bonferroni's multiple comparison test was used for post-ANOVA comparisons. Differences at $P < 0.05$ were considered significant.

RESULTS

*CIDE*C Expression in Lactating Mammary Glands of Dairy Cows

To determine the tissue-specific expression of *CIDE*C in lactating cows, we detected *CIDE*C expression in lactating mammary tissues with high- and low-fat milk. Immunofluorescence staining of sections of lactating mammary glands with high- and low-fat milk (diet-induced milk fat depression) both showed that mammary epithelial cells contained cytoplasmic lipid droplets, most of which were located near the lumen face. As indicated by the images in Figure 1A, *CIDE*C was mainly observed in the cytoplasm of epithelial cells

and localized to lipid droplets. Furthermore, lipid droplets and *CIDE*C protein were also detected in isolated lactating mammary epithelial cells. In addition to its previously established localization on lipid droplets, a substantial portion of *CIDE*C protein was detected in the nucleus (Figure 1B), suggesting that *CIDE*C may participate in regulation of milk lipid synthesis.

*Effect of CIDE*C on Milk Lipid Synthesis in Mammary Glands of Dairy Cows

To investigate whether *CIDE*C has any role in regulating milk lipid synthesis in lactating mammary glands of dairy cows, cultured mammary epithelial cells were transiently transfected either with shRNA directed against *CIDE*C or scrambled shRNA as a negative control. As shown in Figures 2A and 2B, the mRNA and protein levels of *CIDE*C were significantly decreased when cells were transfected with *CIDE*C-specific shRNA ($P < 0.01$; Figures 2A and 2B), indicating the effectiveness of shRNA in reducing *CIDE*C mRNA and

protein abundance. The cellular TAG levels in *CIDECD*-knockdown mammary epithelial cells were reduced ($P < 0.05$; Figure 2C). Consistent with this, the population of lipid droplets was decreased in *CIDECD*-knockdown cells compared with cells transfected with scrambled shRNA (Figure 2D).

Next, we treated cultured mammary epithelial cells with the pcDNA3.1-*CIDECD* vector or empty pcDNA3.1(+) vector as a negative control. Compared with cells carrying the empty pcDNA3.1(+) vector, the mRNA and protein levels of *CIDECD* in dairy cow mammary epithelial cells were significantly increased by transfection with pcDNA3.1-*CIDECD* vector ($P < 0.01$; Figures 3A and 3B). The overexpression of *CIDECD* in mammary epithelial cells markedly enhanced TAG synthesis ($P < 0.01$; Figure 3C). Control cells transfected with the empty pcDNA3.1(+) vector exhibited smaller lipid droplets. In contrast, transfection of mammary epithelial cells with pcDNA3.1-*CIDECD* resulted in lipid droplets with increased size (Figure 3D). Overall, these data support the notion that *CIDECD* is crucial in

controlling milk lipid synthesis in mammary glands of dairy cows.

Effects of *CIDECD* on Expression of Enzymes Involved in Milk Lipid Synthesis

To explore the molecular events by which *CIDECD* mediates milk lipid synthesis, we conducted an analysis

Figure 1. The expression of cell death-inducing DNA fragmentation factor- α -like effector C (*CIDECD*) in lactating mammary tissues and cultured mammary epithelial cells of dairy cows. (A) Immunofluorescence staining showing the localization of *CIDECD* expression in mammary tissues of dairy cows. Scale bar = 45 μ m; H = lactating mammary tissues with high-fat milk; L = lactating mammary tissues with low-fat milk. (B) Immunofluorescence staining showing the localization of *CIDECD* expression in cultured mammary epithelial cells of dairy cows. Scale bar = 10 μ m. For A and B, *CIDECD* was labeled by tetraethyl rhodamine isothiocyanate (red, light gray in the CIDECD and Merge panels in the grayscale version), lipid droplets (LD) were labeled by Bodipy 493/503 (green, light gray in the LD and Merge panels in the grayscale version; Invitrogen, Carlsbad, CA), and nuclei were labeled by 4',6-diamidino-2-phenylindole (blue, dark gray in the Nuclei and Merge panels in the grayscale version). Merge images show the overlap of nuclei, *CIDECD*, and lipid droplets. Color version available online.

