Fatty acid elongase 6 plays a role in the synthesis of long-chain fatty acids in goat mammary epithelial cells

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ABSTRACT

In nonruminants, it is well established that elongation of very long-chain fatty acid-like fatty acid elongase 6 (*ELOVL6*) catalyzes the synthesis of C18:0 from C16:0 in lipogenic tissues like adipose and liver. However, the role of *ELOVL6* in regulating lipid metabolism in ruminant mammary gland remains unknown. In the present study, *ELOVL6* was overexpressed or knocked down via adenoviral transfection to assess its role in goat mammary epithelial cells. Results revealed that *ELOVL6* overexpression had a weak effect on the expression of genes related to triacylglycerol (TAG) synthesis and desaturation. Overexpression of *ELOVL6* increased the content of C18:0 at the expense of C16:0, and increased the elongation index of C16:0. Overexpression of *ELOVL6* had no significant effect on the elongation index of C16:1n-7 and the desaturation indices of C16:0 and C18:0. Knockdown of *ELOVL6* had a negative effect on mRNA expression of the esterification genes *GPAM* and diacylglycerolacyltransferase 2 (*DGAT2*) and TAG concentration; however, it increased the concentration of C16:0 and decreased C18:1n-7 and C18:1n-9 in goat mammary epithelial cells. Accordingly, downregulation of *ELOVL6* significantly decreased the elongation indices of C16:0 and C16:1n-7. The lack of change in the desaturation indices of C16:0 and C18:0 upon knockdown of *ELOVL6* was consistent with the minor change in *SCD1* expression. In conclusion, these are the first results highlighting an important role of *ELOVL6* in long-chain fatty elongation and TAG synthesis in ruminant mammary cells.

Key words: elongase, lactation, fatty acid composition, milk fat

INTRODUCTION

Fat composition is one of the most valuable components in terms of dairy production, in which the high proportion of PUFA contributes a special function for human health (Haenlein, 2004; Shingfield et al., 2008). In nonruminants, the fatty acid elongases are rate-limiting enzymes controlling the synthesis of long-chain fatty acids [**LCFA**; e.g., 16–18 carbons (Green and Olson, 2011)], some of which are substrates for the biosynthesis of PUFA. In the ruminant mammary gland, de novo fatty acid synthesis results in the production of short- and medium-chain fatty acids and a portion of 16:0 (Harvatine et al., 2009). Although the ruminant mammary gland secretes PUFA in milk, it is unknown if elongases play a role in this process.

In rodents, the elongation of very long-chain fatty acid-like fatty acid elongase family (**Elovl**) includes 7 isotypes, *Elovl1* to *Elovl7*. Each elongase exhibits different fatty acid substrate preferences. The enzymes *Elovl1*, *Elovl3*, and *Elovl4* catalyze a broad array of saturated and monounsaturated fatty acids containing up to 24 carbons (Matsuzaka and Shimano, 2009). Substrates of *Elovl2* include PUFA with 20 and 22 carbons (Tvrdik et al., 2000). The *Elovl5* is involved in the elongation of various PUFA containing 18 and 20 carbons (Green et al., 2010). The *Elovl6* catalyzes the chain elongation of palmitate (C16:0) to stearate (C18:0) and the elongation of palmitoleate (C16:1n-7) to vaccinate (C18:1n-7); hence, its activity is closely associated with de novo fatty acid synthesis.

In mice, *Elovl6* is highly expressed in lipogenic organs and is sensitive to dietary, hormonal, and developmental control (Matsuzaka and Shimano, 2009). The mRNA abundance of *Elovl6* in liver and adipose tissue was markedly elevated upon refeeding fasting animals; however, dietary PUFA decreased *Elovl6* profoundly (Matsuzaka et al., 2002). Loss of *Elovl6* function in mice fed a high-sucrose diet increased the content of

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C16:0 and C16:1n-7, but reduced C18:0 and C18:1n-9 in the liver (Matsuzaka et al., 2007). Overexpression of *Elovl6* increased synthesis of 18:0 and 18:1n-9 (Green et al., 2010). Collectively, the data from rodents underscore an important role for *Elovl6* in the overall process of LCFA synthesis.

