Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 26 (2015) 1124–1135

Journal of Nutritional Biochemistry

# A-type ECG and EGCG dimers disturb the structure of 3T3-L1 cell membrane and strongly inhibit its differentiation by targeting peroxisome proliferator-activated receptor γ with miR-27 involved mechanism

Wei Zhu[a], Bo Zou[a,b], Rongzu Nie[a], Ying Zhang[a], Chun-mei Li[a,c,*]

[a]College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, China
[b]Key Laboratory of Functional Foods, Ministry of Agriculture, Guangdong Key Laboratory of Agricultural Products Processing, Sericultural & Agri-Food Research Institute Guangdong Academy of Agricultural Sciences, Guangzhou 510610, China
[c]Key Laboratory of Environment Correlative Food Science (Huazhong Agricultural University), Ministry of Education, Wuhan, China

Received 8 January 2015; received in revised form 28 April 2015; accepted 7 May 2015

**Abstract**

The effects of four proanthocyanidin dimers including epicatechin-(4β→8, 2β→O→7)-epicatechin (A-type EC dimer), epicatechin-(4β→8)-epicatechin (B-type EC dimer), epicatechin-3-gallate-(4β→8, 2β→O→7)-epicatechin-3-gallate (A-type ECG dimer) and epigallocatechin-3-gallate-(4β→8, 2β→O→7)-epigallocatechin-3-gallate (A-type EGCG dimer) on 3T3-L1 preadipocyte cell differentiation and the underlying mechanisms were explored and compared. The results showed that A-type ECG dimer and A-type EGCG dimer significantly reduced the intracellular lipid accumulation in 3T3-L1 preadipocyte cells by targeting miR-27a and miR-27b as well as peroxisome proliferator-activated receptor γ (PPARγ) in the early stage of differentiation, while A-type EC dimer and B-type EC dimer showed little effect. In addition, our results revealed that the inhibitory effects of proanthocyanidin dimers on 3T3-L1 preadipocyte differentiation were highly structure-dependent and the effects were associated with the dimer–membrane interactions. The presence of galloyl moieties and A-type linkage within the structure of proanthocyanidins might be crucial for their inhibitory effect on adipogenesis. The strong disturbing effects of A-type ECG and A type EGCG dimers on the fluidity, hydrophobicity and permeability of membrane of 3T3-L1 preadipocyte cell were at least, in part, responsible for their distinct inhibitory effects on adipocyte hyperplasia.


*Keywords:* Adipogenesis; PPARγ; MicroRNAs; Structural-dependent; Membrane disturbing effects

## 1. Introduction

Obesity is a chronic lifestyle-related disease. It imposes significant risks on metabolic disorders such as type 2 diabetes, fatty liver, hypertension and coronary heart disease and is considered to be a worldwide public health problem [1]. Because obesity is caused not only by adipocyte hypertrophy but also by adipocyte hyperplasia (adipogenesis) [2], inhibition of adipocyte differentiation has been considered as an effective strategy for preventing obesity and its associated diseases. The whole process of adipogenesis includes preadipocyte proliferation and their differentiation into mature adipocytes [3,4], which could be well mimicked by the 3T3-L1 preadipocyte differentiation system *in vitro*. Therefore, preadipocytes 3T3-L1 cell line has become one of the most well-characterized and reliable models for studying preadipocyte differentiation *in vitro*. The conversion of 3T3-L1 preadipocytes cell into adipocytes is regulated by a complicated process encompassing diverse transcription factors [5]. Among the various transcription factors, peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding proteins (C/EBPs) and sterol regulatory-element binding proteins (SREBPs) are most important because they control the expression of a variety of genes involved in the maturation process of adipocytes [5,6]. Therefore, searching for agents that can effectively regulate these transcription factors and thus suppress this process has been extensively carried out.

Dietary polyphenols attracted many scientists' attention due to their significant effects on improving metabolic syndrome such as dyslipidemia [7,8], atherosclerosis [9] and nonalcoholic steatohepatitis [10,11]. Monomeric polyphenols such as apigenin [12] and epigallocatechin-3-*O*-gallate (EGCG) [13], resveratrol [14], curcumin [15], and polyphenolic extracts such as cocoa polyphenols extract and grape seed extract [3,16] were reported to inhibit 3T3-L1 adipocyte differentiation effectively through the suppression of PPARγ. It was reported that some polyphenols suppressed PPARγ expression in 3T3-L1 cells by activating AMP-activated protein kinase (AMPK) [12], while some reports indicated that the expression of PPARγ was modified by several specific microRNAs ( miRNAs) such as miR-27a and miR-27b [17,18]. It is known that bioactivities of proanthocyanidins are largely related to their chemical structures, such as monomer

* Corresponding author at: College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, China. Tel./fax: +86 27 87282966.
*E-mail address:* lichmyl@mail.hzau.edu.cn (C. Li).

http://dx.doi.org/10.1016/j.jnutbio.2015.05.006
0955-2863/

compositions, the linkage type of interflavan bonds and the degree of polymerization [19,20]. Previous reports strongly suggested that the influence of polyphenols on adipocyte hyperplasia and the underlying mechanisms were highly structure-dependent [21,22]. However, little was known about the specific effect of proanthocyanidin dimers on lipid metabolism in 3T3-L1 preadipocytes, as well as the underlying mechanisms and the structure–effect relationship. Therefore, one aim of the present study was to compare the effects of four proanthocyanidin dimers including epicatechin-(4β→8, 2β→O→7)-epicatechin (A-type EC dimer), epicatechin-(4β→8)-epicatechin (B-type EC dimer), epicatechin-3-gallate-(4β→8, 2β→O→7)-epicatechin-3-gallate (A-type ECG dimer) and epigallocatechin-3-gallate-(4β→8, 2β→O→7)-epigallocatechin-3-gallate (A-type EGCG dimer) (Supplementary Fig. 1), on 3T3-L1 preadipocyte cell differentiation and the miRNA expression and the related pathways.

How the structural-relevant behavior of polyphenols on adipocyte hyperplasia as well as the underlying mechanisms can be explained? And how do polyphenols initiate the regulating effects on critical transcription factors of adipogenesis such as PPARγ? Considering that polyphenols interact primarily with the cell membranes in the cell systems and different polyphenols may differ greatly in their structural properties such as polarity, number of H-bonds, topological surface area (tPSA), conformation, etc., we proposed that the structural-relevant behavior of polyphenols on adipocyte hyperplasia may be associated with the different compounds–cell membrane interactions. Thereby, we further investigated how the structural properties such as partition coefficient (logP), number of H-bonds and tPSA correlated with the compound–cell membrane interaction as well as the suppression of the above four dimers on 3T3-L1 preadipocyte differentiation.

## 2. Materials and methods

### 2.1. 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-2yl)-2, 5-diphenyltetrazolium bromide (MTT) and Oil Red O (ORO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Mouse monoclonal antibody PPARγ, 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/EBPα was purchased from Proteintech Group, Inc. (Wuhan, China). Commercial kits used for determination of triglyceride (TG) were from Shanghai Mind Bioengineering Co., Ltd. (Shanghai, China). 3,3′-Dioctadecyloxacarbocyanine perchlorate (Dio cell membrane green fluorochrome) and membrane permeable calcium indicator Fluo-3 AM were purchased from Beyotime Institute of Biotechnology (Nanjing, China). 6-Dodecanoyl-2-dimethyl aminonaphthalene (Laurdan) was purchased from Invitrogen/Molecular Probes Inc. (Eugene, OR, USA)

### 2.2. Samples preparation

Epicatechin-(4β→8, 2β→O→7)-epicatechin (A-type EC dimer; Supplementary Fig. 1) was prepared from peanut (*Arachis hypogaea*) red skins as the method we previously reported [21]. Epicatechin-(4β→8)-epicatechin (B-type EC dimer; Supplementary Fig. 1) was isolated from Granny Smith apples (*Malus domestica*) as previously reported [23]. Epicatechin-3-gallate-(4β→8, 2β→O→7)-epicatechin-3-gallate (A-type ECG dimer; Supplementary Fig. 1) and epigallocatechin-3-gallate-(4β→8, 2β→O→7)-epigallocatechin-3-gallate (A-type EGCG dimer; Supplementary Fig. 1) were separated from persimmon as the method we previously reported [20] and further prepared by preparative high-performance liquid chromatography (HPLC). Their purity and identity were confirmed by HPLC and mass spectrometry. The purity of B-type EC dimer was confirmed by HPLC to be 87.57% using commercial procyanidin B1 as the standard, and the purity of three A-type dimers were analyzed by HPLC and calculated to be 88.72%, 82.56% and 93.28% using procyanidin A2 as the standard. The HPLC profiles of dimers are shown in Supplementary Fig. 2.

### 2.3. 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 maintained in DMEM supplemented with 10% newborn calf serum, 100 U/ml of penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. The medium was changed every other day. 3T3-L1 preadipocytes were seeded in 6-well plates at a density of $2\times10^5$ per well and incubated until confluence. After a 2-day confluence, adipocyte differentiation was induced as described by Zhang et al. [24]. In brief, the cells were incubated by DMEM containing 10% FBS and cocktail (MDI, 0.5 mM isobutyl-1-methylxanthine, 1 μM dexamethasone, 10 μg/ml insulin). After 3 days, the medium was replaced by DMEM with 10% FBS and 10 μg/ml insulin, and the cells were incubated for another 3 days. The medium was then replaced with DMEM containing 10% FBS, and the cells were cultured for 2 days. Dimers were dissolved in dimethyl sulfoxide (DMSO), diluted in sterile media to make a final concentrations of 0–100 μg/ml, and the final concentration of DMSO was 0.1%.

### 2.4. Cell viability

3T3-L1 preadipocytes were seeded in 96-well plates at a density of $6\times10^3$ per well and allowed to attach for 24 h at 37°C. After treatment with various concentrations of A-type EC dimer, B-type EC dimer, A-type ECG dimer and A-type EGCG dimer for 24 and 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.

### 2.5. ORO staining

After 8 days of adipocyte differentiation, the cells were washed with phosphate-buffered saline (PBS) and then fixed with 4% formaldehyde for 1 h, 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.

### 2.6. TG determination

3T3-L1 preadipocytes were subjected to differentiate into mature adipocytes in the presence or absence of A-type EC dimer, B-type EC dimer, A-type ECG dimer and A-type EGCG dimer 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.

