Short communication: Altered expression of specificity protein 1 impairs milk fat synthesis in goat mammary epithelial cells

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ABSTRACT

Specificity protein 1 (encoded by *SP1*) is a novel transcription factor important for the regulation of lipid metabolism and the normal function of various hormones in model organisms. Its potential role, if any, on ruminant milk fat is unknown. Despite the lower expression of the lipolysis-related gene *ATGL* (by 44 and 37% respectively), both the adenoviral overexpression and the silencing of *SP1* [via short interfering (si)RNA] markedly reduced cellular triacylglycerol (TAG) content (by 28 and 25%, respectively), at least in part by decreasing the expression of *DGAT1* (–36% in adenovirus treatment) and *DGAT2* (–81 and –87%, respectively) that are involved in TAG synthesis. Consistent with the markedly lower expression of genes related to lipid droplet formation and secretion (*TIP47* by 19 and 32%, and *ADFP* by 25 and 25%, respectively), cellular lipid droplet content was also decreased sharply, by 9 and 8.5%, respectively, after adenoviral overexpression of *SP1* or its silencing via siRNA. Overall, the results underscored a potentially important role of *SP1* in maintaining milk-fat droplet synthesis in goat mammary epithelial cells.

Key words: dairy goat, *SP1*, fat synthesis, lipid metabolism

Short Communication

Goat milk is widely considered to have a high nutritional value by virtue of its high content of short- and medium-chain fatty acids and smaller fat globules. The negative outcomes of dyspepsia and gastrointestinal dysfunction in humans are partly alleviated by

consumption of goat milk (Haenlein, 2004). Although several genes are known to be important for the coordination of lipid metabolism in ruminants (Hosseini and Loo, 2014), a clearer mechanism of how milk fat formation is regulated in small ruminants such as the goat remains to be determined.

Specificity protein 1 (*SP1*), as a member of the Sp/Kruppel superfamily (Solomon et al., 2008), is a zinc finger transcription factor with important roles in regulating expression of cellular and viral genes that contain GC boxes in their promoters (Huang and Xie, 2012). Although *SP1* is believed to function mainly as a basal transcription factor (Hoey et al., 1993; Gill et al., 1994; Liu et al., 2008; Solomon et al., 2008), several experiments have demonstrated that *SP1* is involved in regulating fatty acid synthesis in human (Lu and Archer, 2010), rat (Fukuda et al., 1997; Jeon et al., 2012), and goat (Zhu et al., 2015b). From that standpoint, it is noteworthy that in humans, 34% of SREBP1 targets are occupied by *SP1* and synergistically regulate gene transcription (Reed et al., 2008) including ATP-citrate lyase (*ACLY*) and acetyl-CoA carboxylase (*ACACA*) (Daniel and Kim, 1996; Moon et al., 1999).

Some evidence indicates that *SP1* can function as a co-factor of signal transducer and activator of transcription 3 (*STAT3*; Huang and Xie, 2012), and also could activate the promoter of acyl-coenzyme-A oxidase (*ACOX1*), the rate-limiting enzyme in peroxisomal β -oxidation of fatty acids, in synergy with peroxisome proliferator-activated receptor (*PPAR*) or retinoid-X receptor (*RXR*) (Krey et al., 1995). Despite the important role of *SP1* in regulating lipid metabolism in nonruminant species, its role (if any) on the regulation of milk fat synthesis in ruminants remains unknown.

The hypothesis of this study was that alteration of *SP1* expression in goat mammary epithelial cells would cause changes in the expression of other lipogenic genes and in triacylglycerol (TAG) synthesis. The specific objective was to increase the expression of *SP1* via adenovirus transfection or downregulate its expression via

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short interfering (si)RNA. In both cases, an important objective was to measure the expression of lipogenic genes and the accumulation of lipid droplets.