Figure 2. The effects of cell death-inducing DNA fragmentation factor- α -like effector C (*CIDECD*) knockdown on milk lipid synthesis. (A) Quantitative real-time PCR (qPCR) analyses of the mRNA levels of *CIDECD* in *CIDECD*-knockdown (RNAi) and scrambled short hairpin RNA (shRNA) control (Con) mammary epithelial cells. The RNA was extracted from control and shRNA-treated cells, and mRNA levels were measured by qPCR and normalized to β -actin mRNA. (B) Western blot analyses of the protein levels of *CIDECD* and β -actin (loading control) in *CIDECD*-knockdown (RNAi) and scrambled shRNA control (Con) mammary epithelial cells. Protein lysates from control and *CIDECD* shRNA-treated cells were loaded at 30 μ g/lane and probed with antibody against *CIDECD* or β -actin. (C) Knockdown of *CIDECD* in cultured mammary epithelial cells of dairy cows (RNAi) decreased triacylglycerol (TAG) content. (D) Immunofluorescence staining showing the lipid droplets in *CIDECD*-knockdown (RNAi) and scrambled shRNA control (Con) mammary epithelial cells. Lipid droplets (LD) were labeled by Bodipy 493/503 (green, light gray in the LD and Merge panels in the grayscale version; Invitrogen, Carlsbad, CA), and nuclei were labeled by 4',6-diamidino-2-phenylindole (blue, dark gray in the Nuclei and Merge panels in the grayscale version). Merge images show the overlap between nuclei and lipid droplets. Scale bar = 30 μ m. Results are shown as the mean $\pm$ SEM from 3 independent experiments. * $P < 0.05$; ** $P < 0.01$. Color version available online.

of gene expression in lactating mammary epithelial cells when *CIDEA* was knocked down or overexpressed. The mRNA expression of acyl-CoA synthetase short-chain family member 2 (*ACSS2*), acetyl-CoA carboxylase (*ACC*), and fatty acid synthase (*FASN*), which encode enzymes involved in the de novo FA synthesis (Bionaz

Figure 3. The effects of cell death-inducing DNA fragmentation factor- α -like effector C (*CIDEA*) overexpression on milk lipid synthesis. (A) Quantitative real-time PCR (qPCR) analyses of the mRNA levels of *CIDEA* in *CIDEA*-overexpressing mammary epithelial cells. The RNA was extracted from control (Con) and *CIDEA*-overexpressing cells, and mRNA levels were measured by qPCR and normalized to β -actin mRNA. (B) Western blot analyses of the protein levels of *CIDEA* and β -actin (loading control) in *CIDEA*-overexpressing mammary epithelial cells. Protein lysates from control and *CIDEA*-overexpressing cells were loaded at 30 μ g/lane and probed with antibody against *CIDEA* or β -actin. (C) Overexpression of *CIDEA* in cultured mammary epithelial cells of dairy cows increased triacylglycerol (TAG) content. (D) Immunofluorescence staining showing the lipid droplets (LD) in *CIDEA*-overexpressing mammary epithelial cells. Lipid droplets were labeled by Bodipy 493/503 (green, light gray in the LD and Merge panels in the grayscale version; Invitrogen, Carlsbad, CA), and nuclei were labeled by 4',6-diamidino-2-phenylindole (blue, dark gray in the Nuclei and Merge panels in the grayscale version). Merge images show the overlap between nuclei and lipid droplets. Scale bar = 30 μ m. In the control group (Con), cells were transfected with the pcDNA3.1(+) vector. In the overexpression group, cells were transfected with the pcDNA3.1-*CIDEA* vector. Results are shown as the mean $\pm$ SEM from 3 independent experiments. ** P < 0.01. Color version available online.

