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Heterologous expression of *C. elegans fat-1* decreases the *n*-6/*n*-3 fatty acid ratio and inhibits adipogenesis in 3T3-L1 cells

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ABSTRACT

In general, a diet enriched in polyunsaturated fatty acids (PUFAs) inhibits the development of obesity and decreases adipose tissue. The specific impacts of *n*-3 and *n*-6 PUFAs on adipogenesis, however, have not been definitively determined. Traditional *in vivo* and *in vitro* supplementation studies have yielded inconsistent or even contradictory results, which likely reflect insufficiently controlled experimental systems. *Caenorhabditis elegans fat-1* gene encodes an *n*-3 fatty acid desaturase, and its heterologous expression represents an effective method both for altering the *n*-6/*n*-3 PUFA ratio and for evaluating the biological effects of *n*-3 and *n*-6 PUFAs. We sought to determine whether a reduced *n*-6/*n*-3 ratio could influence adipogenesis in 3T3-L1 cells. Lentivirus-mediated introduction of the *fat-1* gene into 3T3-L1 preadipocytes significantly reduced the *n*-6/*n*-3 ratio and inhibited preadipocyte proliferation and differentiation. In mature adipocytes, *fat-1* expression reduced lipid deposition, as measured by Oil Red O staining, and induced apoptosis. Our results indicate that a reduced *n*-6/*n*-3 ratio inhibits adipogenesis through several mechanisms and that *n*-3 PUFAs more effectively inhibit adipogenesis (but not lipogenesis) than do *n*-6 PUFAs.

1. Introduction

It is widely accepted that both the quantity and fatty-acid composition of ingested fats are important for human health. Polyunsaturated fatty acids (PUFAs) are required for many homeostatic processes (including adipogenesis) [1,2], and a diet rich in PUFAs can decrease adipose tissue mass and inhibit obesity [1,3–5]. The specific effects of *n*-3 and *n*-6 PUFAs on adipogenesis and lipogenesis, however, remain more controversial. Only a limited number of studies have addressed these questions, often yielding inconsistent and even contradictory results. In a previous review, Madsen concluded that *n*-6 PUFAs are more efficient than *n*-3 PUFAs in inhibiting adipocyte differentiation and *de novo* fatty-acid synthesis within 3T3-L1 cells [1]. In rodents fed high-fat diets, however, *n*-3 PUFAs more effectively prevent obesity than do *n*-6 PUFAs [6,7]. In addition, *n*-6 arachidonic acid (ARA) and *n*-3 eicosapentaenoic acid (EPA) reduce triacylglycerol accumulation to similar degrees [1]. This agrees with Wortma's finding that *n*-3 and *n*-6 PUFAs have similar effects on lipogenesis in 3T3-L1 adipocytes [8].

These previous studies generally used traditional supplementation protocols, which can be difficult to compare because of experimental variability. For example, different supplements can have different total lipid and energy contents. The purity and freshness of the supplement can also affect the results. These confounding factors often result in unreliable, inconsistent, and even conflicting results [9–11]. Because *n*-6 and *n*-3 PUFAs are metabolically and functionally distinct and often have opposing physiological functions [9], the *n*-6/*n*-3 PUFA ratio (rather than the absolute levels of these two PUFAs) is the critical factor during both homeostasis and development. Transgenic mice or cell lines that heterologously express the *Caenorhabditis elegans* gene *fat-1* can convert supplemental *n*-6 PUFAs into *n*-3 PUFAs, thereby altering the *n*-6/*n*-3 ratio through a single supplement [12,13]. These transgenic tools, therefore, represent unique and reliable models for assessing the impact of different *n*-6/*n*-3 ratios. To date, this well-controlled experimental system has been used to ask whether the *n*-6/*n*-3 ratio affects cardioprotection [12], neuroprotection [14], inflammation [15,16], carcinogenesis, tumorigenesis [17–22], and diabetes [23], for example.

