Guava Leaf Extracts Inhibit 3T3-L1 Adipocyte Differentiation *Via* Activating AMPK

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Abstract: The guava tree (*psidium guajava* linn.) is commonly used not only as food but also as folk medicine. In our previous studies, we showed that oral administration of guava leaf extracts (GLE) had beneficial anti-obesity effects using metabolic syndrome model rats. However, we did not clarify molecular mechanism by which GLE administration leads to anti-obesity effect. This study was designed to evaluate the mechanism of anti-obesity by GLE using 3T3-L1 pre-adipocyte cell lines. We found that GLE significantly inhibited 3T3-L1 differentiation *via* down-regulation of adipogenic transcription factors and markers. Mitotic clonal expansion, which is essential for adipose differentiation, was also depressed in the early phase. Interestingly, GLE increased the phosphorylation of AMPK on 3T3-L1 cells and, by pretreatment with AMPK siRNA, the GLE treatment group showed restored adipocyte differentiation. In conclusion, these results showed that GLE is capable of inhibiting adipocyte differentiation *via* AMPK activation and therefore it may prevent obesity *in vivo*.

Keywords: AMP-activated protein kinase, 3T3-L1 differentiation, guava leaf extract, Mitotic clonal expansion, obesity.

INTRODUCTION

The guava tree (*psidium guajava* linn.) is a member of the Myrtaceae family, which ranges over tropical and subtropical countries. It is commonly used not only as food but also as folk medicine with antioxidant, anti-spasmodic, anti-allergy, anti-inflammatory and anti-diabetic properties using extracts from the fruit, leaf, bark or roots [1, 2]. Leaves of this plant have been used as folk medicine and are reported to contain several compounds such as various terpenoids [3, 4], flavonoids [5] and tannins [6]. In our previous studies, we showed that oral administration of guava leaf extract (GLE) had beneficial anti-obesity effects, preventing body weight gain, reducing adipose tissue weight and fatty liver, and also improving insulin resistance using SHRSP.Z-Leprfa/IzmDmcr rats, which were established as a new model of metabolic syndrome [7]. We did not, however, clarify how to improve obesity by GLE administration.

As one of the mechanisms of anti-obesity, reducing fat accumulation through the inhibition of adipocyte differentiation may play a crucial role. Several previous studies have established preadipocyte cell lines such as 3T3-L1, increasing our understanding of adipocyte differentiation. Peroxisome proliferator-activated receptor gamma (PPARγ), from the CCAAT-enhancer-binding protein (C/EBP) family, was reported as a transcription regulator of adipocyte differentiation. In particular, it was revealed that PPARγ is a transcription factor that is indispensable for the differentiation of fat cells, and it is considered that C/EBPα is a transcription factor with an essential role in the development of insulin sensitivity. Of these factors, adipocyte specific fatty acid binding protein (aP2) is a fatty acid binding protein that functions in fatty acid import, storage and export [8, 9]. Growth-arrested preadipocytes reenter the cell cycle and undergo division, known as mitotic clonal expansion, which is necessary for adipogenesis [10]. These are also used as adipogenic markers to detect mature adipocytes.

AMP-activated protein kinase (AMPK) plays a key role in the regulation of energy homeostasis [11]. AMPK activation requires phosphorylation at Thr172 of AMPKα subunit and it acts on metabolic enzymes and transcription factors [12]. Acetyl-CoA carboxylase (ACC), which is a critical enzyme for lipid biosynthesis, can be phosphorylated and inactivated by AMPK [13]. Fatty acid oxidation was increased by inhibition of carnitin palmitoyl-CoA transferase (CPT1) *via* inactivation of ACC [14]. Previous studies have demonstrated that AMPK activation by some compounds were associated with adipocyte differentiation.

In the present study, we used 3T3-L1 cell to determine the effects of GLE on anti-obesity by adipocyte differentiation and changes in the expression

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of PPARγ, C/EBPα and aP2 genes. Also, AMPK siRNA was applied to identify the function of AMPK in the regulation of adipocyte differentiation by GLE.

