Saururus chinensis (Lour.) Baill. extract promotes skeletal muscle cell differentiation by positively regulating mitochondrial biogenesis and AKT/mTOR signaling *in vitro*

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Abstract. Promotion of myoblast differentiation by activating mitochondrial biogenesis and protein synthesis signaling pathways provides a potential alternative strategy to balance energy and overcome muscle loss and muscle disorders. *Saururus chinensis* (Lour.) Baill. extract (SCE) has been used extensively as a traditional herbal medicine and has several physiological activities, including anti-asthmatic, anti-oxidant, anti-inflammatory, anti-atopic, anticancer and hepatoprotective properties. However, the effects and mechanisms of action of SCE on muscle differentiation have not yet been clarified. In the present study, it was investigated whether SCE affects skeletal muscle cell differentiation through the regulation of mitochondrial biogenesis and protein synthesis in murine C2C12 myoblasts. The XTT colorimetric assay was used to determine cell viability, and myosin heavy chain (MyHC) levels were determined using immunocytochemistry. SCE was applied to C2C12 myotube at different concentrations (1, 5, or 10 ng/ml) and times (1,3, or 5 days). Reverse transcription-quantitative PCR and western blotting were used to analyze the mRNA and protein expression change of factors related to differentiation, mitochondrial biogenesis and protein synthesis. Treatment of C2C12 cells with SCE at 1,5, and 10 ng/ml did not affect cell viability. SCE promoted C2C12 myotube formation and significantly increased MyHC expression in a concentration- and time-dependent manner. SCE significantly increased the mRNA and protein expression of muscle differentiation-specific markers, such as MyHC, myogenic differentiation 1, myogenin, Myogenic Factor 5, and β-catenin, mitochondrial biosynthesis-related factors, such as peroxisome proliferator-activated receptor-gamma coactivator-1α, nuclear respirator factor-1, AMP-activated protein kinase phosphorylation, and histone deacetylase 5 and AKT/mTOR signaling factors related to protein synthesis. SCE may prevent skeletal muscle dysfunction by enhancing myoblast differentiation through the promotion of mitochondrial biogenesis and protein synthesis.

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Introduction

Maintaining of skeletal muscle functional homeostasis is an important factor in the quality of life, including posture, movement and breathing, in addition to regulating glucose and protein metabolism and heat production (1,2). In addition, skeletal muscle loss can lead to adverse health outcomes including disability, weakness, fatigue, insulin resistance and mortality (3-5). Therefore, preventing muscle loss and strengthening the muscles have important implications for athletes and individuals who want to maintain a healthy body. Various factors, including acute or chronic disease, biochemical changes due to aging, poor nutrition, or lack of activity can lead to muscle loss, which is difficult to control because it is multifactorial and genetically influenced (3,6). However, decreased protein synthesis, protein degradation, mitochondrial and satellite cell dysfunction, and increased inflammation are associated with muscle loss (7). Previously, research on the development of various functional foods and medicines for the strengthening muscle function and maintaining the balance between protein synthesis and degradation in muscles is being actively conducted. In addition, herbs and medicinal plants, whose various beneficial functions have been proven in ethnopharmacological studies,

have been safely used for a long time. Therefore, they have been the focus of development as natural medicines to prevent and treat muscle loss (8-10).

Saururus chinensis (Lour.) Baill. (SC) is a perennial herbaceous plant of the Saururaceae family, mainly distributed in wet and humid regions of East Asia and rarely found in lowland wetlands on Jeju Island, South Korea. In South Korea, SC is an Endangered Species designated by the Korea Forest Service and a Class 2 endangered wild animal or plant species by the Ministry of Environment (11,12). It has been widely used as traditional Chinese medicine for a wide range of disorders including edema, pneumonia, hypertension, leprosy, jaundice, gonorrhea, rheumatoid arthritis and inflammatory diseases (13-16). SC contains essential oil such as quercetin, quercitrin, isoquercitrin, rutin and tannin as main components, and contains various lignans (17,18), which have several pharmacological activities including anti-asthmatic (19), anti-oxidant (20), anti-inflammatory (21-23), anti-atopic (24), anticancer (25) and hepatoprotective (26) properties. In addition, according to a previous study, sauchinone isolated from the roots of SC protects oxidative stress-induced C2C12 myoblast damage by regulating heat shock protein-70 level (27). However, the effects of SC extract (SCE) on muscle mass, function and metabolic mechanisms have not yet been elucidated.

Therefore, to evaluate how SCE may be beneficial to muscle function, the present study investigated the regulation of muscle differentiation, mitochondrial biogenic factor, energy metabolism and protein synthesis in C2C12 mouse skeletal muscle cells via activation of the PGC-1α, AMP-activated protein kinase (AMPK) and the AKT/mTOR/p70S6K signaling pathway post-SCE treatment.

Materials and methods

Chemical, reagents and antibodies. SCE (cat. no. KPM046-069) was purchased from the Korea Plant Extract Bank of the Korea Research Institute of Bioscience and Biotechnology (Cheongju, Korea). The maximum concentration of DMSO in the cell culture medium was 0.1% (v/v). TRIzol® reagent was purchased from Thermo Fisher Scientific, Inc. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were obtained from Gibco; Thermo Fisher Scientific, Inc. Specific antibodies against myosin heavy chain (MyHC; 1:500; cat. no. sc-376157), myogenic differentiation 1 (MyoD; 1:1,000; cat. no. sc-377460), myogenin and peroxisome proliferator-activated receptor-gamma coactivator-1 α (PGC-1α; 1:1,000; cat. no. sc-518038) were obtained from Santa Cruz Biotechnology, Inc. Antibodies against non-phospho (active) β-catenin (1:1,000; cat. no. 8814), β-catenin (1:1,000; cat. no. 9582), phospho-AMPK (1:1,000; cat. no. 2535), AMPK (1:1,000; cat. no. 2532), phospho-AKT (1:1,000; cat. no. 9271), AKT (1:1,000; cat. no. 9272), phospho-mTOR (1:1,000; cat. no. 2971), mTOR (1:1,000; cat. no. 2983), phospho-ribosomal protein S6 kinase B1(p70S6K1) (1:1,000; cat. no. 9234), p70S6K1 (1:1,000; cat. no. 2708) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1,000; cat. no. 2118) were purchased from Cell Signaling Technology, Inc. Specific antibody against phospho-histone deacetylase 5 (HDAC5; 1:1,000; cat. no. ab47283) was obtained from Abcam. Secondary antibodies, including horseradish peroxidase-conjugated anti-mouse (1:3,000; cat. no. ADI-SAB-100-J) and anti-rabbit IgG (1:3,000; cat. no. ADI-SAB-300-J), were obtained from Enzo Life Sciences, Inc.

