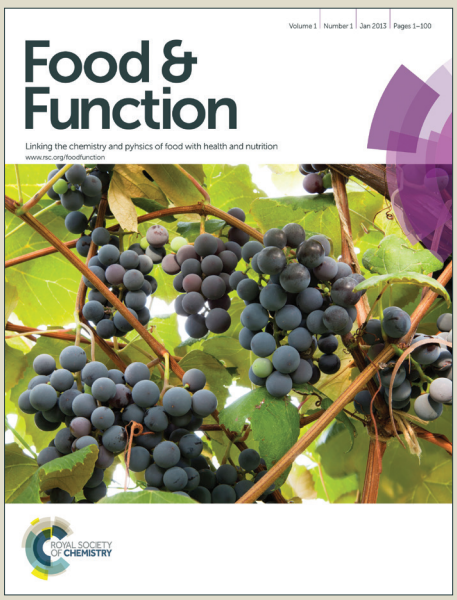
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Inhibitory effects of tannic acid in the early stage of 3T3-L1 preadipocytes differentiation by down-regulating PPARγ expression

Fangyuan Nie[a], Yan Liang[b], Hang Xun[c], Jia Sun[c], Fei He[d], Xiaofeng Ma[a,*]

[a], *College of Life Sciences, University of Chinese Academy of Sciences, No. 19A Yuquan Road, Beijing 100049, China*

[b], *School of Kinesiology and Health, Capital University of Physical Education and Sports, No. 11 Beisanhuanxi Road, Beijing 100191, China*

[c], *State Forestry Administration, International Centre for Bamboo and Rattan Academy of Bioresource Utilization, Beijing 100102, China*

[d], *Xinjiang Technical Institute of Physics & Chemistry, Chinese Academy of Sciences, Urmuqi 830011, China*

* Corresponding author.

Tel. /Fax: +8610-88256353 (X. Ma)

E-mail addresses: maxiaofeng@ucas.ac.cn (X. Ma)

Abstract

Obesity is a medical condition of excess body fat negatively influencing morbidity and mortality via non-communicable disease risks. Adipogenesis, the process of preadipocytes differentiate into adipocytes, plays a pivotal role in obesity. Our previous study has proved that tannic acid (TA) showed anti-adipogenesis effect in 3T3-L1 preadipocytes. However, the precise mechanism involved in the inhibition in adipocytes differentiation by TA is unclear, thus is the subject of the present investigation. In this study, we determined the effect of TA on different stages of 3T3-L1 preadipocytes differentiation, and found that when treating in the early stage of differentiation, TA reduced lipid accumulation significantly. However, TA did not reduce lipid accumulation when treating in mid- and late-stages of adipocyte differentiation. To further study which gene TA had an impact on in the early stage of differentiation, we identified a number of genes associated with lipid metabolism. The results showed that compared to control group, the mRNA levels of FAS, C/EBPα, and PPARγ were significantly decreased ($p<0.05$), while the mRNA levels of adipsin, ap2 were increased ($p<0.05$). However, TA had no effect on mRNA levels of ACC1 and ACC2. Western blot results showed that TA down-regulated the expression of PPARγ, which is a major factor in preadipocyte differentiation. In addition, TA did not affect PI3K/AKT pathway. These results indicate that the anti-adipogenesis effect of TA involves down-regulation of PPARγ in the early stage of 3T3-L1 preadipocyte differentiation. Some potential limitations of this study should be considered. All the results in this study were based on cell experiments. However, the human bioavailability of TA is not clear. In the present study, the concentration of TA was 5 μM, so it was concerned about whether orally intake of TA could reach to the effective concentrations. This important point needs to be clarified *in vivo*.

Key words: tannic acid; PPARγ; fatty acid synthase; inhibit; 3T3-L1 preadipocyte; lipid accumulation.

INTRODUCTION

Obesity is a medical condition in which excess body fat has accumulated to the extent that it may have a negative effect on health, leading to reduced life expectancy and/or increased health problems.[1] Since involved in various serious diseases including type 2 diabetes, hypertension, coronary heart disease, apoplexy, osteoarthritis and cancers, obesity has become a worldwide public health threat.[2-9]

Obesity is resulted from increased adipose tissue mass, which is caused by increased fat-cell numbers (hyperplasia) and size (hypertrophy), accompanied by the imbalance between energy intake and expenditure.[10] Adipose tissue consists of mature adipocytes, preadipocytes, endothelial cells, macrophages, fibroblasts, and adiposederived stem cells.[11] Preadipocytes are capable to propagate and differentiate into mature adipocytes, which determine the number of fat cells throughout their entire lifespan.[12] Meanwhile, the size of fat-cell depends on the lipid accumulation in the adipocytes. Therefore, adipose tissue mass can be reduced by the inhibition of adipogenesis from preadipocytes to mature adipocytes or the prevention of intracellular lipid accumulation.

Adipogenesis, the differentiation process of preadipocytes into adipocytes, plays a pivotal role in fat metabolism.[13] The 3T3-L1 mouse preadipocyte cell line, originally obtained from mouse embryos, has been commonly used to investigate the molecular mechanism of adipogenesis as an *in vitro* model system.[14] The 3T3-L1 preadipocytes differentiated into adipocytes and express adipocytes-specific genes in the presence of adipogenic cocktail, containing 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and insulin.[15] Peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding protein α (C/EBPα) are the major transcription factors expressed during adipogenesis in 3T3-L1 preadipocytes.[16, 17] These major transcription factors together activate the expression of adipocyte-specific genes including adipsin, adipocyte fatty acid-binding protein 2 (aP2), Acetyl-CoA carboxylase 1 (ACC1), ACC2, and fatty acid synthase (FAS)

in adipogenesis.[18-20]

In our previous study, we found that tannic acid (TA) could reduce lipid accumulation of 3T3-L1 adipocytes dose-dependently. Moreover, TA showed strong capability of inactivating FAS with the half maximal inhibitory concentration (IC_{50}) value of 0.14 μM.[21] However, the precise mechanisms involved remain unclear. The purpose of this study is to investigate the possible mechanisms of TA on the regulation of preadipocyte differentiation and/or on the lipid accumulation using the adipogenic cell line 3T3-L1.

