Insulin-induced gene 1 and 2 isoforms synergistically regulate triacylglycerol accumulation, lipid droplet formation, and lipogenic gene expression in goat mammary epithelial cells

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ABSTRACT

The insulin-induced genes *INSIG1* and *INSIG2* (INSIG) are known to regulate adipogenesis in non-ruminants. Although data in bovine mammary tissue underscore a role for *INSIG1* during lactation, regulatory mechanisms of INSIG action in ruminant mammary lipid metabolism are not well known. In the present study, *INSIG1* and *INSIG2* were overexpressed or silenced through adenoviral transfection to evaluate their role in lipid metabolism in goat mammary epithelial cells (GMEC). The INSIG were overexpressed using an adenovirus system with recombinant green fluorescent protein as the control. Downregulation of INSIG was performed via small interfering RNA targeting INSIG with a scrambled small interfering RNA as a negative control. The GMEC were treated with these constructs for 48 h before analyses. Responses to overexpressing *INSIG1* or *INSIG2* included downregulation of *SREBF1*, *ACACA*, *FASN*, *SCD1*, *GPAM*, *DGAT2*, *ATGL*, and *HSL* coupled with a decrease in content of triacylglycerol (TAG), total cholesterol (TC), and lipid droplet accumulation. The marked decrease in content of TAG and TC in response to overexpression of *INSIG2*, along with a modest decrease in content of TAG when *INSIG1* was overexpressed, suggested that TAG synthesis is mainly regulated by *INSIG2*, whereas TC synthesis is equally regulated by *INSIG2* and *INSIG1*. The lack of difference in mRNA expression of genes related to lipid metabolism, content of TAG, and accumulation of lipids in response to interference alone of *INSIG1* or *INSIG2* indicated that INSIG proteins play a biological role in the maintenance of lipid homeostasis. However, in response to simultaneous interference of *INSIG1* and *INSIG2*, the marked increase in content

of TAG and TC and accumulation of lipids along with significant upregulation of *SREBF1*, *ACACA*, *SCD1*, *AGPAT6*, and *DGAT2* suggested that *INSIG1* and *INSIG2* synergistically regulate milk fat synthesis in GMEC. These results highlight an essential role of INSIG in regulating lipid synthesis in dairy goat mammary cells and underscore the complexity of mammary lipid synthesis in ruminants.

Key words: insulin-induced gene, lipid metabolism, RNA interference, overexpression

INTRODUCTION

In nonruminants, the insulin-induced genes *INSIG1* and *INSIG2* (**INSIG**), located in the outer endoplasmic reticulum membrane, play an essential role in regulation of lipid metabolism (Yabe et al., 2003; Bionaz et al., 2012). Previous studies (Dong and Tang, 2010; Smith et al., 2010) in humans on the expression and distribution of *INSIG1* and *INSIG2* revealed that *INSIG1* has the highest mRNA expression in liver, accounting for more than 75% of the total INSIG. In contrast, *INSIG2* is widely expressed in most tissues, and its expression accounts for half of *INSIG1* (Krapivner et al., 2007). In dairy cows, *INSIG1* mRNA in bovine mammary tissue at peak lactation is about 14 times higher than in the dry period (Bionaz and Llor, 2008). Differentiation of murine preadipocytes is associated with a 12-fold upregulation of *INSIG2* mRNA (Krapivner et al., 2008). Regulation of *INSIG1* and *INSIG2* differs, underscoring potential differences in terms of their role in lipid metabolism. For instance, *INSIG1* plays a central role in cholesterol homeostasis in rodent liver (Gong et al., 2006), whereas *INSIG2* controls differentiation of precursor adipocytes (Lee and DeBose-Boyd, 2010). Therefore, available evidence underscores the potential importance of INSIG genes in aspects related to lipid metabolism.

Most of the previous published studies on the relationship between INSIG and lipid metabolism in

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humans and mice have focused on the liver and fat cells (Li et al., 2003; Cheng et al., 2015; Wang et al., 2016; Lee et al., 2017; Linden et al., 2018). Whether these genes play a similar role in goat mammary tissue remains unclear. The hypothesis was that *INSIG1* and *INSIG2* encode proteins with critical roles in the regulation of lipid metabolism in goat mammary epithelial cells (GMEC). Therefore, the objective was to evaluate the function of INSIG in lipid metabolism of GMEC. We measured expression levels of INSIG in dairy goat mammary tissue at different stages of lactation and used adenovirus-mediated overexpression and RNA interference approaches to uncover their role in triacylglycerol (TAG) and cholesterol metabolism.

MATERIALS AND METHODS

Ethics Statement

The Animal Care and Use Committee (permit number 15516) of Northwest A&F University (Yangling, Shaanxi, China) approved all procedures involved in the use of live goats.

Goat Mammary Tissue Collection

Twelve multiparous and mastitis-free Xinong Saanen dairy goats from the experimental farm at Northwest A&F University were selected for the study. Mammary gland tissues from goats ($n = 3$ biological replicates from each lactation stage) in early lactation (15 d postpartum), peak lactation (60 d postpartum), mid lactation (120 d postpartum), and the nonlactating period (60 d before parturition) were harvested via biopsy. Percutaneous biopsies were performed from the right udder according to methods described previously (Farr et al., 1996). Briefly, after making a skin incision, blunt dissection of the mammary capsule was performed to ensure that the tissue obtained was mammary parenchyma during the biopsy. All mammary samples were obtained under sterile conditions and immediately frozen in liquid nitrogen until RNA extraction.

