

Persimmon tannin represses 3T3-L1 preadipocyte differentiation via up-regulating expression of miR-27 and down-regulating expression of peroxisome proliferator-activated receptor- γ in the early phase of adipogenesis

Bo Zou · Zhenzhen Ge · Wei Zhu · Ze Xu · Chunmei Li

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Abstract

Purpose Currently, obesity has become a worldwide health problem. Adipocyte differentiation is closely associated with the onset of obesity. Our previous studies suggested that persimmon tannin might be a potent anti-adipogenic dietary bioactive compound. However, the mechanism of persimmon tannin on adipocyte differentiation is still unknown. The purpose of this study was to investigate the effect of persimmon tannin on adipogenic differentiation in 3T3-L1 preadipocytes and the underlying mechanisms.

Methods Adipogenic differentiation was induced by cocktail in the presence or absence of persimmon tannin. Intracellular lipid accumulation was determined by Oil red O staining and enzymatic colorimetric methods. Gene expression and protein levels were measured by real time RT-PCR and Western blot.

Results Persimmon tannin inhibited intracellular lipid accumulation markedly, and the inhibitory effect was largely limited to the early stage of adipocyte differentiation. Persimmon tannin suppressed the expression of C/EBP α and peroxisome proliferator-activated receptor- γ (PPAR γ), significantly. Furthermore, genes related to lipogenesis, such as sterol regulatory element-binding protein 1, were down-regulated by persimmon tannin. In addition, adipocyte fatty acid binding protein (aP2), which is a target

gene of PPAR γ , was suppressed by persimmon tannin notably. Correspondingly, the expression of miR-27a and miR-27b were up-regulated by persimmon tannin from Day 2 to Day 8 significantly.

Conclusion Persimmon tannin inhibited adipocyte differentiation through regulation of PPAR γ , C/EBP α and miR-27 in early stage of adipogenesis.

Keywords Persimmon tannin · Adipogenesis · PPAR γ · Mitotic clonal expansion

Introduction

Obesity has become a worldwide health problem. It is closely associated with metabolic syndrome, such as hyperlipidemia, type 2 diabetes and hypertension [1]. Obesity is often caused by increased number of adipocytes (hyperplasia) and an increased cell size (hypertrophy). The former is contributed to preadipocyte differentiation, while the latter is due to lipid accumulation. It is suggested that adipocyte hyperplasia occurs not only in children and adolescence, but also in morbidly obese adults [2, 3]. Therefore, inhibition of adipocyte differentiation is one of the important targets for preventing obesity and its associated diseases.

Mouse preadipocytes 3T3-L1 has become the “gold standard” for investigating preadipocyte differentiation in vitro [4, 5]. In the presence of cocktail, the growth-arrested preadipocytes reenter to cell cycle and undergo about two rounds of mitotic clonal expansion (MCE). Multiple transcriptional factors, such as CCAAT/enhancer-binding proteins (C/EBPs), peroxisome proliferator-activated receptor γ (PPAR γ), are induced in MCE. C/EBP α and PPAR γ are two major regulators for controlling adipogenesis. Once activated, they coordinately promote the expression of

B. Zou · Z. Ge · W. Zhu · Z. Xu · C. Li (✉)
College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, China
e-mail: lichmyl@126.com; lichmyl@mail.hzau.edu.cn

C. Li
Key Laboratory of Environment Correlative Food Science,
Huazhong Agricultural University, Ministry of Education,
Wuhan, China

adipocyte-specific genes such as adipocyte fatty acid binding protein (aP2) and lipoprotein lipase (LPL), resulting in generating fully mature adipocyte [6, 7]. A number of studies demonstrated that polyphenols could inhibit adipocyte differentiation through the suppression of PPAR γ [1, 8–10]. It was reported that PPAR γ could be regulated by AMP-activated protein kinase (AMPK) [11]. Some polyphenols such as apigenin suppressed adipogenesis in 3T3-L1 cells by activating AMPK, which down-regulated the expression of PPAR γ and its downstream genes [12]. However, the anti-adipogenic action of some polyphenols, such as (–)-epigallocatechin-3-gallate (EGCG), was reported to be independent of AMPK pathway [13]. The discrepancy could be due to the differences in the polyphenol structure and treatment conditions.

Recently, microRNAs (miRNAs) were reported to play regulatory roles in many biological processes associated with obesity, including adipocyte differentiation [14]. Some studies indicated that miR-27a and miR-27b acted as the negative regulators of adipocyte differentiation through inhibition of PPAR γ [15, 16]. Current data suggested that dietary polyphenols, such as EGCG [17] and cocoa proanthocyanidins [18], could modify expression of miRNAs. Baselga-Escudero et al. [17] indicated that flavonoids inhibited or had no effect on miR-33a and miR-122. On the contrary, the nonflavonoid resveratrol up-regulated the expression of these miRNAs, suggesting that the influence of polyphenols on miRNAs expression may be structure dependent. Persimmon fruit and its extracts were reported to exert potent hypolipidemic effect in animal models [19, 20]. Recently, their anti-hypercholesterolemia effects were confirmed in human subjects [21]. Our previous study demonstrated that persimmon tannin was the main component accounting for the anti-hyperlipidemic effect of consuming persimmon [22]. Moreover, in our preliminary study, we found persimmon tannin notably inhibited adipocyte differentiation. These results suggested that persimmon tannin might be a potent anti-adipogenic dietary bioactive compound. However, to the best of our knowledge, the mechanism of persimmon tannin on adipocyte differentiation is still unknown. Therefore, the purpose of this study was to investigate the underlying mechanisms of persimmon tannin on adipogenesis in 3T3-L1 preadipocyte.

Materials and methods

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) and newborn calf serum were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Sijiqing (Hangzhou, China). 3-Isobutyl-1-methylxanthine,

insulin, dexamethasone, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)], Compound C and Oil red O were obtained from Sigma Chemical Co. (St Louis, MO, USA). Rabbit polyclonal antibodies against AMPK α (Cat. #2532) and phospho-AMPK α (p-AMPK α , Thr-172) were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal antibody PPAR γ , sterol regulatory element-binding protein 1 (SREBP1) and rabbit polyclonal antibodies against β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies C/EBP α were purchased from Proteintech Group, Inc. (Wuhan, China). Commercial kits used for determination of triglyceride (TG) were purchased from Shanghai Mind Bioengineering Co., Ltd. (Shanghai, China).

