Shortened title : Up-regulation of adipogenin during adipogenesis

Title : Up-regulation of adipogenin, an adipocyte plasma transmembrane protein, during adipogenesis

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Abstract

Until now, the various proteins highly expressed in adipose tissues have been identified and characterized by traditional gene cloning techniques. However, methods of computer analysis have been developed that compare levels of expression among various tissues, and genes whose expression levels differ significantly between tissues have been found. Among these genes, we report on the possible function of a new adipose-specific gene, showed higher expression in adipose tissue through 'Search Expression' on Genome Institute of Norvartis Research Foundation (GNF) SymAtlas v0.8.0. This database has generated and analyzed gene expression of each gene in diverse samples of normal tissues, organs, and cell lines. This newly-discovered gene product was named adipogenin because of its role in stimulating adipocyte differentiation and development. Adipogenin mRNA was highly expressed in four different fat depots, and exclusively expressed in adipocytes isolated from adipose tissues. The level of adipogenin mRNA was up-regulated in the subcutaneous and visceral adipose tissues of mice fed a high-fat diet compared to those on the control diet. The expression of adipogenin mRNA is dramatically elevated during adipocyte differentiation of 3T3-L1 cells. Troglitazone, which up-regulated peroxisome proliferators-activated receptor γ2 (PPAR-γ2) expression, increased adipogenin mRNA expression, although this gene was down-regulated by retinoic acid. Confocal image analyses of green-fluorescent protein-adipogenin (pEGFP-adipogenin) transiently expressed in 3T3-L1 adipocytes showed that adipogenin was strictly localized to membranes and was absent from the cytosol.

Moreover, small interfering RNA (siRNA) mediated a reduction of adipogenin mRNA in 3T3-L1 cells and blocked the process of adipocyte differentiation. These results indicate that adipogenin, an adipocyte-specific membrane protein, may be involved with adipogenesis, as one of the regulators of adipose tissue development.

Key words : adipogenin, GNF SymAtlas, adipogenesis, high-fat diet

Introduction

White adipose tissue plays a critical role in the regulation of energy balance and acts as a secretory/endocrine organ that mediates numerous physiological and pathological processes [1, 3]. Excessive adipose tissue mass leads to obesity, which is a major risk factor for diseases like type 2 diabetes mellitus, hypertension, hyperlipidemia and cardiac infarction. Adipose tissue mass is determined by both the size and the number of the fat cells, or adipocytes. Increases in fat cell number (hyperplasia) are accomplished by the differentiation of preadipocytes to mature adipocytes, or adipogenesis [3, 4, 1, 28].

Studies on adipocyte differentiation have been greatly facilitated by availability of *in vitro* models of adipogenesis based on preadipocyte cell lines such as 3T3-L1 and 3T3-F422A [1]. At the molecular level, differentiation of preadipocytes to mature adipocytes results in differential expression of a variety of genes. The major transcription factors in the transcription network of adipogenesis include peroxisome proliferators-activated receptor γ (PPARγ), some of the CAAT/enhancer-binding proteins (C/EBP) family and adipocyte differentiation and determination factor 1 (ADD1)-sterol regulatory element binding protein 1 (SREBP1). It has been known that the expression of C/EBPβ and C/EBPδ contributes to the expression of PPARγ and C/EBPα, and ADD-1/SREBP-1 regulates the generation of PPARγ ligands that enhance the transcriptional activity of PPARγ[1]. Sequential activation of these transcription factors induces expression of adipocyte-specific genes, including enzymes, structural proteins, hormone receptors, and a variety of secreted factors involved in paracrine and

endocrine functions [3, 4].

Until now, several regulating factors specifically expressed in adipocytes and adipose tissue have been identified and characterized by the use of DNA chips, mRNA display and cDNA PCR subtraction. Recently, the mouse DNA (or protein) database provided by the public organizations Genome Institute of Norvartis Research Foundation (GNF), European Bioinformatic Institute (EBI), and Riken Expression Array Database (READ), detailed the expression profile of all genes. SymAtlas v0.8.0, which was provided by GNF (http://symatlas.gnf.org/SymAtlas/), showed the relative expression levels of each gene in all tissues [7, 16].

The purpose of this study is to identify adipogenesis-related factor(s) in adipose tissues and 3T3-L1 cells. To this end, we conducted an expression search to identify genes that were expressed in adipose tissues as highly and specifically as leptin and adiponectin using SymAtlas v0.8.0. We explored the functions of these genes by investigating their expression patterns in adipocyte differentiation and adipose tissues. Here, we report that one of them, which we now name adipogenin for its effect of inducing adipocyte differentiation, increases during adipogenesis and the development of adipose tissues.

