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Effect of *INSIG1* on the milk fat synthesis of buffalo mammary epithelial cells

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Research Article

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Abstract

We hypothesized that insulin-induced gene 1 (*INSIG1*) affects milk fat synthesis in buffalo. For this reason, the protein abundance of INSIG1 in the mammary tissue of buffalo during the peak period of lactation and dry-off period was evaluated. The results showed that the expression of INSIG1 at the peak of lactation was lower than that in the dry-off period. To explore the role of INSIG1 in milk fat synthesis, the buffalo mammary epithelial cells (**BMECs**) were isolated and purified from buffalo mammary tissue, and *INSIG1* gene were overexpressed and knocked down by constructing the recombinant lentivirus vector of *INSIG1* gene and transfecting into BMECs. Results revealed that *INSIG1* overexpression decreased the expression of *INSIG2*, *SREBP*, *PPARG*, *SCD*, *GPAM*, *DGAT2* and *AGPAT6*, which led to reduction of triglycerides (TAG) content in the cell. In contrast, knockdown of *INSIG1* had a positive effect on mRNA expression of the above genes. Overall, the data provide strong support for a key role of INSIG1 in the regulation of milk fat synthesis in BMECs.

The insulin induced gene 1 (INSIG1) plays a regulatory role in fat metabolism and adipocyte differentiation. In 1993, *INSIG1* gene was originally identified and characterized from regenerating liver (Diamond *et al*., 1993). The study showed that INSIG1 could effectively block proteolytic activation of sterol-regulatory element binding protein (**SREBP**) family members (Carobbio *et al*., 2013). INSIG1 is a membrane protein that resides in the endoplasmic reticulum (**ER**) and contains six membrane-spanning helices (Feramisco *et al*., 2004). Recent studies have shown that the expression of *INSIG1* gene is regulated by insulin, free fatty acids, glucose and other nutrients (Dong and Tang, 2010).

As an important transcription factor, SREBP is involved in the regulation of sterol and fatty acid synthesis in mammals, and directly involved in the regulation of the expression of more than 30 genes related to the biosynthesis and uptake of cholesterol, triglycerides, fatty acids and phospholipids (Horton *et al*., 2002, 2003). With the participation of intracellular sterols, INSIG1 regulates the activation of SREBP, thereby feedback-regulating the uptake of exogenous cholesterol in cells (Gong *et al*., 2006; Liou *et al*., 2012). SREBP can only be transported from ER to the Golgi by an escort protein called SREBP cleavage activating protein (**SCAP**), which forms a SCAP/SREBP complex immediately after their synthesis (Tsushima *et al*., 2018). INSIG1 prevents the transport of SREBP/SCAP complex to Golgi by binding to SCAP in the presence of sterols, causing ER retention of the SREBP. In this way, the suppression of SREBP causes a decrease of lipogenic enzyme's gene expression which consequently results in lower rate of fatty acid synthesis in animals. However, in the sterol-depleted cell, SCAP undergoes a conformational change that leads it to dissociate from INSIG1, SCAP escorts SREBP to the Golgi where the SREBP is processed by two membrane-bound proteases that release the active fragment of SREBP, which can enter the nucleus and regulate the transcription of a variety of target genes. Thus, SREBP can play a transcriptional regulatory function and promote the expression of related genes in the process of sterol and fatty acid biosynthesis (Espenshade and Hughes, 2007; Owen *et al*., 2012). Finally, free INSIG1 protein is degraded rapidly by ubiquitination. When cells are incubated with sterols, INSIG1 is stabilized by binding to SCAP, blocking the degradation of INSIG1 (Lee *et al*., 2006).

The synthesis of milk fat has been widely studied, and the main aspects of milk fat metabolism, including de novo synthesis and fatty acids uptake from blood, are defined and quantified (Osorio *et al*., 2016). Milk fat is primarily composed of triglycerides (**TAG**) and a small proportion of other lipids. It is an important energy source in milk. In ruminants, about half of the milk fat is synthesized de novo (Harvatine *et al*., 2009). A network summarizing 45 genes related to milk fat synthesis was previously constructed, and it is suggested that *SREBP* is at the core of the milk fat synthesis network in the bovine mammary gland (Bionaz and Loor, 2008). They also suggested that INSIG1 was an essential protein regulating SREBP. Therefore, INSIG1 also plays an important role in the synthesis of milk fat in bovine. However, the regulatory

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function and the molecular mechanism of *INSIG1* gene in milk fat synthesis of buffalo are not known. The objective of the present study was to examine the role of INSIG1 in the regulation of milk fat synthesis for buffalo. To assess the role of INSIG1, both lentivirus-mediated RNA interference and overexpression were performed in buffalo mammary epithelial cells (**BMECs**).