Samples preparation

Mature and fully colored fruit of the astringent persimmon (*Diospyros kaki Thunb.* Gongcheng Yueshi) was harvested in late October from an orchard in Guang'xi province (China). After harvest, fruit was held at 100 °C for about 5 min to inactivate polyphenol oxidase and then stored deep frozen at –80 °C. High molecular weight persimmon tannin was prepared according to our previous reports [20, 23]. It was characterized by MALDI-TOF, thiolysis–HPLC–ESI–MS and NMR [24]. The mean degree of polymerization of persimmon tannin was estimated to be 26 by thiolysis. The extension units were (epi)catechin, (epi)gallocatechin, EGCG and (epi)catechin-3-*O*-gallate with the relative moles of 2.78, 3.95, 11.0 and 7.58, respectively, and the terminal units were catechin, EGCG and myricetin with the relative moles of 0.29, 0.26 and 0.45. The proposed structure was elucidated in our earlier paper [24]. The content of total polyphenols in persimmon tannin was 98.7 % on a mass basis by Folin–Denis method [25].

Cell culture and treatment

3T3-L1 preadipocytes were purchased from Institute of Cell Biology at the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM supplemented with 10 % newborn calf serum, 100 U/ml of penicillin and 100 μ g/ml streptomycin at 37 °C in a humidified atmosphere of 95 % air and 5 % CO₂. The medium was replaced every 2 days. 3T3-L1 preadipocytes were seeded in 6-well plates at a density of 2×10^5 per well and incubated until confluence. After 2 days of confluence, adipocyte differentiation was induced as described by Zhang et al. [26]. In brief, the cells were stimulated to differentiate by DMEM contained 10 % FBS and cocktail (MDI, 0.5 mM isobutyl-1-methylxanthine, 1 μ M dexamethasone, 10 μ g/ml insulin) for 3 days. Cells were incubated in DMEM with 10 %

FBS and 10 $\mu\text{g/ml}$ insulin for another 3 days, followed by 2 days of additional culture with DMEM containing 10 % FBS (Day 8). Persimmon tannin was dissolved in dimethyl sulfoxide (DMSO), diluted in sterile media to make a final concentrations of 0–100 $\mu\text{g/ml}$, and the final concentration of DMSO was 0.1 %.

To study the effect of persimmon tannin on AMPK pathway, postconfluent 3T3-L1 cells were simultaneously treated with persimmon tannin and Compound C (4 μM) during adipocyte differentiation according to Zhang et al. [26].

Oil red O (ORO) staining

After 8 days of adipocyte differentiation, the cells were washed with PBS and then fixed with 4 % formaldehyde for 1 h and stained with 3 mg/ml ORO (60 % isopropanol and 40 % water) at room temperature for another 1 h. The cells were washed with distilled water to remove excess stain and photographed by an Olympus microscope (Tokyo, Japan) at magnification 200 \times .

Triglyceride determination

3T3-L1 preadipocytes were subjected to differentiation into mature adipocytes in the presence or absence of persimmon tannin for 8 days. The intracellular TG content was determined by enzymatic colorimetric methods with commercial kits. The TG content was normalized to cellular protein, and the results were expressed as the relative TG content compared to the MDI-treated control cells.

Cell viability

The effect of persimmon tannin on cell viability was determined by MTT assay. Briefly, 3T3-L1 preadipocytes were seeded in 96-well plates at a density of 6×10^3 per well and allowed to attach for 24 h at 37 °C. After treatment with various concentrations of persimmon tannin (0–100 $\mu\text{g/ml}$) for 24 or 48 h, the culture medium was replaced by MTT (0.5 mg/ml) and incubated at 37 °C for 4 h. DMSO was added to dissolve the formazan crystals, and then, the absorbance was measured using microplate reader (Thermo Scientific Multiskan GO, Vantaa, Finland) at 570 nm.

Cell cycle assay

Two days after confluence, 3T3-L1 preadipocytes were cultured in MDI medium in the presence or absence of persimmon tannin for 18 and 24 h. The cells were harvested, washed with PBS and fixed with 70 % ethanol for at least 2 h on ice. Next, the cells were washed twice with PBS, stained with 40 $\mu\text{g/ml}$ propidium iodine solution

containing 500 $\mu\text{g/ml}$ of RNase A at 37 °C for 30 min. The quantitation of cell cycle distribution was performed with FACScan cytometer.

Real time RT-PCR

The cells were harvested at indicated time, and total RNA was extracted using Trizol reagent (Invitrogen; Carlsbad, CA, USA). Reverse transcription was performed with a first-strand cDNA synthesis kit (Toyobo, Osaka, Japan). After cDNA synthesis, quantitative real time PCR was performed on SLAN PCR system (Hongshi, Shanghai, China) using the SYBR Green PCR Master Mix (Toyobo) according to the manufacturer's instructions. Reaction mixtures were incubated for an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 20 s and 72 °C for 20 s. Stem-loop primers for miRNAs were as follows: miR-27a, GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACGCGGA (stem-loop), TGCG CTTCA- CAGTGGCTAAGT (Forward), CCAGTGCAGG GTCCGAGGTATT (reverse); miR-27b, GTCGTATCCA- GTGCAGGGTCCGAGGTATTCGCACTGGATACGA CGCAGA (stem-loop), TGCGCTTCACAGTGGCTA- AGT (Forward), CCAGTGCAGGGTCCGAGGTATT (reverse); U6, CGCTTCGGCAGCACATATAC (Forward), AAATATG GAACGCTTCACGA (reverse). Primers for mRNA are shown in Table 1. Expression of mRNA or miRNA values were calculated using the threshold cycle (CT) value. For each sample, the ΔCT sample value was determined by calculating the difference between the CT value of the target gene and the CT value of β -actin reference gene (the expression of miRNA was normalized to U6 small nuclear RNA). The expression levels relative to control were estimated by calculating $\Delta\Delta\text{CT}$ ($\Delta\text{CT}_{\text{sample}} - \Delta\text{CT}_{\text{control}}$) and subsequently using the $2^{-\Delta\Delta\text{CT}}$ method [27].

