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RNA Interference Mediated Knockdown of ATP Binding Cassette Subfamily A Member 1 Decreases the Triglyceride Content of Bovine Mammary Epithelial Cells

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Authors' Contribution
ZZ and PJ designed and conceived the study. PJ and MW reformed the experimental work. PJ, LX and YC analyzed the data. PJ, MW and XL supervised the study. PJ wrote the article. RY and XF helped in preparation of article.

Key words
ABCA1, RNA interference, Bovine mammary epithelial cells, Triglyceride, Lipid metabolism

ABSTRACT

ATP binding cassette subfamily A member 1 (ABCA1) is a membrane protein. As a member of the superfamily of ABC transporters, ABCA1 can transport lipids and cholesterol across cell membranes. To further verify the relationship between the ABCA1 gene and milk fat metabolism of Chinese Holstein dairy cows, we exploited transient transfection mediated-RNA interference technology to specifically knockdown expression levels of the endogenous gene ABCA1 in bovine mammary epithelial cells (BMECs) to analyse the effect on intracellular triglyceride (TG) content by quantitative real-time polymerase chain reaction (qRT-PCR), Western blot, cell TG Assay and RT^2 Profiler PCR array. The results showed that ABCA1 knockdown reduced the TG content in BMECs. On the other hand, the RT^2 Profiler PCR array demonstrated that the expression levels of 12 genes, including fatty acid binding protein 4 (FABP4), acyl-CoA dehydrogenase short/branched chain (ACADSB), ELOVL fatty acid elongase 3 (ELOVL3) and solute carrier family 27 member 1 (SLC27A1), were altered upon ABCA1 down-regulation using RNA interference (RNAi). Overall, ABCA1 expression influenced triglyceride metabolism in BMECs by regulating the expression of related genes in the lipid metabolism pathway.

INTRODUCTION

Milk is a source of nutrient-rich food for human daily life, which contains many other nutrients including fat, protein and lactose. Milk fat and milk protein content are the important indicators for milk quality, in which milk fat is mainly composed of triglycerides (Bauman and Davis, 1974) and it is markedly affected by genetics, the stage of lactation, dietary manipulations and nutrition (Khan *et al*., 2018). With the development of in milk industry, the higher milk quality is required. Therefore, the study of functional genes related milk fat to improve the dairy quality has become a hotspot in dairy quality research.

ATP-binding cassette transporter A1 (ABCA1) is a 2261-amino-acid integral membrane protein which belongs to superfamily of ABC transporters and utilizes ATP as a source of energy for transporting lipids and cholesterol across membranes (Dean *et al*., 2001; Liu and Tang, 2012). and ABCA1 mRNA is widely expressed in multiple tissues including liver, brain, kidney, (Langmann *et al*., 1999). Low expression of ABCA1 was observed in human, rat and mouse BMEC and brain (Warren *et al*., 2009). ABCA1 contains two conserved peptide motifs which known as Walker A and Walker B, which are existed in various proteins that utilize ATP (Oram and Lawn, 2001). ABCA1 uses ATP to transport phospholipids and cholesterol freely to lipid-poor cells or cells lacking apolipoprotein A-1 (apoA1) to promote high-density lipoprotein (HDL) particle formation. In addition, reverse cholesterol transport (RCT) is another process that plays an important role in the body by removing excess lipids (Lee *et al*., 2012; Ghorbanian *et al*., 2013) and ABCA1 can actively remove the cholesterol from peripheral tissues by RCT (Demina *et al*., 2013). ABCA1 knockout mice fed a

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high fat diet showed that the dietary cholesterol absorption was increased 62% in gut (McNeish *et al*., 2000). In addition, *Cuffe et al* (Cuffe *et al*., 2018) suggested that ABCA1 is a key regulator of adipocyte lipogenesis and lipid accretion in adipocytes as it could activate lipogenic transcription factors peroxisome proliferator activated receptor γ (PPAR γ) and sterol regulatory element binding protein 1 (SREBP1) in mice adipose tissue. In our previous study indicated that ABCA1 is a candidate gene which associate with milk fat metabolism according to transcriptome analysis results between high-fat and low-fat BMECs in Chinese Holstein dairy cows (Jiang *et al*., 2018). However, the understanding at the present stage of the function of ABCA1 on bovine mammary epithelial cells (BMECs) and milk quality in bovine remains unclear.

RNAi interference (RNAi) technology, which could silence gene by posttranscriptional regulation of gene expression, has become a common research tool to investigate gene function (Hasselblatt and Offensperger, 2003). Exogenous or endogenous double-stranded RNA is placed into cells, and mRNA homologous with double-stranded RNA is degraded. Thus, the corresponding gene will be suppressed (Moss, 2001; Cerutti, 2003).