Materials and Methods

Animals for dietary fat comparison

Three-week-old C57BL/6J mice were obtained from Charles River**,** Japan. They were housed individually in cages with wire-mesh bottoms at a temperature

of 20°C to 22°C, humidity of 50% to 60%, and under a 12h-light, 12h-dark cycle. The animals had free access to water and food (Oriental Yeast, Chiba, Japan) containing 8.5% (w/w) fat, 43.7% carbohydrate, and 29.7% protein, with an energy content of 3.69 kcal/g, for an acclimatization period of 1 week. Male and female C57BL/6J mice were then weighed and divided into two groups of six, with approximately equal mean body weights. One group was fed the standard diet (see above) and the other received a high-fat diet for 11 weeks (4 to 15 weeks of age). The high-fat diet was obtained from Research Diet and contained 41% fat, 36% carbohydrate, and 23% protein, with an energy content of 4.33 kcal/g; its fat source was the same as that of the standard diet and it contained the same absolute amounts of protein and fiber as did the standard diet. The animals were weighed weekly. At the end of the experimental period, the mice were killed by decapitation. White adipose tissues were rapidly separated from subcutaneous, perirenal, mesenteric, epididymal and parmetrical fat sites, immediately frozen in liquid nitrogen, and stored at -80C until RNA extraction. All experiments were conducted in accordance with the Shinshu University Guide for the Care and Use of Experimental Animals.

Cell culture of 3T3-L1 cells and differentiation

Murine 3T3-L1 preadipocytes were plated and grown to 2 days post-confluence in DMEM with 10% fetal bovine serum (FBS). The medium was changed every 48 h. Cells were induced to differentiate by changing the differentiation medium to methyl-3-isobutylxanthine (IBMX, 0.5mM),

dexamethasone (DEX, 0.25μM), and insulin (1μg/mL) in DMEM containing 10% FBS. Two days later, IBMX and DEX were removed and insulin was maintained for 2 more days. Thereafter, cells were grown in DMEM containing 10% FBS in the absence of differentiating reagents by replacing the media every 2 days. In another set of experiments, troglitazone (5 μM) or all-trans retinoic acid (0.1μM) was added to the differentiation medium on day 0 and this treatment was continued throughout the experimental period.

Isolation of adipocytes and stromal-vascular cells from adipose tissues

White adipose tissue taken from the parametrial regions of 26-week-old female mice was dissected from the connective tissue and blood vessels. The adipose tissue was divided into stromal-vascular cell and adipocyte fractions. Briefly, freshly excised fat pads from 26-week-old female mice were rinsed in Krebs-Ringer bicarbonate (KRB), minced, and digested for 40 min at 37°C in KRB (pH 7.4) with 3.5% bovine serum albumin and 0.5 mg/ml type I collagenase (Worthington, Freehold, NJ). The digested tissue was filtered through a 250μm nylon mesh to remove undigested tissue and centrifuged at 500*g* for 5 min. The floating adipocyte fraction was removed, washed in buffer, and recentrifuged to isolate free adipocytes. The stromal-vascular pellet was resuspended in an erythrocyte lysis buffer (154 mM NH_4Cl, 10 mM $KHCO_3$), filtered through a 28μm nylon mesh to remove endothelial cells, and pelleted at 1500*g* for 5 min. Isolated adipocytes and stromal-vascular cells were used for the measurement of adipogenin mRNA.

Total RNA extraction and semi-quantitative RT-PCR

The total RNA was extracted from fresh dissociated adipocytes, stromal-vascular cells, adipose tissues by the acid guanidium -thiocyanate-phenol-chloroform method. Total RNA from 3T3-L1 cells was extracted using Trizol Reagent (Gibco BRL, Rockville, MD). Semi-quantitative RT-PCR was performed to measure levels of adipogenin and PPAR-γ2 mRNA. ß-actin, the housekeeping gene, was used as an internal control. The primers used for semi-quantitative RT-PCR were: adipogenin forward primer (5'-TATGCGATGTGAAGTACCCTCT-3'), adipogenin reverse primer (5'-CCCCCATTTCTTTAATAGGT-3'); PPAR-γ2 forward primer (5'-TGGGTGAAACTCTGGGAGAT-3'), PPAR-γ2 reverse primer (5'-CCATAGTGGAAGCCTGATGC-3'); ß-actin forward primer (5'-AGGTCATCACTATTGGCAAC-3'), ß-actin reverse primer (5'-ACTCATCGTACTCCTGCTTG-3'). To determine the cycle numbers that would correspond to the amplification range of all PCR products for each reaction, PCR was performed 25-35 cycles on cDNA generated from a single RT reaction. Therefore, amplification of adipogenin, PPAR-γ2 and ß-actin were performed on cycles 28, 30 and 31, respectively, which occurred during linearly increasing phase. Similar results were obtained in at least two or three independent experiments. PCR products were resolved in a 1.2% agarose gel, and the DNA was visualized by ethidium bromide staining and analyzed using NIH image software. The mRNA levels of adipogenin were corrected for using the

transcription level of the ß-actin gene as an internal standard. The amplified cDNAs were subcloned into the pGEM-T easy vector (Promega, Madison, WI), and the sequences were confirmed using an automated DNA sequencer (ABI 310).