MATERIALS AND METHODS

Reagents

TA ($C_{76}H_{52}O_{46}$, 98% HPLC), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), insulin, dimethyl sulfoxide (DMSO), dexamethasone, IBMX and Oil red O were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from local Sijiqing biological engineering material company (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) was a product of Nissui Seiyaku, Tokyo, Japan. Cell Counting Kit (CCK-8) was a product of Dojindo Laboratories, Kumamoto, Japan. The PCR primers for human β-actin, PPARγ, FAS, C/EBPα, adipsin, ap2, ACC1 and ACC2 were synthesized by SBS Genetech Co. Ltd (Beijing, China). AMV and Oligo (dT) were purchased from Promega Corporation (Beijing, China). Other reagents were of analytical grade.

Cell line and cultures

Mouse 3T3-L1 preadipocytes were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells were cultured in low-gucose DMEM containing 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. Medium was renewed every 2 days. When differentiated, 3T3-L1 preadipocytes were seeded in 6-well plate and grown for 2–4 days for differentiation. Two days after reaching confluence, the medium was changed by high-glucose DMEM containing 10% FBS supplemented with 0.5

mM IBMX, 1 μM dexamethasone, and 1.7 μM insulin (d0). The cells were treated for 2 days (d2), and then were cultured in high-glucose DMEM containing 10% FBS and 1.7 μM insulin for another 2 days. Thereafter (d4), the cells were cultured in high-glucose DMEM containing 10% FBS up to day 8, and the medium was renewed every 2 days. TA was conserved in DMSO before added into the culture medium.

CCK8 assay

3T3-L1 preadipocytes were seeded in a 96-well plate (5×10^3 cells/well) and then treated with TA in different concentrations for 24 h. As for mature adipocytes, after 8 days of differentiation, adipocytes were seeded in a 96-well plate (5×10^3 cells/well) and then treated with TA in different concentrations for 24 h. Thereafter, almost 1/10 CCK8 (1 ml CCK8 in 10 ml DMEM) was added into each well of a microtiter plate and incubated for 2 h at 37 °C and then measured at 492 nm by a microplate spectrophotometer. Data were obtained from the average of five experiment wells, and the assay was repeated three times.

Oil red O staining

Cells in 24-well plates were gently washed by phosphate buffer solution (PBS) for three times. 1 ml Oil red O-isopropanol (six parts 0.6% Oil Red O dye in isopropanol and four parts water) was added into each well and kept at room temperature for 15 min. Excess Oil red O was washed by water for three times, and then photographed under the microscope. After that, each well was added 1 ml isopropanol for 15 min, and then 150 μl eluate was transferred to 96-well plates, and measured the optical density at 540 nm using a microplate spectrophotometer (Multiskan, MK3).

Cell proliferation

Cell proliferation was determined by staining cells with propidium iodide (PI) labeling. Briefly, 1.5×10^5 cells/ml were incubated without or with TA (2.5, 5 μM) for 24 h. Afterwards, the cells were washed twice with ice-cold PBS, and then PI was applied to stain cells as the kit directions. The status of cell staining was

analyzed by flow cytometer (Becton Dickinson).

CCK8 was also used to determine cell proliferation. 3T3-L1 preadipocytes were treated with TA (1, 2, 3, 4, 5 μM) for six days and cell viability was detected every day. Cell viability enhancement represents the increase in the number of cells.

RNA preparation from 3T3-L1 cells and RT-PCR

Total RNA was isolated from 3T3-L1 adipocytes using RNAsimple Total RNA Kit (TianGen Biotech, Beijing). 5 μl total RNA from each sample was reverse-transcribed to cDNA with a cDNA synthesis kit (TianGen Biotech, Beijing). The gene expression levels of FAS and PPARγ were analyzed by quantitative real-time PCR (Mx 3000P, USA). The conditions for PCR were: initial denaturation at 95°C for 5 min and followed by 45 cycles (95 °C, 15 s, 55 °C, 15 s, 72 °C, 20 s). The primer sequences were shown in Table 1.

Western blotting

Cells were washed three times with ice-cold PBS and harvested in RIPA lysis buffer with 1 mM PMSF, and then lysed for 5 min on ice. The homogenate was centrifuged at 12,000 rpm for 10 min at 4°C and supernatant was collected for FAS analysis. Equal protein extracts were separated using SDS-PAGE. Proteins were electrophoretically transferred to PVDF membranes. Incubation with primary and secondary antibodies was performed in Tris-buffered saline containing 5% non-fat dry milk for 2 h or more. After incubation, the membranes were washed in Tris-buffered saline containing 0.1% Tween 20. ECL Western Blotting Detection System was used for detection. The relative expression of proteins was quantified densitometrically with the software ImageJ and calculated according to the reference bands of β-actin or GAPDH.

Statistics

Data were expressed as mean ± standard deviation (SD). The unpaired Student's t test was used to compare the means of two groups. The statistical differences among three or more groups were determined by one-way

ANOVA with Tukey's post-test. The gel images were analyzed with the software Quantity One. p values of 0.05 or less were considered to be statistically significant.

RESULTS

TA inhibited the cell viability of preadipocytes, but not mature adipocytes.

In order to elucidate the effect of TA on the viability of 3T3-L1 cells, both undifferentiated preadipocytes and differentiated mature adipocytes were assayed. The results showed that when treating with TA at the concentrations of 3 μM or more, the viability of preadipocytes reduced significantly. However, TA did not affect the viability of adipocytes. As shown in figure 1, the viability of fully differentiated adipocytes did not reduce even when treated with 25 μM TA.

(Figure 1)

TA inhibited cell proliferation of preadipocytes dose-dependently.