Therefore, to explore the functions of ABCA1 gene on triglyceride metabolism in BMECs, our study performed quantitative real-time polymerase chain reaction (qRT-PCR), Western blot, cell triglyceride (TG) assay and RT^2 Profiler PCR array to analysis the BMECs with ABCA1 knockdown by shRNA vector, and this result will provide the molecular basis and reliable authentication for further studies of ABCA1.

MATERIALS AND METHODS

Construction of RNAi vector for ABCA1 silence

A 21-mer siRNA oligonucleotide targeting the coding sequence of ABCA1 is GCAGTAGCACTGTGTCATACC and a sequence of the primer pairs were as follows: (5'CACCGCAGTAGTGTCATACCTTCAAGAGAGGTATGACACAGTGCTACTGCTTTTTTG 3') and (5'GATCCAAAAAAGCAGTAGCACTGTGTCATACCTCTCTTGAAGGTATGACAGTGCTACTGC 3') which were designed using the following website (http://rnaidesigner.thermofisher.com/rnaiexpress/setOption.do?designOption=shrna&pid=7471770366812053752) and synthesized by Genepharma Corporation (Shanghai, China).

Bovine mammary epithelial cell culture and transfection

The BMECs of Chinese Holstein dairy cows in this study were established previously in our laboratory (Lu *et al*., 2012). BMECs were seeded into six-well culture plates (Falcon, Franklin, Lake, NJ, USA) with 0.5×10^5 cells/well and culture with DMEM/F12 (GIBCO, Grand Island, NY, USA) containing 10% (V/V) foetal bovine serum (Hyclone, Logan, USA). When the confluence of BMECs achieved approximately 70%, the mixture of 150 μL of Opti-MEM serum-free media containing 3 μg of plasmid and 7.5 μL of FuGENE HD Transfection Reagent (Promega, Madison, WI, USA) was incubated at room temperature for 15 min and then added into culture medium in each well of BMECs. The cells were incubated at 37°C in a CO_2 incubator (Thermo, Marietta, OH, USA). The cells were placed in a fresh growth medium post-transfection and changed the medium every per days. 24 h later, GFP expression in the cells was observed under a fluorescence microscope (Nikon, T.E. 2000, Tokyo, Japan). The experiment was repeated 3 times for each group.

Detection of ABCA1 by qRT-PCR

BMECs were harvested 48 h post-transfection, 1 μg total RNA of BMECs transfected with ABCA1 RNAi vector and negative control vector were extracted using the trizol reagent (Tiangen, Beijing, China) following the manufacturer's protocols. The quality of RNA was assessed by agarose gel electrophoresis; RNA concentration was measured by a spectrophotometer. cDNA synthesis was performed using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Bio Inc., Dalian, China) according to the manufacturer's protocol. cDNA was stored in an -80 °C refrigerator until use in future experiments.

The primers were designed by Primer Premier 6.0 (Premier Biosoft International, Canada) according to the full-length sequence of the ABCA1 gene in GenBank (NM_001024693.1) and synthesized by GENEWIZ, Inc. (Suzhou, China). qRT-PCR was performed using PCRmax Eco 48 (PCRmax, Staffordshire, UK) in a 10 μL reaction volume including 5.0 μL of 2 × SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara Bio Inc., Dalian, China), 0.2 μL of upstream and downstream primers (10 μM) (sense primer 5' TGTGGTGCTGGCTGAGGTA 3' and antisense primer 5' TGTTGTTCATAGGGTGGATAGC 3'), 1.0 μL of template cDNA, and 4.6 μL of nuclease-free water. The real-time PCR conditions were as follows: 95 °C for 5 min; 40 cycles of 95 °C for 10 s and 59 °C for 30 s. β-*actin* served as an internal reference gene (sense primer 5' AGAGCAAGAGAGGCATCC 3' and antisense primer 5' TCGTTGTAGAAGGTGTGGT 3') (Fang *et al*., 2017).