Preparation of EGFP constructs of adipogenin

A fusion protein construct bearing the coding region of adipogenin was prepared by subcloning an RT-PCR product of adipogenin into an N-terminal pEGFP-N1 vector (BD Biosciences CLONTECH, Palo Alto, CA) in frame with EGFP. Briefly, PCR was performed using cDNA of 3T3-L1 adipocytes as a template and the following primers**:** forward 5'-CCGCTCGAGCGGATGAAGTACCC-3' and reverse 5'-GGAATTCCCCAGTGGAGTCCGTC-3'. The PCR product was purified from an agarose gel, digested with XhoI and EcoRI and cloned into the XhoI/EcoRI sites of the pEGFP vector. Double-stranded sequencing of all cloned cDNA of the adipogenin protein was performed by automated fluorescent dideoxynucleotide sequencing. As a positive control, a pEGFP-N1 vector expressing only EGFP was used.

Cell transfection and visualization of EGFP-adipogenin constructs

3T3-L1 cells cultured on poly-*d*-lysine-coated coverslips were transiently transfected with vectors containing adipogenin protein cDNAs. Transfections

were performed using Lipofectamine (Gibco BRL, Rockville, MD), and transfected cells were used in experiments 24 h following transfection. For visualization of EGFP-tagged adipogenin proteins in 3T3-L1, cells were fixed by treatment with 4% paraformaldehyde for 20 min at room temperature, followed by permeabilization with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and 0.1% Nonidet P-40 for 10 min at room temperature. After permeabilization, cells were treated with PBS containing 100 μg/ml RNase A for 30 min at room temperature prior to staining with 0.5 μg/ml propidium iodide for 10 min, followed by three washes with PBS. Images were captured using laser-scanning confocal microscopy (IX70, Olympus, Tokyo).

siRNA construction and transfection

Short interfering RNA (siRNA) oligonucleotides were designed to interact in adipogenin mRNA using the siRNA design tool provided by Dharmacon Research (Lafayette, CO). Oligonucleotide sequences were: 5' -AAUGCUGUUCUUGACGAUCGU- 3' (adipogenin siRNA), and 5' -AAUCAACUGACUCGACCACUA- 3' (scrambled siRNA). The scrambled siRNA was used as a negative control. The siRNAs were constructed employing the Silencer^{TM} siRNA construction kit (Ambion, Austin, TX, USA) according to the protocol provided by manufacturer. Upon confluence, the 3T3-L1 cell media were changed to growth media without antibiotics. Two days later, cells were transfected with siRNAs (50nM) using the siPORT^{TM} lipid transfection reagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Five

volumes of differentiation medium without antibiotics were added 4 h after transfection and the cells were maintained at normal growing conditions and induced to differentiate as described above. The down-regulation of the adipogenin targeted by siRNA was confirmed by analysis of its levels of expression using RT-PCR.

Statistical analysis

Data in Figures are presented as means ± SEM of 3-6 experiments with the same protocol. Comparisons were tested by ANOVA, followed by Fisher's test for protected least significant difference as a posthoc analysis. Significance was set at $P < 0.05$.

Results

Identification of adipogenin in GNF database

The 'Search Expression' facility in Symatlas provided the relative expression of each gene across all tissues. To profile the genes that were highly expressed in adipose tissue, we chose 'adipose tissue' and entered a value of '100-folds over median'. In the results, which included a few known genes such as adipsin and adiponectin, one gene (Genbank No. BC054059) of unknown function was expressed relatively higher in adipose tissue than in other tissues in the GNF database. The open reading frame of this gene, which we named 'adipogenin',

consists of 240 nucleotides specifying a protein of 80 amino acid residues, corresponding to a predicted molecular weight of 9355 Da. We designed specific base primers according to the sequence from the database, and further analyzed how this gene regulates adipose tissue and 3T3-L1 adipocytes.

Level of adipogenin mRNA in adipose tissue and isolated adipocytes

To confirm that the expression of adipogenin in all mouse tissues matched that shown in the expression profiles in the GNF database, adipogenin mRNA expression was examined. Levels of adipogenin mRNA were higher in four different white adipose tissues than in the other tissues in our RT-PCR results (Figure 1A). Low but detectable adipogenin levels were also found in other tissues such as heart, stomach and muscle, but very low, barely detectable levels were found in kidney and lung. To investigate whether adipogenin was expressed in isolated adipocytes, stromal-vascular cells and adipocytes from adipose tissues of female mice aged 26 weeks were examined. As shown in Figure 1B, the adipogenin transcript was not detected in stromal-vascular cells; it was expressed exclusively in the adipocytes.

Understanding how gene expression is regulated in varying states of nutrition may provide valuable clues about its functional relevance in metabolic disease states. Accordingly, we next examined adipogenin gene expression in adipose tissue from mice fed high-fat and normal diets. Adipose tissue was isolated for analysis from 15-week-old mice that had been fed either a normal diet or a high-fat diet for 11 weeks. At 15 weeks, the body weight of high-fat diet mice

was 21% more than that of normal diet mice. In addition, the amounts of the four different fats in the mice fed the high-fat diet were approximately 2-3 times greater than those in mice fed the normal diet. The level of adipogenin and PPAR-γ2 mRNA were up-regulated in all four adipose tissues of the high-fat diet mice compared to normal diet male and female mice (Figure 2).