Figure 4. Effects of cell death-inducing DNA fragmentation factor- α -like effector C (*CIDEA*) on expression of genes encoding enzymes involved in milk lipid synthesis. (A) The mRNA abundance of genes encoding enzymes involved in de novo fatty acid synthesis in mammary epithelial cells with *CIDEA* knockdown (RNAi). (B) The mRNA abundance of genes encoding enzymes involved in de novo fatty acid synthesis in mammary epithelial cells with *CIDEA* overexpression. (C) The mRNA abundance of genes encoding enzymes involved in fatty acid uptake, activation, transportation, and desaturation in mammary epithelial cells with *CIDEA* knockdown (RNAi). (D) The mRNA abundance of genes encoding enzymes involved in fatty acid uptake, activation, transportation, and desaturation in mammary epithelial cells with *CIDEA* overexpression. (E) The mRNA abundance of genes encoding enzymes involved in triacylglycerol (TAG) synthesis in mammary epithelial cells with *CIDEA* knockdown (RNAi). (F) The mRNA abundance of genes encoding enzymes involved in TAG synthesis in mammary epithelial cells with *CIDEA* overexpression. For A, C, and E, total RNA was extracted from mammary epithelial cells transfected with *CIDEA* short hairpin RNA (RNAi) or scrambled RNA as a negative control (Con) for 24 h. For B, D, and F, total RNA was extracted from mammary epithelial cells transfected with pcDNA3.1-*CIDEA* or pcDNA3.1(+) vector as control (Con) for 24 h. The mRNA levels were measured by quantitative real-time PCR and normalized to β -actin mRNA. Results are shown as the mean $\pm$ SEM from 3 independent experiments, each of which was performed in triplicate. * P < 0.05; ** P < 0.01. *ACSS2* = acyl-CoA synthetase short-chain family member 2; *ACC* = acetyl-CoA carboxylase; *FASN* = fatty acid synthase; *CD36* = cluster of differentiation 36 (fatty acid translocase); *ACSL1* = acyl-CoA synthetase long-chain family member 1; *FABP3* = fatty acid binding protein 3; *SCD1* = stearoyl-CoA desaturase 1; *AGPAT6* = 1-acylglycerol-3-phosphate-O-acyltransferase 6; *DGAT1* = diacylglycerol acyltransferase 1.

and Loor, 2008b), was reduced by ~50, 60, and 65%, respectively, by *CIDE*C shRNA treatment compared with the control (Figure 4A). In contrast, the overexpression of *CIDE*C in dairy cow mammary epithelial cells significantly increased the levels of *ACSS2* ($P < 0.01$), *ACC* ($P < 0.01$), and *FASN* ($P < 0.05$; Figure 4B) mRNA.

The mRNA expression of acyl-CoA synthetase long-chain family member 1 (*ACSL1*), which encodes an enzyme involved in long-chain FA activation, was decreased by ~30% when cells were transfected with *CIDE*C shRNA (Figure 4C). Overexpression of *CIDE*C in the cells significantly increased *ACSL1* mRNA levels ($P < 0.05$; Figure 4D). However, compared with the control, the expression levels of the genes for FA translocator *FAT/CD36* (*CD36*), fatty acid binding protein 3 (*FABP3*), and stearoyl-CoA desaturase 1 (*SCD1*), which are involved in long-chain FA uptake from blood, intracellular FA trafficking, and desaturation, were not changed in *CIDE*C-knockdown cells and in cells overexpressing *CIDE*C (Figures 4C and 4D).

To evaluate the effect of *CIDE*C on the expression of enzymes involved in TAG synthesis, we analyzed the mRNA levels of 1-acylglycerol-3-phosphate-O-acyltransferase 6 (*AGPAT6*), and diacylglycerol acyltransferase 1 (*DGAT1*). The results showed that knockdown of *CIDE*C in mammary epithelial cells led to substantially lower expression of *DGAT1* (~60%, $P < 0.01$), but not of *AGPAT6*. In contrast, the overexpression of *CIDE*C resulted in higher *DGAT1* expression ($P < 0.05$) but similar levels of expression of *AGPAT6* (Figures 4E and 4F).

