The Role of Acetoacetyl-CoA Synthetase, a Ketone Body-Utilizing Enzyme, in 3T3-L1 Adipocyte Differentiation

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Acetoacetyl-CoA synthetase (AACS) is a ketone body-utilizing enzyme that converts acetoacetate to acetoacetyl-CoA in the cytosol and consequently provides acetyl units as the precursors for lipogenesis. To clarify the role of AACS in adipogenesis, we investigated the expression and localization of the AACS protein and the effect of AACS knockdown on 3T3-L1 differentiation. The protein expression of AACS is dramatically induced during 3T3-L1 differentiation and is localized in the cytoplasm of differentiated 3T3-L1 cells. Moreover, knockdown of AACS inhibits differentiation of 3T3-L1 cells and suppresses expression of the adipocyte markers, peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα). These results suggest that AACS has a crucial role in the mechanism of 3T3-L1 differentiation.

Key words ketone body; acetoacetyl-CoA synthetase; adipocyte differentiation; peroxisome proliferator-activated receptor γ; CCAAT/enhancer binding protein α; lipogenesis

Adipose tissue stores lipid droplets, secretes hormones, such as leptin and adiponectin, and is involved in appetite and insulin resistance.[1] Understanding the mechanism of adipocyte differentiation is important for studying obesity and lifestyle-related diseases that are of ever-increasing concern, such as cardiovascular disease and type 2 diabetes.

Ketone bodies, which are increased during fasting and diabetes, are known to be a significant energy source for various tissues, particularly in the brain.[2] Mitochondrial succinyl-CoA:3-ketoacid CoA transferase (SCOT; EC 2.8.3.5) is the enzyme thought to be responsible for ketone body utilization for energy production[3] and to be essential for energy balance.[4] On the other hand, recent studies have shown that ketone bodies are directly activated in the cytosol by a novel acetoacetate-specific ligase, acetoacetyl-CoA synthetase (AACS, acetoacetate-CoA ligase, EC 6.2.1.16), for the synthesis of physiologically important lipidic substances such as cholesterol and fatty acid.[5]

We purified this enzyme for the first time as the discrete acetoacetate-specific ligase from rat liver[6] and cloned its cDNA using the 3′-rapid amplification of cDNA ends (RACE) method.[7] Furthermore, we showed that AACS is highly expressed in lipogenic tissues, such as white adipose tissue, brain and liver,[8] and found that mRNA levels of AACS are dramatically increased during adipogenesis in primary rat preadipocytes and 3T3-L1 cells.[9] AACS mRNA levels are transcriptionally regulated by CCAAT/enhancer-binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) during adipogenesis.[8,10] Moreover, the pattern of AACS expression during adipogenesis is similar to that of acetyl-CoA carboxylase-1 (ACC-1), the rate-limiting enzyme of fatty acid synthesis.[9] These data suggest that ketone body utilization through AACS plays an important role in adipogenesis.

To elucidate the role of AACS, we analyzed the protein expression of AACS during 3T3-L1 differentiation and the localization of AACS in adipocytes. Moreover, we assessed the effect of AACS knockdown on the differentiation of 3T3-L1 cells.

The authors declare no conflict of interest.

MATERIALS AND METHODS

Cell Culture 3T3-L1 cells (JCRB No. IFO50416) were purchased from the Health Science Research Resources Bank (Osaka, Japan). The cells were maintained in Dulbecco's modified Eagle's medium Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen) supplemented with 10% newborn calf serum (CS) (Invitrogen) at 37°C in an atmosphere of 5% CO_2. For the induction of adipocyte differentiation, confluent 3T3-L1 cells were grown in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 0.25 μM dexamethasone (Nacalai tesque), and 5 μg/mL insulin (Sigma) for 2 d. Then the media were replaced with DMEM/F-12 and 10% FBS containing insulin (5 μg/mL). The media were changed every 2 d until the cells were collected.

