Background: Naringenin and its glycoside naringin are well known citrus flavonoids with several therapeutic benefits. Although the anti-adipogenic effects of naringenin and naringin have been reported previously, the detailed mechanism underlying their anti-adipogenesis effects is poorly understood.

Objectives: This study examined the anti-adipogenic effects of naringenin and naringin by determining differential gene expression patterns in these flavonoids-treated 3T3-L1 adipocytes.

Methods: Lipid accumulation and triglyceride (TG) content were determined by Oil red O staining and TG assay. Glucose uptake was measured using a 2-[N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-d-glucose fluorescent d-glucose analog. The phosphorylation levels of AMP-activated protein kinase (AMPK) and acetyl Co-A carboxylase (ACC) were observed via Western blot analysis. Differential gene expressions in 3T3-L1 adipocytes were evaluated via RNA sequencing analysis.

Results: Naringenin and naringin inhibited both lipid accumulation and TG content, increased phosphorylation levels of both AMPK and ACC and decreased the expression level of 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) in 3T3-L1 adipocytes. RNA sequencing analysis revealed that 32 up-regulated (> 2-fold) and 17 down-regulated (< 0.6-fold) genes related to lipid metabolism, including Acaca, Fasn, Scd1, Mogat1, Dgat, Lipin1, Cpt1a, and Lepr, were normalized to the control level in naringenin-treated adipocytes. In addition, 25 up-regulated (> 2-fold) and 25 down-regulated (< 0.6-fold) genes related to lipid metabolism, including Acaca, Fasn, Fabp5, Scd1, Srebfl, Hmgcs1, Cpt1c, Lepr, and Lrp1, were normalized to the control level by naringin.

Conclusions: The results indicate that naringenin and naringin have anti-adipogenic potentials that are achieved by normalizing the expression levels of lipid metabolism-related genes that were perturbed in differentiated 3T3-L1 cells.

Keywords: Naringenin; naringin; adipogenesis; gene expression; RNA sequencing
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Conflict of Interest
The authors declare no conflicts of interest.

Author Contributions
Conceptualization: Han CH, Dayarathne LA; Data curation: Ranaweera SS; Funding acquisition: Han CH; Investigation: Dayarathne LA, Han CH; Resources: Han CH; Software: Natraj P, Rajan P; Supervision: Han CH, Lee YJ; Visualization: Dayarathne LA, Han CH; Writing - original draft: Dayarathne LA; Writing - review & editing: Natraj P, Rajan P.

is stored as a lipid, specifically triglyceride (TG), in white adipose tissues [2]. Obesity is a major contributor to the global burden of chronic diseases and complications, including cardiovascular diseases, diabetes, and cancers [3].

AMP-activated protein kinase (AMPK) is an energy sensor that regulates glucose and lipid metabolism to modulate energy homeostasis in the body. The activation of AMPK inhibits preadipocyte differentiation and suppresses transcription factors, including peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer-binding protein alpha (C/EBPα), and sterol regulatory element-binding protein 1 (SREBP-1c), which are necessary for adipogenesis [4]. In addition, the activation of AMPK inactivates key metabolic enzymes, including acetyl Co-A carboxylase (ACC) and 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase (HMGCR), involved in fatty acid and cholesterol synthesis [5]. Therefore, AMPK is considered a target in the treatment of obesity and associated metabolic diseases.

Previous studies investigated anti-adipogenic agents as potential therapeutics for preventing obesity and associated disorders [6]. Recently, the use of natural bioactive compounds as alternative methods for treating obesity and related diseases is increasing because of their low risks of side effects compared to those of synthetic drugs [4].

Flavonoids are plant-derived compounds with multiple therapeutic effects, including anti-hyperglycemic and anti-hyperlipidemic activities [7]. Flavonoids mediate these biological effects via cell signaling pathways that are based on molecular interactions with numerous enzymes [8]. For naringin or its aglycon naringenin, which are citrus-derived flavonoids, diverse biological activities of therapeutic interests have been described, including anti-diabetic and anti-dyslipidemic effects [9]. However, the detailed mechanism associated with the actions of naringenin and naringin on anti-adipogenesis has not yet been fully described.

In this context, the present study hypothesized that naringenin and naringin exert anti-adipogenic potential by normalizing the activities of key enzymes related to lipid metabolism and restoring the expression of genes related to lipid metabolism to a normal level. To that end, the effects of naringenin and naringin on the phosphorylation levels of AMPK, ACC, and the expression levels of HMGCR were observed. In particular, RNA sequencing analysis was performed to examine the anti-adipogenic effects of naringenin and naringin, in which they normalized the expressions of lipid metabolism-related genes that had been perturbed in differentiated 3T3-L1 adipocytes.

MATERIALS AND METHODS

Induction of adipocyte differentiation and incubation with test compounds
The 3T3-L1 fibroblasts (KCLB 42835) obtained from the Korean Cell Line Bank (KCLB, Korea) were cultured Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (1% PS) (Gibco, USA) in a humidified atmosphere containing 5% CO₂ at 37°C.

