Gene Expression, Vol. 15, pp. 199–206
Printed in the USA.

1052-2166/13 $90.00 + .00
DOI: http://dx.doi.org/10.3727/105221613X13571653093123
E-ISSN 1555-3884
www.cognizantcommunication.com

RNA Interference-Mediated Knockdown of *DGAT1* Decreases Triglyceride Content of Bovine Mammary Epithelial Cell Line

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Diacylglyceroltransferase-1 (DGAT1) expresses in nearly all tissues, including the mammary gland. Mice lacking DGAT1 exhibit decreased triglyceride content in mammary tissue, and are resistant to diet-induced obesity and diabetes mellitus. Thus, DGAT1 has received considerable attention. In the present study, the function of *DGAT1* was examined by liposome mediated RNA interference (RNAi) to knockdown the expression of endogenous DGAT1 expression in bovine mammary epithelial cells (BMEC) and the changes of the biological functions of cells were analyzed. The mRNA and protein levels, intracellular triglyceride (TG) content, and total protein of BMECs were analyzed by real-time PCR, Western blot, TG kit, and ultraviolet spectrophotometer, respectively, before and after RNAi treatment. The results indicated that knockdown of *DGAT1* expression significantly reduced TG content in BMECs. This study further confirmed the importance of *DGAT1* in triglyceride synthesis in bovine mammary tissue.

Key words: *DGAT1*; RNA interference (RNAi); Triglyceride (TG); Mammary epithelial cell

INTRODUCTION

DGAT catalyzes the final step in triglyceride synthesis by using diacylglycerol and fatty acyl-CoAs as substrates (5–7). Two isoforms of DGAT have been discovered, DGAT1 and DGAT2, which are members of different families of genes. *DGAT1* is expressed ubiquitously, with the highest expression levels in the small intestine, testis adipose tissue, mammary gland, and skin (19). In milk, DGAT1 may play an important role in triglyceride (TG) synthesis.

The gene encoding DGAT1, which has been mapped within the region of the marbling QTL (14, 15), has been demonstrated affecting the fat content of milk. In cattle, the lysine variant of *DGAT1* was associated with elevated milk fat content (24,25). It was hypothesized that a lysine residue at position 232 of the DGAT1 gene, as was found in all non-bovine mammalian species studied so far, could confer more efficient binding of acyl-coenzyme A than an alanine residue at this position (4,10). However, proof of the causal involvement of the lysine/alanine polymorphism in the variation of milk fat content has not been provided yet. Recently, a lysine/alanine polymorphism in the gene encoding diacylglycerol *O*-acyltransferase (DGAT1), a microsomal enzyme that catalyses the final step of triglyceride synthesis, has been shown to be associated with milk fat content (8).

RNA interference (RNAi) is a regulatory cellular process that controls posttranscriptional gene silencing. During RNAi double-stranded RNA (dsRNA) induces sequence-specific degradation of homologous

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mRNA via the generation of smaller dsRNA oligomo(er) (1). RNAi uses dsRNA to target the destruction of the homologous messenger RNAs via short interfering RNA (siRNA) (13,23).

However, the function of *DGAT1* during TG synthesis (4) in mammary tissue has not been verified by RNAi technology yet. In our present study, a shRNA plasmid vector targeting *DGAT1*, which could persistently generate siRNA inside cells, was transfected into mammary epithelial cells of Chinese Holstein cow and its effect on TG contents in the cell line was investigated.

MATERIALS AND METHODS

Construction of RNAi Vector Targeting DGAT1

A 21-mer siRNA oligonucleotide targeting the coding sequence of *DGAT1* was designed and synthesized by Genepharma Corporation (Shanghai, China). An empty vector with GFP as a negative control also was synthesized by the corporation.

According to the *DGAT1* gene full-length sequence, a pair of primers was designed. Primers used in the reaction of construction of the RNAi vector for *DGAT1* were as follows: sense primer 5′-CAAATT CAAGACGTTTTTTG-3′ and antisense primer 3′-AGCTCAAAAAACGTCTTGAA-5′. The annealed double-stranded oligonucleotides were ligated into *Eco*31I and *Sac*I digested pGenesil-1.1plasmid (Genesil Biotechnology, Wuhan, China).

