Overexpression of SREBP1 (sterol regulatory element binding protein 1) promotes de novo fatty acid synthesis and triacylglycerol accumulation in goat mammary epithelial cells

H. F. Xu,* J. Luo,*¹ W. S. Zhao,* Y. C. Yang,* H. B. Tian,* H. B. Shi,* and M. Bionaz†¹

*Shaanxi Key Laboratory of Molecular Biology for Agriculture, College of Animal Science and Technology, Northwest A&F University, Yangling, 712100, China

†Department of Animal and Rangeland Sciences, Oregon State University, Corvallis 97331

ABSTRACT

Sterol regulatory element binding protein 1 (SREBP1; gene name *SREBF1*) is known to be the master regulator of lipid homeostasis in mammals, including milk fat synthesis. The major role of SREBP1 in controlling milk fat synthesis has been demonstrated in bovine mammary epithelial cells. Except for a demonstrated role in controlling the expression of *FASN*, a regulatory role of SREBP1 on milk fat synthesis is very likely, but has not yet been demonstrated in goat mammary epithelial cells (GMEC). To explore the regulatory function of SREBP1 on de novo fatty acids and triacylglycerol synthesis in GMEC, we overexpressed the mature form of SREBP1 (active NH₂-terminal fragment) in GMEC using a recombinant adenovirus vector (Ad-nSREBP1), with Ad-GFP (recombinant adenovirus of green fluorescent protein) as control, and infected the GMEC for 48 h. In infected cells, we assessed the expression of 20 genes related to milk fat synthesis using real time-quantitative PCR, the protein abundance of SREBP1 and *FASN* by Western blot, the production of triacylglycerol, and the fatty acid profile. Expression of *SREBF1* was modest in mammary compared with the other tissues in dairy goats but its expression increased approximately 30-fold from pregnancy to lactation. The overexpression of the mature form of SREBP1 was confirmed by >200-fold higher expression of *SREBF1* in Ad-nSREBP1 compared with Ad-GFP. We observed no changes in amount of the precursor form of SREBP1 protein but a >10-fold increase of the mature form of SREBP1 protein with Ad-nSREBP1. Compared with Ad-GFP cells (control), Ad-nSREBP1 cells had a significant increase in expression of genes related to long-chain fatty acid activation (*ACSL1*), transport

(*FABP3*), desaturation (*SCD1*), de novo synthesis of fatty acids (*ACSS2*, *ACLY*, *IDH1*, *ACACA*, *FASN*, and *ELOVL6*), and transcriptional factors (*NR1H3* and *PPARG*). We observed a >10-fold increase in expression of *INSIG1* but *SCAP* was downregulated by Ad-nSREBP1. Among genes related to milk fat synthesis and lipid droplet formation, only *LPIN1* and *DGAT1* were upregulated by Ad-nSREBP1. Compared with the Ad-GFP, the cellular triacylglycerol content was higher and the percentage of C16:0 and C18:1 increased, whereas that of C16:1, C18:0, and C18:2 decreased in Ad-nSREBP1 cells. Overall, the data provide strong support for a central role of SREBP1 in the regulation of milk fat synthesis in goat mammary cells.

Key words: sterol regulatory element binding protein 1 (SREBP1), milk fat synthesis, adenovirus, goat

INTRODUCTION

The sterol regulatory element binding protein 1 (SREBP1) belongs to the basic helix-loop-helix leucine zipper transcription factor superfamily (Yokoyama et al., 1993). Two isoforms of SREBP1 (gene name *SREBF1*) have been identified in mammals, SREBP-1a and SREBP-1c, which differ by use of alternative promoters (Horton, 2002). The SREBP isoforms are synthesized as inactive endoplasmic reticulum (ER) membrane-bound protein precursors (Tontonoz et al., 1993; Brown and Goldstein, 1997). Under low sterol concentrations, SREBP cleavage activating protein (SCAP) escorts the SREBP precursors from ER to Golgi apparatus, where SREBP precursors are cleaved to release the N-terminal active fragment (Brown and Goldstein, 1997; Espenshade, 2006; Sato, 2010). The active fragment enters into the nucleus, where it binds the SREBP response element (SRE) in the promoter region of many lipid metabolism-related genes to increase their transcription (Horton, 2002; Eberlé et al., 2004). Under high intracellular sterol concentrations, insulin-induced gene (INSIG) is activated and binds

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¹Corresponding authors: luojun@nwsuaf.edu.cn and massimo.bionaz@oregonstate.edu

SCAP. The SREBP-SCAP-INSIG complex is retained in the ER membrane, inhibiting the activity of SREBP1 (Nohturfft et al., 1998).

The synthesis of milk fat involves many proteins including transporters and enzymes (Moore and Christie, 1979; Clegg et al., 2001). A model summarizing all main proteins involved in milk fat synthesis was previously proposed (Bionaz and Loo, 2008b). Central to the transcriptional regulation of milk fat synthesis is SREBP1, as suggested by studies performed in bovine (Harvatine and Bauman, 2006; Ma and Corl, 2012; Oppi-Williams et al., 2013) and mouse (Rudolph et al., 2010). Recently, it was demonstrated in bovine mammary epithelial cells by gene overexpression and knockdown that SREBP1 is central in transcription regulation of many genes related to milk fat synthesis and secretion (Li et al., 2014a).

Compared with rodents, humans, and bovines, much less is known about the role of SREBP1 in the regulation of milk fat synthesis in mammary epithelial cells of dairy goats. An initial suggestion of a role of SREBP1 in milk fat synthesis in goat was provided by a very recent study, where overexpression of *SREBF1* increased the expression of *FASN* and *SREBF1* in primary goat mammary epithelial cells (Li et al., 2015); however, no expression of additional genes or synthesis of triacylglycerol (TAG) was assessed in that study.

The objective of the present study was to examine the role of SREBP1 in the regulation of milk fat synthesis in goat. To accomplish our objective, we overexpressed the mature form of SREBP1 (i.e., active NH₂-terminal fragment) in primary goat mammary epithelial cells (GMEC) and assessed the effect on expression of a large number of milk fat-related genes and the effect on cellular fatty acid profile and TAG content.