The 3T3-L1 cells were cultured in 96-well plates (1 × 10⁵ cells/well), and after reaching post-confluence, cells were induced for differentiation for 48 h by adding differentiation initiation media (DIM) containing DMEM, 1% PS, 10% fetal bovine serum (FBS; Gibco), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma, USA), 0.5 μM dexamethasone (Sigma), 0.5 μM 3-isobutyl-1-methylxanthine (Sigma, USA), 0.5 μM dexamethasone (Sigma),...
2 μM rosiglitazone (Sigma) and 10 μg/mL insulin (Sigma). Subsequently, the DIM was changed to a normal medium (DMEM containing 1% PS and 10% FBS) containing 10 μg/mL insulin. Media were changed to normal growth media after 48 h and every 2 days thereafter until differentiation to mature adipocytes. To examine the effects of test samples on lipid accumulation, cells were cultured in DIM with or without the following test samples: 5 μM of simvastatin (Sigma) and different concentrations of naringenin and naringin (Sigma) diluted in DMSO until differentiation to mature adipocytes. At day 8, completely differentiated adipocytes were assessed for lipid accumulation through Oil red O staining and Western blot analysis.

Monitoring cellular viability: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay
The 3T3-L1 cells were seeded in 96-well plates at the cell density of 1 × 10^5 cells/well and cultured overnight using routine culture media. After reaching confluence, the cells were treated with different concentrations (0–100 μM) of the naringenin and naringin flavonoids for 48 h. Cell culture media was removed and fresh media containing 10% Ez-Cytox (DogenBio, Korea) was added into each well, according to the manufacturer’s instructions. Plates were incubated for 3 h at 37°C and 5% CO₂. Cell viability, as indicated by formazan production, was measured with an enzyme-linked immunosorbent assay microplate reader (TECAN, Austria) at 450 nm wavelength.

Oil red O staining
Post-confluence preadipocytes were differentiated into adipocytes as described above. According to a previously described method, on day 8 after adipocyte differentiation, cells were stained with Oil red O [10] with slight modifications. The fully differentiated adipocytes were fixed in 10% (v/v) formaldehyde (Biosesang, Korea) for 1 h at room temperature. Next, the cells were rinsed twice with phosphate-buffered saline (PBS; Gibco) and stained with 0.5% Oil red O solution (Sigma) (60% of Oil red O stock solution and 40% distilled water) for 1 h in the dark. The cells were washed with distilled water to remove the unbound dye, and images were captured using an IncuCyte ZOOM (Essen BioScience, USA) at 20× magnification. Quantitative analysis was performed using IncuCyte ZOOM processing software.

Measurement of TG content
Cellular TG contents were quantified using a commercially available colorimetric TG assay kit (BioAssay Systems, USA). Differentiated 3T3-L1 cells at day 8 were washed with PBS and harvested to cell lysis buffer containing 5% Triton X100 (Bio-Rad Laboratories, USA). Lysed cells were homogenized and centrifuged at 3,000 × g for 5 min. The TG content of the diluted supernatants was analyzed according to the manufacturer’s instructions. The protein concentration of each sample was measured using the Bio-Rad DC protein assay (Bio-Rad Laboratories). TG contents were normalized to the respective protein concentration as detected by using bovine serum albumin (Sigma) as the calibration standard.

2-[N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-d-glucose (2-NBDG) glucose uptake assay
Glucose uptake assays were performed on fully differentiated 3T3-L1 adipocytes. Briefly, preadipocytes were cultured in 96-well plates and induced to differentiate using the DIM protocol described above. Adipocytes were serum-starved for 12 h and then incubated with samples for 24 h. The cells were incubated with 40 μM 2-NBDG (Carlsbad, USA) for 30 min at 37°C. Cells were then washed 3 times with ice-cold PBS, and fluorescent images were obtained using an IncuCyte ZOOM fluorescence microscope (Essen BioScience). IncuCyte

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ZOOM fluorescence processing software (Essen BioScience) was used to analyze the total fluorescence intensities of each well.

**Western blot analysis**
Cells were washed with PBS and lysed with ice-cold RIPA buffer containing protease inhibitor mixture. The whole-cell lysates were centrifuged at 12,000 rpm for 10 min. The supernatant was separated, and the amount of protein determined by Bradford assay (Bio-Rad Laboratories). Equal amounts of protein was mixed with 20% loading buffer, separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, and subjected to Western blot with AMPK (Cell Signaling Technology, USA), ACC (Cell Signaling Technology), p-AMPK (Cell Signaling Technology), and p-ACC (Cell Signaling Technology), and β-actin (Thermo Fisher, USA) antibodies. A chemiluminescence bioimaging instrument (NeoScience, Korea) was used to detect the proteins of interest. Densitometry analysis was performed using the ImageJ analysis software.