MATERIALS AND METHODS

Cell Line

3T3-L1 preadipocytes were purchased from The Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan).

Reagents

Guava leaf 70% ethanol extracts were supplied by Beijing University of Chinese Medicine, which identified the biological materials. Dulbecco's modified Eagle's medium (DMEM) and antibiotic solution were obtained from Nacalai Tesque (Kyoto, Japan). Insulin was also obtained from Nacalai Tesque. Isobutylmethylxanthine (IBMX) and dexamethasone (DEX) were purchased from Wako Pure Chemicals (Osaka Japan). Antibodies against AMPKα-Thr172, AMPKα, ACC-Ser79, and ACC were purchased from Cell Signaling Technology (Beverly, MA, USA). Real-time PCR kits were from TOYOBO (Tokyo, Japan) and primers were provided by Invitrogen (CA, USA).

Cell Culture and Adipocyte Differentiation

The 3T3-L1 cells were cultured in DMEM containing 10% FBS under 37 °C, 5% CO_2 and 95% humidity. Two days after cells reached confluence, adipocyte differentiation was induced with insulin, DEX and IBMX in DMEM. After 2 days, medium was replaced with DMEM containing only insulin, and after a further 2 days, the medium changed with regular medium. 3T3 cells were treated with different concentrations of GLE (10, 50, 100 μg/mL) during the differentiation process.

Oil Red O Staining of 3T3-L1 Cells

Lipid accumulation in differentiated adipocytes can be assessed by staining neutral fats using the dye Oil red O. Adipocytes were fixed in 10% formalin and then stained for 20 min with dye. Lipid staining was quantified by adding isopropanol to extract the dye and measuring absorbance at 510 nm.

Primary Antibodies Used

Immunoblotting was performed with the following commercially available antibodies: anti-rabbit AMPK, anti-rabbit phosphor-AMPK, anti-rabbit ACC, anti-rabbit phosphor-ACC from Cell Signaling Technology. Anti-mouse β-actin was obtained from Sigma (St. Louis, MO, USA).

Western Blot Analysis

The cells were homogenized with ice-cold homogenized buffer containing 50mM Tris-HCl (pH7.4), 100mM NaCl, 1%Nonidet-P40, 0.25% Na deoxycholate, 0.1% SDS, 1mM EDTA, 50mM NaF, 2mM Na_3VO_4, 30mM Na pyrophosphate, 2mM PMSF, 1mM benzamidine, 0.02g/mL trypsin inhibitor, 0.02g/mL leupeptin, and 0.02g/mL aprotinin. After incubation for 2 hours, lysates were centrifuged at 15,000 rpm for 20 min and supernatants were isolated. Proteins were extracted by boiling the tissues in 0.5mmol/l Tris/HCl, pH6.8, glycerol, 10% SDS, 0.1% bromophenol blue and 2-mercaptethanol. The proteins (25μg/lean) were electrophoresed using 7.5%–12.5% SDS-PAGE gel at 100V for 2 hours. After fractionating, the proteins were transferred onto a PVDF membrane (Amersham Life Science Inc.) at 100mA for 2 hours. The membrane was blocked in Blocking One (Nacalai Tesque) for 20 min. After appropriate blocking, the blot was incubated with the primary antibody in antibody solution 1 (Toyobo) overnight. It was then washed with TTBS and finally incubated for 1h with a 1:5000 dilution of anti-rabbit and mouse IgG–horseradish peroxidase. Detection was performed using an ECL kit (Amersham Inc.). β-actin was used as an internal control. The density of the bands was measured using NIH Image.