MATERIALS AND METHODS

All experimental procedures involving dairy goats were conducted under the approval of the Animal Care and Use Committee of Northwest A&F University of Agriculture and Technology (Yangling, Shaanxi, China).

Tissue Sample Collection

Three-year-old Xinong Saanen dairy goats of Northwest A&F University experimental farm were selected and killed for mammary gland tissue collection during lactation (120 d after parturition, *n* = 3) and the dry period (10 d after dry-off and 50 d before expected parturition, *n* = 3). Samples were washed with diethylpyrocarbonate (DEPC)-treated water and then immediately snap-frozen in liquid nitrogen.

Generation of Recombinant Adenoviruses

The transcriptionally active NH₂-terminal fragment (AA 1–403) of goat SREBP1 was amplified and used to generate recombinant adenovirus according to previously published reports (He et al., 1998; Shi et al., 2014). Briefly, the cDNA length of 1–1,209 nucleotides of SREBP1 (**nSREBP1**) was amplified and subcloned into the shuttle vector pAdTrack-CMV. The recombinant plasmid pAdTrack-CMV-nSREBP1 linearized by restriction endonuclease *PmeI* was transformed into *Escherichia coli* BJ5183 competent cells containing the backbone vector pAdEasy-1. Homologous recombination occurred between shuttle vector and backbone vector. Positive recombinants of plasmid (pAd-nSREBP1) were identified by kanamycin resistance and digestion of restriction endonuclease *PacI*. Then, the linearized product of pAd-nSREBP1 digested by *PacI* was transfected into HEK293 cells to amplify high-titer recombinant adenovirus (**Ad-nSREBP1**). The recombinant adenovirus of green fluorescent protein (GFP) was used as control (**Ad-GFP**). Detailed procedures on purification, titer determination, and storing conditions of adenovirus were previously provided (Shi et al., 2013a).

Cell Culture and Treatments

The HEK293 cells were cultured in basal RPMI-1640 medium (Hyclone Laboratories, Beijing, China) supplemented with 10% fetal bovine serum (Hyclone) and penicillin/streptomycin (10 kU/L, Harbin Pharmaceutical Group, Harbin, China). Cells were cultured at 37°C in 5% CO₂ and medium was changed every 24 h.

The GMEC were isolated, purified, and cultured from 3 lactating goats according to our previously described protocol (Shi et al., 2013a). Isolated cells were maintained in a basal Dulbecco's modified Eagle medium/F12 medium (Hyclone) supplemented with 10% fetal bovine serum (Hyclone), bovine insulin (5 µg/mL; Sigma, St. Louis, MO), hydrocortisone (5 mg/L; Sigma), penicillin/streptomycin (10 kU/L; Harbin Pharmaceutical Group), and epidermal growth factor (10 ng/mL; Sigma). The medium contained an estimated 315 mg/dL of glucose (i.e., a supraphysiological level in goat, where the norm is between 40 and 75 mg of glucose/dL in blood) and did not contain acetate, with the exception of a negligible dose provided through the fetal bovine serum. Cells were incubated at 37°C in 5% CO₂ and culture medium was changed every 24 h. The GMEC isolated from the 3 goats were cultured individually to passage 5 and pooled before starting the experiment. For RNA extraction, cells were seeded in 6-well cell culture plate (Corning Inc., Corning, NY). For protein extraction and quantification of total

cellular TAG, cells were seeded in 60-mm cell culture dishes (Corning Inc.). Once the culture reached 80 to 90% confluence, cells were washed twice with PBS, and Ad-nSREBP1 or Ad-GFP recombinant adenovirus was added to the cells with the basal culture medium supplemented with prolactin (1.5 µg/mL; Sigma). Cells were harvested after 48 h of incubation. Each treatment was performed in 3 replicates.

RNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted from tissue and cells using RNeasy pure cell/bacteria kit (Qiagen Biotech Co. Ltd., Beijing, China) according to the manufacturer's instructions. The purity and concentration of RNA were determined by using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), the ratio of optical density at 260 nm:280 nm was ≥ 2 for all samples. Integrity of RNA was assessed by electrophoretic analysis of 28S and 18S rRNA subunits stained with ethidium bromide. Results were analyzed by Gel-Pro Analyzer software (Beijing Junyi-Dongfang Electrophoresis Equipment Co. Ltd., Beijing, China) and the ratio of 28S:18S was approximately 2:1 (Supplemental Figure S1; <http://dx.doi.org/10.3168/jds.2015-9736>). For the analysis of mRNA abundance of *SREBF1* between tissues, the same amount of RNA extracted from each type of tissue was pooled from the 3 goats before cDNA synthesis. For mammary tissue during the 2 stages of lactation and for GMEC, cDNA was synthesized from each individual extracted sample. First-strand cDNA was synthesized (0.5 µg of RNA per 10-µL reaction) using PrimeScript RT reagent Kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) was performed using SYBR Premix Ex Taq II Kit (Takara Bio Inc.) on a CFX96 Real-Time PCR Detection system (Bio-Rad Laboratories Inc., Hercules, CA). The amplification conditions were as follows: 1 cycle at 95°C for 30 s, followed by 39 cycles at 95°C for 5 s, and 60°C for 30 s; a dissociation curve of each sample was generated at 95°C for 10 s and then from 65°C to 95°C with an increment of 0.5°C. Gene-specific primers for *SREBF1* between tissues and different lactating stages were as follows: 5'-GCTGAGTGCCCTGAACC-3' and 5'-CCATC-CACGAAGAAACG-3' (i.e., spanning exon 11 to 12, common to all *SREBF1* isoforms). The primer pair for the internal control, *GAPDH*, was 5'-GCAAGTTC-CACGGCACAG-3' and 5'-GGTTCACGCCCATCA-CAA-3'. Gene-specific primers to assess expression of genes in GMEC were designed using Primer Premier 5 (Premier Biosoft, Palo Alto, CA; Table 1). Two primer

pairs were designed for *SREBF1*, one to specifically amplify *SREBF1a* transcript (spanning the exon 1, present only in isoform SREBP-1a) and one to measure simultaneously all *SREBF1* isoforms (spanning exon 3, common to all *SREBF1* isoforms). All primers were tested for presence of a single band at the expected size using a 2% agarose gel and amplicons were sequenced according to methods described previously (Kadegowda et al., 2009; Shi et al., 2013a).