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Here we report the lentivirus-mediated transfer of *fat-1* into 3T3-L1 preadipocytes, a widely used tool for lipid research. This resulted in the efficient and stable expression of *fat-1* in both preadipocytes and mature adipocytes, which effectively reduced the *n*-6/*n*-3 ratio. As a result, both adipogenesis and lipid deposition were inhibited, whereas the level of adipocyte-specific apoptosis was elevated. This implies that an optimized dietary intake of PUFAs may combat a propensity toward obesity.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), newborn bovine calf serum (NBCS), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from GIBCO (BRL Life Technologies, Grand Island, NY, USA). Insulin, dexamethasone, 3-isobutyl-1-methyl-xanthine (IBMX), 14% BF3/MeOH reagent, and Oil Red O were purchased from Sigma–Aldrich (St. Louis, MO, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). M-MLV reverse transcriptase was purchased from Promega (Madison, WI, USA). Maxima SYBR Green qPCR Master Mix was purchased from Fermentas Life Sciences (Hanover, MD, USA). Cell Counting Kit-8 (CCK-8) was purchased from DOJINDO (DOJINDO Lab, Kumamoto, Japan). Single-stranded DNA (ssDNA) apoptosis ELISA kit was purchased from Chemicon (Chemicon International, Darmstadt, Germany).

2.2. Cell culture and MDI-induced differentiation

3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) were placed in high-glucose DMEM supplemented with 10% NBCS and incubated at 37 °C with 5% CO_2 until confluent. As described previously [24], 2 days after reaching confluence (referred to as Day 0) cells were placed in differentiation medium, which is high-glucose DMEM supplemented with 10% FBS, 10 mg/L insulin, 1 μmol/L dexamethasone, and 0.5 μmol/L IBMX (MDI) for 2 days (Day 2). Cells were then placed in post-differentiation medium (10% FBS and 10 mg/L insulin) for an additional 2 days (Day 4). Cells were then maintained in 10% FBS/DMEM for an additional 5 days (Day 9), at which time the cells exhibited a lipid-filled phenotype, which is characteristic of mature adipocytes. Each culture medium was supplemented with 10 μmol/L linoleic acid (LA, 18:2*n*-6) and ARA (20:4*n*-6) as substrates for *fat*-1-encoded *n*-3 desaturase.

2.3. Production of recombinant lentiviruses and transduction

Plasmids for the production of lentiviruses were kindly provided by Dr. Carlos Lois (Division of Biology, California Institute of Technology, Pasadena, CA). These included FUGW, delta 8.9, and the VSV-G plasmids. The cDNA for *fat-1* (which encodes an *n*-3 fatty acid desaturase) was kindly provided in the pCE8 vector by Dr. John Browse (Washington State University, Pullman, WA). EGFP driven by the human *ubiquitin C* promoter (*UbiC*) was released from the FUGW vector by *Bam*HI and *Eco*RI double digestion. The *fat-1* gene was amplified from pCE8 using high-fidelity PCR with primers having the sequences 5′-TATGGATCCACCATGGTCGCTCATTCCTC-3′ containing a *Bam*HI restriction enzyme site (forward), and 5′-CGCGAATTCTTACTTGGCCTTTGCCTT-3′ containing a *Eco*RI restriction enzyme site and a Kozak consensus sequence (CCACC) to ensure efficient translation. The *fat-1* amplification product was then inserted into the FUW vector (LV-*fat-1*). For the control vector, linearized FUW was self-circularized following T4 DNA polymerase blunt-ending (LV-empty).

All viruses were produced as described [25]. Briefly, 293T cells were infected with vectors using the calcium phosphate method. Supernatants were harvested 60 h after infection and concentrated using ultracentrifugation (70,000*g* for 2 h). Viral titers were then determined. Concentrated viral supernatants were then added to fresh medium. The cells were incubated overnight, and the medium was replaced with fresh medium the next day.

2.4. Lipid analysis

A simplified method for lipid analysis was performed as described [26]. Briefly, an aliquot of a cell pellet was placed into a glass methylation tube and mixed with 1 mL of hexane and 1 mL of 14% BF3/MeOH reagent. After the mixture was blanketed with nitrogen, it was heated at 100 °C for 1 h and then cooled to room temperature. Methyl esters were extracted in the hexane phase following the addition of 1 mL H_2O. Samples were then centrifuged at (2000*g* for 1 min), and the upper hexane layer was removed and concentrated under nitrogen. Fatty acid methyl esters were analyzed by gas chromatography.