Western blot

The total proteins in the cells were extracted with lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM β -glycerophosphate, 2 $\mu\text{g/ml}$ leupeptin, 2 $\mu\text{g/ml}$ aprotinin, 2 $\mu\text{g/ml}$ pepstatin and 1 mM PMSF). Protein concentrations were determined by BCA protein assay kit (Jiancheng, Nanjing, China). For Western blotting, 40 μg of protein were separated by 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then electro-transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5 % nonfat dry milk in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween 20) for 1 h at room temperature and incubated overnight at 4 °C with

Table 1 Primer sequences for real time RT-PCR

Gene	Forward (5′–3′)	Reverse (5′–3′)
C/EBP β	TGGACAAGCTGAGCGACGAG	GAACAAGTTCGCGAGGGTGC
C/EBP δ	CACGACTCCTGCCATGTACG	GCCGCTTTGTGGTTGCTGTT
C/EBP α	CCACTTGACAGTTCCAGATCG	CCACCGACTTCTTGGCTTTG
PPAR γ 2	GCACTGCCATGAGCACTTC	CCATTGGGTCAGCTCTTGTG
SREBP1C	GTCAAAACCAGCCTCCCAAG	GTCCCCGTCCACAAAGAAAC
aP2	AAGGTGAAGAGCATCATAACCCT	TCACGCCTTTCATAACACATTCC
FAS	CCGTCGTCTATACCACTGCT	GGCAAAGCTGGTGTATCAA
SCD1	ATGTCTGACCTGAAAGCCGA	GAAGGTGCTAACGAACAGGC
ACC1	ATGGGCGGAATGGTCTCTTTC	TGGGGACCTTGTCTTCATCAT
HSL	TTCGCCATAGACCCAGAGTT	TGTGCCAAGGGAGGTGAGAT
LPL	GCCCAGCAACATTATCCAGT	TGGTCAGACTTCCTGCTACG
PPAR α	TTCAATGCCTTAGAACTGGATG	GCAACTTCTCAATGTAGCCTATG
Actin	CACGATGGAGGGGCCGACTCATC	TAAAGACCTCTATGCCAACACAGT

primary antibodies in TBST (AMPK α , 1:1,000; p-AMPK α , 1:1,000; PPAR γ , 1:200; C/EBP α , 1:1,000; SREBP1C, 1:1,000; β -actin, 1:1,000), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:10,000) for 45 min at room temperature. The transferred proteins were visualized by the enhanced chemiluminescent reagents (Beyotime, Shanghai, China).

Statistical analysis

All data were presented as mean \pm standard error (mean \pm SE). Comparisons between groups were made using one-way ANOVA of SPSS 17.0 followed by Tukey's multiple-range test. Two groups were compared with an independent samples Student's *t* test. *P* value < 0.05 was considered statistically significant.

Results

Effect of persimmon tannin on 3T3-L1 cell viability and adipocyte differentiation

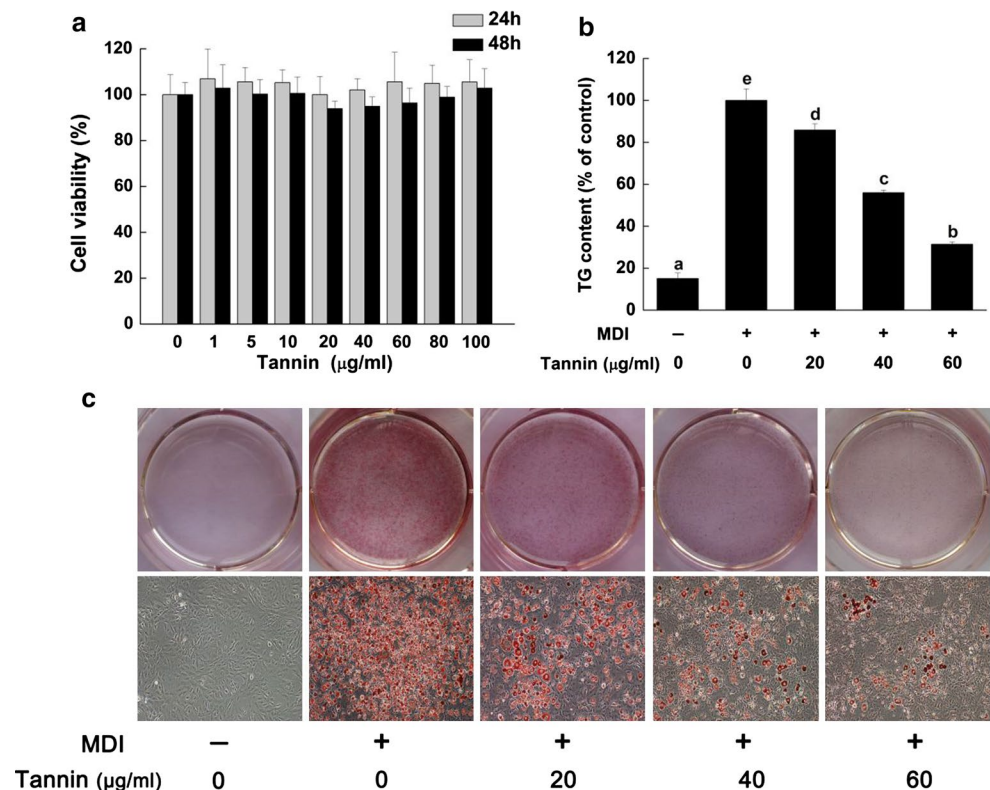
To investigate the cell toxicity of persimmon tannin on preadipocytes, cells were incubated with various concentrations of persimmon tannin (0–100 μ g/ml) for 24 and 48 h, respectively. The cell viability was measured by MTT assay. As shown in Fig. 1a, no significant effect of persimmon tannin on cell viability was observed upon 48 h with concentrations less than 100 μ g/ml, suggesting persimmon tannin showed no cytotoxicity on 3T3-L1 cell viability with concentration below 100 μ g/ml. 3T3-L1 preadipocytes were induced with MDI for 8 days in the presence or absence of persimmon tannin (0–60 μ g/ml). On Day 8, the cells were stained with ORO, and intracellular TG concentration was quantified by enzymatic colorimetric method.

