

# Shikonin Inhibits Fat Accumulation in 3T3-L1 Adipocytes

Haeyong Lee, Ryunhwa Kang, and Yoosik Yoon\*

Department of Microbiology, College of Medicine, Chung-Ang University, Seoul 156–756, Korea

Shikonin, 5,6-dihydroxyflavone-7-glucuronic acid, is the main ingredient of *Lithospermum erythrorhizon* Sieb. et Zucc. and was reported to have various biological activities including antiinflammatory, anticancer, antimicrobial and others. This study aimed to elucidate, for the first time, the antiobesity activity of shikonin and its mechanism of action. Shikonin was found to inhibit fat droplet formation and triglyceride accumulation in 3T3-L1 adipocytes. The half inhibitory concentration,  $IC_{50}$ , for the inhibition of triglyceride accumulation was found to be 1.1  $\mu$ M. The expression of genes involved in lipid metabolism, such as FABP4 and LPL, were significantly inhibited following shikonin treatment. Shikonin also inhibited the ability of PPAR $\gamma$  and C/EBP $\alpha$ , the major transcription factors of adipogenesis, to bind to their target DNA sequences. The expressions of mRNA and protein of PPAR $\gamma$  and C/EBP $\alpha$  were significantly down-regulated following shikonin treatment. Among the upstream regulators of adipogenesis, only SREBP1C was found to be down-regulated by shikonin. The results of this study suggest that shikonin down-regulates the expression of SREBP1C and subsequently the expression of PPAR $\gamma$  and C/EBP $\alpha$ . Together, these changes result in the down-regulation of lipid metabolizing enzymes and reduced fat accumulation. Copyright © 2009 John Wiley & Sons, Ltd.

**Keywords:** obesity; adipogenesis; shikonin; PPAR $\gamma$ ; C/EBP $\alpha$ ; SREBP1C.

## INTRODUCTION

Shikonin (see structure in Fig. 1A) is a purple naphthoquinone dye isolated from the root of a medicinal plant, *Lithospermum erythrorhizon* Sieb. et Zucc. This plant has long been used as a traditional herbal medicine for a variety of inflammatory and infectious diseases including macular eruption, measles, sore-throat, carbuncles, burns and wound healing. The antiinflammatory activities of shikonin were reported to involve the inhibition of leukotriene B4 biosynthesis, inhibition of cyclooxygenase-2 transcription, suppression of mast cell degranulation, inhibition of neutrophil respiratory burst, impairment of phosphatidylinositol signaling and blockage of chemokine binding to its receptor (Wang *et al.*, 1994, 1995; Chen *et al.*, 2001; Subbaramaiah *et al.*, 2001). The anticancer activities of shikonin were reported to involve the induction of apoptosis and the inhibition of DNA topoisomerase (Fuji *et al.*, 1992; Yoon *et al.*, 1999). Other activities include antimicrobial activity, antiplatelet activity, wound healing activity and so on, as reviewed by Chen *et al.* (2002). However, the antiobesity activity of shikonin has not been reported until now.

Adipogenesis is a differentiation process by which undifferentiated preadipocytes are converted to fully differentiated adipocytes, i.e. fat cells. Adipogenesis is known to be closely related to the etiologies of obesity and obesity-related metabolic disorders (Spiegelman *et al.*, 1993). 3T3-L1 cells, originally derived from mouse