Real-Time RT PCR

Total RNA was extracted from cells using Sepasol-RNA I Super G (Nacalai Tesque). From each sample,

Table 1:   Primer Sequences

Gene	Forward	Reverse
PPAR-γ	CCAACTTCGGAATCAGCTCTGT	AGGCTCTTCATGTGGCCTGTT
C/EBP-α	CGCCTTCAACGACGAGTTC	TTGGCCTTCTCCTGCTGTC
aP2	AGCATCATAACCCTAGATGGCG	CATAACACATTCCACCACCAGC
36B4	AGCCTTCCTTCTTGGGTATG	CAGGAGGAGCAATGATCTTG

total RNA was reverse transcribed to cDNA using a ReverTra Ace qPCR RT Kit (Toyobo). THUNDERBIRD SYBR qPCR Mix was used for quantitative real-time RT-PCR analysis of each gene expression. The primers are listed in Table **1**. Amplification was performed with a real-time PCR system (ABI Prism 7000). Results are expressed as a relative value after normalization to 36B4 expression.

AMPKα siRNA Transfection

Preadipocytes were seeded onto the plate and siRNA transfection was carried out 2 days after the confluence of preadipocytes. Transfection reagents and siRNA were individually diluted and mixed. Mixtures were added to each well and incubated for 6 hours. Normal growth medium was added, incubated

for 24 hours, and media were removed and replaced with induction medium or GLE.

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical analysis of the data was performed by Student's *t*-test for the difference between two groups or Dunnett's test for multiple comparisons. A *p*-value less than 0.05 was considered significant.

RESULTS

GLE Inhibited 3T3-L1 Differentiation

The effects of GLE on adipocyte differentiation were examined by Oil red O staining. The results showed that GLE inhibited the accumulation of lipid droplets in
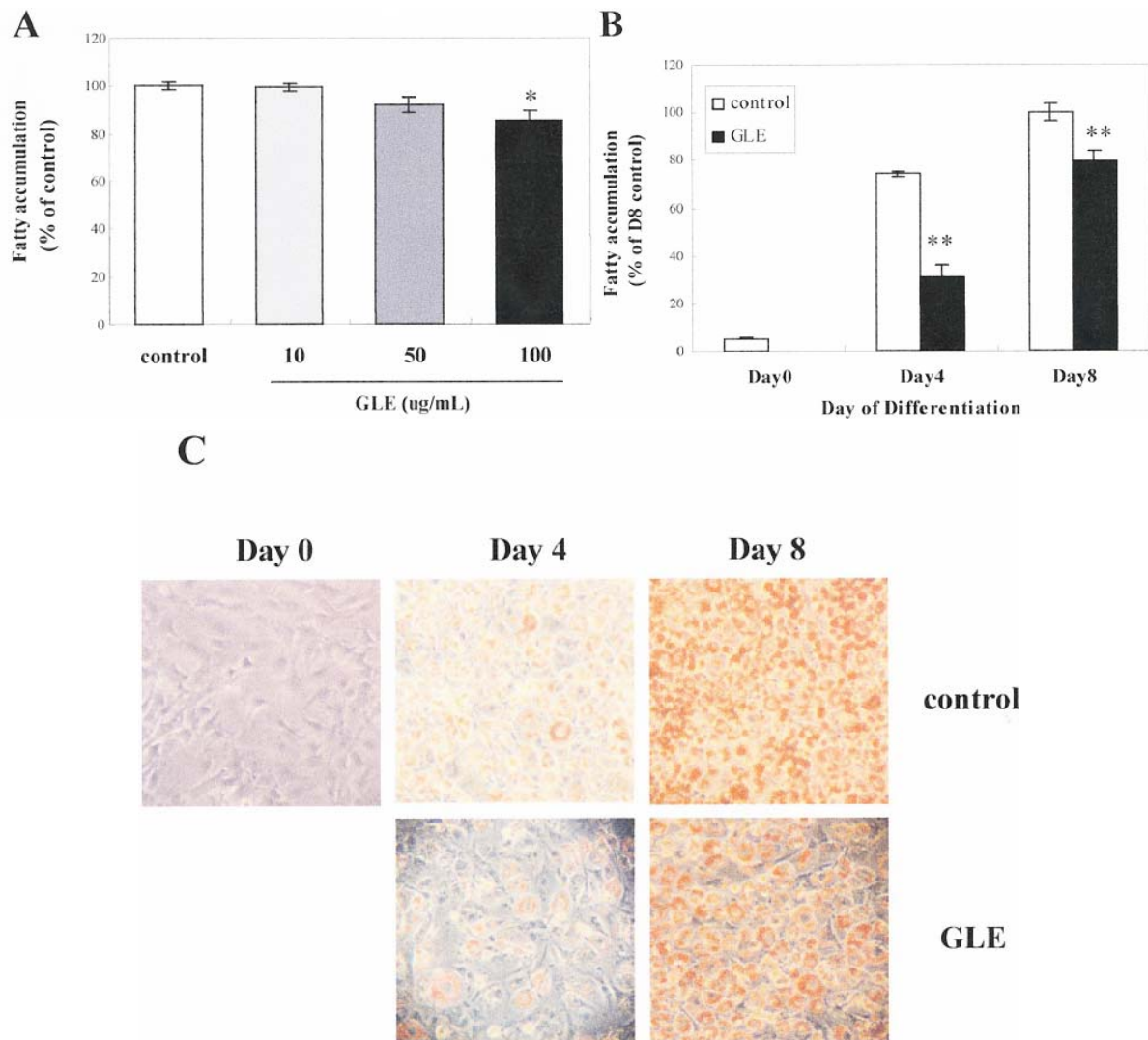