The recent demonstration that *ELOVL6* is expressed in goat and bovine mammary tissue (Bionaz et al., 2012a,b; Shi et al., 2015) and its expression is altered by dietary lipid supplementation in cow mammary tissue (Leroux et al., 2016) suggested that *ELOVL6* may play a role in fatty acid elongation. Compared with rodents and humans, the role of *ELOVL6* in the ruminant mammary gland is not known. Whether *ELOVL6* is essential for the synthesis and alteration of LCFA composition in ruminant mammary cells remains to be determined. We hypothesized that *ELOVL6* has a role in the overall process of LCFA synthesis in goat mammary tissue. To assess the role of *ELOVL6*, both adenoviral-mediated RNA interference and overexpression were performed in goat mammary epithelial cells (GMEC).

MATERIALS AND METHODS

Adenovirus Generation

The whole process for generation and proliferation of recombinant adenovirus expressing *ELOVL6* (Ad-*ELOVL6*) was carried out as previously described (Shi et al., 2013a). Briefly, the *ELOVL6* cDNA (GenBank no. KF667508) was subcloned into the adenoviral plasmic (pAd) Track-CMV plasmid vector to generate pAdTrack-CMV-*ELOVL6* vectors. The vector was inserted into an adenoviral vector (pAdEasy-1) to generate adenoviral plasmids in BJ5183 cells. The adenoviral plasmids linearized by *PacI* (New England Biolabs, Ipswich, MA) were transfected into 293A cells to generate the adenovirus pAd-*ELOVL6*.

To knock down *ELOVL6*, a short hairpin RNA sequence (**shELOVL6**; described in Supplemental Table S1; <https://doi.org/10.3168/jds.2016-12159>) was designed and synthesized with the *XhoI* and *KpnI* restriction sites (Invitrogen Biotechnology Co. Ltd., Shanghai, China). The sequence was constructed into the pENTR/CMV-GFP/U6 vector and then switched into an adenoviral vector (pAd/PL-DEST) using the Gateway technique (Invitrogen, Carlsbad, CA) to generate the pAd-shELOVL6 vector. The *Pac I* linearized adenoviral plasmids were transfected into 293A cells to generate the adenovirus (Ad-shELOVL6). The process of Ad-shELOVL6 generation was described previously (Shi et al., 2013b).

Cell Culture

The GMEC were isolated from peak lactation Xinong Saanen goats as described previously (Hu et al., 2009; Wang et al., 2010; Shi et al., 2014). Details of cell culture were described recently (Lin et al., 2013; Shi et al., 2013b). Briefly, cells were incubated at 37°C in 5% CO₂ and air. Culture medium was composed of Dulbecco's modified Eagle medium/F12 (Hyclone, Beijing, China) containing insulin (5 mg/L, Sigma-Aldrich, St. Louis, MO), hydrocortisone (5 mg/L, Sigma), penicillin/streptomycin (10k unit/L, Harbin Pharmaceutical Group, China), epidermal growth factor (1 mg/L, Sigma-Aldrich), and fetal bovine serum (10%, Gibco, Waltham, MA). To promote lactogenesis, GMEC were cultured in medium described above plus prolactin (2 µg/mL, Sigma-Aldrich) for 24 h before initial experiments. The 293A cells for adenovirus generation were cultured in basal Dulbecco's modified Eagle medium (Gibco) containing 10% fetal bovine serum.

The GMEC at about 80% confluence were transfected with adenovirus supernatant (Ad-*ELOVL6*, Ad-shELOVL6, Ad-GFP, or Ad-shNC). The transfected GMEC were collected after 48 h of culture for lipid extraction, total RNA extraction, and triacylglycerol (TAG) analysis.

Total RNA Extraction and Quantitative Real-Time PCR

Total RNA from GMEC was extracted using the RNA Prep pure cell Kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's protocol (<http://www.tiangen.com/asset/imsupload/up0203129001467351047.pdf>). Genomic DNA contamination was removed using DNase provided with the kit. Synthesis of cDNA was conducted using the PrimeScript RT kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions (http://www.clontech.com/CN/Products/Real-Time_qPCR_and_Reverse_Transcription/Reagents_and_Standards/Two-Step_RT-qPCR/PrimeScript_RT_with_gDNA_Eraser?sitex=10022:22372:US). The quantitative real-time PCR (qPCR) was performed according to the manufacturer's instructions using SYBR Green (SYBR Premix Ex Taq II, Perfect Real Time, Takara Bio Inc.).