Virus Production and Transduction Lenti-X 293T cells (Takara) were seeded in 100 mm poly-L-lysine plates (IWAKI) and transfected with pGreenPuro shRNA vector (System Biosciences) and packaging mix (Invitrogen) according to the manufacturer's protocol. The media were replaced with 10%FBS/D-MEM at approximately 14 h post-transfection, and the viral supernatants were collected 48 h after transfection.

To knock down AACS expression, 3T3-L1 cells were infected with lentiviruses encoding shRNA sequences against 4 different mouse AACS sequences (shAACS #1, GTTCAG TGGAATCGTCTAC; shAACS #2, CCGTGTGGTCGG CTATCTA; shAACS #3 ACAGTGTGTTCCTGGATGA; shAACS #4 GGCAGAGAGGAGATCGTGA) or control viruses encoding shRNA sequences against pGL3 (shcontrol #1, CTTACGCTGAGTACTTCGA) and LacZ (shcontrol #2, ATCGCTGATTTGTGTAGTC). After 2 d, the culture media were replaced with differentiation mix. The culture medium was changed every 2 d, and total RNA was extracted at the indicated time.

Oil Red O 3T3-L1 cells were fixed with 10% formalin (Wako) for 10 min at room temperature on day 8 after experimental treatment. Cells were then washed with 60% isopropanol followed by treatment with oil red O (1.8 mg/mL) for 20 min. After examining the plates microscopically, they were treated with 100% isopropanol to extract the oil red O. The

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solution was then measured for absorbance at 520 nm.

Microscopic Procedures 3T3-L1 cells were grown on cover glasses. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. The cells were permeabilized with 0.1% (v/v) Triton X-100 for 5 min and blocked with 3% (w/v) bovine serum albumin (BSA) for 30 min. After blocking, cells were incubated in Can Get Signal immunostain solution A (TOYOBO) containing anti-AACS[11] overnight at 4°C. Then cells were washed three times with 0.1% BSA in PBS and incubated with Alexa-fluor 488 secondary antibody (Invitrogen) at room temperature for 1h in the dark. After being washed in 0.1% BSA in PBS, the cells were covered with Mowiol mounting solution.

For visualization of DNA, preparations were stained with propidium iodide (Calbiochem). After incubation with the secondary antibodies, the cells were incubated with 1 mg/mL RNase in PBS. Then the cells were stained with propidium iodide for 15 min. After being washed in 0.1% BSA in PBS, the cells were covered with Mowiol mounting solution. Digital images of fixed cells were taken with a Confocal Laser Scanning Microscope (Bio-Rad).

For visualization of lipid droplets, preparations were stained with Nile red (Wako). After incubation with the secondary antibodies, the cells were incubated with 5 μg/mL Nile red. After washing in 0.1% BSA in PBS, the cells were covered with Mowiol mounting solution.

Protein Extraction and Western Blotting 3T3-L1 cells were lysed in RIPA buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 1% Triton X-100, 1% sodium lauryl sulfate and 0.1% sodium deoxycholate). Cell debris was removed by centrifugation at 14000×***g*** for 15 min at 4°C, and the resulting supernatant (cell lysate) was used for western blot analysis. The protein concentrations of the cell lysates were measured using a Bio-Rad protein assay kit.

For Western blotting, 15 μg of protein was separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were probed with specific anti-bodies, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies, and the proteins were detected with Immobilon Western Chemiluminescent HRP Substrate (Millipore).