Persimmon tannin treatment significantly and dose-dependently reduced the lipid accumulation in MDI-induced preadipocyte cells, as evidenced by the ORO staining and intracellular TG concentration analyses (Fig. 1b, c). Persimmon tannin, at the concentration of 60 μ g/ml, reduced the TG content in 3T3-L1 cells by 68.6 %. Cocoa polyphenol extract reduced MDI-induced lipid accumulation in 3T3-L1 cells by 30 % at the dose of 100 μ g/ml [2]. Lipid accumulation in 3T3-L1 adipocytes exposed to 250 μ g/ml of grape seed extract was inhibited by 48 % [28]. Our results indicated that persimmon tannin exerted stronger inhibitory effect on adipogenesis than cocoa polyphenol extract and grape seed extract, and its effect was independent of its cytotoxicity.

The inhibitory effect of persimmon tannin on 3T3-L1 preadipocyte differentiation mainly occurred in the early stage of differentiation

3T3-L1 preadipocyte differentiation induced by MDI consists of three stages, including early (Days 0–3), intermediate (Days 4–6) and terminal (after Day 6) phases. In order to investigate the key adipogenic phase primarily influenced by persimmon tannin, the cells were treated with 60 μ g/ml of persimmon tannin at various phases of adipogenesis during 3T3-L1 preadipocyte differentiation (Fig. 2a). It was observed that the presence of persimmon tannin in the whole stage of adipogenesis (treatment 5) showed a dramatic (*P* < 0.001) reduction in lipid accumulation (Fig. 2b, c). Moreover, identical results were observed in cells incubated with persimmon tannin in the early stage (Days 0–3, treatment 3) or in the early and intermediate stages (Days 0–6, treatment 4). However, the presence of persimmon tannin only in the intermediate stage (Days 4–6, treatment 6) or only in the terminal stage (Days 7–8, treatment 8) or in both of these two stages (Days 4–8,

Fig. 1 Effect of persimmon tannin on 3T3-L1 cell viability and lipid accumulation. **a** Cells were incubated with various concentrations of persimmon tannin (0–100 $\mu\text{g/ml}$) for 24 and 48 h, cell viability was measured by MTT assay. Two-day postconfluent 3T3-L1 preadipocytes were subjected to adipocyte differentiation by MDI medium in the presence or absence of persimmon tannin (20–60 $\mu\text{g/ml}$) for 8 days. **b** TG content was measured by enzymatic kit. Results were expressed as mean \pm SE of three independent experiments. Different letters among groups indicated significant difference ($P < 0.05$) by Tukey's test. **c** Cells were stained with ORO and then photographed by digital camera and microscope ($\times 200$)



treatment 7) exhibited limited inhibition on lipid accumulation (Fig. 2b, c). Our results indicated that the inhibitory effect of persimmon tannin on 3T3-L1 preadipocyte differentiation was largely limited to the early phase of adipocyte differentiation, which played a pivotal role in cell fate determination of preadipocytes.

Because ORO and intracellular TG concentration analyses suggested that the anti-adipogenic effect of persimmon tannin largely occurred in the early stage of differentiation, we further used flow cytometry assay to test the inhibitory effect of persimmon tannin on cell cycle process. According to previous reports [29], induction of 3T3-L1 with MDI promoted the cell cycle of differentiating preadipocytes to S (G2/M) phase in 18 h (24 h). Treatment of 3T3-L1 preadipocytes with persimmon tannin clearly ($P < 0.05$) exhibited a delayed entry of cells into S and G2/M phases after 18 and 24 h of incubation, respectively, compared to the control cells (Fig. 2d, e). Thus, flow cytometry assay provided further supporting evidence for that persimmon tannin blocked 3T3-L1 preadipocyte differentiation in the early stage of adipocyte differentiation.

Effect of persimmon tannin on gene expression during 3T3-L1 preadipocyte differentiation

To elucidate molecular mechanism of persimmon tannin in adipogenesis, we examined the levels of some