2.5. RNA preparation, reverse-transcriptase polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from 3T3-L1 cells using TRIzol according to the manufacturer's instructions. cDNA was synthesized according to the manufacturer's protocol. Briefly, the reverse transcription reaction was carried out in M-MVL RT 5× buffer, 0.5 mM dNTPs, and 200 U of M-MLV reverse transcriptase in a final volume of 20 μL. cDNA was then used as the template for PCR analysis. Primers were ordered from Invitrogen (Beijing, China) as follows: *fat-1*, 5′-CGGCACTCACAATTCTCTAC-3′ (forward) and 5′-GCCAGAAGTGAGATCCATC-3′ (reverse); adipocyte P2 (aP2), 5′-TGGGAGTGGGCTTTG-3′ (forward) and 5′-TGTCGTCTGCGGTGAT-3′ (reverse); β-actin, 5′-AGGTCATCACTATTGGCAAC-3′ (forward) and 5′-CTCATCGTACTCCTGCTTG-3′ (reverse). PCR products then were separated by 1.5% agarose gel electrophoresis.

2.6. Quantitative Real-time RT-PCR (Real-time RT-qPCR)

cDNA was synthesized and analyzed using the Maxima SYBR Green qPCR Master Mix. Real-time RT-qPCR was performed on a Bio-Rad Real-time PCR System (CFX96). Primers were ordered from Invitrogen (Beijing, China) as follows: peroxisome proliferator-activated receptor γ (PPARγ), 5′-TTGACCCAGAGCATGGTGC-3′ (forward) and 5′-GAAGTTGGTGGGCCAGAATG-3′ (reverse); adipocyte P2 (aP2), 5′-TGGGAGTGGGCTTTG-3′ (forward) and 5′-TGTCGTCTGCGGTGAT-3′ (reverse); uncoupling protein-2 (UCP2), 5′-AATCTCGGGAGGCACCTTTC-3′ (forward) and 5′-GAGAATGGGACTGGGCAGAG-3′ (reverse); fatty acid synthase (FAS), 5′-GTGAAGAAGTGTCTGGACTGTGTCAT-3′ (forward) and 5′-TTTTCGCTCACGTGCAGTTTA-3′ (reverse).

2.7. Cell proliferation assay

3T3-L1 cell proliferation was monitored using CCK-8. Preadipocytes were seeded at 1000 cells/well in 96-well plates and allowed to attach for 24 h. Cells were then mixed with 10 μL of the CCK-8 solution, and the plate was incubated at 37 °C for 1 h. The level of cell proliferation was then determined by measuring the absorbance of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophynyl)-2*H*-tetrazolium product at 450 nm with a reference at 600 nm.

2.8. Oil Red O staining

Mature adipocytes were stained with Oil Red O. Briefly, cell monolayers were washed twice with PBS and then fixed with 10% formalin in PBS (pH 7.4). The cells were then washed twice with distilled water and stained for at least 1 h at room temperature in freshly diluted Oil Red O solution. For each dish, three images were captured and analyzed. The stained lipid area was measured as the sum of integrated optical density (IOD) values using the Image-Pro-Plus Program (IPP; Media Cybernetics, Inc., Bethesda, MD, USA).

2.9. Apoptosis assay

Preadipocytes were seeded at 1000 cells/well in 96-well plates and then induced to differentiate into mature adipocytes. Rates of apoptosis were measured at different stages using the ssDNA apoptosis ELISA kit. This assay is based on the ability of formamide to selectively denature DNA within apoptotic cells. Denatured DNA was then detected using a monoclonal antibody to ssDNA. Following this reaction, 3-ethylbenziazoline-6-sulfonic acid was added to color the products of this reaction. Reaction intensity (between ssDNA and the mAb) was determined by the absorbance at