The synthesis of long-chain fatty acids is associated with desaturation and the resulting fatty acids are incorporated into TAG (Harvatine et al., 2009; Green et al., 2010). Thus, several genes related to TAG synthesis [diacylglycerolacyltransferase 1 (*DGAT1*), diacylglycerolacyltransferase 2 (*DGAT2*), glycerol-3-phosphate acyltransferase (*GPAM*), perilipin2 (*PLIN2*)] and fatty

acid desaturation [stearoyl-CoA desaturase 1 (*SCD1*)] were selected. All the qPCR reactions were performed in a Bio-Rad CFX96 (Bio-Rad Laboratories Inc., Hercules, CA) sequence detector, and data were normalized to ubiquitously expressed transcript (*UXT*), mitochondrial ribosomal protein L39 (*MRPL39*), and ribosomal protein S9 (*RPS9*). The primer sequences of the genes are described in the Supplemental Table S2 (<https://doi.org/10.3168/jds.2016-12159>).

Fatty Acid Analysis

After washing 3 times with PBS, treated GMEC were scraped off the culture dish with a 2-mL aliquot of 2.5% (vol/vol) vitriol:methanol. Total lipid extraction and methylation was performed according to Shi et al. (2013a). Methylated lipid samples were analyzed using a Gas Chromatography-Mass Spectrophotometer (Agilent Technologies, Santa Clara, CA) installed with an HP-5 column following a published procedure (Yao et al., 2016). The relative proportions of fatty acids were determined as the ratio of each fatty acid peak relative to the total fatty acid peaks identified.

Cellular TAG Assays

The protocol for cellular TAG assay was described previously (Kang et al., 2015). Briefly, total cellular TAG was extracted using the GPO-Trinder triglyceride assay kit (<http://www.applygen.com/a/meixueyushenghuaceding/276.html>; Applygen Technologies, Beijing, China). The concentration of TAG was determined according to the manufacturer's instructions on a plate reader (Thermo Fisher Scientific, Waltham, MA). The concentrations were calculated using the equation obtained from a linear regression of the standard curve which was established according to the manufacturer's procedure (<http://www.applygen.com/a/meixueyushenghuaceding/276.html>). The quantification of total cellular TAG was normalized to the cellular protein concentration. Protein concentration of each well was determined using a BCA protein assay kit (Pierce, Thermo Fisher Scientific) according to the manufacturer's instructions (<https://www.thermofisher.com/order/catalog/product/23225>).

Statistical Analysis

Treatments were replicated at least 3 times in culture wells, and the PCR was performed in triplicate. Results are expressed as mean $\pm$ standard error of the mean. Data of qPCR were analyzed using the $2^{-\Delta\Delta C_t}$ method. The desaturation index was defined as the ratio between the desaturation product and the substrate. For

example, desaturation index of C16:1 = C16:1n-7/C16:0, desaturation index of C18:1 = C18:1n-9/C18:0. The elongation index was calculated as the ratio between the elongation products and the substrates. For example, elongation index of C16:0 = (C18:0 + C18:1n-9)/C16:0, elongation index of C16:1n-7 = C18:1n-7/C16:1n-7. Significance of RNA expression, relative concentration of fatty acids, elongation index, desaturation index, and cellular TAG content after *ELOVL6* knockdown or overexpression was determined via Student's *t*-test. Significance was declared at $P < 0.05$.

RESULTS

Overexpression of *ELOVL6* Had Little Effect on Genes Related to TAG Synthesis

Compared with controls, the expression of *ELOVL6* after 48 h increased markedly in cells infected with Ad-*ELOVL6* ($P < 0.01$, Figure 1A). However, overexpression of *ELOVL6* had no significant effect on genes related to TAG synthesis (*GPAM*, *DGAT1*, and *DGAT2*), lipid formation (*PLIN2*), and fatty acid desaturation (*SCD1*; Figure 1A).