RNA Extraction and Real-Time Polymerase Chain Reaction (PCR) Total RNA was purified from 3T3-L1 cells using an Illustra RNAspin Midi RNA Isolation Kit (GE Healthcare) according to the manufacturer's protocol. Total RNA from 3T3-L1 cells was analyzed using real-time PCR. One microgram of total RNA was reverse transcribed with the PrimeScript RT reagent kit with gDNA Eraser (Takara, Japan). The cDNA and gene-specific primers were added to SYBR Green PCR Master Mix (SYBR Premix Ex Taq, Takara) and subjected to PCR amplification in an Applied Biosystems StepOne (Applied Biosystems) instrument. The amplified transcripts were quantified using the standard curve method using the 18S ribosomal RNA (rRNA) as an internal control. The real-time PCR primers were based on data from GenBank (according to accession numbers) using the Primer Express software (Applied Biosystems). The sequences of the primers used are the following: 18S; forward primer 5′-TTC GTA TTG CGC CGC TAG A-3′ and reverse primer 5′-CTT TCG CTC TGG TCC GTC TT-3′, AACS; forward primer 5′-CTG GTC TGT CCG GTC GTA TAT G-3′ and reverse primer 5′-GTG AGT AGA CGA TTC CAC TGA ACT TC-3′, PPARγ; forward primer 5′-GCC CAC CAA CTT CGG AAT C-3′ and reverse

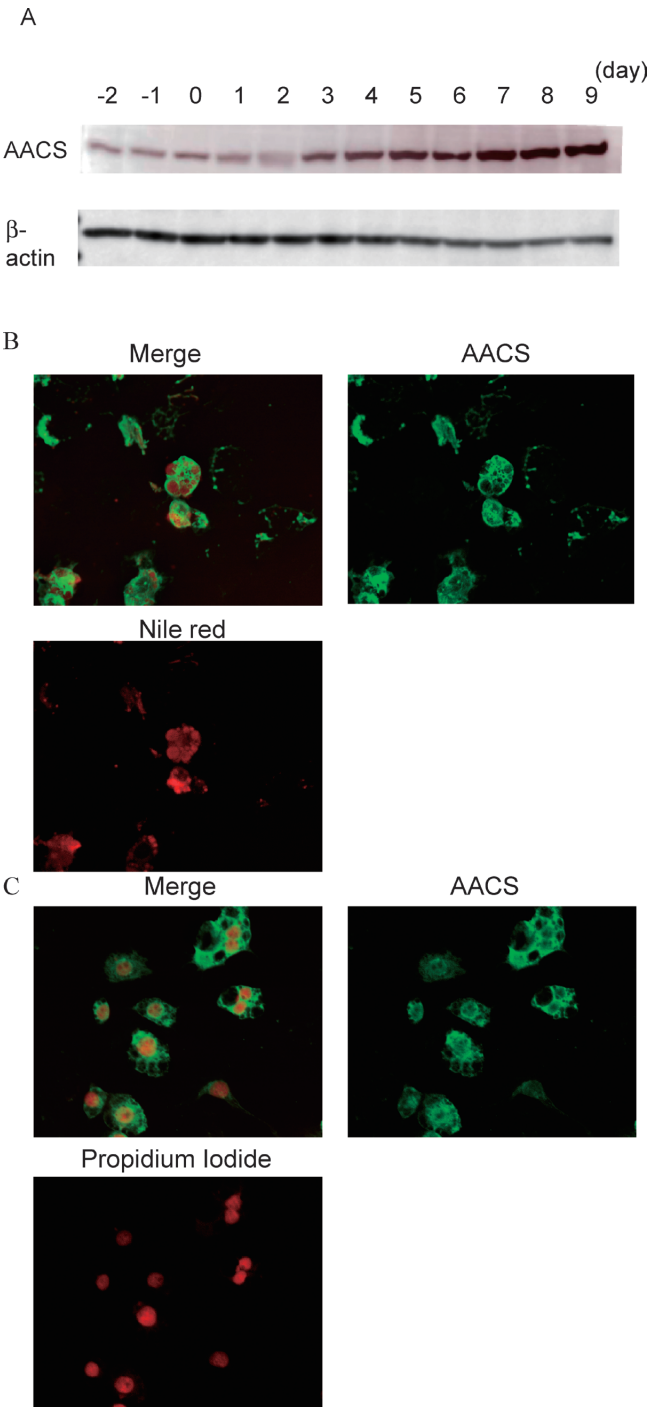

Fig. 1. Protein Expression and Localization of AACS in 3T3-L1 Cells

(A) Proliferating 3T3-L1 cells were cultured in 10% CS until they reached confluence (day −2). Two days post-confluence (day 0), cells were induced to differentiate by exposure to adipocyte differentiation inducers. The numbers indicate the days on which the cells were harvested. AACS and β-actin were detected by western blotting. (B) 3T3-L1 cells on day 8 after the initiation of differentiation were stained for AACS (green). For visualization of lipid droplets, preparations were stained with Nile red (red) as described in Materials and Methods. (C) 3T3-L1 cells on day 8 were stained for anti-AACS (green). For visualization of DNA, preparations were stained with propidium iodide (red) as described in Materials and Methods.