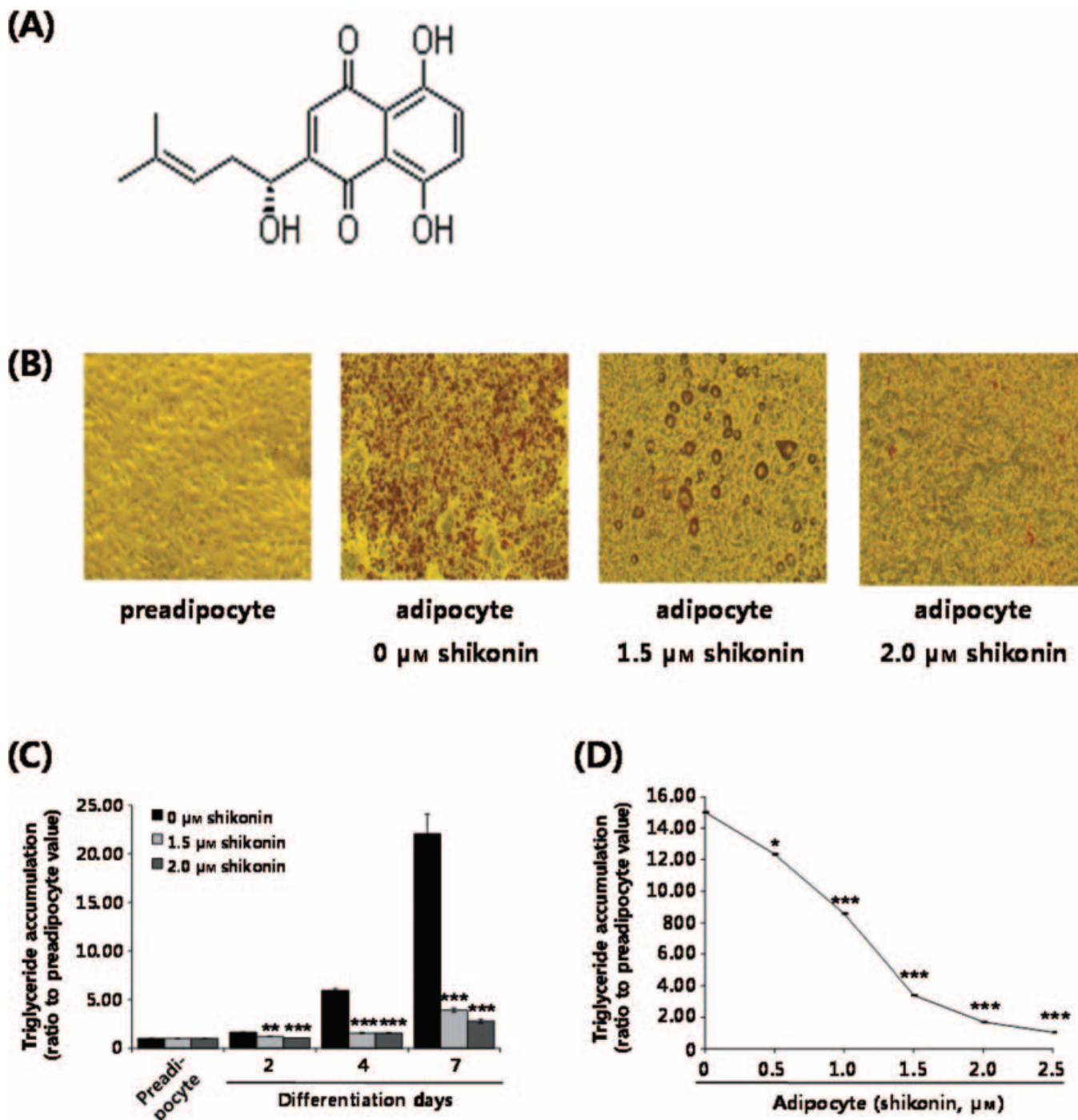
embryos, can be differentiated into adipocytes when stimulated with dexamethasone, methylisobutylxanthine and insulin, and have been widely used as *in vitro* models for obesity (Green and Meuth, 1974; Harmon and Harp, 2001; Madsen *et al.*, 2005; Rahman *et al.*, 2008). During the adipogenesis of 3T3-L1 cells, a number of morphological and physiological changes occur. The 3T3-L1 cells change from fibroblast-like preadipocytes to spherical adipocytes and accumulate large fat droplets containing triglyceride (Jessen and Stevens, 2002). Decades of studies on the molecular mechanism of adipogenesis have elucidated many genes and proteins which play important roles during the formation of fat cells (Rosen and MacDougald, 2006). In the process of adipogenesis, upstream regulators, including C/EBP $\beta$ , C/EBP $\delta$  and SREBP1C, regulate the expression of PPAR $\gamma$  and C/EBP $\alpha$  (Rosen *et al.*, 2002). PPAR $\gamma$  and C/EBP $\alpha$  are major transcription factors of adipogenesis and activate the expression of adipocyte marker genes such as fatty acid binding protein (FABP) 4, lipoprotein lipase (LPL), fatty acid synthetase, adiponectin, glucose transporter 4, leptin and others (Rosen and MacDougald, 2006).

In this study, for the first time, the antiobesity activity of shikonin and its mechanism of action were elucidated during the adipogenesis of 3T3-L1 cells.

## MATERIALS AND METHODS

**Chemicals and reagents.** Shikonin was purchased from Calbiochem (San Diego, CA, USA). Cell culture reagents were obtained from Life Technologies Inc. (Gibco, Grand Island, NY, USA). Anti-FABP antibody, anti-PPAR $\gamma$  antibody, anti-C/EBP $\alpha$  antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz,

\* Correspondence to: Professor Yoosik Yoon, Department of Microbiology, College of Medicine, 221 Heukseok-dong, Dongjak-gu, Seoul, 156–756, Korea.  
E-mail: thanks@cau.ac.kr



**Figure 1.** Inhibition of fat accumulation in 3T3-L1 cells following shikonin treatment. (A) Chemical structure of shikonin. (B) Effects of shikonin on fat droplet formation. 3T3-L1 cells were differentiated for 7 days with or without shikonin. Each culture dish was stained with Oil Red O and examined using a light microscope. (C) Triglyceride contents of 3T3-L1 cells differentiated for 2, 4 or 7 days with or without shikonin treatment. (D) Dose response of shikonin on the inhibition of triglyceride accumulation. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared with untreated adipocytes.

CA, USA). Anti-FABP antibody, anti-PPAR $\gamma$  antibody, anti- $\beta$ -Actin antibody and anti-rabbit secondary antibody were purchased from Cell Signaling (Beverly, MA, USA). All other chemicals were purchased from Sigma (St Louis, MO, USA).

**Cell culture.** 3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 100  $\mu\text{g}/\text{mL}$  streptomycin and 100 units/mL penicillin at

37  $^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . The cells were subcultured after reaching a confluence of 80%. To induce adipogenesis, 3T3-L1 cells were cultured until confluent, and 2 days after reaching confluence, called day 0, the culture medium was changed into a differentiation/induction medium containing 1  $\mu\text{g}/\text{mL}$  insulin, 0.25  $\mu\text{M}$  dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine in DMEM with 10% fetal bovine serum for 2 days. Next, the cells were maintained in a differentiation maintenance medium containing 1  $\mu\text{g}/\text{mL}$  insulin in DMEM with 10% fetal bovine serum. The differentiation main-

tenance medium was changed every 2 days until the cells were harvested. To test the effects of shikonin on adipogenesis, it was added to the medium until the cells were harvested. Cytotoxicity was measured by the 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) method as described (Freshney, 1994).

**Oil red O staining.** After being cultured in differentiation induction medium for 2 days and in differentiation maintenance medium for 5 days, the cells were washed with phosphate-buffered saline, fixed with 4% (w/v) paraformaldehyde for 1 h and stained with Oil Red O as described (Kasturi and Joshi, 1982). The cells were washed with phosphate-buffered saline, and fat droplets in the cells were observed using a light microscope.