Mammary Epithelial Cell Culture and Transfection

The mammary epithelial cells of Chinese Holstein cow in this experiment were established by our laboratory (9). Twenty-four hours before transfection, BMECs were plated 1×10^5–1×10^6/well on six-well culture plates (Falcon, Franklin Lakes, NJ, USA) with 500 μl of DMEM/F12 (GIBCO, Grand Island, NY, USA) with 10% (v/v) fetal bovine serum (FBS; PAA, Pasching, Austria) without antibiotics. When the cells reached more than 80% confluency, the growth medium was replaced by Opti-MEM serum-free media (GIBCO). For transfection, plasmid was diluted in 100 μl Opti-MEM serum-free media, and then mixed gently with FuGENE HD Transfection Reagent (Invitrogen, Carlsbad, CA, USA). Before use, the appropriate amount was diluted into 100 μl Opti-MEM serum-free media. The diluted plasmid was combined with diluted FuGENE HD Transfection Reagent (total volume = 200 μl) and was mixed gently and incubated for 15 min at room temperature. Plasmid-FuGENE mixture (100 μl) was added to each well containing the cells and medium. The cells were incubated at 37°C in a CO_2 incubator (Thermo, Marietta, OH, USA). The cells were split into a 1:10 or higher dilution with fresh growth medium 24 h after transfection.

The transfected cells and untransfected negative control cells were selected using G418 (Invitrogen). Twelve hours later, the expression of GFP in the cells was observed under a fluorescence microscope (NikonTE2000, Japan), and the number of GFP-positive cells was counted under high power magnification every 24 h.

Analysis of DGAT1 *mRNA by RT-PCR*

Stable mammary epithelial cells colonies were harvested after selection with G418. Total RNA was extracted from cultured cells using Trizol reagent (Tiangen, Beijing, China) for reverse transcription and cDNA was synthesized using Superscript First-Strand Synthesis Kit (Invitrogen) following the manufacturer's protocols. PCR amplification cycles for standard curve was performed as follows: 95°C for 3 min; 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and a final extension period at 72°C for 5 min. The PCR products were purified and recovered using the agarose gel DNA recovery kit (Tiangen). The purified *DGAT1* gene was ligated into pMD19-T vector (Takara, Dalian, China) and then transformed into DH5α competent cells (Tiangen). The positive bacterial clones were picked up and shaken overnight at 37°C. The plasmid was extracted using AxyPrep Plasmid Miniprep Kit (Axygen, Hangzhou, China) from positive clones following the manufacturer's instruction. The plasmid was diluted into different concentrations as the template and a stranded curve was drawn with the software from Eppendorf. Quantitative real-time polymerase chain reaction (RT-PCR) assays were carried out using SYBR Green Real-Time PCR Master Mix (Tiangen) using specific primers. According to the *DGAT1* gene full-length sequence, a pair of primers was designed. Primers used in the RT-PCR for DGAT1 were: sense primer 5′-GGCAGCCAGGACGGCAGTGTT-3′ and antisense primer 5′-TGAAGGCCCAGAGGCGGAA C-3′. The total length of the PCR product was 92 bp. The expression level was determined by normalization of the threshold cycle of the *DGAT1* gene to the negative control.

Total Protein Isolation and Western Blot Analysis

For total protein extraction, cells at a confluency of about 90% were incubated in RIPA buffer (10 mM sodium phosphate, PH 7.2, 150 mmol NaCl, 1% Nonidet P40, 1% Na deoxycholate, 0.1% SDS, 2 mmol EDTA, and 1 mmol DTT supplemented with 1 mmol

PMSF, 100 U/ml benzonase, proteinase inhibitor). Cells were incubated on ice for 30 min and the lysate was cleared by centrifugation at $15{,}000 \times g$ for 10 min at 4°C. The supernatant was then collected.

Total protein (50 μg per sample) was resolved by SDS-PAGE and transferred onto PVDF membranes (Bio-Red Laboratories Inc., USA). Immunoblotting was carried out using the following primary antibodies with the suggested dilutions from the manufacturer: anti-DGAT1 (Abcam, USA), anti-β-actin (Abcam). Immunoblots were developed using an ECL Advanced Western Blotting Detection Kit (Invitrogen).