Regulation of adipogenin mRNA during the differentiation

To examine the expression pattern of adipogenin during the process of adipocyte differentiation, the levels of adipogenin mRNA were examined during differentiation in 3T3-L1 cells (Figure 3). Adipogenin mRNA was induced and up-regulated after the differentiation medium was changed; its expression was not detected in confluent cells before differentiation. The expression of PPAR-γ2 gradually increased after differentiation. As the expression of PPAR-γ2 is significantly increased in differentiated adipocytes and it is a well-known master regulator of adipogenesis, it has been widely used as an index of preadipocyte differentiation. To investigate whether up- and down-regulation of PPAR-γ2 is related to the expression of adipogenin mRNA, 3T3-L1 cells were treated by troglitazone and retinoic acid during adipocyte differentiation. Troglitazone treatments stimulated PPAR-γ2 mRNA expression during differentiation for 7 days**,** during which time the level of adipogenin mRNA was significantly up-regulated (Figure 4A). However, retinoic acid, which inhibited the PPAR-γ2 expression, down-regulated the expression of adipogenin (Figure 4B).

Localization of adipogenin fusion protein in 3T3-L1 cells

SOSUI (Secondary Structure Prediction of Membrane Proteins; http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) analysis of the deduced adipogenin amino acid sequence predicted a membrane localization of the protein. To determine whether adipogenin protein is, in fact, located in the membrane, an expression vector for the adipogenin open reading frame-GFP fusion protein was transfected into 3T3-L1 cells and its localization analyzed by confocal microscopy. As shown in Figure 5, the pattern of EGFP-adipogenin appears more compact and near the periphery of the plasma membrane. By contrast, EGFP alone was distributed diffusely throughout both the nuclear and cytoplasmic compartments. The distributions of the EGFP-adipogenin constructs were consistent and the same in stably transfected cells.

Inhibitory effect of adipogenin siRNA during adipocyte differentiation

Adipogenin siRNA silenced its target mRNA specifically and effectively in 3T3-L1 cells. To determine if the reduction of adipogenin involves alteration in the expression of genes encoding a key adipogenic transcriptor factor, we examined the expression of PPAR-γ2 and adipocyte fatty acid binding protein (aP2) during the differentiation of cells that had been transfected with adipogenin siRNA. PPAR-γ2 and aP2 mRNA levels declined in the transfected cells compared with the control cells (Figure 6A and 6B). In addition, the number of Oil-Red-O staining cells was dramatically less in adipogenin siRNA-transfected cells (Figure 6C).

Discussion

These experiments demonstrated that adipogenin, one candidate gene that was relatively highly expressed in adipose tissues in the GNF database, may be involved and required in the development of adipose tissues and in the differentiation process of adipocytes. So far, there are several techniques, mRNA differential display, cDNA subtraction, and DNA microarray, that have been developed to discover new functional genes in adipose tissue. Preadipocyte factor-1 [4], adiponutrin [36], fad158 [33] and ADSF/resistin [14] were identified and characterized using these tools. However, the present study suggested that computer analysis based on searching an expression profile could be a new alternative tool to discover a candidate gene specifically expressed in adipose tissues. Adipogenin, which was obtained and identified from Symatlas, might be one of the regulating factors in the development of adipose tissue and adipogenesis.

White adipose tissue is the major energy reserve in higher eukaryotes, used for storing triacylglycerol in periods of energy excess and for mobilizing it during energy deprivation. By expressing of a variety of proteins involved in lipid metabolism, as well as several secreted molecules, this tissue plays a key role in the control of energy balance. Adipogenin mRNA, which was highly expressed in adipose tissues, was up-regulated in four different white fats in mice fed a high-fat diet compared to control diet. Adipogenin mRNA was up-regulated on adipocyte differentiation and was not detected in confluent 3T3-L1 cells. Furthermore,

adipogenin mRNA was not detected in stromal-vascular cells but, rather, was found exclusively in the adipocytes.

The interesting point to consider is the regulation of adipogenin under the transcription network controlling adipocyte differentiation. The present study showed that the expression of adipogenin mRNA was elevated during the adipogenesis of 3T3-L1 cells, and up- and down-regulation of PPAR-γ2 regulated this gene. The nuclear hormone receptor PPARγ in already well understood to play central roles in this process . As with some other nuclear receptors, it heterodimerizes with the retinoid X receptor to regulate adipogenesis as well as to control the expression of adipocyte-specific genes such as aP2, which are involved in the maintenance of adipocyte phenotype [4, 3, 12, 7]. We performed a functional analysis of adipogenesis using the siRNA knockdown method. When adipogenin expression was knocked down, the accumulation of oil droplets decreased and further expression of PPAR-γ2, an adipocyte differentiation marker**,** declined during adipogenesis. These results suggest that adipogenin has an additional, obligatory role in adipogenesis in the transcriptional cascade of PPAR-γ2.