Adenovirus Generation and Overexpression of *INSIG1* and *INSIG2*

Generation and proliferation of the adenoviruses were performed as previously described (Luo et al., 2007; Shi et al., 2017). Briefly, the cDNA sequences of dairy goat *INSIG1* (GenBank no. NM_001286088.1) and *INSIG2* (GenBank no. NM_001285738.1) were subcloned into the pAdTrack-CMV shuttle plasmid vector between the *SaI* and *Bgl*II (New England BioLabs Inc., Ipswich, MA) restriction sites to generate pAdTrack-CMV-INSIG1

and pAdTrack-CMV-INSIG2 vectors. Subsequently, these 2 vectors were inserted into an adenoviral vector (pAdEasy-1) to generate adenoviral plasmids in BJ5183 bacterial cells. The adenoviral plasmids linearized by *PacI* (New England BioLabs Inc.) were transfected into 293A cells to generate the adenovirus pAd-INSIG1 and pAd-INSIG2. The recombinant adenovirus with green fluorescent protein (Ad-GFP) was used as a negative control.

RNA Interference

To knock down expression of *INSIG1* and *INSIG2*, small interfering RNA (siRNA) were designed using online software (<http://sirna.wi.mit.edu/home.php>), and synthesized by Invitrogen Corp. (Carlsbad, CA). Two siRNA [small interfering RNA target *INSIG1* (siINSIG1)-100, siINSIG1-547; small interfering RNA target *INSIG2* (siINSIG2)-175, siINSIG2-244] for each gene were designed to obtain an ideal transfection efficiency (Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-15492>). A scrambled siRNA (siNC) was designed and used as a negative control. The siRNA sequences are listed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-15492>).

For INSIG mRNA interference, cells cultured in 6-well plates were transfected with 50 nM siRNA using transfection reagent (Lipofectamine RNAiMAX, Thermo Fisher Scientific Inc., Waltham, MA) in lactogenic medium without antibiotic. The transfection was performed according to the manufacturer's instructions. Transfected GMEC were collected after 24 h of initial culture and then harvested at 48 h (24 h later) for RNA extraction and subsequent assays.

Cell Culture and Treatment

The GMEC were isolated from Xinong Saanen dairy goats at peak lactation as previously described (Wang et al., 2010; Shi et al., 2014). Details of cell culture were also described in our previous studies (Lin et al., 2013; Shi et al., 2013). Briefly, cells were incubated at 37°C in 5% CO₂. Culture medium comprised Dulbecco's modified Eagle medium/F12 (Hyclone, Beijing, China) containing insulin (5 mg/L; Sigma-Aldrich, St. Louis, MO), hydrocortisone (5 mg/L; Sigma-Aldrich), penicillin/streptomycin (10,000 units/L; Harbin Pharmaceutical Group, Harbin, 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 transfected 293A cells were cultured in the basal DMEM medium (Gibco) contain-

ing 10% fetal bovine serum. The GMEC at about 80% confluence were transfected with adenovirus supernatant [recombinant adenovirus expressing *INSIG1* (**Ad-INSIG1**), recombinant adenovirus expressing *INSIG2* (**Ad-INSIG2**), and Ad-GFP]. The transfected GMEC were collected after 48 h of culture for lipid extraction, total RNA extraction, and TAG synthesis.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from goat mammary tissue via the Trizol method (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. 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. The RNA was treated with DNase (Tiangen Biotech Co. Ltd.) to remove genomic DNA contamination. The RNA quality was examined by agarose gel electrophoresis analysis of 28S and 18S rRNA. Synthesis of cDNA was conducted using the PrimeScript RT kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's directions. Quantitative real-time PCR was performed according to the manufacturer's instructions using SYBR Green (SYBR Premix Ex TaqII, Perfect Real Time, Takara Bio Inc., Dalian, China) on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The PCR amplification was carried out at 95°C for 4 min followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The presence of a single PCR product was confirmed by the dissociation protocol using incremental temperatures to 95°C for 15 s and 65°C for 5 s.

Genes evaluated include those related to fatty acid (FA) de novo synthesis and desaturation [fatty acid synthase (*FASN*), acetyl-CoA carboxylase α (*ACACA*), and stearoyl-CoA desaturase (*SCD1*)], transcription factors [cleavage activating protein (*SCAP*) and sterol regulatory element-binding transcription factor 1 (*SREBF1*)], TAG synthesis [glycerol-3-phosphate acyltransferase (*GPAM*), 1-acylglycerol-3-phosphate O-acyltransferase 6 (*AGPAT6*), and diacylglycerol O-acyltransferase 2 (*DGAT2*)] and TAG hydrolysis [adipose triglyceride lipase (*ATGL*) and hormone sensitive lipase (*HSL*)]. The gene expression values were calculated using the relative quantification ($2^{-\Delta\Delta C_t}$) method (Oppi-Williams et al., 2013); ubiquitously expressed transcript (*UXT*), mitochondrial ribosomal protein L39 (*MRPL39*), and ribosomal protein S9 (*RSP9*) were used as endogenous references for normalization of targeted RNA profiles, and 3 technical replicates were analyzed per sample (Bionaz and Looor, 2007; Kadegowda et al., 2009; Bonnet et al., 2013). The primer sequences of the above

genes are included in Supplemental Table S2 (<https://doi.org/10.3168/jds.2018-15492>).