The goat mammary epithelial cells (GMEC) and 293A cells were cultured following the protocols described in the supplementary material (<http://dx.doi.org/10.3168/jds.2015-10733>). The GMEC were seeded in 10-cm² cell-culture clusters (Nunc, Shanghai, China) for 24 h (approximately 70–80% confluence) before treatment with adenovirus (containing negative sequence as control, Ad-GFP: adenovirus expressing green fluorescent protein) or siRNA (with the negative siRNA as control, Scramble; using Lipofectamine RNAiMAX; Invitrogen, Carlsbad, CA) for 48 h in Dulbecco's modified Eagle medium/nutrient mixture F-12 complete medium (DMEM/F-12; Zhu et al., 2015b). Synthesis of TAG (Applygen, Beijing, China) and formation of lipid droplet (oil red O staining; Sigma, St. Louis, MO) was performed as detailed in the supplementary material (Zhu et al., 2015b). Before adenovirus or siRNA transfection, cells were washed twice with PBS and once with serum-free DMEM/F-12 without antibiotics, and 1.5 µg/mL of prolactin (CAS 9002-62-4, Sigma) was supplemented to the culture medium (Peterson et al., 2004; Kadegowda et al., 2009a; Zhu et al., 2015a). Using the same method, we also cultured GMEC in 4.5-cm² clusters (Nunc) with adenovirus or siRNA for determination of TAG accumulation. The SYBR Premix Ex TaqII (Takara, Dalian, China) was then used to measure mRNA expression by quantitative real-time PCR (RT-qPCR) according to the manufacturer's manual on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), mitochondrial ribosomal protein L39 (*MRPL39*), and ubiquitously expressed transcript (*UXT*) were used as internal control genes for normalization of target genes (Kadegowda et al., 2009b; Lin et al., 2013; Shi et al., 2013b). The primers for RT-qPCR are reported in Supplementary Table S1 (<http://dx.doi.org/10.3168/jds.2015-10733>). The software SPSS 19.0 (SPSS Inc./IBM Corp. Armonk, NY) was used for the statistical analysis, as described in the supplementary material.

As shown previously (Zhu et al., 2015b), compared with the control group (Ad-GFP), the expression of *SP1* increased significantly after Ad-*SP1* treatment. At the same time, overexpression of *SP1* was associated with decreased expression of genes associated with TAG synthesis, including diacylglycerol acyltransferase 1 (*DGAT1*) by 36% and diacylglycerol acyltransferase 2 (*DGAT2*) by 81% compared with the control. In contrast, we detected no obvious change in the expression of 1-acylglycerol-3-phosphate O-acyltransferase 6

(*AGPAT6*) and phosphatidic acid lipin 1 (*LPIN1*). The higher expression of *SP1* was also associated with lower expression of the lipolytic gene adipose triglyceride lipase (*ATGL*) and with a greater expression of hormone-sensitive lipase (*HSL*). In addition, overexpression of *SP1* was associated with downregulation of genes related to lipid-droplet formation and secretion, including tail-interacting protein 47 gene (*TIP47*), adipose differentiation-related protein (*ADFP*), and xanthine oxidoreductase (*XDH*), which decreased by 19, 25, and 81%, respectively (Figure 1A). These changes in gene expression were accompanied by a lower content of cellular TAG (−28%; $P < 0.05$) with the overexpression of *SP1* (Figure 2A). Consistent with this reduction in TAG, the accumulation of lipid droplets (Figure 2B) decreased significantly (−9%; $P < 0.05$) compared with the control group.