Protein-protein BLAST searches of various databases did not provide clues to the potential role of the adipogenin. On the other hand, the SOSUI system predicted a conserved single transmembrane domain spanning 23 amino acid residues, indicating that adipogenin might be an integral membrane protein. This clearly confirmed the results of our transfection experiment using pEGFP-adipogenin: that this gene was localized to membranes and was absent from the cytosol. Recent reports have explained that various transmembrane

proteins have an important role in development of adipose tissue and obesity. Liu et al. [6] reported that adiponutrin, a transmembrane protein, is regulated by the energy balance in human adipose tissue. Transmembrane tumor necrosis factor (mTNF)-alpha is reported to be present in adipose tissue and its production is significantly increased in different rodent obesity models as well as in obese humans [6]. TPRA40 (a transmembrane domain protein of 40 kDa, regulated in adipocytes) represents a novel membrane-associated protein whose expression in white adipose tissue is altered with aging and the presence of type 2 diabetes [6]. Like these membrane proteins, adipogenin - as a membrane protein of adipocytes - may have an important role in adipocyte differentiation and development.

The results described herein highlight the involvement of adipogenin in adipocyte differentiation and adipocyte development, and indicate that adipogenin is highly up-regulated during adipocyte differentiation and that it is regulated by the expression of PPAR-γ2 mRNA. In addition, these results strongly suggest that adipogenin has an important role in facilitating the maturation of 3T3-L1 cells into adipocytes and adipose tissue depots.

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Acknowledgements

This work was partly supported by Grant-in-Aid (No.15780178 to Sang-Gun Roh; No.15580246 to Shinichi Sasaki) for scientific research from the Ministry of Education, Science, Sports and Culture of Japan.

Figure legends

Figure 1. (A) The levels of adipogenin mRNA in various adult mouse tissues. Upper panel: representative ethidium bromide-stained agarose gel showing amplified adipogenin (483 bp) and β-actin (363 bp) with molecular markers. Lower panel: data were normalized using β-actin mRNA and expressed as fold of the value obtained for lung. All data represent means±SEM of six independent experiments. M: 100 bp molecular size ladder; WAT: white adipose tissue; N.D: No detected *P<0.05 vs lung. (B) The levels of adipogenin mRNA in adipocytes and stromal-vascular cells isolated from mouse parametrial adipose tissue. Upper panel: representative ethidium bromide-stained agarose gel showing amplified adipogenin (483 bp) and β-actin (363 bp) with molecular markers. Lower panel: data were normalized using β-actin mRNA and expressed as fold of the value obtained for adipocytes. All data represent means±SEM of three independent experiments. M: 100 bp molecular size ladder; SV: stromal-vascular cells; AD: adipocytes; N.D: No detected *P<0.05 vs adipocytes.

Figure 2. The levels of adipogenin mRNA in adipose tissues of male and female mice fed normal (N) and high-fat (H) diets. Upper panel: representative ethidium bromide-stained agarose gel showing amplified adipogenin (483 bp), PPAR-γ2 (454 bp) and β-actin (363 bp) with molecular markers. Lower panel: data were normalized using β-actin mRNA and expressed as fold of the value obtained for subcutaneous adipose tissue of mice fed the normal diet. All data represent means±SEM of six independent experiments. M: 100 bp molecular size ladder;

Sub: subcutaneous adipose; Per: perirenal adipose; Mes: mesenteric adipose; Epi: epididymal adipose; Par: parametrial adipose. *P<0.05 vs mice fed normal diet.

Figure 3. The levels of adipogenin and PPAR-γ2 mRNA during the differentiation of 3T3-L1 cells. 3T3-L1 cells were proliferated to confluence and then were allowed to differentiate to adipocytes in differentiation medium. Total RNA was extracted from 1 h to day 8 after the differentiation medium was changed. Upper panel: representative ethidium bromide-stained agarose gel showing amplified adipogenin (483 bp), PPAR-γ2 (454 bp) and β-actin (363 bp) with molecular markers on different days of differentiation indicated on top. Lower panel: data were normalized using β-actin mRNA and expressed as fold of the value obtained at day 3. All data represent means±SEM of three independent experiments. M: 100 bp molecular size ladder. ^{abc}Mean values with different superscripts are significantly different (P < 0.05).

Figure 4. (A) The levels of adipogenin in 3T3-L1 adipocytes treated with or without troglitazone (5 μM) during differentiation. Upper panel: representative ethidium bromide-stained agarose gel showing amplified adipogenin (483 bp), PPAR-γ2 (454 bp) and β-actin (363 bp) with molecular markers on different days of differentiation indicated on top. Lower panel: data were normalized using β-actin mRNA and expressed as fold of the value obtained at day 3 with no treatment. (B) The levels of adipogenin (483 bp) mRNA in 3T3-L1 adipocytes treated with retinoic acid (RA, 0.1μM) during differentiation. Upper panel:

representative ethidium bromide-stained agarose gel showing amplified adipogenin (483 bp), PPAR-γ2 (454 bp) and β-actin (363 bp) with molecular markers on different days of differentiation indicated on top. Lower panel: data were normalized using β-actin mRNA and expressed as fold of the value obtained at day 3 with no treatment. All data represent means±SEM of three independent experiments. M: 100 bp molecular size ladder; N.D: No detected *P<0.05 vs no treatment.