**Triglyceride content assay.** Cultured and differentiated cells in each well of six-well plates were harvested in 500  $\mu$ L of phosphate-buffered saline (PBS), frozen and then sonicated. The triglyceride contents of the cell lysates were measured by using the TG-S reaction kit (Asan Pharm. Co., Seoul, Korea) according to the manufacturer's instructions.

**Real-time reverse transcription polymerase chain reaction.** Total RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany). One  $\mu$ g of RNA was reverse-transcribed at 37 °C using the cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA, USA). Real-Time RT-PCR was performed using the 7000 Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA, USA). PCR was performed in triplicate in a final volume of 20  $\mu$ L and included: the TaqMan gene expression master mix, 250 nM TaqMan probe, an optimized concentration of each primer and 2  $\mu$ L of cDNA. First, the reaction mixtures were preheated at 95 °C for 10 min to activate the enzyme, then subjected to 40 cycles of: melting at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. The PCR primer and probe sequences for C/EBP $\alpha$  and C/EBP $\beta$  were kindly provided by Gustafson and Smith (2006), and the assay-on-demand gene expression products (Applied Biosystems, Inc., Foster City, CA, USA) were used for other genes. The level of 18S rRNA was used as an endogenous control as described (Gustafson and Smith, 2006). For each sample, the levels of each mRNA were normalized to the abundance of 18S rRNA, and the ratios of normalized mRNA amounts of each samples to that of the pre-adipocyte (day 0) were presented according to the comparative *Ct* method (Livak and Schmittgen, 2001).

**DNA binding activity assay of PPAR $\gamma$  and C/EBP $\alpha$  in nuclear extract.** 3T3-L1 cells were scraped in phosphate-buffered saline and centrifuged for 10 min at 2000  $\times$  g. The pelleted cells were dissolved in buffer A, containing 25 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM EDTA and 5 mM dithiothreitol and homogenized in a tight homogenizer. The homogenate was pelleted by centrifugation at 3300  $\times$  g for 10 min and washed twice with buffer A. High salt extraction of nuclear proteins was performed by incubating nuclei with buffer B containing 25 mM Tris-HCl (pH 7.5), 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.5 mM EDTA and 25% sucrose for 30 min on ice. After centrifugation at 20000  $\times$  g for 30 min, the supernatants were used as nuclear extracts. DNA-binding activity of PPAR $\gamma$  in the nuclear extracts to its target sequence,

i.e. peroxisome proliferators response element (PPRE: 5'-AACTAGGTCAAAGGTCA-3') was measured using the TransAM PPAR $\gamma$  ELISA kit (ActiveMotif, Carlsbad, CA, USA). DNA-binding activity of C/EBP $\alpha$  to its target sequence, 5'-GCAAT-3', were measured by a similar method using a TransAM C/EBP ELISA kit (ActiveMotif, Carlsbad, CA, USA).

**Protein extraction and Western blotting.** Cultured and differentiated cells were harvested using a cell scraper and lysed with ice-cold RIPA buffer, containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS and a protease inhibitor cocktail (Sigma, St Louis, MO, USA). The cell lysates were sonicated and centrifuged at 14000 rpm for 20 min at 4 °C to remove insoluble materials. The protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Fifty  $\mu$ g of protein extracts was resolved by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes at 150 mA for 1 h. The membranes were then blocked for 2 h at room temperature with phosphate buffered saline containing 5% skim milk and 0.1% Tween 20 and incubated with a 1:1000-dilution of primary antibody overnight at 4 °C and subsequently with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (diluted 1:1000) for 1 h at room temperature. Peroxidase activity was visualized using the ECL kit (Pierce, Rockford, IL, USA). An anti- $\beta$ -actin antibody (diluted 1:1000) was used as an endogenous control to confirm that equal amounts of proteins were loaded.

**Statistical analysis.** The data were expressed as mean  $\pm$  SE from at least three independent experiments. Statistical significances for the comparisons between two groups, shikonin-treated sample and corresponding untreated sample, were determined by unpaired *t*-test. All analyses were performed using SPSS ver. 14 (SPSS Inc., Chicago, IL, USA).

## RESULTS

### Shikonin inhibits fat accumulation in 3T3-L1 cells

When 3T3-L1 cells were induced to differentiate into adipocytes, fat droplets formed inside the cells. These fat droplets can be stained with Oil Red O dye as shown in Fig. 1B. It was shown that shikonin inhibited the formation of fat droplets in a dose-dependent manner, and that fat droplet formation was almost completely blocked by treatment with 2.0  $\mu$ M of shikonin. The main component of the fat droplet is lipid in the form of triglyceride. The triglyceride contents of the cells increased dramatically during adipocyte differentiation for 7 days, and the addition of shikonin into the differentiation induction medium and differentiation maintenance medium almost completely blocked triglyceride accumulation (Fig. 1C). The inhibitory effects of shikonin on triglyceride accumulation during adipogenesis were dose-dependent and the half inhibitory concentration (IC<sub>50</sub>) was observed to be 1.1  $\mu$ M (Fig. 1D). Shikonin was dissolved in dimethylsulfoxide (DMSO) just before treatment, and the final concentration of DMSO in culture medium was adjusted to 0.02% (v/v). The sample