Materials and methods

All procedures for sample collection were performed in accordance with the Guide for Animal Care and Use of Experimental Animals and approved by the Yunnan Provincial Experimental Animal Management Committee under Contract 2007-0069.

Buffalo mammary gland tissue collection

Six adult female Binglangjiang buffalo with the same management conditions, of which three were in non-lactating stage (the dry-off period) and another three were in the peak of lactation, were euthanized and mammary gland tissue samples were collected. Total protein was isolated from each tissue sample using RIPA buffer (Beyotime, Shanghai, China) supplemented with PMSF (Beyotime, Shanghai, China) according to the manufacturer's instructions. Protein concentrations were determined using the BCA protein kit (Beyotime, Shanghai, China) according to the manufacturer's instructions.

Vector construction

The lentiviral construct that expressed buffalo *INSIG1* was pLVX-IRES-ZsGreen1 bicistronic shuttle vector (CLONTECH Laboratories, Inc.). Buffalo *INSIG1* cDNA (Accession no. JX853922) was prepared by reverse transcription polymerase chain reaction (RT-PCR). The primers used for cDNA cloning are presented in online Supplementary Table S1. The open reading frame sequence of buffalo *INSIG1* gene was inserted into the vector through *Xho*I and *BamH*I sites to obtain the recombinant pLVX-INSIG1-IRES-ZsGreen1 (Lv-INSIG1).

To knock down buffalo *INSIG1*, three short hairpin RNA sequences (shRNA103, shRNA547 and shRNA681) were designed by the BLOCK-iT™ RNAi Designer (https://rnaidesigner.thermofisher.com/rnaiexpress/) using the buffalo *INSIG1* gene sequence (Accession no. JX853922). We selected the shRNA sequences with the highest interference efficiency and those sequences were synthesized with the *EcoR*I and *Age*I restriction sites (Sangon Biotech Co. Ltd., Shanghai, China; Supplementary Table S2). The shRNA sequences were constructed into the pLKO.1-TRC cloning vector through *EcoR*I and *Age*I sites to generate pLKO.1-shRNA recombinants (Lv-sh*INSIG1*). Meanwhile, the *INSIG1* gene open reading frame sequence was amplified and inserted into the vector through *Xho*I and *Hand*III sites to get pEGFP-INSIG1-N1 (CLONTECH Laboratories, Inc.) construct. Detailed primer information is described in online Supplementary Table S1.

Cell culture

The details of 293T cell culture were previously described (Zu *et al.*, 2007). Briefly, the cells were cultured in basal Dulbecco's modified Eagle medium/F12 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 2% penicillin/streptomycin (Gibco). Routine cultures were incubated at 37 °C in 5% CO_2 and medium was changed every 24 h.

The BMECs were isolated from peak lactation buffalo and identified by cytokeratin 18 (Sigma, USA) as described previously (Hu *et al.*, 2009; Shi *et al.*, 2014). The purified BMECs were cultured in basal DMEM/F12 supplemented with 10% FBS and 2% penicillin/streptomycin. To promote the synthesis of milk protein and fat, the induction medium was basal medium containing 5 μg/ml insulin (Sigma), 5 μg/ml Holotransferrin (Sigma), 5 μg/ml hydrocortisone (Sigma), 1 μg/ml epidermal growth factor (Sigma). The BMECs were incubated at 37 °C in 5% CO_2 and medium was changed every 24 h.

Preliminary screening of shRNA sequences

In order to obtain the most effective shRNA for targeting *INSIG1* gene, we screened the shRNA sequences in 293T cells. The 239T cells at about 80% confluence in 6 plates were transiently transfected with 4 μg of three pLKO.1-shRNAs with pEGFP-INSIG1-N1, at a ratio of 3 : 1 using TransIntro™ EL Transfection Reagent (TransGen Biotech, Beijing, China) in accordance with the manufacturer's protocol. The pLKO.1-TRC control (Lv-shNC) and pEGFP-INSIG1-N1 also were transfected together as a negative control in the same amount as above. The green fluorescent protein was observed using fluorescent microscope (Nikon, Japan). Meanwhile, the expression of *INSIG1* gene was further verified by real-time quantitative PCR (RT-qPCR).