Figure 5. Visualization of cellular localization of the adipogenin-GFP fusion protein in 3T3-L1 cells. 3T3-L1 cells were transfected with pEGFP-adipogenin or a control expression vector. The GFP (green) fluorescence and propidium iodide (PI; red) are presented on the merged image. The localization of the fusion protein was assessed by confocal microscopy. The scale bars represent 50.0μm.

Figure 6. Adipocyte differentiation in siRNA-transfected 3T3-L1 cells. (A) The levels of adipogenin, PPAR-γ2 and aP2 mRNA in 3T3-L1 cells transfected with adipogenin and scrambled siRNAs during differentiation for 7 days. Representative ethidium bromide-stained agarose gel showing amplified adipogenin (483 bp), PPAR-γ2 (454 bp), aP2 (366 bp) and β-actin (363 bp) with molecular markers. M: 100 bp molecular size ladder. (B) The mRNA levels of adipogenin, PPAR-γ2 and aP2 in siRNA-transfected 3T3-L1 cells. Data were normalized using β-actin mRNA and expressed as fold of the value obtained for controls. All data represent means±SEM of three independent experiments. *P<0.05 vs control. (C) 3T3-L1 cells were cultured and transfected with 50 nM

scrambled siRNA (control) and adipogenin siRNA (siAdipogenin) prior to induction of differentiation. After 7 days, the cells were fixed and stained with Oil-Red-O to detect oil droplets.

Figure

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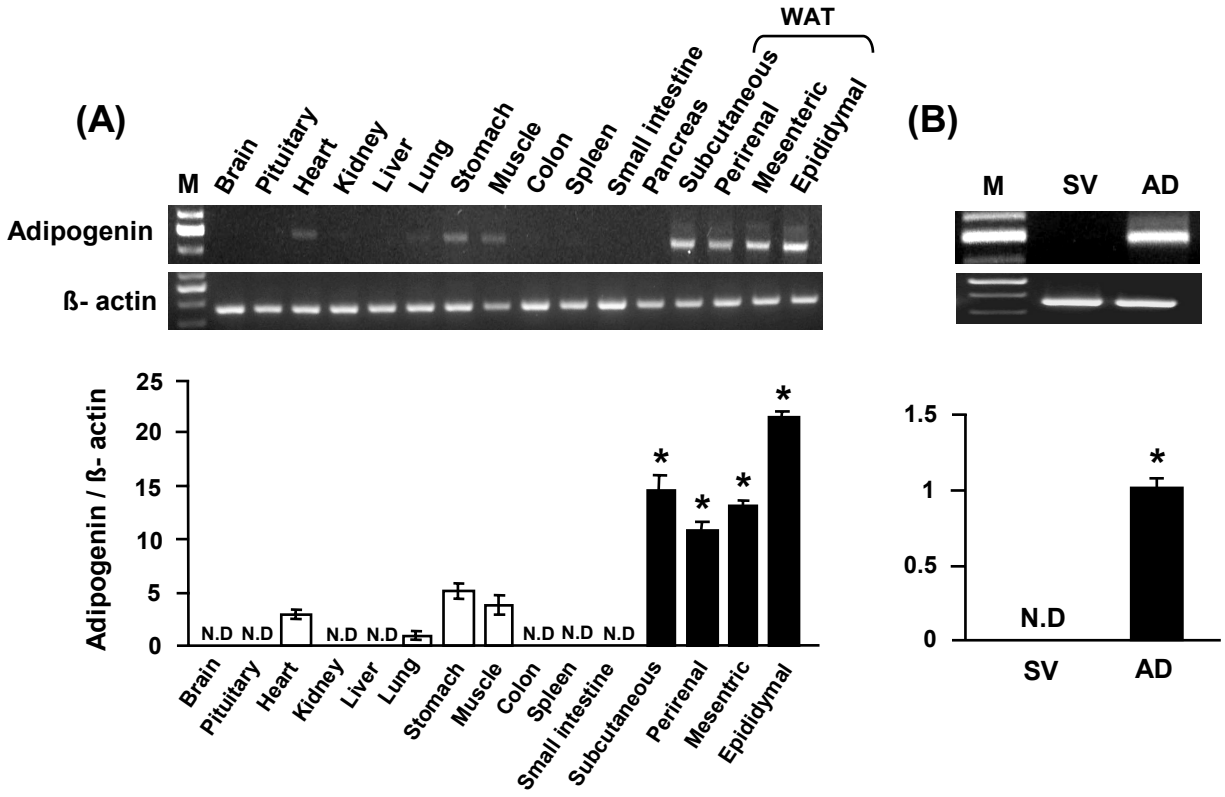

Figure 2. Hong YH et al.