Cellular TAG and Total Cholesterol Assay

Concentration of TAG was measured with a commercial kit (Applygen Technologies Inc., Beijing, China) according to the manufacturer's instructions. Briefly, adenovirus-infected or siRNA-transfected cells were washed 3 times with PBS and harvested in lysis buffer (50 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1% Triton X-100; Beijing Solarbio Science & Technology Co., Ltd, Beijing, China), and cell lysates then extracted. The TAG and total cholesterol (TC) contents were measured by enzymatic colorimetry using a commercial kit (GPO Trinder reaction, Applygen Technologies Inc.). The BCA protein kit (Pierce, Thermo Fisher Scientific Inc.) was used to determine the total protein concentration. The contents of TAG and TC were determined by normalization to the total protein of each sample, and data were reported as milligrams per gram of protein.

Oil Red O Staining

The GMEC cultured in 6-well plates (Nunc, Roskilde, Denmark) were incubated with adenovirus supernatant for 48 h and then washed with PBS and fixed in 10% (vol/vol) paraformaldehyde for 1 h at 4°C. After rinsing again with PBS, cells were stained with 5% Oil Red O in isopropanol for 30 min at room temperature and then rinsed repeatedly to remove impurities. A system incorporated in a microscope (Leica, Wetzlar, Germany) was applied to capture representative photomicrographs at 200 $\times$ magnification, and pictures were taken according to a published procedure (Mehlem et al., 2013).

Statistical Analysis

Data for all measurements are from triplicate incubations, and all the experimental data are reported as means $\pm$ standard error of the mean. An ANOVA (SAS Institute Inc., Cary, NC) was used for statistical comparisons and for determining the differences of mRNA expression of genes and total TAG or TC among treatments. The statistical model was

$$x_{ij} = \mu + \alpha_i + \varepsilon_{ij},$$

where x_{ij} is the experimental variable, μ is the overall mean response, α_i is the effect of the i th level A_i of factor A, and ε_{ij} is the random residual. For instance, if x_{ij} refers to relative mRNA expression of *INSIG1* or *INSIG2*, α_i pertains to the effect of 4 different lacta-

tion stages; if x_{ij} refers to mRNA expression of genes, α_i pertains to the treatment effect of control and overexpression or interference of *INSIG1/INSIG2*; and if x_{ij} refers to cellular TAG and TC content, α_i pertains to the treatment effect of control and overexpression or interference of *INSIG1/INSIG2*. The statistical significance of differences was evaluated with either Student's 2-tailed *t*-test or the 1-way ANOVA when appropriate. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Transcript Abundance of *INSIG* Isoforms in Mammary Tissue of Dairy Goats at Different Stages of Lactation

As shown in Figure 1, the expression level of *INSIG1* was higher than that of *INSIG2*. A significant upregulation of *INSIG1* mRNA was detected in early, peak, and mid lactation compared with the dry period ($P < 0.05$). No differences were detected among lactation periods (Figure 1A). Relative to mid lactation, the expression level of *INSIG2* in early and peak lactation was dramatically increased ($P < 0.05$), whereas no significant change was observed relative to the dry period (Figure

1B). During the whole lactation, *INSIG1* and *INSIG2* mRNA reached the highest expression at mid and peak lactation, respectively (Figure 1A and B).

Overexpression of *INSIG* Suppresses mRNA Expression of Key Genes Related to Lipogenesis

As shown in Figures 2 and 3, mRNA expression of *INSIG1* in the cultures with Ad-*INSIG1* was almost 500-fold ($P < 0.01$) greater than that of Ad-GFP (Figure 2A), and expression of *INSIG2* was greater ($P < 0.01$) in cultures with Ad-*INSIG2* (Figure 3A). Compared with the Ad-GFP control, no significant effect on the expression of *SCAP* and *SREBF1* in cells infected with Ad-*INSIG1* was observed (Figure 2B). Overexpression of *INSIG2* had no effect on *SCAP* but had a tendency to reduce the expression of *SREBF1* and markedly suppressed mRNA abundance of *INSIG1* ($P < 0.05$; Figure 3B). Compared with the Ad-GFP control, the overexpression of *INSIG1* markedly downregulated the expression of *ACACA*, *FASN*, and *SCD1* ($P < 0.05$; Figure 2C). A tendency for *FASN* downregulation was observed when *INSIG2* was overexpressed, whereas expression of *ACACA* and *SCD1* was markedly downregulated ($P < 0.05$) compared with the negative control (Figure 3C).

Figure 1. Relative mRNA expression of (A) *INSIG1* and (B) *INSIG2* in goat mammary tissues at different lactation stages. Samples were collected in 4 lactation stages (early lactation, peak lactation, mid lactation, and dry period). Quantitative real-time PCR was applied and data were analyzed using the relative quantification ($2^{-\Delta\Delta C_t}$) method. Values are presented as means $\pm$ SE. The different letters (a–c) represent significant differences ($P < 0.05$) in gene expression.

