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Altered *Gelidium elegans* Extract-stimulated Beige-like Phenotype Attenuates Adipogenesis in 3T3-L1 Cells

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Abstract Previously, we showed that Gelidium elegans extract (GE) suppresses oxidative stress and lipid accumulation. However, the molecular mechanism underlying the anti-adipogenic ability of GE is still unclear. The levels of adipogenesis markers and triglyceride synthesis enzymes were measured by western blot. To evaluate the lipid accumulation in 3T3-L1 cells, oil red o staining was performed. We investigated whether GE induces lipolysis by measuring adipocyte triglyceride lipase (ATGL) during adipocyte differentiation. We also examined the expression of beige cell-associated genes and the production of carbon dioxide in 3T3-L1 cells. We showed that GE increased the protein expression of CAAT/enhancer binding protein (C/EBP) homologous protein 10 and inhibited the expression of C/EBPβ. GE discouraged triglyceride synthesis via deregulation of lysophosphatidic acid acyltransferase-θ (LPAAT0) and diacylglycerolacyltransferase 1 (DGAT1) during late-stage adipogenesis in 3T3-L1 cells. GE also dramatically increased ATGL in 3T3-L1 cells. Finally, in 3T3-L1 cells treated with GE, markers of beige adipocytes such as PRDM16 and UCP1 were upregulated, and large amounts of carbon dioxide were produced. These data indicate that GE suppresses adipogenesis by stimulating a beige-like phenotype in 3T3-L1 cells.

Keywords: *gelidium elegans, hesperidin, obesity, transdifferentiation, brown adipocyte, energy expenditure*

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1. Introduction

Obesity has emerged as a problematic disease worldwide and is associated with multiple post-modern metabolic diseases [32]. Fat accumulation is caused by two different types of white adipocyte development processes, hyperplasia (adipocyte number increase) and hypertrophy (adipocyte size increase) in white adipocyte tissue [10]. Adipogenic stem cell differentiation and intracellular lipid droplet accumulation contribute to the development of white adipose tissue [6].

For adipocyte differentiation, CAAT/enhancer binding protein (C/EBP) homologous protein (CHOP10), a negative regulator of C/EBPβ, is decreased during mitotic clonal expansion [7]. Subsequently, C/EBPβ can coordinate transcriptional regulation by turning on crucial adipogenic factors, including the expression of C/EBPα and peroxisome proliferator-activated receptor-γ (PPARγ). C/EBPα and PPARγ stimulate triglyceride synthesis via the activation of lysophosphatidic acid acyltransferase-θ (LPAAT0) and diacylglycerolacyltransferase 1 (DGAT1) during late-stage adipogenesis [31].

A potential target for anti-obesity therapies is increasing beige adipocyte number [3]. Beige adipocytes exhibit an increased expression of thermogenesis-associated genes, including a unique protein called uncoupling protein 1 (UCP1) and the zinc-finger protein PRDM16 during adipogenesis *in vitro* [17], resulting in thermogenesis, energy expenditure, and carbon dioxide production. Thus, regulation of both white and beige adipocyte differentiation-associated key factors is important for decreasing the development of adipocytes in obesity.

3T3-L1 cells have most commonly been used to investigate both the anti-obesity properties and molecular mechanism of drugs or phytochemicals *in vitro*. In previous studies, we and others have shown that phytochemical and functional food consumption significantly suppressed adipocyte differentiation and adipocyte-mediated systemic inflammation in 3T3-L1 cells [15,16,18,23,29]. Similar to white, beige, and brown adipocytes *in vitro*, 3T3-L1 cells have all isoforms of the beta adrenergic receptor (AR) [5]. Further, it has been shown that proper environmental conditions and certain types of chemical such as isoproterenol stimulate this characteristic change by up-regulating UCP1 transcription in 3T3-L1 cells [1,24]. Despite this evidence, very little attention has been paid to beige cell differentiation in 3T3-L1 cells.

Recently, we showed that *Gelidium elegans* extract (GE) attenuates the production of reactive oxygen species (ROS) and adipogenesis [11,12]. However, the underlying detailed molecular mechanism of the anti-adipogenic effects of GE is still unclear. In addition, no other study

has been published showing how GE influences the change towards a beige cell phenotype.