The mRNA abundance of *SREBF1* between tissues and in mammary tissue in the 2 different lactation stages was determined using the $2^{-\Delta\Delta C_q}$ method, where C_q is the quantitation cycle, and *GAPDH* was used as internal control. For the measurement of gene expression in GMEC, the RDML (real-time PCR data markup language; <http://www.rdml.org/>) files were exported and uploaded into the LinRegPCR software (www.linregpcr.nl) for quantification analysis (Ramackers et al., 2003). The normalization factor was calculated as the geometrical mean of the RT-qPCR data obtained from LinRegPCR analysis of mitochondrial ribosomal protein L39 (*MRPL39*), ribosomal protein S9 (*RSP9*), and ubiquitously expressed transcript (*UXT*). The stability of the internal control genes and the reliability of the calculated normalization factor were evaluated using geNorm (Vandesompele et al., 2002). The M-value (expression stability) was < 0.28 for all 3 internal control genes and the V-value (pairwise variation) was 0.11; thus, all 3 internal control were very stable and the normalization factor reliable according to the threshold suggested by Vandesompele and collaborators (2002; i.e., ≤ 1.5 for M-value and ≤ 0.15 for V-value). The final value of each target gene was obtained by dividing the RT-qPCR data obtained from LinRegPCR analysis by the normalization factor. The percentage RNA abundance of each genes relative to *SREBF1a* was calculated as $[E^{-C_q \text{ gene}}/E^{-C_q \text{ SREBF1a}}] \times 100$. The efficiency (E) of PCR amplification was obtained using LinRegPCR (Table 1).

Protein Extraction and Western Blot

Cells were washed twice with cold PBS before harvest. Total protein was extracted with radioimmuno-precipitation assay (RIPA) buffer (Solarbio Tech Co. Ltd., Beijing, China) supplemented with phenylmethanesulfonyl fluoride (PMSF; Pierce, Rockford, IL) at a final concentration of 1 mM. The protein concentration was measured using a bicinchoninic acid (BCA) kit (Thermo Fisher Scientific) according to the manufacturer's instruction, and 25 µg of total protein per lane was separated by SDS-PAGE. The blot was transferred to nitrocellulose membrane (Pall Corp., Port Washing-

Table 1. Genes, accession numbers, sequences, amplicon length of primer pairs, efficiency of amplification of PCR, cycle quantitation (Cq), and mRNA abundance of each gene relative to *SREBF1a* in Ad-GFP-transfected goat mammary epithelial cells

Gene and accession no.	Primer ¹	Sequence (5' to 3')	Amplicon size, bp	Efficiency ²	Cq ³	% mRNA ⁴	Reference
<i>ACACA</i> XM_005693156.1	F.3609 R.3779	CTCCAACCTCAACCACTAGGG GGGAATCAGAGAACGAGCC	171	2.15	23.0	126.6	Shi et al., 2013a
<i>ACLY</i> XM_005693833.1	F.738 R.1013	TTACCCAGAGAACGCTAC AGGATCTTGCCATCTGGTGC	276	1.95	20.7	5,519.7	van Dorland et al., 2009
<i>ACSL1</i> BC119914	F.1855 R.1974	GTGGCTCCTTTGAAGAACTGT ATAGATGCTTTGACCTGTCAAAT	120	2.03	23.0	492.5	Bionaz and Loor, 2008a
<i>ACSS2</i> BC134532	F.1822 R.1921	GGCAATCTTCTCTAATCTGCTT AAGCAAGTTGCCATCTCTCA	100	2.13	28.1	3.2	Bionaz and Loor, 2008b
<i>AGPAT6</i> NM_001083669.1	F.955 R.1036	AACTGTGGTCCAAATTCGA GGAGCTTATCCAGGCCAATG	101	2.30	27.1	0.8	Shi et al., 2013a
<i>ATGL</i> NM_001285739.1	F.408 R.587	TGCGGCAGATGTCACCTCT CCACTGGGACCTGAGGTGTC	180	1.91	19.6	16,938.4	Lin et al., 2013
<i>DGAT1</i> XM_005688895.1	F.657 R.757	GCATCACACACACCAATCA GGAAGCCTTTAGTCTCTGGTC	101	1.94	24.9	380.9	Bionaz and Loor, 2008b
<i>ELOVL6</i> NM_001102155.1	F.173 R.377	ATTGTATCTCTAGTTGGGTGTC GATGAGACACGCGAGATG	205	2.18	20.2	746.9	This article
<i>FABP3</i> NM_001285701.1	F.214 R.333	GTCAACTATTCCCGCACAAAG GGCTCCACACCGTGTTCCTCA	120	1.98	27.3	43.0	Shi et al., 2013a
<i>FASN</i> DQ915966.3	F.6762 R.6987	GCTCTGTGGCGCTGCAGCTG ATTGACCTTTGGCAGATAG	226	2.08	21.6	742.2	Shi et al., 2013a
<i>GPAM</i> AY515690	F.15 R.202	AAAGACCTTCCCAACAAG CGATGAGAAGAGAGTGGAGGA	188	2.02	23.1	477.3	Ma and Corl, 2012
<i>IDH1</i> NM_181012	F.234 R.361	CAAGCGGGGTATATTTTG AGCTCACAAAGTTCAAGCG	128	1.84	27.0	403.1	Ma and Corl, 2012
<i>INSIG1</i> NM_001286088.1	F.455 R.586	ACAGTGTCTAATGTCAAGG GAGGGAAGAAACACCAAA	132	2.05	21.8	879.3	This article
<i>LIPIN1</i> XM_002707716	F.1686 R.1887	GATGCTGACGCTGGACAACA AGGTCTCTTTTGTGGCATCC	202	1.91	25.4	307.5	Ma and Corl, 2012
<i>MRPL39</i> XM_005674737.1	F.370 R.470	TTGGTCAGAGCCCAAGAGT CATCAACCCCATCTTCAGATT	101	1.98	22.0	1,607.8	Kadegowda et al., 2009
<i>NR1H3</i> GU332719	F.1005 R.1167	CAGGCCTCCACATATGTGT TGGTCTCCTCGCTTACATC	163	2.13	23.5	107.6	Shi et al., 2013a
<i>PLIN2</i> NM_173980	F.83 R.350	TCCTTGGCCCAAGTCAAGC CCTCACCCACCGTTGACTTCT	268	1.96	21.1	3,866.3	Ma and Corl, 2012
<i>PPARG</i> HQ589347.1	F.101 R.245	GATACAGGCTCCACTTTGATTGC CCTCGACCAAGAGCTGAAG	145	1.93	32.4	3.0	Shi et al., 2013a
<i>RP59</i> XM_005709411.1	F.72 R.135	CCATGTCACCTCAAGGAGGA TGTCGATCTTGGGTGTGGAG	64	2.26	17.3	4,116.7	Bionaz and Loor, 2007
<i>SCAP</i> XM_005696121.1	F.816 R.922	CCATGTCACCTCAAGGAGGA TGTCGATCTTGGGTGTGGAG	107	1.99	21.9	1,557.7	Shi et al., 2013a
<i>SCD1</i> GU947654	F.357 R.612	CCATGTCACCTCAAGGAGGA GTCGGATAATCTAGCGTAGCA	256	2.02	21.3	1,673.4	Shi et al., 2013a
<i>SREBF1a</i> HM443643.1	F.73 R.153	CTGCTGACCGACATAGAAGACAT GTAGGGCGGGTCAACACAGG	81	2.00	25.7	100	Shi et al., 2013a
<i>SREBF1</i> HM443643.1	F.986 R.1166	ACGCCATCGAGAAACGCTAC GTGCGCAGACTCAGGTTCTC	181	2.04	23.9	215.0	Li et al., 2014a
<i>UXT</i> XM_005700842.1	F.270 R.370	TGTGCGCCTTGGATATGGTT GGTTCTCGCTGAGCTCTGTG	101	2.06	21.2	1,232.7	Bionaz and Loor, 2007