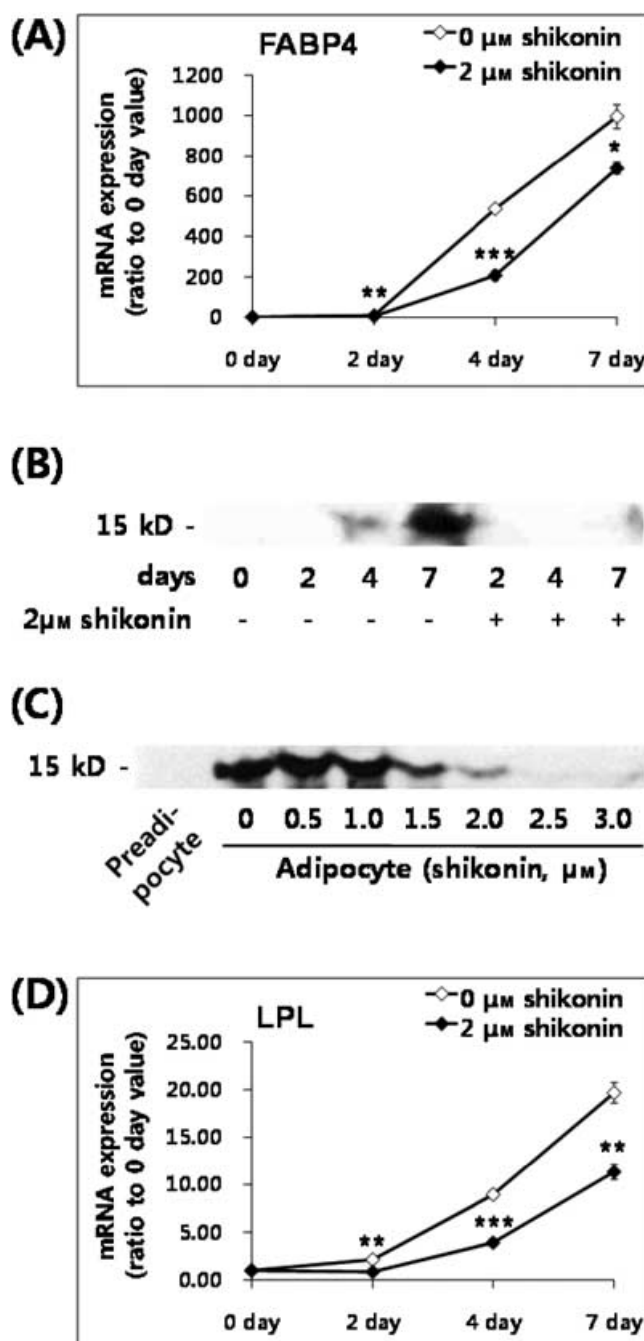
for vehicle control was treated with 0.02% (v/v) of DMSO alone, and it showed no difference with non-treated sample (data not shown). To test the cytotoxic effects, MTT assays were conducted after the treatment of various concentrations of shikonin for 7 days during the adipogenesis of 3T3-L1 cells. The results of the MTT assay showed that shikonin did not decrease the cell viability up to the concentration of 3.0  $\mu\text{M}$ , which suggests that the inhibitory effects of shikonin on fat droplet formation and triglyceride accumulation were not due to its cytotoxicity (data not shown).

### Shikonin inhibits the expression of genes involved in lipid metabolism

Fatty acid binding protein (FABP) 4, which binds fatty acids with high affinity and transports them to various compartments within the cell, is a key mediator of intracellular transport and metabolism of fatty acids in adipose tissues. FABP4 is massively expressed during adipogenesis and comprises up to 6% of total cytosolic proteins in a mature fat cell (Spiegelman and Green, 1980). The mRNA of FABP4 was up-regulated more than 1000-fold during adipogenesis, but, when 2.0  $\mu\text{M}$  shikonin was added to the medium, the expression of FABP4 was significantly inhibited (Fig. 2A). The protein levels of FABP4 showed a similar expression pattern to its mRNA levels (Fig. 2B). The inhibitory effect of shikonin on FABP4 protein expression was dose-dependent, as shown in Fig. 2C. Lipoprotein lipase (LPL), a key enzyme regulating the disposal of lipid in the body, is expressed in fat and muscle cells, and its role is to hydrolyse triglyceride circulating in the lipoprotein particles in order to uptake fatty acids into the cells (Fielding and Frayn, 1998). Its expression was increased more than 20-fold during adipogenesis, but significantly inhibited by shikonin treatment (Fig. 2D).