Cell culture. Murine C2C12 skeletal muscle cell line (cat. no. CRL-1772) were purchased from American Type Culture Collection. Cells were cultured in growth medium (GM; DMEM containing 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin) in a humidified incubator at 37˚C with 5% CO_2 until they reached 70% confluence. To induce differentiation, nearly confluent C2C12 cells were incubated in DMEM containing 2% heat-inactivated horse serum (differentiation medium; DM) for varying lengths of time as previously described (27).

Induction of myogenic differentiation and observation of morphologic changes. C2C12 myoblasts were seeded in 6-well plates at a density of $5x10^4$ cells/well. For the myogenic differentiation, GM was replaced with DM when the cells reached 80% confluence. Then, the cells were treated with or without SCE at final concentrations of 0,1,5, or 10 ng/ml for 5 days. Fresh SCE was added to the DM every 2 days. The morphology of myotubes was observed using a phase-contrast microscope (Nikon TS2; Nikon Instruments Inc.). Images were captured at x50 magnification.

Measurement of cell viability. To determine cytotoxicity, C2C12 myoblasts were seeded in 96-well plates ($1x10^3$ cells/well) and incubated in the culture medium until they reached 70-80% confluence as previously described (28). The medium was then changed to a DM, and the cells were treated with or without SCE (0,1,5, or 10 ng/ml). Following incubation for 5 days, after adding XTT solution (50 μl) to each well and incubating for 4 h at 37˚C, cell viability was determined by absorbance at 450 nm using a multi-detection microplate reader (Molecular Devices, LLC).

Immunofluorescence staining and determination of the diameter. C2C12 myoblasts cultured in 48-well plates ($3x10^4$ cells/well) were fixed using 3.7% paraformaldehyde [in phosphate-buffered saline (PBS)], permeabilized in 0.1% Triton X-100 for 15 min and blocked in 5% bovine serum albumin (BSA; MiliporeSigma) for 3 h at room temperature as previously described (27). After the cells were blocked in 5% BSA, they were incubated with the primary antibody at 4˚C for 24 h. Mouse anti-MyHC antibody was used at a 1:200 dilution. MyHC was detected by incubating the cells with anti-mouse secondary antibody Alexa Fluor 488-conjugated (1:200; cat. no. A32723; Thermo Fisher Scientific, Inc.) at 4˚C for 24 h, and then 4'-6-diamidino-2-phenylindol (DAPI; 1 mg/ml) was used to label the nuclei. Cells were observed using fluorescence Leica DM IRE2 microscope (Leica Microsystems GmbH) and Nikon Eclipse 50I microscope (Nikon Instruments Inc.) and images of myotubes were captured using IM50 software (Leica Microsystems GmbH) and Nis-Elements D 4.00 software (Nikon Instruments Inc.), respectively, for size comparison. For myotube diameter, the average measurement on each slide was calculated from ~25 myotubes; three fields were randomly selected, and all MyHC-positive multinucleated cells containing at least three nuclei in each field were

quantified. The data were then converted to a percentage increase compared with the control (DM).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from C2C12 cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions and cDNA was synthesized, followed by qPCR using SYBR® Green Premix (Bioneer Corp.) with specific primers as previously described (29). qPCR primers were designed using Primer3 software (Whitehead Institute for Biomedical Research). The amplification conditions were 95˚C for 5 min, followed by 40 cycles of 95˚C for 1 min, 60˚C for 30 sec, and 72˚C for 1 min. Using *GAPDH* as an internal control, the relative gene expression was determined using the quantification cycle (C_q) value (30). The primer sequences were as follows: *GAPDH* forward, 5'-TCAAGAAGGTGGTGAAGCAG-3' and reverse, 5'-AGTGGGAGTTGCTGTTGAAGT-3'; MyHC forward, 5'-GCCCAGTGGAGGACAAAATA-3' and reverse, 5'-TCTACGTGCTCCTCAGCAT-3'; *MyoD* forward, 5'-CGCTCCAACTGCTCTGATG-3' and reverse, 5'-TAGTAGGCGGTGTCGTAGCC-3'; Myogenin forward, 5'-CTACAGGCCTTGCTCAGCTC-3' and reverse, 5'-AGATTGTGGGCGTCTGTAGG-3'; Myogenic Factor 5 (Myf5*)* forward, 5'-AGGAAAAGAAGCCCTGAAGC-3' and reverse, 5'-GCAAAAAGAACAGGCAGAGG-3'; PGC-1α forward, 5'-CACCAAACCCACAGAAAACAG-3' and reverse, 5'-GGGTCAGAGGAAGAGATAAGTTG-3'; and nuclear respiratory factor (NRF)-1 forward, 5'-AGGGCGGTGAAATGACCATC-3' and reverse, 5'CGGCAGCTTCACTGTTGAGG-3'.