Lentivirus generation and transduction

The 239T cells in log-phase growth were transiently transfected with 8 μg of pLKO.1-shRNA with psPAX2 (packaging plasmid) and pMD2.G (envelope plasmid), at a ratio of 5 : 2 : 3 in accordance with the manufacturer's protocol. The pLKO.1-TRC control was used as a negative control in the same amount as above. Analogously, the 293T cells were transfected by sequencing-verified pLVX-INSIG1-IRES-ZsGreen1 for packaging lentiviral particles. Lentivirus containing green fluorescent protein pLVX-IRES-ZsGreen1 (Lv-GFP) was used as a negative control. These lentivirus particles were directly collected and concentrated from cell culture media 52 h after transduction by multi-steps of ultracentrifugation (50 000 × ***g***, 2 h at 4 °C). The BMECs at about 80% confluence were transfected with lentivirus supernatant (Lv-INSIG1, Lv-GFP, Lv-shINSIG1 and Lv-shNC) as described previously (Zhou *et al.*, 2017). The transfected BMECs were harvested after 52 h of culture for total RNA extraction and TAG analysis.

RT-qPCR and western blot

The process of total RNA extraction and cDNA synthesis was described previously (Song *et al.*, 2016). The RT-qPCR was performed according to the manufacturer's instruction using SYBR Green (TaKaRa, Dalian, China). Specific primers were designed for several genes related to the synthesis of milk fat: *INSIG2*, *SREBP*, *PPARG*, *SCD*, *GPAM*, *DGAT2* and *AGPAT6* (Supplementary Table S3). The insulin induced gene 2 (**INSIG2**) and INSIG1 belong to the same family. The peroxisome proliferator-activated receptor-γ (**PPARG**) plays a crucial role in fatty acid (**FA**) metabolism through regulation of stearoyl-CoA desaturase (**SCD**) gene expression, which is the rate-limiting enzyme for the biosynthesis of monounsaturated FA (Shi *et al.*,

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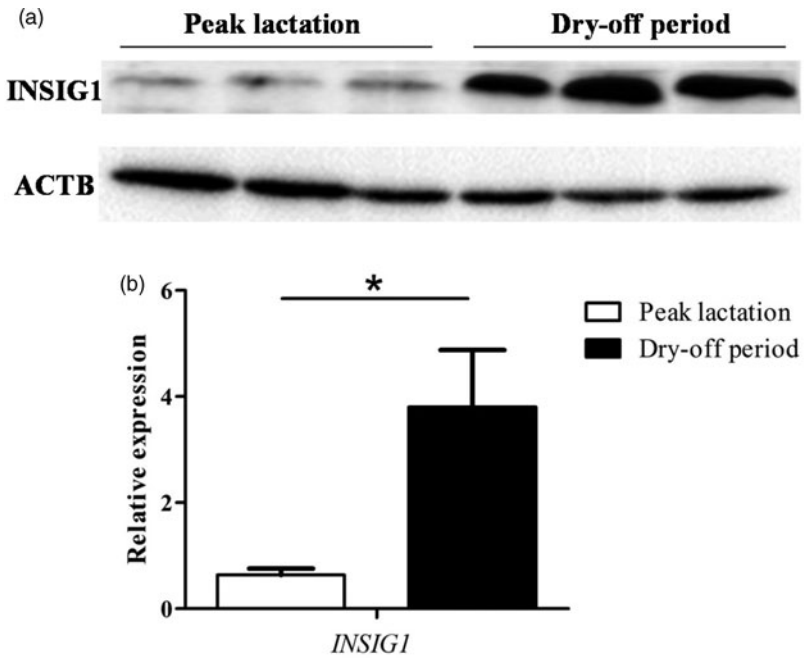

Fig. 1. INSIG1 expression in buffalo mammary gland tissue. a. representative immunblots; b. corresponding mean gray values of INSIG1 protein. Protein abundance was calibrated with β-actin. Values are presented as means ± SEM; *$P < 0.05$.

2013). The glycerol-3-phosphate acyltransferase (**GPAM**), diacylglycerol acyltransferase 2 (**DGAT2**) and 1-acylglycerol-3-phosphate O-acyltransferase 6 (**AGPAT6**) are related to TAG synthesis (Shi *et al.*, 2017). The beta-actin (***ACTB***) was selected as an internal control gene. All the RT-qPCR reactions were performed in a Mastercycler ep realplex (Eppendorf, Germany) Real-time qPCR detector and data were normalized to *ACTB*.