For the cell cycle progression, PI staining was used and flow cytometry analysis revealed that TA administration resulted in a significant reduction in the cell population in S phase (from 25.1 to 18.8%) and G2/M phase (from 26.8 to 17.6%) with a concomitant increase in the cell population in G0/G1 phase (from 48.1 to 63.7%) at 48 h after induction (Figure 2A). These data suggested that TA inhibited the proliferation of preadipocytes through inducing G0/G1 arrest.

CCK8 assay was used to detect cell proliferation case between untreated and TA treated 3T3-L1 preadipocytes. Relative growth rate of the cells treated with different concentrations (0, 1, 2, 3, 4, 5 μM) of TA was recorded in six consecutive days. The results showed that TA inhibited the proliferation of preadipocytes dose- and time-dependently, as Figure 2B shown.

(Figure 2)

TA inhibited the lipid accumulation of preadipocytes mainly in the early stage of differentiation.

In our previous study, we have reported that TA could suppress lipid accumulation in 3T3-L1 adipocytes.[18] To further understand the role of TA on the process of adipocyte differentiation, we added 5 μM TA in different stages of adipocyte differentiation, and stained cells with Oil red O after 8 days, then the stained lipids were extracted with isopropanol to record the relative number of lipid droplets quantitatively, as shown in Figure 3. Result showed that TA had the most significant inhibitory effect on the accumulation of lipid droplets when added in the early stage of differentiation (primary 2 days).

(Figure 3)

TA down-regulated the mRNA levels of FAS, C/EBPα, and PPARγ.

In the initial stage of cell differentiation, many genes associated with lipid synthesis were generated and began to play its biological role in lipid synthesis. To further study which gene TA had an impact on in the early stage of differentiation, we identified a number of genes associated with lipid metabolism. The results showed that compared to control group, the mRNA levels of FAS, C/EBPα, and PPARγ were significantly decreased ($p<0.05$), while the mRNA levels of adipsin, ap2 were increased ($p<0.05$). However, TA had no effect on mRNA levels of ACC1 and ACC2 (Figure 4).

(Figure 4)

As we know, PPARγ gene started to transcript and translate in the primary 2 days of differentiation. In order to prove whether the reduction of lipid accumulation caused by TA was related to PPARγ pathway, we detected the expression of PPARγ by western blot. As shown in Figure 5A, TA administration in the early stage of differentiation significantly down-regulated PPARγ expression compared to control group. Similar to the results of lipid accumulation, PPARγ expression was not changed when treating with TA in middle and late stage of differentiation.

TA did not affect PI3K/AKT pathway in 3T3-L1 preadipocytes.

PI3K/AKT pathway mediates PPARγ and C/EBPα gene expression in adipocyte differentiation. In order to determine whether TA affected the PI3K/AKT pathway, we detected p-AKT, p-ERK1/2 as well as total AKT and ERK1/2 expression by western blot. As shown in Figure 5B, both of the two proteins were not influenced. (Figure 5)

DISCUSSION

The development of obesity is characterized by increased number and size of adipocytes,[22] which are regulated by genetic, metabolic and nutritional factors.[23, 24] Therefore, deciphering the mechanism of how certain nutrients affect adipocyte differentiation and adipogenesis is important for the prevention of obesity and related diseases. Polyphenols, abundant in functional foods (i.e. tea, vegetables and fruits), have recently attracted public attention due to their beneficial health effects.[25-28] TA is a natural polyphenol existed in tea, coffee, immature fruits, etc. and has also been used as a food additive. In our previous study, TA was found to inhibit adipogenesis by down regulating the gene expression of PPARγ and FAS.[21] In the present study, the detailed mechanism of TA on adipocyte differentiation was investigated.

FAS is a key enzyme in *de novo* fatty acid synthesis *in vivo* primarily in the fat cells. During preadipocytes differentiation period, the expression level and relative activity of FAS were both increased continuously. However, in the early stage of differentiation, FAS expression level is relatively low.[29] Our previous study showed that TA could inhibit FAS activity and reduce lipid accumulation of preadipocytes. However, in this present study, we found that TA exhibited strong activity of reducing lipid accumulation mainly in the early stage of differentiation. Therefore, the inhibitor effect of TA on FAS may be not the main reason of its anti-adipogenesis activity.

PPARs are ligand-activated transcription factors which regulate genes transcription in cell differentiation and lipid homeostasis.[30-34] PPARs are a family including isoform of PPARα, PPARβ, PPARγ, PPARδ, in which

PPARγ mainly contributes to energy storage by enhancing adipogenesis.[35] PPARγ is regarded as the main regulator of adipogenesis because no other factor was found to rescue adipocyte formation when PPARγ is knockout.[36] PPARγ rapidly increases in expression during early adipogenesis,[37-39] when is the day 0-day 2 during the 3T3-L1 cells differentiation. Semenkovitch and colleagues have proven that FAS promotes PPARγ activation and adipogenesis which is demonstrated by FAS knockout experiments which shows FAS is necessary to generate endogenous ligands for PPARγ.[40] In this study, we found that TA down-regulated PPARγ expression significantly. This result indicated that the anti-adipogenesis effect of TA may be related to PPARγ.

Adipsin, also known as complement factor D, is the first adipocytokine described.[41] As a fat cell derived protein associated with lipid metabolism, adipsin mRNA levels in obese rats were lower than those of lean rats.[42] It has been previously established that there was a negative correlation between the mRNA levels of adipsin and FAS. In the present study, consistent with previous study, we found that TA down-regulated mRNA levels of FAS but up-regulated mRNA levels of adipsin. As a key regulator of adipogenesis, ap2 facilitates intracellular mobilization of free fatty acids.[43] It was reported that disruption of ap2 gene impaired fat cell lipolysis and increased cellular fatty acid levels.[43] The results of this study indicated that, after treated with TA, the expression levels of PPARγ, FAS and ap2 genes were not positively correlated. The detailed mechanism involved was still unknown, however, these genes were regulated toward the same direction of decreasing intracellular fatty acids.