¹Primer direction (F = forward, R = reverse) and hybridization position on the sequence.

²Efficiency of amplification as calculated by LinRegPCR (www.linregpcr.nl).

³Cycle quantitation as calculated by LinRegPCR for the pAd-GFP group only, where Ad-GFP = recombinant adenovirus of green fluorescent protein as control.

⁴The percentage mRNA abundance of each measured gene in Ad-GFP group is relative to the abundance of *SREBF1a* and it was calculated as follows: $[(E^{-Cq_{\text{Gene}}}) / (E^{-Cq_{\text{SREBF1a}}})] \times 100$, where E denotes efficiency of amplification.

ton, NY) by a Bio-Rad Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories Inc.). Membranes were blocked for 1 h using 5% skim milk in Tris-buffered saline/Tween-20 (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% Tween-20) before incubation overnight at 4°C with primary antibodies for β -actin (CW0096, 1:1,000; CW Biotech, Beijing, China), SREBP1 (ab3259, 1:500; Abcam, Cambridge, UK), and FASN (1:300; Li et al., 2014a). Horseradish peroxidase (HRP)-conjugated goat anti-mouse (CW0102, 1:1,000; CW Biotech) and goat anti-rabbit IgG (CW0103, 1:1,000; CW Biotech) were used as secondary antibodies. Signal was measured using the chemiluminescent ECL Western blot detection system (Thermo). The grayscale data of each band were analyzed using ImageJ software (<http://imagej.nih.gov/ij/>) and relative protein abundance was normalized to β -actin.

Measurement of Total Cellular TAG

After 48 h of incubation with adenovirus, cells were rinsed twice with cold PBS and the total content of cellular TAG was extracted by using a tissue/cell triacylglycerol assay kit, according to the manufacturer's instruction (Applygen Technologies Inc., Beijing, China), and the data were read on a microtiter plate reader (Bio-Rad Laboratories Inc.). Total TAG content was normalized to total cellular protein quantified by using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific).

Measurement of Fatty Acid in GMEC

Total lipid was extracted from 60-mm culture dish of GMEC using 2 mL of 2.5% (vol/vol) sulfuric acid:methanol (Sigma) in an 8-mL glass tube. After ultrasonication for 10 min, the glass tubes were incubated at 80°C for 1 h. After cooling the tube to room temperature, 2 mL of 0.1 M HCl and 800 μ L of hexyl hydride (Sigma) were added and the tube was vortexed for 30 s followed by centrifugation for 5 min at $900 \times g$. Supernatant was collected in a 2-mL silicified tube with 0.5 g of water-free sodium sulfate. After acute shock, the tube was centrifuged for 3 min at $13,800 \times g$, and the liquid supernatant was collected for fatty acid analysis by GC-MS (Thermo Fisher Trace GC ultra-DSQII-AI3000) using an BP-5MS (30 m $\times$ 0.25 mm $\times$ 0.25 μ m) column (Agilent Technologies, Santa Clara, CA). A commercial C19:0 fatty acid (Sigma) was used as an internal control. The relative proportion of each fatty acid was determined as percentage of the total peak area that could be identified (Wang et al., 2012; Shi et al., 2013b).

Statistical Analysis

For GMEC, normalized RT-qPCR data were divided by the average of the control group, and transformed data were checked for normal distribution using Univariate Proc of SAS (version 9.4, SAS Institute Inc., Cary, NC) before statistical analysis. This was performed using GLM of SAS with treatment (Ad-nSREBP1 and Ad-GFP) or tissue as main effect and replicates as random. Least squares means (LSM) were separated using Tukey's test. Differences were considered statistically significant when $P < 0.05$, and data are reported as $\text{LSM} \pm \text{SE}$.