Western blotting. C2C12 myoblasts were washed in a culture dish with cold phosphate-buffered saline (PBS) and lysed in cold lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, 1% deoxycholate and protease inhibitors as previously described (29). Samples were incubated in ice for 30 min with lysis buffer and cell debris were separated by centrifugation at 10,000 x g for 15 min at 4˚C. Protein concentrations were determined using the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories Inc.). Total protein extracts (20-30 μg) were then separated using 10% SDS-PAGE, transferred to PVDF membranes (Bio-Rad Laboratories, Inc.), blocked with 5% non-fat milk in TBST buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5 and 0.1% Tween-20) for 1 h at room temperature, and incubated with specific primary antibody overnight at 4˚C. The membranes were then washed thrice and incubated with secondary antibodies for 1 h at room temperature. The membranes were further washed three times and visualized using Immobilon Western Chemiluminescent HRP Substrate (MilliporeSigma). GAPDH was used as a loading control. The density of western blotting bands was quantified using ImageJ software (version 2; National Institutes of Health).

Statistical analysis. All experimental tests were performed at least three times (n=3 per group) and all quantitative data are presented as the mean ± standard deviation (SD). The data were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test using SPSS 14.0 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

SCE promotes myoblast differentiation. Before investigating the effects of SCE on myoblast differentiation, its cytotoxicity during myoblast differentiation was examined. No significant difference was observed in myoblast viability with the addition of up to 10 ng/ml SCE (Fig. 1A). Subsequently, the morphological changes in C2C12 cells according to SCE concentration during their differentiation were examined. During differentiation for 5 days, treatment with SCE led to the formation of larger and more elongated myotubes compared with untreated cells (Fig. 1B). MyHC immunofluorescence staining was performed to confirm the effect of SCE on myotube formation. As demonstrated in Fig. 2A, SCE treatment promoted C2C12 differentiation, as evaluated by the visualization of MyHC-positive myotubes. DAPI staining was performed to assess cell density and to determine myotube formation, as observed in myotubes containing three or more nuclei (Fig. 2A). On the 5th day of differentiation, the myotube diameter was significantly increased in the 1, 5 and 10 ng/ml SCE treatment groups compared with that in the control group (Fig. 2B).

SCE increases the expression of muscle-specific factors related to myogenesis. It was investigated whether SCE affects the expression of muscle-specific genes involved in myogenic differentiation. As revealed in Fig. 3A, RT-qPCR results revealed that SCE gradually increased the mRNA expression of MyHC, MyoD, myogenin and Myf5 in a dose-dependent manner. Protein expression of MyHC, MyoD and myogenin was also significantly increased compared with that in the control group, even after treatment with SCE 1 ng/ml for 5 days, as expected (Fig. 3B). To confirm changes in the expression of myogenesis factors during the myoblast differentiation period, C2C12 myoblasts were treated with 10 ng/ml SCE for 5 days. Myf5 mRNA level and the mRNA and protein levels of MyHC and myogenin peaked on day 5 compared with those in the control (Fig. 3C and D). Notably, MyoD mRNA levels in SCE-treated cells were significantly higher than those in control cells on days 1 and 3 (Fig. 3C). Furthermore, MyoD protein levels were significantly upregulated in SCE-treated cells from day 3 to 5 compared with the control (Fig. 3D). Therefore, activation of MyoD is essential for the early stages of myogenic determination. Next, the activity of β-catenin, a major protein that regulates myoblast proliferation and myotube formation, was confirmed according to dose and time via western blotting (Fig. 3E and F). The activity of β-catenin was significantly increased at 1, 5 and 10 ng/ml (Fig. 3E) and was activated compared with the control group on days 1 and 3 after treatment with 10 ng/ml SCE (Fig. 3F).

SCE upregulates the expression of mitochondria biogenesis factors. To demonstrate the effects of SCE on mitochondrial biogenesis during myogenic differentiation, the expression of the mitochondrial biogenesis transcription factors PGC-1α and NRF-1 at the mRNA and protein levels were measured using quantitative RT-qPCR and western blotting. Treatment of myotubes with 5 or 10 ng/ml SCE for 5 days increased the expression of PGC-1α mRNA (Fig. 4A) and protein (Fig. 4B) compared with the control. In addition, treatment with SCE

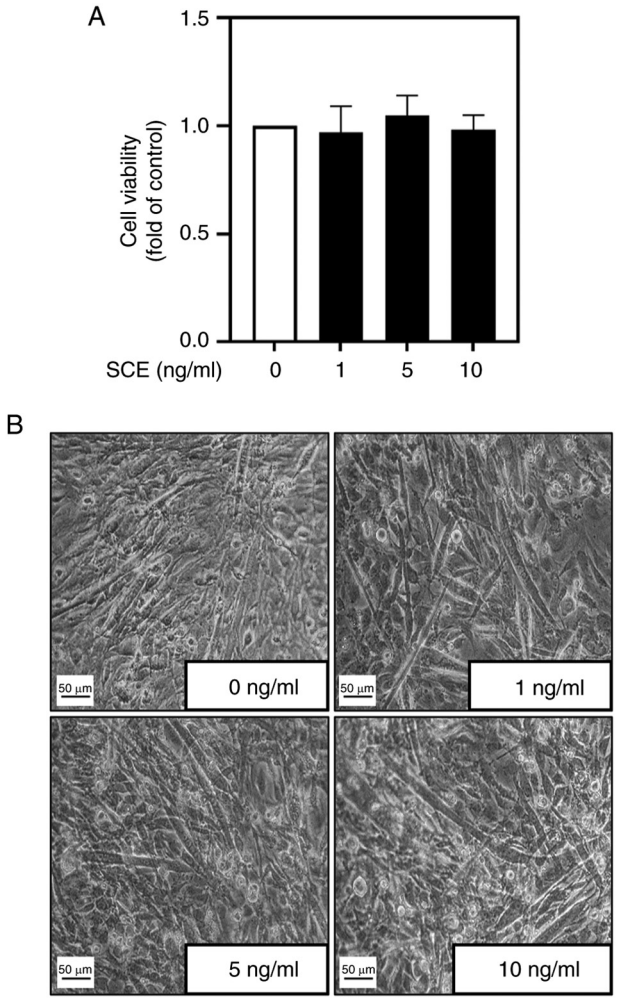

Figure 1. SCE changes cell morphology during myogenic differentiation. C2C12 myoblasts were cultured in growth medium until 80% confluence and then changed to DMEM containing 2% horse serum and 1, 5 and 10 ng/ml SCE for 5 days. The medium was changed every 2 days and fresh SCE was added. (A) Cell viability was assessed using the XTT assay (n=6 per group). (B) Images of morphological changes were obtained using light microscopy at the end of the experiment (5 days) (n=4 per group). Bar, 50 μm. SCE, *Saururus chinensis* (Lour.) Baill. Extract.