Detection of TG Content and Total Protein Content in Positive and Control Cells

A TG kit (BHKT, Beijing, China) was used for detecting TG content in both positive cells (cells were transfected with an RNAi vector targeting *DGAT1*) and control cells (cells were transfected with a GFP vector). Then 1×10^5 cells/well were seeded in six-well flat-bottom culture plates with DMEM/F12 medium without serum. After 48 h, the cells were collected, and the concentration of cells was adjusted to ensure the equal numbers between positive and control cells. The cells were broken by a transonic machine and the total protein was extracted as previously described. The concentration of total protein was determination by an ultraviolet spectrophotometer (UNIC2802H, Shanghai, China).

Statistical Analysis

Experimental data were shown as mean ± SD. Statistically significant differences in the mean value were determined using *t*-tests and statistically significant differences were defined as $p < 0.05$.

RESULTS

The Result of RNAi Vector Construction for DGAT1

The *DGAT1*-shRNA interference vector was confirmed by restriction enzyme digestion and DNA sequencing (Fig. 1). It is suggested by the company that the interference efficiency of the vector could reach 70% generally.

Transfection of Mammary Epithelial Cells With DGAT1-*shRNA Vector*

To specifically silence *DGAT1* gene expression in BMECs, cells were transiently transfected with appropriate siRNA vectors (shRNA targeting DGAT1 and GFP as control). After transfections, both the mammary epithelial cells transfected with DGAT1 RNAi vector and cells transfected with GFP empty vector, but not the untransfected cells, showed green fluorescence (Fig. 2).

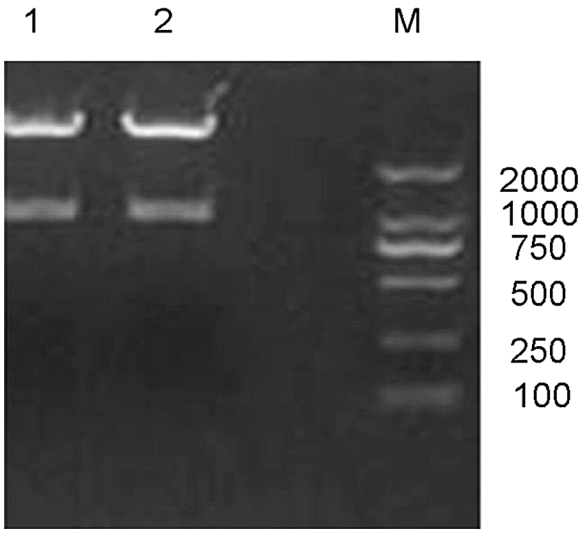


Figure 1. Restriction enzyme digestion of *DGAT1*-shRNA vector by Sac*I*. A fragment about 1000 bp was cut out as expected. Lane M: D2000 molecular weight marker; lane 1 and 2: vectors digested by Sac*I*.

shRNA Targeting DGAT1 *Inhibited* DGAT1 *mRNA Expression in Mammary Epithelial Cells*

To investigate the effect of *DGAT1* specific RNAi on mRNA expression, the mRNA of *DGAT1* was analyzed by real-time PCR. The stranded curve and melting curve were drawn by the software from Eppendorf (Figs. 3 and 4). The equation of the stranded curve was $C_t = -2.591 \times \log\ C_0 + 30.25$ ($R^2 = 0.997$). The mRNA copy number (mean ± SD) of cells transfected with *DGAT1* RNAi vector was 84.21 ± 4.39, and the mRNA copy number of cells transfected with GFP empty vector was 142.94 ± 4.11 (Fig. 5).

The statistical analysis showed that *DGAT1* mRNAs of mammary epithelial cells in the test group were downregulated significantly after transfection with RNAi vector plasmids, compared with the GFP control cells ($p < 0.05$). The interference efficiency was 41.6%. The possible reason for this low efficiency may be that the selected positive cells may still contain some negative cells.