### 2.7. Cell cycle analysis

Two days after confluence, 3T3-L1 preadipocytes were cultured in MDI medium in the presence or absence of A-type EC dimer, B-type EC dimer, A-type ECG dimer and A-type EGCG dimer for 18 and 24 h. The cells were harvested, washed with PBS and fixed with 70% for at least 2 h on ice. Next, the cells were washed twice with PBS and stained with 40 μg/ml propidium iodine solution containing 500 μg/ml of RNase A at 37°C for 30 min. The quantitation of cell cycle distribution was performed with FACScan cytometry.

### 2.8. Real-time reverse transcriptase polymerase chain reaction

The cells were harvested at indicated time, and total RNA was extracted using Trizol Reagent (Invitrogen; Carlsbad, CA, USA). Reverse transcription (RT) was performed with a first-strand cDNA synthesis kit (Toyobo, Osaka, Japan). After cDNA synthesis, quantitative real-time polymerase chain reaction (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, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCGGA (stem-loop), TGCGCTTCA-CAGTGGCTAAGT (Forward), CCAGTGCAGGGTCCGAGGTATT (reverse); miR-27b, GTCGTATCCA-GTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCAGA (stem-loop), TGCGCTTCACAGTGGCTA-AGT (Forward), CCAGTGCAGGGTCCGAGGTATT (reverse); U6, CGCTTCGGCAGCACATATAC (Forward), AAATATGGAACGCTTCACGA (reverse). Primers for mRNA are shown in Supplementary Table 1. Expression of mRNA or miRNA values was 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 ΔΔCT ($\Delta CT_{sample}-\Delta CT_{control}$) and subsequently using the $2^{-\Delta\Delta CT}$ method [25].

### 2.9. 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% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM β-glycerolphosphate, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/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 was separated by 10% SDS-polyacrylamide gel electrophoresis and then electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat dry milk in Tris Buffered Saline with Tween (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 primary antibodies in TBST (PPARγ, 1: 200; C/EBPα, 1: 1000; SREBP1C, 1:1000; β-actin, 1:1000), 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).

### 2.10. Membrane fluidity

3T3-L1 cells were seeded overnight at 50,000 cells per 2 ml per well in a 6-well culture plates. The growth medium was removed and the cells were incubated with fresh medium in the absence or in the presence of 60 μg/ml of the four dimers (A-type EC dimer, B-type EC dimer, A-type ECG dimer and A-type EGCG dimer) for 6 h. After medium removal, cells were washed twice with PBS and followed by adding 1 ml of prewarmed PBS (pH 7.4) containing 5 μM of the fluorescent probe Dio [26]. After 15 min of incubation at 37°C to allow the incorporation of the probe into the membrane, cells were washed twice with ice-cold PBS and then imaged with an inverted fluorescence microscope (OLYMPUS IX70, Tokyo, Japan) using FITC channel, and fluorescence anisotropy was measured at 501 nm (λ excitation: 484 nm) in a spectrofluorometer (Tecan, Switzerland).

### 2.11. Membrane hydration

After cell incubation in the conditions described above, the culture medium was removed and replaced by 1 ml of PBS containing 5 μM of the fluorescent probe Laurdan [27]. After 15 min of incubation at 37°C, Laurdan generalized polarization (GP) was calculated using the equation:

$$GP = \frac{I380 - I350}{I380 + I350}$$

where $I_{350}$ and $I_{380}$ are the fluorescence intensities at 430 nm after exciting samples at 350 and 380 nm, respectively.

### 2.12. Membrane permeability

After cell incubation in the conditions described above, the culture medium was removed and replaced by 1 ml of Hank's balanced salt solution containing 10 μM of the fluorescent probe Fluo-3 AM [28]. After 30 min of incubation at 37°C, the fluorescence intensity of calcein in the cells was measured at 526 nm (λ excitation: 488 nm).

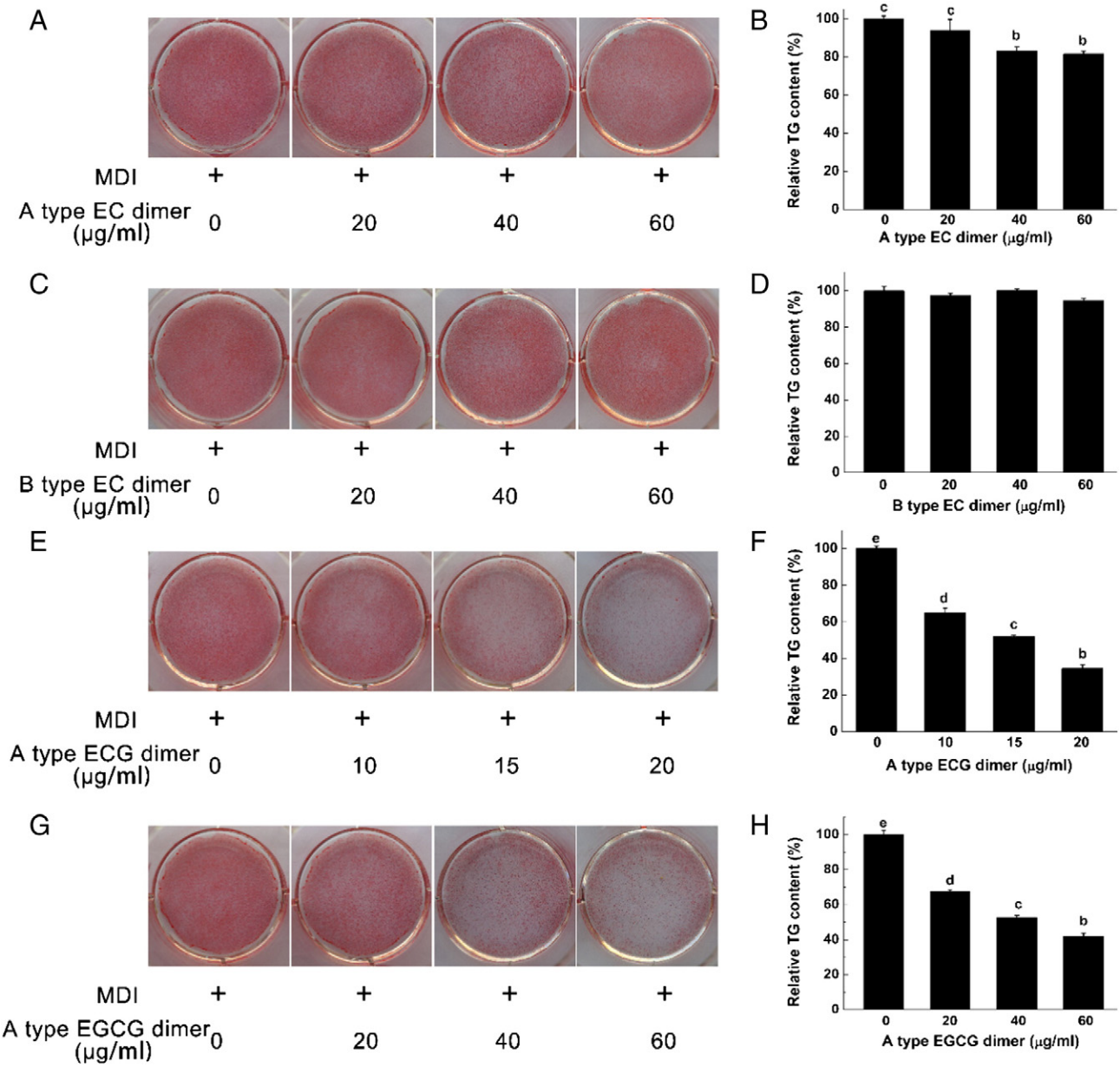


Fig. 1. Effect of dimers on MDI-induced adipocyte differentiation in 3T3-L1 cells. (A, C, E, G) Dimers inhibited intracellular lipid accumulation as indicated by staining with ORO. (B, D, F, H) Intracellular TG content was measured by enzymatic kit. Results were expressed as mean±S.E. of three replications. Different letters among groups indicated significant difference ($P$<.05).

