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trans-10,*cis*-12 conjugated linoleic acid alters lipid metabolism of goat mammary epithelial cells by regulation of de novo synthesis and the AMPK signaling pathway

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ABSTRACT

The *trans*-10,*cis*-12 isomer of conjugated linoleic acid (t10c12-CLA) is a biohydrogenation intermediate in the rumen and has been shown to cause milk fat depression in dairy goats. However, few studies have focused on the in vitro molecular mechanisms involved in the response of the goat mammary gland to t10c12-CLA. In the present study, RNA sequencing technology was used to investigate the effects of t10c12-CLA on goat mammary epithelial cells. From the data, 25,153 annotated transcripts were obtained, and differentially expressed genes were selected based on a false discovery rate <0.05. Candidate genes and potent cellular signaling pathways were identified through Gene Ontology (GO) and pathway analysis. Next, real-time quantitative PCR and Western blot analyses were used to verify the results of the RNA sequencing data. The results indicated that t10c12-CLA inhibits fatty acid synthesis through downregulation of genes involved in de novo fatty acid synthesis, and this process is likely correlated with the activation of the AMP-activated protein kinase signaling pathways.
Key words: *trans*-10,*cis*-12 conjugated linoleic acid, mammary, RNA sequencing, AMP-activated protein kinase (AMPK)

INTRODUCTION

The mammalian body can adjust its metabolism in response to a variety of nutritional conditions, and the regulation of gene expression and altered enzyme activities in metabolic pathways play critical roles in this adaptive response (Nakamura et al., 2004). Milk content and composition can be affected by diet (Peterson et al., 2004), and significant changes occur in milk FA composition in response to altered feeding conditions, especially in dairy cattle and goat (Chilliard et al., 2007). The *trans*-10,*cis*-12 isomer of conjugated linoleic acid (**t10c12-CLA**), a biohydrogenation intermediate in the rumen, has been known to decrease milk fat synthesis (Kadegowda et al., 2010; Hussein et al., 2013; Harvatine et al., 2014). In dairy cattle, t10c12-CLA is a primary factor causing milk fat depression (Baumgard et al., 2000, 2002).

Studies in bovine MAC-T cells indicated that inhibition of de novo fatty acid synthesis by t10c12-CLA (100 μM) may be due to reduced proteolytic activation of sterol regulatory element-binding transcription factor 1 (**SREBF-1**), downregulation of thyroid hormone responsive (**THRSP**), and changes to a series of lipogenic genes (Peterson et al., 2004; Harvatine and Bauman, 2006). Gene expression data associated with fatty acid uptake, intracellular activation, intracellular transport, de novo synthesis, and esterification after treatment with t10c12-CLA indicate negative effects on lipogenic gene networks in bovine mammary cells (Kadegowda et al., 2009). In vivo investigations using the microarray method in murine mammary tissue indicated that the effects of t10c12-CLA might encompass inhibition of peroxisome proliferator-activated receptor gamma (**PPARγ**) signaling and fatty acid biosynthesis, as well as induction of inflammation and endoplasmic reticulum stress (Kadegowda et al., 2013).

However, few studies have focused on the molecular mechanisms underlying those responses in the goat mammary gland. Although previous murine and bovine studies provide guidance for similar studies in dairy goat, direct application of those results are limited. First, those reports may not directly represent the effects of t10c12-CLA in dairy goat due to species-specific differences. Second, methods such as real-time quantitative PCR (**RT-qPCR**) and microarray might not detect the global dynamic range of gene expression in response to t10c12-CLA. RNA is a dynamic and diverse biomol-

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ecule, playing an essential role in numerous biological processes (Byron et al., 2016). Thus, capturing whole transcriptome information is critical in a comprehensive investigation of the response to t10c12-CLA in the goat mammary gland. RNA sequencing provides an in-depth view of the transcriptome, allowing study of global gene expression changes and alternative splicing in any cell population, overcoming limits of microarray assays that are unable to detect novel changes or identify novel isoforms of known genes (Mortazavi et al., 2008; Jakhesara et al., 2013). In the present study, RNA sequencing technology was applied to evaluate the effects of t10c12-CLA on goat mammary epithelial cells (**GMEC**). Important genes and potential cellular signaling pathways involved in the response were identified and verified by RT-qPCR and Western blot.