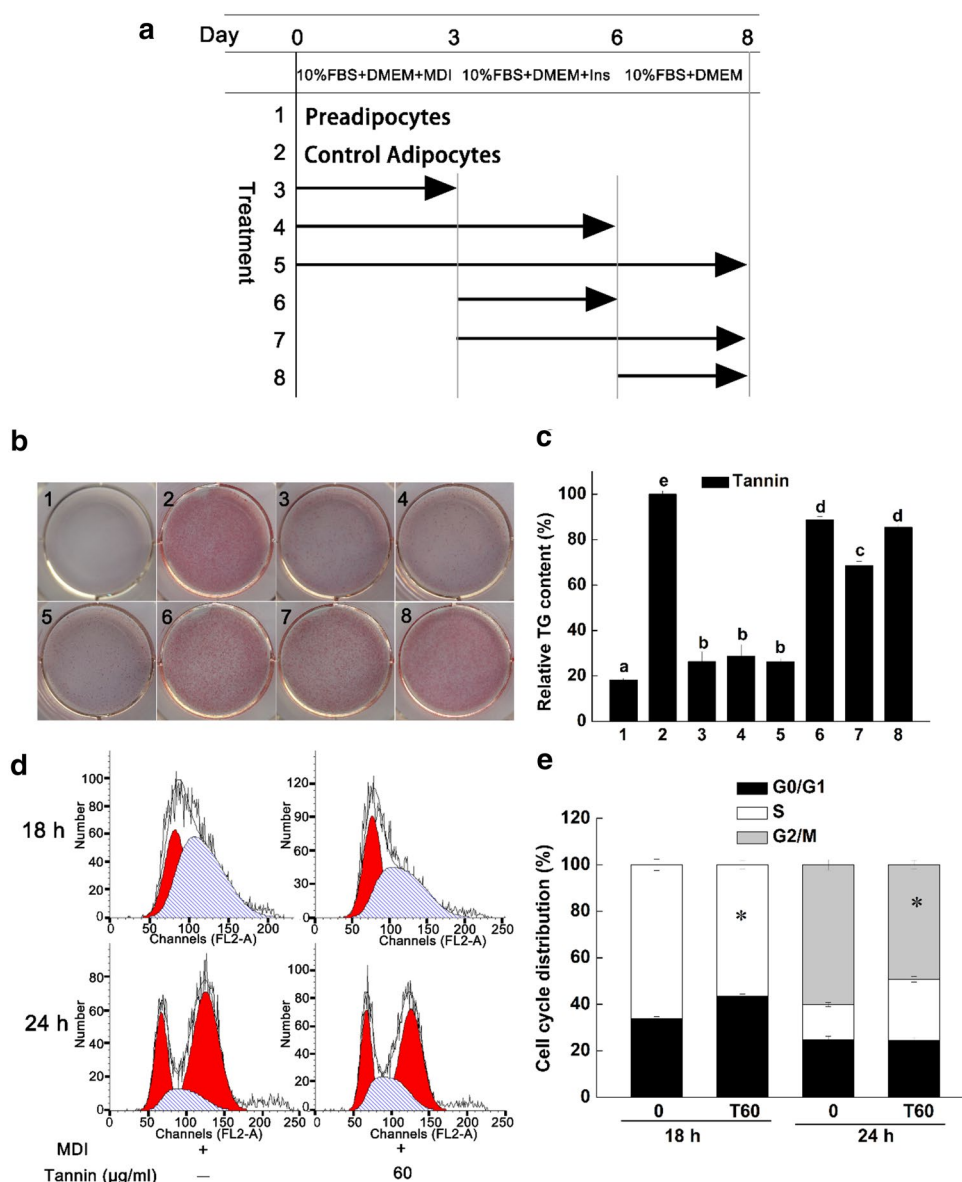
transcription factors such as C/EBPs and PPAR γ by real time RT-PCR. It was clear that the expressions of C/EBP β and C/EBP δ were significantly ($P < 0.05$) increased by MDI treatment for 24 (Fig. 3a) and 48 h (Fig. 3b), while C/EBP δ expression was notably repressed in the presence of persimmon tannin (40–60 $\mu\text{g/ml}$). The expression of C/EBP β was also decreased by treatment of persimmon tannin on Day 1 and 2, but without statistical differences. We also observed that the mRNA levels of C/EBP α and PPAR γ 2 were significantly ($P < 0.05$) up-regulated in the differentiated cells on Day 2, but the gene expression levels of these factors were markedly ($P < 0.05$) reduced in a dose-dependent manner by treatment of persimmon tannin (40–60 $\mu\text{g/ml}$). These results indicated that persimmon tannin suppressed the expression of marked transcription factors including C/EBP δ , C/EBP α and PPAR γ 2 in the early phase of adipocyte differentiation, thus blocking 3T3-L1 preadipocyte differentiation.

We further examined the mRNA levels of transcription factors on Day 5. In the persimmon tannin (60 $\mu\text{g/ml}$) treated cells, PPAR γ 2, C/EBP α and SREBP1C mRNA levels were decreased approximately by 89, 85, and 66 %, respectively, as compared with those of the differentiated cells (Fig. 3c). Subsequently, we examined the target genes of SREBP1C. As shown in Fig. 3d, the expression levels of the lipogenic genes such as

Fig. 2 Persimmon tannin blocked 3T3-L1 intracellular lipid accumulation and MCE process in the early stage of adipocyte differentiation. Two-day postconfluent 3T3-L1 preadipocytes were subjected to adipocyte differentiation by MDI medium in the presence or absence of 60 $\mu\text{g/ml}$ persimmon tannin for indicated period.

a The indicated time points with persimmon tannin. **b** Cells were stained with ORO. **c** TG content was measured by enzymatic kit. Different letters among groups indicated significant difference ($P < 0.05$) by Tukey's test.

d After 18- and 24-h treatment, the differentiation cells were harvested, stained with PI and then subjected to FACSscan for cell cycle analysis. **e** Quantitative analysis of the percentages of cells in different phases in cell cycle. Results were expressed as mean \pm SE of three independent experiments. * $P < 0.05$ versus control group by Student's *t* test