Effects of *CIDE*C on Expression of Transcription Factors Involved in Milk Lipid Synthesis

Several transcription factors regulate milk lipid synthesis in dairy cows. The mRNA expression of sterol regulatory element-binding protein 1 (*SREBP1*) and *SREBP* cleavage activating protein (*SCAP*) were reduced by ~45 and 66%, respectively, by *CIDE*C shRNA treatment compared with control ($P < 0.01$; Figure 5A). In contrast, the overexpression of *CIDE*C in dairy cow mammary epithelial cells significantly increased *SREBP1* and *SCAP* expression levels ($P < 0.05$; Figure 5B). However, compared with the control, the expression of insulin-induced gene 1 (*INSIG1*), which interacts with *SCAP* and regulates the responsiveness of *SREBP1* processing via *SCAP*, was not changed in *CIDE*C-knockdown cells or in cells overexpressing *CIDE*C (Figures 5A and 5B). Moreover, the knockdown or overexpression of *CIDE*C did not affect the expression levels of *PPAR* γ and its coactivator, peroxisome

Figure 5. Effects of cell death-inducing DNA fragmentation factor- α -like effector C (*CIDE*C) on expression of genes encoding transcription factors involved in milk lipid synthesis. (A) The mRNA abundance of genes encoding transcription factors involved in milk lipid synthesis in mammary epithelial cells with *CIDE*C knockdown (RNAi). Total RNA was extracted from mammary epithelial cells transfected with *CIDE*C short hairpin RNA (RNAi) or scrambled RNA as a negative control (Con) for 24 h. The mRNA levels were measured by quantitative real-time PCR (qPCR) and normalized to β -actin mRNA. (B) The mRNA abundance of genes encoding transcription factors involved in milk lipid synthesis in mammary epithelial cells with *CIDE*C overexpression. Total RNA was extracted from mammary epithelial cells transfected with pcDNA3.1-*CIDE*C or pcDNA3.1(+) vector as the control (Con) for 24 h. The mRNA levels were measured by qPCR and normalized to β -actin mRNA. Results are shown as the mean $\pm$ SEM from 3 independent experiments, each of which was performed in triplicate. * $P < 0.05$; ** $P < 0.01$. *PPAR* γ = peroxisome proliferator-activated receptor- γ ; *PPARGC1A* = peroxisome proliferator-activated receptor gamma coactivator-1 α ; *INSIG1* = insulin-induced gene 1; *SREBP1* = sterol regulatory element-binding protein 1; *SCAP* = *SREBP* cleavage activating protein.

proliferator-activated receptor gamma coactivator-1 α (*PPARGC1A*; Figures 5A and 5B).

DISCUSSION

Milk lipid synthesis plays a central role in determining dairy product quality. Understanding the biosynthesis of milk lipids and the factors that influence milk lipid quantity and quality are important for the dairy industry. Cell death-inducing DNA fragmentation factor- α -like effector C is known to be crucial in promoting formation of large lipid droplets in brown and white adipocytes (Nishino et al., 2008; Toh et al., 2008). However, little is known about its expression and function in dairy cow mammary gland. Our study demonstrates that *CIDEA* is expressed in lactating mammary tissues and cultured mammary epithelial cells of dairy cows. We also observed *CIDEA* expression in dairy cow mammary tissues during diet-induced milk fat depression. The nuclear localization of *CIDEA* suggests that it participates in controlling milk lipid synthesis in lactating dairy cows.

In the current study, we observed that *CIDEA* deficiency resulted in markedly reduced milk lipid content as well as reduction in the transcription of key genes involved in milk lipid synthesis. In contrast, the overexpression of *CIDEA* in mammary epithelial cells of dairy cows markedly increased the expression of key genes involved in milk lipid synthesis and increased milk lipid concentration. These data are in agreement with a previous report that forced expression of *CIDEA* by adenovirus in mouse hepatocytes leads to increased TAG levels, whereas knockdown by adenovirus expression of *CIDEA* shRNA results in lower accumulation of hepatic TAG compared with control adenovirus-infected liver (Matsusue et al., 2008).