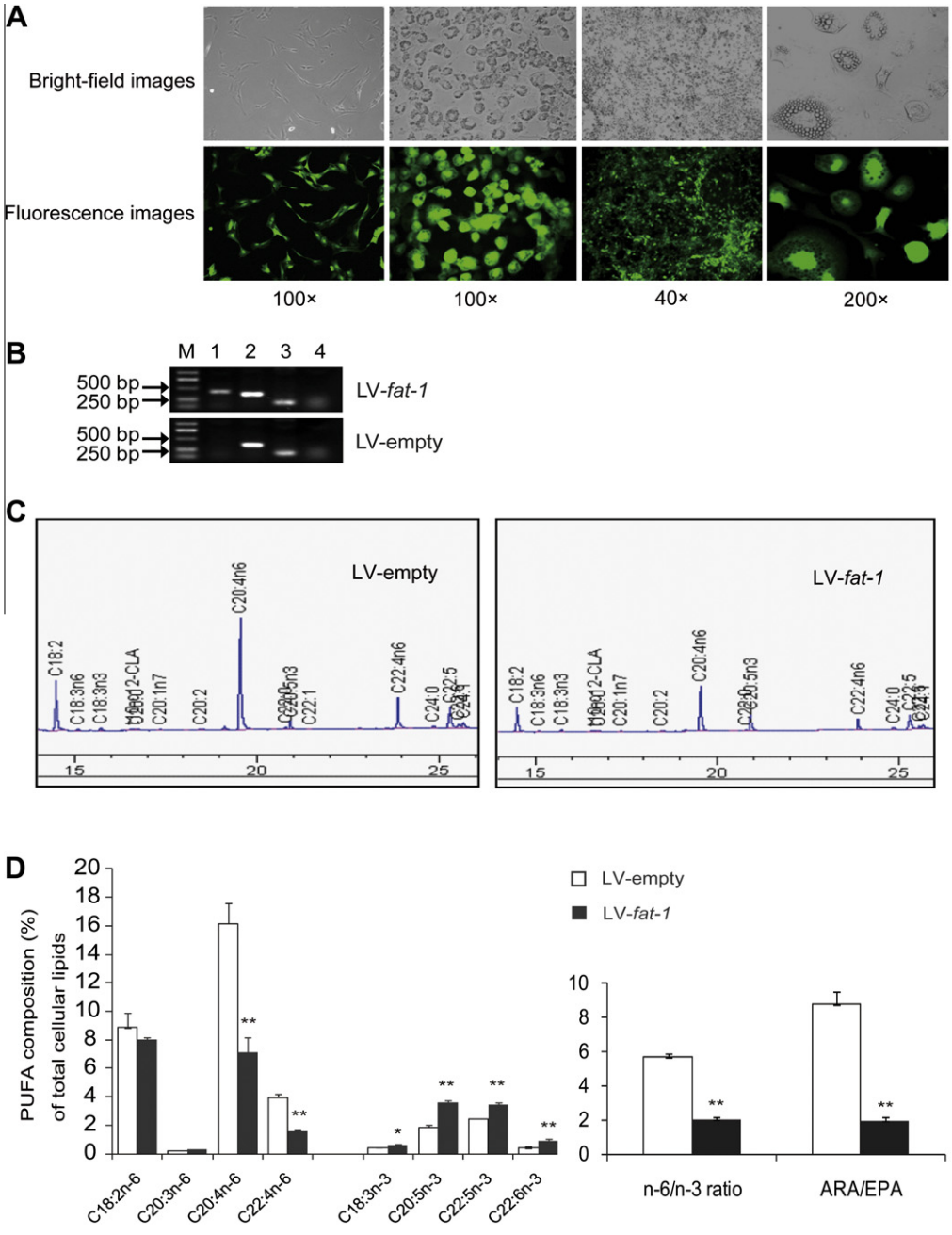

Fig. 1. Lentivirus-mediated expression of *fat-1* in 3T3-L1 cells affects the PUFA profile. (A) Lentiviruses provided an efficient and stable system for delivering exogenous genes into 3T3-L1 cells. Expression of EGFP in 3T3-L1 preadipocytes (left-most upper and lower images) and mature 3T3-L1 adipocytes (remaining images) following infection. (B) Detection of *fat-1* mRNA in mature adipocytes. M, DM 2000 Marker; lane 1, *fat-1* (411 bp); lane 2, *β-actin* (357 bp); lane 3, *aP2* (165 bp); lane 4, total RNA input (to confirm no genomic-DNA contamination). *β-actin* and *aP2* were used as a housekeeping gene and adipocyte-specific marker, respectively. (C) Partial gas chromatograph traces showing changes of cellular fatty-acid profile that resulted from expression of *fat-1* in 3T3-L1 cells. (D) PUFA composition (%) of total cellular lipids extracted from cells transfected with LV-empty or LV-*fat-1*. The *n*-6/*n*-3 PUFA ratio and the ARA/EPA ratio are also shown. Values are expressed as the mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$.

405 nm. Time points for the apoptosis assay are as follows: −4, −2, 4, and 8 days relative to Day 0.

2.10. Statistical analysis

Data are expressed as the mean ± SD. Differences between controls and treated groups were evaluated using Student's *t*-test. Results were considered significant for values of $p < 0.05$ (∗) and $p < 0.01$ (∗∗).