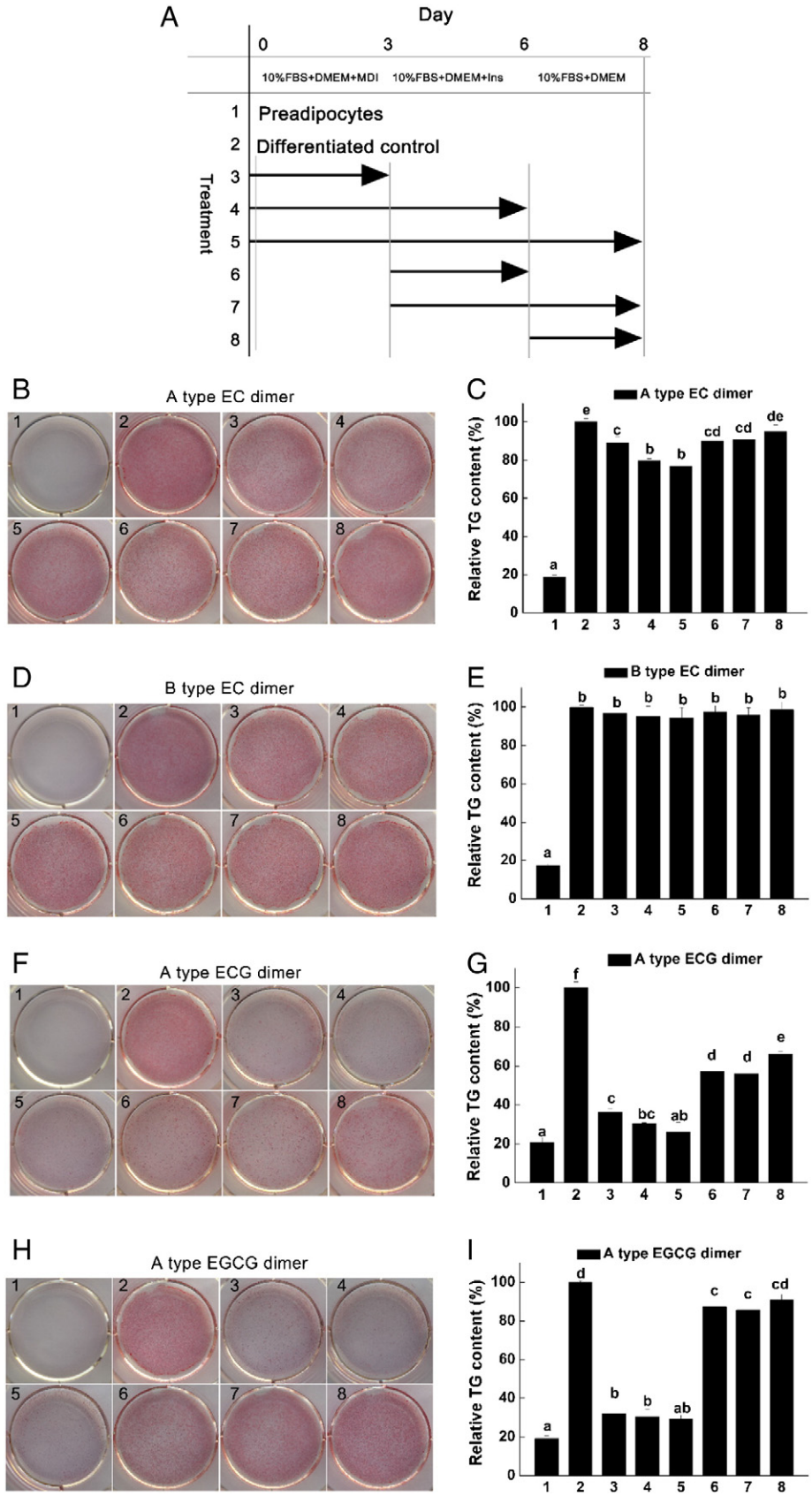


Fig. 2. Dimers blocked 3T3-L1 intracellular lipid accumulation and MCE process in the early stage of adipocyte differentiation. Two-day postconfluent 3T3-L1 preadipocytes were subjected to adipocyte differentiation by MDI medium in the presence A-type EC dimer (60 μg/ml), B-type EC dimer (60 μg/ml), A-type ECG dimer (20 μg/ml) and A-type EGCG dimer (60 μg/ml) for indicated period. (A) The indicated time points with dimers. (B) Cells were stained with ORO. (C) TG content was measured by enzymatic kit. Different letters among groups indicated significant difference ($P<.05$).

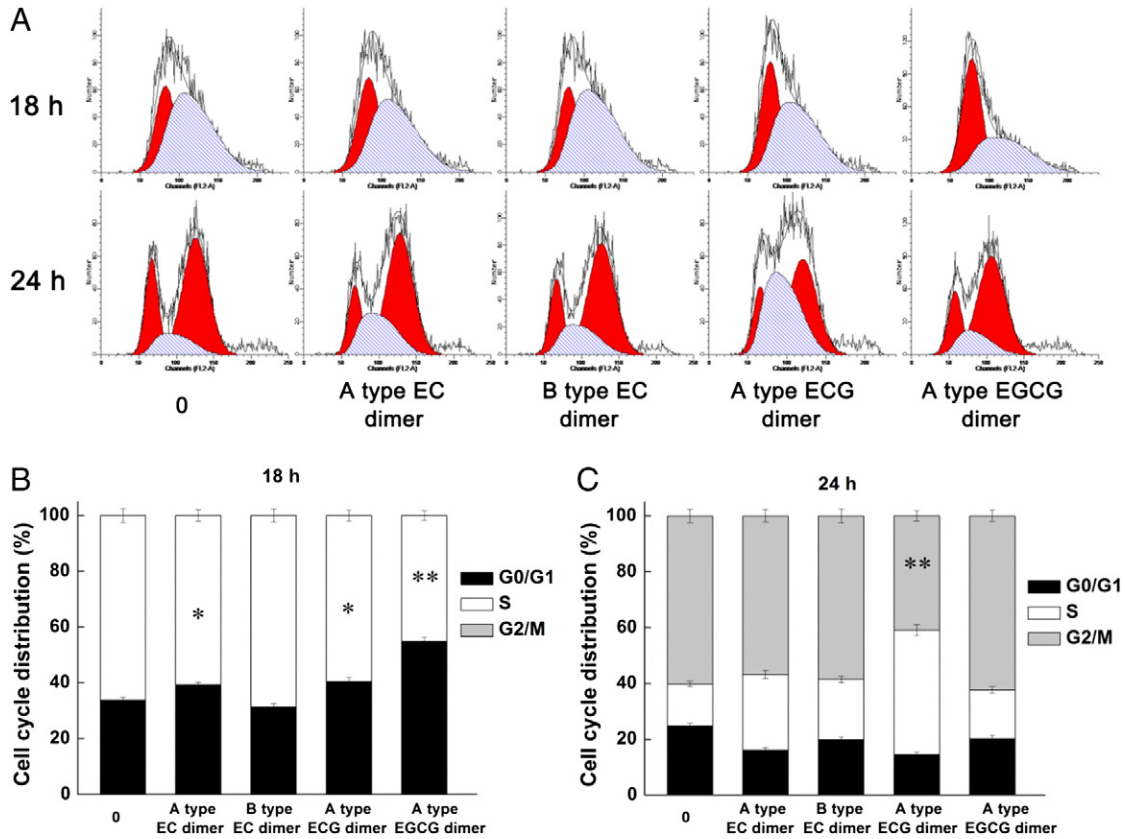


Fig. 3. Two-day postconfluent 3T3-L1 preadipocytes were subjected to adipocyte differentiation by MDI medium in the presence of A-type EC dimer (60 μg/ml), B-type EC dimer (60 μg/ml), A-type ECG dimer (20 μg/ml) and A-type EGCG dimer (60 μg/ml). (A) After 18- and 24-h treatment, the differentiation cells were harvested, stained with propidium iodine (PI), and then subjected to FACScan for cell cycle analysis. (B, C) Quantitative analysis of the percentages of cells in different phases in cell cycle. Results were expressed as mean±S.E. of three replications. *$P$<.05 and **$P$<.01 vs. control group.

### 2.13. Computer simulation

Chem Draw & Chem 3D Pro 14.0 (Cambridge Soft Corporation, Cambridge, MA, USA) was the software used to simulate the molecular structures and associated properties [29]. Using Chem 3D Pro, energy calculations with MM2 force field can be carried out. MM2 methods include (1) energy minimization for locating stable conformation (global minimum) and (2) molecular surface properties for studying molecular motion of atoms. The logP values were predicted by ChemProp. The basic structural information including the formula, the molecular weight, the types of atom and functional groups (hydrophilic and hydrophobic functional groups such as hydroxyl groups and aromatic rings) was used for calculating the logP values, and pH values of 7.4 and temperature of 37°C were set for logP value prediction for each dimer. The ChemBio 2D structure was then put into the ChemBio 3D window, after loading the MM2 job, and the stable 3D conformation and the number of H-bonds could be obtained.

### 2.14. Statistical analysis

All data were presented as means±standard error (means±S.E.) of three replications. Comparisons between groups were made using one-way analysis of variance of SPSS 17.0 followed by Tukey's multiple-range test. A $P$ value <.05 was considered statistically significant.

## 3. Results

### 3.1. A-type ECG dimer and A-type EGCG dimer exerted significant effects against 3T3-L1 adipocyte differentiation, while A-type EC dimer and B-type EC dimer displayed little effect

To investigate the cell toxicity of the four dimers on preadipocytes, cells were incubated with various concentrations of dimers (0–100 μg/ml) for 24 and 48 h, respectively. The cell viability was measured by MTT assay. The results showed that A-type EC dimer and B-type EC dimer showed no cytotoxicity on 3T3-L1 cell viability at concentration below 100 μg/ml, and A-type ECG dimer and A-type EGCG dimer showed no cytotoxicity at concentration below 80 μg/ml (Supplementary Fig. 3).

3T3-L1 preadipocytes were induced with MDI for 8 days in the presence or absence of dimers. On day 8, the cells were stained with ORO. As shown in Fig. 1, the intracellular lipid contents were reduced significantly by treatment with A-type ECG dimer and A-type EGCG dimer. In contrast, non-gallate forms of proanthocyanidin A-type EC dimer and B-type EC dimer displayed little effect against cellular lipid accumulation. Among these dimers, A-type ECG dimer was the most effective. At concentration of 20 μg/ml, A-type ECG dimer reduced the TG content in 3T3-L1 cells by 65%. A-type EGCG dimer inhibited 3T3-L1 cells TGs accumulation by 58% at the concentration of 60 μg/ml. However, A-type EC dimer only reduced about 20% of the TGs accumulation at the concentration of 60 μg/ml and no inhibitory effect was observed on B-type EC dimer even with the concentration up to 80 μg/ml. Cocoa polyphenol extract reduced MDI-induced lipid accumulation in 3T3-L1 cells by 30% at the dose of 100 μg/ml [3]. Our data indicated that A-type ECG dimer and A-type EGCG dimer were more potent in inhibiting MDI-induced lipid accumulation in 3T3-L1 cells than Cocoa polyphenols, while A-type EC dimer and B-type EC dimer exerted little effect.