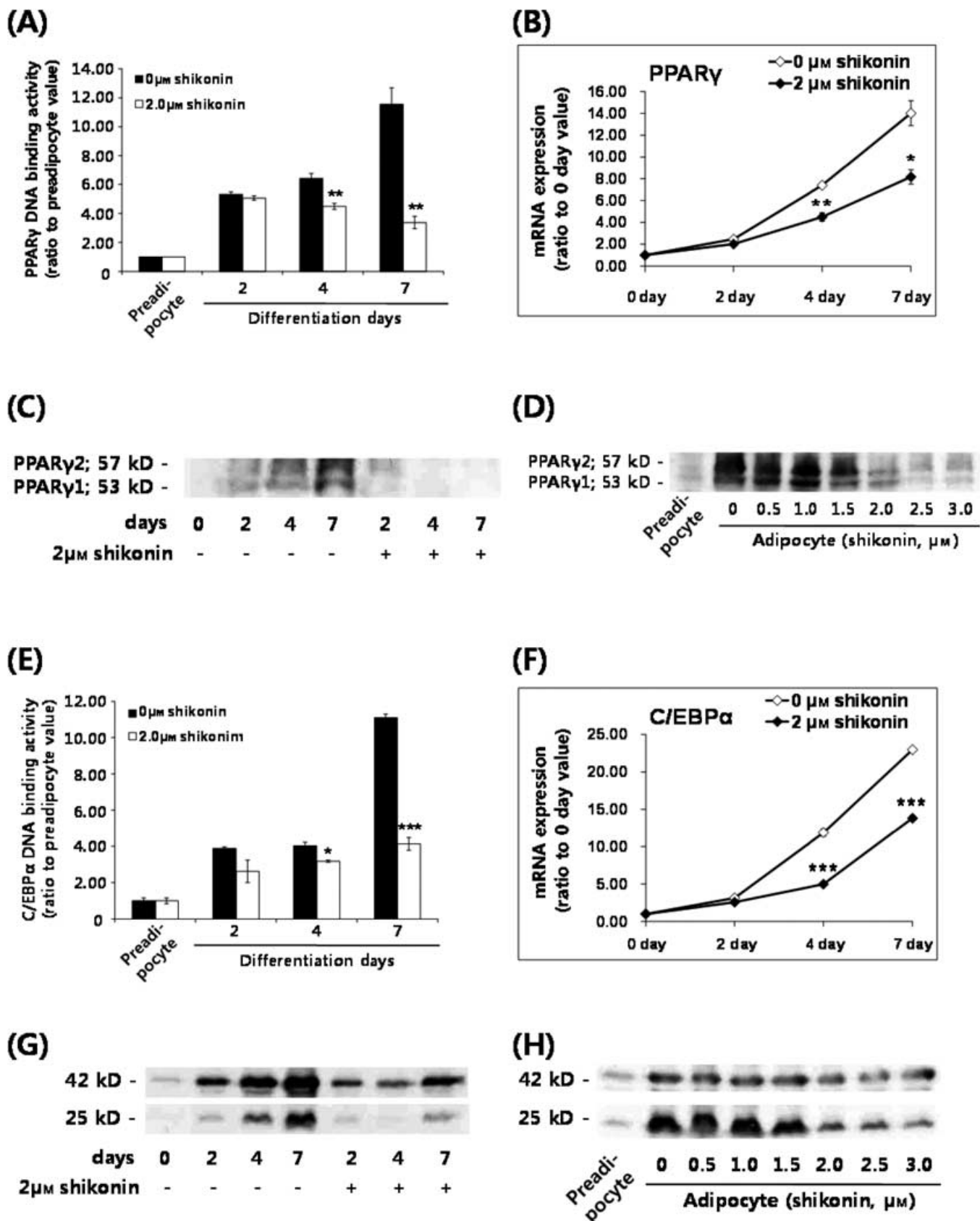
### Shikonin inhibits the major transcription factors of adipogenesis, PPAR $\gamma$ and C/EBP $\alpha$

PPAR $\gamma$ , a transcription factor of the nuclear-receptor superfamily, is a master regulator of adipogenesis and is both necessary and sufficient for adipogenesis (Rosen *et al.*, 2000). Expression of PPAR $\gamma$  alone is shown to induce adipogenesis in fibroblasts (Tontonoz *et al.*, 1994). In this study, it was found that the binding of PPAR $\gamma$  to its target DNA sequence, PPARE (5'-AAC-TAGGTCAAAGGTCA-3') was increased 12 fold during adipogenesis (Fig. 3A). Shikonin treatment significantly reduced the amounts of PPAR $\gamma$  bound to PPARE. Reduced binding of PPAR $\gamma$  to PPARE can be explained by the reduction of its mRNA and protein level as shown in Fig. 3B and 3C. The inhibitory effect of shikonin on PPAR $\gamma$  expression was dose-dependent (Fig. 3D). It was reported that functional PPAREs exist in the promoter regions of adipocyte-expressed genes, including FABP4 (Tontonoz *et al.*, 1994; Schachtrup *et al.*, 2004), LPL (Schoonjans *et al.*, 1996; Li *et al.*, 2002), acyl-CoA synthetase (Schoonjans *et al.*, 1995), phosphoenolpyruvate carboxykinase (Tontonoz *et al.*, 1995), adiponectin (Iwaki *et al.*, 2003) among others. Shikonin-induced reduction of FABP4 and LPL shown in Fig. 2 can be explained by the reduced binding of PPAR $\gamma$  on PPARE in their promoter regions.



**Figure 2.** Effects of shikonin on the expression of genes involved in lipid metabolism. (A) Expression of FABP4 mRNA in 3T3-L1 cells differentiated for 2, 4 or 7 days with or without shikonin treatment. (B) Expression of FABP4 protein in 3T3-L1 cells differentiated for 2, 4 or 7 days with or without shikonin treatment. (C) Dose response of shikonin on the inhibition of FABP4 protein expression. (D) Expression of LPL mRNA in 3T3-L1 cells differentiated for 2, 4 or 7 days with or without shikonin treatment. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared with untreated adipocytes.

It was also found that the binding of C/EBP $\alpha$  to its target DNA sequence, 5'-GCAAT-3', increased 11 fold during adipocyte differentiation (Fig. 3E). Shikonin treatment significantly reduced the amounts of C/EBP $\alpha$  bound to its target DNA; this effect can be explained by the reduction of its mRNA and protein level as shown in Fig. 3F and 3G. The inhibitory effect of shikonin on C/EBP $\alpha$  expression was also dose-dependent (Fig. 3H). PPAR $\gamma$  and C/EBP $\alpha$  are two major transcription factors of adipogenesis and their expressions are



