Negative effect of insulin-induced gene 2 on milk fat synthesis in buffalo mammary epithelial cells

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SUPPLEMENTARY FILE

Materials and methods

Isolation of BuMECs

Briefly, the buffalo mammary tissues were stripped of connective tissues. After washing with D-Hank's solution, the tissue sections were cut into 1 mm3 pieces and were transferred onto the cell dishes (Corning, USA) coated with collagen. After 1 hour of culture, 1 mL basal medium consisting of DMEM containing 10% FBS, 5 μg/mL insulin (Sigma, USA), 5 μg/mL hydrocortisone (Sigma), and 2% penicillin/streptomycin was added to the culture dish. The medium was changed with fresh medium every 24 h until the BuMECs were migrated out of the tissue. The purified BuMECs were cultured separately in the same medium as above to passage five and then pooled before the start of the experiment.

Preliminary screening of shRNA sequences

Three pLKO.1-shRNAs and pEGFP-INSIG2-N1 were respectively co-transfected into 239T cells at 70-80% confluence in 12 plates through TransIntroTM EL Transfection Reagent (TransGen Biotech, China) following the manufacturer's instruction. By the same method mentioned above, the pLKO.1-TRC vector and pEGFP-INSIG2-N1 were co-transfected as a positive control (Lv-shPC). The green fluorescent protein was assessed by fluorescent microscope (Carl Zeiss, Germany). In addition, the mRNA abundance of the *INSIG2* was further determined by RT-qPCR.

Genes related to milk fat synthesis

INSIG1, insulin-induced gene 1; *SREBP*, sterol regulatory element-binding protein; *PPARG*, peroxisome proliferator-activated receptor-γ; *FASN*, fatty acid synthase; *ELOVL6*, fatty acid elongase 6; *SCD*, stearoyl-CoA desaturase; *GPAM*, glycerol-3-phosphate acyltransferase; *DGAT2*, diacylglycerol acyltransferase 2; *AGPAT6*, 1-acylglycerol-3-phosphate O-acyltransferase 6; *TIP47*, tail-interacting protein of 47 kDa.

Table S1 Primer pairs used for CDS generation of buffalo *INSIG2*

Name of fragment	Sequence (5' to 3') [1]	Annealing temperature (℃)
pLVX-INSIG2-CDS	ctcgagATGGCAGAAGGAGAGACCGA ggatccTTCTTGATGAGATTTTTCTG	52.4
pEGFP-INSIG2-CDS	ctcgagATGGCAGAAGGAGAGACCGA aagcttTTCTTGATGAGATTTTTCTG	52.4

[1]The lowercase letters indicates the restriction sites

Table S2 Characteristics of shRNA used in this study

Name of shRNA	Sequence (5' to 3') [1]
shRNA1	ccggGCCGGAGTGTGAACTTGATGACTCAGGTCATCAAGTTCACACTCCGGCTTTTTG
	aattcAAAAAGCCGGAGTGTGAACTTGATGACTCAGGTCACTAAGTTCCAACTCCGGC
shRNA2	ccggGCATCTAGGAGAACCACATAACTCAGGTTATGTGGTTCTCCTAGATGCTTTTTG
	aattcAAAAAGCATCTAGGAGAACCACATAACTCAGGTTATGTGGTTCTCCTAGATGC
shRNA3	ccggGCATAACAATGGGAAACATTGCTCGAGCAATGTTTCCCATTGTTATGCTTTTTG
	aattcAAAAAGCATAACAATGGGAAACATTGCTCAGGCAATGTTTCCCATTGTTATGC

[1]Three shRNAs were designed, and each shRNA was added with restriction sites *EcoR*I and *Age*I.

Table S3 Primer information used for real-time quantitative PCR.