For Western blot, the proteins extracted from mammary tissue were separated by SDS-PAGE, transferred to PVDF membrane (Millipore, Billerica, MA, USA) and probed with the primary antibodies polyclonal rabbit anti-INSIG1 (1 : 500; Absin, Shanghai, China; Lot: abs125485a) and monoclonal mouse anti-ACTB (1 : 5000; TransGen Biotech; Lot: #K20912). Polyclonal goat anti-rabbit IgG (1 : 5000; Millipore; Lot: #2491145) and polyclonal goat anti-mouse IgG (1 : 5000; Millipore; Lot: #2517746) were used as secondary antibody. All antibodies were used according to the manufacturer's recommendations. The results were visualized by the chemiluminescent ECL Western blot detection system (Pierce, USA).

Quantification of cellular TAG

Cellular TAG was extracted according to the manufacturer's instructions of GPO-Trinder triglyceride assay kit (Applygen Technologies, Beijing, China). The concentrations were calculated by the equation obtained from a linear regression of the standard curve, which was established according to the manufacturer's procedure. And TAG content was normalized by cellular protein concentration assessed using a BCA protein assay kit.

Data analysis

All the treatments were executed in triplicates and all the experimental data are presented as means ± standard error of the mean (SEM). Data of RT-qPCR were analyzed using the $2^{-\Delta\Delta Ct}$ method. Significance of mRNA expression, cellular TAG content after *INSIG1* overexpression or knock down and Western blot results was determined *via* Student's *t*-test. Significance was declared at $P < 0.05$.

Results

Insig1 expression level at 2 stages of lactation

The expression of INSIG1 protein in mammary tissue during peak lactation and dry-off period was detected. Relative to the dry-off period, the expression level of INSIG1 at peak lactation decreased significantly ($P < 0.05$) (Fig. 1 and Fig. S1).

Isolation of mammary gland epithelial cells

Buffalo mammary epithelial cells were isolated by tissue culture method. The process of cell separation is shown in online Supplementary Fig. S2. After the tissue was cultured for 7 d, primary fibroblasts were first migrated out from mammary tissue (Fig. S2A). Primary epithelial cells were emigrated from mammary tissue after 10 d in culture. Fibroblast and epithelial cells were observed in the dish, most being mixed together (Fig. S2B). The BMECs were separated after about 3–5 passages and purified. The resulting BMECs were present as a cellular island (Fig. S2C) and cobblestone form (Fig. S2D). Furthermore, we identified the purified cells by cytokeratin 18. The results showed that purified cells exhibited intense positive staining of the cytoplasmic meshwork of cytokeratin fibrils when incubated with the antibody, indicating that the purified cells were buffalo mammary gland epithelial cells (Fig. S3).

Overexpression of INSIG1 inhibits expression of lipogenic genes and synthesis of intracellular TAG

Compared with controls (Lv-GFP), the mRNA abundance of *INSIG1* gene increased markedly in BMECs incubated with Lv-*INSIG1* (Fig. 2a). Overexpression of *INSIG1* dramatically downregulated the mRNA levels of genes related to synthesis of milk fat (Fig. 2b). As a member of the same family of *INSIG1*,

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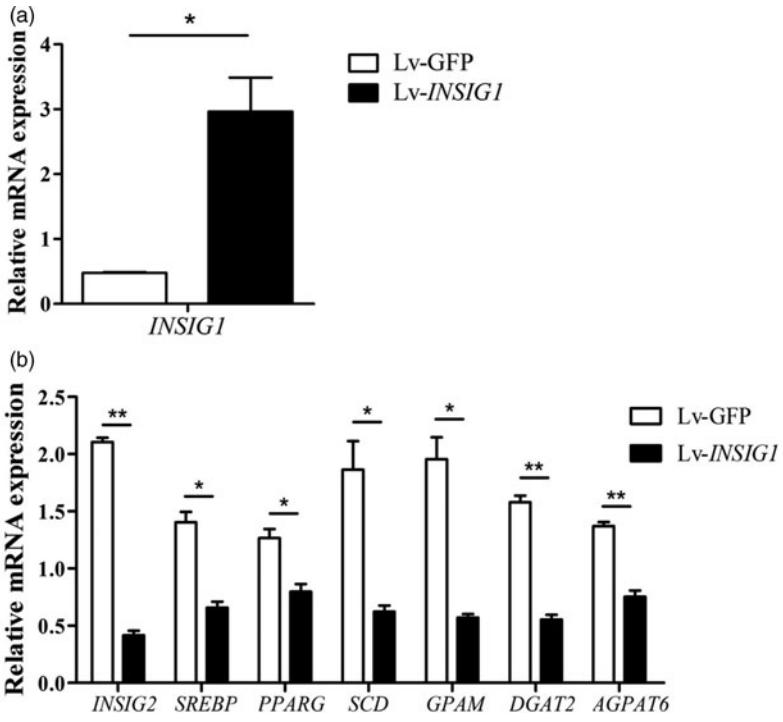