After the siRNA treatment targeting the *SP1* sequence, the expression of *SP1* was significantly decreased (Zhu et al., 2015b). Similar to the effect with *SP1* overexpression, the expression of *AGPAT6*, *LPIN1*, *DGAT1*, *DGAT2*, and *ATGL* was decreased ($P < 0.05$) by 46, 74, 34, 87, and 37%, respectively, in response to *SP1* silencing compared with the control group (scramble; $P < 0.05$). In contrast, the expression of *HSL* was decreased ($P < 0.05$) by 54% due to silencing of *SP1*. Similar to the results with Ad-*SP1*, the reduction in *SP1* gene expression was associated with lower ($P < 0.05$) expression of *TIP47* (by 32%) and *ADFP* (by 25%). However, the expression of *XDH* was unaffected by siRNA treatment ($P > 0.05$; Figure 1B). The loss of *SP1* was associated with a significant reduction in the abundance of TAG (−25%; $P < 0.05$; Figure 2C). This decrease was confirmed by the reduction in accumulation of lipid droplets (Figure 2D) within GMEC (−8.5%; $P < 0.05$).

The role of *SP1* as a basal transcriptional factor is unequivocal (Safe and Abdelrahim, 2005; Huang and Xie, 2012). Similarly, there is evidence of an important role for *SP1* in inducing the expression of genes associated with lipid metabolism in response to hormones (Solomon et al., 2008), including insulin (Samson and Wong, 2002), prolactin (Hu et al., 1998), and estrogen (Lu and Archer, 2010). However, a clear mechanism for how *SP1* may affect milk fat synthesis in the mammary gland of ruminants, especially in dairy goats, remains unknown. The present study, as shown in Supplementary Table S2 (<http://dx.doi.org/10.3168/jds.2015-10733>), is novel in that the overexpression and silencing of *SP1* indicated that alterations in the expression of *SP1* impair the accumulation of TAG and subsequently diminishes lipid droplet formation by reducing esterification in goat mammary epithelial cells.

Our previous study revealed that *SP1* may be involved in regulating the expression of lipogenic genes via the control of PPAR γ and LXR α (Zhu et al., 2015b), with a positive correlation in expression with cluster of differentiation 36 (*CD36*), acyl-CoA synthetase long-chain family member 1 (*ACSL1*), and heart type fatty acid bind protein (*FABP3*) (Zhu et al., 2015b). In contrast, expression of *SP1* was inversely related to expression of acetyl-CoA carboxylase (*ACACA*). In the present study, except for *AGPAT6* and *LPIN1*, consistent with the reduction of cellular TAG, both Ad-*SP1* and siRNA treatment decreased the mRNA expression of genes related to TAG synthesis. However, *ATGL* and *HSL* (except for the increase of *HSL* with Ad-*SP1* treatment) decreased with the alteration of expression of *SP1*, a response to counteract the reduction of cellular TAG. Therefore, despite a decrease in lipolysis, the negative effect of SP1 on de novo synthesis could have been the main reason for the final reduction of cellular TAG in GMEC.

The *DGAT1* and *DGAT2* genes encode enzymes that are required for lipid droplet formation during adipogenesis (Harris et al., 2011). These enzymes share simi-

lar activity and account for almost all TAG synthesis in adipocytes and the vast majority of other tissues (Ganji et al., 2004). A positive correlation between the expression of these genes and TAG synthesis has been documented in adipocytes (Smith et al., 2000), small intestine (Buhman et al., 2002), liver (Meegalla et al., 2002), and white adipose tissue (Cases et al., 2001; Banilas et al., 2011). Although we previously reported that inhibition of *FASN* in GMEC decreased expression of *DGAT2* (Zhu et al., 2015a), the exact function of *DGAT* in the mammary gland of ruminants remains unclear. In the present study, consistent with the reduction of cellular TAG, both Ad-*SP1* and siRNA treatment decreased the mRNA expression of *DGAT1* and *DGAT2*. Although the decreased expression of *AGPAT6* and *LPIN1* contributed to the reduction of TAG synthesis upon siRNA treatment, their expression did not change after treatment with Ad-*SP1*. These data seem to indicate that decreased expression of *DGAT1* and *DGAT2* mainly contribute to the suppression of cellular lipid accumulation.