**RNA sequencing analysis**
RNA sequencing analysis was performed as previously described [11]. Briefly, total RNA was isolated from 3T3-L1 adipocytes using an Easy-blue RNA extraction kit (iNtRON Biotechnology, Korea). RNA quality was assessed by Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, Netherlands). Based on the manufacturer’s instructions, libraries for RNAs were constructed using Quantsseq 3’mRNA-Seq Library Prep Kit (Lexogen, Austria). High-throughput sequencing was performed as single-end 75 sequencings using the NextSeq 500 (Illumina, USA). QuantSeq 3’mRNA-Seq reads were aligned using Bowtie2 version 2.1.0 [12]. Differentially expressed genes were determined based on counts from unique and multiple alignments using EdgeR within R version 3.2.2 and Bioconductor version 3.0 [13]. The RT (read count) data were processed based on the quantile normalization method using the Genowiz version 4.0.5.6 (Ocimum Biosolutions, India). Gene classification was performed using the Medline database (National Center for Biotechnology Information, USA).

**Statistical analysis**
Values were expressed as means ± SE of 3 independent experiments. Data were statistically analyzed with the aid of IBM SPSS Statistics (Ver.17.0; IBM, USA). The statistical differences among groups were assessed using a 1-way analysis of variance followed by Tukey’s test. The $p < 0.05$, $p < 0.005$, and $p < 0.0005$ indicate statistically significant differences from the differentiated cell control group.

**RESULTS**

**Cell viability of 3T3-L1 cells**
The MTT assay was performed to assess the effect of naringenin and naringin on 3T3-L1 cell viability (Fig. 1D and E). Significant cellular toxicity was not observed for up to 25 µM concentrations of both naringenin and naringin in 3T3-L1 cells. Accordingly, the following experiments were conducted using non-toxic concentrations (10 and 20 µM) of the 2 flavonoids.

**Lipid accumulation and TG content**
The effects of naringenin and naringin on lipid accumulation and TG content in 3T3-L1 adipocytes were observed by Oil red O staining and TG assay (Fig. 2). Differentiated
adipocytes significantly increased lipid accumulation and TG content compared to those of undifferentiated adipocytes (Fig. 2B and C). In contrast, treatment with simvastatin (positive control) and the higher concentrations (20 µM) of naringenin and naringin significantly inhibited ($p < 0.0005$) both lipid accumulation and TG content (Fig. 2B and C). The treatment of naringin shows more significant effectiveness ($p < 0.0005$) in inhibiting lipid accumulation and TG content of 3T3-L1 adipocytes than naringenin even at the low concentration (10 µM). Therefore, the results suggest that both naringenin and naringin suppress both lipid accumulation and TG content in differentiated 3T3-L1 adipocytes.
Glucose uptake in 3T3-L1 adipocytes

The glucose uptake effects of naringenin and naringin in 3T3-L1 adipocytes were assessed using the 2-NBDG glucose uptake assay (Fig. 3). Differentiated 3T3-L1 adipocytes showed slightly increased glucose uptake when compared with undifferentiated adipocytes. However, simvastatin treatment significantly increased \((p < 0.005)\) glucose uptake compared to that of the differentiated control. In particular, both naringenin and naringin significantly increased \((p < 0.005)\) glucose uptake compared to the differentiated control when used at the 20 µM concentration (Fig. 3A and B). Naringin at the low concentration (10 µM) also produced a significant increase in glucose uptake \((p < 0.005)\); however, that was not observed with naringenin treatment at the low concentration. These results indicate that both naringenin and naringin stimulate cellular glucose uptake in differentiated 3T3-L1 adipocytes.

Phosphorylation levels of AMPK pathway molecules

Western blot analysis was conducted to observe the effects of naringenin and naringin on the regulation of key enzymes in the AMPK signaling pathway (Fig. 4). The phosphorylation levels of both AMPK (Thr172) and ACC (Ser79) in 3T3-L1 adipocytes were significantly decreased \((p < 0.05)\) in differentiated adipocytes compared to undifferentiated adipocytes. In contrast, the phosphorylation levels of both AMPK (Thr172) and ACC (Ser79) were significantly increased by the treatment of simvastatin, naringenin, and naringin in differentiated 3T3-L1 adipocytes (Fig. 4A and B). The effects of naringenin on the phosphorylation levels of AMPK (Thr172) and ACC (Ser79) were greater \((p < 0.0005)\) than those of naringenin and were similar to those of simvastatin. In addition, compared to the differentiated control treatment, naringenin and naringin treatment significantly reduced...
The protein expression of HMGCR (Fig. 4C). The results suggest that both naringenin and naringin activate the AMPK signaling pathway in 3T3-L1 adipocytes.

Differential gene expression

RNA sequencing analysis was performed to observe the effects of naringenin and naringin on the expression levels of genes related to lipid metabolism in 3T3-L1 adipocytes. Based on the results of gene ontology analysis, a large proportion of the genes related to lipid metabolism was either up- or down-regulated in naringin and naringenin-treated 3T3-L1 adipocytes (Fig. 5). Thirty-two up-regulated (> 2-fold) genes, including Scd1, Scd3, Elovl3, Ppara, Lipin 1, and Cebpa, and 17 down-regulated (< 0.6-fold) genes, including Cpt1α, Lrp1, Lrp5, Adh7, and Lepr were normalized to the control level in naringenin-treated adipocytes (Tables 1 and 2). In addition, 25 up-regulated (> 2-fold) genes, including Scd1, Apoa4, Ppara, Cebpa, Fabp5, and G6pc and 25 down-regulated (< 0.6-fold) genes, including Pitpnc1, Spns2, Cpt1c, Lepr, and Pigv were normalized to the control level in naringin-treated adipocytes (Tables 3 and 4).