3. Results and discussion

3.1. Highly efficient and stable expression of a foreign gene in 3T3-L1 cells using lentivirus-mediated transfection

Although the 3T3-L1 cell line is widely used as model system for adipogenesis and lipogenesis, genetic modification of these cells has proven difficult. Methods of transfection that involve electroporation, liposomes, and adenoviruses have not yielded satisfying results [27,28]. Carlotti indicated, however, that 3T3-L1 cells (both preadipocytes and mature adipocytes) could be efficiently transduced using lentiviral vectors, without generating adverse side effects. They used a four-plasmid system that contains a *phosphoglycerate kinase-1* (*PGK*) promoter [27], whereas we used a three-plasmid lentiviral system that contains a ubiquitin promoter. We first evaluated the efficiency and stability of our transfection protocol using an EGFP transgene. Nearly all 3T3-L1 preadipocytes that were transduced using this method expressed EGFP (left panel in Fig. 1A). Moreover, EGFP expression was maintained as the preadipocytes differentiated into mature adipocytes (Fig. 1A). Lentivirus-mediated transfection itself did not compromise the differentiation potential of preadipocytes, as the majority of induced cells could differentiate into mature adipocytes (second panel in Fig. 1A). These mature cells were morphologically normal and contained large numbers of lipid droplets (fourth panel in Fig. 1A).

3.2. Expression of fat-1 in 3T3-L1 cells affects the PUFA profile

After preadipocytes were transfected with lentiviruses carrying either an empty vector (LV-empty) or the *fat-1* cDNA (LV-*fat-1*), cells were cultured in medium supplemented with 10 μmol/L LA and 10 μmol/L ARA. On Day 9, RT-PCR analysis indicated that *fat-1* was stably expressed in mature 3T3-L1 adipocytes (Fig. 1B). To measure the functional consequences of *fat-1* expression in 3T3-L1 cells, total cellular lipids from control (LV-empty) and *fat-1*-expressing cells were analyzed using gas chromatography. Expression of *fat-1* significantly altered the cellular PUFA profile (Fig. 1C). Relative amounts of PUFAs within the total cellular lipids are shown in Fig. 1D. In *fat-1*-expressing cells, most *n*-6 PUFAs were converted to corresponding *n*-3 PUFAs. This significantly reduced the *n*-6/*n*-3 PUFA ratio from 5.66 to 2.01. The ratio between ARA (20:4*n*-6) and EPA (20:5*n*-3) was also reduced (8.77 to 1.97; Fig. 1D). It is interesting to note that although *fat-1* encodes an *n*-3 desaturase that recognizes a range of 18- and 20-carbon *n*-6 substrates [29], it converts ARA more efficiently than other *n*-6 substrates in present study. This result agrees with a similar study in human lung cancer cells [18]. Finally, cells infected with the LV-empty vector had a fatty acid profile that was indistinguishable from uninfected controls (data not shown). This indicates that the lentiviral infection itself did not affect PUFA metabolism.

3.3. fat-1 expression in 3T3-L1 preadipocytes affects both proliferation and differentiation

In lung and breast cancer cells, decreased *n*-6/*n*-3 ratio after heterologous expression of *fat-1* inhibits cell proliferation [18,19]. In supplementation studies, *n*-3 PUFAs, or reductions in the *n*-6/*n*-3 ratio, also inhibit proliferation in many cell types [30–32]. Here we used the CCK-8 assay to assess the proliferation of pre-confluent 3T3-L1 preadipocytes. Preadipocytes that expressed *fat-1* had lower rates of proliferation, as cell numbers were significantly reduced at Day 4 and later time points (Fig. 2A). On Day 7, for example, control preadipocytes were confluent, whereas cells expressing *fat-1* were not. This result implies that a lower *n*-6/*n*-3 ratio may inhibit hyperplasia in preadipocytes.

To assess cellular differentiation, the expression of three genes involved in adipogenesis was measured in the two groups of transfected cells (LV-*fat-1* and LV-empty). After cells were subjected to the MDI induction protocol, expression of *PPARγ*, *aP2,* and *UCP-2* was significantly lower in cells transfected with LV-*fat-1* (Fig. 2B). The reduced level of differentiation observed in *fat-1*-expressing 3T3-L1 preadipocytes (together with the reduced rate of proliferation described above) implies that low *n*-6/*n*-3 ratios attenuate adipogenesis. These results are similar to observations made by Kim et al. [24]. Finally, based on comparisons to an uninfected control, the lentiviral transfection itself did not affect proliferation or differentiation of 3T3-L1 preadipocytes (data not shown).