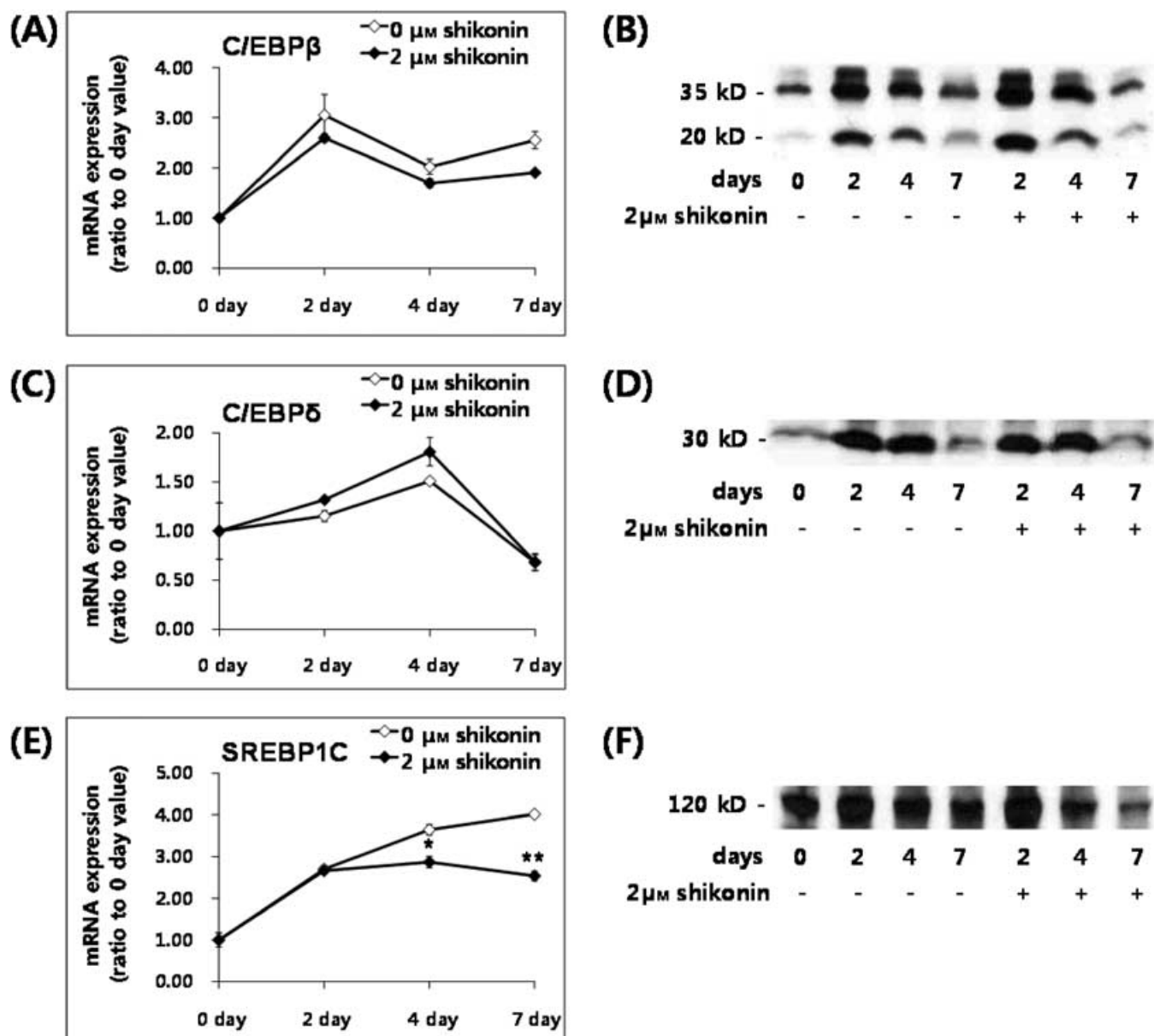
**Figure 3.** Effects of shikonin on the major transcription factors of adipogenesis. (A) Binding of PPAR $\gamma$  to PPARE sequence. Nuclear extracts were prepared during adipogenesis and their PPARE-binding activities were measured. The data were normalized to the value of preadipocyte. (B) Effects of shikonin on the mRNA levels of PPAR $\gamma$ . (C) Effects of shikonin on the protein levels of PPAR $\gamma$ . (D) Dose response of shikonin on the inhibition of PPAR $\gamma$  protein expression. (E) Binding of C/EBP $\alpha$  to its target sequence. The data were normalized to the value of preadipocytes. (F) Effects of shikonin on the mRNA levels of C/EBP $\alpha$ . (G) Effects of shikonin on the protein levels of C/EBP $\alpha$ . (H) Dose response of shikonin on the inhibition of C/EBP $\alpha$  protein expression. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 compared with untreated adipocytes.

mutually reinforced by forming a positive feedback loop between themselves. Among them, however, PPAR $\gamma$  has a more crucial role than C/EBP $\alpha$ . PPAR $\gamma$  can promote adipogenesis in the absence of C/EBP $\alpha$ , but C/EBP $\alpha$  has no ability to promote adipogenesis in the absence of PPAR $\gamma$  (Rosen *et al.*, 2000). PPAR $\gamma$  is also known to be responsible for inducing the expression of C/EBP $\alpha$  by binding to its promoter region (Rosen *et al.*, 2002). The shikonin-induced reduction of C/EBP $\alpha$  mRNA may be the result of a reduction in binding of PPAR $\gamma$  to its target DNA sequence in the promoter region of the C/EBP $\alpha$  gene.

#### The effects of Shikonin on the upstream regulators of adipogenesis pathway

C/EBP $\beta$ , C/EBP $\delta$  and SREBP1C are well-known upstream regulators of PPAR $\gamma$  in the adipogenesis pathway (Rosen *et al.*, 2000). To identify the upstream

regulators involved in the shikonin-induced inhibition of PPAR $\gamma$ , levels of these regulators were measured during the adipogenesis of 3T3-L1 cells with or without shikonin treatment (Fig. 4). C/EBP $\beta$  and C/EBP $\delta$  are known to be expressed in the early phase of adipogenesis and their major function is to induce the expression of PPAR $\gamma$  and C/EBP $\alpha$  (Wu *et al.*, 1996). Interestingly, their mRNA and protein levels were only slightly changed following shikonin treatment (Fig. 4A–D). On the contrary, the mRNA and protein levels of SREBP1C were markedly decreased following shikonin treatment (Fig. 4E, F). These results suggest that the shikonin-induced reduction of PPAR $\gamma$  is caused by a decrease in the expression of upstream SREBP1C. SREBP1C is a basic helix-loop-helix transcription factor that binds to an E-box motif, a nucleotide sequence of CANNTG, which is located in the promoter of its target gene (Tontonoz *et al.*, 1993). The PPAR $\gamma$  gene has three promoters,  $\gamma$ 1,  $\gamma$ 2 and  $\gamma$ 3, and SREBP1C was shown to activate the PPAR $\gamma$  expression by binding to E-box