PI3K/AKT pathway is up-stream of adipocyte differentiation pathways including PPARγ pathway. TA and other tannins were proven many biological functions, including anti-oxidation, antibacterial and anti-inflammation mainly through PI3K/AKT pathway. Thus, in this study, to verify whether the lipid reduction effect of TA was related to PI3K/AKT pathway, we measured the expression of p-AKT and

p-ERK1/2 in TA treated 3T3-L1 preadipocytes. Results showed that TA administration did not affect PI3K/AKT pathway in 3T3-L1 preadipocytes.

As a main active compound of pomegranate, TA was reported to have the activities of reducing body-weight and food intake.[44, 45] However, the detailed mechanisms involved have not been fully clarified. From the results above, we proposed that a possible way was that TA down regulated the expression of PPARγ, FAS and C/EBPα in the early stage of preadipocyte differentiation. Subsequently, the newly synthesized fatty acids, which were main component of lipid droplets, were reduced significantly.

Preliminary experiments in our laboratory have found evidence that the inhibitory effect of TA on 3T3-L1 preadipocytes did not relate to non-specific sedimentation, while low concentration of TA (up to 36.7 μM) did not cause the protein sedimentation.[21] In the present research, the highest concentration of TA used was much lower than that could induce protein sedimentation, which demonstrated that the inhibitory activity of TA on adipocytes did not due to protein non-specific sedimentation.

Some potential limitations of this study should be considered. All the results in this study were based on cell experiments. Indeed, in regard to aiming to target intracellular genes for therapeutic applications, an important issue is the bioavailability of TA. However, although studies have revealed the bioactivities of TA in human and rats, the human bioavailability of TA is not clear.[46] Bravo et al reported that rats were given diets containing 20 g TA per Kg chow during a three week experimental period, only 4.6% of TA was found in the faeces.[47] That is, the apparent digestibility of TA was 95.4%. So the bioavailability of TA is relative low. In the present study, the concentration of TA was 5 μM, so it was concerned about whether orally intake of TA could reach to the effective concentrations. This important point needs to be clarified *in vivo*. To this aim, it may be useful the study of the effect of TA on obese mouse or rat models.

In conclusion, TA could inhibit the differentiation and lipid accumulation of 3T3-L1 preadipocytes when treat

in the early stage of differentiation. TA down regulated the mRNA levels of three important markers (PPARγ, FAS and C/EBPα) that were induced when the cells have just starting to differentiate. In contrast, TA administration in the middle and/or the late stage of differentiation program had a much weaker inhibitory effect on lipid accumulation. Therefore, the results of this study suggest that the down-regulation of PPARγ be major roles on its anti-adipogenesis effect of TA.

ACKNOWLEDGMENTS

This work was supported by the Special Science and Technology Projects for Outstanding Young in Life Sciences (KSCX2-EW-Q-19), the "12th five-year plan" period: the basic frontier project in life sciences (KSCX2-EW-J-29), Youth Innovation Promotion Association, CAS, as well as High-Tech Research and Development Program of Xinjiang (No. 201315108) and China Postdoctoral Science Foundation (No. 2013M540785).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. D.W. Haslam, and W.P. James, Obesity, *Lancet*, 2005, **366**, 1197-1209.
2. C.L. Ogden, M.D. Carroll, B.K. Kit, and K.M. Flegal, Prevalence of obesity and trends in body mass index among US children and adolescents, 1999-2010, *JAMA.*, 2012, **307**, 483-490.
3. K.G. Alberti, R.H. Eckel, S.M. Grundy, P.Z. Zimmet, J.I. Cleeman, K.A. Donato, J.C. Fruchart, W.P. James, C.M. Loria, and S.C. Jr Smith, Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity, *Circulation*, 2009, **120**, 1640-1645.
4. M. Zeyda, and T.M. Stulnig, Obesity, inflammation, and insulin resistance – a mini-review, *Gerontology*, 2009, **55**, 379-386.
5. S.S. du Plessis, S. Cabler, D.A. McAlister, E. Sabanegh, and A. Agarwal, The effect of obesity on sperm disorders and male infertility, *Nat. Rev. Urol.*, 2010, **7**, 153-161.
6. P.G. Kopelman, Obesity as a medical problem, *Nature*, 2000, **404**, 635-643.
7. U.N. Das, Obesity: genes, brain, gut and environment, *Nutrition*, 2010, **26**, 459-473.
8. Y.E. Bogaert, and S. Linas, The role of obesity in the pathogenesis of hypertension, *Nat. Clin. Pract. Nephrol.*, 2009, **5**, 101-111.
9. J.S. Rana, M. Nieuwdorp, J.W. Jukema, and J.J. Kastelein, Cardiovascular metabolic syndrome–an interplay of, obesity, inflammation, diabetes and coronary heart disease, *Diabetes Obes. Metab.*, 2007, **9**, 218-232.
10. C. Couillard, P. Mauriège, P. Imbeault, D. Prud'homme, A. Nadeau, A. Tremblay, C. Bouchard, and J.P. Després, Hyperleptinemia is more closely associated with adipose cell hypertrophy than with adipose tissue hyperplasia, *Int. J. Obes. Relat. Metab. Disord.*, 2000, **24**, 782-788.
11. A. Armani, C. Mammi, V. Marzolla, M. Calanchini, A. Antelmi, G.M. Rosano, A. Fabbri, and M. Caprio, Cellular models for understanding adipogenesis, adipose dysfunction, and obesity, *J. Cell Biochem.*, 2010, **110**, 564-572.
12. J.K. Sethi, and A.J. Vidal-Puig, Thematic review series: adipocyte biology. Adipose tissue function and plasticity orchestrate nutritional adaptation, *J. Lipid Res.*, 2007, **48**, 1253-1262.
13. J.J. Cao, Effects of obesity on bone metabolism, *J. Orthop. Surg. Res.*, 2011, **6**, 30.
14. H. Song, K.C. O'Connor, K.D. Papadopoulos, and D.A. Jansen, Differentiation kinetics of in vitro 3T3-L1 preadipocyte cultures, *Tissue Eng.*, 2002, **8**, 1071-1081.
15. Q.Q. Tang, T.C. Otto, and M.D. Lane, Mitotic clonal expansion: a synchronous process required for adipogenesis, *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 44-49.
16. K.M. Sprott, M.J. Chumley, J.M. Hanson, and R.T. Dobrowsky, Decreased activity and enhanced nuclear export of CCAAT enhancer-binding protein beta during inhibition of adipogenesis by ceramide, *Biochem. J.*, 2002, **365**, 181-191.
17. P.C. Liu, M.A. Phillips, and F. Matsumura, Alteration by 2,3,7,8-tetrachlorodibenzo-p-dioxin of CCAAT/enhancer binding protein correlates with suppression of adipocyte differentiation in 3T3-L1 cells, *Mol. Pharmacol.*, 1996, **49**, 989-997.
18. S.Y. Cho, P.J. Park, H.J. Shin, Y.K. Kim, D.W. Shin, E.S. Shin, H.H. Lee, B.G. Lee, J.H. Baik, and T.R. Lee, (-)-Catechin suppresses expression of Kruppel-like factor 7 and increases expression and secretion of adiponectin protein in 3T3-L1 cells, *Am. J. Physiol. Endocrinol. Metab.*, 2007, **292**, E1166-E1172.