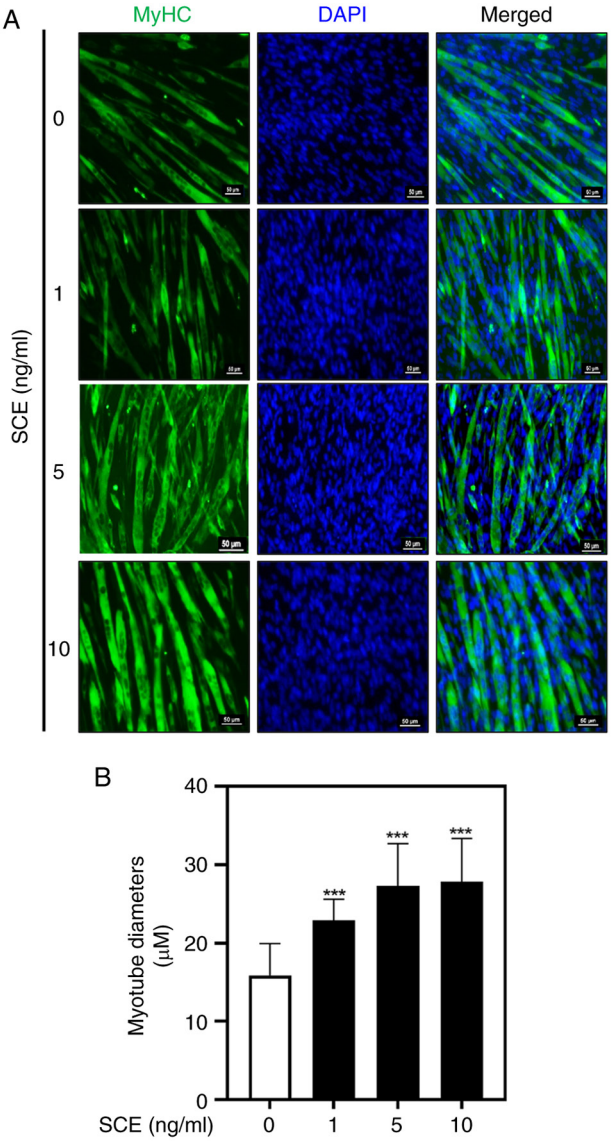

Figure 2. SCE promotes myotube formation by C2C12 cells. C2C12 myoblasts were cultured in a DMEM containing 2% horse serum and SCE (0,1, 5 and 10 ng/ml) for 5 days. (A) Immunofluorescence staining for the myotube marker, MyHC (green), and the nuclei marker, DAPI (blue), revealed myotube formation by C2C12 cells (n=4 per group). (B) Myotube diameters in randomly selected fields were quantified using an image analysis program. ***P<0.01 vs. the control group. Scale bar, 50 μm. SCE, *Saururus chinensis* (Lour.) Baill. Extract.

10 ng/ml significantly increased PGC-1α in a time-dependent manner during myoblast differentiation (Fig. 4C). As for the protein levels, the expression of PGC-1α significantly increased in a time-dependent manner (Fig. 4D), suggesting the involvement of PGC-1α in myogenic differentiation. Similar to PGC-1α, the expression of NRF-1 mRNA was also significantly increased when cells were treated with 5 or 10 ng/ml SCE for 5 days (Fig. 4E), and upregulated at 3 or 5 days when cells were treated with 10 ng/ml SCE during myogenesis (Fig. 4F).

SCE enhances the AMPK-HDAC5 pathways in myotubes. Next, the effects of SCE on the AMPK-HDAC5 signaling pathway that activates energy metabolism in myotubes were tested. Treatment with 1, 5 and 10 ng/ml SCE resulted in increased phosphorylation of AMPK in the myotubes (Fig. 5A). Treatment with 10 ng/ml SCE significantly increased the phosphorylation of AMPK compared with that in the control (Fig. 5B). Moreover, the phosphorylation of HDAC5 was increased at 1 ng/ml (Fig. 5C) and was significantly activated compared with the control group on day 1 (Fig. 5D). These results indicated that SCE increases energy metabolism associated with mitochondrial biogenesis in myotubes by activating the AMPK-HDAC5 signaling pathway.

SCE activates muscle protein synthesis-related biomarkers. To further prove the mechanism by which SCE promotes formation of C2C12 myotubes, muscle protein turnover-related biomarkers were evaluated. Western blotting was performed to confirm whether SCE enhanced the AKT/mTOR pathway, an essential regulator of protein synthesis and degradation. As demonstrated in Fig. 6A, the phosphorylation of AKT, mTOR and p70S6K was increased in a dose-dependent manner following treatment with SCE. In addition, SCE significantly increased AKT and