### 3.2. A-type ECG dimer and A-type EGCG dimer exerted significant effects against 3T3-L1 adipocyte differentiation by targeting miR-27a and miR-27b as well as PPARγ 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

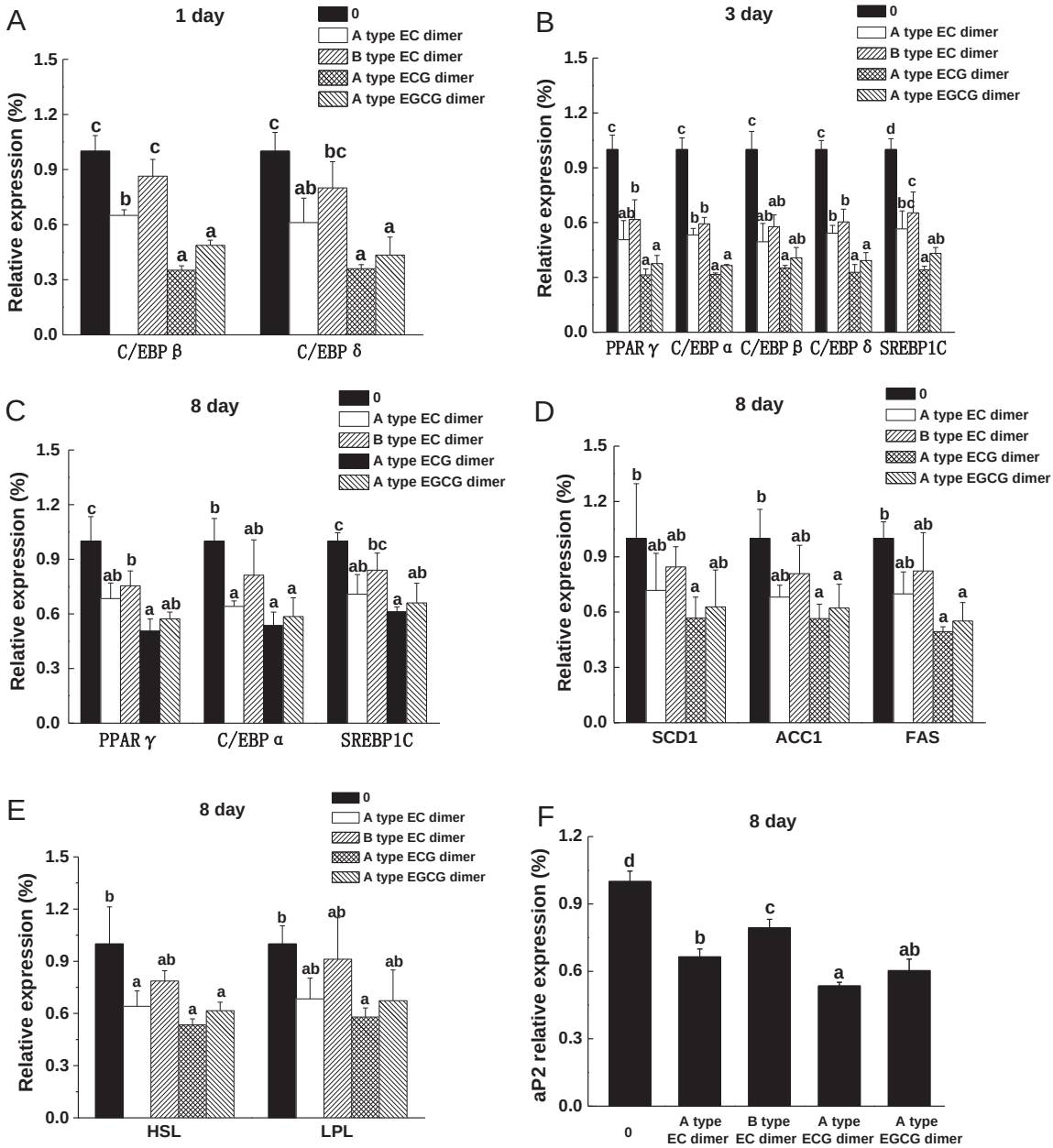


Fig. 4. Effect of dimers on gene expression in 3T3-L1 cells during differentiation. Two-day postconfluent 3T3-L1 preadipocytes were subjected to adipocyte differentiation by MDI medium in the presence or absence of A-type EC dimer (60 μg/ml), B-type EC dimer (60 μg/ml), A-type ECG dimer (20 μg/ml) and A-type EGCG dimer (60 μg/ml). (A) Cells were treated with dimers for 1 day, relative mRNA expression of the adipogenic transcription factors. (B) Cells were treated with dimers for 3 days, relative mRNA expression of the adipogenic transcription factors. (C) Cells were treated with dimers for 8 days, relative mRNA expression of the adipogenic transcription factors. (D, E, F) Cells were treated with dimers for 5 days, relative mRNA expression of downstream genes of adipogenic transcription factors. Results were expressed as mean±S.E. of three replications. Different letters among groups indicated significant difference ($P<.05$).

adipogenic phase primarily influenced by dimers, the cells were treated with dimers at various phases of adipogenesis during 3T3-L1 preadipocyte differentiation (Fig. 2A). It was observed that the presence of A-type ECG dimer and A-type ECGG dimer in the whole stage of adipogenesis (treatment 5) showed a dramatic ($P<.001$) reduction in lipid accumulation (Fig. 2F–I). Moreover, similar results were observed in cells incubated with A-type ECG dimer and A-type ECGG dimer 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 A-type ECG dimer and A-type ECGG dimer 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, treatment 7) exhibited limited inhibition on lipid accumulation in adipocyte differentiation (Fig. 2F–I). A-type EC dimer exerted weak antiadipogenic activity in the early stage of differentiation (Fig. 2B and C), while B-type EC dimer had no effect on adipogenesis in different stage of cell differentiation (Fig. 2D and E). Our results indicated that the inhibitory effect of A-type dimers 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.

Considering that mitotic clonal expansion (MCE) is necessary for the differentiation of 3T3-L1 preadipocytes, we further attempted to elucidate the effect of the four dimers on MCE during adipocyte differentiation. The inhibitory effects of dimers on cell cycle process

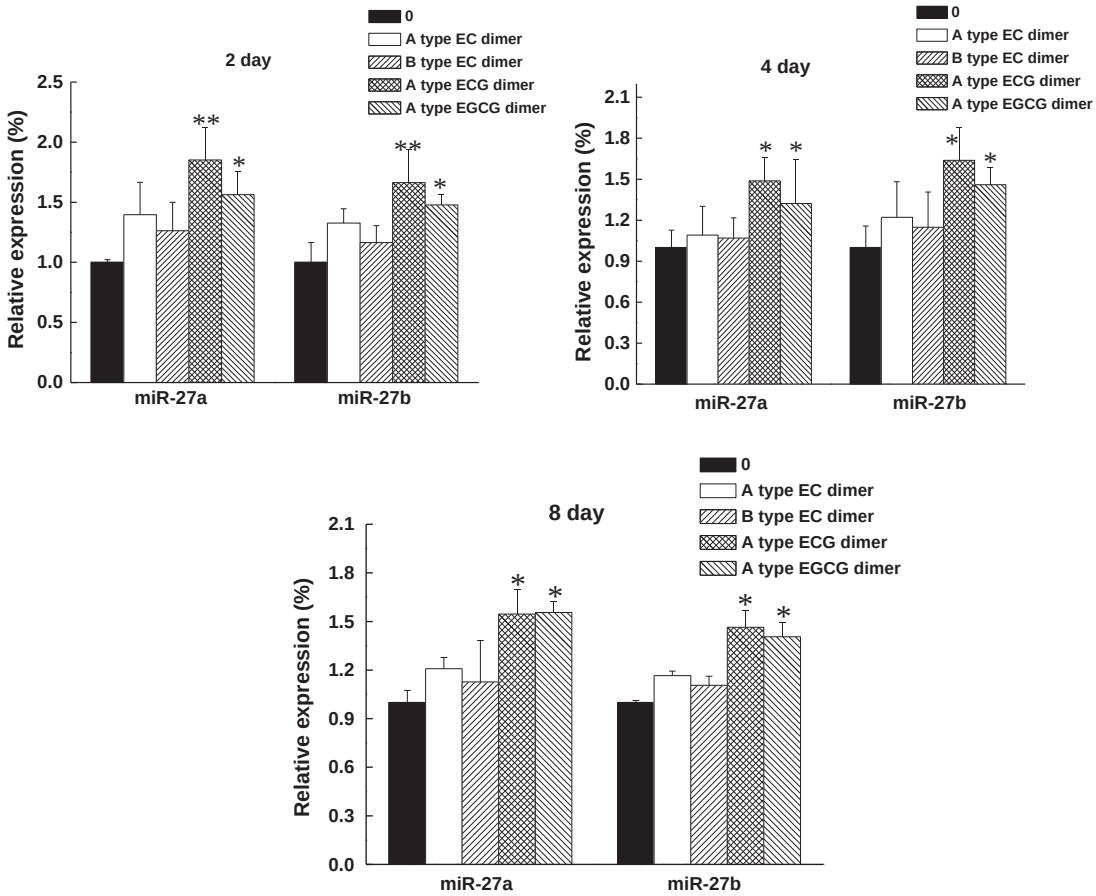


Fig. 5. Effect of dimers on miR-27a and miR-27b in 3T3-L1 cells during differentiation. Two-day postconfluent 3T3-L1 preadipocytes were subjected to adipocyte differentiation by MDI medium in the presence or absence of A-type EC dimer (60 μg/ml), B-type EC dimer (60 μg/ml), A-type ECG dimer (20 μg/ml) and A-type EGCG dimer (60 μg/ml). The expression of miR-27a and miR-27b was determined by real-time RT-PCR. Results were expressed as mean±S.E. of three replications. *$P$<.05 and **$P$<.01 vs. control group.

were examined by flow cytometry assay. According to previous reports [15], 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 all three A-type dimers clearly exhibited a significant ($P$<.05) delayed entry of cells into S phase after 18 h compared to the control cells (Fig. 3A, B), while B-type EC dimer showed no effect. In addition, after 24 h of treatment, significant ($P$<.01) suppression of cell cycle entry to G2/M phase was also observed in cells treated with A-type ECG dimer (Fig. 3A, C). Thus, flow cytometry assay provided further supporting evidence for that A-type dimers blocked 3T3-L1 preadipocyte differentiation in the early stage of adipocyte differentiation.

To elucidate the molecular mechanisms of dimers in adipogenesis, we firstly examined the levels of some transcription factors such as C/EBPs and PPARγ in the early stage by real-time RT-PCR. It was clear that the expressions of C/EBPβ and C/EBPδ were significantly ($P$<.05) decreased by A-type dimers treatment for 1 day (Fig. 4A). We also observed that A-type ECG dimer (A-type EGCG dimer) treatment for 3 days significantly ($P$<.05) reduced the mRNA levels of C/EBPα, PPARγ and SREBP1C by about 68% (63%), 69% (62%) and 66% (57%), respectively, while the expressions of these genes were suppressed by about 50% by A-type EC dimer treatment. Also, only about 40% of inhibition was observed in cells treated with B-type EC dimer on day 3, but little effect of B-type EC dimer on expression of C/EBPβ and C/EBPδ on day 2 was seen. These results indicated that A-type dimer suppressed the expression of marked transcription factors including C/EBPβ, C/EBPδ, C/EBPα, PPARγ and SREBP1C in the early phase of adipocyte differentiation, with A-type ECG dimer being the most potential.