19. P.C. Jou, B.Y. Ho, Y.W. Hsu, and T.M. Pan, The effect of Monascus secondary polyketide metabolites, monascin and ankaflavin, on adipogenesis and lipolysis activity in 3T3-L1, *J. Agric. Food Chem*., 2010, **58**, 12703-12709.
20. G.M. Thompson, D. Trainor, C. Biswas, C. LaCerte, J.P. Berger, and L.J. Kelly, A high-capacity assay for PPARgamma ligand regulation of endogenous aP2 expression in 3T3-L1 cells, *Anal. Biochem.,* 2004, **330**, 21-28.
21. H. Fan, D. Wu, W. Tian, and X. Ma, Inhibitory effects of tannic acid on fatty acid synthase and 3T3-L1 preadipocyte, *BBA-Mol. Cell Biol. L.*, 2013, **1831**, 1260-1266.
22. S. de Ferranti, and D. Mozaffarian, The perfect storm, obesity, adipocyte dysfunction: and metabolic consequences, *Clin. Chem.*, 2008, **54**, 945-955.
23. P.G. Kopelman, Obesity as a medical problem, *Nature*, 2000, **404**, 635-643.
24. S.R. Farmer, and J. Auwerx, Adipose tissue: new therapeutic targets from molecular and genetic studies: IASO Stock Conference 2003 report, *Obes. Rev.*, 2004, **5**, 189-196.
25. O.Y. Kim, S.M. Lee, H. Do, J. Moon, K.H. Lee, Y.J. Cha, and M.J. Shin, Influence of quercetin-rich onion peel extracts on adipokine expression in the visceral adipose tissue of rats, *Phytother. Res*., 2012, **26**, 432-437.
26. M.G.L. Hertog, E.J.M. Feskens, P.C.H. Hollman, M.B. Katan, and D. Kromhout, Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study, *Lancet*, 1993, **342**, 1007-1011.
27 P. Knekt, J. Kumpulainen, R. Järvinen, H. Rissanen, M. Heliövaara, A. Reunanen, T. Hakulinen, and A. Aromaa, Flavonoid intake and risk of chronic diseases, *Am. J. Clin. Nutr.*, 2002, **76**, 560-568.
28. P.J. Mink, C.G. Scrafford, L.M. Barraj, L. Harnack, C.P. Hong, J.A. Nettleton, and D.R. Jacobs Jr., Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women, *Am. J. Clin. Nutr.*, 2007, **85**, 895-909.
29. N. Moustaïd, and H.S. Sul, Regulation of expression of the fatty acid synthase gene in 3T3-L1 cells by differentiation and triiodothyronine, *J. Biol. Chem.*, 1991, **266**, 18550-18554.
30. B. Desvergne, and W. Wahli, Peroxisome proliferator-activated receptors: nuclear control of metabolism, *Endocr. Rev.*, 1999, **20**, 649-688.
31. I. Issemann, and S. Green, Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators, *Nature*, 1990, **347**, 645-650.
32. A. Rogue, C. Lambert, R. Jossé, S. Antherieu, C. Spire, N. Claude, and A. Guillouzo, Comparative gene expression profiles induced by PPAR γ and PPAR α/γ agonists in human hepatocytes, *PLoS One*, 2011, **6**, e18816.
33. A. Rogue, C. Spire, M. Brun, N. Claude, and A. Guillouzo, Gene expression changes induced by PPAR gamma agonists in animal and human liver, *PPAR Res.*, 2010, **2010**, 325183.
34. T.M. Willson, P.J. Brown, D.D. Sternbach, and B.R. Henke, The PPARs: from orphan receptors to drug discovery, *J. Med. Chem.*, 2000, **43**, 527-550.
35. M.S. Rao, P. Kashireddy, S. Musunuri, and A. Okonkwo, Prevention/reversal of choline deficiency-induced steatohepatitis by a peroxisomeproliferator- activated receptor alpha ligand in rats, *In Vivo*, 2002, **16**, 145-152.
36. A. Chawla, and M.A. Lazar, Peroxisome proliferator and retinoid signaling pathways co-regulate preadipocyte phenotype and survival, *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 1786-1790.
37. M. Adams, C.T. Montague, J.B. Prins, J.C. Holder, S.A. Smith, L. Sanders, J.E. Digby, C.P. Sewter, M.A. Lazar, V.K. Chatterjee, and S. O'Rahilly, Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentiation, *J. Clin. Invest.*,