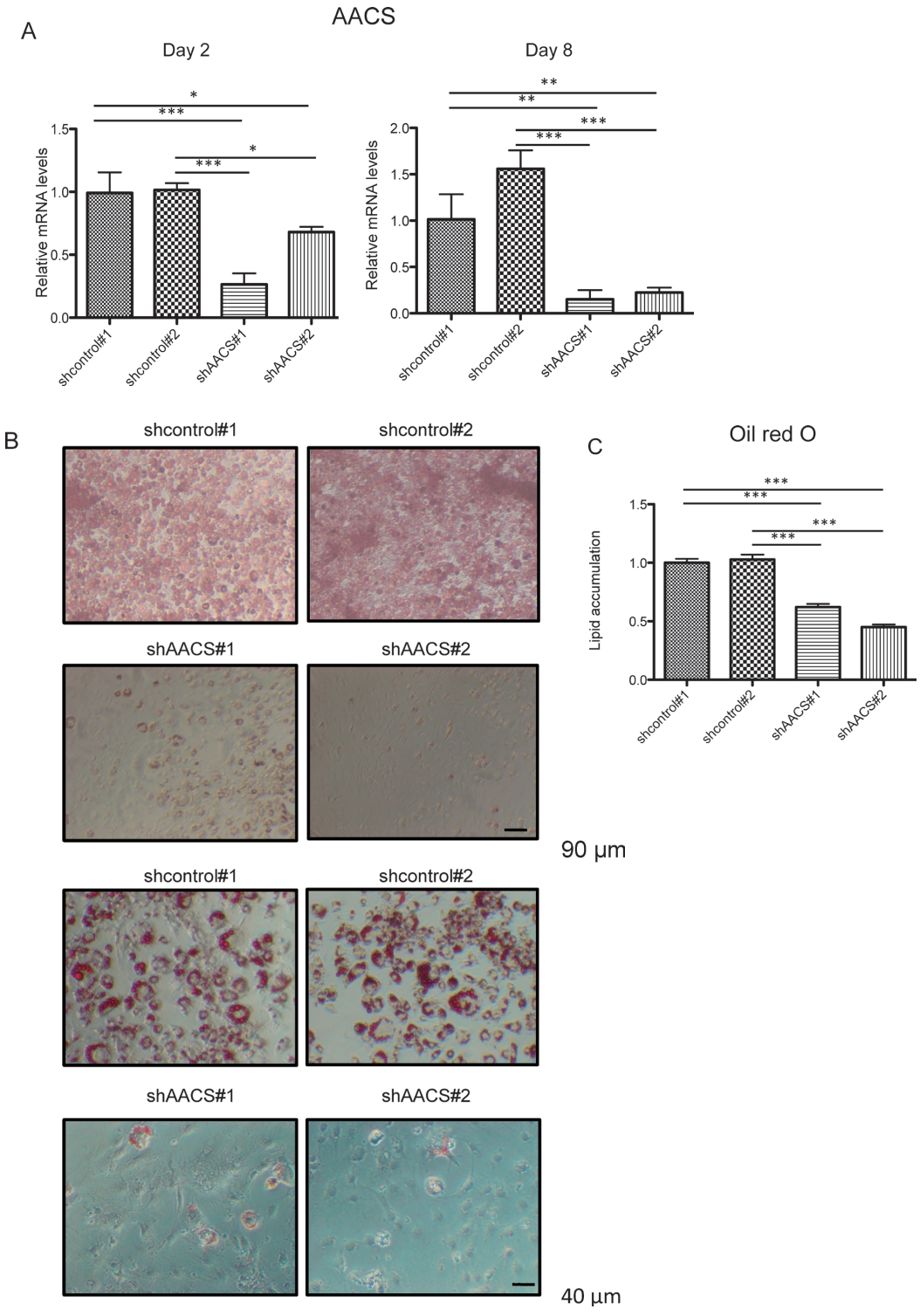

Fig. 2. The Effect of AACS Knockdown on 3T3-L1 Differentiation

(A) 3T3-L1 cells were infected with shcontrol or shAACS targeted lentivirus when cells reached confluence. Total RNA was extracted from 3T3-L1 cells at the indicated time. The expression of AACS was analyzed by real-time PCR, and the mRNA levels were normalized to 18S rRNA. The average expression values in the control are indicated as 1.0. Error bars indicate the standard deviation (S.D., $n=3$); *$p<0.05$; **$p<0.01$; ***$p<0.001$. (B) The 3T3-L1 cells were infected with shcontrol or shAACS targeted lentivirus when cells reached confluence. The cells were fixed with formalin on day 8 after the initiation of differentiation. Lipid droplets were stained with oil red O, and the cells were microscopically examined. Upper four panels; scale bar=90 μm. Lower four panels; scale bar=40 μm. (C) 3T3-L1 cells were infected with shcontrol or shAACS targeted lentivirus. After 8 d of differentiation, cells were stained with oil red O and treated with 100% isopropanol to extract the oil red O. The solution was then measured for absorbance at 520 nm. The error bars indicate the S.D. ($n=3$); ***$p<0.001$.