In cows, about half of the FA, including short-chain FA (4–8 carbons), medium-chain FA (10–14 carbons), and a portion of 16-carbon FA, come from de novo FA synthesis (Bauman and Griinari, 2003). As the precursor for de novo FA synthesis, acetate needs to be activated by acyl-CoA synthetase to produce acetyl-CoA (Ma and Corl, 2012). The *ACSS2* is the isoform responsible for acetate activation during de novo FA synthesis in dairy cow mammary gland (Bionaz and Loor, 2008b). The transcription pattern of *ACSS2* corresponds with bovine mammary acetyl-CoA production throughout lactation (Mellenberger et al., 1973). In a sequence of steps, de novo synthesis is carried out by *ACC* and *FASN* utilizing acetyl-CoA. The *ACC* is the regulatory enzyme in FA synthesis as its activity is lower than other enzymes involved in lipogenesis and it possesses numerous kinetic properties characteristic of a regulatory enzyme (Mellenberger et al., 1973). The

FASN performs the majority of enzymatic steps of FA synthesis (Martin et al., 1961), and it is responsible for the production of short- and medium-chain FA as well as a substantial portion of long-chain FA (Smith, 2009). Gene expression studies have revealed that the activation of *ACSS2*, *ACC*, and FA synthesis in dairy cow mammary epithelium occurs in response to parturition and the onset of lactation (Bionaz and Loor, 2008b). In the current study, transcript abundance of *ACSS2*, *ACC*, and *FASN* was decreased significantly with *CIDEA* shRNA, in agreement with TAG content data, indicating that de novo FA synthesis might be regulated by *CIDEA* in mammary epithelial cells of dairy cows.

Long-chain FA in milk lipids, including a portion of 16-carbon FA, are derived from the uptake of circulating lipids (Bauman and Griinari, 2003). It has been reported that *CD36* is involved in FA uptake in bovine mammary cells, and its mRNA shows a large increase in expression during lactation (Bionaz and Loor, 2008b). The activation of long-chain FA is catalyzed by *ACSL* in the inner face of the plasma membrane before participating in TAG synthesis (Mashek and Coleman, 2006). The *ACSL1* mRNA is predominant among *ACSL* isoforms in bovine mammary tissue (Bionaz and Loor, 2008a). Long-chain FA or long-chain-acyl-CoA within cells can be captured by *FABP*, which transports them to specific intracellular organelles for utilization. The *FABP3* is the most abundant isoform in lactating bovine mammary gland, and the important function of *FABP3* in bovine mammary gland is to provide long-chain FA for *SCD1* to produce UFA or to directly provide long-chain-acyl-CoA for TAG synthesis (Bionaz and Loor, 2008b). In this study, we observed a reduction in *ACSL1* mRNA in mammary epithelial cells treated with *CIDEA* shRNA for 24 h, indicating that long-chain FA activation is regulated transcriptionally by *CIDEA*. However, *CD36*, *FABP3*, and *SCD1* expression were not affected by *CIDEA* knockdown or overexpression, suggesting that *CIDEA* is not involved in the transcriptional regulation of long-chain FA uptake, trafficking, and desaturation.

During milk lipid synthesis, *AGPAT6* and *DGAT1* are required for TAG production in bovine mammary gland (Bionaz and Loor, 2008a). The role of *AGPAT6* is to add FA to the sn-2 position of acylglycerol-3-phosphate. When *AGPAT6* is knocked out in lactating mice, they fail to synthesize milk lipids (Beigneux et al., 2006). In the bovine mammary gland, *AGPAT6* expression is markedly upregulated during lactation (Bionaz and Loor, 2008a). In our study, although the expression of *SREBP1* was increased with *CIDEA* overexpression, *AGPAT6* expression was not affected. This result is consistent with the report that *AGPAT6* is not regu-

lated by SREBP1 in bovine mammary epithelial cells (Ma and Corl, 2012). Diacylglycerol acyltransferase 1 is expressed in almost all tissues, including the mammary gland (Farese et al., 2000). Female *DGAT1*-deficient mice are unable to lactate (Smith et al., 2000). Diacylglycerol acyltransferase 1 catalyzes the final step in TAG synthesis. It has high affinity for butyryl-CoA and even higher affinity for palmitoyl-CoA (Marshall and Knudsen, 1979). In our study, the reduction in *DGAT1* mRNA with *CIDEA* shRNA treatment indicates that *DGAT1* is controlled by *CIDEA* to affect milk lipid synthesis.