1997, **100**, 3149-3153.
38. S. Muruganandan, A.A. Roman, and C.J. Sinal, Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: cross talk with the osteoblastogenic program, *Cell Mol. Life Sci.*, 2009, **66**, 236-253.
39. I. Tzameli, H. Fang, M. Ollero, H. Shi, J.K. Hamm, P. Kievit, A.N. Hollenberg, and J.S. Flier, Regulated production of a peroxisome proliferator-activated receptor-gamma ligand during an early phase of adipocyte differentiation in 3T3-L1 adipocytes, *J. Biol. Chem.*, 2004, **279**, 36093-36102.
40. I.J. Lodhi, L. Yin, A.P.L. Jensen-Urstad, K. Funai, T. Coleman, J.H. Baird, M.K. El Ramahi, B. Razani, H. Song, F. Fu-Hsu, J. Turk, and C.F. Semenkovich, Inhibiting adipose tissue lipogenesis reprograms thermogenesis and PPARγ activation to decrease diet-induced obesity, *Cell Metab.*, 2012, **16**, 189-201.
41. K.S. Cook, H.Y. Min, D. Johnson, R.J. Chaplinsky, J.S. Flier, C.R. Hunt, and B.M. Spiegelman, Adipsin: a circulating serine protease homolog secreted by adipose tissue and sciatic nerve, *Science*, 1987, **237**, 402-405.
42. G. Shillabeer, J. Hornford, J.M. Forden, N.C. Wong, J.C. Russell, and D.C. Lau, Fatty acid synthase and adipsin mRNA levels in obese and lean JCR:LA-cp rats: effect of diet, *J. Lipid Res.*, 1992, **33**, 31-39.
43. N.R. Coe, M.A. Simpson, D.A. Bernlohr, Targeted disruption of the adipocyte lipid-binding protein (aP2 protein) gene impairs fat cell lipolysis and increases cellular fatty acid levels, *J. Lipid Res.*, 1999, **40**, 967-972.
44. F. Lei, X.N. Zhang, W. Wang, D.M. Xing, W.D. Xie, H. Su, and L.J. Du, Evidence of anti-obesity effects of the pomegranate leaf extract in high-fat diet induced obese mice, *Int. J. Obes. (Lond)*, 2007, **31**, 1023-1029.
45. M.N. Al-Muammar, and F. Khan, Obesity: the preventive role of the pomegranate (Punica granatum), *Nutrition*, 2012, **28**, 595-604.
46. H. Gin, V. Rigalleau, O. Caubet, J. Masquelier, and J. Aubertin, Effects of red wine, tannic acid, or ethanol on glucose tolerance in non-insulin-dependent diabetic patients and on starch digestibility in vitro, *Metabolism*, 1999, **48**, 1179-1183.
47. L. Bravo, R. Abia, M.A. Eastwood, and F. Saura-Calixto, Degradation of polyphenols (catechin and tannic acid) in the rat intestinal tract. Effect on colonic fermentation and faecal output, *Br. J. Nutr.*, 1994, **71**, 933-946.

Figure captions

Figure 1. Inhibitory effect of TA on the viability of 3T3-L1 preadipocytes and mature adipocytes.

A 3T3-L1 preadipocytes were treated with TA (0-7 μM) for 24 h. B Mature 3T3-L1 adipocytes were treated with TA (0-25 μM) for 24 h. Cell viabilities were determined by the CCK-8 assay as described in "Materials and Methods". The percentage of cell viability was calculated as the ratio of TA treated cells to control cells. Data represented the mean ±SD of three independent experiments. *$p<0.05$ compared to control (0 μM); **$p<0.01$ compared to control (0 μM).

Figure 2. Effects of TA on proliferation of 3T3-L1 preadipocytes.

A Cells were treated with TA (0, 2.5, 5 μM) for 24 h, and then Flow Cytometry was used to determine cell proliferation with a DNA-PI staining kit.

B Growth curves of 3T3-L1 preadipocytes treated with TA (0, 1, 2, 3, 4, 5 μM) for six days.

Cell viability was determined every day by CCK8 assay as described in "Materials and methods". The values were from three times and triplicate tests.

Figure 3. TA exhibited different effect on 3T3-L1 adipocytes at different stages of cellular maturation.

A Cell culture was performed as described in the "Materials and methods". Photos of differentiated cells were taken at day 8 after Oil red O staining.

B Quantitative analysis of lipid accumulation. After Oil red O staining, lipid and Oil red O were dissolved in isopropanol and absorbance was measured at the wavelength of 492 nm. Control means no TA treatment; d0 means treatment with TA during the first two days of cell differentiation, and d02 means treatment with TA during the first 4 days of cell differentiation and so forth. The concentrations of TA were 5 μM.

Figure 4. Changes in lipid metabolism-related genes after TA treatment

A-G Expression of PPARγ, FAS, C/EBPα, adipsin, ap2, ACC1 and ACC2 in TA treated cells. Cells were

400 incubated with 5 μM TA for 48 h (the first two days during cell differentiation) and the mRNA expression of
401 lipid metabolism-related genes were detected by RT-PCR.

402 Figure 5. Effect of TA on the expression of PPARγ, p-AKT, and p-ERK1/2 in 3T3-L1 adipocytes at different
403 stages of cellular maturation.