RESULTS

Transcript Abundance of *SREBF1* in Mammary of Dairy Goats in Pregnancy and Lactation

The raw RT-qPCR data of *GAPDH* were not different between nonlactating (i.e., pregnant) and lactating mammary tissue in goat (data not shown), and the RT-qPCR data of *SREBF1* normalized with *GAPDH* revealed a ~ 30 -fold increase in expression from pregnancy to lactation (see insert in Supplemental Figure S2A; <http://dx.doi.org/10.3168/jds.2015-9736>). Overall, mammary tissue had an intermediate mRNA abundance of *SREBF1* (Supplemental Figure S2; <http://dx.doi.org/10.3168/jds.2015-9736>). Abundance of *SREBF1* between tissues is reported in Supplemental Figure S2 (<http://dx.doi.org/10.3168/jds.2015-9736>).

Ad-SREBP1 Overexpresses Mature SREBP1 in GMEC

The mRNA abundance of *SREBF1a* increased >300 -fold and *SREBF1* increased >250 -fold in Ad-nSREBP1-infected cells compared with Ad-GFP-infected cells (Figure 1A). Overexpression of *SREBF1* transcriptionally active NH_2 -terminal fragment (nSREBP1) led to a >10 -fold increase of SREBP1 mature protein, whereas the precursor form of SREBP1 protein was not affected (Figure 1B).

Overexpression of *SREBF1* Promotes Expression of Lipogenic Genes and Increases Cellular TAG

Compared with Ad-GFP infected cells, overexpression n-SREBP1 dramatically upregulated the mRNA levels of all measured genes coding for proteins involved in fatty acid activation, transport, and desaturation, encompassing fatty acid binding protein 3 (*FABP3*; >100 -fold), acyl-CoA synthase long-chain family member 1 (*ACSL1*; ~ 4 -fold), ELOVL fatty acid elongase 6 (*ELOVL6*; >2 -fold), and stearoyl-CoA desaturase

(*SCD1*; >6-fold; Figure 2). Overexpression of nSREBP1 increased the transcription of all genes measured involved in de novo fatty acid synthesis, with >50-fold increase for acyl-CoA synthetase short-chain family member 2 (*ACSS2*), >9-fold increase for fatty acid synthase (*FASN*), >2-fold increase for acetyl-CoA carboxylase α (*ACACA*), >1.5-fold increase for isocitrate dehydrogenase 1 (NADP+) soluble (*IDH1*), and ~6-fold increase for ATP citrate lyase (*ACLY*). The increase in

expression of *FASN* was also confirmed by the increase in its protein abundance (Figure 2; Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2015-9736>). The expression of most of the transcription regulation-related genes, including *INSIG1*, *NR1H3*, and *PPARG*, was upregulated by overexpression of nSREBP1 but the expression of *SCAP* was downregulated (Figure 2).

Few genes coding for proteins related to TAG synthesis and lipid droplet formation were affected by over-

Figure 1. Adenovirus-mediated overexpression of nSREBP1 (the transcriptionally active NH₂-terminal fragment of the sterol regulatory element binding protein 1 gene, *SREBF1*) in primary goat mammary epithelial cells. (A) Real-time quantitative PCR analysis of *SREBF1* expression after 48 h of infection with Ad-nSREBP1 adenovirus. Values are presented as LSM $\pm$ SE for 3 replicates; ** P < 0.01. (B) Western blot analysis of precursor (125 kDa) and mature (68 kDa) SREBP1 protein levels after 48 h infection with Ad-nSREBP1 adenovirus. Protein abundance was normalized to β -actin. The blots are representative of 3 independent experiments.

expression of nSREBP1 (Figure 3). Among these were lipin 1 (*LPIN1*), which increased >15-fold, and diacylglycerol O-acyltransferase 1 (*DGAT1*), which increased >1.5-fold. No effects were detected for 2 of the enzymes involved in TAG synthesis: glycerol-3-phosphate acyltransferase 1 (*GPAM*) and 1-acylglycerol-3-phosphate O-acyltransferase 6 (*AGPAT6*), or for genes coding for perilipin 2 (*PLIN2*), involved in lipid droplet formation, and adipose triacylglycerol lipase (*ATGL*), responsible for the lipid droplet TAG depletion (Figure 3).

Overexpression of SREBF1 Affects Fatty Acid Composition in GMEC

Because of increased expression of TAG synthesis-related genes, total cellular content of TAG also increased in cells transfected with Ad-nSREBP1 (Figure 3). The fatty acid profile in GMEC was affected with an increase in the proportion of C16:0, total C18:1, and *cis*-9 C18:1 and a decrease in proportion of C16:1, C18:0, and C18:2, whereas relative abundance of 2

Figure 2. Adenovirus-mediated overexpression of nSREBP1 (the transcriptionally active NH₂-terminal fragment of the sterol regulatory element binding protein 1 gene, *SREBF1*) upregulates the expression of genes related to fatty acid synthesis in goat mammary epithelial cells. Reported is the effect of nSREBP1 overexpression on main genes coding for proteins with key roles in long-chain fatty acid activation (*ACSL1*), transport (*FABP3*), desaturation (*SCD1*), de novo fatty acid synthesis (*FASN*, *ACACA*, *ACSS2*, and *IDH1*), regulation of SREBP1 activity (*INSIG1* and *SCAP*), and transcriptional factors important for regulation of milk fat synthesis (i.e., *PPARG* and *NR1H3*). Expression of genes with unclear or not yet demonstrated role in mammary fatty acid synthesis (*ACLY* and *ELOVL6*) are also reported. The effect of nSREBP1 overexpression on the protein level of fatty acid synthase (*FASN*) is shown. Real-time quantitative PCR measurements of gene expression are expressed as fold change compared with Ad-GFP-transfected (recombinant adenovirus of green fluorescent protein as control) cells. Values are presented as LSM ± SE for 3 replicates; **P < 0.01.