Protein isolation and western blot analysis

To isolate total proteins from BMECs transfected with the RNAi vector and negative control vector, cells at 48 h post-transfection were washed 3 times with PBS and then resuspended in RIPA buffer (Boster, Wuhan, China) on ice for 30 min. The lysate was centrifuged at 12,000 g

for 5 min at 4 °C, and then the supernatant was collected, protein was mixed with loading buffer and denatured for 10 minutes. And then, 20 μg total proteins were resolved by SDS-PAGE and transferred onto PVDF membranes (Bio-Red laboratories Inc., CA, USA). The concentration was determined by a spectrophotometer and adjusted to a set total protein concentration that was equal among samples for the follow-up assay. The total protein concentration was determined using a Nano Drop 2000 (Thermo, DE, USA), and Western blotting was performed using the following primary antibodies: anti-ABCA1 (ab18180, Abcam, MA, USA) and anti-β-actin (ab8227, Abcam, MA, USA) and the specific operations follows the instruction manual (Liu *et al*., 2018).

Detection of the intracellular TG content in BMECs

The intracellular of TG content in BMECs transfected with ABCA1 gene RNAi and negative control vector were detected by an assay Kit (APPLYGEN, Beijing, China) following the manufacturers protocols. Cellular TG content of BMECs was adjusted by quantity of total protein. Each sample was repeated 3 times.

Lipid and fatty acid metabolism RT2 Profiler PCR array

Total RNA was extracted from BMECs using an RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany). After generating cDNA from 2.0 μg of total RNA was used for cDNA synthesis with RT² First Strand Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. qRT-PCR was performed using Mx3005p (Agilent Stratagene, CA, USA). Transcript levels of genes in lipid and fatty acid metabolism pathways were analysed using the RT² Profiler PCR Array (CLAB24070A, Qiagen, Hilden, Germany). 5 different housekeeping genes, β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tyrosine 3-monooxygenase (YWHAZ), hypoxanthine phosphoribosyltransferase 1 (HPRT1) and TATA-box binding protein (TBP) were used as internal controls.

Statistical analysis

Experimental data were analysed using T-tests and presented as the mean ± SEM. Statistically significant differences were defined as p <0.05. The expression levels of genes were performed after the Ct values were obtained using the $2^{-\Delta\Delta Ct}$ method. Data regarding gene expression levels were analysed using the RT² Profiler PCR Array data analysis online software (https://www.qiagen.com/cn/shop-old/data-interpretation-systems/bioinformatics/geneglobe-data-analysis-center/).

RESULTS

Transfection results of mammary epithelial cells with ABCA1-shRNA vector

Cell morphology and expression of green fluorescent protein (GFP) in BMECs observed in the RNAi vector transfected group was consistent with the negative control group at 24 h post-transfection as expected (Fig. 1), and the transfected cells were used for subsequent experiments. In contrast, cells that were not transfected did not exhibit green fluorescence under the fluorescence microscope.

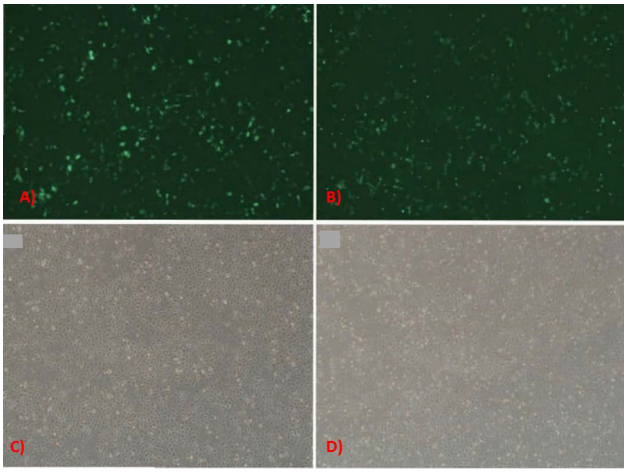

Fig. 1. ABCA1 RNAi vector and the negative control vector were transfected into BMECs; A, Cells transfected with *ABCA1-shRNA*; B, cells transfected with negative control vector.

ABCA1 mRNA and protein expression levels in RNAi and control groups

Results revealed that ABCA1 mRNA in BMECs was down-regulated significantly after transfection with the RNAi vector compared with the negative control vector (p < 0.05) (Fig. 2A). Western blot analysis results indicated that protein expression in the *ABCA1-shRNA* group was down-regulated compared with that in the negative control group, and a significant difference was noted between the *ABCA1-shRNA* and negative control groups of BMECs (p < 0.05) (Fig. 2C).

Effect of ABCA1 on intracellular TG content

To examine the effect of ABCA1 gene on intracellular TG synthesis, the TG content was compared between the *ABCA1-shRNA* group and the negative control group. The results indicated that the TG content was reduced by the shRNA vector to inhibit the interest gene, the BMECs of ABCA1 knockdown have a 50% reduction of TG content compared with control group. But the statistical

significance of differences in mean valves was not significant ($p = 0.0941$, Fig. 2B).