**Figure 4.** Effects of shikonin on the upstream regulators of adipogenesis. The mRNA and protein levels of C/EBP $\beta$  (A and B), C/EBP $\delta$  (C and D) and SREBP1C (E and F) were measured by real time PCR and Western blotting during adipogenesis with or without shikonin treatment. \* $p$  < 0.05. \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 compared with untreated adipocytes.

motifs in the  $\gamma 1$  and  $\gamma 3$  promoters of the PPAR $\gamma$  gene (Fajas *et al.*, 1999). It was also reported that SREBP1C activates PPAR $\gamma$  by generating the ligand of PPAR $\gamma$  (Kim *et al.*, 1998).

The results of this study suggest that shikonin down-regulates the expression of SREBP1C and the subsequent expression of PPAR $\gamma$  and C/EBP $\alpha$ , resulting in the down-regulations of lipid metabolizing genes and reduced fat accumulation.

## DISCUSSION

Shikonin was reported to have various pharmacological activities on diverse cell types including antiinflammatory, anticancer, antimicrobial, antiplatelet and so on. Half inhibitory concentration, IC<sub>50</sub>, is a widely used parameter for the comparison of pharmacological activities of compounds with inhibitory functions. Shikonin was reported to inhibit phorbol 12-myristate 13-acetate-induced superoxide generation in neutrophils at an IC<sub>50</sub> of 2.0  $\mu$ M (Yoshimi *et al.*, 1992), and to inhibit leukotriene B<sub>4</sub> synthesis at an IC<sub>50</sub> of 0.62  $\mu$ M (Wang *et al.*, 1994). Shikonin also reacts directly with reactive oxygen species including singlet oxygens, superoxides and hydroxyl radicals at an IC<sub>50</sub> of 9.0  $\mu$ M (Gao *et al.*, 2000), and selectively blocks the binding of chemokines, CCL5 and CCL4, to their receptors on monocytes with an IC<sub>50</sub> of 3.6  $\mu$ M and 2.8  $\mu$ M, respectively (Chen *et al.*, 2001). In this study, shikonin was found to inhibit fat accumulation in 3T3-L1 adipocytes with an IC<sub>50</sub> of 1.1  $\mu$ M (Fig. 1D). Various cellular activities of shikonin imply that multiple cellular targets can be inhibited by shikonin, even though the exact identities of shikonin's targets remain to be elucidated. Recently, Kamei *et al.* (2002)

also reported that treatment with 10–50  $\mu$ M of shikonin stimulated glucose uptake in 3T3-L1 cells. They proposed that shikonin may activate as yet unidentified tyrosine kinase, resulting in the activation of AKT kinase, and the stimulation of glucose uptake by GLUT4 translocation via an insulin-independent pathway. When the results of Kamei *et al.* (2002) were compared with the results of this study, in which treatment with 2.0  $\mu$ M of shikonin almost completely inhibited fat accumulation in 3T3-L1 cells, it can be suggested that at least two targets of shikonin exist in 3T3-L1 cells: (i) a target for the activation of glucose uptake and (ii) a target for the inhibition of fat accumulation. The target for the inhibition of fat accumulation can be efficiently inhibited at a much lower concentration than the target for the activation of glucose uptake. The comparisons of IC<sub>50</sub>s for various cellular activities of shikonin suggest that the inhibition of fat accumulation is one of the strongest activities of shikonin.

In this study, for the first time, shikonin was shown to inhibit fat accumulation of adipocytes. The IC<sub>50</sub> value of 1.1  $\mu$ M suggested that the inhibition of fat accumulation is among the strongest activities of shikonin, a compound which has diverse cellular activities. The experimental data showed that shikonin down-regulates the expression of SREBP1C and the subsequent expression of PPAR $\gamma$  and C/EBP $\alpha$ , thereby resulting in the down-regulation of lipid metabolizing enzymes and in the reduction of fat accumulation.

## Acknowledgements

This research was supported by a grant of Biogreen 21 project (20070301034031) from Rural Development Administration of Korea and a grant of the Oriental Medicine R&D Project (B080020) from Ministry of Health, Welfare and Family Affairs, Republic of Korea.