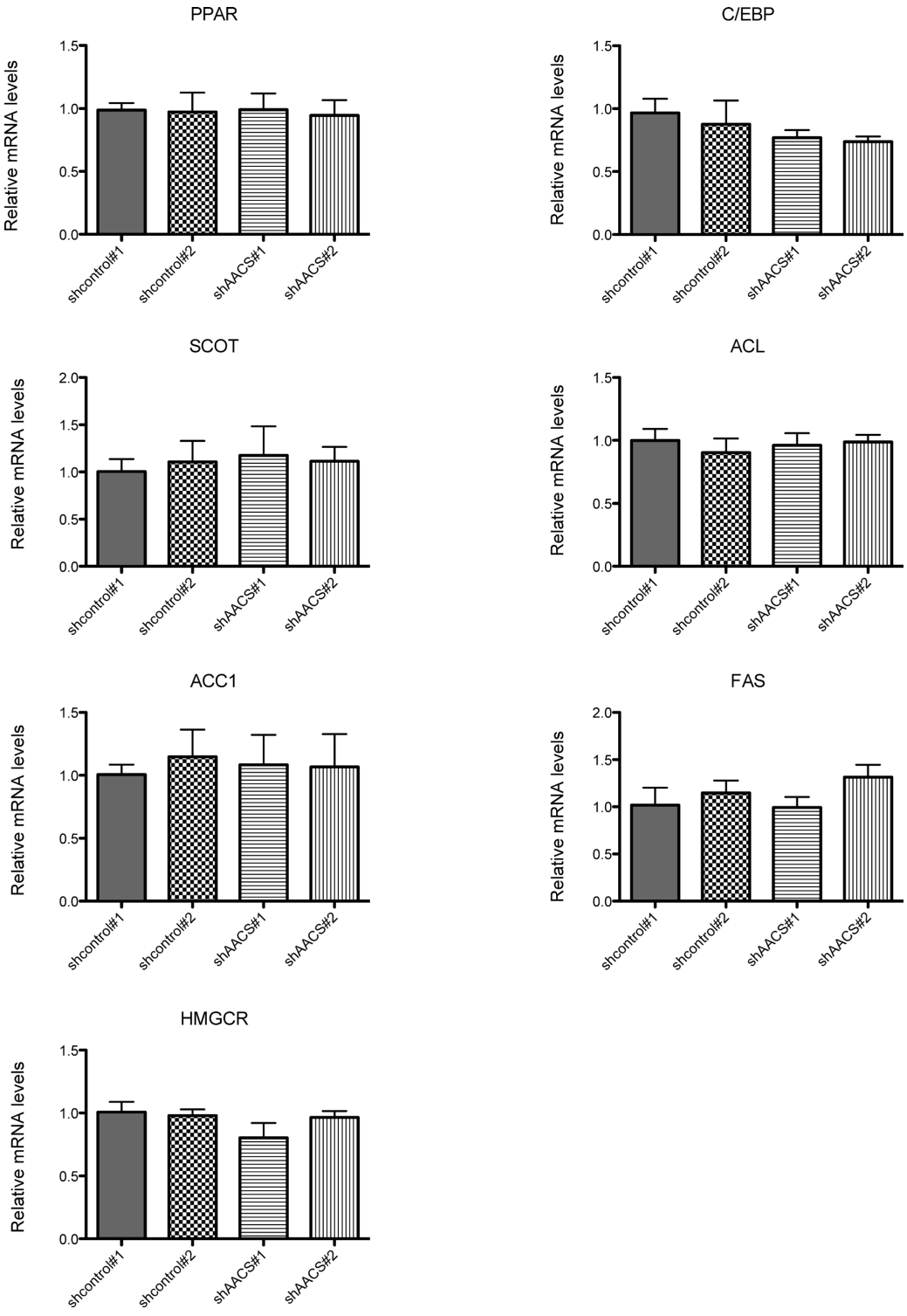

Fig. 3. Effect of shAACS on Gene Expression of Lipogenic Enzymes and Adipocytes Markers in 3T3-L1 Cells

3T3-L1 cells were infected with shcontrol or shAACS targeted lentivirus when cells reached confluence. Two days post-confluence (day 0), cells were induced to differentiate by exposure to adipocyte differentiation inducers. Total RNA was extracted from 3T3-L1 cells on day 2 after the initiation of differentiation. The mRNA levels of PPARγ, C/EBPα, SCOT, ACL, ACC-1, FAS and HMGCR were analyzed by real-time PCR. Gene expression was normalized to 18S rRNA. The average expression values in the control are indicated as 1.0. The data are shown as the mean±S.D. (n=3).

primer 5′-TGC GAG TGG TCT TCC ATC AC-3′, C/EBPα; forward primer 5′-AAA GCC AAG AAG TCG GTG GAC-3′ and reverse primer 5′-CTT TAT CTC GGC TCT TGC GC-3′.

RESULTS

Expression and Localization of the AACS Protein in 3T3-L1 Cells To investigate the function of AACS in adipocytes, we assessed the protein expression and localization of AACS in 3T3-L1 cells. The protein expression of AACS was

