Effect of diacylglycerol acyltransferase 2 overexpression in 3T3-L1 is associated to an increase in mono-unsaturated fatty acid accumulation

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Abstract

Background: Fatty acid (FA) composition is the most important parameter affecting the flavor and nutritional value of the meat. The final and the only committed step in the biosynthesis of triglycerides is catalyzed by diacylglycerol acyltransferase 2 (DGAT2). The role of DGAT2 in lipid accumulation has been demonstrated in adipocytes. However, little is known about the effect of DGAT2 on the FA composition of these cells.

Methods: To investigate the role of DGAT2 in regulating lipid accumulation, FA composition and the expression of adipogenic genes, we cloned the open reading frame of the porcine DGAT2 gene and established 3T3-L1 cells that overexpressed DGAT2. Cells were then cultured in differentiation medium (DM) without FA, with a mixture of FAs (FA-DM), or containing a 13C stable isotope-labeled FA mixture (IFA-DM). The FA composition of adipocytes was analyzed by gas chromatography–mass spectrometry and gas chromatography-isotope ratio mass spectrometry. Quantitative PCR and western blotting were employed to detect expression of adipogenic genes in 3T3-L1 adipocytes cultured with FA-DM for 12 d.

Results: The triacylglyceride (TAG) content was significantly higher in 3T3-L1 adipocytes overexpressing DGAT2 than in control cells. When cultured in DM or FA-DM for 12 d, cells overexpressing DGAT2 showed a higher proportion of unsaturated FAs (C16:1 and C18:1). However, when cells overexpressing DGAT2 were cultured with FA-DM for 30 min, the FA composition was almost identical to that of controls. Further, the proportion of stable isotope-labeled FAs were similar in 3T3-L1 adipocytes overexpressing DGAT2 and control cells cultured in IFA-DM for 12 d. These results collectively indicate that the higher proportion of mono-unsaturated FAs, C16:1 and C18:1, may originate from de novo FA synthesis but not from the uptake of specific FAs from the medium. This hypothesis is further supported by evidence that both mRNA and protein expression of genes involved in FA synthesis (ACACA, FASN, SCD1, and A-FABP) were significantly higher in cells overexpressing DGAT2 than in control cells.

Conclusions: In conclusion, our study revealed that TAG accumulation, the proportion of MUFAs, and the expression of adipogenic genes were higher in 3T3-L1 cells overexpressing DGAT2 than in control cells.

Keywords: DGAT2, Fatty acid composition, Overexpressing, Pig
Introduction

Meat quality depends on various sensory and chemical parameters, including color, tenderness, and the content of flavoring substances [1]. Fatty acid (FA) composition is one of the most important parameters affecting meat quality. The proportion of saturated, monounsaturated, and polyunsaturated FAs in the diet is reported to have important effects on human health. For instance, high intake of saturated FAs can elevate plasma cholesterol, which can have harmful cardiovascular effects [2]. Further, foods rich in monounsaturated FAs (MUFAs) may decrease platelet aggregation [3], increase bleeding time [4], and increase fibrinolysis [5]; thereby preventing thrombogenesis.

The FA composition of cells is determined by phospholipid metabolism [6,7], FA synthesis [8], and FA transport [9-13]. Diacylglycerol acyltransferase (DGAT1 and DGAT2) catalyzes the final step in triacylglyceride (TAG) formation through the acylation of diacylglycerol (DAG) [14,15]. DGAT1 plays an important role in incorporating oleoyl-CoA into TAG [15,16]. In COS-7 cells, DGAT2 overexpression has been reported to significantly increase lipid accumulation [17]. In contrast, lipid storage in 3T3-L1 adipocytes was markedly decreased by DGAT2 knockdown [18]. The effect of DGAT2 overexpression on the FA composition of cells is unknown.

This study investigated the effect of DGAT2 overexpression on the FA composition of 3T3-L1 preadipocytes. mRNA and protein expression of adipogenic genes in cells overexpressing DGAT2 was also investigated. Our results revealed a crucial role of DGAT2 in the regulation of FA composition and adipogenic gene expression.