Search Tool for the Retrieval of Interacting Genes/Proteins analysis was performed to identify protein-protein interactions (PPIs) among the normalized genes induced by naringenin and naringin, and the results were visualized as a set of nodes and edges (Fig. 6A and B). The proteins related to lipid metabolism, including Acaca, Fasn, Scd1, Mogat1, Dgat, and Lipin1, were closely localized and interacted directly with each other within the PPI network of naringenin. In addition, the normalized down-regulated genes, including Cpt1α, Lepr, and Lrp1, were closely localized in the functional hub in the PPI network of naringenin. In the naringin PPI network, the normalized up-regulated genes, including Acaca, Fasn, Fabp5, Scd1, Srebf1, and
Hmgcs1, were closely localized and formed a functional cluster. In addition, normalized down-regulated genes, including Cpt1c, Lrp1, and Lepr, were located in the functional hub of the naringin PPI network. Overall, RNA sequencing analysis reveals that naringenin and naringin normalize the expression levels of lipid metabolism-related genes that were perturbed in differentiated 3T3-L1 adipocytes.

**Fig. 4.** Effects of naringenin and naringin on the expression of AMPK pathway molecules. The 3T3-L1 preadipocytes were differentiated in the absence or presence of flavonoids for 8 days. The p-AMPK and p-ACC and the expression of HMGCR were then determined by Western blot analysis. Immunoblots of p-AMPK (A), p-ACC (B), and HMGCR (C). Relative protein levels were quantified using densitometry analysis. Values represent mean ± SE. p-AMPK, phosphorylation of AMP-activated protein kinase; p-ACC, phosphorylation of acetyl Co-A carboxylase; HMGCR, 3-hydroxy-3-methylglutaryl CoA reductase; DIM, differentiation initiation media; Con, concentration. *p < 0.05, **p < 0.005 and ***p < 0.0005 vs. the differentiated control.
Fig. 5. Effects of naringenin and naringin on differential gene expression. Gene ontology analysis of 3T3-L1 adipocytes compared to normal cells (A). Naringenin-treated 3T3-L1 adipocytes compared to normal cells (B). Naringin-treated 3T3-L1 adipocytes compared to normal cells (C). The pie chart indicates functional categorization of the differentially expressed genes in 3T3-L1 adipocytes, and the bar graph represents the number of genes that were up- and down-regulated.
The present study demonstrated the anti-adipogenic effects of naringenin and naringin on differentiated 3T3-L1 adipocytes. The naringenin and naringin treatments suppressed both