We further examined the mRNA levels of transcription factors in late stage of differentiation (on day 8). In A-type ECG dimer-treated cells, PPARγ, C/EBPα and SREBP1C mRNA levels were decreased approximately by 50%, 47% and 39%, respectively, as compared with those of the differentiated cells (Fig. 4C). A-type EGCG dimer induced an approximately 43%, 42% and 34% of decrease in the expression of PPARγ, C/EBPα and SREBP1C, respectively. A-type EC dimer also decreased expression of these genes, but its effect was not as strong as that of A-type ECG dimer and A-type EGCG dimer, while B-type EC dimer showed little effect on the expressions of these genes. We subsequently investigated the effects of the dimers on downstream lipid biosynthesis-related target genes of SREBP1C including stearoyl-CoA desaturase 1 (SCD1), acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS). As shown in Fig. 4, the expressions of the lipogenic genes such as SCD1, ACC1 and FAS were significantly ($P$<.05) suppressed by A-type ECG dimer and A-type EGCG dimer (Fig. 4D), while A-type EC dimer and B-type EC dimer exerted little effect on the expressions of these lipogenic genes. The aP2, which participated in fatty acid transport and metabolism [30], and genes involved in the lipolysis such as hormone-sensitive lipase (HSL) and lipid protein lipase (LPL) were also significantly ($P$<.05) attenuated by A-type ECG dimer and A-type EGCG dimer. Taken together, A-type ECG dimer and A-type EGCG dimer, particularly A-type ECG dimer, exhibited potent inhibitory effects on lipogenic genes expressions in late stage of differentiation.

Although our findings indicated that A-type ECG dimer and A-type EGCG dimer suppressed 3T3-L1 preadipocyte differentiation in the early phase of adipogenesis through regulating C/EBPs, PPARγ,

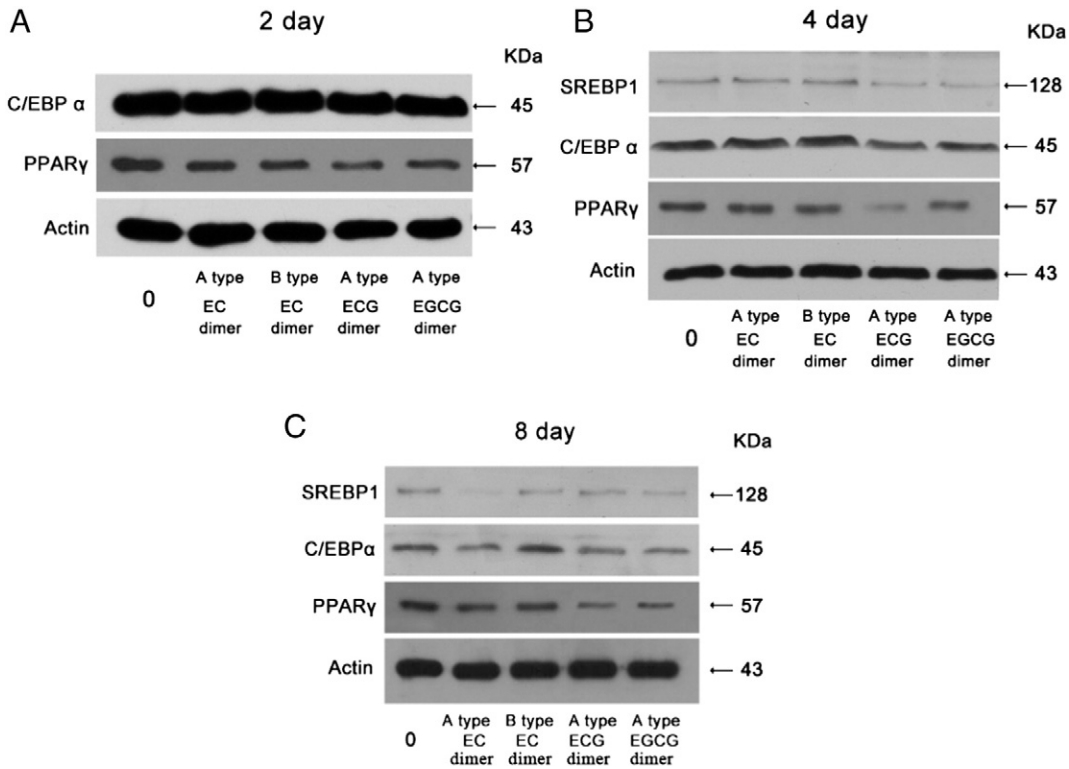


Fig. 6. Effect of dimers on adipogenic transcription factors in 3T3-L1 cells during differentiation. Two-day postconfluent 3T3-L1 preadipocytes were subjected to adipocyte differentiation by MDI medium in the presence or absence of A-type EC dimer (60 μg/ml), B-type EC dimer (60 μg/ml), A-type ECG dimer (20 μg/ml) and A-type EGCG dimer (60 μg/ml). Protein levels of PPARγ, SRBBP1 and C/EBPα were determined by Western blot at indicated time. A representation blot from three replications was shown.

SREBP1C and their target genes, the up-stream regulators of these transcription factors were unclear. Some reports demonstrated that polyphenols could suppress the expression of PPARγ in 3T3-L1 cells by activating AMPK, but our previous study suggested that the suppression of the four dimers on PPARγ in 3T3-L1 cells was independent of AMPK pathway (data not shown). Recently, miRNAs have been demonstrated to play vital regulatory roles in adipogenic differentiation and obesity [31]. Overexpression of miR-27a [18] and miR-27b [17] repressed the adipocyte differentiation by reducing PPARγ expression. We speculated that the expression of miR-27 might be modulated by A-type ECG dimer and A-type EGCG dimer. Therefore, we examined the expression of miR-27 on days 2, 4 and 8 after differentiation induction. As shown in Fig. 5, the expression of miR-27a and miR-27b was significantly ($P<.05$) increased by A-type ECG dimer and A-type EGCG dimer compared to the cells treated with MDI alone. A-type EC dimer and B-type EC dimer did not affect miR-27a and miR-27b expressions. Correspondingly, protein expression of PPARγ on days 2, 4 and 8 were notably suppressed in cells treated with A-type ECG dimer and A-type EGCG dimer, and the protein levels of C/EBPα and SREBP1C were also reduced by these two dimers on both days 4 and 8 (Fig. 6B and C). In contrast, A-type EC dimer and B-type EC dimer had limited effect on the protein levels of PPARγ, C/EBPα and SREBP1C.

### *3.3. The different effects of diverse dimers on 3T3-L1 preadipocyte differentiation may be associated with the dissimilar compound–cell membrane interactions*

The above results strongly indicated that the effects of polyphenols on adipocyte hyperplasia were highly related to their structures. But how such different behaviors can be explained? Considering that polyphenols interacted primarily with the cell membranes in cell systems and many cellular process such as cell singling and cell cycle were membrane-dependent [32,33], we speculated that the different effects of different dimers on 3T3-L1 preadipocyte differentiation might be due to their different modes of action on the cell membrane.

Effects of different dimers on the cell membrane fluidity were measured using Dio as a probe. As shown in Fig. 7A, all A-type dimers resulted in a decreased fluorescence intensity of cell membrane, indicating a decrease of membrane fluidity (membrane rigidification), while B-type EC significantly increased the fluorescence intensity, indicative of increased membrane fluidity. It seemed that the membrane fluidity decrease effects of A-type dimers correlated with their adipocyte differentiation inhibitory effects. Besides the fluidity, membrane hydration is another membrane property associated with functions of cell membranes [34]. Laurdan, which is a fluorescent probe incorporated in lipophlilic regions of the membrane lipid bilayer, is highly sensitive to water relaxation of the surrounding phospholipids. A decrease of the GP values of Laurdan indicated an increase of hydration [27]. As seen from Fig. 7B, A-type dimers increased the hydration of 3T3-L1 cells membrane significantly in the order of A-type ECG dimer>A-type EGCG dimer>A-type EC dimer, while B-type EC dimer exerted no effect. When the membrane hydration increase effect of the dimers correlated with their adipocyte differentiation inhibitory effects, we could clearly see that the higher hydration increase effects of dimers, the stronger inhibitory effect on adipocyte differentiation. The interaction of dimers with membrane might not only change the membrane fluidity and hydration, but also affect the permeability of the membrane, which could be measured using fluorescent probe Fluo-3 AM. It was clear that A-type dimers notably increased the permeability of the membrane, but B-type EC dimer did not affect the membrane permeability, which correlated with their adipocyte differentiation inhibitory effects well.

## 4. Discussion

In the present study, we compared the effects of four proanthocyanidin dimers on differentiation of 3T3-L1 preadipocytes. Our data

strongly suggested that the structures of polyphenols greatly affected their effects on adipocyte hyperplasia. Kim et al. [22] reported that ECG showed a stronger inhibitory effect on differentiation of 3T3-L1 cells than did EGCG. Results of Lin et al. [21] also suggested that gallated tea polyphenols, such as EGCG and ECG, significantly decreased intracellular lipid content in human HepG2 cells, while non-gallate forms of tea polyphenols, such as C and EC, displayed little effect. In the present study, we observed that A-type proanthocyanidin dimers, especially A-type ECG dimer and A-type EGCG dimer, significantly decreased intracellular lipid accumulation, while A-type EC dimer showed less inhibitory potential, and B-type EC dimer exerted little effect. Similar results were also obtained for the four dimers in L02 cells as we previously reported [35]. These results strongly indicated that the presence of galloyl moieties within the structures of proanthocyanidins might be a crucial structural requirement for their potent inhibition on adipocyte hyperplasia and intracellular lipid accumulation. In addition, the A-type interflavan linkage in proanthocyanidins molecular had great contribution to their adipocyte hyperplasia inhibitory effect.

Many polyphenols were reported to inhibit adipocyte differentiation through suppressing the key transcription factors involving in the maturation process of adipocytes such as PPARγ, C/EBPs and SREBPs [22,36]. In the current study, we found that A-type ECG dimer and A-type EGCG dimer exerted significant effects against 3T3-L1 adipocyte differentiation by suppressing mRNA levels of PPARγ, C/EBPα and SREBP1C as well as the mRNA levels of PPARγ target genes including HSL, LPL and aP2 and SREBP1C downstream lipid biosynthesis-related genes including SCD1, ACC1 and FAS. However, A-type EC and B-type EC dimers showed little effect on the expressions of these genes. As reported by Furuyashiki et al. [37], ECG and EGCG were stronger than catechin and EC for greatly decreasing expression of the adipocyte PPARγ2 and C/EBPα. Kim et al. [22] showed that catechins (CG, EGCG, ECG, EGC), especially ECG and EGCG, significantly reduced intracellular lipid accumulation and repressed the expression of SREBP1c, and ECG was found to be more potential than EGCG. These results suggested that the presence of galloyl moieties and A-type linkage within the structure of proanthocyanidins might be crucial for their inhibitory effect on adipogenesis.