markedly increased by day 4 following the induction of differentiation (Fig. 1A). Then we assessed localization of AACS protein in 3T3-L1 adipocytes on day 8 following the induction of differentiation. The lipid droplets were stained with Nile red (Fig. 1B), and the nuclei were stained with propidium iodide (Fig. 1C). The AACS protein was mainly localized in the cytoplasm, especially near the nucleus. These results suggest that AACS supplies metabolic substrates for lipogenesis near the nucleus of 3T3-L1 cells.

Effect of AACS Knockdown on Differentiation of 3T3-L1 Next, we investigated the effects of AACS knockdown on adipocyte differentiation. After reaching confluence, 3T3-L1 preadipocytes were infected with lentivirus encoding short hairpin (sh) RNA targeting the AACS gene (shAACS). mRNA levels of AACS were significantly decreased on day 2 and 8 following shAACS treatment (Fig. 2A and Supplementary Fig. S1A). The number of differentiated 3T3-L1 cells was assessed using oil red O staining. Figures 2B and S1B show that 3T3-L1 cells following treatment of short hairpin control (shcontrol) were fully differentiated on day 8, whereas the rate of adipocyte differentiation was significantly reduced in the AACS knockdown cells. Correspondingly, the degree of oil red O absorption was significantly reduced in shAACS-treated cells compared with control cells (Fig. 2C and Supplementary Fig. S1C). These results suggest that AACS is an important factor in 3T3-L1 differentiation.