Fig. 2. Overexpression of *INSIG1* altered genes related to milk fat synthesis. The buffalo mammary epithelial cells were transfected with a lentivirus expressing *INSIG1* (Lv-*INSIG1*) or the control (Lv-GFP) for 52 h and then extracted RNA. a. mRNA expression of *INSIG1*; b. mRNA expression of genes related to milk fat synthesis (*INSIG2*, *SREBP*, *PPARG*, *SCD*, *GPAM*, *DGAT2* and *AGPAT6*). Values are means ± SEM from 3 individual cultures; **P* < 0.05, ***P* < 0.01.

the expression of *INSIG2* decreased by 80.2% ($P < 0.01$). The relative expressions of *SREBP* ($P < 0.05$), *PPARG* ($P < 0.05$) and *SCD* ($P < 0.05$) were also significantly decreased. Meanwhile, overexpression of *INSIG1* decreased the transcription of genes measured involved in TAG synthesis, with 70.9% decrease for *GPAM*, 65.0% decrease for *DGAT2*, and 45.1% decrease for *AGPAT6*. Accordingly, the TAG content of BMECs transfected with Lv-*INSIG1* was decreased significantly ($P < 0.05$) compared with the negative control (Fig. 3).

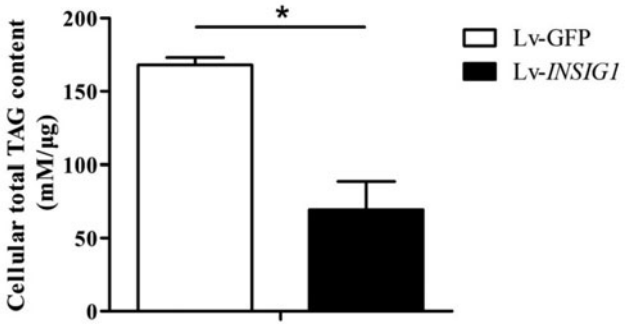

Fig. 3. *INSIG1* inhibited the synthesis of cellular TAG. The buffalo mammary epithelial cells were transfected with a lentivirus expressing *INSIG1* (Lv-*INSIG1*) or the control (Lv-GFP) and collected at 52 h for cellular TAG analysis. Values are means ± SEM from 3 individual cultures. **P* < 0.05 compared with control.

Preliminary shRNA screening

To obtain the highest knockdown of *INSIG1* transcript, three lentiviruses with sh*INSIG1* sequence were co-transfected in 293T cells with pEGFP-INSIG1-N1, respectively. The GFP protein on the pEGFP-INSIG1-N1 vector was used to assess the interference efficiency *via* intensity of green fluorescence inside the cells (Fig. 4), and in addition the mRNA of *INSIG1* gene was verified *via* RT-qPCR in the cells (Fig. 5). The shRNAs that were specific for *INSIG1* would inhibit green fluorescence expression, in other words, they would inhibit the expression of *INSIG1* gene. Thus, the interference efficiency of shRNA was assessed by the disappearance of green fluorescence inside the cells and mRNA expression level of *INSIG1*. As shown in Fig. 5, shRNA103 and shRNA681 were more efficient than shRNA547 to down-regulate *INSIG1* expression. Compared with shRNA103 (98.8%), the shRNA681 (99.7%) was more efficient in knocking down buffalo *INSIG1* by the RT-qPCR detecting (Fig. 5).