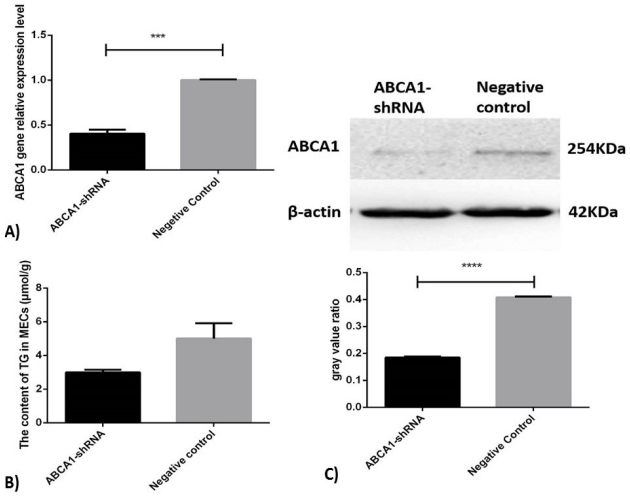

Fig. 2. A, ABCA1 mRNA expression levels in BMECs; B, Comparison of the TG content of cells transfected with RNAi vector or negative control vector; C, ABCA1 protein expression levels using a grey value analyser to calculate grey value ratios (target protein content/β-actin content).

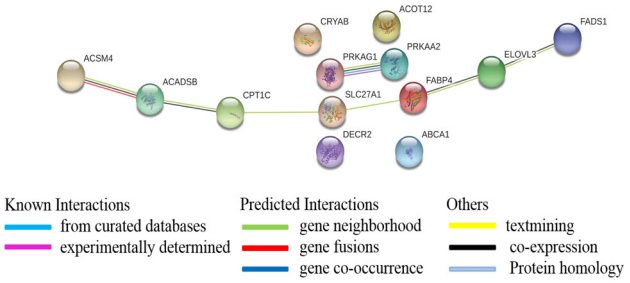

Fig. 3. The protein-protein interaction between ABCA1 gene and related fatty acid metabolism pathway genes was obtained by STRING.

Regulation of genes in lipid and fatty acid metabolism by ABCA1

The fold change of mRNA expression of genes involved in lipid and fatty acid metabolism pathways are summarized in Table I, and the protein-protein interaction network of these genes was obtained and visualized by STRING (Fig. 3).The RT^2 Profiler PCR array data suggested that ABCA1 RNAi resulted in the up-regulation of 8 genes, including carnitine palmitoyltransferase-1C (CPT1C), fatty acid binding protein 4 (FABP4), short/branched chain acyl-CoA dehydrogenase (ACADSB), and acyl-CoA thioesterase 12 (ACOT12). In addition, 4 genes were deregulated, including ELOVL fatty acid elongase 3 (ELOVL3), fatty acid desaturase 1 (FADS1), and solute carrier family 27 member 1 (SLC27A1). Among that, AMP-activated catalytic subunit alpha 2 (PRKAA2) is the most significant up-regulated gene and SLC27A1 is the most obvious negatively down-regulated gene.

Table I. Fold change in the expression of genes involved in lipid and fatty acid metabolism mediated by ABCA1.

Gene name	Ref-sequence	Fold change
ACADSB	NM_001017933, XM_005225840	1.61
ACOT12	NM_001192607	1.62
CRYAB	NM_174290	1.61
CPT1C	XM_002695120	1.73
DECR2	NM_001078122	1.62
FABP4	NM_174314	1.54
PRKAA2	NM_001205605	2.66
PRKAG1	NM_174586	1.62
ACSM4	XM_002698016	0.61
ELOVL3	NM_001192306	0.57
FADS1	XM_002699285	0.65
SLC27A1	NM_001033625	0.29

Abbreviation used: ACADSB, short/branched chain acyl-CoA dehydrogenase; ACOT12, acyl-CoA thioesterase 12; CRYAB, crystallin alpha B; CPT1C, carnitine palmitoyltransferase-1C; DECR2, 2,4-dienoyl-CoA reductase 2; FABP4, fatty acid binding protein 4; PRKAA2, protein kinase, AMP-activated, alpha 2 catalytic subunit; PRKAG1, protein kinase AMP-activated non-catalytic subunit gamma 1; ACSM4, acyl-CoA synthetase medium-chain family member 4; ELOVL3, elongation of very long chain fatty acids; FADS1, fatty acid desaturase 1; SLC27A1, solute carrier family 27.