Transcriptional regulation of TAG synthesis is mediated by several different factors. Sterol regulatory element-binding protein 1 is a transcription factor that controls genes involved in FA and TAG synthesis (Brown and Goldstein, 1997). In mouse hepatocytes, SREBP1 and its regulatory protein, SCAP, combine to regulate expression of mRNAs for the de novo enzymes that synthesize FA from precursor molecules (Osborne, 2001; Horton et al., 2003). In vivo deletion of *SCAP* in the mouse mammary gland significantly decreases FA synthesis and the growth rate of nursing pups (Rudolph et al., 2010). The bovine mammary gland also uses the SCAP-SREBP regulatory system to establish de novo FA synthesis and to integrate dietary regulation of lipogenic gene expression during lactation (Harvatine and Bauman, 2006). Insulin-induced gene 1 is a key regulator in the processing of SREBP1, which mediates its posttranslational maturation and activation (Li et al., 2003). Anchoring of SREBP1 in the endoplasmic reticulum (ER) as part of a tripartite complex with SCAP and INSIG1 (Eberlé et al., 2004) exerts a permissive retaining effect on SREBP1, preventing its maturation and activation. If the ER-anchoring activity of INSIG1 is diminished, SCAP escorts SREBP1 from the ER to the Golgi to be cleaved, producing a soluble and transcriptionally active mature SREBP1 protein (Osborne, 2001). In 3T3-L1 adipocytes, overexpression of *INSIG1* inhibits both adipogenesis and lipid accumulation (Li et al., 2003). In the bovine mammary gland during lactation, the upregulation of *INSIG1* can dampen SREBP1 activity (Bionaz and Loo, 2008b). In this study, *CIDEA* regulated the expression of only *SCAP* and *SREBP1* in mammary epithelial cells of dairy cows. However, *INSIG1* expression was not affected by *CIDEA*.

Nuclear receptor PPAR γ is also upregulated in bovine mammary tissue during lactation (Kadegowda et al., 2009). Treatment of bovine mammary epithelial cells with rosiglitazone, a specific PPAR γ agonist, results in coordinated upregulation of expression of genes involved in FA import, de novo FA synthesis, and TAG synthesis (Kadegowda et al., 2008). However, we ob-

served that expression of PPAR γ and its coactivator, *PPARG1A*, was not affected by *CIDEA* in cultured mammary epithelial cells of dairy cows, suggesting that *CIDEA* expression does not feed back on PPAR γ expression in dairy cow mammary gland.

CONCLUSIONS

Cell death-inducing DNA fragmentation factor- α -like effector C is expressed in lactating mammary tissues of dairy cows. It is capable of regulating the expression of genes involved in milk lipid synthesis and modulating mammary epithelial cell TAG levels in lactating mammary tissue of dairy cows.

ACKNOWLEDGMENTS

The authors declare that no competing interests exist. This work was supported by grants from the National Natural Science Foundation of China (31401109 to Y. L., and 31671285 to X. H.), the University Nursing Program for Young Scholars with Creative Talents in Heilongjiang Province (UNPYSCT-2015010, China), and Young Talents Project of Northeast Agricultural University (14QC03, China).