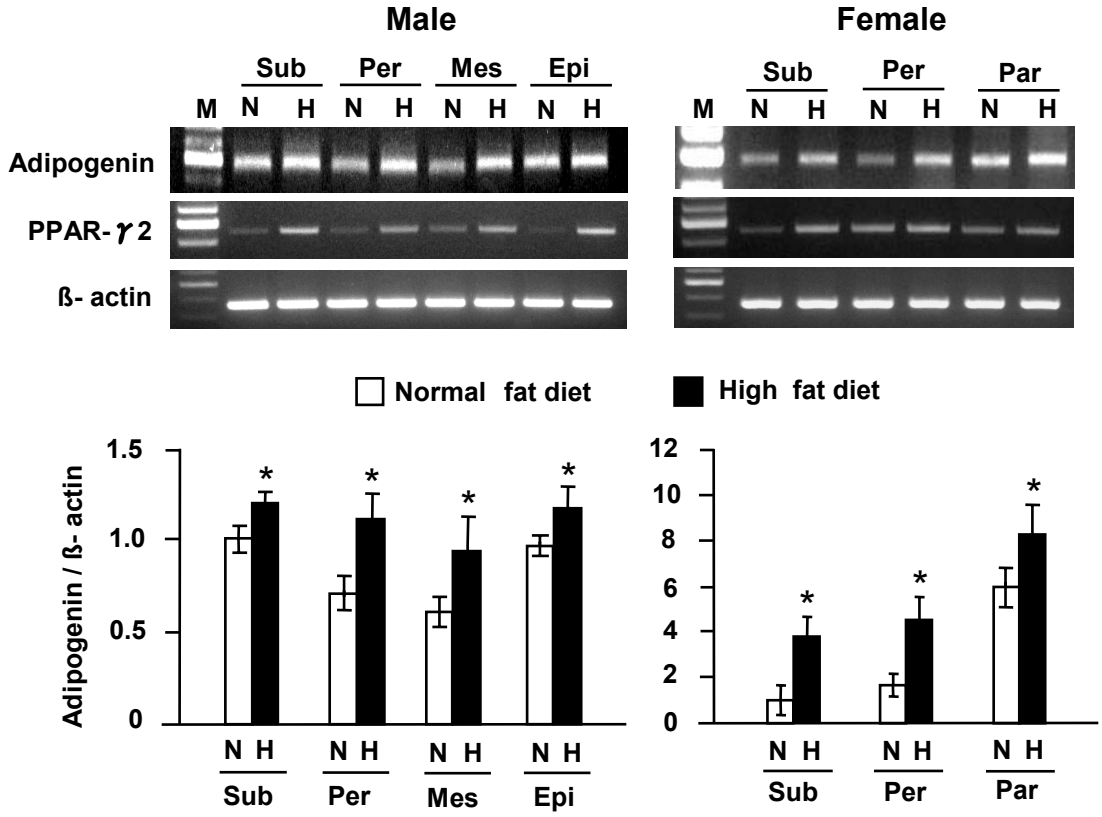

Figure 3. Hong YH et al.

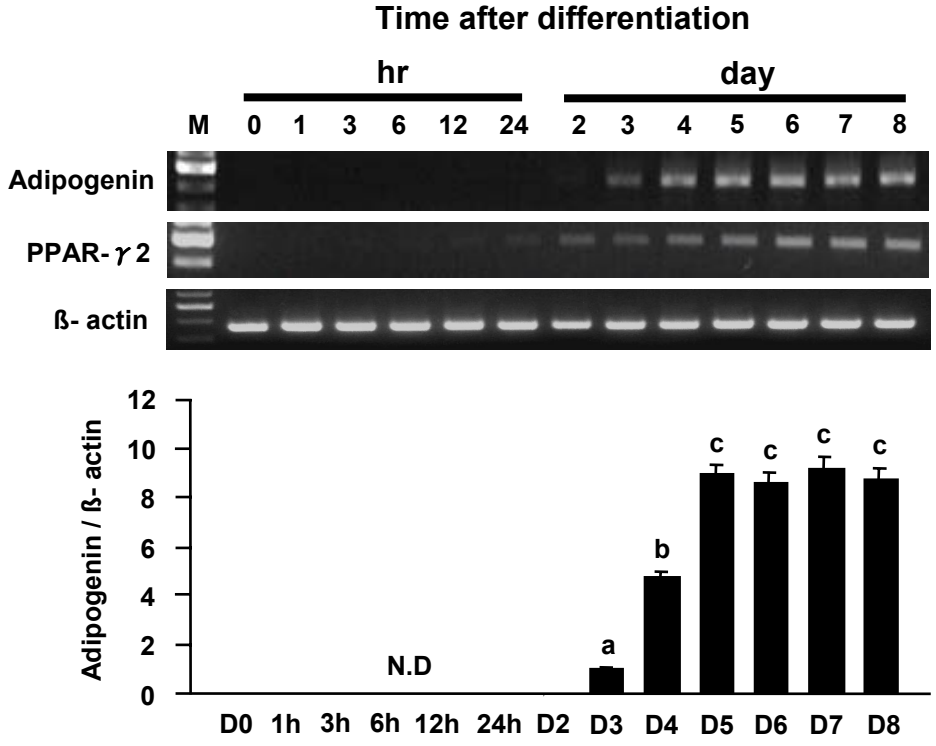

Figure 4. Hong YH et al.