Effect of shAACS on mRNA Levels of Lipogenic Enzymes and Adipocyte Markers Adipocyte markers, such as PPARγ and C/EBPα, and lipogenic enzymes are important factors for adipocyte differentiation and lipid storage.[12,13] Therefore, we investigated the effect of shAACS on the expression of lipogenic genes and adipogenesis markers using real-time PCR (Fig. 3). PPARγ and C/EBPα mRNA levels were not altered in shAACS-treated cells compared with those in shcontrol-treated cells on day 2 following the induction of differentiation. SCOT, another ketone body-utilizing enzyme for energy production, was unchanged in shAACS-treated cells *versus* shcontrol-treated cells. Moreover, we analyzed the mRNA levels of fatty acid synthesis-related genes, such as ATP citrate lyase (ACL), ACC-1 and fatty acid synthase, and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), the rate-limiting enzyme of cholesterol synthesis, in each cell. We observed no difference in lipogenic gene expression between shAACS-treated cells and shcontrol-treated cells. These results suggest that the shAACS lentivirus treatment has no off-target effects on the other lipogenic genes on day 2 following the induction of differentiation.

Next, we analyzed mRNA levels of adipocyte markers on day 8 following the initiation of differentiation. Total RNA was extracted from 3T3-L1 cells on day 8, and PPARγ and C/EBPα were analyzed using real-time PCR (Fig. 4). Gene expression of PPARγ and C/EBPα were dramatically reduced by treatment with the shAACS lentivirus.

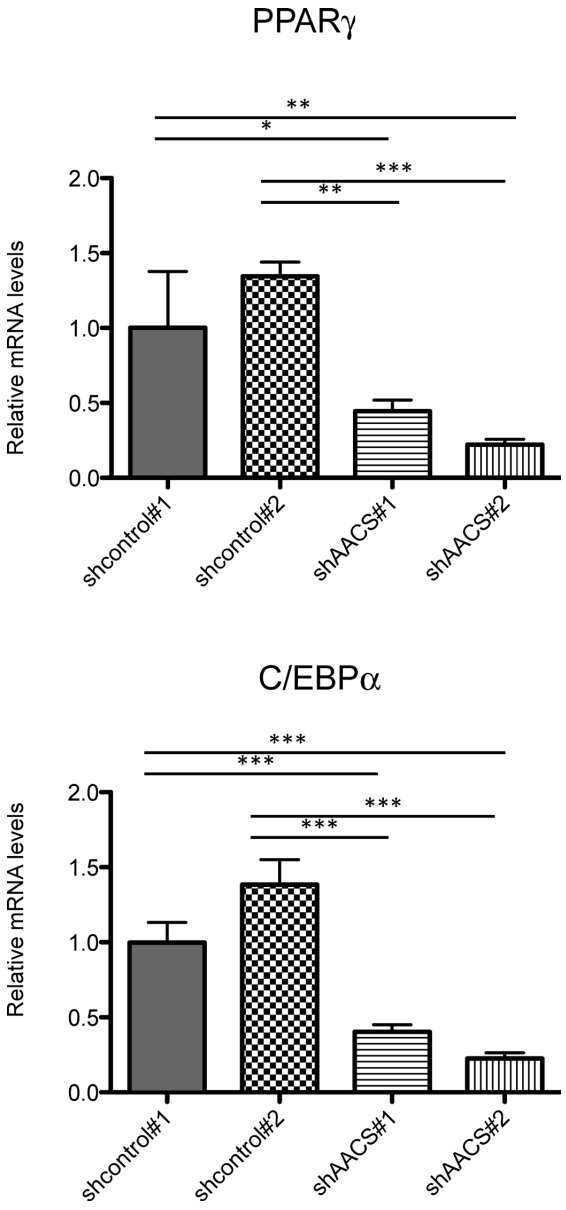

Fig. 4. Effect of shAACS on Gene Expression of Adipocyte Markers on Day 8 in 3T3-L1 Cells

3T3-L1 cells were infected with shcontrol or shAACS targeted lentivirus when cells reached confluence. Total RNA was extracted from 3T3-L1 cells on day 8 after the initiation of differentiation. The mRNA levels of PPARγ and C/EBPα were analyzed by real-time PCR. Gene expression was normalized to 18S rRNA. The average expression values in the control are indicated as 1.0. The data are shown as the mean±S.D. (n=3); *p<0.05; **p<0.01; ***p<0.001.