Overexpression of *ELOVL6* Altered the Content of Fatty Acids and Elongation Index

To investigate the effect of *ELOVL6* on fatty acid synthesis in GMEC, the relative of concentration of LCFA was measured. Compared with controls, a decrease of C16:0 ($P < 0.05$) and an increase of C18:0 ($P < 0.05$) was detected for 48 h in GMEC treated with Ad-*ELOVL6* (Figure 2A). However, no significant differences were detected for C16:1n-7, C18:1n-7, C18:1n-9, and C18:2.

Compared with the control group (Ad-GFP), overexpression of *ELOVL6* significantly upregulated the elongation of C16:0 in GMEC, but had a minor effect on elongation of C16:1n-7 (Figure 2B). No significant change in the desaturation indices of C16:0 and C18:0 were detected in GMEC transfected with Ad-*ELOVL6* (Figures 2C and 2D; $P > 0.05$).

Knockdown of *ELOVL6* Downregulated Genes Related to TAG Synthesis

To further investigate the role of *ELOVL6*, an adenovirus with sh*ELOVL6* sequence was generated and incubated with GMEC. Compared with controls (Ad-shNC), the mRNA abundance of *ELOVL6* decreased more than 80% in GMEC incubated with Ad-sh*ELOVL6* (Figure 3A). The relative expression of *GPAM*

and *DGAT2* was downregulated significantly, but the mRNA abundance of *DGAT1*, *PLIN2*, and *SCD1* was not altered significantly upon knockdown of *ELOVL6* (Figure 3B).

Knockdown of *ELOVL6* Altered the Fatty Acid Content and Decreased the Elongation Index

Compared with the control group incubated with Ad-shNC, the relative concentration of C18:1n-7 and C18:1n-9 in GMEC incubated with Ad-sh*ELOVL6* decreased significantly. In contrast, a marked increase of C16:0 ($P < 0.05$) was observed (Figure 4A). No

significant changes were observed in the concentration of C16:1n-7, C18:0, and C18:2 after knockdown of *ELOVL6*. Concomitant with the changes in fatty acid composition, the elongation indices of C16:0 and C16:1n-7 were markedly decreased in cells incubated with Ad-sh*ELOVL6* (Figure 4B). However, knockdown of *ELOVL6* had no effect on the desaturation indices of C16:0 and C18:0 (Figures 4C and 4D).

ELOVL6 Increased Concentration of Cellular TAG in GMEC

After overexpression of *ELOVL6* in GMEC, the concentration of cellular TAG increased significantly ($P < 0.05$; Figure 5A). In contrast, the concentration of TAG in GMEC incubated with Ad-sh*ELOVL6* was decreased ($P < 0.05$) markedly compared with controls (Figure 5B).

Figure 1. Overexpression of elongation of very long-chain fatty acid-like fatty acid elongase 6 (*ELOVL6*) had no significant change on genes related to triacylglycerol synthesis and desaturation. The goat mammary epithelial cells were transfected with a recombinant adenovirus expressing *ELOVL6* (Ad-*ELOVL6*) or the control (Ad-GFP) for 48 h and then collected for mRNA extraction. (A) mRNA expression of *ELOVL6*; (B) mRNA expression of genes related to triacylglycerol synthesis [diacylglycerolacyltransferase 1 (*DGAT1*), diacylglycerolacyltransferase 2 (*DGAT2*), glycerol-3-phosphate acyltransferase (*GPAM*), and perilipin2 (*PLIN2*)] and desaturation (stearoyl-CoA desaturase 1; *SCD1*). Values are means $\pm$ SEM from 3 individual cultures. ** $P < 0.01$ compared with control (Ad-GFP).

DISCUSSION

The *ELOVL* protein family is essential for fatty acid elongation and synthesis of key saturated and unsaturated LCFA (Sun et al., 2013). Although evidence from *Elovl6*-deficient mice illustrated that its fatty acid substrates range from 12 to 16 carbons (Matsuzaka et al., 2007), the effect of altered expression of this enzyme on fatty acid composition in ruminant mammary cells has not been addressed. Our findings using knockdown and overexpression approaches reveal a crucial role of *ELOVL6* in mammary cells in catalyzing the elongation of 16-carbon to 18-carbon fatty acids.