**Table 1. Up-regulated genes normalized by naringenin in 3T3-L1 adipocytes**

| Gene symbol | Differentiated/normal | Simvastatin/normal | Naringenin/normal | Gene name                                                                 |
|-------------|-----------------------|--------------------|------------------|---------------------------------------------------------------------------|
| Scd3        | 1.031                 | 1.031              | 1.317            | Stearoyl-coenzyme A desaturase 3                                           |
| Elovl3      | 1.002                 | 1.002              | 1.002            | Elongation of very long chain fatty acids like 1                           |
| Scd1        | 1.160                 | 1.160              | 1.031            | Stearoyl-coenzyme A desaturase 1                                           |
| Ppara       | 1.578                 | 1.578              | 1.002            | Peroxisome proliferator-activated receptor alpha                           |
| Lip1        | 1.283                 | 1.283              | 1.002            | Lipin 1                                                                    |
| Aooa4       | 1.001                 | 1.001              | 1.001            | Apolipoprotein A-IV                                                        |
| Mogat1      | 1.001                 | 1.001              | 1.001            | Monoacylglycerol O-acyltransferase 1                                       |
| Cebpα       | 1.182                 | 1.182              | 1.002            | CCAAT/enhancer-binding protein, alpha                                       |
| Cyp2f2      | 1.081                 | 1.081              | 1.001            | Cytochrome P450, family 2, subfamily f, polypeptide 2                     |
| Dgat1       | 1.444                 | 1.444              | 1.001            | Diacylglycerol O-acyltransferase 1                                         |
| Pla2g5      | 1.001                 | 1.001              | 1.001            | Phospholipase A2, group V                                                  |
| Acsf2       | 1.071                 | 1.071              | 1.001            | Acyl-coa synthetase family member 2                                        |
| Cyp27a1     | 1.000                 | 1.000              | 1.000            | Cytochrome P450, family 27, subfamily a, polypeptide 1                     |
| G6pc        | 1.383                 | 1.383              | 1.002            | Glucose-6-phosphatase, catalytic                                           |
| Pparg       | 1.082                 | 1.082              | 1.001            | Peroxisome proliferator-activated receptor gamma                           |
| Dbi         | 1.244                 | 1.244              | 1.001            | Diazepam binding inhibitor                                                 |
| Adipor2     | 1.267                 | 1.267              | 1.001            | Adiponectin receptor 2                                                     |
| Fasn        | 1.267                 | 1.267              | 1.001            | Fatty acid synthase                                                        |
| Awt1        | 1.000                 | 1.000              | 1.000            | Acyl-coa wax alcohol acyltransferase 1                                     |
| HacΔ2       | 1.383                 | 1.383              | 1.001            | Peroxisome proliferator-activated receptor gamma                           |
| Accα1       | 1.042                 | 1.042              | 1.001            | Acyl-Coenzyme A oxidase 1, palmitoyl                                       |
| Hmgcs1      | 1.214                 | 1.214              | 1.001            | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1                           |
| Nkx6.2      | 1.000                 | 1.000              | 1.000            | Phosphoinositide-3-kinase, regulatory subunit 6                             |
| Cyp3α57     | 1.000                 | 1.000              | 1.000            | Cytochrome P450, family 3, subfamily a, polypeptide 57                     |
| Mogat2      | 1.000                 | 1.000              | 1.000            | Monoacylglycerol O-acyltransferase 2                                       |
| Apoα2       | 1.000                 | 1.000              | 1.000            | Apolipoprotein A-1                                                         |
| Scp2        | 0.818                 | 0.818              | 1.000            | Sterol carrier protein 2, liver                                            |
| Oxsm        | 0.936                 | 0.936              | 1.000            | 3-oxoacyl-ACP synthase, mitochondrial                                      |
| Fads2       | 0.946                 | 0.946              | 1.000            | Fatty acid desaturase 2                                                    |
| Cebpb       | 0.964                 | 0.964              | 1.000            | CCAAT/enhancer-binding protein, beta                                       |
| PpardCα1    | 1.129                 | 1.129              | 1.000            | Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha   |
| Galc        | 1.056                 | 1.056              | 1.000            | Galactosylceramidase                                                       |

**Table 2. Down-regulated genes normalized by naringenin in 3T3-L1 adipocytes**

| Gene symbol | Differentiated/normal | Simvastatin/normal | Naringenin/normal | Gene name                                                                 |
|-------------|-----------------------|--------------------|------------------|---------------------------------------------------------------------------|
| Akr1c14     | 0.792                 | 0.792              | 0.792            | Aldo-keto reductase family 1, member C14                                   |
| Gata2       | 1.007                 | 1.007              | 0.792            | GATA binding protein 2                                                     |
| Npyr1       | 1.072                 | 1.072              | 0.792            | Neuropeptide Y receptor Y1                                                 |
| Hmtn1I      | 0.765                 | 0.765              | 0.792            | Myotubularin related protein 1                                             |
| Cptα1       | 1.050                 | 1.050              | 0.792            | Carnitine palmitoyltransferase 1a                                          |
| Adh7        | 0.949                 | 0.949              | 0.792            | Alcohol dehydrogenase 7 (class IV)                                        |
| Rhd4        | 0.792                 | 0.792              | 0.792            | Phospholipase D1                                                          |
| HacΔ4       | 0.792                 | 0.792              | 0.792            | 3-hydroxyacyl-coa dehydratase 4                                            |
| Lrp1        | 0.792                 | 0.792              | 0.792            | Low density lipoprotein receptor-related protein 1                         |
| Asah1       | 0.850                 | 0.850              | 0.792            | N-aclysphingosine amidohydrolase 1                                         |
| Lepr        | 1.224                 | 1.224              | 0.792            | Leptin receptor                                                           |
| Lrp5        | 0.792                 | 0.792              | 0.792            | Low density lipoprotein receptor-related protein 5                         |
| Lmna        | 0.726                 | 0.726              | 0.792            | Lamin A                                                                   |
| E2f1        | 1.045                 | 1.045              | 0.792            | E2F transcription factor 1                                                 |
| Ccnd1       | 0.718                 | 0.718              | 0.792            | Cyclin D1                                                                 |
| Gla         | 1.083                 | 1.083              | 0.792            | Galactosidase, alpha                                                      |
| Cyb5r3      | 0.792                 | 0.792              | 0.792            | Cytochrome b5 reductase 3                                                  |

Hierarchical clustering results for differentially expressed genes normalized by naringenin in 3T3-L1 adipocytes. The expression levels of these genes were normalized by naringenin treatment compared to differentiated and normal conditions.