Methods

Generation of 3T3-L1 preadipocytes overexpressing DGAT2

The open reading frame region of DGAT2 was subcloned into pcDNA3.1(+) to produce pcDNA3.1(+) -DGAT2, which was then linearized and transfected into 3T3-L1 cells (CL-173, ATCC, USA). Cells transfected with pcDNA3.1(+) -DGAT2 and pcDNA3.1(+) were treated with G418 (350 μg/mL) for 14 d until all non-transfected cells died. The selected transfected cells were then cultured in growth medium or differentiation medium supplemented with G418 (150 μg/mL). The selected transfected cells were then cultured in growth medium or differentiation medium until the cells matured (12 d): DMEM/F12, 10% charcoal-stripped FBS, and 0.174 μmol/L insulin (DM) (Life Technologies, Grand Island, NY, USA), DM supplemented with unlabeled FA (FA-DM), or DM supplemented with one of the stable isotope-labeled FA mixtures (FAM-DM). FA-DM contained 10 μmol/L C16-13C16-palmic acid, C16-13C18-palmitoleic acid, C18-13C18-stearic acid, C18-13C18-oleic acid, C18-13C18-linoleic acid, and C20-13C20-arachidic acid (Sigma-Aldrich, St. Louis, MO, USA). IFA-DM contained 10 μmol/L 13C16-palmic acid, 13C18-stearic acid, 13C18-oleic acid, (Cambridge Isotope Laboratories, Tewksbury, MA, USA) C16-13C18-palmitoleic acid, C18-13C18-linoleic acid, and C20-13C20-arachidic acid. All FA mixtures were pre-complexed with 60 μmol/L FA-free bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA).

Triglyceride analysis and Oil Red O staining

The cells were washed twice with Ca2+- and Mg2+-free PBS and lysed using 150 μL of RIPA lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride). The TAG and total protein content of the cells in each well were determined from cell lysates using commercial kits (Biosino Bio-Technology and Science Inc., Beijing, China) on a microplate reader (Thermo Labsystems MK3, Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol. The TAG content of cells in each well was then normalized to the total protein. Mature adipocytes were rinsed twice with Ca2+- and Mg2+-free PBS and then fixed in 4% paraformaldehyde in PBS (w/v) for 30 min at room temperature to facilitate Oil Red O staining. A stock solution of 0.35% Oil Red O (Amresco, Inc., Solon, OH, USA) in isopropanol (w/v) was diluted in water (6:4, v/v) and added to the fixed cells for 1 h at room temperature. The cells were then washed with water and photographed.

Analysis of cellular FA composition

Cellular lipids were extracted according to a previously described procedure [20], converted to FA methyl esters using BF3 and methanolic potassium hydroxide [21], and analyzed using gas chromatography (GC; model MSD-6890; Agilent, USA) equipped with an automatic injector. Aliquots of 1 μL were injected into the capillary column (30 m × 0.32 mm × 0.25 μm; DB-5 MS; Agilent) with cyanopropyl methyl silicone as the stationary phase. The column oven temperature was programmed to hold at 130°C for 1 min, increase from 130°C to 200°C at 5°C/min, and then hold at 200°C for 5 min. Helium was used as the carrier gas at a flow rate of 1 mL/min. The proportions of
individual FAs were determined by measuring the peak area using ChemStation software.

**Stable isotope-labeled FA profile**
Cellular lipids were extracted and converted to FA methyl esters, which were resolved by GC using a chromatograph (Model 6890, Agilent, USA) equipped with a 30 m × 0.32 mm × 0.25 μm DB-5 MS capillary column (Agilent, USA) and burned to generate CO₂ and to detect the molecular ions of masses 44, 45, and 46 using isotope ratio mass spectrometry (IR-MS) (GV Instruments, UK). A mixed nominal sample (C₁₄-myristic acid, C₃₀-FA, C₂₁-diolefin, C₂₆-diolefin, and C₃₆-diolefin; University of Illinois, USA) was used with a standardized isotope value of CO₂ in the cylinder. The carbon isotope value of each FA was calculated using the formula $\delta = \frac{(R_s/R_R) - 1}{1} \times 1,000$, where $\delta$ and $R_s/R_R$ represent the carbon isotope and ¹³C/¹²C values, respectively, of an international nominal sample (Pee Dee Belemnite, South Carolina, USA). The data are relative to control cells transfected with pcDNA3.1(+).