Knockdown of INSIG1 *promotes expression of lipogenic genes*

Based on the above results, the Lv-shRNA681 (Lv-sh*INSIG1*) was selected to block the expression of *INSIG1* in BMECs, and expression analysis of genes related to milk fat synthesis was evaluated (Fig. 6). Results demonstrated that the mRNA expression of *INSIG1* decreased markedly ($P < 0.05$) in the Lv-sh*INSIG1* group compared with the Lv-shNC group. The expression of *INSIG2* was increased significantly ($P < 0.01$) by the loss of *INSIG1*. *INSIG1* gene silencing upregulated the mRNA expression levels of *SREBP*, *PPARG* and *SCD* (all $P < 0.01$) in the cells transfected with Lv-sh*INSIG1* compared with the cells transfected with Lv-shNC. Meanwhile, *INSIG1* gene knockdown increased the transcription of all the genes measured involved in TAG synthesis, with a 6.5-fold increase for *GPAM*, 2.3-fold increase for *DGAT2*, and 2.3-fold increase for *AGPAT6*.

Discussion

In humans, the *INSIG1* mRNA is highly expressed in the liver (Krapivner *et al.*, 2008). The expression of *INSIG1* mRNA in mouse is very high in mammary glands during lactation (Han *et al.*, 2010), and the mRNA expression of buffalo *INSIG1* is high in the mammary gland and liver and moderate in the

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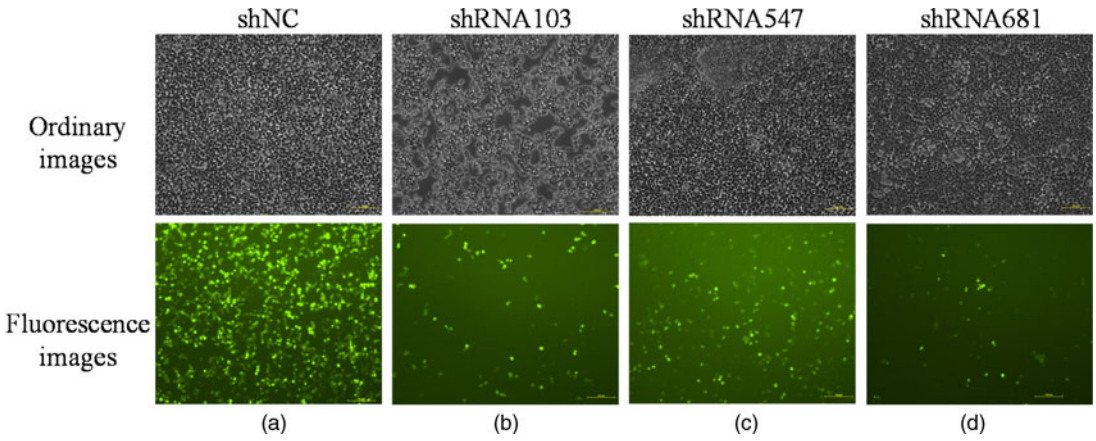

Fig. 4. Efficiency screening of the three designed shRNA *via* images analysis. The pLKO.1-TRC control vector was co-transfected with pEGFP-INSIG1-N1 vector as a control (a). The three tested shRNA (shRNA103, shRNA547 and shRNA681) as pLKO.1-shRNA vector were co-transfected with pEGFP-INSIG1-N1 (b–d). Shown are representative images of the *INSIG1* expression (in green) after a 48 h co-transfection. The images show that the shRNA103 and shRNA681 had a higher effect on *INSIG1* vector expression (B and D).

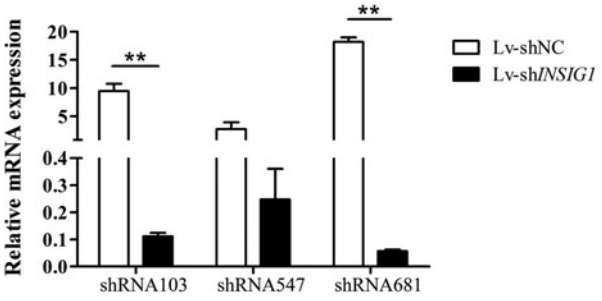

Fig. 5. Efficiency screening of the three designed shRNA *via* RT-qPCR. The efficiency of Lv-sh*INSIG1* (co-transfected with pEGFP-INSIG1-N1 for 52 h) in decreasing *INSIG1* expression in 293T cells was assessed by RT-qPCR. The data revealed that Lv-shRNA681 had the highest knockdown of *INSIG1* transcript; thus, it was used in the subsequent experiments.