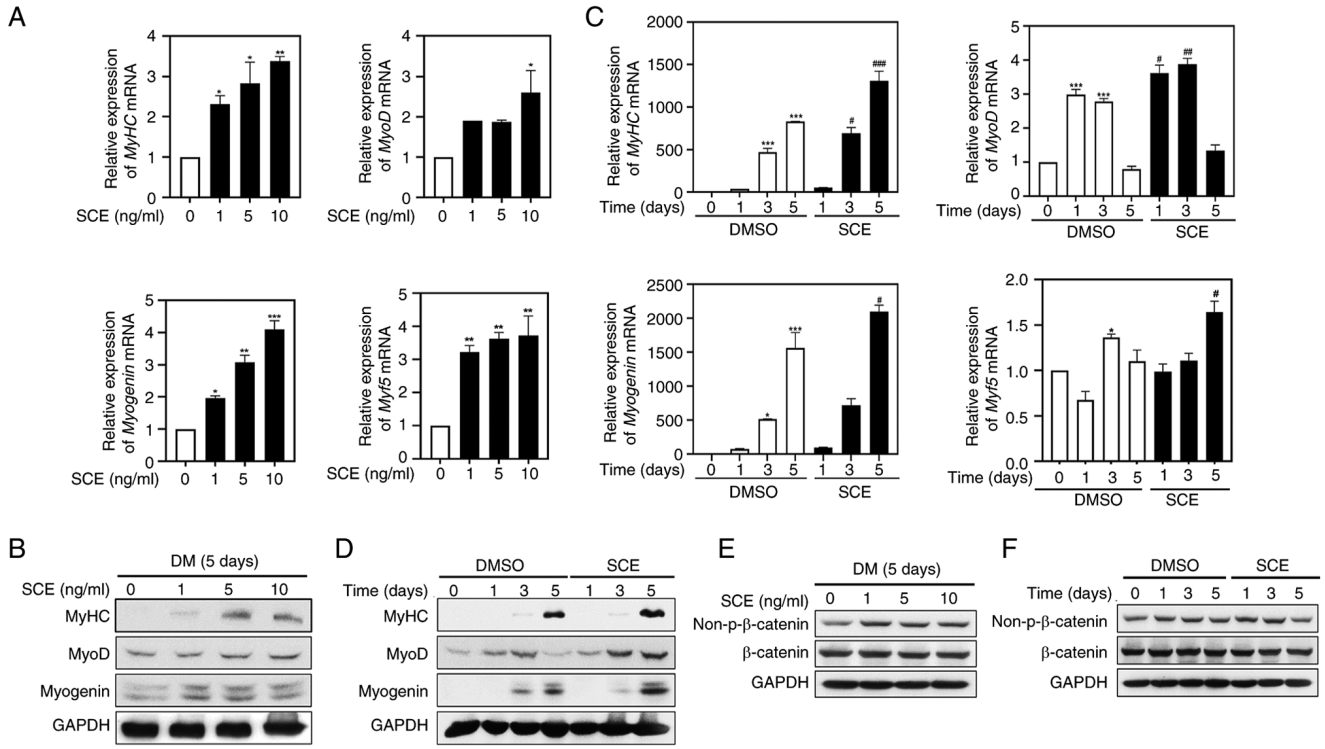

Figure 3. SCE increases the expression of muscle-specific factors related to myogenesis. C2C12 myoblasts were induced to differentiate in DMEM containing 2% horse serum, treated for 5 days with different concentrations of SCE (1, 5 and 10 ng/ml), and analyzed by (A) RT-qPCR (n=3 per group) and (B) western blotting (n=3 per group). The expression of MyHC, MyoD, myogenin and Myf5 over the time course of myogenesis was detected through (C) RT-qPCR (n=3 per group) and (D) western blotting (n=3 per group) in cells treated with SCE. *P<0.05, **P<0.01 and ***P<0.001 vs. the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the SCE-treated group at the corresponding indicated times. Non-β-catenin and β-catenin were detected by western blotting depending on (E) the concentration or (F) the time of SCE. SCE, *Saururus chinensis* (Lour.) Baill. extract; RT-qPCR, reverse transcription-quantitative PCR; MyHC, myosin heavy chain; MyoD, myogenic differentiation 1; Myf5, Myogenic Factor 5.