**DISCUSSION**

The present study demonstrated the anti-adipogenic effects of naringenin and naringin on differentiated 3T3-L1 adipocytes. The naringenin and naringin treatments suppressed both...
lipid accumulation and TG content and increased glucose uptake in 3T3-L1 adipocytes. Both naringenin and naringin increased the phosphorylation levels of AMPK and ACC and inhibited the expression level of HMGCR, which are the key enzymes in lipogenesis and

### Table 3. Up-regulated genes normalized by naringin in 3T3-L1 adipocytes

| Gene symbol | Differentiated/normal | Simvastatin/normal | Naringin/normal | Gene name |
|-------------|-----------------------|-------------------|----------------|-----------|
| Scd1        | 45.49                 | 8.10             | 1.327          | Stearoyl-Coenzyme A desaturase 1 |
| Ppara       | 77.170                | 39.919           | 1.670          | Peroxisome proliferator-activated receptor alpha |
| Apor4       | 91.349                | 7.713            | 1.043          | Apolipoprotein A-IV |
| Cebp4       | 18.250                | 12.830           | 1.161          | CCAAT/enhancer-binding protein alpha |
| Fabp5       | 15.580                | 12.573           | 1.001          | Fatty acid binding protein 5, epidermal |
| Lpl         | 7.613                 | 7.816            | 1.152          | Lipoprotein lipase |
| G6pc        | 5.879                 | 1.000            | 1.012          | Glucose-6-phosphatase, catalytic |
| Pparg       | 5.716                 | 5.128            | 1.670          | Peroxisome proliferator-activated receptor gamma |
| Adipor2     | 5.577                 | 4.734            | 1.174          | Adiponectin receptor 2 |
| Fasn        | 5.001                 | 3.137            | 1.289          | Fatty acid synthase |
| Bmp4        | 3.927                 | 1.000            | 1.008          | Bone morphogenetic protein 4 |
| Ghr         | 3.901                 | 3.893            | 1.018          | Ghrelin |
| Hmgcs1      | 3.370                 | 3.956            | 1.326          | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 |
| Cyp3a57     | 2.950                 | 1.000            | 1.005          | Cytochrome P450, family 3, subfamily a, polypeptide 57 |
| Mogo2       | 2.950                 | 1.000            | 1.005          | Monoacylglycerol O-acyltransferase 2 |
| Srebfl      | 2.907                 | 2.027            | 1.271          | Sterol regulatory element-binding transcription factor 1 |
| Lia         | 2.898                 | 2.812            | 1.050          | Lipid acid synthetase |
| Eci3        | 2.828                 | 1.684            | 1.234          | Enoyl-Coenzyme A delta isomerase 3 |
| Socs2       | 2.756                 | 2.257            | 1.058          | Suppressor of cytokine signaling 2 |
| G6pdx       | 2.737                 | 2.347            | 1.264          | Glucose-6-phosphate dehydrogenase X-linked |
| Oxsm        | 2.656                 | 2.235            | 1.020          | 3-oxoacyl-ACP synthase, mitochondrial |
| Fads2       | 2.342                 | 1.908            | 0.974          | Fatty acid desaturase 3 |
| Cebp        | 2.161                 | 1.297            | 1.055          | CCAAT/enhancer-binding protein (C/EBP), beta |
| Ppargc1a    | 2.101                 | 2.743            | 0.903          | Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha |
| Galc        | 2.037                 | 1.562            | 1.256          | Galactosylceramidase |

### Table 4. Down-regulated genes normalized by naringin in 3T3-L1 adipocytes

| Gene symbol | Differentiated/normal | Simvastatin/normal | Naringin/normal | Gene name |
|-------------|-----------------------|-------------------|----------------|-----------|
| Akr1c14     | 0.151                 | 0.296             | 0.993          | Aldo-keto reductase family 1, member C14 |
| Prtncl      | 0.164                 | 0.204             | 0.899          | Phosphatidylinositol transfer protein, cytoplasmic 1 |
| Spro2       | 0.280                 | 0.232             | 0.775          | Spinster homolog 2 |
| Gba         | 0.288                 | 0.379             | 0.707          | Glucosidase, beta, acid |
| Npy1r       | 0.290                 | 0.489             | 1.069          | Neuropeptide Y receptor Y1 |
| Mtom11      | 0.291                 | 0.381             | 0.956          | Myotubularin related protein 11 |
| Cptlc       | 0.302                 | 0.257             | 1.102          | Carnitine palmitoyltransferase 1c |
| Adh7        | 0.316                 | 0.635             | 1.001          | Alcohol dehydrogenase 7 (class IV) |
| Pd1         | 0.333                 | 0.462             | 0.972          | Phospholipase D1 |
| Hadc4       | 0.355                 | 0.211             | 1.025          | 3-hydroxyacyl-coa dehydratase 4 |
| Piv         | 0.360                 | 0.735             | 0.802          | Phosphatidylinositol glycan anchor biosynthesis, class V |
| Hexa        | 0.375                 | 0.491             | 0.870          | Hexosaminidase A |
| Lrp3        | 0.379                 | 0.387             | 0.861          | Low density lipoprotein receptor-related protein 1 |
| Lepr        | 0.394                 | 0.984             | 1.056          | Leptin receptor |
| Fgfl0       | 0.403                 | 0.615             | 0.998          | Fibroblast growth factor 10 |
| Lrp5        | 0.407                 | 0.348             | 1.096          | Low density lipoprotein receptor-related protein 5 |
| Lmna        | 0.412                 | 0.347             | 0.784          | Lamin A |
| Scpep1      | 0.438                 | 0.533             | 0.772          | Serine carboxypeptidase 1 |
| Ezfl        | 0.440                 | 0.517             | 1.112          | E2F transcription factor 1 |
| Lina1       | 0.457                 | 0.334             | 1.008          | LIM domain and actin binding 1 |
| Fgfl7       | 0.463                 | 0.480             | 1.167          | Fibroblast growth factor 7 |
| Gla         | 0.474                 | 0.714             | 1.101          | Galactosidase, alpha |
| Pigt        | 0.491                 | 0.587             | 1.124          | Phosphatidylinositol glycan anchor biosynthesis, class T |
| Soc5        | 0.494                 | 0.584             | 0.921          | Suppressor of cytokine signaling 5 |
| Tmem150a    | 0.497                 | 0.640             | 0.782          | Transmembrane protein 150A |