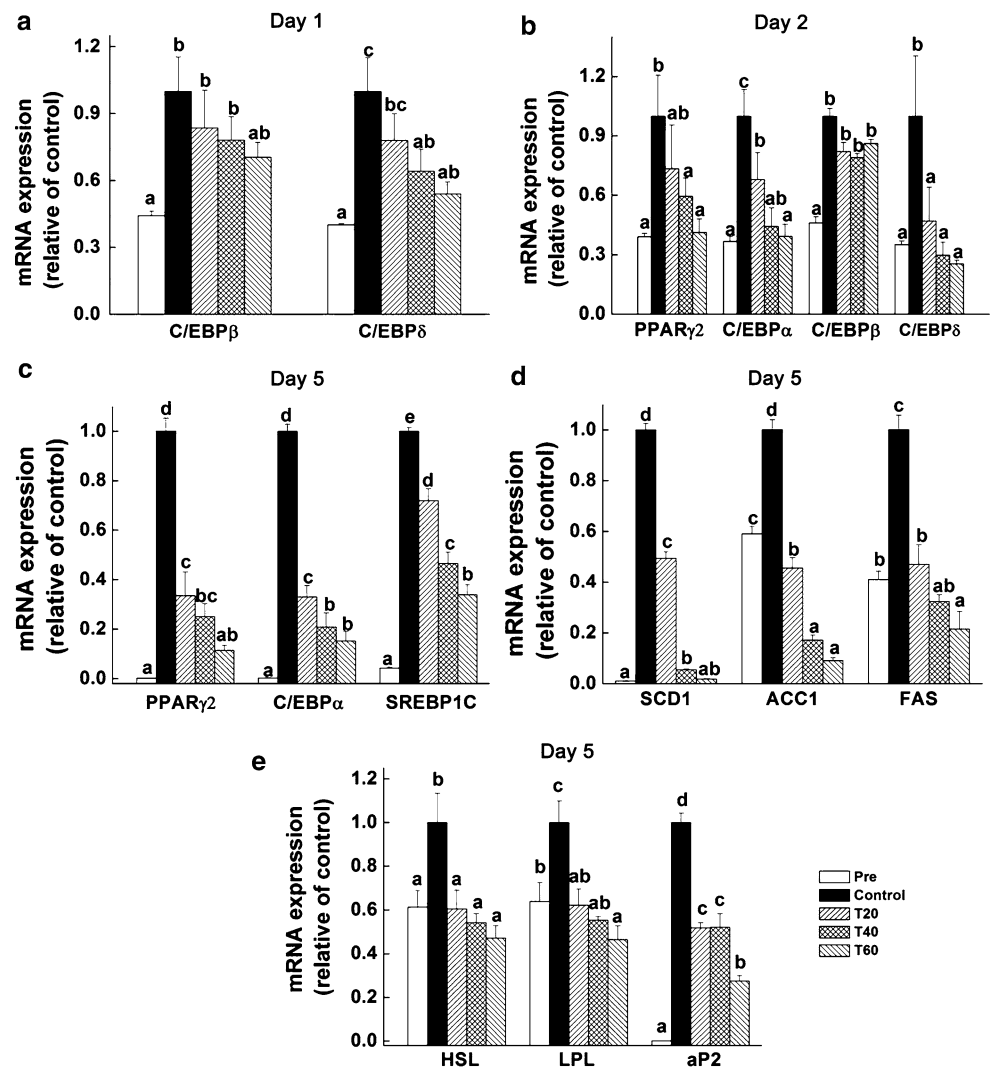


stearoyl-CoA desaturase 1 (SCD1), acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS) in the differentiated cells increased notably ($P < 0.05$) compared with those of the undifferentiated cells. In contrast, the expressions of these genes were significantly ($P < 0.05$) decreased by treatment of persimmon tannin in a dose-dependent manner (Fig. 3c). Since the expression of PPAR γ was inhibited by persimmon tannin, we speculated that PPAR γ target genes including aP2, hormone sensitive lipase (HSL) and LPL might also be suppressed in differentiated cells by treatment of persimmon tannin. The aP2, which participated in fatty acid transport and metabolism [30], and genes involved in the lipolysis, such as HSL and LPL, were significantly ($P < 0.05$) attenuated by persimmon tannin.

The inhibitory effect of persimmon tannin on adipocyte differentiation was through PPAR γ and miR-27a/b, but not AMPK pathway

Although our findings indicated that persimmon tannin suppressed 3T3-L1 preadipocyte differentiation in the early phase of adipogenesis through C/EBP α -PPAR γ -SREBP1C pathway, the up-stream regulators of C/EBP α -PPAR γ -SREBP1C were unclear. It was reported that activation of AMPK could decrease PPAR γ , C/EBP α and SREBP1C expression [11, 31]. To further elucidate the molecular mechanism of the inhibitory effect of persimmon tannin on 3T3-L1 preadipocyte differentiation, we investigated the possibility of persimmon tannin acting as an AMPK activator. The protein levels of AMPK and phosphorylation of

Fig. 3 Effect of persimmon tannin on gene expression in 3T3-L1 cells during differentiation. Two-day postconfluent 3T3-L1 preadipocytes were subjected to adipocyte differentiation by MDI medium in the presence or absence of persimmon tannin (20–60 $\mu\text{g}/\text{ml}$). **a** Cells were treated with persimmon tannin for 1 day, relative mRNA expression of the adipogenic transcription factors. **b** Cells were treated with persimmon tannin for 2 days, relative mRNA expression of the adipogenic transcription factors. **c** Cells were treated with persimmon tannin for 5 days, relative mRNA expression of the adipogenic transcription factors. **d, e** Cells were treated with persimmon tannin for 5 days, relative mRNA expression of PPAR γ downstream target genes. Results were expressed as mean \pm SE of three independent experiments. Different letters among groups indicated significant difference ($P < 0.05$) by Tukey's test



AMPK were decreased by treatment of persimmon tannin for 5 days (Fig. 4a). Subsequently, we employed Compound C (an AMPK inhibitor) to treat cells, and the results indicated that Compound C did not prevent the inhibitory effect of persimmon tannin on adipocyte differentiation and adipogenesis (Fig. 4b, c). These results indicated that the anti-adipogenic activity of persimmon tannin was independent of AMPK pathway.

Recently, miRNAs have been demonstrated to play vital regulatory roles in adipogenic differentiation and obesity [14]. Overexpression of miR-27a [16] and miR-27b [15] could repress the adipocyte differentiation by reducing PPAR γ expression. These findings further promoted us to examine the expression of miR-27 on Day 2, 4 and 8 after differentiation induction. As shown in Fig. 5, both miR-27a and miR-27b were down-regulated during adipocyte differentiation when compared to undifferentiated cells. However, the expression of miR-27a and miR-27b were significantly ($P < 0.05$) increased by persimmon tannin

compared to the cells treated with MDI alone, especially on Day 2 ($P < 0.001$). Although the expression of miR-27 showed a decline tendency during adipocyte differentiation by treatment of persimmon tannin, the miR-27a and miR-27b expression on Day 8 were also significantly ($P < 0.05$) up-regulated compared with those of control cells. Correspondingly, protein expression of PPAR γ and C/EBP α on Day 2, 4 and 8 were notably suppressed in cells treated with persimmon tannin, and the protein level of SREBP1C was also reduced by persimmon tannin on both Day 4 and 8 (Fig. 5b, c).

Discussion

The results of the present study revealed that persimmon tannin exerted strong inhibitory effect on differentiation of 3T3-L1 preadipocytes. As it is well known, when growth-arrested 3T3-L1 preadipocytes are stimulated by MDI, the