Evaluation of DGAT1 *Expression by Western Blot*

To investigate the effect of *DGAT1* RNAi on protein expression, total protein was isolated from both positive cells and control cells. Results of Western blot analysis indicated that protein expression in test cells was downregulated, compared with normal cells

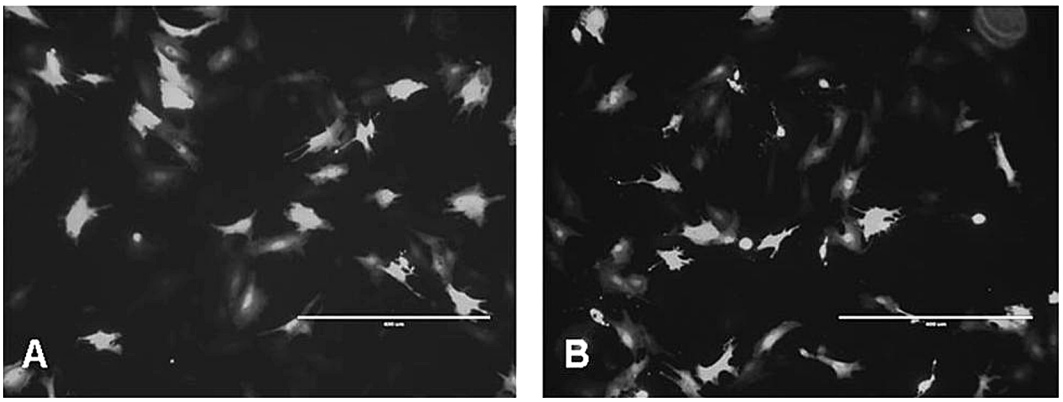
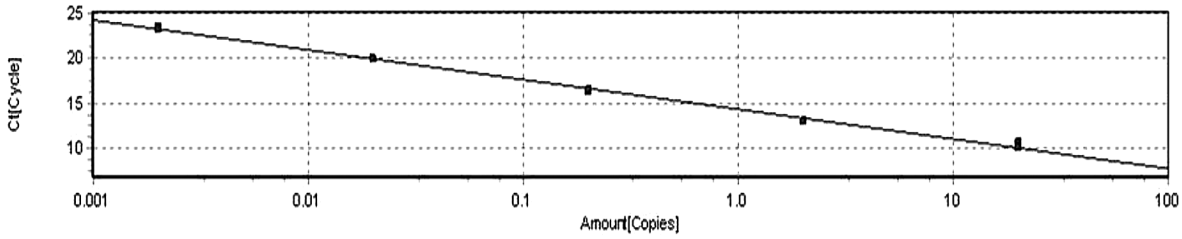


Figure 2. Green fluorescence from BMECs transfected with the *DGAT1* RNAi vector and the GFP empty vector. Twenty-four hours after transfection with the *DGAT1* RNAi vector and the GFP empty vector, the expression of GFP was observed under a fluorescence microscope. (A) Cells transfected with the *DGAT1* RNAi vector. (B) Cells transfected with the GFP empty vector.



Slope: -3.293
Y-Intercept: 14.37
Efficiency: 1.01
R^2: 0.997

Figure 3. The stranded curve of plasmid for real time RT-PCR for *DGAT1*. Absolute quantitation with real time RT-PCR was employed to investigate the effect of *DGAT1* RNAi on mRNA expression. From the equation of stranded curve ($C_t = -2.591 \log C_0 + 30.25$), the R^2 was 0.997. The value of R^2 indicated that the stranded curve could be used in the next step.