DISCUSSION

In this study, we presented evidence that ketone body utilization through AACS is a crucial step in adipocyte differentiation. Previous studies showed that gene expression of AACS is regulated by PPARγ and C/EBPα during adipogenesis.[8,10] In the present study, we showed that this regulation is an important step for adipocyte differentiation.

Because AACS was located in the cytoplasm near the nucleus and, to a lesser extent, in surrounding lipid droplets (Figs. 1B and C), AACS may supply acetyl units for lipogenesis in the cytoplasm surrounding the nucleus. Moreover, our data indicated that AACS is an important factor for 3T3-L1 differentiation (Figs. 2 and S1). A recent study showed that ACL has an important role in adipocyte differentiation and chromatin modification in the nucleus.[14] Cytosolic acetoacetyl-CoA is formed from two units of acetyl-CoA by cytosolic acetoacetyl-CoA thiolase,[15] which is a crucial substrate for the lipogenesis pathway, as is acetyl-CoA. Because thiolase activity is approximately 10-fold higher than AACS activity,[16]

it could be stipulated that direct activation of acetoacetate to acetoacetyl-CoA by AACS in the cytosol is an alternative reaction for the synthesis of lipidic substances. However, knockdown of AACS dramatically reduced the rate of adipocyte differentiation. Taken together, ACL and AACS may play different roles in the mechanism of 3T3-L1 differentiation.

Figures 2A and S1A show that knockdown of AACS is more effective in shAACS#1 and shAACS#4 treated-cells compared to shAACS#2 and shAACS#3 treated-cells, however, attenuation of adipogenesis seems to be more remarkable in shAACS#2 and shAACS#3 treated-cells (Figs. 2C, 4 and S1C). Moreover, gene expression of fatty acid synthase was inclined to increase in the shAACS#2-treated cells but not in the shAACS#1-treated cells (Fig. 3). We obtained the similar results in other cell types treated with shAACS against other sites of AACS gene (data not shown). These results suggest that they differ from one another in the effect on the cells. Database of Esembl predicts the existence of AACS transcript variants in many species. Taken together with our data, it is possible that mouse AACS gene has some transcript variants and that the different extent of attenuation of adipogenesis is due to the differential effect of shAACS on AACS transcript variants.

A previous study showed that the pattern of AACS expression during adipogenesis is similar to that of ACC-1, suggesting that the function of AACS is related to fatty acid synthesis.[9] However, the degree of ketone body incorporation into fatty acids in adipocytes is smaller than that in the liver[17] although AACS is abundantly expressed in adipocytes. Recent studies have shown that the HMGCR inhibitors, lovastatin and simvastatin, inhibit the differentiation of 3T3-L1 cells.[18,19] Moreover, farnesyl pyrophosphate, which is the precursor of almost all isoprenoids activates PPARγ, and promotes lipid accumulation during adipocyte differentiation.[20] These results suggest that the cholesterol biosynthetic pathway has an important role in adipocyte differentiation. As AACS expression is up-regulated by cholesterol depletion and ketone bodies are incorporated into cholesterol synthesis,[5,7] ketone body utilization by AACS may play an important role in cholesterol synthesis. Hence, the suppression of adipocyte differentiation can be attributed to the inhibition of cholesterol synthesis by knockdown of AACS. To understand the mechanisms of obesity and life style-related diseases, further studies on the role of acetyl unit utilization in lipogenesis during 3T3-L1 differentiation are now under way.

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