**Quantitative PCR**
Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After treatment with DNase I (Takara Bio Inc., Shiga, Japan), total RNA (2 μg) was reverse-transcribed to cDNA in a final volume of 20 μL using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) and oligo-dT₁₈ random primers according to the manufacturer's instructions. β-actin was used as a standard for gene expression. All primers for the selected genes were designed by Primer Premier 5 (Table 1). SYBR Green real-time PCR Master Mix reagents (Toyobo Co., Ltd., Osaka, Japan), cDNA, ddH₂O, the sense and antisense primers (200 nmol/L for each gene) were used for quantitative PCR, which was performed using an Mx3005p instrument (Stratagene, La Jolla, CA, USA). The thermal cycling conditions were as follows: 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at different temperature for 15 s, and extension at 72°C for 40 s. Melting curve and sequence analyses were performed for each product to confirm the specific amplification. mRNA expression levels in cells overexpressing DGAT2 are presented as a ratio of those in control cells transfected with pcDNA3.1(+).

**Immunoblot analysis**
The cells were lysed in RIPA lysis buffer. Homogenates were centrifuged at 12,000 rpm for 5 min at 4°C, and the protein concentration in the supernatants was determined.

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**Table 1 Primers used in RT-PCR analysis**

| Gene     | GenBank access no. | Product, bp | Sequence (5’→3’)                  |
|----------|--------------------|-------------|-----------------------------------|
| HSL      | U08188.1           | 184         | F: AGTGCCCTATTTCAAGGACAGA         |
|          |                    |             | R: TGGGGATGTTGCTTT               |
| ACACA    | AY451393           | 172         | F: GACAGAGGAGAAGTGCCCGTC         |
|          |                    |             | R: TACACCTTCTGCTGCTTGGG          |
| a-FABP   | NM_024406.2        | 93          | F: GCTCATAGCACCCTCTCTG           |
|          |                    |             | R: TCCAGGTTCCCCACAAAGG           |
| LPL      | NM_008509.2        | 211         | F: ACTGCCACTTCAACCCACC           |
|          |                    |             | R: GCCACATCAATTTCCACC            |
| ATGL     | NM_025802.3        | 169         | F: GACCTGATGACACCCCTTTC          |
|          |                    |             | R: GGCTACCCGTCGCTCTT             |
| DGAT2    | NM_001160080.1     | 96          | F: GGCTCAATAGGTCAAGAGGA          |
|          |                    |             | R: GGCCGTGTCGACCTCAA             |
| SCD1     | NM_009127.4        | 103         | F: GCTTACACCTGCCTCTTC           |
|          |                    |             | R: CCCTGCTCTGTAAAGTCTG           |
| β-actin  | NM_007393.3        | 422         | F: TAAAGGCAAACGTTGAAAGAATGAC     |
|          |                    |             | R: ACCGCCTGTGCTCAAGTATGAT         |
| PPARγ    | NM_001127330.1     | 232         | F: TCAAGGAGGTGAGCAGTTG           |
|          |                    |             | R: GGCTTCCGGACGGCTTT            |
| FASN     | NM_007988.3        | 280         | F: CCAAAGCTGACTCGGCCCTACT        |
|          |                    |             | R: GCCAGTTCTGCAAGTTCTAT          |
| FAT/CD36 | NM_001159556.1     | 214         | F: CTGGGCTCATGGCTGG              |
|          |                    |             | R: GCCCAAGCTCATGGGGTTT           |
using a BCA protein assay reagent kit (Pierce, Rockford, IL). Protein samples, subjected to a 20% SDS (Beyotime, Shanghai, China), were degenerated for 10 min at 99°C. A total of 30 μg protein were resolved by sodium dodecyl sulfate (SDS)-poly-acrylamide gel electrophoresis (30% acrylamide, 1.5 mol/L Tris (pH8.8), 10% SDS, 10% ammonium persulfate, TEMED; 10% SDS-PAGE) and separated by electrophoresis at 110 V for 75 min using Tris-glycine running buffer (0.025 mol/L Tris base, 0.192 mol/L glycine, and 0.1% SDS, pH 8.3). Proteins then were subsequently electrotransferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) using transfer buffer containing 25 mmol/L Tris base, 192 mmol/L glycine, and 10% methanol at pH 8.1-8.3. The membranes were blocked with 5% nonfat milk in PBS for 1 h at room temperature. The primary antibodies [goat anti-LPL, rabbit anti-ATGL, rabbit anti-ACACA, goat anti-SCD1, rabbit anti-CD36 (Santa Cruz Biotechnology Inc., Dallas, Texas, USA); rabbit anti-β-actin (Cell Signaling, Danvers, MA, USA); goat anti-DGAT2, mouse anti-FASN (Lifespan, Providence, RI, USA) were incubated at 4°C overnight, followed by incubation with the appropriate secondary antibody (1:1,000, Bioss, Beijing, China) for 1 h at room temperature. Protein expression was measured using a FluorChem M Fluorescent Imaging System (ProteinSimple, Santa Clara, CA, USA) and normalized to β-actin expression.