Figure 1: Effect of GLE on adipocyte differentiation in 3T3-L1 cells.
Confluent cells were exposed to a differentiation cocktail and GLE at the indicated concentrations (**A**) or cells were exposed to GLE (100 ug/mL) for the indicated time period (**B**). Fat drops were stained by Oil red O dye staining (**C**). Data are the means ± SEM * *P*< 0.05 ** *P*< 0.01 vs control group; n = 3.
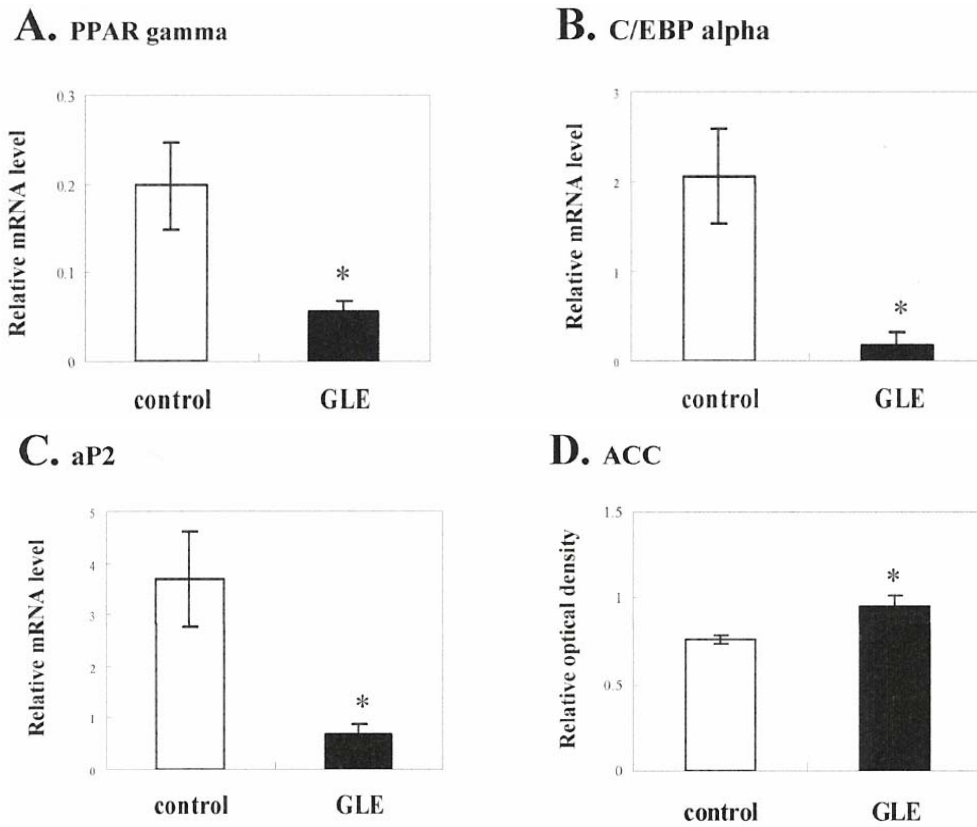
Figure 2: GLE Inhibited the Expression of Adipogenesis Related Transcription Factors and Markers.
3T3-L1 cells were induced to differentiate and were treated with or without GLE (100 ug/mL). Total RNA and protein were extracted on day 4 and the effects on mRNA levels of PPAR-gamma (**A**), C/EBP-alpha (**B**) and aP2 (**C**) were investigated using real-time PCR. The protein level of ACC and its phosphorylated state (**D**) were determined by Western blotting. Data are the means ± SEM ; * $P < 0.05$ vs control group; n = 3.