Table 2. Effects of overexpression of nSREBP1 (transcriptionally active NH₂-terminal fragment of the sterol regulatory element binding protein 1 gene, *SREBF1*) on fatty acid composition (%; means $\pm$ SD) in goat mammary epithelial cells¹

Fatty acid	Ad-GFP	Ad-nSREBP1	P-value
C16:0	22.40 $\pm$ 0.70	25.29 $\pm$ 0.53	0.042
<i>cis</i> -11 C16:1 + <i>trans</i> -9 C16:1	1.69 $\pm$ 0.38	0.79 $\pm$ 0.04	0.047
C18:0	19.04 $\pm$ 0.69	12.5 $\pm$ 0.60	0.004
C18:1 (total)	37.85 $\pm$ 0.72	43.79 $\pm$ 0.57	0.006
<i>cis</i> -9 C18:1	32.03 $\pm$ 0.88	37.50 $\pm$ 1.26	0.043
C18:2	2.78 $\pm$ 0.25	1.49 $\pm$ 0.10	0.006
C20:4	8.90 $\pm$ 0.89	8.82 $\pm$ 0.31	0.923
C22:6	7.34 $\pm$ 0.06	7.29 $\pm$ 0.03	0.481

¹Ad-GFP = recombinant adenovirus of green fluorescent protein as control; Ad-nSREBP1 = adenovirus-mediated overexpression of the transcriptionally active NH₂-terminal fragment of the sterol regulatory element binding protein 1 gene.

PUFA, C20:4 and C22:6, was not affected (Table 2; see Supplemental Figures S3 and S4 for examples of chromatographs; <http://dx.doi.org/10.3168/jds.2015-9736>).

DISCUSSION

Expression of *SREBF1* Between Physiological States

The SREBP proteins were first identified and isolated from nuclear extracts of cultured human HeLa cells in 1993 (Briggs et al., 1993; Wang et al., 1993; Brown and Goldstein, 1997). The gene expression profile of SREBP-1a and SREBP-1c is different, as observed in mice and humans, where the *SREBP-1c* gene is highly

expressed in the liver, white adipose tissue, skeletal muscle, adrenal gland, and brain, whereas *SREBP-1a* is highly expressed in cell lines and tissues with high capacity for cell proliferation, such as spleen and intestine (Desvergne et al., 2006; Chen et al., 2013). Relative mRNA abundance of *SREBF1* between tissues is reported in the Supplemental File (<http://dx.doi.org/10.3168/jds.2015-9736>).

The large increase in expression of *SREBF1* from pregnancy to lactation in mammary tissue of goats confirmed the previously observed increase in expression of this gene in mammary tissue of other species in the same physiological changes, such as in murine (Rudolph et al., 2010) and bovine (Bionaz and Loor, 2008b), but

Figure 3. Adenovirus-mediated overexpression of nSREBP1 (the transcriptionally active NH₂-terminal fragment of the sterol regulatory element binding protein 1 gene, *SREBF1*) increases cellular triacylglycerol content and expression of related genes in primary goat mammary epithelial cells (GMEC). Reported is the effect of nSREBP1 overexpression on main genes coding for proteins with key roles in triacylglycerol synthesis (*LPIN1*, *GPAM*, *AGPAT6*, and *DGAT1*) and lipid droplet formation (*PLIN2* and *ATGL*). The amount of triacylglycerol (TAG) in GMEC is also shown. Values are presented as LSM $\pm$ SE for 3 replicates; **P < 0.01.

the magnitude of increase was much larger than that in other species (~2-fold for mouse and bovine vs. ~30-fold for goat). The high expression of *SREBF1* during lactation is thought to be related to the increased milk fat synthesis in sheep, bovine, and mouse (Bauman et al., 2006; Rudolph et al., 2010; Carcangiu et al., 2013), and SREBP1 is considered a central hub in the transcriptional network controlling milk fat synthesis in bovine (Bionaz and Looor, 2008b). This role has been previously suggested by investigation of the mechanism behind milk fat depression, which is partly caused by *trans*-10, *cis*-12 CLA. Conjugated linoleic acid depresses milk fat synthesis by decreasing the expression of milk fat synthesis-related genes via inhibition of SREBP1 activity and expression (Peterson et al., 2004; Bauman et al., 2008; Harvatine et al., 2009; Ma and Corl, 2012). Recent studies have established that SREBP1 is a central player in the regulation of milk fat synthesis in bovine (Lengi and Corl, 2010; Ma and Corl, 2012; Li et al., 2014a). Taken together, these results strongly support a key role of SREBP1 in milk fat synthesis in bovine. Initial evidence for a similar role in goats was provided by a recent study in which overexpression of SREBP1 increased the expression of *FASN* in GMEC (Li et al., 2015). The very large increase in expression from pregnancy to lactation in mammary tissue in our experiment provides additional support for a key role for SREBP1 in goats.

Overexpression of nSREBP1 Increases Expression of *SREBF1*

In this study, adenovirus-mediated overexpression technology was used to successfully overexpress the mature form of SREBP1, which is the functional form entering the cell nucleus and increasing the expression of its target genes. The successful overexpression was demonstrated by >10-fold increase in protein abundance of the mature form of SREBP1 and >250-fold increase in the expression of *SREBF1*. The primer pair used in the present experiment to amplify *SREBF1a* by RT-qPCR spans nucleotides 73 to 153 of the *SREBP1* mRNA. This segment codes for positions 24 to 50 of the AA sequence of the SREBP1a protein, as estimated using data from a previous study in bovine (Lengi and Corl, 2010). Thus, we designed a primer pair that amplifies only the gene for *SREBF1a* and one primer pair that amplifies all *SREBF1* isoforms. We did not design primer pairs to specifically measure SREBP-1c transcript. The overexpression of nSREBP1 increased the expression of *SREBF1a* >300-fold, which was similar to the increase in expression observed when the primer to amplify all *SREBF1* isoforms was used; thus, it is likely that the increase in expression of *SREBF1* observed in

our study was mostly due to the *SREBF1a* gene. This is consistent with the observed fact that isoform 1a is expressed in cell culture in vitro (Desvergne et al., 2006).