It was reported that AMPK and some kinds of miRNAs such as miR-27a and miR-27b could act as the negative regulators of adipocyte differentiation by inhibition of PPARγ [17,18]. Apigenin [12] and resveratrol [12] were reported to down-regulate the expressions of PPARγ and its down-stream genes through activating AMPK in 3T3-L1 cells. Our previous study revealed that the prevention of both A-type ECG dimer and A-type EGCG dimer in L02 cells was independent of AMPK pathway [35]. In the present study, we also found that the protein levels of AMPK and its phosphorylation were not increased by treatment of A-type ECG dimer and A-type EGCG dimer in 3T3-L1 preadipocyte cells (data not shown). Instead, the expressions of miR-27a and miR-27b were significantly up-regulated in cells treated with A-type ECG dimer and A-type EGCG dimer (Fig. 5). Some authors suggested that polyphenols may bind to miRNAs directly. For example, resveratrol and EGCG were shown to bind to miRNAs directly by $^{1}$H NMR spectroscopic method [38]. However, Lin et al. [39] proposed that miR-27 might not directly repress PPARγ or C/EBPαin 3T3-L1 cells, but target an unknown gene that negatively regulates the two key transcription factors (PPARγ and C/EBPα) of adipogenic differentiation. Although the detailed pathway by which miR-27 inhibited the induction of PPARγ and C/EBPα was not clear yet, our data demonstrated that A-type ECG dimer and A-type EGCG dimer blocked MCE process in the early phase of adipogenesis by targeting PPARγ as well as miR-27a and miR-27b, while A-type EC and B-type EC dimers showed little effect on the expressions of these genes. Current data suggested that grape proanthocyanidin extracts, EGCG [38] and cocoa proanthocyanidins [40], differed in their effects on modulating expression of miRNAs. These results and our current data suggested that the effects of polyphenols on miRNAs expression were structure-dependent. Our results indicated that A-type of linkage and the galloyl moieties existing in proanthocyanidin moleculars might play very important roles in up-regulating the expressions of miR-27a and miR-27b.

The ability of a compound to interact with the membrane and to change the biophysical parameters of the membrane is thought to be critical for its biological effects [41]. Selvaraj et al. [42] suggested that trans-resveratrol could alter the membrane rigidity and thus inhibiting cell proliferation. This result implied that different inhibitory effects of different dimers on 3T3-L1 preadipocyte cell might be due to their dissimilar membrane interactions. Therefore, we further investigated the influence of different dimers on membrane parameters to examine possible correlations with the inhibitory effects.

It is well known that good fluidity, suitable hydrophobicity and permeability of membrane are very important for cells to maintain their normal growth and proliferation. Alterations of membrane properties can affect the activity of membrane-associated enzymes [43] and membrane receptors [44], leading to some changes in intracellular signaling pathways [45,46]. It was reported that the decrease of membrane fluidity could also lead to alterations in functionality of membrane-associated cell singling and cell cycle [27,43,46]. Some food constituents were reported to inhibit the proliferation of tumor cells by decreasing the membrane fluidity [33,42]. Previous studies suggested that cell hydration regulated cell function and a decrease in cellular hydration in liver triggered the protein catabolic states that accompanied diseases [47]. Hepatocyte swelling might open stretch-activated nonselective cation channels, which allowed passage of $Ca^{2+}$ into the cell and activated AMPK [48]. Our data suggested that A-type procyanidin dimers, particularly A-type ECG dimer, strongly disturbed the structures of cell membrane by decreasing the fluidity and hydrophobicity and increasing the permeability of membrane of 3T3-L1 preadipocyte cell, thus affecting the membrane-associated intracellular signaling pathways and inhibiting its differentiation. The different interactions between the dimers and the membrane could be explained, at least in part, by the different structural properties of the dimers such as polarity, number of H-bonds, tPSA and conformation. The partition coefficient (logP), the number of H-bonds formed and the tPSA of the four dimers were calculated using a software of CS ChemProp (2014) and shown in Table 1. LogP was related with lipid penetration, while tPSA and H-bonds number showed the interaction of dimers in the lipid–water interface. The order of the logP values of the four tested dimers was as follows: A-type ECG dimer (5.24)>A-type EGCG dimer (4.17)>A-type EC dimer (3.32)>B-type EC dimer (2.56). This order was in line with the relative inhibitory potency of four dimers on 3T3-L1 cell differentiation. The higher the values of logP, the higher hydrophobic of the molecular, and the higher affinity of a compound for the hydrophobic region of cell membrane. Previous report showed that green tea catechins with a galloyl moiety had a higher affinity for hydrophobic lipid bilayers of membrane than free catechins [49]. Our data were in line with this as well. In consequence, this could partially explain the strongest inhibition of 3T3-L1 cell differentiation by A-type ECG dimer. Compared to B-type EC dimer, beside the higher relative hydrophobicity, A-type dimers were more elongated and disordered due to the additional 2β→O→7 ether bond in the molecular (Supplementary Fig. 4), which made A-type dimers having a greater number of contact sites with the membrane, thus resulting in a stronger interaction with the membrane. According to previous reports [50], apart from the interaction with the hydrophobic region, proanthocyanidins oligomers could interact with the polar headgroups of lipids at the lipid–water interface via formation of hydrogen bonds. Huh et al. [51] suggested that the existence of H-bonds formed between catechins and adjacent lipids decreased hydration repulsion of lipid bilayers. In the present study, we observed that A-type dimers

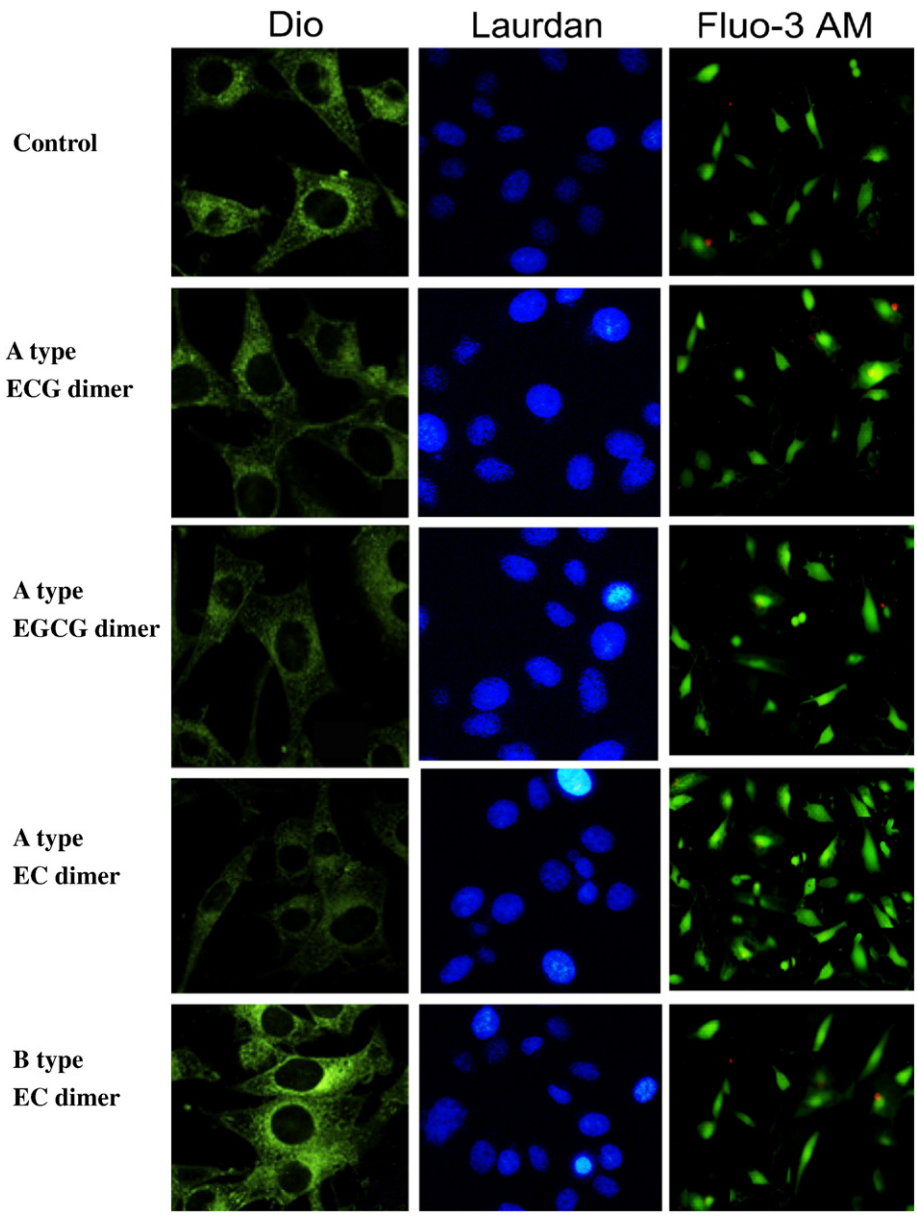


Fig. 7. Effect of dimers on 3T3-L1 preadipocytes membrane physical properties (fluidity, hydration, permeability). Representative fluorescence photomicrograph of Dio, Laurdan, Fluo-3 AM probe output by fluorescence microscope (magnification ×200; scale bars represent 100 μm). Relative fluorescence intensity of dimers on 3T3-L1 preadipocytes membrane physical properties: (A) fluidity, (B) hydration and (C) permeability. Results were expressed as mean±S.E. of three replications. *$P$<.05 and **$P$<.01 vs. control group.

increased the hydration of 3T3-L1 cells membrane significantly in the order of A-type ECG dimer>A-type EGCG dimer>A-type EC dimer, while B-type EC dimer exerted no effect on the hydration of 3T3-L1 cells membrane. As shown in Table 1, the relative potencies of both the number of H-bonds and the tPSA of the four dimers were as follows: A-type EGCG dimer>A-type ECG dimer>B-type EC dimer>A-type EC dimer. Our data indicated that gallated dimers (A-type ECG/EGCG dimer) which had higher tPSA and could form more hydrogen bonds significantly increased membrane hydration than that of non-gallated dimers (A/B-type EC dimer). The result correlated with their adipocyte differentiation inhibitory effects well. It was suggested that catechins with galloyl moiety (EGCG, ECG) had higher tPSA and could form more H-bonds and exerted more potential inhibitory effect on bacteria and cancer cells [52,53]. Our results agreed with this as well because A-type dimers, especially the A-type ECG dimer, could strongly interact with the lipid bilayers of the membrane and disturb its structure, thus notably increasing the permeability of the membrane. Our data indicated that the membrane permeability increase effects of the four dimers correlated with their adipocyte differentiation inhibitory effects well.