a dose-dependent manner during 3T3-L1 differentiation (Figure **1A**). On Day 4, the addition of GLE cells decreased intracellular fatty accumulation as compared with the control (Figure **1B**). This result was further supported by photographs of lipid staining (Figure **1C**).

GLE Inhibited the Expression of Adipogenesis-Related Transcription Factors and Markers

3T3-L1 adipogenic differentiation requires the synergistic action of multiple transfection factors and adipogenic markers including PPARγ, C/EBPα and
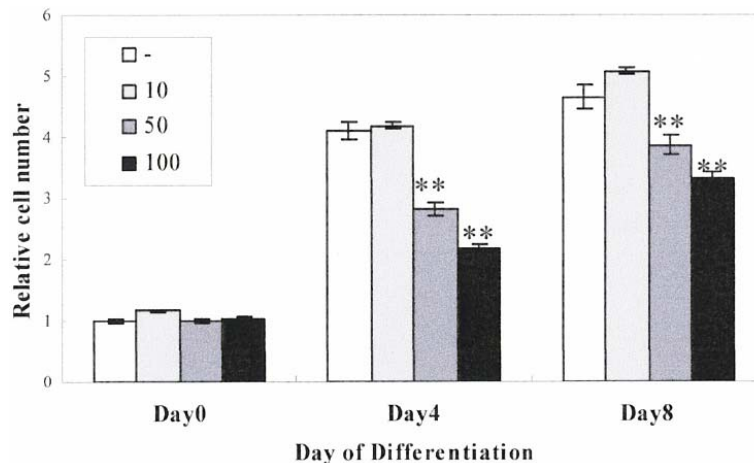

Figure 3: Effect of GLE on clonal expansion in 3T3-L1 cells.
Cells were differentiated at the indicated GLE concentration (10, 50, 100 ug/mL). On Day 0, 4, 8, cell numbers were counted using the MTT assay. Data are the means ± SEM; ** $P < 0.01$ vs control group; n = 3.

aP2. Cells incubated with 100 µg/mL GLE exhibited markedly decreased mRNA expression of PPARγ, C/EBPα and aP2 compared with the control, as demonstrated by real-time PCR (Figure **2A**, **B**, **C**). GLE treatment also resulted in a significant increase in the phosphorylation of ACC, which is a late adipogenic marker enzyme in 3T3-L1 (Figure **2D**).

GLE Inhibited Clonal Expansion

In order to examine whether the site of action of GLE is within this initiation phase of adipogenesis, cell numbers were measured beginning on Day 0, 4 and 8. On Day 0, cell numbers were not different among treatments; however, by Day 4, cell number had decreased with GLE treatment as compared to the control. Day 8 showed the same results as Day 4 (Figure **3**).

GLE Enhanced AMPK Activity and Inhibited 3T3-L1 Adipogenesis

Activity of AMPK was assessed by measuring the phosphorylated AMPK (p-AMPK) level. Compared with the control, the GLE-treatment group exhibited significantly increased p-AMPK protein (Figure **4A**). Moreover, after treatment with AMPKα siRNA and restoration of differentiation by GLE, the relative fatty accumulation of each group was determined. The results suggested that AMPKα siRNA can restore adipocyte differentiation with GLE treatment (Figure **4B**).