abomasum, brain and muscle (Wu *et al*., 2014). To investigate the role of *INSIG1*, we first demonstrated that INSIG1 protein was expressed in buffalo mammary tissue, with expression level of dry-off period being higher than that of peak lactation. The enrichment of INSIG1 at the dry-off period may be a reflection of its physiological role in inhibiting the synthesis of milk fat. However, previous studies have shown that the change of *INSIG1* mRNA expression during bovine lactation was >12-fold in magnitude compared with non-lactating period (Bionaz and Loor, 2008). This is probably due to the post-transcriptional regulation. Furthermore, buffalo milk is richer in milk fat than cow milk, suggesting that there are differences in fat metabolism of the mammary gland between the two species. To understand milk fat regulation in buffalo mammary gland, a representative model of cells is vital. In this study, mammary epithelial cells from a peak lactation buffalo were obtained to meet the demand of further research in buffalo lactation.

Lipogenesis is regulated by sterols and insulin by regulating the expression and activation of SREBP, but this process is more sensitive to cholesterol and less sensitive to insulin (Attie, 2004). In addition, the regulation of lipid homeostasis by insulin is mediated to some extent by the enhanced transcription of *SREBP* gene. The stable expression of *INSIG1* enhances the ability of sterols to inhibit activation of SREBP, which activates transcription of genes involved in fatty acid synthesis (Leichner *et al*., 2009). In INSIGs deleted mutant Chinese hamster ovary cells, sterols could not inhibit the processing of SREBP, but sterol regulation of SREBP processing was fully restored when they were transfected with expression plasmids encoding INSIG1. These results reveal the absolute demand for INSIG1 in the regulatory system that regulates lipid homeostasis in animal cells.

Previous results from overexpression and knockdown of *INSIG1* in mice indicated that INSIG1 protein could regulate the transcription of SREBP (Engelking *et al*., 2004; McFarlane *et al*., 2014). In this study, lentivirus-mediated overexpression and knockdown were used to successfully overexpress and knock down the *INSIG1* in BMECs, which altered mRNA expression level of *SREBP* in a fashion consistent with data in transgenic mice (Engelking *et al*., 2004; McFarlane *et al*., 2014). In this study, the results that *INSIG1* overexpression decreased the expression of *SREBP* mRNA confirmed the role of INSIG1 in inhibiting SREBP transcription in buffalo mammary cells. And significant increase of *SREBP* mRNA after knockdown of *INSIG1* further underscored the function of INSIG1 in inhibiting SREBP processing.

INSIG2 is a close homolog of INSIG1. Both INSIGs can bind SCAP in the endoplasmatic reticulum, inhibiting the SREBP processing (Krapivner *et al*., 2008). In primary cultures of rat hepatocytes, insulin-stimulated SREBP processing is mediated by selective depletion of *INSIG2* by promoting decay of its homologous mRNA. Therefore, the decrease of INSIG2 protein induced by insulin leads to the enhancement of export of SCAP/SREBP complex from ER to the Golgi (Yellaturu *et al*., 2009). A previous study demonstrated that these two INSIG proteins have complementary roles in the regulation of the SREBP pathway (Engelking *et al*., 2005), *i.e*. decrease of one INSIG protein is compensated for by an increase in the other INSIG protein. Consistent with that previous finding, we observed a significant decrease in the mRNA expression level of *INSIG2* upon overexpression of *INSIG1* in BMECs. Similarly, knockdown of *INSIG1* increased the mRNA expression of buffalo *INSIG2*.

PPARG is an important central regulator of lipid metabolism in mammary cells. SCD involves desaturation in the de novo synthesis of fatty acids (Shi *et al*., 2013). *INSIG1* is a *PPARG* target gene, and its regulation by PPARG can indirectly regulate SREBP protein activity (Kast-Woelbern *et al*., 2004).