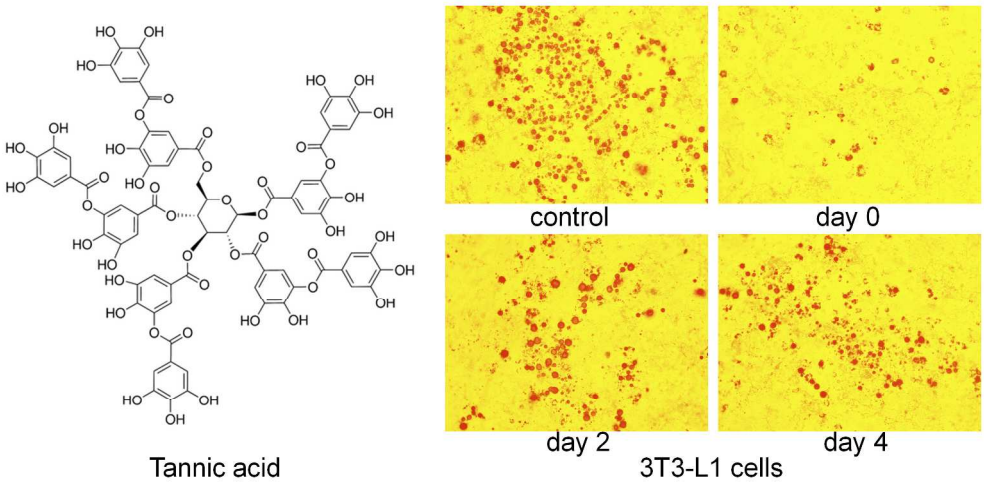
404 Cells were incubated with 5 μM TA during different stage (d0, d2, d4, d6) or full stage (d0246) for 8 days.
405 Then the levels of PPARγ, AKT, ERK1/2, p-AKT, p-ERK1/2 were detected by Western blot.
406

407 Table 1. Primers used for RT-PCR

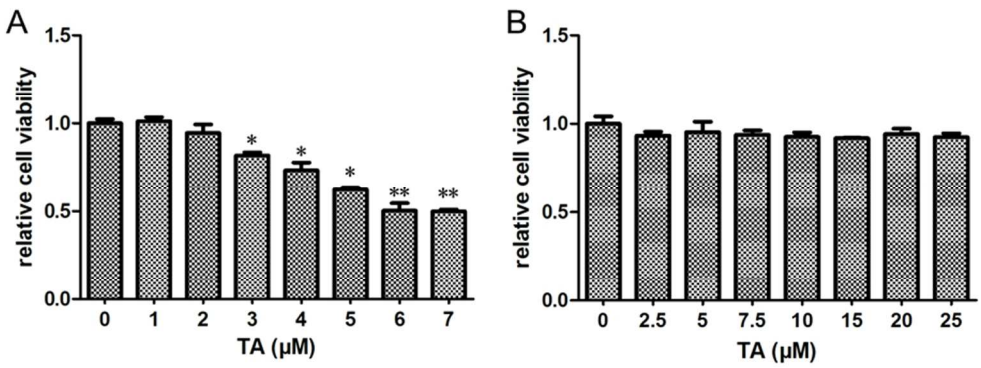
408

Gene	Sequences (forward/reverse)
β-actin	GTGGGCCGCTCTAGGCACCAA
	CTCTTTGATGTCACGACGATTTC
FAS	TTCGTACCTCCTTGGCAAAC
	GGCTGCAGTGAATGAATTTG
C/EBPα	GCCGAGATAAAGCCAAACAA
	CCTTGACCAAGGAGCTCTCA
PPARγ	GCATGGTGCCTTCGCTGA
	TGGCATCTCTGTGTCAACCATG
ap2	ACACCGAGATTTCCTTCAAACTG
	CCATCTAGGGTTATGATGCTCTTC
ACC1	GCTGTTCCTCAGGCTCACAT
	ACACCATGTTGGGAGTTGTG
ACC2	CGTGTCGATATCGTTGTTCTG
	CATGGTAGTGGCTTTGAAGGA
Adipsin	CATGCTCGGCCCTACATGG
	CACAGAGTCGTCATCCGTCAC

409



Tannic acid inhibits lipid accumulation in the early stage of 3T3-L1 preadipocytes differentiation by down-regulating PPARγ.
800x400mm (96 x 96 DPI)



329x121mm (72 x 72 DPI)

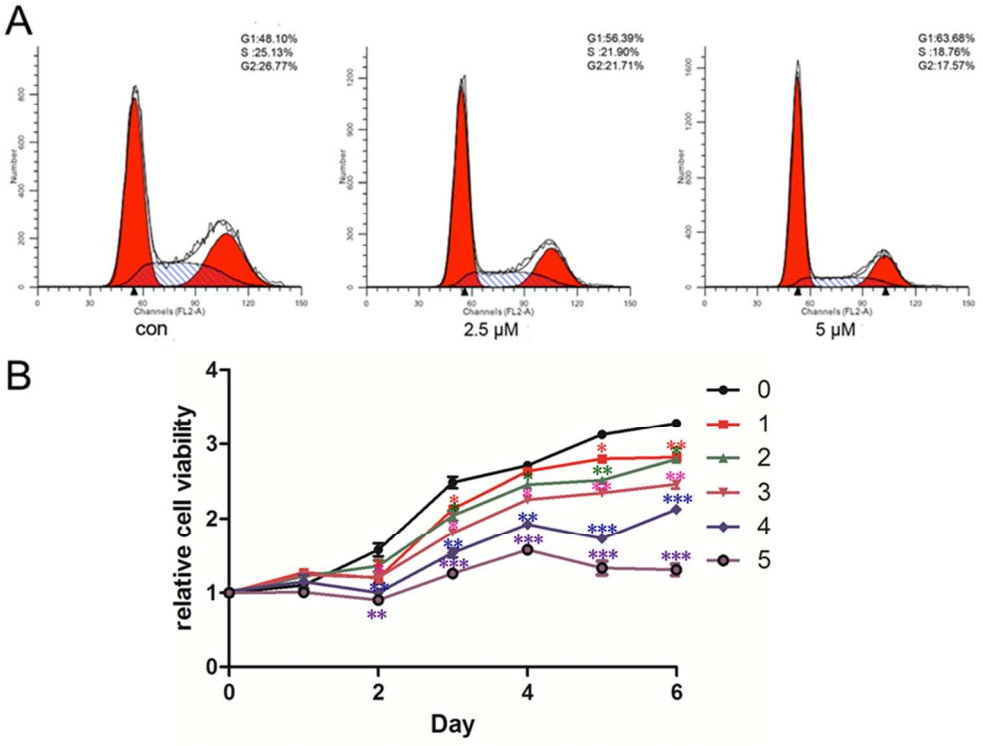


Figure 2
254x190mm (96 x 96 DPI)