Previous findings from knockdown and overexpression of *Elovl-6* in murine cell lines indicated that *Elovl-6* drove synthesis of 18:0 and 18:1n-9 using 16:0 as a substrate (Green et al., 2010). The alterations of elongation indices after knockdown and overexpression of *ELOVL6* are consistent with data in insulin-secreting-1 β -cells (Green et al., 2010). The findings in this study that *ELOVL6* overexpression increased the concentration of C18:0 at the expense of C16:0 confirmed the role of *ELOVL6* in elongating C16:0 to C18:0 in ruminant mammary cells. The significant decrease of elongation indices of C16:0 and C16:1 after knockdown of *ELOVL6* further underscored the role of *ELOVL6* in catalyzing the elongation of 16 to 18 carbon.

The decrease in C18:1n-7 concentration after knockdown of *ELOVL6* confirmed the hypothesis from work with *Elovl6*^{-/-} mice that C16:1n-7 is also a substrate for *Elovl6* (Moon et al., 2014). Because C16:0 is the predominant 16-carbon fatty acid synthesized in mammary cells, our data suggest that *ELOVL6* is the rate-limiting enzyme controlling the elongation of 16-carbon fatty acids to longer chain fatty acids.

Figure 2. Overexpression of very long-chain fatty acid-like fatty acid elongase 6 (*ELOVL6*) altered fatty acid composition and elongation index. The goat mammary epithelial cells (GMEC) were transfected with a recombinant adenovirus expressing *ELOVL6* (Ad-ELOVL6) or the control (Ad-GFP) for 48 h and then collected for fatty acid analysis. (A) Concentration of C16–C18 carbon fatty acids in GMEC. Data are reported as ratio of total fatty acids. * $P < 0.05$ compared with Ad-GFP. (B) Elongation index for C16:0 and C16:1n-7. * $P < 0.05$ compared with Ad-GFP. (C) Desaturation index for C16:0. (D) Desaturation index for C18:0. Values are means $\pm$ SEM from 3 individual cultures.

Oleic acid (C18:1n-9) is the preferred substrate for synthesis of TAG (Green et al., 2010). This fatty acid is synthesized during the desaturation of C18:0. Thus, we speculate that a function of *ELOVL6* is to provide additional substrates for TAG synthesis. This idea is supported by the fact that the concentration of TAG was increased after overexpression of *ELOVL6* but decreased after knockdown of *ELOVL6*. The down-regulation of *GPAM* and *DGAT2* after knockdown of *ELOVL6* might have been induced by the decrease in availability of substrates. Furthermore, the decrease in *DGAT2* after knockdown of *ELOVL6* is consistent with the hypothesis in mice that *DGAT2* is prone to respond to availability of endogenous fatty acids (Smith et al.,

2000; Harris et al., 2011). The fact that accumulation of C16:0 would result in endoplasmic reticulum stress and apoptosis (Green and Olson, 2011) could mean that the elongation of C16:0 to C18:0 might serve to protect the mammary cell from lipotoxicity.

In nonruminants, the synthesis of 18:1n-9 requires desaturation after elongation of 16:0 to 18:0. In insulin-secreting-1 β -cells, overexpression of *ELOVL6* increased the total desaturation and knockdown of *ELOVL6* decreased the total desaturation (Green et al., 2010). The lack of change in the present study for the desaturation indices of C16:0 and C18:0 agreed with the lack of change *SCD1* expression when *ELOVL6* was knocked down or upregulated. Furthermore, the lack of change in *SCD1* expression after altering *ELOVL6* in this study was consistent with data from rat liver (Moon et al., 2014). The minor effect of *ELOVL6* manipulation in GMEC on *SCD1* could partly be explained by the low expression of *SCD1* mRNA in GMEC cultured in vitro (data not shown). In addition, the lower concentration of C18:1n-9 after knockdown of *ELOVL6* in this study may be due to less synthesis of C18:0 from C16:0.