REFERENCES

- Bauman, D. E., and J. M. Griinari. 2003. Nutritional regulation of milk fat synthesis. *Annu. Rev. Nutr.* 23:203–227.
- Beigneux, A. P., L. Vergnes, X. Qiao, S. Quatela, R. Davis, S. M. Watkins, R. A. Coleman, R. L. Walzem, M. Philips, K. Reue, and S. G. Young. 2006. *Agpat6*—A novel lipid biosynthetic gene required for triacylglycerol production in mammary epithelium. *J. Lipid Res.* 47:734–744.
- Bionaz, M., and J. J. Loo. 2008a. ACSL1, AGPAT6, FABP3, LPIN1, and SLC27A6 are the most abundant isoforms in bovine mammary tissue and their expression is affected by stage of lactation. *J. Nutr.* 138:1019–1024.
- Bionaz, M., and J. J. Loo. 2008b. Gene networks driving bovine milk fat synthesis during the lactation cycle. *BMC Genomics* 9:366.
- Brown, M. S., and J. L. Goldstein. 1997. The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* 89:331–340.
- Connor, E. E., S. Siferd, T. H. Elsasser, C. M. Evock-Clover, C. P. Van Tassel, T. S. Sonstegard, V. M. Fernandes, and A. V. Capuco. 2008. Effects of increased milking frequency on gene expression in the bovine mammary gland. *BMC Genomics* 9:362.
- Eberlé, D., B. Hegarty, P. Bossard, P. Ferré, and F. Foufelle. 2004. SREBP transcription factors: Master regulators of lipid homeostasis. *Biochimie* 86:839–848.
- Emery, R. S. 1973. Biosynthesis of milk fat. *J. Dairy Sci.* 56:1187–1195.
- Farese, R. V. Jr., S. Cases, and S. J. Smith. 2000. Triglyceride synthesis: Insights from the cloning of diacylglycerol acyltransferase. *Curr. Opin. Lipidol.* 11:229–234.
- Harvatine, K. J., and D. E. Bauman. 2006. SREBP1 and thyroid hormone responsive spot 14 (S14) are involved in the regulation of bovine mammary lipid synthesis during diet-induced milk fat depression and treatment with CLA. *J. Nutr.* 136:2468–2474.
- Hillerton, J. E., C. H. Knight, A. Turvey, S. D. Wheatley, and C. J. Wilde. 1990. Milk yield and mammary function in dairy cows milked four times daily. *J. Dairy Res.* 57:285–294.