Figure 2. Overexpression of *INSIG1* suppressed mRNA expression of genes related to lipid synthesis in goat mammary epithelial cells (GMEC). The GMEC were transfected with a recombinant adenovirus expressing *INSIG1* (Ad-INSIG1) or the control (adenovirus containing green fluorescent protein; Ad-GFP) for 48 h and then collected for mRNA extraction. (A) *INSIG1* mRNA expression level. The mRNA expression levels of genes related to (B) transcription factors, (C) de novo fatty acid synthesis and desaturation, (D) triacylglycerol hydrolysis, and (E) triacylglycerol synthesis in GMEC. Values are presented as means $\pm$ SE from 3 individual cultures. * $P < 0.05$ and ** $P < 0.01$ compared with control (Ad-GFP).

Compared with the Ad-GFP control, overexpression of *INSIG1* did not affect the mRNA abundance of *AGPAT6* but decreased the expression of *GPAM* and *DGAT2* ($P < 0.05$; Figure 2E). Overexpressing *INSIG2* markedly downregulated mRNA abundance of *DGAT2* and *AGPAT6* ($P < 0.05$), whereas no change in *GPAM* mRNA was observed (Figure 3E). Relative to the Ad-GFP control, the cells overexpressing *INSIG1* had downregulation of *ATGL* ($P < 0.05$), but *HSL* was not affected (Figure 2D). Treatment with Ad-INSIG2 markedly downregulated *HSL* ($P < 0.05$), and *ATGL* mRNA tended to be downregulated relative to the Ad-GFP control (Figure 3D). Collectively, these data suggested that overexpression of *INSIG1* and *INSIG2* suppressed lipid accumulation and simultaneously inhibited lipolysis in GMEC.

Overexpression of *INSIG* Affects TAG Accumulation, TC Content, and Lipid Droplet Formation

Relative to the Ad-GFP control, lipid droplet accumulation was markedly decreased upon *INSIG2* overexpression ($P < 0.05$) and Ad-INSIG1 treatment

(Figure 4A). Compared with the Ad-GFP control, the overexpression of *INSIG2* markedly reduced total cellular TAG ($P < 0.01$; Figure 4D). However, no significant change in TAG was detected in cells overexpressing *INSIG1* compared with the control Ad-GFP (Figure 4B). Both Ad-INSIG1 and Ad-INSIG2 treatments markedly decreased TC content compared with the Ad-GFP infected cells ($P < 0.05$; Figure 4C and E).

Simultaneous Interference of *INSIG* Enhances mRNA Expression of Key Genes Related to Lipogenesis

Compared with the siNC control, siRNA for siINSIG1-547 and siINSIG2-175 effectively knocked down endogenous expression of *INSIG1* and *INSIG2* by an average of 80 and 85%, respectively ($P < 0.05$; Supplemental Figure S1, <https://doi.org/10.3168/jds.2018-15492>). Thus, these 2 siRNA were used in the subsequent interference experiment. The quantitative PCR results (Figure 5A) indicated that incubation with siINSIG1 decreased abundance of *INSIG1* and had no effect on the mRNA abundance of *INSIG2*. Compared with the siNC control, incubation with siINSIG2 de-

creased *INSIG2* and simultaneously reduced abundance of *INSIG1* by approximately 28% (Figure 5A). In addition, compared with the siNC control, the GMEC treated with siINSIG1 and siINSIG2 together decreased the mRNA expression of *INSIG1* and *INSIG2* by more than 80%. Therefore, as depicted in Figure 5A, the selected siRNA effectively knocked down endogenous *INSIG1* and *INSIG2* gene expression.

Relative to the siNC control, incubation with siINSIG1 or siINSIG2 alone had no significant effect on expression of genes associated with lipid metabolism, including *SCAP*, *SREBF1*, *ACACA*, *FASN*, *SCD1*, *GPAM*, *AGPAT6*, and *DGAT2* (Figure 5B–D). However, compared with the siNC control, simultaneous incubation with siINSIG1 and siINSIG2 led to a marked upregulation of mRNA abundance of *SREBF1*, *ACACA*, *SCD1*, *AGPAT6*, and *DGAT2* ($P < 0.05$). Furthermore, there was a tendency for upregulation of *FASN* mRNA, whereas expression of *GPAM* was down-regulated (Figure 5B–D).

Interference of *INSIG* Increases TAG Accumulation, TC Content, and Lipid Droplet Formation

Compared with the siNC control, incubation with siINSIG1 or siINSIG2 alone induced lipid droplet accumulation, whereas siINSIG1 and siINSIG2 together led to a marked increase in lipid droplet accumulation (Figure 6A). Compared with the siNC control (Figure 6B), knockdown of *INSIG1* or *INSIG2* alone had no significant effect on cellular TAG content, whereas interference of *INSIG1* and *INSIG2* together markedly increased ($P < 0.05$) TAG accumulation. Relative to the siNC control, the cellular TC content was enhanced regardless of interference of *INSIG* alone or in combination (Figure 6C).