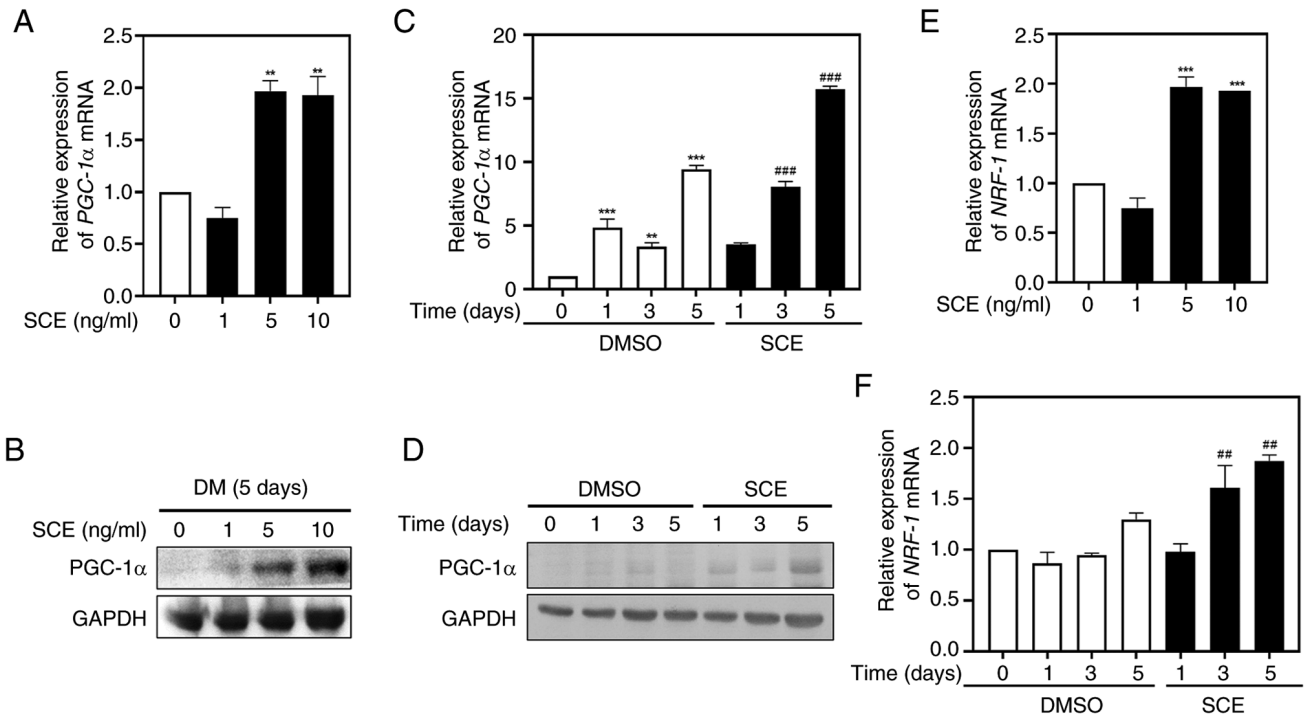

Figure 4. SCE upregulates the expression of biogenesis-regulating factors during C2C12 differentiation. C2C12 myoblasts were induced to differentiate in a DMEM containing 2% horse serum, treated for 5 days with different concentrations of SCE (1, 5 and 10 ng/ml) and analyzed using (A and E) RT-qPCR (n=3 per group) and (B) western blotting (n=3 per group). Cells were treated with 10 ng/ml SCE in DM for 1, 3 and 5 days and subjected to (C and F) RT-qPCR (n=3 per group) and (D) western blotting (n=3 per group). GAPDH was used as a loading control. **P<0.01 and ***P<0.001 vs. the control group; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the SCE-treated group at the corresponding indicated times. SCE, *Saururus chinensis* (Lour.) Baill. extract; RT-qPCR, reverse transcription-quantitative PCR; PGC-1a, peroxisome proliferator-activated receptor-gamma coactivator-1 α; NRF1, nuclear respiratory factor 1.

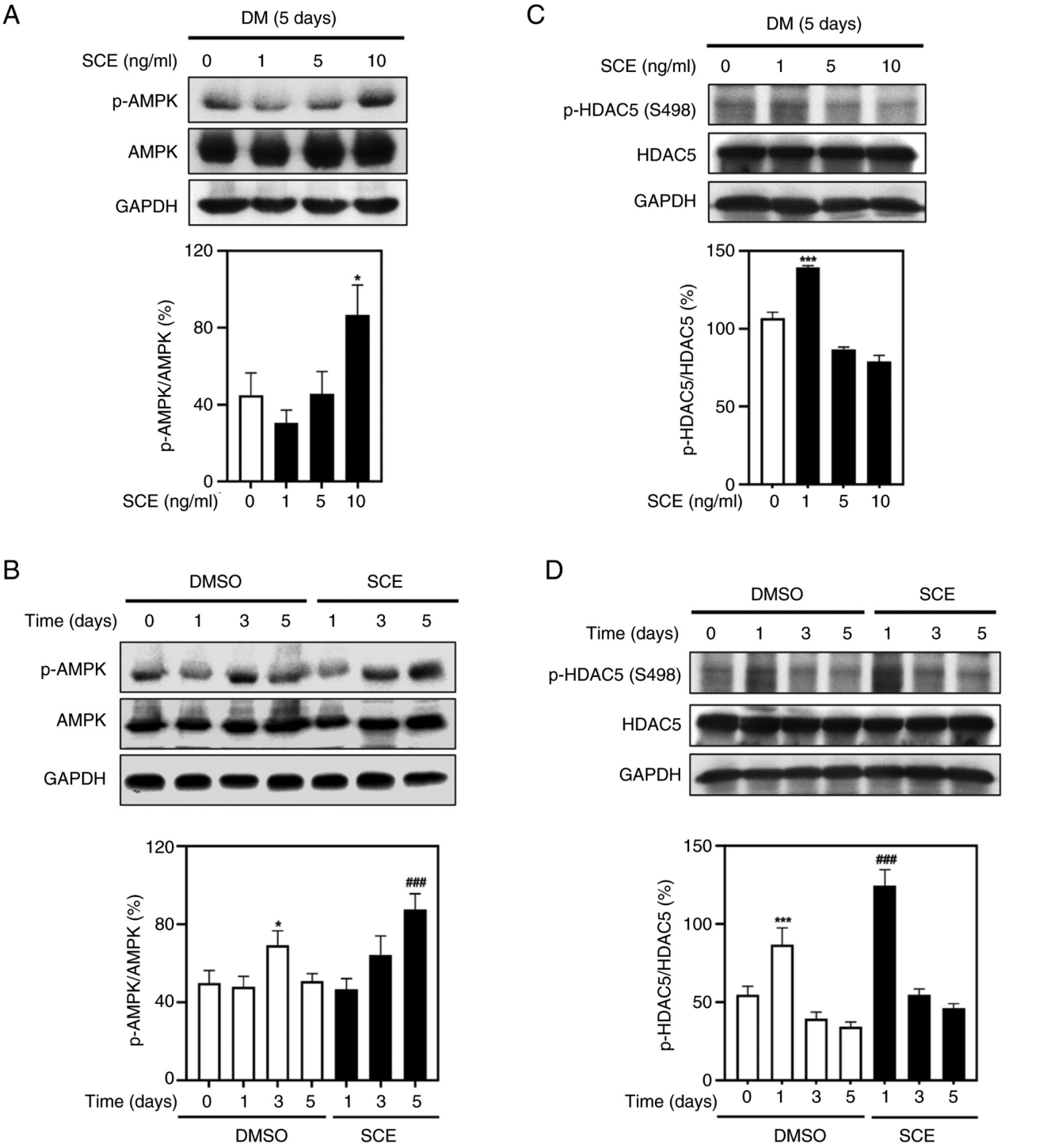

Figure 5. SCE activates the AMPK-HDAC5 pathways in myotubes. (A and C) C2C12 myoblasts were induced to differentiate in a DMEM containing 2% horse serum, treated for 5 days with different concentrations of SCE (1, 5, 10 ng/ml) and analyzed by western blotting (upper panel). Quantification of protein expression levels (n=3 per group) (lower panel). *P<0.05 and ***P<0.01 vs. the control. (B and D) Cells were treated with 10 ng/ml SCE in DM for 1, 3 and 5 days and western blotting was performed using the indicated antibodies (upper panel). Quantification of protein expression levels (n=3 per group) (down). *P<0.05 and ***P<0.01 vs. the DMSO; $^{\#\#\#}$P<0.001 vs. the DMSO at the indicated time points. SCE, *Saururus chinensis* (Lour.) Baill. extract; AMPK, AMP-activated protein kinase; HDAC5, histone deacetylase 5; p-, phosphorylated.

mTOR phosphorylation during myogenesis. Specifically, on day 5 of treatment, SCE increased the phosphorylation of AKT and mTOR and subsequently activated p70S6K1, a key downstream target of the AKT/mTOR signaling cascade (Fig. 6B). These data revealed that SCE enhances differentiation of C2C12 cells by regulating the AKT/mTOR signaling pathway, which is important for protein synthesis.