DISCUSSION

ABCA1 was obtained in our previous study as a candidate gene associated with milk fat by RNA-seq analysis in BMECs of Chinese Holstein. Milk is produced by the mammary glands of dairy cattle, thus, mammary epithelial cells is a widely used model for the study of functional genes in milk quality.

Adipocyte-specific ABCA1 knockout mice with a high-fat, high-cholesterol diet had a significantly lower body weight, epididymal fat pad weight, and adipocyte size compared with control mice, and ABCA1 silencing by siRNA also reduce peroxisome proliferator-activated receptor γ (PPARγ) expression and triglyceride content during 3T3-L1 pre-adipocyte differentiation which consist with our result in BMECs (Cuffe *et al.*, 2018). Moreover,

ABCA1 affects the expression levels of genes mainly involved in TG and fatty acid metabolism according to PCR array results in the current study. Among them, FABP4 is an important gene in the lipid and fatty acid metabolism pathway with roles in fat deposition and fatty acid transport in animals. FABP4 gene polymorphisms are associated with fat content, fatty acid composition, and meat and carcass traits in many breeds of beef cattle (Michal *et al*., 2006; Pannier *et al*., 2010; Narukami *et al*., 2011; Goszczynski *et al*., 2017), as well as milk protein percentage and milk yield in Holstein-Friesian × Jersey cows (Kulig *et al*., 2013; Zhou *et al*., 2015). These results indicate that the ABCA1 gene may influence milk traits by altering the intracellular fat content by regulating FABP4. ACOT12 is a crucial enzyme known to hydrolyse the thioester bond of acetyl-coA in the cytosol of liver cells. Cytosolic acetyl-CoA levels in the liver are affected by lipid metabolites and lead to allosteric enzymatic and transcriptional regulation of ACOT12 (Kulig *et al*., 2013); hence, altering the TG content by ABCA1 knockdown also affects the transcriptional level of ACOT12 in BMECs. ELOVL3 is a critical enzyme for lipid accumulation in brown adipocytes and can increase fatty acid oxidation in brown adipocytes. ELOVL3 expression increased gradually during skeletal muscle-derived satellite cell differentiation in cattle (Liu *et al*., 2017). In our study, the silence of ABCA1 led to the decreasing of ELOVL3 mRNA expression in BMECs. ACADSB is a member of the acyl-CoA dehydrogenase family of enzymes and catalyses the dehydrogenation of acyl-CoA derivatives in the metabolism of fatty acids. ACADSB is also important gene involved in TG synthesis in BMECs (Jiang *et al*., 2018), the silence of ABCA1 led to the increasing of ACADSB mRNA expression in BMECs, which may both mean that ABCA1 may affect the milk fat by regulating the differential genes. In addition, CPT1 is the rate-limiting enzyme in fatty acid β-oxidation. This gene family includes CPT1A, CPT1B, and CPT1C in mammals and regulates the entry of long-chain fatty acids into the mitochondria (Virmani *et al*., 2015). Increases in the transcriptional level of CPT1C may affect long-chain fatty acid transport and metabolism, thus altering the TG content and fatty acid component (Lee and Wolfgang, 2012).

However, only the PRKAA2 and SLC27A1 genes exhibited greater than 2-fold changes in expression. AMPK is a heterotrimeric complex comprised of catalytic α-, β-, and γ- subunits. The α-subunits confer kinase activity, and the protein kinase PRKAA2 catalytic subunit is expressed in multiple hypothalamic neurons (Torsoni *et al*., 2016). SLC27A1 is also called fatty acid transport protein 1 (FATP1) and is a major regulator of trans-membrane transportation and oxidative metabolism of free fatty acid (Qi *et al*., 2016). SLC27A1 is involved in docosahexaenoic acid transport and regulates the transport of taurine and biotin from blood to brain (Ochiai *et al*., 2017). A previous study revealed that SLC27A1 mutants were associated with milk production (Lv *et al*., 2011) and milk fat content (Ordovas *et al*., 2008). The above results suggest that these genes are very important in the lipid and fatty acid metabolism pathway and may serve as a regulatory network to reduce TG levels after ABCA1 knockdown in BMECs. However, the specific molecular mechanism and the protein-protein interactions of these genes require further exploration.

CONCLUSION

Overall, this study demonstrates that ABCA1 could influence TG deposition at the cellular level by regulating the genes in lipid metabolism and ABCA1 expression may correlate with milk traits. Therefore, ABCA1 represents a potential candidate gene to improve milk fat traits in dairy cattle breeding and the functional mechanism in BMECs of TG metabolism is also important for further study.

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Statement of conflict of interest

The authors declare that they have no conflict of interest.

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