Statistical analysis
The data are presented as the mean ± SEM. An independent t-test was used for statistical analysis of the differences between the means, and the cut-off point for significance was set at \( P < 0.05 \).

Results
Lipid accumulation in 3T3-L1 adipocytes overexpressing DGAT2
3T3-L1 cells transfected with pcDNA3.1(+)−DGAT2 had 63-fold higher DGAT2 mRNA expression than control cells transfected with empty vector (\( P < 0.01 \)) (Figure 1). Oil Red O staining and TAG content analysis similarly demonstrated that lipid accumulation was much higher in 3T3-L1 adipocytes overexpressing DGAT2 (Figure 2).

FA composition in 3T3-L1 adipocytes overexpressing DGAT2 cultured with FA-DM
When cells were cultured with FA-DM for 12 d, the proportion of palmitoleic acid (C16:1; \( P < 0.05 \)), oleic acid (C18:1; \( P < 0.01 \)), and linoleic acid (C18:2; \( P < 0.01 \)) was significantly higher in 3T3-L1 adipocytes overexpressing DGAT2 than in control cells (Table 3). The reverse was true for stearic acid (C18:0) and arachidonic acid (C20:4) (\( P < 0.01 \); Table 3). When the cells were incubated...
in FA-DM for 30 min, the proportion of palmitoleic acid (C16:1) was slightly elevated in cells overexpressing DGAT2 ($P < 0.05$) (Table 3).

### Table 2 FA composition of 3T3-L1 adipocytes overexpressing DGAT2 cultured with DM

| Fatty acids          | Control    | DGAT2     | $P$ value |
|----------------------|------------|-----------|-----------|
| Palmitoleic acid (C16:1) | 2.17 ± 0.52 | 4.20 ± 0.27 | $P < 0.05$ |
| Palmitic acid (C16:0)  | 48.03 ± 2.90 | 41.76 ± 2.10 | $P > 0.05$ |
| Linoleic acid (C18:2)  | 3.17 ± 0.35  | 2.94 ± 0.13  | $P > 0.05$ |
| Oleic acid (C18:1)  | 18.43 ± 0.13  | 21.87 ± 0.96  | $P < 0.05$ |
| Stearic acid (C18:0)  | 20.79 ± 0.76  | 19.97 ± 0.54  | $P < 0.05$ |
| Arachidonic acid (C20:4)  | 7.40 ± 0.75  | 9.26 ± 0.83  | $P > 0.05$ |

After 12 d of differentiation, cells were collected for FA analysis. Cells were then subjected to transesterification and injected into a GC. The results are expressed as the percent of the total FA content (100%). The data are presented as the mean ± SEM of six independent wells. *$P < 0.05$. 