DISCUSSION

The change in the profile of *INSIG1* and *INSIG2* in mammary tissue across stages of lactation agrees with

Figure 3. Overexpression of *INSIG2* repressed mRNA expression of genes related to lipid synthesis in goat mammary epithelial cells (GMEC). The GMEC were transfected with a recombinant adenovirus expressing *INSIG2* (Ad-INSIG2) or the control (adenovirus containing green fluorescent protein; Ad-GFP) for 48 h and then collected for mRNA extraction. (A) *INSIG2* mRNA expression level. The mRNA expression levels of genes related to (B) transcription factors, (C) de novo fatty acid synthesis and desaturation, (D) triacylglycerol hydrolysis, and (E) triacylglycerol synthesis in GMEC. Values are presented as means $\pm$ SE from 3 individual cultures. * $P < 0.05$ and ** $P < 0.01$ compared with control (Ad-GFP).

the dynamics in goat milk fat composition and abundance of genes related to lipid metabolism (Romero et al., 2013). In addition, the much higher expression

of *INSIG1* mRNA at mid lactation and the higher expression of *INSIG2* at peak lactation underscore the potentially different biological functions for these 2

Figure 4. Overexpression of *INSIG1* and *INSIG2* decreased the accumulation of lipid droplet, concentration of cellular triacylglycerol (TAG), and total cholesterol (TC). The goat mammary epithelial cells (GMEC) were transfected with a recombinant adenovirus expressing *INSIG1* (Ad-INSIG1), adenovirus expressing *INSIG2* (Ad-INSIG2), and the control (adenovirus containing green fluorescent protein; Ad-GFP) and collected at 48 h for lipid droplet measurement and TAG and TC analysis. (A) Lipid droplet formation after Oil Red O treatment (scale bar: 100 μ m). (B) Total TAG content in GMEC incubated with Ad-INSIG1. (C) Total TC content in GMEC incubated with Ad-INSIG1. (D) Total TAG content in GMEC incubated with Ad-INSIG2. (E) Total TC content in GMEC incubated with Ad-INSIG2. Values are presented as means $\pm$ SE from 3 individual cultures. The contents of TAG and TC are presented as milligrams per gram of protein. * P < 0.05 and ** P < 0.01 compared with control (Ad-GFP).

isoforms during the lactation cycle. These results led us to hypothesize that INSIG plays a crucial role in mammary lipogenesis and TAG synthesis.

Work in rodent models has revealed that INSIG binds *SCAP* and inhibits the activation of SREBF protein but has little effect on their mRNA level (Yang et al., 2002). The lack of change in mRNA expression of *SCAP* and *SREBF1* when *INSIG1* was overexpressed agrees with in vivo studies in mice (Engelking et al., 2004; Takaishi et al., 2004). Despite the lack of effect on *SCAP* mRNA when *INSIG2* was overexpressed, the tendency for downregulation of *SREBF1* mRNA along with the downregulation of *INSIG1* mRNA suggested a role of *INSIG2* in transcriptional regulation in GMEC.

The downregulation of *ACACA*, *FASN*, and *SCD1* (target genes of *SREBF1*) in response to overexpres-

sion of *INSIG1* agrees with previous studies in mice (Engelking et al., 2004; Takaishi et al., 2004). Thus, the present data suggest that activation of *INSIG1* transcription inhibits the activation of SREBF protein along with the transcription of its target genes. An important function of *INSIG1* appears to be the inhibition of de novo synthesis and desaturation of FA. The downregulation of *ACACA* and *SCD1* in response to *INSIG2* overexpression also agrees with previous data in mice (Takaishi et al., 2004). Together, the data suggest that both *INSIG1* and *INSIG2* could affect de novo synthesis and desaturation of FA.

Milk fat TAG synthesis is partly controlled by 3 key enzymes: GPAM is involved in the first step reaction (rate-limiting step), AGPAT catalyzes the esterification of the *sn*-2 position of glycerol-3-phosphate, and DGAT

Figure 5. Simultaneous interference of *INSIG1* and *INSIG2* promotes mRNA expression of genes related to lipid synthesis in goat mammary epithelial cells (GMEC). The GMEC were incubated with small interfering RNA target *INSIG1* (siINSIG1) or small interfering RNA target *INSIG2* (siINSIG2) or a negative control (scrambled small interfering RNA; siNC) for 48 h and then collected with mRNA extraction. Interference of *INSIG1* is presented as 50% siNC + 50% siINSIG1; interference of *INSIG2* is presented as 50% siNC + 50% siINSIG2; simultaneous interference of *INSIG1* and *INSIG2* is presented as 50% siINSIG1 + 50% siINSIG2. (A) The interference efficiency of *INSIG1* and *INSIG2*. The mRNA expression levels of genes related to (B) transcription factors, (C) de novo fatty acid synthesis and desaturation, and (D) triacylglycerol synthesis in GMEC. Values are presented as means $\pm$ SE from 3 individual cultures. * $P < 0.05$ compared with control (siNC).

adds an FA acyl-CoA to diacylglycerol to form TAG (Abeni et al., 2005; Bichi et al., 2013). The concentration of TAG in cells changes dynamically, with lipolysis also playing a role in terms of providing FA for mem-

brane lipid synthesis and as sources of energy via oxidation. Both ATGL and HSL are key enzymes involved in the hydrolysis of TAG. The downregulation of *GPAM* and *DGAT2* in response to overexpression of *INSIG1*