In the present study, we investigated whether GE regulates the aforementioned expression of genes, including mitotic clonal expansion, terminal differentiation makers, and triglyceride synthesis enzymes in 3T3-L1 cells. Moreover, we sought to further investigate whether GE stimulates beige-like adipocyte differentiation by regulating the UCP1 and PRDM16 levels in 3T3-L1 cells.

2. Materials and Methods

2.1. Reagents

Gelidium elegans extract was kindly provided from NEWTREE Inc. (Seongnam, Kyonggi, South Korea). Hesperidin was purchased from LKT Laboratories Inc. (St. Paul, MN, USA). Dulbecco's modified Eagle's medium (DMEM), bovine calf serum (BCS), fetal bovine serum (FBS), penicillin-streptomycin, phosphate buffered saline (PBS), and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Gaithersburg, MD, USA). Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), insulin, modified Griess reagent, and Oil red O were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA): C/EBPα (SC-61), PPARγ (sc-7273), C/EBPβ (SC-150), GAPDH (sc-25778), LPAATθ (SC-68372), SREBP-1 (SC-366), DGAT1 (SC-32861), CHOP10 (SC-575), Lipin1 (SC-98450), and UCP1 (SC-6529). ATGL (ab99532) and PRDM16 (ab106410) were obtained from Abcam (Cambridge, MA, USA).

2.2. Determination of Chemical Composition

The phenol–sulfuric acid method was used to determine the total carbohydrate content [8]. The protein concentration was measured by the Lowry method [22] using the DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). The moisture was determined by drying the samples in a drying oven for 2 h at 131 °C, and the ash content was determined by heating and drying the sample in a furnace at 600 °C for 8 h.

2.3. Determination of the Total Phenol Content in *Gelidium elegans* Extract

The total phenol content was determined according to the modified Folin-Ciocalteu method [19]. The sample solution (1 mL) was placed in a test tube with distilled water (7.5 mL) and Folin-Ciocalteu reagent (0.5 mL) that was saturated with sodium carbonate solution (1 mL) and then allowed to stand for 30 min. The absorbance was measured at 760 nm. The total phenol content was calculated as tannic acid equivalents (TAE). All experiments were repeated three times.

2.4. Cell culture

Murine 3T3-L1 pre-adipocytes (CL-173, ATCC, Manassas, VA, USA) were cultured in DMEM containing 10 % BCS and 1 % penicillin-streptomycin solution. 3T3-L1 cells were differentiated with DMEM containing 10% FBS and MDI mixture (10 μM dexamethasone, 0.5 mM IBMX, and 2 μg/mL insulin) when cells reached confluence after 2 days. To investigate the effects of GE on fully differentiated adipocytes, GE or hesperidin (HP) was applied to the cell. GE or HP was supplemented at 2-day intervals when the culture medium was replenished.

2.5. Cell Viability Assay

Cells were seeded on 96-well plates. The cells were incubated with various concentrations of GE for 24 h. Cell viability was measured using an ELISA reader, Wallac 140 Victor 2 plate reader, (Perkin-Elmer, Boston, MA, USA) at 450 and 690 nm after the reaction for 4 h at 37 °C, followed by the addition of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reagent.

2.6. Oil Red O Staining

3T3-L1 cells were washed with PBS, fixed with 10 % formaldehyde, and allowed to stand for 1 h at 4 °C. After washing with PBS, cells were stained with 0.5 % Oil red O solution containing isopropanol and water (60:40, vol/vol) for 2 h at room temperature. Lipid content was quantified using the Image J software (Bethesda, MD, USA).

2.7. Western Blot Analysis

Cells were lysed in Pro-Prep solution (Intron, Seoul, Korea) containing protease and phosphatase inhibitor cocktail 2 and 3 (Sigma, St Louis, MO, USA). The lysates were clarified by centrifugation at 12,000 g for 20 min at 4 °C, and the protein concentration in the supernatant was determined by the Bradford Assay (Bio Legend, San Diego, CA, USA). Protein samples (25 μg) were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked in a blocking buffer containing 5 % skim milk and then immunoblotted with primary antibodies. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA, USA) for 1 h, and signals were detected using the LAS image software (Fuji, New York, NY, USA).

2.8. Measurement of Triglyceride Content in 3T3-L1 Cells

Triglycerides were determined using a total triglyceride assay kit according to the manufacturer's instructions (Zen-Bio, Research Triangle Park, NC, USA) with a Wallac 140 Victor 2 plate reader (Perkin-Elmer) at a wavelength 540 nm.