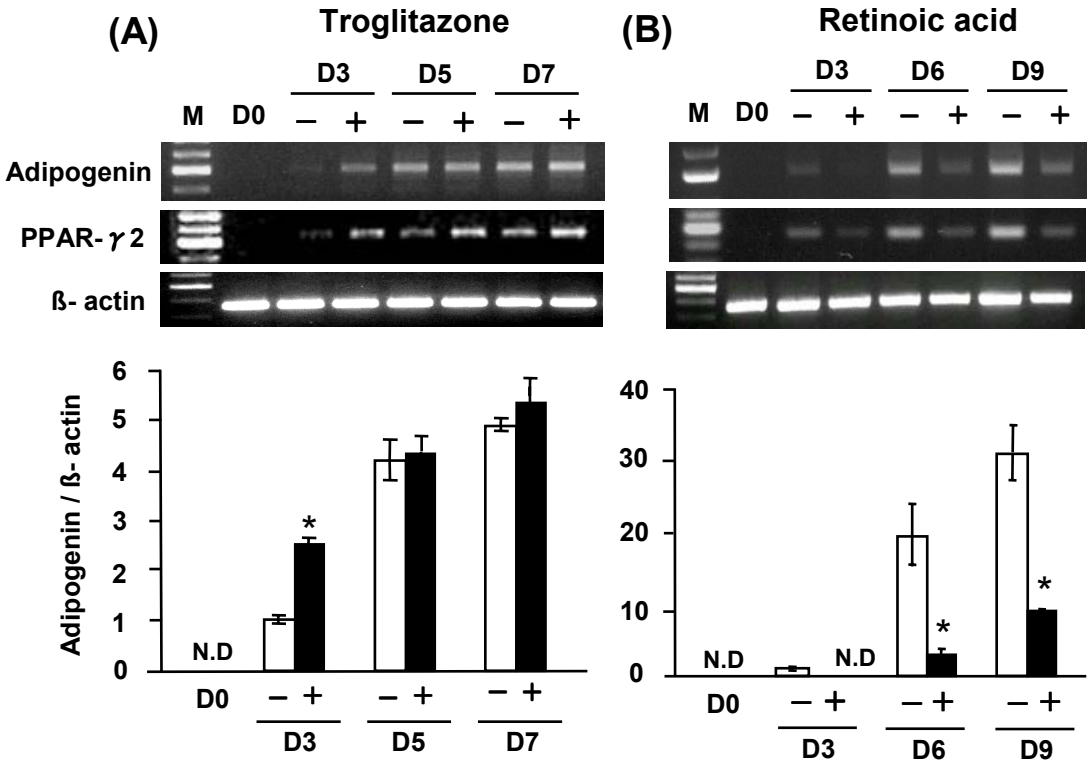

Figure 5. Hong YH et al.

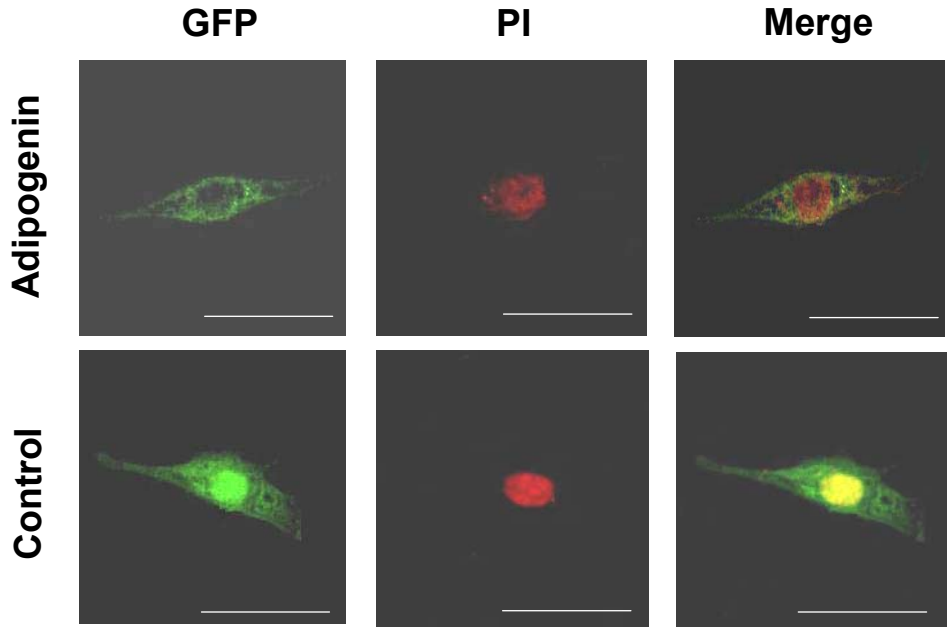

Figure 6. Hong YH et al.