Figure 6. Simultaneous interference of *INSIG1* and *INSIG2* increased the accumulation of lipid droplet, concentration of cellular triacylglycerol (TAG), and total cholesterol (TC). The goat mammary epithelial cells (GMEC) were incubated with small interfering RNA target *INSIG1* (siINSIG1) or small interfering RNA target *INSIG2* (siINSIG2) or a negative control (scrambled small interfering RNA; siNC) and collected at 48 h for lipid droplet measurement and TAG and TC analysis. (A) Lipid droplet formation after Oil Red O treatment (scale bar: 100 μ m). (B) The effect of interfering *INSIG1* and *INSIG2* on total TAG content in GMEC. (C) The effect of interfering *INSIG1* and *INSIG2* on total TC content in GMEC. Values are presented as means $\pm$ SE from 3 individual cultures. The contents of TAG and TC are presented as milligrams per gram of protein. * $P < 0.05$ and ** $P < 0.01$ compared with control (siNC).

indicated an inhibitory effect on TAG synthesis, also supported by the decrease in TAG content. This idea is further supported by previous data with mice in which overexpression of *INSIG1* suppressed the transcription of genes related to TAG synthesis in liver (Engelking et al., 2004; Takaishi et al., 2004). Similar to lipogenesis, the data suggest that activation of *INSIG1* also could decrease TAG synthesis.

The downregulation of *AGPAT6* and *DGAT2* coupled with a lack of change in *GPAM* in response to overexpression of *INSIG2* reinforces the existence of complex mechanisms and regulatory pathways coordinating lipid metabolism (Loor et al., 2013). Although INSIG proteins decrease the activation of SREBF and the degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) in nonruminants (Theesfeld and Hampton, 2013; Jiang et al., 2018; Johnson and DeBose-Boyd, 2018), other factors may regulate mRNA expression of *GPAM*. The decrease in expression of *HSL* and *ATGL* in response to INSIG upregulation may represent a self-protecting mechanism within mammary epithelial cells to control the intracellular concentration of lipid at a normal level.

The marked decrease in content of TAG and TC in response to overexpression of *INSIG2* along with a modest decrease in content of TAG when *INSIG1* was overexpressed suggested that TAG synthesis is mainly regulated by *INSIG2*, whereas TC synthesis is equally regulated by *INSIG2* and *INSIG1*. This suggestion is supported by a previous study in which overexpression of *INSIG2* compared with overexpression of *INSIG1* significantly downregulated TAG content in murine plasma and liver (Takaishi et al., 2004). Furthermore, overexpression of *INSIG2* resulted in a strong inhibition of adipogenic differentiation and lipid biosynthesis in adipose-derived stem cells (Chen et al., 2017).

The lack of change in mRNA expression of *SCAP* and *SREBF1* and TAG content in response to interference of *INSIG1* agrees with results in murine plasma and liver (McFarlane et al., 2014), which indicated a compensatory effect of *INSIG2* on lipid synthesis. The lack of difference in mRNA expression of genes related to lipid metabolism along with the lack of changes in content of TAG and accumulation of lipids when *INSIG1* or *INSIG2* were downregulated alone suggested that INSIG1 and INSIG2 proteins help maintain lipid homeostasis. Thus, we speculate that interference of one INSIG subtype may trigger a compensatory effect by the other subtype, resulting in no net changes in lipid synthesis. The significant increase in lipid accumulation in response to simultaneous interference of *INSIG1* and *INSIG2* agrees with previous studies in mice (McFarlane et al., 2014). The significant increases in total TAG and TC contents are consistent with a reported

4- to 6-fold increase in liver of hamsters (Engelking et al., 2005). The marked increase in content of TAG and TC and accumulation of lipids along with significant upregulation of *SREBF1*, *ACACA*, *SCD1*, *AGPAT6*, and *DGAT2* indicated that *INSIG1* and *INSIG2* synergistically regulate lipid metabolism in GMEC.

CONCLUSIONS

The genes *INSIG1* and *INSIG2* induced a widespread suppression of TAG accumulation, lipolysis, and lipid droplet formation partly through controlling gene transcription in GMEC. Thus, *INSIG1* and *INSIG2* synergistically regulate various aspects of lipid metabolism in GMEC to control homeostasis. Understanding the regulatory role of INSIG in lipid metabolism in the lactating ruminant may contribute to the efforts in manipulating milk fat synthesis and secretion.