2.9. Measurement of Carbon Dioxide Production

Carbon dioxide production was determined using a carbon dioxide assay kit according to the manufacturer's instructions (Crystal Chem, Downers, IL, USA) with a Wallac 140 Victor 2 plate reader (Perkin-Elmer) at a wavelength 420 nm.

2.10. Statistical Analysis

All values are expressed as the mean ± standard deviation (SD). All data were obtained in triplicate, quadruplicate or hexaplicate experiments. Significant differences were determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests using the SAS 9.0 software (SAS Institute, NC, USA). Means with the same letter are not significantly different. Values with different letters are significantly different, $p<0.05$.

3. Results and Discussion

3.1. Preparation of *Gelidium elegans* Extracts (GE) and Composition Analysis

In general, the chemical composition of *Gelidium elegans* differs depending on the anatomical region, growing conditions, extraction procedures and analytical methods used [2,4]. To produce a homogenous *Gelidium elegans* composition, we established a standard procedure for the preparation of GE. As shown in Figure 1, *Gelidium elegans* was washed three to six times to completely remove the sea salt. *Gelidium elegans* was extracted with 70 % ethanol. After this process, GE was extracted with deionized water at 90 °C. Both the ethanol and deionized water extracts were mixed together and filtered with 50 μm size filter. The spray drying method was used to yield the total GE concentrate. GE was stored at 25 °C until use.

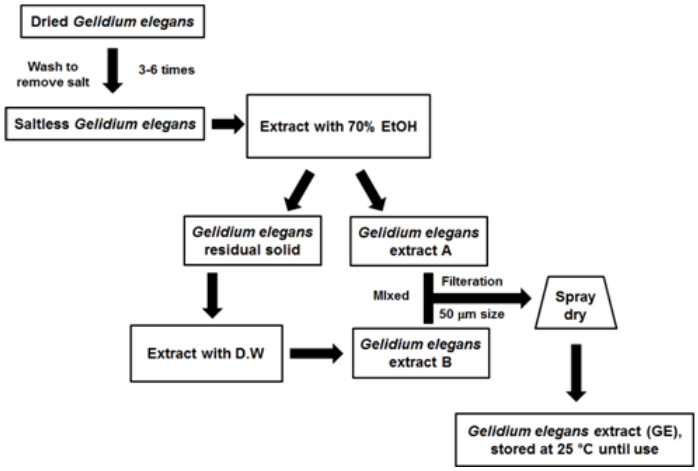

Figure 1. Preparation procedure of *Gelidium elegans* extract

The composition analysis showed that GE is made up of 47.6% carbohydrate, 16.9 % protein, 5.1 % moisture, and 24.1 % crude ash as shown in The total polyphenol was approximately 8.79 mg in 1 g of GE.

Table 1. The composition analysis of *Gelidium elegans* extract

Compositions	*Gelidium elegans* extract.
Carbohydrate	47.6 %
Crude protein	16.9 %
Moisture	5.1 %
Crude ash	24.1 %
Total polyphenols	8.79 mg per 1 g

3.2. Evaluation of *Gelidium elegans* Extract on Cell Viability in 3T3-L1 Cells

Hesperidin (HP) is known to be one of the major bioactive compounds in *Gelidium elegans* [33]. Thus, HP was used as a positive control to compare against GE in this study. First, we performed the cell viability assay in 3T3-L1 cells with 0, 5, 10, 20, and 40 μM of HP by using the XTT assay as described in the Materials and Methods. We also performed the XTT assay to analyze the viability of 3T3-L1 cells treated with GE for 24 h. As shown in Figure 2A and 2B, 40 μM of HP was found to be toxic to 3T3-L1 cells. In addition, treatment of GE with a 200 μg/mL concentration significantly decreased the cell viability. As one of the major bioactive compounds of GE, HE has been shown to contain approximately 88 ng/1 μg in GE [33].