Both naringenin and naringin increased the phosphorylation levels of AMPK and ACC and inhibited the expression level of HMGCR, which are the key enzymes in lipogenesis and
cholesterol synthesis. RNA sequencing results revealed that naringenin treatment normalizes the expression of genes involved in lipid metabolism, including \textit{Acaca}, \textit{Fasn}, \textit{Scd1}, \textit{Mogat1}, \textit{Dgat}, \textit{Lipin1}, \textit{Cpt1a}, and \textit{Lepr}. In addition, the treatment of naringin normalizes the expression of genes related to lipid metabolism, including \textit{Acaca}, \textit{Fasn}, \textit{Fabp5}, \textit{Scd1}, \textit{Srebf1}, \textit{Hmgcs1}, \textit{Cpt1c}, \textit{Lepr}, and \textit{Lrp1} in 3T3-L1 adipocytes.

The present study shows that naringenin and naringin treatments reduce intracellular lipid accumulation and TG content in 3T3-L1 adipocytes, as was observed previously [6,14]. Naringenin reduces diet-induced weight gain and improves glucose and lipid metabolism in animal models [15]. In addition, naringin-rich pomelo (\textit{Citrus grandis} (L.) Osbeck) peel extract has been shown to reduce body weight, TG, and total cholesterol level in obese rats [16]. Therefore, the present study of differentiated 3T3-L1 adipocytes confirms that both naringenin and naringin have significant roles in inhibiting lipid accumulation and TG synthesis.

Furthermore, the present study in differentiated 3T3-L1 adipocytes confirmed that both naringenin and naringin significantly increase glucose uptake in 3T3-L1 adipocytes. Many flavonoids are known to increase glucose uptake via the activation of AMPK, which enhances glucose transporter type 4 translocation in 3T3-L1 adipocytes [17,18]. Naringenin and naringin enhance glucose utilization through increased glycolysis, which ultimately reduces the utilization of lipids [19,20]. Therefore, the previous and present findings suggest that naringenin and naringin produce anti-adipogenic effects by increasing glucose uptake, which might enhance glucose metabolism in differentiated 3T3-L1 adipocytes.

In the present study, the naringenin and naringin treatments increased the phosphorylation levels of both AMPK and ACC and decreased the expression level of HMGCR. Activation of AMPK suppresses lipid synthesis [21] through downregulation of key adipogenic factors, including PPAR\(\gamma\), C/EBP\(\alpha\), and SREBP-1c [4]. Phosphorylation of ACC inhibits fatty acid synthesis by blocking the conversion of acetyl-CoA to malonyl-CoA [22]. Naringenin is known to reduce the levels of plasma cholesterol and hepatic TG that accompany the decreased expression of HMGCR in animal models [23]. In addition, naringin is known to activate AMPK [20] and suppress HMGCR expression in type 2 diabetic mice [24]. The
previous and present findings suggest that naringenin and naringin treatments regulate lipid metabolism by activating the AMPK pathway in 3T3-L1 adipocytes, which potentially contributes to a reduction of adipogenesis.

RNA sequencing results revealed that both naringenin and naringin treatments normalized the expressions of genes involved in lipid metabolism in 3T3-L1 adipocytes. The treatment of naringenin normalized the expressions of up-regulated genes, including *Acaca*, *Fasn*, *Scd1*, *Mogat1*, *Dgat*, and *Lipin1*. The *Acaca* encoding enzyme catalyzes the carboxylation of acetyl-CoA to malonyl-CoA [25], while *Fasn* catalyzes the conversion of malonyl-CoA into palmitate [26]. *Scd1* is a rate-limiting enzyme in the synthesis of mono-unsaturated fatty acids [27], and *Mogat* catalyzes the synthesis of diacylglycerol, the precursor of triacylglycerol [28]. *Dgat* catalyzes the covalent addition of a fatty acyl chain to diacylglycerol [29] and reduces TG levels in *Dgat* knockout mice [30]. *Lipin1* is a key regulator of TG metabolism and lipoprotein synthesis [31]. In this regard, normalization of genes related to lipid metabolism, including *Acaca*, *Fasn*, *Scd1*, *Mogat1*, *Lipin1*, and *Dgat*, by naringenin contributes to reducing adipogenesis in 3T3-L1 adipocytes.