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REFERENCES

- Abeni, F., L. Degano, F. Calza, R. Giangiacomo, and G. Pirlo. 2005. Milk quality and automatic milking: Fat globule size, natural creaming, and lipolysis. *J. Dairy Sci.* 88:3519–3529.
- Bichi, E., G. Hervás, P. G. Toral, J. J. Loor, and P. Frutos. 2013. Milk fat depression induced by dietary marine algae in dairy ewes: Persistency of milk fatty acid composition and animal performance responses. *J. Dairy Sci.* 96:524–532. <https://doi.org/10.3168/jds.2012-5875>.
- Bionaz, M., and J. J. Loor. 2007. Identification of reference genes for quantitative real-time PCR in the bovine mammary gland during the lactation cycle. *Physiol. Genomics* 29:312–319.
- Bionaz, M., and J. J. Loor. 2008. Gene networks driving bovine milk fat synthesis during the lactation cycle. *BMC Genomics* 9:366.
- Bionaz, M., B. J. Thering, and J. J. Loor. 2012. Fine metabolic regulation in ruminants via nutrient-gene interactions: saturated long-chain fatty acids increase expression of genes involved in lipid metabolism and immune response partly through PPAR- α activation. *Br. J. Nutr.* 107:179–191. <https://doi.org/10.1017/S0007114511002777>.
- Bonnet, M., L. Bernard, S. Bes, and C. Leroux. 2013. Selection of reference genes for quantitative real-time PCR normalisation in adipose tissue, muscle, liver and mammary gland from ruminants. *Animal* 7:1344–1353.
- Chen, C. C., L. W. Hsu, K. T. Huang, S. Goto, C. L. Chen, and T. Nakano. 2017. Overexpression of Insig-2 inhibits atypical antipsychotic-induced adipogenic differentiation and lipid biosynthesis in adipose-derived stem cells. *Sci. Rep.* 7:10901.
- Cheng, C., P. Ru, F. Geng, J. Liu, J. Y. Yoo, X. Wu, X. Cheng, V. Euthine, P. Hu, J. Y. Guo, E. Lefai, B. Kaur, A. Nohturfft, J. Ma, A.