## REFERENCES

- Chen X, Oppenheim J, Howard OM. 2001. Shikonin, a component of antiinflammatory Chinese herbal medicine, selectively blocks chemokine binding to CC chemokine receptor-1. *Int Immunopharmacol* **1**: 229–236.
- Chen X, Yang L, Oppenheim JJ *et al.* 2002. Cellular pharmacology studies of shikonin derivatives. *Phytother Res* **16**: 199–209.
- Fajas L, Schoonjans K, Gelman L *et al.* 1999. Regulation of peroxisome proliferator-activated receptor  $\gamma$  expression by adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1: Implications for adipocyte differentiation and metabolism. *Mol Cell Biol* **19**: 5495–5503.
- Fielding BA, Frayn KN. 1998. Lipoprotein lipase and the disposition of dietary fatty acids. *Br J Nutr* **80**: 495–502.
- Freshney RI. 1994. *Culture of Animal Cells: A Manual of Basic Technique*, 3rd edn. Wiley-Liss Inc: New York.
- Fuji N, Yamashita Y, Arima Y *et al.* 1992. Induction of topoisomerase II-mediated DNA cleavage by the plant naphthoquinones plumbagin and shikonin. *Antimicrob Agents Chemother* **36**: 2589–2594.
- Gao D, Kakuma M, Oka S *et al.* 2000. Reaction of beta-alkannin (shikonin) with reactive oxygen species: detection of beta-alkannin free radicals. *Bioorg Med Chem* **8**: 2561–2569.
- Green H, Meuth M. 1974. An established pre-adipose cell line and its differentiation in culture. *Cell* **3**: 127–133.
- Gustafson B, Smith U. 2006. Cytokines promote Wnt signaling and inflammation and impair the normal differentiation and lipid accumulation in 3T3-L1 preadipocytes. *J Biol Chem* **281**: 9507–9516.
- Harmon AW, Harp JB. 2001. Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis. *Am J Physiol Cell Physiol* **280**: C807–C813.
- Iwaki M, Matsuda M, Maeda N *et al.* 2003. Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. *Diabetes* **52**: 1655–1663.
- Jessen BA, Stevens GJ. 2002. Expression profiling during adipocyte differentiation of 3T3-L1 fibroblasts. *Gene* **299**: 95–100.
- Kamei R, Kitagawa Y, Kadokura M *et al.* 2002. Shikonin stimulates glucose uptake in 3T3-L1 adipocytes via an insulin-independent tyrosine kinase pathway. *Biochem Biophys Res Commun* **292**: 642–651.
- Kasturi R, Joshi VC. 1982. Hormonal regulation of stearoyl coenzyme A desaturase activity and lipogenesis during adipose conversion of 3T3-L1 cells. *J Biol Chem* **257**: 12224–12230.
- Kim JB, Wright HM, Wright M *et al.* 1998. ADD1/SREBP1 activates PPAR $\gamma$  through the production of endogenous ligand. *Proc Natl Acad Sci USA* **95**: 4333–4337.
- Li L, Beauchamp MC, Renier G. 2002. Peroxisome proliferator-activated receptor  $\alpha$  and  $\gamma$  agonists upregulate human macrophage lipoprotein lipase expression. *Atherosclerosis* **165**: 101–110.
- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**: 402–408.
- Madsen L, Petersen RK, Kristiansen K. 2005. Regulation of adipocyte differentiation and function by polyunsaturated fatty acids. *Biochim Biophys Acta* **1740**: 266–286.

- Rahman Md A, Kumar SG, Kim SW *et al.* 2008. Proteomic analysis for inhibitory effect of chitosan oligosaccharide on 3T3-L1 adipocyte differentiation. *Proteomics* **8**: 569–581.
- Rosen ED, Hsu CH, Wang X *et al.* 2002. C/EBP $\alpha$  induces adipogenesis through PPAR $\gamma$ : a unified pathway. *Genes Dev* **16**: 22–26.
- Rosen ED, MacDougald OA. 2006. Adipocyte differentiation from the inside out. *Nat Rev Mol Cell Biol* **7**: 885–896.
- Rosen ED, Walkey CJ, Puigserver P *et al.* 2000. Transcriptional regulation of adipogenesis. *Genes Dev* **14**: 1293–1307.
- Schachtrup C, Emmeler T, Bleck B *et al.* 2004. Functional analysis of peroxisome-proliferator-responsive element motifs in genes of fatty acid-binding proteins. *Biochem J* **382**: 239–245.
- Schoonjans K, Peinado-Onsurbe J, Lefebvre AM *et al.* 1996. PPAR and PPAR $\gamma$  activators direct a tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J* **15**: 5336–5348.
- Schoonjans K, Watanabe M, Suzuki H *et al.* 1995. Induction of the acyl-coenzyme A synthetase gene by fibrates and fatty acids is mediated by a peroxisome proliferator response element in the C promoter. *J Biol Chem* **270**: 19269–19276.
- Spiegelman BM, Choy L, Hotamisligil GS *et al.* 1993. Regulation of adipocyte gene expression in differentiation and syndromes of obesity/diabetes. *J Biol Chem* **268**: 6823–6826.
- Spiegelman BM, Green H. 1980. Control of specific protein biosynthesis during the adipose conversion of 3T3 cells. *J Biol Chem* **255**: 8811–8818.
- Subbaramaiah K, Bulic P, Lin Y *et al.* 2001. Development and use of a gene promoter-based screen to identify novel inhibitors of cyclooxygenase-2 transcription. *J Biomol Screen* **6**: 101–110.
- Tontonoz P, Hu E, Devine J *et al.* 1995. PPAR $\gamma$ 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. *Mol Cell Biol* **15**: 351–357.
- Tontonoz P, Hu E, Graves RA *et al.* 1994. mPPAR $\gamma$ 2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev* **8**: 1224–1234.
- Tontonoz P, Kim JB, Graves RA *et al.* 1993. ADD1: a novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation. *Mol Cell Biol* **13**: 4753–4759.
- Wang WJ, Bai JY, Liu DP *et al.* 1994. The antiinflammatory activity of shikonin and its inhibitory effect on leukotriene B<sub>4</sub> biosynthesis. *Yao Xue Xue Bao* **29**: 161–165.
- Wang JP, Raung SL, Chang LC *et al.* 1995. Inhibition of hind-paw edema and cutaneous vascular plasma extravasation in mice by acetylshikonin. *Eur J Pharmacol* **272**: 87–95.
- Wu Z, Bucher NL, Farmer SR. 1996. Induction of peroxisome proliferator-activated receptor gamma during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBP $\beta$ , C/EBP $\delta$ , and glucocorticoids. *Mol Cell Biol* **16**: 4128–4136.
- Yoon Y, Kim YO, Lim NY *et al.* 1999. Shikonin, an ingredient of *Lithospermum erythrorhizon* induced apoptosis in HL60 human promyelocytic leukemia cell line. *Planta Med* **65**: 532–535.
- Yoshimi N, Wang A, Morishita Y *et al.* 1992. Modifying effects of fungal and herb metabolites on azoxymethane-induced intestinal carcinogenesis in rats. *Jpn J Cancer Res* **83**: 1273–1278.