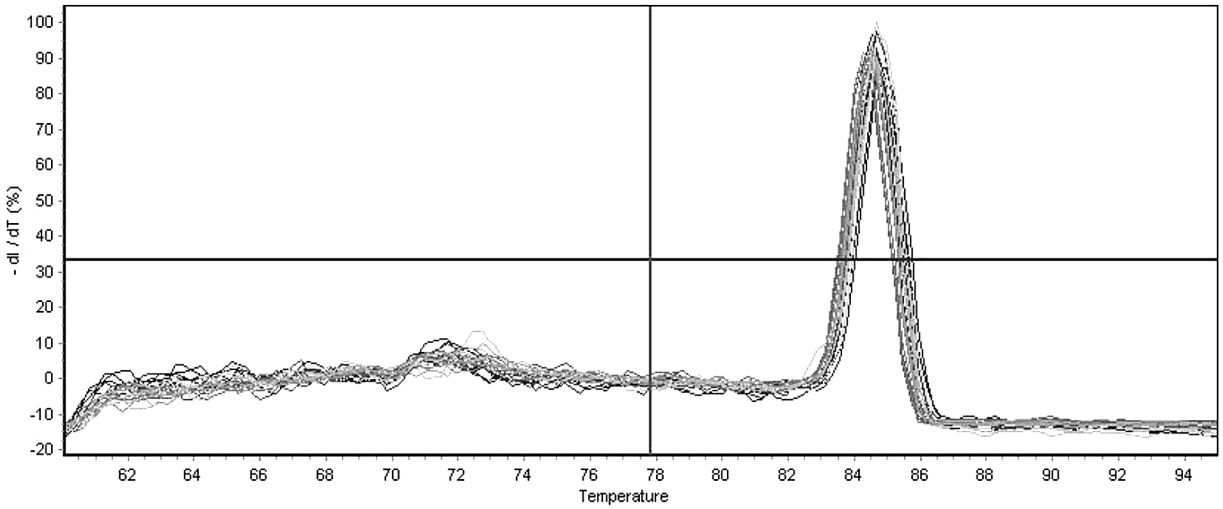


Figure 4. The melting curve of *DGAT1* RT-PCR. The melting curve of *DGAT1* RT-PCR showed that the reaction condition was qualified.

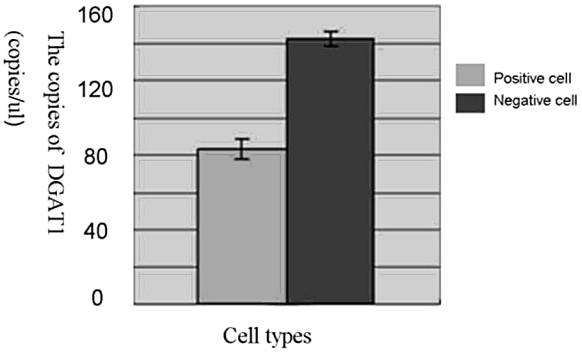


Figure 5. *DGAT1* mRNA expression in mammary epithelial cell after shRNA transfection. The expression of *DGAT1* was detected by real-time PCR. The copy number of positive cells (cells transfected with *DGAT1* RNAi vector) was 84.21 ± 4.39, and the copy number of negative cells (cells transfected with GFP empty vector) was 142.91 ± 4.11. The interference efficiency was 41.6%.

as a control. β-Actin was used as an internal control. There was no significant difference between *DGAT1* RNAi cells and normal mammary epithelial cells (Fig. 6).

The Effect of siRNA Targeting DGAT1 *on Intracellular TG Content and Intracellular Total Protein Content*

To examine the effect of *DGAT1* siRNA on intracellular fat content, a TG kit was employed. The difference in TG content between positive cells and the negative control cells was statistically significant ($p < 0.05$) (Table 1, Fig. 7). The results indicated that TG content was decreased due to the inhibition of *DGAT1* gene by RNAi vector, which suggests that *DGAT1* is essential for triglyceride synthesis in bovine mammary epithelial cells.

The concentrations of total protein of positive cells and negative control cells were determined by an ultraviolet spectrophotometer. There was no significant difference between the two cell populations (Table 2). The results indicated that *DGAT1* is not essential for total protein synthesis in bovine mammary epithelial cells.

DISCUSSION

In milk, DGAT1 may play an important role in triglyceride synthesis (16). To gain more insight into the function of *DGAT1* in mammary tissues, *DGAT1* expression in mammary epithelial cells of Chinese Holstein cow was selectively knocked down using RNAi. The results indicated that knockdown of *DGAT1* expression significantly reduced the TG contents but no significant effect to total protein content in bovine mammary epithelial cells. The results suggested that *DGAT1* is essential for triglyceride synthesis in bovine mammary tissue.