Discussion

Herbal medicines used as dietary supplements have numerous useful effects and have long been known to improve health, stamina and abnormalities in the elderly (31). SCE is widely used for medicinal purposes owing to various physiological activities including anti-oxidant, anticancer, anti-aging and

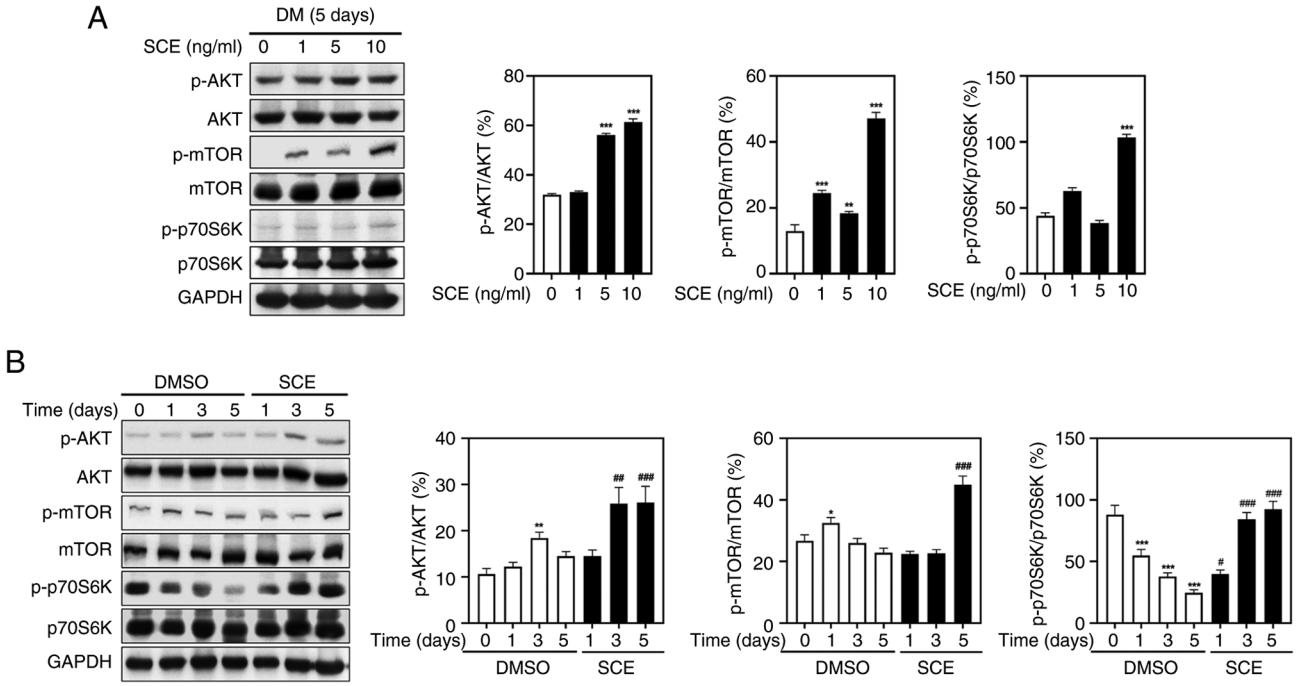

Figure 6. SCE regulates AKT/mTOR and its downstream effectors in myotubes. (A) C2C12 myoblasts were induced to differentiate in a DMEM containing 2% horse serum, treated for 5 days with different concentration of SCE (1, 5 and 10 ng/ml) and analyzed using western blotting (left). Quantification of protein expression levels (n=3 per group) (right). **P<0.01 and ***P<0.001 vs. the control. (B) Cells were treated with 10 ng/ml SCE in DM for 1, 3 and 5 days and western blotting was performed using the indicated antibodies (left). Quantification of protein expression levels (n=3 per group) (right). *P<0.05, **P<0.01 and ***P<0.001 vs. the DMSO; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the DMSO at the indicated time points. SCE, *Saururus chinensis* (Lour.) Baill. extract; p-, phosphorylated; p70S6K1, ribosomal protein S6 kinase B1.

anti-inflammatory (13-26). In traditional medicine, SCE is used for the prevention and treatment of cardiovascular diseases, hypertension and angina because it clears the blood and the walls of blood vessels, along with toxin discharge and diuretic action (16,32). In addition, SCE protects against oxidative stress-induced myoblast damage by downregulating ceramide (27). However, the effects and mechanisms of action of SCE on skeletal muscle cell differentiation and function have not yet been investigated. In the present study, for the first time SCE was investigated for its potential ability to improve myotube muscle function. SCE promoted the differentiation of myoblasts into myotubes by increasing MyHC, MyoD, myogenin, Myf5 and β-catenin levels. These effects are due to an increase in mitochondrial biogenesis by upregulating the mitochondrial transcription factors PGC-1α and NRF-1 through activation of the AMPK and AKT/mTOR signaling pathways and promotion of protein synthesis.