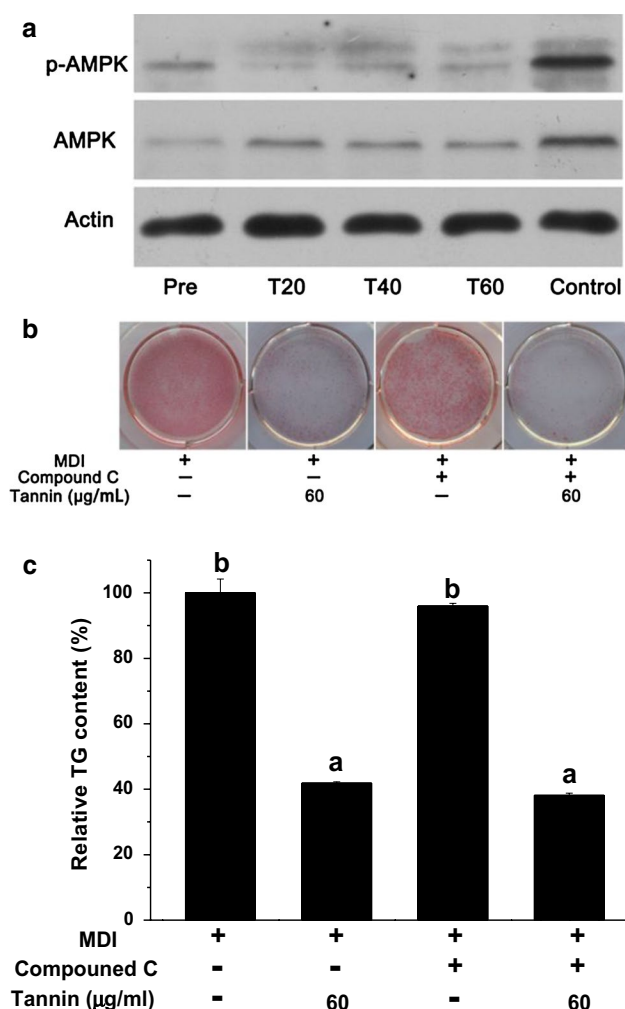


Fig. 4 Effect of persimmon tannin on AMPK in 3T3-L1 cells during differentiation. **a** Two-day postconfluent 3T3-L1 preadipocytes were subjected to adipocyte differentiation by MDI medium in the presence or absence of persimmon tannin (20–60 µg/ml) for 5 days. Protein levels of AMPK and p-AMPK were determined by Western blot. A representation blot from three independent experiments was shown. Cells were treated with Compound C for 8 days in the presence or absent of persimmon tannin, then stained with ORO (**b**), TG content was measured by enzymatic kit (**c**). Results were expressed as mean \pm SE of three independent experiments. Different letters among groups indicated significant difference ($P < 0.05$) by Tukey's test

cells enter into MCE, which is a prerequisite for 3T3-L1 preadipocyte differentiation and occurs in the early stage of differentiation [5, 29]. We observed that persimmon tannin blocked MCE process in the early phase of adipogenesis as evidenced by a reduced number of cells in S and G2/M phases after 18 h and 24 h of incubation. Similar results were found in resveratrol [32], curcumin [29], EGCG [33], curcumin [29] and cocoa polyphenols [2].

MCE, which induced in the early stage of adipogenic program, is a prerequisite step for transcriptional activation and terminal adipocyte differentiation [5, 29]. Within

48 h of MDI treatment, the expression of early adipogenic transcriptional factors such as C/EBP β and C/EBP δ are activated, followed by activation of C/EBP α and PPAR γ [29]. The two transcription factors, C/EBP β and C/EBP δ , which are required for adipocyte differentiation, expressed rapidly (within 4 h) by exposure to differentiation cocktail. Their vital role for adipogenesis in preadipocyte cells resulted from their ability to promote adipogenesis partly by inducing C/EBP α and PPAR γ [5, 7]. C/EBP α can activate itself and PPAR γ . C/EBP α and PPAR γ function together as promoters of many adipogenic genes such as FAS and as maintainers of the differentiated state during adipocyte maturation [5]. PPAR γ exists in two isoforms (PPAR γ 1 and PPAR γ 2), PPAR γ 1 expresses in other cell types besides adipocytes, while PPAR γ 2 is the primary adipocyte-specific isoform [7]. A number of studies indicated that PPAR γ 2 acted as a master regulator which mediated adipocyte differentiation and maintained the terminal differentiation of adipocytes [5, 34]. In this study, we analyzed the expression of adipogenic transcriptional factors in the early stage of adipocyte differentiation. We found that the expression of C/EBP δ , C/EBP α and PPAR γ 2 were reduced by treatment of persimmon tannin in a dose-dependent manner. These results indicated that persimmon tannin inhibited the expression of transcription factors in the early phase of adipocyte differentiation, which resulted in blocking adipogenesis.

The activation of C/EBP α and PPAR γ is not only necessary for adipocyte differentiation in the early stage, but also important for terminal adipocyte differentiation [7]. Furthermore, it was suggested that lipid synthesis notably increased in mature adipocytes. SREBP1C and its downstream lipid biosynthesis-related enzymes including SCD1, ACC1 and FAS participate in adipocyte maturation [35]. Our result showed that the mRNA levels of transcription factors, including PPAR γ 2, C/EBP α and SREBP1C, were significantly decreased by persimmon tannin on Day 5. Cells treated with persimmon tannin also reduced the expression levels of the lipogenic genes such as FAS, as well as PPAR γ target genes including aP2 significantly. Collectively, our data suggested that persimmon tannin inhibited the expression of adipogenic transcription factors in the early stage and subsequently repressed later stages of adipocyte differentiation through C/EBP α –PPAR γ –SREBP1C pathway.

It was reported that some polyphenols such as apigenin [12] and EGCG [36] could inhibit adipocyte differentiation by activating AMPK signaling pathway. In this paper, we attempted to study the effect of persimmon on AMPK. We found that the effect of persimmon tannin on adipocyte differentiation was independent of AMPK pathway. Licochalcone A was reported to prevent adipocyte differentiation and lipogenesis through suppression of PPAR γ and