DISCUSSION

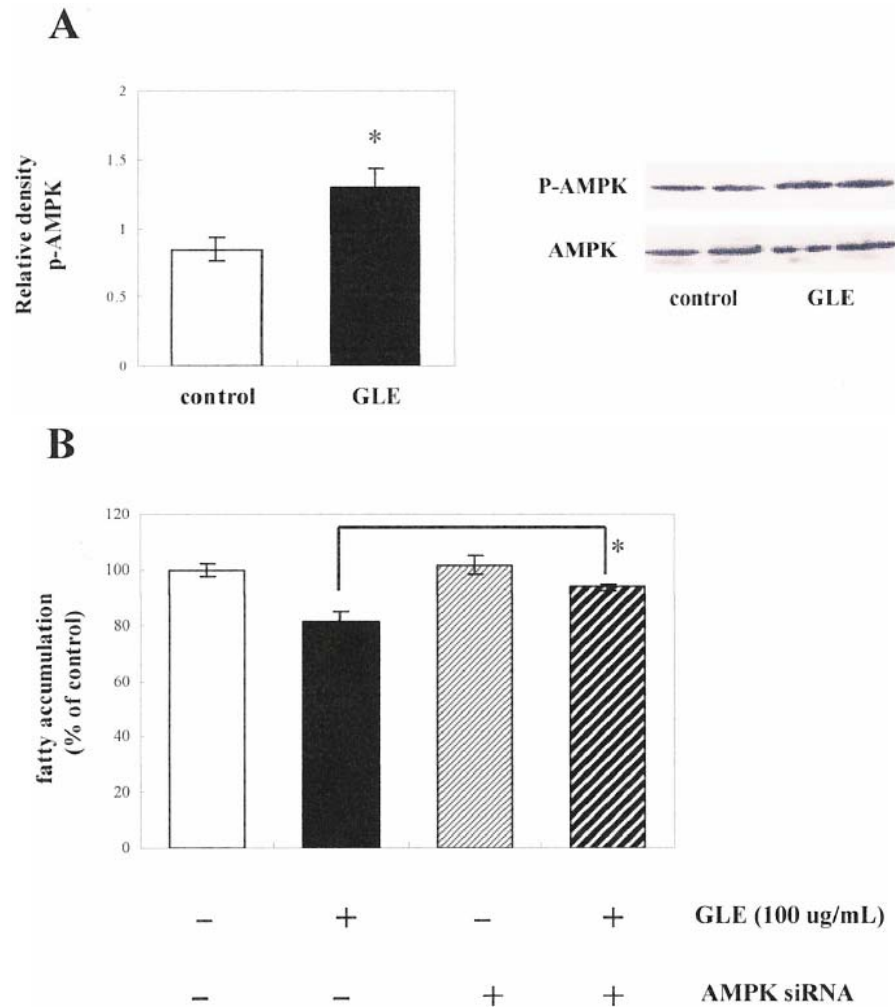In our previous studies, we showed that oral administration of guava leaf extract (GLE) had

Figure 4: Effect of GLE on AMPK in 3T3-L1 cells.
After 24 hr of GLE (100 ug/mL) treatment to confluence cells, cells were collected and lysed for Western blotting to determine the effect of GLE on AMPK activation (**A**). Phosphorylated AMPK was normalized to AMPK. After AMPK siRNA pretreatment and GLE-induced differentiation, the relative intracellular fatty accumulation of 3T3-L1 cells in each group was measured at Day 8 (**B**). Data are the means ± SEM;. * $P< 0.05$ vs control group. n = 3

beneficial anti-obesity effects using SHRSP.Z-Leprfa/IzmDmcr rats. We did not, however, clarify how to improve obesity by GLE administration. This study was designed to evaluate the mechanism of anti-obesity by GLE using 3T3-L1 pre-adipocyte cell lines and we show that GLE inhibited 3T3-L1 adipocyte differentiation which is a key player in obesity and metabolic syndrome and may serve as a potential therapeutic target for treating obesity.