A previous study confirmed that *XDH* is crucial for lipid droplet secretion (Chong et al., 2011). Ablation of

Figure 1. Effect of specificity protein 1 (*SP1*) expression alteration on the mRNA abundance of genes related to milk fat synthesis in mammary epithelial cells. Mammary epithelial cells (GMEC) were incubated with Ad-*SP1* (A) or transfected with short interfering (si)RNA for 48 h (B). Then, total RNA was extracted for gene expression determination by real-time quantitative (q)PCR. Ad-*SP1* = adenovirus containing *SP1* sequence; Ad-GFP = adenovirus for overexpression control containing green fluorescent protein; siRNA = silencing of *SP1* gene; scramble for silencing control; *AGPAT6* = 1-acylglycerol-3-phosphate O-acyltransferase; *LPIN1* = lipin 1; *DGAT* = diacylglycerol acyltransferase; *ATGL* = adipose triglyceride lipase; *HSL* = hormone-sensitive lipase; *TIP47* = tail-interacting protein 47 gene; *ADFP* = adipose differentiation-related protein; *XDH* = xanthine oxidoreductase. Columns are average of 3 repeats; error bars represent SD. * $P < 0.05$.

Figure 2. Effect of specificity protein 1 (*SP1*) expression alteration on cellular triglyceride content and lipid droplets accumulation. Mammary epithelial cells (GMEC) were incubated with Ad-*SP1* (A, B) or transfected with short interfering (si)RNA for 48 h (C, D). Then, triglyceride content (A, C) and lipid droplet accumulation (B, D) were determined. Ad-*SP1* = adenovirus containing *SP1* sequence; Ad-GFP = adenovirus for overexpression control containing green fluorescent protein; siRNA = silencing of *SP1* gene; scramble for silencing control. Columns are average of 6 repeats; error bars represent SD. * $P < 0.05$. Color version available online.

BTN1A1 and *XDH* in mice resulted in impairment of cellular lipid droplet envelopment and secretion (Ogg et al., 2004). In the present study, although both Ad-*SP1* and siRNA treatments decreased lipid droplet accumulation, *XDH* expression remained insensitive to the silencing of *SP1*. Considering the significant decrease in expression of *TIP47* (Shi et al., 2014; Kang et al., 2015) and *ADFP* (Listenberger et al., 2007), both of which are crucial for lipid droplet formation, the decrease in production of lipid droplets could be the main factor responsible for the reduction of milk fat synthesis in response to alterations in expression of *SP1*. However, the exact mechanism for the suppression of cellular lipid droplet synthesis remains unknown.

From a cellular function standpoint, we must acknowledge the general limitation of gene expression data for determining mechanisms. Considering that most genes studied in the present study are terminal enzymes, it is likely that their mRNA expression is positively correlated with protein expression. Clearly, protein expression analyses will be required in the future, once suitable antibodies are obtained.

Our previous study demonstrated that *SP1* may regulate the expression of lipogenic genes via control

of *PPAR* γ and *LXR* α . Activation of *PPAR* γ in GMEC also affected expression of *AGPAT6*, *DGAT1*, *TIP47*, and *ADFP* (Shi et al., 2013a). Activation of *LXR* α is reported to regulate the expression of *AGPAT6* and *DGAT1* in bovine mammary epithelial cells (Oppi-Williams et al., 2013). Although we could not propose an exact mechanism for *SP1*, it is likely that changes in expression of *PPAR* γ and *LXR* α partly explain the regulation of lipogenic genes. Furthermore, due to the basal regulatory role in transcription, *SP1* may also bind directly to the promoters of the lipogenic genes. More controlled experiments, such as promoter analysis, fatty acid detection, and protein expression of the target genes, will need to be performed in the future to better address the mechanisms of *SP1* in controlling fat formation.

In conclusion, the combination of overexpression and silencing suggested that abnormal expression of *SP1* can induce widespread suppression of TAG accumulation and the expression of genes related to TAG synthesis and lipid-droplet formation. A better understanding of the regulatory role of *SP1* in fat formation may contribute to efforts in improving the efficiency and quality of goat milk production.

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