### Table 3 FA composition of 3T3-L1 adipocytes overexpressing DGAT2 cultured with FA-DM

| Fatty acids          | 30 min     | 12 d       |
|----------------------|------------|------------|
| Myristic acid (C14:0) | 2.70 ± 0.15 | 2.84 ± 0.42 |
| Palmitoleic acid (C16:1) | 2.67 ± 0.15  | 2.61 ± 0.17  |
| Palmitic acid (C16:0)  | 39.11 ± 4.55  | 31.17 ± 1.100 |
| Linoleic acid (C18:2)  | 5.14 ± 0.38 | 8.44 ± 0.29 |
| Oleic acid (C18:1)  | 14.18 ± 1.19 | 8.66 ± 0.32 |
| Stearic acid (C18:0)  | 22.95 ± 2.33 | 26.76 ± 0.42 |
| Arachidonic acid (C20:4)  | 12.29 ± 1.88  | 15.65 ± 0.46  |
| Arachidic acid (C20:0) | 0.96 ± 0.04  | 4.73 ± 0.21  |

3T3-L1 adipocytes were incubated with FA-DM (10 μmol/L C16-palmic acid, C16-palmitoleic acid, C18-stearic acid, C18-oleic acid, C18-linoleic acid, and C20-arachidic acid bound to 60 μmol/L FA-free BSA) for 30 min or 12 d. Cells were then subjected to transesterification and injected into a GC for FA analysis. The results are expressed as the percent of the total FA content (100%). The data are presented as the mean ± SEM of six independent wells. *$P < 0.05$; **$P < 0.01$.

FA composition in 3T3-L1 adipocytes overexpressing DGAT2 cultured with FA-DM

When cells were cultured with IFA-DM for 12 d, the proportion of 13C FAs were the same in 3T3-L1 adipocytes overexpressing DGAT2 and control cells ($P > 0.05$) (Figure 3). These results indicate that the higher proportion of C16:1 and C18:1 may originate from de novo FA synthesis but not from the uptake of specific FAs from the medium.

Adipogenic gene expression in 3T3-L1 adipocytes overexpressing DGAT2

As shown in Figures 4 and 5, mRNA and protein expression of adipose triglyceride lipase, acetyl CoA carboxylase (ACACA), FA synthase (FASN), stearoyl-CoA desaturase-1 (SCD1), and FA-binding protein (a-FABP) was significantly higher in 3T3-L1 adipocytes overexpressing DGAT2 than in control cells. In addition, mRNA expression of FA translocase (FAT/CD36) and peroxisomal proliferator-activated receptor γ (PPARγ) was higher in cells overexpressing DGAT2.

Discussion

Previous studies have proved that DGAT2 plays an important role in lipid accumulation [17]. However, little is known about the influence of DGAT2 on the FA composition of adipocytes. Our study revealed that TAG accumulation, the proportion of MUFAs, and the expression of adipogenic genes were all higher in 3T3-L1 cells overexpressing DGAT2 than in control cells.

The existence of DGAT2 was first implicated from the finding that mice lacking DGAT1 had an abundance of TAG in their tissues [22]. Further research revealed that DGAT2 mRNA was highly expressed in several lipid metabolism tissues, including liver [23], mammary glands...
and adipose tissue [25]. DGAT2 overexpression enhanced the accumulation of lipid droplets in COS-7 cells [17]. In contrast, DGAT2 knockdown decreased lipid storage in 3T3-L1 adipocytes [18]. Consistent with these studies, our results showed that the TAG content was significantly higher in 3T3-L1 adipocytes overexpressing DGAT2 than in control cells.