Naringenin treatment normalized the expressions of down-regulated genes, including *Cpt1a*, and *Lepr*. *Cpt1* catalyzes fatty acid oxidation by converting acyl-CoAs into acylcarnitines [32], and *Cpt1* knockout mice are reported to be susceptible to high-fat-diet-induced obesity [33]. *Lepr* regulates energy expenditure and reduces lipid accumulation in the body [34]. A previous study showed attenuation of obesity via *Lepr* gene therapy [35]. Naringenin has been reported to stimulate the mRNA expression of *Cpt1* [36], an observation supported by the present findings. Therefore, the previous and present findings suggest that naringenin reduces adipogenesis by normalizing lipid metabolism-related genes in 3T3-L1 adipocytes.

Naringin treatment normalized the expressions of up-regulated genes, including *Acaca*, *Fasn*, *Scd1*, *Fabp5*, *Srebf1*, and *Hmgcs1*. As described previously, the expression of *Acaca* catalyzes the first committed step in fatty acid synthesis [25]. *Fasn* is the central enzyme involved in *de novo* lipogenesis [26], and *Scd1* is the rate-limiting enzyme in the synthesis of mono-unsaturated fatty acids [37]. In addition, *Fabp5* is involved in lipid trafficking and intracellular fatty acid storage [38]. *Srebf1* regulates SREBP1a and SREBP1c that are involved in fatty acid and cholesterol synthesis [39]. *Hmgcs1* is involved in cholesterol synthesis, converting acetyl-CoA and acetoacetyl-CoA into HMG-CoA [40]. In this regard, normalization of genes related to lipid metabolism, including *Acaca*, *Fasn*, *Fabp5*, *Scd1*, *Srebf1*, and *Hmgcs1*, by naringin treatment contributes to reducing adipogenesis in 3T3-L1 adipocytes.

Naringin treatment normalized the expressions of down-regulated genes, including *Cpt1c*, *Lepr*, and *Lrp1*. *Cpt1* is involved in fatty acid oxidation [32], and *Lepr* regulates energy expenditure, which reduces lipid accumulation [34]. *Lrp1* is a key factor in maintaining lipid homeostasis, insulin sensitivity, and glucose homeostasis [41]. Overall, the results suggest that naringin restores adipogenesis to the normal level by normalizing the expression of *Cpt1c*, *Lepr*, and *Lrp1* genes in 3T3-L1 adipocytes.

In the present study, both naringenin and naringin showed significant anti-adipogenic effects, with naringin having a higher potency than naringenin in mitigating obesity. Naringenin and naringin are major flavonoids present in grapefruit and are abundant in many other citrus fruits [42]. Naringin is a 7 O-glycoside in which 2 rhamnose units are attached to its aglycon portion, naringenin, at the 7-carbon position [43]. The glycoside
forms of flavonoids are the most abundant polyphenols in plants [44]. Glycosides are known to be more biologically active than the respective aglycone, since the bound sugar moiety of O-glycosides is known to influence their bioavailability [45]. Attaching a glycosidic moiety increases hydrophilicity, which influences the pharmacokinetic properties of the respective compounds [46]. Solubility has a major role in the therapeutic efficacy of flavonoids. Low solubility of flavonoid aglycones in water, coupled with its short residence time in the intestine, results in their low absorption. The low solubility of the flavonoids in water often presents a problem in medicinal applications [47]. Further, O-glycosylation can enhance some biological benefits, including anti-adipogenic activity, as reported previously [48]. However, as the glycoside linkages are less stable than most glucuronide linkages and might not withstand the acidic environment in the stomach; moreover, glycosidase from intestinal bacteria may cleave sugar residues, which will generate the aglycone naringenin [49,50]. Therefore, the absorption of naringin inside the body remains to be clarified further [50]. However, being a 7 O-glycoside, naringin shows higher anti-adipogenic potential than its aglycone naringenin in 3T3-L1 adipocytes.

Overall results of the present study indicate that both naringenin and naringin treatments reduce lipid accumulation and TG content in 3T3-L1 adipocytes by increasing the phosphorylation of both AMPK and ACC while reducing the expression of HMGCR. In particular, both naringenin and naringin normalize the expression of genes involved in lipid metabolism in 3T3-L1 adipocytes (Fig. 7). Further studies are needed to delineate the potential role of these genes as therapeutic targets in adipogenesis. The overall results suggest that naringenin and naringin treatments have potent anti-adipogenic effects related to normalizing the expression of lipid metabolism-related genes that are perturbed in differentiated 3T3-L1 adipocytes.

**Fig. 7.** Proposed model of the anti-adipogenic effects of naringenin and naringin on 3T3-L1 adipocytes. GLUT4, glucose transporter type 4; AMPK, AMP-activated protein kinase; HMGCR, 3-hydroxy-3-methylglutaryl CoA reductase; ACC, acetyl Co-A carboxylase; CPT1, carnitine palmitoyltransferase I.
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