3.4. Effect of fat-1 expression on lipid deposition in 3T3-L1 adipocytes

Adipogenesis can lead to lipogenesis and the accumulation of lipids within adipocytes. Oil Red O staining was used to examine lipid accumulation in mature adipocytes that were derived from the two groups of transfected cells. Representative images of stained cells showed substantially less lipid deposition in *fat-1*-expressing cells, as compared with LV-empty controls (Fig. 3A).

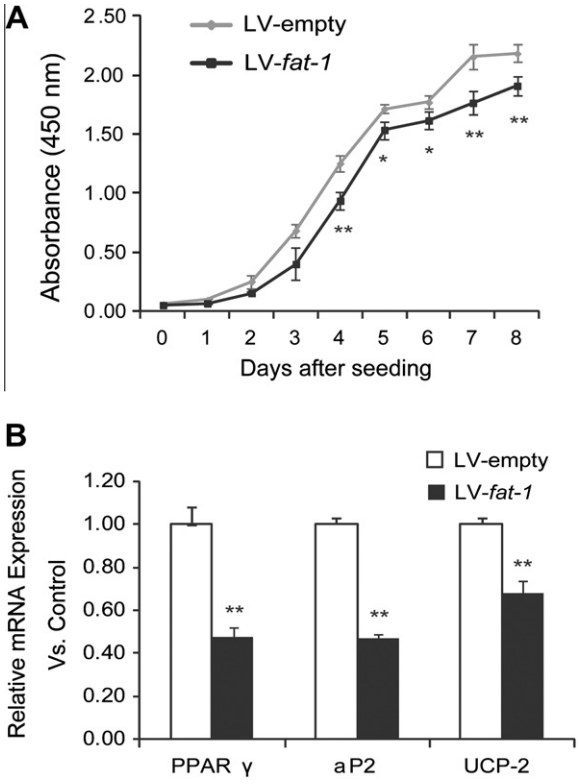

Fig. 2. *fat-1* expression affects both proliferation and differentiation of 3T3-L1 preadipocytes. (A) Proliferation rates for 3T3-L1 preadipocytes that were infected with LV-empty or LV-*fat-1*. (B) Quantitative gene expression analysis of genes involved in adipogenesis, which include *PPARγ*, *aP2*, and *UCP-2*. Values are expressed as the mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$.

Quantification of stained lipid levels (i.e., the lipid area) also revealed significantly less lipid area in *fat-1*-expressing adipocytes (Fig. 3B). FAS is the central enzyme in *de novo* lipogenesis [33,34], and has been used as a marker for lipogenesis [8]. RT-qPCR analysis determined that levels of FAS expression were not affected by *fat-1* expression in adipocytes (Fig. 3C). This finding was supported by images of mature adipocytes from the two groups of transfected cells (insets in Fig. 3A), which revealed comparable levels of lipid deposition within adipocytes. Therefore, it is likely that the reduced level of lipid accumulation that we measured in *fat-1*-expressing adipocytes (Fig. 3B) resulted from an inhibition of adipogenesis rather than lipogenesis.

3.5. Effect of fat-1 expression on apoptosis in 3T3-L1 cells

In transgenic mice, *fat-1*-induced decreases in the *n*-6/*n*-3 ratio result in apoptosis in lung cancer cells [18], prostate cancer cells [20], and epithelial cells of the colon [22]. In traditional supplementation studies, *n*-3 PUFAs (or reduced *n*-6/*n*-3 ratios) increase apoptosis in many cell types, including 3T3-L1 preadipocytes