The large increase in expression of *SREBF1* detected in our study was likely due to the overexpression of the *SREBF1* sequence coding for the mature form of SREBP1. Thus, our experiment does not allow us to test whether SREBP1 controls the expression of *SREBF1* in GMEC. However, the *SREBP-1c* gene is known to have an SREBP1 response element (SRE) in the promoter region as observed in mouse (Amemiya-Kudo et al., 2000) and bovine (Lengi and Corl, 2010). We have analyzed the promoter region of the sheep *SREBF1a* up to 5,000 bp upstream of the transcription starting site (TSS; gene link ENSOARG00000001504) and analyzed the sequence using the free web-based tool LASAGNA (Lee and Huang, 2013) and found a total of 11 SRE motifs, with one motif at 1,547 bp upstream of the TSS being considered very strong. We could not retrieve the promoter region of *Capra hircus SREBF1*; however, based on the sheep data, it is possible that the upstream region of *Capra SREBF1* also presents one or more strong SRE. Based on these data, it is reasonable to expect that the large increases in expression of *SREBF1* and *SREBF1a* are partly due to the activity of the more-abundant mature SREBP1. The increase in protein abundance of immature SREBP1 in our study was only numerically larger in Ad-nSREBP1 compared with Ad-GFP (Figure 1B), which seems to contrast with the above postulated effect of SREBP1 in its own gene expression in goat. The reason for this apparent contrasting finding is not clear, but it is possible that the effect of SREBP1 on *SREBF1* expression is minor in GMEC.

SREBP1 Controls Expression of Milk Fat-Related Genes and Augments TAG Synthesis in GMEC

It has been demonstrated that *SREBF1c* overexpression in bovine hepatocytes increases TAG synthesis (Li et al., 2014b). Similarly, overexpression of *SREBF1* in mammary epithelial cells of bovine increased the expression of genes related to fatty acid and TAG synthesis and increased lipid droplet formation and TAG production (Li et al., 2014a). Consistent with those previous findings, we observed a significant increase in cellular TAG upon overexpression of nSREBP1 in GMEC. The increase in cellular TAG was consistent with the increase in expression of several genes coding for proteins involved in milk fat synthesis. Interestingly, our data strongly indicate that SREBP1 controls expression of genes in all the metabolic steps for the TAG formation in GMEC.

The overexpression of nSREBP1 likely increased the generation of acetyl-CoA through greater expression of *ACLY* and *ACSS2* (Yoshii et al., 2009; Chypre et al., 2012). By providing acetyl-CoA from citrate coming from the tricarboxylic acid cycle, ATP citrate lyase connects glucose/glutamine metabolism to de novo fatty acid synthesis (Hatzivassiliou et al., 2005); however, its role in mammary gland of ruminants does not seem very important, as also demonstrated in goats (Hardwick, 1966).

The positive effect of SREBP1 on de novo fatty acid synthesis is also supported in our study by the increase in expression of *ACACA* and *FASN*, 2 genes coding for rate-limiting enzymes of de novo fatty acid synthesis (Mashima et al., 2009), in agreement with previous studies in mammary tissue of human (Barber et al., 2003), mice (Rudolph et al., 2010), bovine (Oppi-Williams et al., 2013), and goat (Li et al., 2015). The role of SREBP1 in de novo fatty acid synthesis is further supported in our study by the observed increase in expression of *IDH1*. This gene codes for isocitrate dehydrogenase 1, a protein generating NADPH that is used by enzymes involved in de novo fatty acid synthesis, such as *FASN*, and TAG synthesis. Even though the expression of genes coding for proteins involved in de novo fatty acid synthesis was upregulated by overexpression of SREBP1, we cannot conclude that the amount of short- and medium-chain fatty acid was indeed substantially increased, considering the lack of detection of short- and medium-chain fatty acids in our experiment (Table 2). The lack of detection of short- and medium-chain fatty acids was likely caused by the extremely small amount of acetate in the medium used. However, we detected a significant increase in C16:0, indicating that some increase in the de novo synthesis of fatty acids happened in cells with overexpression of SREBP1. It is likely that addition of acetate to the medium would have increased further the production of TAG and production of short- and medium-chain fatty acids.

The large increase in expression of *ACSL1*, the main enzyme involved in the activation of long-chain fatty acids (LCFA) in bovine mammary, of *FABP3*, the main LCFA transporter in bovine mammary tissue, and of *SCD1*, the main desaturation enzyme for LCFA, especially C16:0 and C18:0 (Bionaz and Loor, 2008b), indicated a large increase in the formation of LCFA in Ad-nSREBP1-transfected cells. This was supported by the observed increase in proportions of C18:1 and C16:0 (Table 2). The increase in expression of *ELOVL6* due to overexpression of nSREBP1 further supports a role of SREBP1 in the production of LCFA in goat milk. The *ELOVL6* gene codes for an enzyme involved in the elongation of LCFA starting from C16:0 and pro-

ducing from C18:0 to C26:0 (Jakobsson et al., 2006). No studies have investigated the role of LCFA elongases in mammary tissue of ruminants and their importance remains unclear.

The increased proportions of C16:0 and C18:1 and decreased proportion of C18:0 in TAG in GMEC overexpressing nSREBP1 is in agreement with the above demonstrated regulatory role of SREBP-1 in de novo fatty acid synthesis, including desaturation by *SCD1*, which was likely the cause of the decreased proportion of C18:0 and increased proportion of *cis*-9 C18:1 observed. These data suggest that SREBP1 plays a role in the regulation of the synthesis of MUFA, a role that was previously attributed to peroxisome proliferator-activated receptor gamma (**PPAR** γ ; Shi et al., 2013b).