Diacylglycerol acyltransferase (DGAT) catalyzes the last step of the glycerol phosphate pathway of TG synthesis in mammalian cells. DGAT produces TG from its two substrates, DAG and fatty acyl-CoA. In this capacity, DGAT promotes TG storage but may also decrease FA substrates. Two DGAT genes have been cloned (6,7); both *DGAT1* and *DGAT2* expressed in many types of tissues, including adipose tissue, liver, intestines, and skeletal muscles, although differences in relative abundance are also evident. *DGAT1* and *DGAT2* bear no sequence homology to each other. DGAT1 is a member of the mammalian acyl-CoA cholesterol acyltransferase family, whereas DGAT2 belongs to a new family and was cloned by a homolog search based on the sequence of the fungal DGAT2 (15). Although both enzymes catalyze the same reaction in TG synthesis, they are functionally distinguished by their differences in regulation,

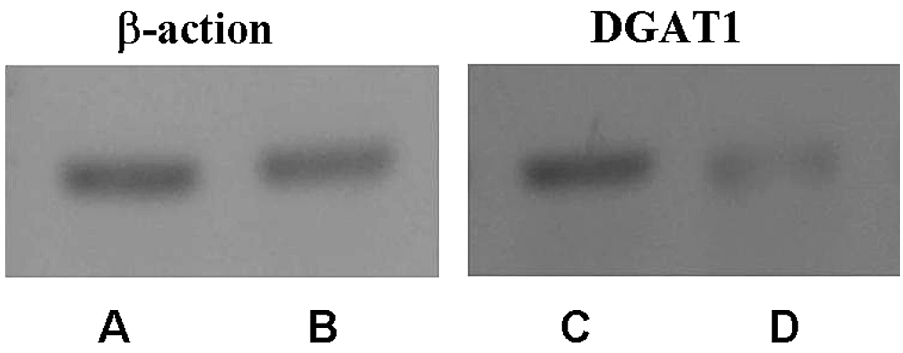


Figure 6. *DGAT1* expression in mammary epithelial cell detected by Western blot analysis. Western blot analysis indicated that protein expression in test cells was downregulated, compared with normal cells as a control (β-actin was used as an internal control). (A) β-Actin expression in normal mammary epithelial cells. (B) β-Actin expression in positive cells (cells transfected with *DGAT1* RNAi vector). (C) *DGAT1* expression in normal mammary epithelial cells. (D) *DGAT1* expression in positive cells (cells transfected with *DGAT1* RNAi vector).

TABLE 1
COMPARISON OF THE TG CONTENTS BETWEEN POSITIVE AND CONTROL CELLS

Sample	Positive 1	Positive 2	Positive 3	Control 1	Control 2	Control 3
TG content (mmol/L)	0.4217 ± 0.017	0.4421 ± 0.030	0.4081 ± 0.0098	0.5645 ± 0.015	0.5373 ± 0.019	0.5985 ± 0.029

Values are mean ± SE. Positive cells: cells transfected with the *DGAT1* RNAi vector. Control cells: cells transfected with the GFP empty vector.

phenotypic consequences when rendered deficient and additional functions.

Recently, a lysine/alanine polymorphism in the gene encoding diacylglycerol *O*-acyltransferase 1 (*DGAT1*), a microsomal enzyme that catalyses the final step of triglyceride synthesis, has been shown to be associated with milk fat content (percentage of lipid content of milk). Diacylglycerol *O*-acyltransferase 1 became a functional and positional candidate gene for milk fat content, based on the phenotype of *DGAT1*-deficient mice and its position close to a milk fat QTL on bovine chromosome 14, respectively (3,17). Mice lacking *DGAT1* do not lactate, most likely because of deficient triglyceride synthesis (24). In cattle, the lysine variant of *DGAT1* was associated with elevated milk fat content. It was hypothesized that a lysine residue at position 232 of the DGAT1 protein (20), as is found in all non-bovine mammalian species studied so far, could confer more efficient binding of acyl-coenzyme A than an alanine residue at this position (22). However, proof of the causal involvement of the lysine/alanine polymorphism in the variation of milk fat content has not yet been provided.