- Chakravarti, and D. Guo. 2015. Glucose-mediated N-glycosylation of SCAP is essential for SREBP-1 activation and tumor growth. *Cancer Cell* 28:569–581.
- Dong, X. Y., and S. Q. Tang. 2010. Insulin-induced gene: A new regulator in lipid metabolism. *Peptides* 31:2145–2150.
- Engelking, L. J., H. Kuriyama, R. E. Hammer, J. D. Horton, M. S. Brown, J. L. Goldstein, and G. Liang. 2004. Overexpression of Insig-1 in the livers of transgenic mice inhibits SREBP processing and reduces insulin-stimulated lipogenesis. *J. Clin. Invest.* 113:1168–1175.
- Engelking, L. J., G. Liang, R. E. Hammer, K. Takaishi, H. Kuriyama, B. M. Evers, W. P. Li, J. D. Horton, J. L. Goldstein, and M. S. Brown. 2005. Schoenheimer effect explained—Feedback regulation of cholesterol synthesis in mice mediated by Insig proteins. *J. Clin. Invest.* 115:2489–2498.
- Farr, V. C., K. Stelwagen, L. R. Cate, A. J. Molenaar, T. B. McFadden, and S. R. Davis. 1996. An improved method for the routine biopsy of bovine mammary tissue. *J. Dairy Sci.* 79:543–549.
- Gong, Y., J. N. Lee, M. S. Brown, J. L. Goldstein, and J. Ye. 2006. Juxtamembranous aspartic acid in Insig-1 and Insig-2 is required for cholesterol homeostasis. *Proc. Natl. Acad. Sci. USA* 103:6154–6159.
- Jiang, L. Y., W. Jiang, N. Tian, Y. N. Xiong, J. Liu, J. Wei, K. Y. Wu, J. Luo, X. J. Shi, and B. L. Song. 2018. Ring finger protein 145 (RNF145) is a ubiquitin ligase for sterol-induced degradation of HMG-CoA reductase. *J. Biol. Chem.* 293:4047–4055.
- Johnson, B. M., and R. A. DeBose-Boyd. 2018. Underlying mechanisms for sterol-induced ubiquitination and ER-associated degradation of HMG CoA reductase. *Semin. Cell Dev. Biol.* 81:121–128.
- Kadegowda, A. K., M. Bionaz, L. S. Piperova, R. A. Erdman, and J. J. Loo. 2009. Peroxisome proliferator-activated receptor- γ activation and long-chain fatty acids alter lipogenic gene networks in bovine mammary epithelial cells to various extents. *J. Dairy Sci.* 92:4276–4289.
- Krapivner, S., E. Chernogubova, M. Ericsson, C. Ahlbeck-Glader, A. Hamsten, and F. M. van't Hooft. 2007. Human evidence for the involvement of insulin-induced gene 1 in the regulation of plasma glucose concentration. *Diabetologia* 50:94–102.
- Krapivner, S., S. Popov, E. Chernogubova, M. L. Hellenius, R. M. Fisher, A. Hamsten, and F. M. van't Hooft. 2008. Insulin-induced gene 2 involvement in human adipocyte metabolism and body weight regulation. *J. Clin. Endocrinol. Metab.* 93:1995–2001.
- Lee, J. H., H. S. Kang, H. Y. Park, Y. A. Moon, Y. N. Kang, B. C. Oh, D. K. Song, J. H. Bae, and S. S. Im. 2017. PPAR α -dependent Insig2a overexpression inhibits SREBP-1c processing during fasting. *Sci. Rep.* 7:9958.
- Lee, P. C., and R. A. DeBose-Boyd. 2010. Intramembrane glycine mediates multimerization of Insig-2, a requirement for sterol regulation in Chinese hamster ovary cells. *J. Lipid Res.* 51:192–201.
- Li, J., K. Takaishi, W. Cook, S. K. McCorkle, and R. H. Unger. 2003. Insig-1 “brakes” lipogenesis in adipocytes and inhibits differentiation of preadipocytes. *Proc. Natl. Acad. Sci. USA* 100:9476–9481.
- Lin, X. Z., J. Luo, L. P. Zhang, W. Wang, H. B. Shi, and J. J. Zhu. 2013. MiR-27a suppresses triglyceride accumulation and affects gene mRNA expression associated with fat metabolism in dairy goat mammary gland epithelial cells. *Gene* 521:15–23.
- Linden, A. G., S. Li, H. Y. Choi, F. Fang, M. Fukasawa, K. Uyeda, R. E. Hammer, J. D. Horton, L. J. Engelking, and G. Liang. 2018. Interplay between ChREBP and SREBP-1c coordinates postprandial glycolysis and lipogenesis in livers of mice. *J. Lipid Res.* 59:475–487.
- Loor, J. J., M. Bionaz, and J. K. Drackley. 2013. Systems physiology in dairy cattle: Nutritional genomics and beyond. *Annu. Rev. Anim. Biosci.* 1:365–392. <https://doi.org/10.1146/annurev-animal-031412-103728>.
- Luo, J., Z. L. Deng, X. Luo, N. Tang, W. X. Song, J. Chen, K. A. Sharff, H. H. Luu, R. C. Haydon, K. W. Kinzler, B. Vogelstein, and T. C. He. 2007. A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. *Nat. Protoc.* 2:1236–1247.
- McFarlane, M. R., G. Liang, and L. J. Engelking. 2014. Insig proteins mediate feedback inhibition of cholesterol synthesis in the intestine. *J. Biol. Chem.* 289:2148–2156.
- Mehlem, A., C. E. Hagberg, L. Muhl, U. Eriksson, and A. Falkevall. 2013. Imaging of neutral lipids by oil red O for analyzing the metabolic status in health and disease. *Nat. Protoc.* 8:1149–1154.
- Oppi-Williams, C., J. K. Suagee, and B. A. Corl. 2013. Regulation of lipid synthesis by liver X receptor α and sterol regulatory element-binding protein 1 in mammary epithelial cells. *J. Dairy Sci.* 96:112–121.
- Romero, T., M. C. Beltran, M. Rodriguez, A. M. De Olives, and M. P. Molina. 2013. Short communication: Goat colostrum quality: Litter size and lactation number effects. *J. Dairy Sci.* 96:7526–7531.
- Shi, H., J. Luo, J. Zhu, J. Li, Y. Sun, X. Lin, L. Zhang, D. Yao, and H. Shi. 2013. PPAR γ regulates genes involved in triacylglycerol synthesis and secretion in mammary gland epithelial cells of dairy goats. *PPAR Res.* 2013:310948.
- Shi, H., H. Shi, J. Luo, W. Wang, A. B. Haile, H. Xu, and J. Li. 2014. Establishment and characterization of a dairy goat mammary epithelial cell line with human telomerase (hT-MECs). *Nihon Chikusan Gakkaiho* 85:735–743.
- Shi, H. B., C. H. Zhang, W. Zhao, J. Luo, and J. J. Loo. 2017. Peroxisome proliferator-activated receptor δ facilitates lipid secretion and catabolism of fatty acids in dairy goat mammary epithelial cells. *J. Dairy Sci.* 100:797–806.
- Smith, E. M., Y. Zhang, T. M. Baye, S. Gawrieh, R. Cole, J. Blangero, M. A. Carless, J. E. Curran, T. D. Dyer, L. J. Abraham, E. K. Moses, A. H. Kissebah, L. J. Martin, and M. Olivier. 2010. INSIG1 influences obesity-related hypertriglyceridemia in humans. *J. Lipid Res.* 51:701–708.
- Takaishi, K., L. Duplomb, M. Y. Wang, J. Li, and R. H. Unger. 2004. Hepatic insig-1 or -2 overexpression reduces lipogenesis in obese Zucker diabetic fatty rats and in fasted/refed normal rats. *Proc. Natl. Acad. Sci. USA* 101:7106–7111.
- Theesfeld, C. L., and R. Y. Hampton. 2013. Insulin-induced gene protein (INSIG)-dependent sterol regulation of Hmg2 endoplasmic reticulum-associated degradation (ERAD) in yeast. *J. Biol. Chem.* 288:8519–8530.
- Wang, H., M. Zhao, N. Sud, P. Christian, J. Shen, Y. Song, A. Pashaj, K. Zhang, T. Carr, and Q. Su. 2016. Glucagon regulates hepatic lipid metabolism via cAMP and Insig-2 signaling: Implication for the pathogenesis of hypertriglyceridemia and hepatic steatosis. *Sci. Rep.* 6:32246.
- Wang, Z., J. Luo, W. Wang, W. Zhao, and X. Lin. 2010. Characterization and culture of isolated primary dairy goat mammary gland epithelial cells. *Sheng Wu Gong Cheng Xue Bao* 26:1123–1127.
- Yabe, D., R. Komuro, G. Liang, J. L. Goldstein, and M. S. Brown. 2003. Liver-specific mRNA for Insig-2 down-regulated by insulin: Implications for fatty acid synthesis. *Proc. Natl. Acad. Sci. USA* 100:3155–3160.
- Yang, T., P. J. Espenshade, M. E. Wright, D. Yabe, Y. Gong, R. Aebersold, J. L. Goldstein, and M. S. Brown. 2002. Crucial step in cholesterol homeostasis: Sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. *Cell* 110:489–500.