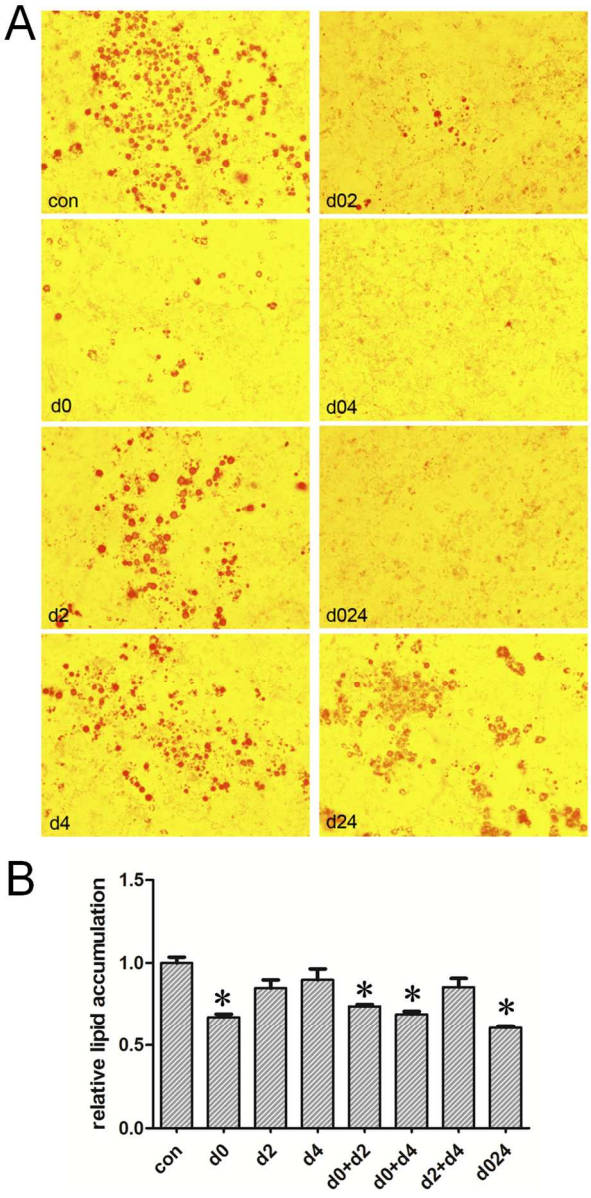


Figure 3
328x641mm (72 x 72 DPI)

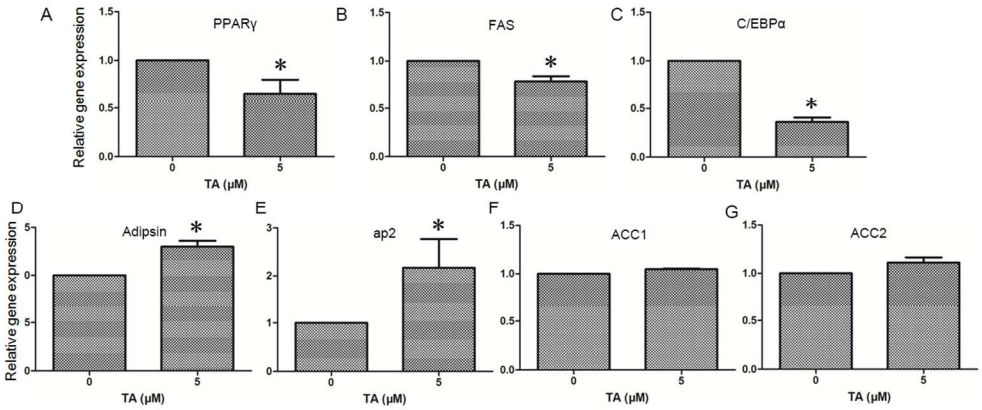


Figure 4
468x192mm (72 x 72 DPI)

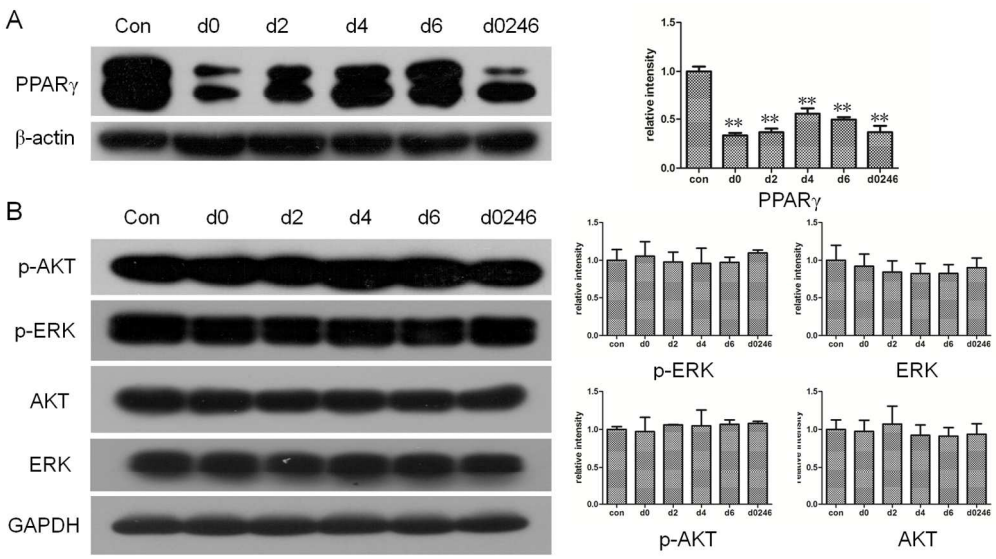


Figure 5
597x336mm (72 x 72 DPI)