TAG formation is catalyzed by both DGAT1 and DGAT2, through the acylation of diacylglycerol [14,15]. In mice, DGAT1 is known to have a strong substrate preference for incorporating oleoyl-CoA into TAG [15,16]. Gene polymorphisms of DGAT in cows were reported to be closely related to the content of oleic acid in muscle [26]. Our results also demonstrated the novel function of porcine DGAT2 on the FA composition of adipocytes. When cultured in either DM or FA-DM for 12 d, 3T3-L1 adipocytes overexpressing DGAT2 showed a higher proportion of MUFAs (C16:1 and C18:1); when incubated in FA-DM for 30 min, the proportion of only palmitoleic acid (C16:1) was significantly elevated in cells overexpressing DGAT2 than in controls. However, when the cells were incubated with IFA-DM containing a stable IFA mixture, the proportion of 13C FAs was unchanged. These observations indicate that the long-term effects of DGAT2 overexpression in the FA profile may originate from de novo FA synthesis but not from the uptake of specific FAs from the culture medium.

To investigate the possible mechanism underlying the effects of DGAT2 on FA composition, we studied the expression of adipogenic genes in cells overexpressing DGAT2 mRNA and protein expression of ACACA, FASN, A-FABP, and SCD1 was significantly higher in 3T3-L1 adipocytes overexpressing DGAT2 than in control cells, as was the mRNA expression of FAT/CD36 and PPARγ. Of these genes, ACACA and FASN are the two key enzymes in de novo FA synthesis. This evidence suggests that the higher levels of lipid accumulation observed in cells overexpressing DGAT2 may result from de novo FA synthesis.
Several studies have demonstrated that FAT/CD36, a-FABP, and SCD1 are associated with the uptake of MUFAs [11,13,27]. DGAT2 and SCD1 co-localize to a compartment involved in activating lipid synthesis, suggesting that SCD1 and DGAT2 play a coordinated role in TAG synthesis [28]. Thus, SCD1 may participate in TAG synthesis by producing an easily accessible pool of MUFA [29].

Although the function of DGAT2 has been described solely as the catalysis of TAG formation, we found that the expression of a great number of adipogenic genes was affected by DGAT2 overexpression. DAG, an important cellular second messenger, may be involved in this phenomenon. Considering that DGAT catalyzes TAG synthesis through DAG, numerous reports have shown that lower DGAT2 expression resulted in lower DAG content [30,31], subsequently activating protein kinase Cε (PKCε) [32-35]. Therefore, we suspect that the expression of various adipogenic genes may be modulated by PKCε, which could increase the transcription of genes involved in FA biosynthesis by activating the sterol regulatory element binding protein 1c (SREBP1) [36-39]. The elucidation of the precise mechanism whereby DGAT2 affects adipogenic gene expression will require further study.

In conclusion, our study revealed that TAG accumulation, the cellular proportion of MUFAs, and the expression of adipogenic genes were higher in 3T3-L1 adipocytes overexpressing DGAT2 than in control cells. This information may be helpful in producing and selecting animals with a desirable FA profile.

Abbreviations
FA: Fatty acid; DGAT2: Diacylglycerol acyltransferase 2; DM: Differentiation medium; FA-DM: Differentiation medium with a mixture of FA; IFA-DM: Differentiation medium with 13C stable isotope-labeled FA mixture; MUFAs: Monounsaturated fatty acids; TAG: Triacylglyceride; GC: Gas chromatography; FBS: Fetal bovine serum; BSA: Bovine serum albumin; IR-MS: Isotope ratio mass spectrometry; ACACA: Acetyl CoA carboxylase; FASN: FA synthase; SCD1: Stearoyl-CoA desaturase-1; a-FABP: FA-binding protein.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
GJM participated in the design of the study, carried out the experiments and statistical analysis, and wrote the first draft of the manuscript. SG and JQY participated in the design of the study and the statistical analysis, and oversaw manuscript preparation. ZXT and CH participated in the cell experiments and plasmid construction. WSB, WLN, GP, XQY, ZYL, and YL participated in the study design and coordination. ZZG participated in writing the final versions of the manuscript. All authors have read and approved the final manuscript.

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