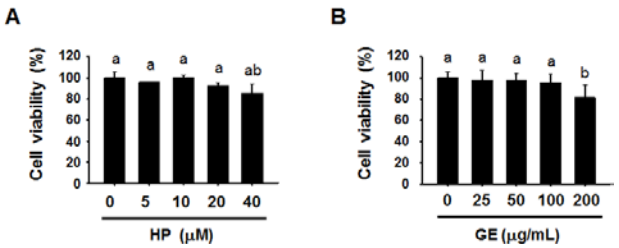

Figure 2. Evaluation of *Gelidium elegans* extract on cell viability in 3T3-L1 cells. 3T3-L1 pre-adipocytes ($1x10^4$ cells/well) were seeded on 96-well plates and maintained with (A) hesperidin (HP) or (B) *Gelidium elegans* extract (GE) for 24 h. Cell viability was determined by the XTT assay. These data were measured as the standard deviation of hexaplicates. Values with different letters are significantly different, $p<0.05$

This report indicates that 100 μg/mL GE included approximately 8.8 μg/mL of HP, which is approximately 29 μM of the HP concentration. To avoid such an HP-dependent effect, we selected the concentrations of 12.5 and 25 μg/mL of GE for further investigation.

3.3. *Gelidium elegans* Extract Suppresses Fat Accumulation and Its Key Regulatory Factors in 3T3-L1 Cells

Previous studies from our lab have shown that GE suppresses adipogenic transcription factors and subsequently decreased adipogenic differentiation markers in 3T3-L1 cells [12,19]. To evaluate the effect of GE on fat accumulation

in adipocytes, lipid accumulation was visualized by Oil Red O staining, as shown in Figure 3A. The HP (20 μM) treated 3T3-L1 cells had inhibited lipid accumulation. The concentrations of 12.5 and 25 μg/mL of GE more markedly abrogated adipocyte differentiation in a dose-dependent manner compared with HP (20 μM). These results indicated that polyphenol-enriched GE efficiently inhibits adipogenesis compared with a high concentration of single polyphenol treatment in 3T3-L1 cells.

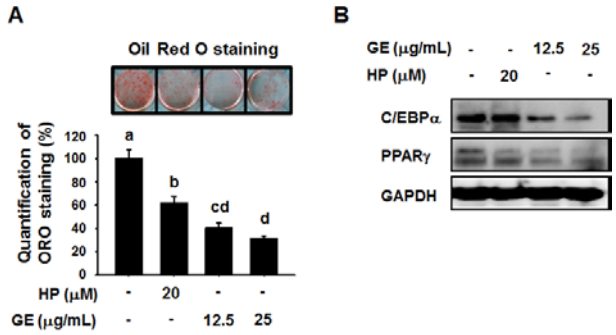

Figure 3. Effect of *Gelidium elegans* extract on fat accumulation and the expression of key adipogenic factors in 3T3-L1 cells. (A) Oil Red O staining was used to assess adipocyte differentiation at 8 days in the presence or absence of *Gelidium elegans* extract (GE) or hesperidin (HP). These data were measured as the standard deviation of quadruplicates. Values with different letters are significantly different, $p<0.05$. (B) Total protein was assessed after 8 days, and each parameter was measured using western blot analysis with specific antibodies. The protein expression levels were normalized against GAPDH. A representative western blot of C/EBPα and PPARγ in 3T3-L1 cells

Next, we analyzed the western blot results to determine the protein expression of adipocyte differentiation constituents, including C/EBPα and PPARγ in 3T3-L1 cells. As shown in Figure 3B, 12.5 and 25 μg/mL of GE dramatically inhibited the expression of adipogenic differentiation factors, C/EBPα and PPARγ. HP (20 μM) also decreased the expression of the PPARγ protein in 3T3-L1 cells, but not in C/EBPα. These results indicated that GE attenuated lipid accumulation by regulating the expression of C/EBPα and PPARγ and inhibited the progression of the terminal differentiation of 3T3-L1 cells.

3.4. Effect of *Gelidium elegans* Extract on Intracellular Triglyceride Content and the Expression of Triglyceride Synthesis Enzymes in 3T3-L1 Cells

Both C/EBPα and PPARγ stimulate the expression of SREBP-1, which is an essential transcription factor for cholesterol metabolism and triglyceride synthesis [20,27]. It has been suggested that the accumulation of intracellular triglycerides is associated with obesity [9,14].