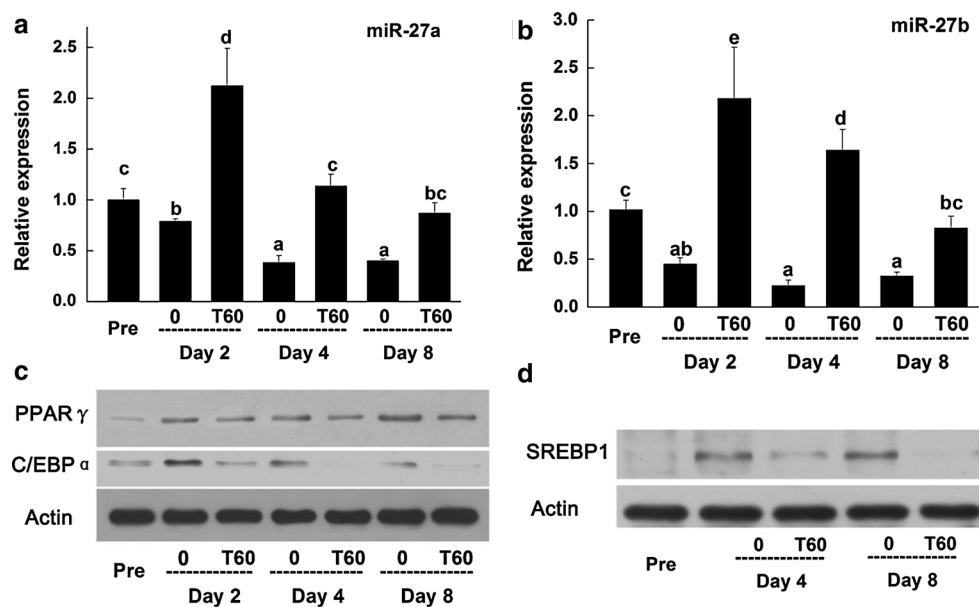


Fig. 5 Effect of persimmon tannin on miR-27a, miR-27b and adipogenic transcription factors in 3T3-L1 cells during differentiation. Two-day postconfluent 3T3-L1 preadipocytes were subjected to adipocyte differentiation by MDI medium in the presence or absence of persimmon tannin (60 μ g/ml). **a** 3T3-L1 preadipocytes were treated with persimmon tannin for 2, 4 and 8 days, respectively, the expression of miR-27a and miR-27b were determined by real time RT-PCR.

Results were expressed as mean \pm SE of three independent experiments. *Different letters* among groups indicated significant difference ($P < 0.05$) by Tukey's test. **b** Protein levels of PPAR γ and C/EBP α were determined by Western blot on Day 2, 4 and 8, respectively. **c** Protein levels of SREBP1 were determined by Western blot on Day 4 and 8, respectively. A representation blot from three independent experiments was shown

SREBP1C pathways, but not activation of AMPK [9]. Roobos, which contains a rich complement of polyphenols, was reported to inhibit adipogenesis and affect adipocyte metabolism, accompanying with decreasing mRNA expression of PPAR γ , FAS and the protein levels of phosphorylation of AMPK [10]. Recently, EGCG was also reported to inhibit 3T3-L1 preadipocyte mitogenesis independent of AMPK pathway [13]. Our results were in line with these studies. AMPK can be activated by increased intracellular AMP/ATP ratio and pathological stresses, such as glucose deprivation and reactive oxygen species (ROS). Nobiletin activated AMPK in 3T3-L1 cells through increasing intracellular ROS generation [30]. On the contrary, roobos increased ATP levels and resulted in decreasing phosphorylation of AMPK [10]. The different effects of polyphenols on AMPK may be due to their differences in chemical structures, antioxidant abilities, etc. In the present study, we did not determine the ROS, AMP and ATP levels. Our previous studies showed that persimmon tannin exerted potent antioxidant activities both in vivo and in vitro [37]. We proposed that the decrease in phosphorylation of AMPK by treatment of persimmon tannin may be attributed to its potent antioxidant activity [37].

PPAR γ is the master regulator of adipocyte differentiation, and it is both necessary and sufficient for adipogenesis [6]. Indeed, the transcription factors such as C/EBP δ and

C/EBP α could not modulate adipogenesis directly. They exerted the differentiation promoting effects through induction of PPAR γ [7]. PPAR γ is predicted to contain a putative binding motif for miR-27a and miR-27b. A luciferase reporter assay demonstrated that the 3'-untranslated region (3'-UTR) of PPAR γ harbored a putative miRNA binding site that specifically bound to miR-27a [16]. Another family member miR-27b could also bind to the 3'-UTR of PPAR γ and decrease PPAR γ mRNA during human adipocyte differentiation [15]. However, results of Lin et al. [38] revealed that miR-27 might not directly repress PPAR γ or C/EBP α mRNA in 3T3-L1 cells, but target an unknown gene or pathway that negatively regulates the two key transcription factors (PPAR γ and C/EBP α) of adipogenic differentiation. Our results indicated that expression of miR-27a and miR-27b were gradually decreased during 3T3-L1 preadipocyte differentiation, while the expression of PPAR γ was increased, but these effects were prevented by persimmon tannin treatment. Although the detailed pathway by which miR-27 inhibited the induction of PPAR γ and C/EBP α was not clear yet, our results indicated that persimmon tannin may inhibit 3T3-L1 preadipocyte differentiation along with up-regulating expression of miR-27a and miR-27b, and down-regulating PPAR γ expression.

Currently, the mechanism of proanthocyanidins regulate miRNA levels is unclear. Resveratrol and EGCG were

shown to bind to miRNAs directly by ^1H NMR spectroscopic method [17]. EGCG bound to miR-122 through an interaction with all of the rings in the molecule, while resveratrol bound to miR-33a and miR-122 primarily through an A ring interaction [17]. Persimmon tannin, which contained high content of EGCG and ECG in the extension units, might also bind to miR-27a and miR-27b. Furthermore, high molecular weight proanthocyanidins can regulate cell signaling by interacting with cell membrane lipids and proteins, inducing changes in membrane biophysics. Hexameric procyanidins were demonstrated to interact with membrane lipid rafts through bonding to cholesterol [39]. The highly polymerized persimmon tannin can be easily extracted with acidic methanol, however, once incubated with the cells, it could be hardly extracted from the cells, and we observed that cells became brown. We speculated that persimmon tannin might exert inhibitory effect on adipocyte differentiation through interacting with membranes. The detailed mechanisms need further research.

In summary, persimmon tannin could inhibit 3T3-L1 preadipocyte differentiation through delaying MCE process and down-regulating expression of adipogenic transcription factors such as PPAR γ , C/EBP α in the early stage of adipogenesis. Our researches further showed that persimmon tannin suppressed PPAR γ expression with modulating the expression of miR-27a and miR-27b, but independent of AMPK pathway. Our findings supported that persimmon tannin had the potential to treat obesity and its related disorders.

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