Pre-adipocytes exposed to multiple hormones and cytokines, including cAMP, insulin, and IGF-1, were able to fully mature into adipocytes [15]. Several transcription factors have been identified that act in a sequential fashion to promote adipocyte differentiation. PPARγ and C/EBP families served as early markers of adipocyte differentiation. The C/EBP families include C/EBPα, C/EBPβ and C/EBPδ. Transient expression of C/EBPβ and C/EBPδ occurs during the early stages of differentiation followed by induced expression of PPARγ and C/EBPα. They then induce the expression of multiple adipocyte-specific genes and are important for terminal adipocyte differentiation [16]. Cells develop the fat accumulation ability at differentiation. Our studies demonstrated that GLE inhibits 3T3-L1 adipogenesis at a relatively early stage by regulating the expression of PPARγ and C/EBPα mRNA. Moreover, GLE significantly suppressed aP2, which is a fatty acid binding protein downstream of PPARγ, so we used late adipogenic markers.

On the other hand, previous studies have identified that mitotic clonal expansion was essential for 3T3-L1 differentiation. During the clonal expansion phase, cells are relieved of the growth arrest signal initiated upon cell confluence and undergo multiple rounds of DNA synthesis and mitosis, followed by exit from the cell cycle and the deduction of several adipose-specific genes. Tang *et al*. reported that C/EBPβ was required for clonal expansion [10]. In order to determine of effects on clonal expansion of GLE, we quantitated cell numbers and found that GLE had inhibitory effects on clonal expansion of 3T3-L1 cells.

Most interestingly, we identified that GLE treatment increased the expression of p-AMPK, while AMPKα siRNA pretreatment increased adipocyte fatty accumulation. AMPK, an energy sensor, plays a key role in the regulation of fatty acid. Several researches have demonstrated that AMPK activation reduced body weight gain and decreased liver and plasma triglyceride *in vivo* [17]. Furthermore, *in vitro*, it was reported to affect AMPK in adipocyte differentiation.

Zhou *et al*. reported that A-769662, which is an AMPK activator, inhibited 3T3-L1 adipogenic differentiation by down-regulating the expression of C/EBPβ and C/EBPδ, and hence inhibiting clonal expansion. Moreover, it blocked the expression of PPARγ and C/EBPα and downstream adipogenic genes. This compound also activated AMPK and hence phosphorylated and inactivated ACC, which is essential for lipid biosynthesis [18]. Susan *et al*. also demonstrated that an AMPK activator, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), inhibited the differentiation of 3T3-L1 adipocytes by blocking the expression of adipogenic markers and early clonal expansion [19]. The AMPK cascade is an important therapeutic target for obesity because it promotes the expression or phosphorylation of downstream proteins involved in energy metabolism. Some phytochemicals, such as epigallocatechin-3-gallate [20], resveratrol [21], capsaicin and genistein [22], have demonstrated the activation of AMPK and inhibition of 3T3-L1 adipocyte lipogenesis. GLE may have a similar mechanism.

Taken together, these results suggest that GLE inhibited adipocyte differentiation by down-regulating the expression of PPARγ and C/EBPα and downstream adipogenic genes, which are essential for adipocytes, and inhibited clonal expansion *via* AMPK activity; therefore, oral administration of GLE may lead to beneficial anti-obesity effect.

COMPETING INTERESTS

The authors declare that they have no competing interests.

ABBREVIATIONS

ACC   = Acetyl-CoA carboxylase

AMPK   = AMP-activated protein kinase

aP2   = adipocyte fatty acid binding protein

C/EBP-α = CCAAT/enhancer- binding protein α

FFA   = free fatty acid

GLE   = Guava leaf extract

IL-6   = interleukin-6

PCR   = polymerase chain reaction

PPAR-γ = peroxisome proliferators-activated receptor γ

TNF   = Tumor Necrosis Factor

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