The higher permeability increase effects of A-type dimers, especially A-type ECG and EGCG dimers, might lead to the higher cell entry of

Table 1
Topological polar surface area (tPSA), number of H-bonds, number of intramolecular H-bond (IMHBs) and partition coefficient for octanol water mixture (logP) for the four characteristic units (CS ChemProp, 2014)

| Characteristic units | tPSA | H-bonds | IMHBs | LogP |
|---|---|---|---|---|
| A-type ECG dimer | 343.28 | 33 | 7 | 5.24 |
| A-type EGCG dimer | 374.51 | 36 | 9 | 4.17 |
| A-type EC dimer | 209.76 | 21 | 3 | 3.32 |
| B-type EC dimer | 220.76 | 22 | 2 | 2.56 |

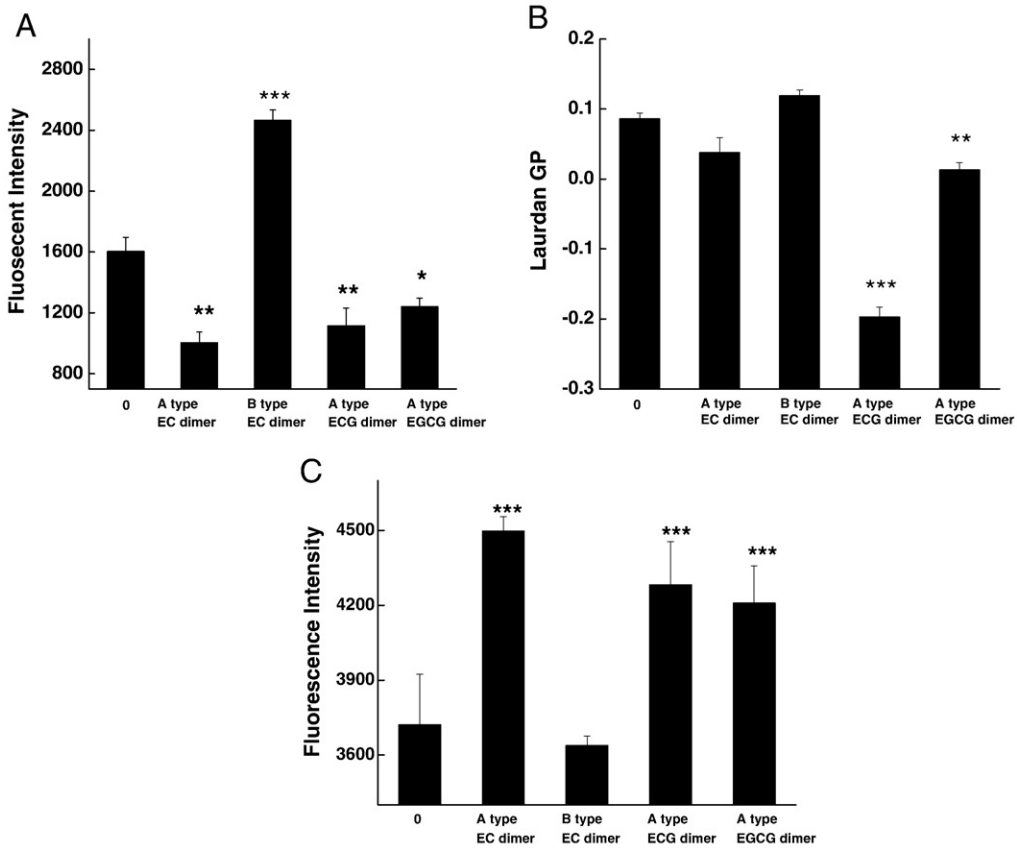


**Fig. 7** (*continued*).

proanthocyanidins, thus becoming the major regulator over the other properties measured in our experiments. Our further work on determining the cell uptake of the dimers by HPLC confirmed our hypothesis. As we expected, the absorption values of A-type ECG dimer, A-type EGCG dimer and A-type EC dimer by the cell were 5.8, 3.5 and 1.0 nmol/mg protein, respectively, after incubating for 4 h, but we failed to detect the B-EC dimer inside the cell (Supplementary Fig. 5). This might be due to the more stability and the higher affinity for the cell membrane of A-type dimers than B-type dimers. Our results were in line with that of Appeldoorn et al. [54], who indicated that although both are poorly absorbed (<5%), A-type dimers ($A_1$ and $A_2$) were better absorbed than B-type dimer $B_2$ in rats. Our results also indicated that galloylated A-type proanthocyanidins were more bioavailable than non-gallated A-type ones and B-type ones, thus exerting better biological activities. B-type proanthocyanidins are abundant in many foods such as apple, pear, cocoa, grape, blueberries, and so on, while A-type proanthocyanidins only exist in a limited number of foods, such as cranberries, persimmon fruits, plum and peanuts. Appeldoorn et al. [54] suggested that high DP oligomers might enhance the absorption of $B_2$ but not $A_1$. Whether the presence of A-type proanthocyanidins could help B-type proanthocyanidins to exert biological activities by facilitating their entry to cells needs further study.

As reported in the literatures, the concentrations of proanthocyanidins used in most cell models varied from 10 to 200 μg/ml, depending on its cell toxicity, and the dosages of proanthocyanidins used in animal models ranged from 10 to 250 mg/kg body weight. It was reported that a concentration of 25 μg/ml of proanthocyanidins is needed *in vitro* to reach the significant regulation of miRNA [55]. Results from Qiu et al. [56] indicated that after a single oral administration of 100 mg/kg body weight for each compound, the plasma concentrations of EGC and EGCG dimer were 7.5 and 9.3 nmol/l, separately. Although the *in vivo* and *in vitro* conditions are not comparable, Baselga-Escudero et al. [55] suggested that when the rats were administrated with 250 mg/kg body weight of proanthocyanidins, the plasma levels of proanthocyanidins and their metabolites (about 17.18 μmol/l in total) were five times higher than that in the cell media at 25 μg/ml of proanthocyanidins. Therefore, the concentrations (20–60 μg/ml) we used in the present study were enough to modulate miRNA levels *in vivo*.

In summary, our study clearly suggested that A-type ECG dimer and A-type EGCG dimer inhibited 3T3-L1 preadipocyte differentiation through down-regulating expression of adipogenic transcription factors, such as PPARγ and C/EBPα and up-regulating expression of miR-27a and miR-27b in the early stage of adipogenesis. Our data also suggested that different membrane interactions of structural different dimers were, at least in part, responsible for their distinct inhibitory effects on adipocyte hyperplasia. A-type ECG and A-type EGCG dimers strongly disturbed the structures of cell membrane by decreasing the fluidity and hydrophobicity and increasing the permeability of membrane of 3T3-L1 preadipocyte cell, thus displaying significant inhibition on its differentiation.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2015.05.006.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgment

This study was supported by the Chinese Ministry Program for New Century Excellent Talents in University (NCET-12-0865), the National Natural Science Foundation of China (No. 31271833), Special Fund for

Agro-scientific Research in the Public Interest (No. 201203047) and Fundamental Research Funds for the Central Universities (2013PY022).