Gene[1]	Primers (5' to 3')	Product length (bp)	Efficiency[2]	Accession number
INSIG1	F: ACGTTCAGCTCTCCTTGACATT R: CTGTCGTCCTATGTTTCCCAC	239	2.11	JX853922
INSIG2	F: GGATTGTGGTGGACTTTTGATA R: ATTCATACATTGCCAGTTGTCG	217	2.09	NM_001290944
SREBP	F: GCACCGAGGCCAAGTTGAATAA R: CAGGTCCTTCAGCGATTTGCTT	146	2.14	KU517671
PPARG	F: GCTCCAAGAGTACCAAAGTG R: GTCCTCCTGAAGAAACCCTT	204	2.03	NM_001290893
FASN	F: AGGCCAGCTCCGAAGGCAACA R: TACCACGTCGGCCACTTGTGTC	209	2.01	NM_001012669
ELOVL6	F: TTTTCCGCTCTGTATGCT R: ACCCTGGTCACAAACTGAATGCT	201	2.05	XM_006076909
SCD	F: CGTGCCGTGGTATCTGTGG R: AAAGGTGTGGTGGTAGTTGTGG	217	2.10	NM_001290915
GPAM	F: ACTACGGATGTGTCAGAGTGGAT R: CACTGGGTCTTGAGGGAAGTATAG	141	2.17	XM_006043939
DGAT2	F: GTCCTGTCTTTCCTCGTGCT R: CCTCCTGCCACCTTTCTT	141	2.19	MK651507
AGPAT6	F: CTTTGCGTGGGCTACCTTG R: TCTTGGTCACCTCGTCGTC	242	2.02	JX518941
TIP47	F: GCTGTCACAGACGCTTATCCT R: CTGGGTGATGTCACGGAACA	193	2.04	XM_006067126
ACTB	F: TCTTGGTCACCTCGTCGTC R: GGCGCGATGATCTTGAT	196	2.05	NM_001290932
GAPDH	F: ATGGAGAAGGCTGGGGCTCA R: GCAGGAGGCATTGCTGACAA	144	2.07	XM_006065800
RPS23	F: ACCGACGAGACCAGAAGT R: CTCCAGGAATGTCACCAA	306	2.00	XM_006059350

[1]Annealing temperature of all primers is 60 ℃.
[2]Efficiency of amplification as calculated by LinRegPCR (www.linregpcr.nl).

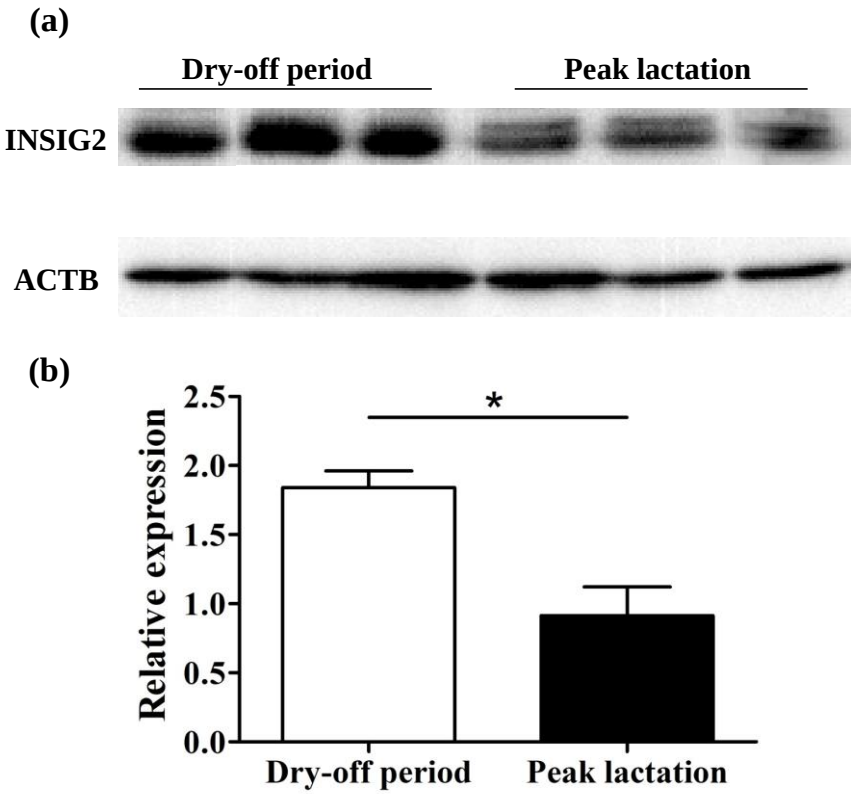

Fig. S1 The protein abundance of INSIG2 in buffalo mammary gland tissue. (a): representative immunblots; (b): corresponding mean gray values of INSIG2 which was expressed in relevance to beta-actin (ACTB). The values are displayed with means ± SEM; **P*< 0.05.