In order to inhibit muscle wasting, it is necessary to either stimulate muscle-building pathways or inhibit the signaling pathways responsible for muscle wasting in the regulation of muscle metabolism. Satellite cells, such as C2C12 myoblasts, differentiate into multinucleated fibers and myotubes through myoblast fusion (33). Muscle tissue contains satellite cells, which are skeletal muscle-derived stem cells, and myogenic differentiation begins when satellite cells are activated (33). During differentiation, satellite cells and myoblasts are orchestrated by various myogenic regulatory factors (MRFs), including MyoD, Myf5 and myogenin, and structural muscle proteins, including MyHC (33,34). Mature myotubes express structural muscle proteins such as MyHC, which are motor proteins and specific maturation marker proteins of thick muscle filaments (33,34). Furthermore, β-catenin acts as a molecular switch that regulates the transition from cell proliferation to myogenic differentiation (35). In the present study, SCE treatment significantly increased the mRNA and/or protein levels of MyHC, MyoD, myogenin, Myf5 and β-catenin in C2C12 myotubes (Fig. 3), suggesting that SCE promotes myoblast differentiation. However, further experiments on MRFs and the regulation of their signaling are needed to improve elucidation of the impact of SCE on myogenesis and metabolism.

PGC-1α is a key transcriptional regulator of several genes involved in various physiological responses related to energy homeostasis, thermoregulation, lactate and fatty acid metabolism, muscle growth and mitochondrial biogenesis (36). In skeletal muscle, PGC-1α increases energy expenditure by increasing the rates of respiration and mitochondrial biogenesis and interacts with NRF-1, a transcription factor regulating the expression of several mitochondrial genes (36,37). AMPK acts as an energy switch, which is a key energy sensor controlling metabolic homeostasis at cellular and systemic levels, regulating processes such as cell growth, lipid-glucose metabolism and autophagy (38,39). Specifically, in skeletal muscles, mitochondrial biogenesis provides cells with ATP, which ultimately promotes AMPK activation (38,39). AMPK inhibition downregulates myogenin transcription and myogenesis, mainly through phosphorylation of HDAC5 mediated by AMPKα1, meaning that AMPK is a key molecular target to promote myogenesis and muscle regeneration (40). In the present study, SCE treatment of C2C12 myotubes increased

the activation of PGC-1α, NRF-1, AMPK and HDAC5 in a time- and concentration-dependent manner (Figs. 4 and 5), indicating that the promotion of myogenic differentiation by SCE is related to the enhancement of mitochondrial biogenesis. However, changes in mitochondrial biogenesis, caused by SCE treatment need to be further confirmed to improve understanding of the role of the drug in muscle energy metabolism.

AMPK is involved not only in mitochondrial biogenesis but also in the synthesis and wasting metabolism of skeletal muscle through the regulation of several downstream targets, such as the PI3K/AKT pathway. AMPK plays an important role in regulating skeletal muscle development and growth and regulating muscle mass and regeneration by influencing anabolic and catabolic cellular processes (41). AKT plays an important role as a promyogenic kinase, and AKT signaling is contributed to heterodimerization of MyoD/E-proteins and alteration in chromatin remodeling at muscle-specific loci (42,43). AKT/mTOR/p70S6K pathway is important for the differentiation of myoblasts and hypertrophy of myotubes (44). mTOR is a kinase downstream of AKT, which phosphorylates 4E-BP1 and p70S6K thereby inducing initiation of protein synthesis (45). In addition, AKT pathways prevent muscle atrophy by inhibiting atrophy-related ubiquitin ligases and FoxO transcriptional factors (45). In myostatin knockout mice, an increase in AKT/mTOR/p70S6K signaling was observed (46). SCE induced the phosphorylation of AKT, mTOR and p70 S6K in a concentration- and time-dependent manner (Fig. 6). These results indicated that SCE enhanced myogenic differentiation through the AKT/mTOR protein synthesis signaling pathway.

In conclusion, the present study is the first, to the best of the authors' knowledge, to reveal that SCE regulates muscle differentiation, mitochondrial biogenesis and protein synthesis. SCE significantly increased the expression of MyoD, myogenin and MyHC in myotubes, as well as the expression of PGC-1α and NRF-1, through the activation of AMPK and AKT/mTOR signaling pathways. These results suggested that SCE improves muscle function by enhancing myoblast differentiation and energy metabolism. However, further *in vitro*/*in vivo* studies on the mechanism of action of SCE in pathological conditions such as aging, energy and nutritional imbalance and muscle loss are needed to clearly understand the mechanisms by which SCE regulates muscle differentiation, mitochondrial biogenesis and protein synthesis. A limitation of the present study was that more specific experimental evidence is needed to support the role of the AMPK-HDAC5 and AKT/mTOR/p70S6K pathways, such as the use of inhibitors or activators of these pathways. In addition, it will be necessary to confirm the effectiveness of SCE in suppressing muscle loss and enhancing muscle strength through additional *in vivo* experiments.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JYK and MSL designed the study and revised the manuscript. SYE, CHC, YHC and GDP performed the experiments. SYE, CHC and CHL analyzed the data. SYE and CHC drafted figures. JYK and MSL wrote the manuscript. JYK and MSL confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All methods were performed in accordance with relevant international and national guidelines and regulations. Because the present study was an *in vitro* experiment that did not involve animals or humans, no animal or human ethics approval was required. SC is stored at the 'National Institute of Biological Resources' (Institution identification number: 50520), and the specimen was collected from farms on Jeju Island, not from its native habitat, grown for medicinal purposes. SC Methanolic (99%) whole-plant extract (KPM046-069) was obtained from the Korea Plant Extract Bank, Korea Research Institute of Bioscience and Biotechnology (Cheongju) for research purposes. Experimental studies were conducted according to the Natural Products Central Bank (NPCB)'s 'Standard operating procedure (2023 revised edition)' (https://www.kobis.re.kr/npcb/uss/notice/library.do). The NPCB does not collect or damage internationally endangered species designated and announced by the Minister of Environment in accordance with the Convention on International Trade in Endangered Species of Wild Plants (CITES) (http://www.cites.org) and the international Union for Conservation of Nature's (IUCN) standards (https://s3.amazonaws.com/iucnredlist-newcms/staging/public/attachments/3154/reg_guidelines_en.pdf), and complies with permit procedures for protected species.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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