RNAi is a ubiquitous mechanism of eukaryotic gene regulation and an excellent strategy for specific gene silencing. Recently, the vector-based approach of shRNA interference has been developed in order to achieve stable, long-term, and highly specific suppression of gene expression in mammalian cells (11,12). RNAi interference ?RNAi?is a regulatory cellular process that controls post-postranscriptional gene silencing (2). During RNAi double-stranded RNA (dsRNA) induces sequence-specific degradation of homologous mRNA via the generation of smaller dsRNA oligomo (er) (18). RNAi uses dsRNA to target the destruction of the homologous messenger RNAs via siRNA (11).

In the present study, *DGAT1*-RNAi approach was used to reduce the TG contents in mammary epithelial cells, aiming to examine whether *DGAT1* is essential for triglyceride synthesis in bovine mammary tissue. The relationship of *DGAT1* and TG synthesis has been studied previously with other methodologies, such as a positional cloning approach to identify a substitution (K232A) in bovine *DGAT1* gene affecting milk fat composition (14); transplantation studies demonstrating that the impaired development resulting from a deficiency of *DGAT1* in both the stromal and epithelial tissues (3); *DGAT1* overexpression in muscle by in vivo DNA electroporation to increase intramyocellular lipid content (21).

Results from the present study showed that the RNAi efficiency was 41.6%. The possible reason for

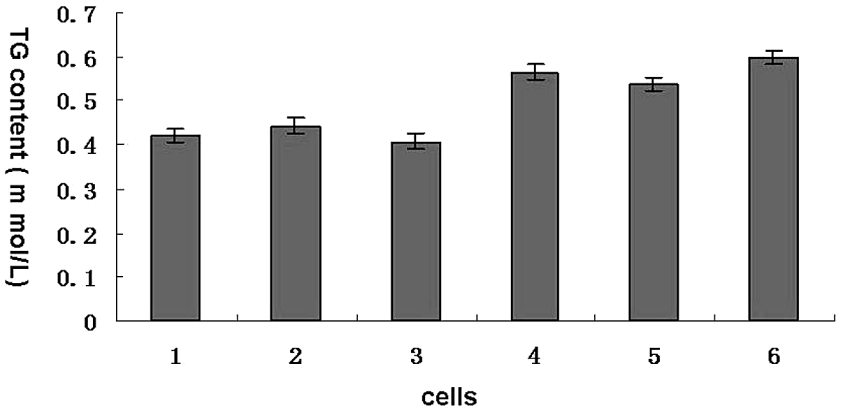


Figure 7. Comparison of the TG content in positive and control cells. The TG contents of positive cells and the negative control cells were analyzed with a TG kit and compared. The difference was statistically significant ($p < 0.09$). 1, 2, 3: the TG contents in the control cells (cells transfected with GFP empty vector) and 4, 5, 6: the TG content in positive cells (cells transfected with DGAT1 RNAi vector).

TABLE 2
COMPARISON OF THE TOTAL PROTEIN CONTENTS BETWEEN POSITIVE AND CONTROL CELLS

Sample	Positive 1	Positive 2	Positive 3	Control 1	Control 2	Control 3
Total protein content (mg/ml)	6.75 ± 0.59	6.67 ± 0.77	6.60 ± 0.98	6.71 ± 0.76	6.80 ± 0.88	6.56 ± 0.52

Values are mean ± SE. Positive cells: cells transfected with the *DGAT1* RNAi vector. Control cells: cells transfected with the GFP empty vector.

this low efficiency compared to 70% in general claimed by the company could be that the positive mammary epithelial cells were not pure.

In conclusion, the results indicated that knockdown of *DGAT1* expression significantly reduced the TG contents in BMECs, suggesting that *DGAT1* is essential for TG synthesis in bovine mammary tissue.

ACKNOWLEDGMENTS

This work was supported by the National R&D Project of Transgenic Organisms of Ministry of Science and Technology of China (Nos. 2011ZX08007-001, 2009ZX08007-005B, and 2009ZX08009-156B), the National Natural Science Foundation of China (Nos. 31000991 and 30972085), and the Doctoral Program Foundation of Institutions of Higher Education of China (No. 20100061120039), and the National High Technology Development Project (2013AA102505). We thank the anonymous reviewers for their time and energy in reviewing this article.

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