Therefore, we examined whether GE affected the triglyceride content and the biosynthesis of triglyceride-associated enzymes. We observed that 12.5 and 25 μg/mL of GE treatment significantly inhibited triglyceride accumulation compared with the absence of GE or HP (20 μM), as shown in Figure 4A. Consistently, the biosynthesis of triglyceride-associated enzymes, including SREBP-1, was inhibited in 3T3-L1 cells with the presence of GE in a dose-dependent manner, as shown in Figure 4B. Moreover, SREBP-1 downstream triglyceride biosynthetic enzymes, including LPAATθ and DGAT, were inhibited in 3T3-L1 cells treated with GE compared with the GE non-treated group.

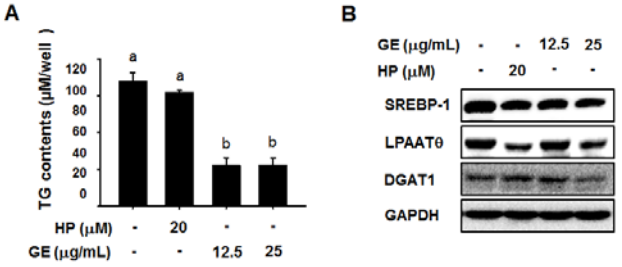

Figure 4. *Gelidium elegans* (GE) discourages increased triglyceride content via inhibition of triglyceride synthesis enzymes in 3T3-L1 cells. Fully confluent pre-adipocytes were induced by MDI to differentiate into mature adipocytes in the presence or absence of *Gelidium elegans* extract (GE) or hesperidin (HP) for 8 days. Total protein was harvested after 8 days, and the triglyceride (TG) content was measured using a triglyceride content assay kit, as described in the Materials and Methods, and with western blot analysis with specific antibodies. (A) The level of TG in 3T3-L1 cells. These data were measured as the standard deviation of quadruplicates. Values with different letters are significantly different, $p<0.05$. (B) The protein expression levels were normalized against GAPDH. Representative western blot of triglyceride synthesis enzymes including SREBP-1, LPAATθ, and DGAT1 in 3T3-L1 cells

These results showed that GE inhibited adipocyte differentiation by attenuating the expression of key adipogenesis factors and the activity of their triglyceride synthesis enzymes. In addition, the aforementioned evidence possibly suggests that GE may stimulate the lipolysis pathway and/or suppress mitotic clonal expansion, which is an essential step to progress adipogenic differentiation in 3T3-L1 cells.

3.5. *Gelidium elegans* Extract Stimulates Lipolysis and Blocks Terminal Differentiation in 3T3-L1 Cells

To gain insight into the mechanism of GE on adipogenesis, we examined whether GE affects adipose triglyceride lipase (ATGL), which is one of the major lipolysis pathway-involved enzymes. As demonstrated in Figure 5A, GE strongly increased the protein activity of ATGL in a dose-dependent manner compared with the absence of GE. Here, we found that GE stimulated the lipolysis pathway by activating ATGL.

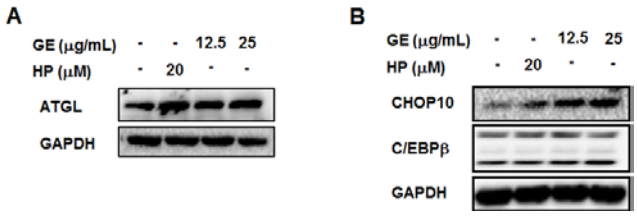

Figure 5. *Gelidium elegans* extract stimulates lipolysis and blocks terminal differentiation via activation of CHOP10 in 3T3-L1 cells. Fully confluent pre-adipocytes were induced by MDI to differentiate into mature adipocytes in the presence or absence of *Gelidium elegans* extract (GE) or hesperidin (HP) for 8 days. Total protein was harvested after 8 days, and each parameter was measured via western blot analysis with specific antibodies. The protein expression levels were normalized against GAPDH. (A) Representative western blot of adipose triglyceride lipase (ATGL) in 3T3-L1 cells. (B) Representative western blot of CHOP10 and C/EBPβ in 3T3-L1 cells

Based on the aforementioned evidence, we hypothesized that GE represses ongoing terminal differentiation by modulating mitotic clonal expansion-associated proteins. Thus, we next evaluated CHOP10 and C/EBPβ, which are displayed in pre-adipocytes until the 3T3-L1 cells enter mitotic clonal expansion [28]. As shown in Figure 5B, western blot analysis demonstrated that HP (20 μM) induced an increase in CHOP10 expression, but not C/EBPβ, compared with the absence of HP or GE. In contrast, 12.5 and 25 μg/mL GE markedly stimulated the expression of the CHOP10 protein and subsequently decreased its downstream target C/EBPβ compared with the absence of GE.