MATERIALS AND METHODS

Preparation of t10c12-CLA

A stock solution of 30 m*M* t10c12-CLA (Sigma-Aldrich, St, Louis, MO) was dissolved in 95% ethanol and 30 m*M* NaOH as previously described (Kadegowda et al., 2009). A solution of 95% ethanol and 30 m*M* NaOH served as the control.

Cell Culture and Treatments

The experiment was conducted under the approval of the Institutional Animal Use and Care Committee of Northwest A&F University (Shaanxi, China). The GMEC were isolated from Xinong Saanen goats during peak lactation and cultured in a basal Dulbecco's modified Eagle medium/F12 (DMEM/F12) medium (Hyclone, South Logan, UT) containing 1 μg/mL hydrocortisone (Sigma-Aldrich), 5 μg/mL insulin, 100 U/mL penicillin, 100 mg/mL streptomycin (Harbin Pharmaceutical Group, Harbin, China), 5 m*M* sodium acetate, 10 ng/mL epidermal growth factor 1 (Invitrogen Corp., Frederick, MD), and 10% fetal bovine serum (Gibco/Thermo Fisher Scientific, Waltham, MA) at 37°C in a humidified atmosphere with 5% CO_2. The serum-free DMEM/F12 medium contained no serum, 1 mg/mL BSA (Sigma-Aldrich), and 2 μg/mL prolactin (Sigma-Aldrich). For the experimental treatment of GMEC, the basal medium was changed to serum-free medium containing CLA or the control. The GMEC were grown in 6-well culture plates (for RNA extraction) or 60-mm culture plates (for Western blot) until approximately 80% confluence, and were then treated with serum-free medium containing 0 (control; **CTR**), 100 (treatment **TR1**), or 200 (treatment **TR2**) μ*M* t10c12-CLA for 12 h.

RNA Extraction, Library Construction, and Sequencing

For RNA sample preparation, each treatment had 8 replicates. The total RNA of each group was isolated using the Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The extracted samples were separated on 1% agarose gels to detect RNA degradation and contamination. The RNA concentration was measured using the Qubit RNA Assay Kit in Qubit2.0 Fluorometer (Life Technologies). The purity and integrity of RNA was detected using the Nano 6000 Assay kit of the Bioanalyzer Agilent 2100 system (Agilent Technologies, Palo Alto, CA). Finally, 5 samples of homogenized total RNAs with RNA integrity number values >8 for each treatment were mixed to make one sample at the same concentration. Three sequencing libraries were prepared: control group (CTR), 100 μ*M* t10c12-CLA (TR1), and 200 μ*M* t10c12-CLA (TR2), using the IlluminaTruSeq RNA Sample Preparation kit (Illumina, San Diego, CA) according to the manufacturer's instructions and adding 4 index codes to attribute sequence to each sample. After library construction, the Qubit2.0 system was used to measure the concentration of cDNA libraries. The libraries were then diluted to 1 ng/μL, and the insert size was measured using the Agilent 2100 system. Then, the cDNA libraries were quantified by RT-qPCR (library effective concentration >2 n*M*) to ensure the quality. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBotHS (Illumina) according to the manufacturer's instructions. The library preparations were sequenced on an Illumina HiSeq 2500 platform and 150-bp paired-end reads were generated. The sequenced RNA-seq data for CTR, TR1, and TR2 are available from National Center for Biotechnology Information Sequences Read Archive with accession numbers SRR5714864, SRR5714865, and SRR5714866.

Mapping and Assembly of Sequence Reads

The raw data (in fastq format) were first processed using in-house Perl scripts, and then clean data were obtained by removal of adapter sequences, reads containing poly-N (>10%), and low quality reads (more than 50% bases in which phred quality score ≤5 in a read) from the raw data. The indices of clean bases, error rate, Q20 (proportion of bases with a phred base quality score >20), Q30 (proportion of bases with a phred base quality score >30), and GC content from the sequence reads were assessed to ensure high quality of the clean data. Using the *Capra hircus* (goat) genome (ID: 10731) as reference genome, an index of