The large increase in expression of *INSIG1* and *LPIN1* and the decrease in expression of *SCAP* are interesting findings in our study. The *INSIG1* protein can be considered an inhibitor of SREBP1 activity by retaining the inactive SREBP1 in the ER (Bionaz and Loor, 2008b). Lipin 1 also appears to inhibit the nuclear activity of SREBP1, as demonstrated in the mouse (Peterson et al., 2011); in contrast, *SCAP* is considered a positive activator of SREBP1 (Matsuda et al., 2001). Overall, it appears that the overexpression of SREBP1 induces a negative feedback regulation; thus, SREBP1 controls its own activity.

Potential SREBP1 Target Genes in Goat

Our study also provides some insights into genes that are targets of SREBP1, albeit indicative rather than definitive, because the increase in expression of genes due to overexpression of nSREBP1 could be caused by a direct effect of SREBP1 but could also be due to secondary effects (i.e., SREBP1 induces expression of other transcription factors that in turn increase the expression of their target genes).

Based on the large increase due to nSREBP1 overexpression, *FABP3* and *ACSS2* are likely direct targets of SREBP1. The *ACSS2* gene is known to be a direct target of SREBP1 in humans (Luong et al., 2000) and our data confirm this. Our findings are further supported by the observation that expression of *FABP3* and *ACSS2* increases substantially during lactation in bovine mammary tissue (Bionaz and Loor, 2008a) and decreases dramatically by SREBP1 knockdown and *trans*-10, *cis*-12 CLA supplementation in bovine (Kadegowda et al., 2009; Ma and Corl, 2012).

In the current study, the mRNA level of *ACSL1* was significantly upregulated by nSREBP1 overexpression in GMEC, in accordance with previous studies (Maxwell et al., 2003; Li et al., 2014a). However, knockdown of SREBP1 by small interfering (si)RNA in Mac-T cells

and *trans*-10,*cis*-12 CLA treatment in bovine kidney cells had no effect on *ACSL1* expression (Bionaz et al., 2012; Ma and Corl, 2012). Thus, *ACSL1* may not be a direct target of SREBP1, at least in ruminants.

The *SCD1* and *ELOVL6* genes were upregulated by overexpression of nSREBP1 in our study suggesting that those are SREBP1 target genes. The *SCD1* gene is downregulated by *trans*-10,*cis*-12 CLA treatment in bovine cells (Peterson et al., 2004; Kadegowda et al., 2009) and its promoter region contains an SRE in bovine (Peterson et al., 2004). Further support comes from a study in which *SREBF1* expression was significantly induced by insulin (Wang et al., 2006).

The *LPIN1* and *DGAT1* genes were the only ones coding for enzymes responsible for TAG synthesis in mammary tissue (Bionaz and Loor, 2008b). The expression of *LPIN1* and *DGAT1* genes was upregulated by overexpression of nSREBP1. Previous research has demonstrated that *LPIN1* is directly regulated by SREBP1c in hepatoblastoma cells (Ishimoto et al., 2009) and its mRNA expression is downregulated by *trans*-10,*cis*-12 CLA in bovine cells (Kadegowda et al., 2009). These previous studies and the upregulation of *LPIN1* in our study provide support for *LPIN1* being an SREBP1 target gene in goat, at least in vitro; however, *LPIN1* is also a known PPAR γ target gene (Kadegowda et al., 2009). The expression of *DGAT1* is not affected by the inhibition of SREBP1 by *trans*-10,*cis*-12 CLA in bovine cells (Kadegowda et al., 2009), implying that *DGAT1* may not be a SREBP1 target but the increase in expression observed in our study may be due to SREBP1 activation or increase in expression of a secondary transcription factor. One such factor could be PPAR γ , because its expression was increased by nSREBP1 overexpression in our study and because activation of this nuclear receptor by a specific synthetic agonist increased the expression of *DGAT1* in bovine mammary cells (Kadegowda et al., 2009).

In addition to SREBP1, other transcription factors including INSIG1, SCAP, LXR α , and PPAR γ can be involved in the transcriptional regulation of milk fat synthesis. Both INSIG1 and SCAP play important roles in the proteolytic process of SREBP; SCAP works as activator, whereas INSIG1 works as an inhibitor of SREBP activity (Yang et al., 2002; Radhakrishnan et al., 2007). The expression of *INSIG1* was strongly upregulated by nSREBP1 overexpression, in support of this gene being a target of SREBP1, as previously shown in mouse (Horton et al., 2003), whereas *SCAP* is not an SREBP1 target (Figure 2).

Liver X receptor α (LXR α) and PPAR γ are ligand-dependent nuclear receptors with a potential role in controlling milk fat synthesis in bovine (Bionaz and Loor, 2008b; Kadegowda et al., 2009; McFadden and

Corl, 2010). Both nuclear receptors in bovine regulate the expression of *SREBF1* (Kadegowda et al., 2009; Lengi and Corl, 2010). In the present study, we observed a dramatic increase of *NR1H3* and *PPAR γ* expression by nSREBP1 overexpression, indicating that these nuclear receptors are targets of SREBP1 in goats and supporting cross-talk between transcription factors in the regulation of milk fat synthesis in goat, as previously suggested for bovine (Bionaz and Loor, 2008b). The upregulation of *PPAR γ* in the present study might be a confounding factor in determining SREBP1 target genes because many of the genes measured in the present experiment are putative or demonstrated PPAR γ target genes in bovine and goat (Bionaz and Loor, 2008b; Bionaz et al., 2013; Zhao et al., 2014).

CONCLUSIONS

We observed a large increase in *SREBF1* from pregnancy to lactation in goat mammary tissue. Overexpression of nSREBP1 increased the expression of genes related to milk fat synthesis, cellular TAG content, and the amounts of C16:0 and C18:1. A model summarizing the findings from the present experiment is reported in Figure 4 (See color figure in Supplemental Figure S5; <http://dx.doi.org/10.3168/jds.2015-9736>). Our results provide strong support for SREBP1 being a key regulator for the expression of genes coding for proteins involved in milk fat synthesis, particularly de novo fatty acid and triacylglycerol synthesis, in goat mammary epithelial cells. Furthermore, the positive effect on expression of other transcriptional factors provides support for interactive networks of transcriptional factors involved in the control of milk fat synthesis in goat.

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