## References


[1] Wang S, Moustaid-Moussa N, Chen L, Mo H, Shastri A, Su R, et al. Novel insights of dietary polyphenols and obesity. J Nutr Biochem 2014;25:1–18.
[2] de Ferranti S, Mozaffarian D. The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences. Clin Chem 2008;54:945–55.
[3] Min SY, Yang H, Seo SG, Shin SH, Chung MY, Kim J, et al. Cocoa polyphenols suppress adipogenesis in vitro and obesity in vivo by targeting insulin receptor. Int J Obes (Lond) 2013;37:584–92.
[4] Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, et al. Dynamics of fat cell turnover in humans. Nature 2008;453:783–7.
[5] Tang QQ, Lane MD. Adipogenesis: from stem cell to adipocyte. Annu Rev Biochem 2012;81:715–36.
[6] Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM. Transcriptional regulation of adipogenesis. Gene Dev 2000;14:1293–307.
[7] Hsu CL, Yen GC. Effect of gallic acid on high fat diet-induced dyslipidaemia, hepatosteatosis and oxidative stress in rats. Br J Nutr 2007;98:727–35.
[8] Liu Y, Wang D, Zhang D, Lv Y, Wei Y, Wu W, et al. Inhibitory effect of blueberry polyphenolic compounds on oleic acid-induced hepatic steatosis in vitro. J Agric Food Chem 2011;59:12254–63.
[9] Zang M, Xu S, Maitland-Toolan KA, Zuccollo A, Hou X, Jiang B, et al. Polyphenols stimulate AMP-activated protein kinase, lower lipids, and inhibit accelerated atherosclerosis in diabetic LDL receptor-deficient mice. Diabetes 2006;55:2180–91.
[10] Zou B, Ge Z, Zhang Y, Du J, Xu Z, Li C-m. Persimmon Tannin accounts for hypolipidemic effects of persimmon through activating of AMPK and suppressing NF-kappaB activation and inflammatory responses in high-fat diet rats. Food Funct 2014;5:1536–46.
[11] Baselga-Escudero L, Blade C, Ribas-Latre A, Casanova E, Salvado MJ, Arola L, et al. Chronic supplementation of proanthocyanidins reduces postprandial lipemia and liver miR-33a and miR-122 levels in a dose-dependent manner in healthy rats. J Nutr Biochem 2014;25:151–6.
[12] Ono M, Fujimori K. Antiadipogenic effect of dietary apigenin through activation of AMPK in 3T3-L1 cells. J Agric Food Chem 2011;59:13346–52.
[13] Hung PF, Wu BT, Chen HC, Chen YH, Chen CL, Wu MH, et al. Antimitogenic effect of green tea (−)-epigallocatechin gallate on 3T3-L1 preadipocytes depends on the ERK and Cdk2 pathways. Am J Physiol Cell Physiol 2005;288:C1094–108.
[14] Kwon JY, Seo SG, Yue S, Cheng JX, Lee KW, Kim KH. An inhibitory effect of resveratrol in the mitotic clonal expansion and insulin signaling pathway in the early phase of adipogenesis. Nutr Res 2012;32:607–16.
[15] Kim CY, Le TT, Chen C, Cheng J-X, Kim K-H. Curcumin inhibits adipocyte differentiation through modulation of mitotic clonal expansion. J Nutr Biochem 2011;22:910–20.
[16] Jeong YS, Hong JH, Cho KH, Jung HK. Grape skin extract reduces adipogenesis- and lipogenesis-related gene expression in 3T3-L1 adipocytes through the peroxisome proliferator-activated receptor-gamma signaling pathway. Nutr Res 2012;32:514–21.
[17] Karbiener M, Fischer C, Nowitsch S, Opriessnig P, Papak C, Ailhaud G, et al. microRNA miR-27b impairs human adipocyte differentiation and targets PPARgamma. Biochem Biophys Res Commun 2009;390:247–51.
[18] Kim SY, Kim AY, Lee HW, Son YH, Lee GY, Lee J-W, et al. miR-27a is a negative regulator of adipocyte differentiation via suppressing PPARgamma expression. Biochem Biophys Res Commun 2010;392:323–8.
[19] Kimura H, Ogawa S, Akihiro T, Yokota K. Structural analysis of A-type or B-type highly polymeric proanthocyanidins by thiolytic degradation and the implication in their inhibitory effects on pancreatic lipase. J Chromatogr A 2011;1218:7704–12.
[20] Dong X, Zou B, Zhang Y, Ge Z, Du J, Li C-m. Preparation of A-type proanthocyanidin dimers from peanut skins and persimmon pulp and comparison of the antioxidant activity of A-type and B-type dimers. Fitoterapia 2013;91:128–39.
[21] Lin CL, Huang HC, Lin JK. Theaflavins attenuate hepatic lipid accumulation through activating AMPK in human HepG2 cells. J Lipid Res 2007;48:2334–43.
[22] Kim H, Hiraishi A, Tsuchiya K, Sakamoto K. (-) Epigallocatechin gallate suppresses the differentiation of 3T3-L1 preadipocytes through transcription factors FoxO1 and SREBP1c. Cytotechnology 2010;62:245–55.
[23] Li C, Trombley J, Schmidt M, Hagerman A. Preparation of an acid butanol standard from fresh apples. J Chem Ecol 2010;36:453–60.
[24] Zhang T, Sawada K, Yamamoto N, Ashida H. 4-Hydroxyderricin and xanthoangelol from Ashitaba (*Angelica keiskei*) suppress differentiation of preadiopocytes to adipocytes via AMPK and MAPK pathways. Mol Nutr Food Res 2013;57:1729–40.
[25] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods 2001;25:402–8.
[26] Wang T, GZ, Shi X, Deng H, Lin Z. Effect of quercetin on HaCaT membrane fluidity by UVB irradiation. Prog Mod Biomed 2012;12.
[27] Verstraeten SV, Jaggers GK, Fraga CG, Oteiza PI. Procyanidins can interact with Caco-2 cell membrane lipid rafts: involvement of cholesterol. Biochim Biophys Acta 1828;2013:2646–53.
[28] Yin ST, Tang ML, Deng HM, Xing TR, Chen JT, Wang HL, et al. Epigallocatechin-3-gallate induced primary cultures of rat hippocampal neurons death linked to calcium overload and oxidative stress. Naunyn Schmiedebergs Arch Pharmacol 2009;379:551–64.
[29] Colon M, Nerin C. Molecular interactions between caffeine and catechins in green tea. J Agric Food Chem 2014;62:6777–83.
[30] Choi Y, Kim Y, Ham H, Park Y, Jeong HS, Lee J. Nobiletin suppresses adipogenesis by regulating the expression of adipogenic transcription factors and the activation of AMP-activated protein kinase (AMPK). J Agric Food Chem 2011;59:12843–9.
[31] Chen L, Song J, Cui J, Hou J, Zheng X, Li C, et al. microRNAs regulate adipocyte differentiation. Cell Biol Int 2013;37:533–46.
[32] Delehanty James B, BJJ, Hickey Thomas E, Pons Thomas, Ligler Frances S. Binding and neutralization of lipopolysaccharides by plant proanthocyanidins. J Nat Prod 2007;70:1718–24.
[33] Tsuchiya H, Nagayama M, Tanaka T, Furusawa M, Kashimata M, Takeuchi H. Membrane-rigidifying effects of anti-cancer dietary factors. Biofactors 2002;16:45–56.
[34] Zhou Zhe, Sayer Brian G, DWH, Stark Ruth E, Epand Richard M. Studies of phospholipid hydration by high-resolution magic-angle spinning nuclear magnetic resonance. Biophys J 1999;76:387–99.
[35] Zou B, Nie R, Zeng J, Ge Z, Xu Z, Li C. Persimmon tannin alleviates hepatic steatosis in L02 cells by targeting miR-122 and miR-33b and its effects closely associated with the A type ECG dimer and EGCG dimer structural units. J Funct Foods 2014;11:330–41.
[36] Noh JR, Kim YH, Hwang JH, Gang GT, Yeo SH, Kim KS, et al. Scoparone inhibits adipocyte differentiation through down-regulation of peroxisome proliferators-activated receptor gamma in 3T3-L1 preadipocytes. Food Chem 2013;141:723–30.
[37] Furuyashiki T, Nagayasu H, Aoki Y, Bessho H, Hashimoto T, Kanazawa K, et al. Tea catechin suppresses adipocyte differentiation accompanied by down-regulation of PPARgamma2 and C/EBPalpha in 3T3-L1 cells. Biosci Biotechnol Biochem 2004;68:2353–9.
[38] Baselga-Escudero L, Blade C, Ribas-Latre A, Casanova E, Suarez M, Lluis Torres J, et al. Resveratrol and EGCG bind directly and distinctively to miR-33a and miR-122 and modulate divergently their levels in hepatic cells. Nucleic Acids Res 2014;42:882–92.
[39] Lin Q, Gao Z, Alarcon RM, Ye J, Yun Z. A role of miR-27 in the regulation of adipogenesis. FEBS J 2009;276:2348–58.
[40] Arola-Arnal A, Blade C. Proanthocyanidins modulate microRNA expression in human HepG2 cells. PLoS One 2011;6:e25982.
[41] Da Silva M, Jaggers GK, Verstraeten SV, Erlejman AG, Fraga CG, Oteiza PI. Large procyanidins prevent bile-acid-induced oxidant production and membrane-initiated ERK1/2, p38, and Akt activation in Caco-2 cells. Free Radic Biol Med 2012;52:151–9.
[42] Selvaraj S, Mohan A, Narayanan S, Sethuraman S, Krishnan UM. Dose-dependent interaction of trans-resveratrol with biomembranes: effects on antioxidant property. J Med Chem 2013;56:970–81.
[43] Muriel P, Sandoval G. Nitric oxide and peroxynitrite anion modulate liver plasma membrane fluidity and Na(+)/K(+)-ATPase activity. Nitric Oxide 2000;4:333–42.
[44] Conforti G, Zanetti A, Pasquali-Ronchetti I, Quaglino Jr D, Neyroz P, Dejana E. Modulation of vitronectin receptor binding by membrane lipid composition. J Biol Chem 1990;265:4011–9.
[45] Verstraeten SV, Fraga CG, Oteiza PI. Interactions of flavan-3-ols and procyanidins with membranes: mechanisms and the physiological relevance. Food Funct 2015;6:32–41.
[46] Reiss K, Cornelsen I, Husmann M, Gimpl G, Bhakdi S. Unsaturated fatty acids drive disintegrin and metalloproteinase (ADAM)-dependent cell adhesion, proliferation, and migration by modulating membrane fluidity. J Biol Chem 2011;286:26931–42.
[47] Haussinger D, Roth E, Lang F, Gerok W. Cellular hydration state: an important determinant of protein catabolism in health and disease. Lancet 1993;341:1330–2.
[48] Haussinger D. The role of cellular hydration in the regulation of cell function. Biochem J 1996;313:697–710.
[49] Kajiya K, Kumazawa S, Nakayama T. Steric effects on interaction of tea catechins with lipid bilayers. Biosci Biotechnol Biochem 2001;65:2638–43.
[50] Poklar Ulrih N, Ota A, Šentjurc M, Kure S, Abram V. Flavonoids and cell membrane fluidity. Food Chem 2010;121:78–84.
[51] Huh Nam-Won, Porter NA, TJM, Simon SA. The interaction of polyphenols with bilayers: conditions for increasing bilayer adhesion. Biophys J 1996;71:3261–77.
[52] Sirk TW, Brown EF, Sum AK, Friedman M. Molecular dynamics study on the biophysical interactions of seven green tea catechins with lipid bilayers of cell membranes. J Agric Food Chem 2008;56:7750–8.
[53] Sirk TW, Brown EF, Friedman M, Sum AK. Molecular binding of catechins to biomembranes: relationship to biological activity. J Agric Food Chem 2009;57:6720–8.
[54] Appeldoorn MM, Vincken JP, Gruppen H, Hollman PC. Procyanidin dimers A1, A2, and B2 are absorbed without conjugation or methylation from the small intestine of rats. J Nutr 2009;139:1469–73.
[55] Baselga-Escudero L, Blade C, Ribas-Latre A, Casanova E, Salvado MJ, Arola L, et al. Grape seed proanthocyanidins repress the hepatic lipid regulators miR-33 and miR-122 in rats. Mol Nutr Food Res 2012;56:1636–46.
[56] Qiu J, Kitamura Y, Miyata Y, Tamaru S, Tanaka K, Tanaka T, et al. Transepithelial transport of theasinensins through Caco-2 cell monolayers and their absorption in Sprague–Dawley rats after oral administration. J Agric Food Chem 2012;60:8036–43.