- Horton, J. D., N. A. Shah, J. A. Warrington, N. N. Anderson, S. W. Park, M. S. Brown, and J. L. Goldstein. 2003. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proc. Natl. Acad. Sci. USA* 100:12027–12032.
- Hou, X., L. Lin, W. Xing, Y. Yang, X. Duan, Q. Li, X. Gao, and Y. Lin. 2016. Spleen tyrosine kinase regulates mammary epithelial cell proliferation in mammary glands of dairy cows. *J. Dairy Sci.* 99:3858–3868.
- Kadegowda, A. K., M. Bionaz, L. S. Piperova, R. A. Erdman, and J. J. Loo. 2008. Lipogenic gene expression in MAC-T cells is affected differently by fatty acids and enhanced by PPAR- γ activation. *J. Dairy Sci.* 91(E-Suppl. 1):566.
- Kadegowda, A. K., M. Bionaz, L. S. Piperova, R. A. Erdman, and J. J. Loo. 2009. Peroxisome proliferator-activated receptor- γ activation and long-chain fatty acids alter lipogenic gene networks in bovine mammary epithelial cells to various extents. *J. Dairy Sci.* 92:4276–4289.
- Kang, Y., S. Hengbo, L. Jun, L. Jun, Z. Wangsheng, T. Huibin, and S. Huaiping. 2015. PPAR γ modulated lipid accumulation in dairy GMEC via regulation of ADRP gene. *J. Cell. Biochem.* 116:192–201.
- Keller, P., J. T. Petrie, P. De Rose, I. Gerin, W. S. Wright, S. H. Chiang, A. R. Nielsen, C. P. Fischer, B. K. Pedersen, and O. A. MacDougald. 2008. Fat-specific protein 27 regulates storage of triacylglycerol. *J. Biol. Chem.* 283:14355–14365.
- Li, J., K. Takaishi, W. Cook, S. K. McCorkle, and R. H. Unger. 2003. Insig-1 “brakes” lipogenesis in adipocytes and inhibits differentiation of preadipocytes. *Proc. Natl. Acad. Sci. USA* 100:9476–9481.
- Ma, L., and B. A. Corl. 2012. Transcriptional regulation of lipid synthesis in bovine mammary epithelial cells by sterol regulatory element binding protein-1. *J. Dairy Sci.* 95:3743–3755.
- Marshall, M. O., and J. Knudsen. 1979. Specificity of diacylglycerol acyltransferase from bovine mammary gland, liver and adipose tissue towards acyl-CoA esters. *Eur. J. Biochem.* 94:93–98.
- Martin, D. B., M. G. Horning, and P. R. Vagelos. 1961. Fatty acid synthesis in adipose tissue. I. Purification and properties of a long chain fatty acid-synthesizing system. *J. Biol. Chem.* 236:663–668.
- Mashek, D. G., and R. A. Coleman. 2006. Cellular fatty acid uptake: The contribution of metabolism. *Curr. Opin. Lipidol.* 17:274–278.
- Matsusue, K., T. Kusakabe, T. Noguchi, S. Takiguchi, T. Suzuki, S. Yamano, and F. J. Gonzalez. 2008. Hepatic steatosis in leptin-deficient mice is promoted by the PPAR γ target gene Fsp27. *Cell Metab.* 7:302–311.
- McManaman, J. L. 2009. Formation of milk lipids: A molecular perspective. *Clin. Lipidol.* 4:391–401.
- Mellenberger, R. W., D. E. Bauman, and D. R. Nelson. 1973. Metabolic adaptations during lactogenesis. Fatty acid and lactose synthesis in cow mammary tissue. *Biochem. J.* 136:741–748.
- Nishino, N., Y. Tamori, S. Tateya, T. Kawaguchi, T. Shibakusa, W. Mizunoya, K. Inoue, R. Kitazawa, S. Kitazawa, Y. Matsuki, R. Hiramatsu, S. Masubuchi, A. Omachi, K. Kimura, M. Saito, T. Amo, S. Ohta, T. Yamaguchi, T. Osumi, J. Cheng, T. Fujimoto, H. Nakao, K. Nakao, A. Aiba, H. Okamura, T. Fushiki, and M. Kasuga. 2008. FSP27 contributes to efficient energy storage in murine white adipocytes by promoting the formation of unilocular lipid droplets. *J. Clin. Invest.* 118:2808–2821.
- Osborne, T. F. 2001. CREating a SCAP-less liver keeps SREBPs pinned in the ER membrane and prevents increased lipid synthesis in response to low cholesterol and high insulin. *Genes Dev.* 15:1873–1878.
- Puri, V., S. Konda, S. Ranjit, M. Aouadi, A. Chawla, M. Chouinard, A. Chakladar, and M. P. Czech. 2007. Fat-specific protein 27, a novel lipid droplet protein that enhances triglyceride storage. *J. Biol. Chem.* 282:34213–34218.
- Rudolph, M. C., J. Monks, V. Burns, M. Phistry, R. Mariani, M. R. Foote, D. E. Bauman, S. M. Anderson, and M. C. Neville. 2010. Sterol regulatory element binding protein and dietary lipid regulation of fatty acid synthesis in the mammary epithelium. *Am. J. Physiol. Endocrinol. Metab.* 299:E918–E927.
- Schmittgen, T. D., and K. J. Livak. 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 3:1101–1108.
- Smith, S. 2009. Mechanism of chain length determination in biosynthesis of milk fatty acids. 1980. *J. Mammary Gland Biol. Neoplasia* 14:245–260.
- Smith, S. J., S. Cases, D. R. Jensen, H. C. Chen, E. Sande, B. Tow, D. A. Sanan, J. Raber, R. H. Eckel, and R. V. Jr. Farese. 2000. Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat. *Nat. Genet.* 25:87–90.
- Steinberg, R. S., L. Pereira, G. A. Lacorte, M. G. Peixoto, R. S. Verneque, R. L. Teodoro, M. A. Machado, C. G. Fonseca, and M. R. Carvalho. 2009. Technical note: A new and cost-effective method for detection of the bovine acyl-CoA:diacylglycerol acyltransferase 1 K232A polymorphism in cattle. *J. Dairy Sci.* 92:773–776.
- Tan, J. S., C. J. Seow, V. J. Goh, and D. L. Silver. 2014. Recent advances in understanding proteins involved in lipid droplet formation, growth and fusion. *J. Genet. Genomics* 41:251–259.
- Toh, S. Y., J. Gong, G. Du, J. Z. Li, S. Yang, J. Ye, H. Yao, Y. Zhang, B. Xue, Q. Li, H. Yang, Z. Wen, and P. Li. 2008. Up-regulation of mitochondrial activity and acquirement of brown adipose tissue-like property in the white adipose tissue of fsp27 deficient mice. *PLoS One* 3:e2890.