3.6. *Gelidium elegans* Extract Stimulates the Change to a Brown Adipocyte-like Phenotype Change in 3T3-L1 Cells

We also investigated the expression of Lipin1 which plays a crucial role in early stage adipocyte differentiation [26,34]. A Lipin1 knockout produced lipodystrophy, whereas a gain of Lipin1 function in mice promotes an obesity-associated phenomenon and insulin resistance.

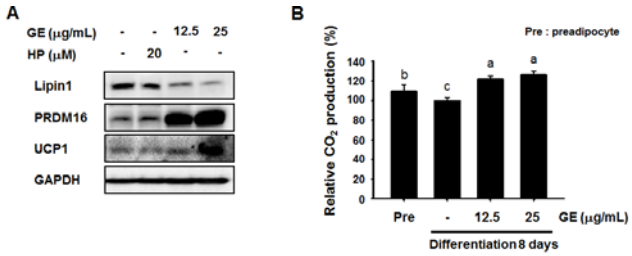

Figure 6. *Gelidium elegans* extract stimulates a beige cell-like phenotype and increases the production of carbon dioxide in 3T3-L1 cells. Fully confluent pre-adipocytes were induced by MDI to differentiate into mature adipocytes in the presence or absence of *Gelidium elegans* extract (GE) or hesperidin (HP) for 8 days. Total protein was harvested after 8 days, and each parameter was measured using western blot analysis with specific antibodies. The protein expression levels were normalized against GAPDH. (A) Representative western blot of Lipin1, PRDM16, and UCP1 in 3T3-L1 cells. (B) The production of carbon dioxide (CO2) was used to assess adipocyte energy expenditure at 8 days in the presence or absence of GE. These data were measured as the standard deviation of triplicates. Values with different letters are significantly different, $p<0.05$

As shown in Figure 6A, GE inhibited the expression of Lipin1 in 3T3-L1 cells compared with the absence of GE. Recently, the knockout of Lipin1 was shown to promote altered adipocyte characteristics, such as beige cell or brown adipocyte development [25]. This result indicates that GE possibly increases beige cell development-associated proteins such as PRDM16 and UCP1.

To examine the effect of GE on the trans-differentiation from pre-adipocyte to beige-like cells, we performed western blot analysis. PRDM16 activates brown fat phenotype-associated genes, including PGC1α and UCP1 [30]. As shown in Figure 6A, GE significantly activated the expression of PRDM16 and its downstream target UCP1 in 3T3-L1 cells.

Beige cells and brown adipocytes are a major site of thermogenesis and produce an apparent increase in the production of carbon dioxide [21]. Thus, we measured the levels of carbon dioxide from the cultured media in 3T3-L1 cells with or without GE. As shown in Figure 6B, the production of carbon dioxide was significantly increased in GE-treated 3T3-L1 cells compared to 3T3-L1 cells in both the pre-adipocyte and fully differentiated adipocyte states. These results indicated that GE also enhances the change to a beige-like phenotype in 3T3-L1 cells.

4. Conclusions

In conclusion, we show that GE suppresses adipocyte differentiation by regulating mitotic clonal expansion constituents and subsequently repressing terminal differentiation markers. ATGL was significantly increased in 3T3-L1 cells in the presence of GE. Moreover, the data from this study add to the striking evidence that GE up-regulates UCP1 and PRDM16 protein expression, leading to measurable changes in the beige-like phenotype differentiation of 3T3-L1 cells.

Although further molecular mechanistic studies will be required to clarify the function of GE on the beige-like adipocyte changes in 3T3-L1 cells, we provide partial evidence that seaweed extract and phytochemicals potentially stimulate the beige-like phenotype in 3T3-L1 adipocytes.

In addition, GE shows a better inhibitory ability against adipocyte differentiation and the stimulation of beige-like phenotype development compared with HP in 3T3-L1 cells. Our results showed that a more varied polyphenol combination has more beneficial effects that improve the physiological condition compared to a single high concentration of bioactive compound extracted from *Gelidium elegans* in 3T3-L1 adipocytes. Together, we suggest that GE has potential as a functional food ingredient extracted from *Gelidium elegans* to increase energy expenditure and anti-obesity efficacy.

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