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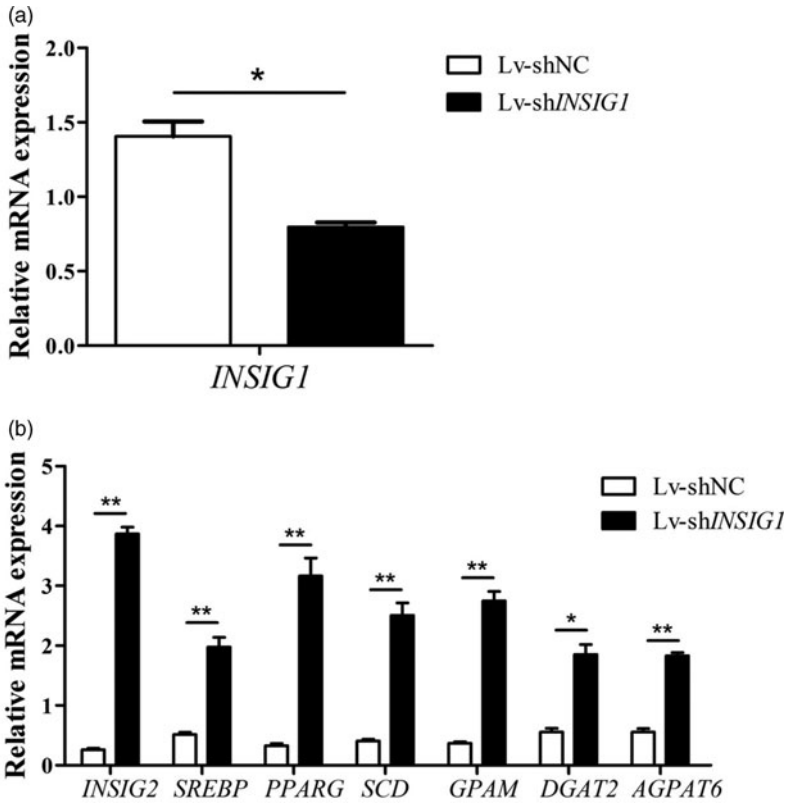

Fig. 6. Effect of *INSIG1* knockdown on genes involved in milk fat synthesis in BMECs. The buffalo mammary epithelial cells were transfected with lentivirus containing small hair RNA knocking down *INSIG1* (Lv-sh*INSIG1*) or the lentivirus containing negative control (Lv-GFP) for 52 h and then extracted RNA. a. mRNA expression of *INSIG1*; b. mRNA expression of genes related to milk fat synthesis (*INSIG2*, *SREBP*, *PPARG*, *SCD*, *GPAM*, *DGAT2* and *AGPAT6*). Values are means ± SEM from three individual cultures; $^{*}P < 0.05$, $^{**}P < 0.01$.

Furthermore, overexpression of *SREBP* significantly increased the expression of *PPARG* and *SCD*, indicating that they are target genes of *SREBP* (Xu *et al.*, 2016). In this study, the change of *INSIG1* significantly altered the mRNA expression level of *PPARG* and *SCD*. It is speculated that *INSIG1* regulates *PPARG* and *SCD* by controlling *SREBP*. Thus, our study supported crosstalk between transcription factors in the regulation of milk fat synthesis (Bionaz and Loor, 2008). However, *INSIG1* interference had no effect on the expression of *SCD* in goat mammary epithelial cells (Li *et al.*, 2019), which may reflect a different molecular mechanism of milk fat metabolism in buffalo. Together, the data suggest that *INSIG1* gene could affect desaturation of fatty acid.

TAG is a key component of milk fat, formed by long chain fatty acids and glycerol. In the process of TAG synthesis, there are three key enzymes: GPAM participates in the first step of catalytic reaction, AGPAT catalyzes the esterification of TAG *sn*-2 position, and DGAT makes diacylglycerol plus fatty acid acyl coenzyme A to form triacylglycerol, which is the rate-limiting enzyme for TAG synthesis (Abeni *et al.*, 2005). In this study, overexpression of *INSIG1* decreased the gene expression involved in the synthesis of TAG and knockdown of *INSIG1* increased the gene expression. This is consistent with the results in rats that overexpression of *INSIG1* can inhibit the expression of TAG synthesis genes (Takaishi *et al.*, 2004). Thus, it is confirmed that a function of INSIG1 protein is to inhibit the TAG synthesis in buffalo. This idea is supported by the fact that the content of total TAG was decreased after overexpression of *INSIG1*.

In conclusion, we observed a significant difference in INSIG1 protein between peak lactation and dry-off period in buffalo mammary tissue. We successfully isolated the primary mammary epithelial cells from buffalo mammary tissue. The overexpression of *INSIG1* decreased the expression of genes related to milk fat synthesis and cellular total TAG content, whereas knockdown increased the expression of genes. Our results provide a support for INSIG1 inhibiting SREBP processing. Furthermore, these results demonstrate a negative effect of *INSIG1* on milk fat synthesis.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029920000710

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