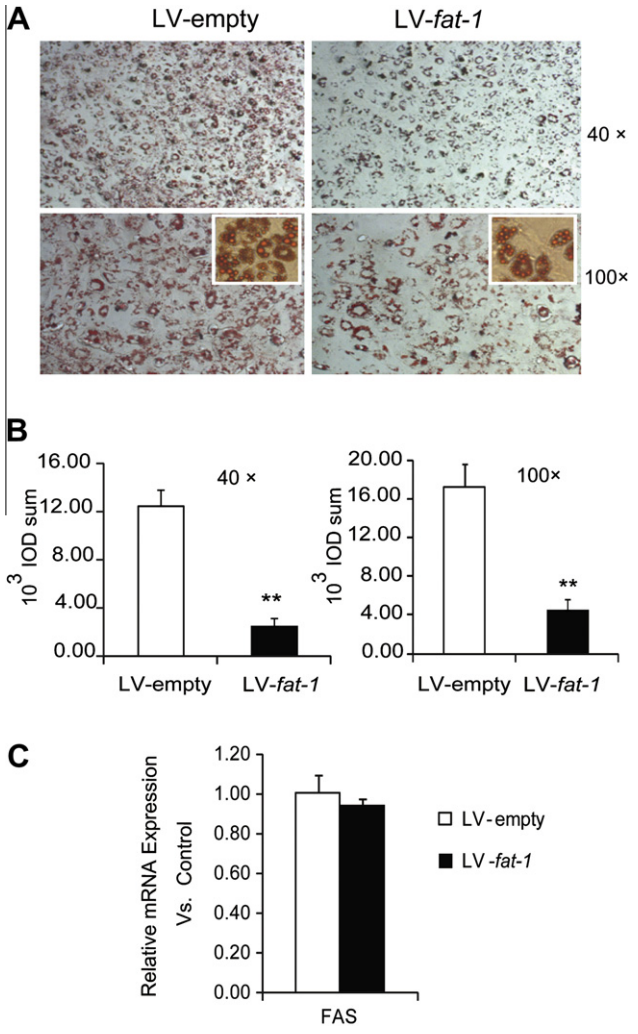

Fig. 3. *fat-1* affects lipid deposition in 3T3-L1 adipocytes. (A) Representative images of Oil Red O-stained adipocytes at 40× and 100× magnification, 3T3-L1 cells were transfected with LV-empty or LV- *fat-1*. Insets show the comparable levels of lipid deposition within adipocytes from two groups. (B) Lipid area quantification of images of two groups at 40× and 100× magnification. (C) Quantitative analysis of FAS expression (a gene involved in lipogenesis). Values are expressed as the mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$.

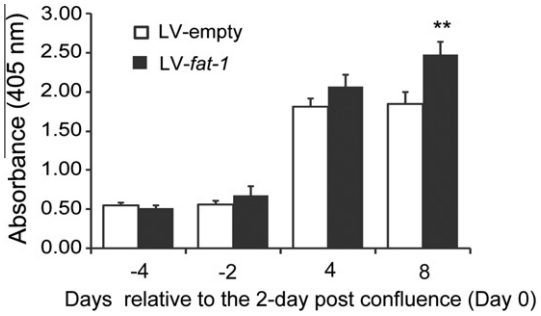

Fig. 4. The effect of *fat-1* expression on apoptosis in 3T3-L1 cells. Day 0 is 2 days post-confluence. Values are expressed as the mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$.

[24]. We used a ssDNA ELISA kit to measure apoptosis levels within *fat-1*-expressing 3T3-L1 cells. Both groups of transfected cells exhibited very low levels of apoptosis during preadipocyte and confluent stages (i.e., Day – 4 and Day – 2; [Fig. 4]). After cells were induced to differentiate, however, apoptosis rates were elevated in both groups. On Day 8, significantly higher rates of apoptosis were detected in *fat-1*-expressing adipocytes as compared with those for controls. This implies that reduced *n*-6/*n*-3 ratios may accelerate adipocyte-specific apoptosis. Our results do not completely agree with those of Kim et al. [24], as we observed elevated levels of apoptosis in mature adipocytes but not in preadipocytes. The adipocyte-specific apoptosis suggests that apoptosis may occur in a dose-dependent manner. Lentiviral transfection itself did not affect apoptosis in 3T3-L1 cells, as differences in cell death were not detected between LV-empty cells and an untransfected control (data not shown). This is similar to results reported by Carlotti et al. [27].

In summary, we have used a well-controlled system to evaluate the effects of *n*-6 and *n*-3 PUFAs on adipogenesis in 3T3-L1 cells. We showed that expression of *fat-1* reduced the *n*-6/*n*-3 ratio, and adversely affected 3T3-L1 cells in a number of ways. *fat-1* inhibited the proliferation and differentiation of preadipocytes and increased the level of apoptosis in adipocytes. Lipogenesis within mature adipocytes, however, was not likely affected. These findings suggest that a relatively small decrease in the dietary ratio between *n*-6 and *n*-3 PUFAs could reduce the incidence of obesity, primarily by inhibiting hyperplasia.

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