In rodent liver, the expression of *ELOVL6* is regulated by transcription factors including PPARA, nuclear receptor subfamily 1 group H member 3, and sterol regulatory element binding transcription factor 1 (Wang et al., 2006). In bovine, the expression of *ELOVL6* is sensitive to diet and was associated with the expression of peroxisome proliferator activated receptor gamma in intramuscular fat (Moisá et al., 2014), subcutaneous fat (Ji et al., 2012), and mammary tissue (Leroux et al., 2016). Based on the fact that LCFA are natural ligands of these transcription factors, these data are suggestive that *ELOVL6* could be regulated by dietary LCFA via transcription factors.

Our recent experiment with RNA sequencing of goat mammary gland revealed that 4 isoforms of the *ELOVL* family (*ELOVL1*, *ELOVL5*, *ELOVL6*, and *ELOVL7*) are expressed in lactating mammary tissue (Shi et al., 2015). Similar transcriptome data revealed various *ELOVL* isoforms also are expressed in the bovine mammary gland (Bionaz et al., 2012a,b). Their roles (if any) in fatty acid elongation in ruminant mammary tissue remain unknown; thus, additional experiments should be performed to explore their involvement in controlling fatty acid elongation. Collectively, the data from the present study suggest an important role of *ELOVL6* in controlling LCFA elongation in goat mammary cells.

CONCLUSIONS

This study is the first to directly assess the role of *ELOVL6* in regulating fatty acid elongation in rumi-

Figure 3. Knockdown of elongation of very long-chain fatty acid-like fatty acid elongase 6 (*ELOVL6*) altered genes related to triacylglycerol synthesis and desaturation. The goat mammary epithelial cells were transfected with adenovirus containing small hair RNA knocking down *ELOVL6* (Ad-shELOVL6) or adenovirus containing negative control (Ad-shNC) for 48 h and then collected for mRNA extraction. (A) mRNA expression of *ELOVL6*; (B) mRNA expression of genes related to triacylglycerol synthesis [diacylglycerolacyltransferase 1 (*DGAT1*), diacylglycerolacyltransferase 2 (*DGAT2*), glycerol-3-phosphate acyltransferase (*GPAM*), and perilipin2 (*PLIN2*)] and desaturation (*SCD1*). Values are means $\pm$ SEM from 3 individual cultures. * P < 0.05 and ** P < 0.01 compared with control (Ad-GFP).

Figure 4. Knockdown of elongation of very long-chain fatty acid-like fatty acid elongase (*ELOVL6*) altered fatty acid composition and elongation index. The goat mammary epithelial cells (GMEC) were transfected with adenovirus containing small hair RNA knocking down *ELOVL6* (Ad-sh*ELOVL6*) or adenovirus containing negative control (Ad-shNC) for 48 h and then collected for fatty analysis. (A) Concentration of C16–C18 carbon fatty acids in GMEC. Data are reported as ratio of total fatty acids. * $P < 0.05$ compared with control (Ad-GFP). (B) Elongation index for C16:0 and C16:1n-7. * $P < 0.05$ compared with control (Ad-GFP). (C) Desaturation index for C16:0. (D) Desaturation index for C18:0. Values are means $\pm$ SEM from 3 individual cultures.

Figure 5. Elongation of very long-chain fatty acid-like fatty acid elongase (*ELOVL6*) promoted the accumulation of cellular triacylglycerol (TAG). The goat mammary epithelial cells were transfected with a recombinant adenovirus expressing *ELOVL6* (Ad-*ELOVL6*), adenovirus containing small hair RNA knocking down *ELOVL6* (Ad-sh*ELOVL6*), control (Ad-GFP), and adenovirus containing negative control (Ad-shNC) and collected at 48 h for cellular TAG analysis. Values are means $\pm$ SEM from 3 individual cultures. * $P < 0.05$ compared with control (Ad-GFP or Ad-shNC).

nant mammary cells. The upregulation of *ELOVL6* increased the elongation indices of C16:0 to C18:0, whereas knockdown decreased the elongation index. Those data were associated with the accumulation of C16:0. Upregulation of *ELOVL6* increased the synthesis of TAG via controlling the synthesis of C18 carbon fatty acids. At least in vitro, these data demonstrate a direct role for *ELOVL6* in modulating LCFA synthesis in goat mammary cells.

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