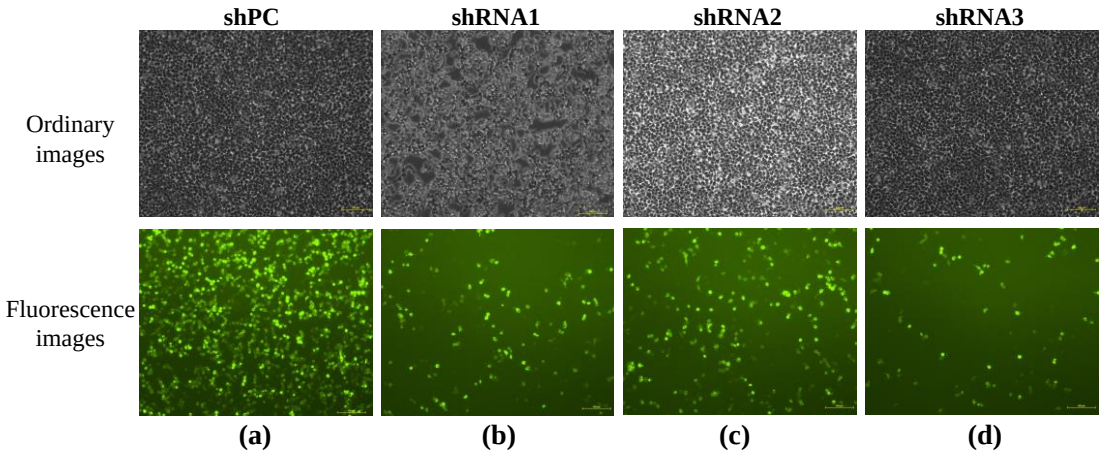

Fig. S2 Efficiency evaluation of the shRNA via images analysis. The pLKO.1-TRC and pEGFP-INSIG2-N1 were co-transfected as a positive control (a). The three pLKO.1-shRNAs (shRNA1, shRNA2 and shRNA3) were co-transfected with pEGFP-INSIG2-N1 (b, c and d).

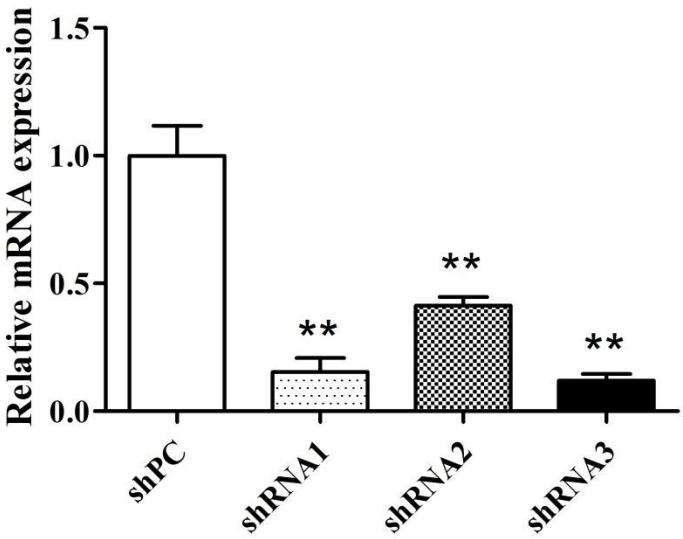

Fig. S3 Efficiency evaluation of the shRNAs by RT-qPCR. The efficiency of three pLKO.1-shRNAs (co-transfected with pEGFP-INSIG2-N1) in inhibiting *INSIG2* expression was determined by RT-qPCR.