

Schisandrae fructus enhances myogenic differentiation and inhibits atrophy through protein synthesis in human myotubes

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Abstract: *Schisandrae fructus* (SF) has recently been reported to increase skeletal muscle mass and inhibit atrophy in mice. We investigated the effect of SF extract on human myotube differentiation and its acting pathway. Various concentrations (0.1–10 µg/mL) of SF extract were applied on human skeletal muscle cells in vitro. Myotube area and fusion index were measured to quantify myotube differentiation. The maximum effect was observed at 0.5 µg/mL of SF extract, enhancing differentiation up to 1.4-fold in fusion index and 1.6-fold in myotube area at 8 days after induction of differentiation compared to control. Phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 and 70 kDa ribosomal protein S6 kinase, which initiate translation as downstream of mammalian target of rapamycin pathway, was upregulated in early phases of differentiation after SF treatment. SF also attenuated dexamethasone-induced atrophy. In conclusion, we show that SF augments myogenic differentiation and attenuates atrophy by increasing protein synthesis through mammalian target of rapamycin/70 kDa ribosomal protein S6 kinase and eukaryotic translation initiation factor 4E-binding protein 1 signaling pathway in human myotubes. SF can be a useful natural dietary supplement in increasing skeletal muscle mass, especially in the aged with sarcopenia and the patients with disuse atrophy.

Keywords: *Schisandrae fructus*, mTOR signaling, human skeletal muscle cells, myotubes

Introduction

Sarcopenia, a syndrome of progressive and generalized loss of skeletal muscle mass and strength, poses a huge health care burden in the elderly.^{1–5} It has raised a great interest in the search for dietary ingredients that can help increase and maintain skeletal muscle mass.^{6–8} The dried *Schisandrae fructus* (SF) has been traditionally used in herbal medicine as the therapy for asthma, night sweats, insomnia, dry coughs, urinary disorders, involuntary ejaculation, poor memory, hyperacidity, chronic diarrhea, hepatitis, diabetes, etc.^{9–12} Previous studies on SF extract have reported various biological activities as antioxidant, antiviral, antitumor, and anti-inflammatory agent.^{13–18} Recently, SF has been shown to increase skeletal muscle mass and ameliorate atrophy in the mouse models of sciatic neurectomy and dexamethasone (DEX) treatment.^{19–24}

Muscle hypertrophy is affected by balancing between protein synthesis and degradation.²⁵ Several materials, such as leucine and ursolic acid, as well as exercise, have been known to induce skeletal muscle hypertrophy stimulating v-akt murine thymoma viral oncogene homolog (Akt) or mammalian target of rapamycin (mTOR) signaling.^{26–32} Activation of mTOR generates upregulation of phosphorylated eukaryotic translation initiation factor 4E-binding protein 1 (p-4E-BP1) or phosphorylated 70 kDa

ribosomal protein S6 kinase (p-P70S6K). Those signals induce hypertrophy by enhancing translation of mRNAs.

On the other hand, inhibition of protein degradation (muscle proteolysis) has an important role for muscle hypertrophy and atrophy.²⁵ Muscle RING finger 1 (MuRF1) is a key regulator for muscle proteolysis through ubiquitin–proteasome pathway.^{33–35} MuRF1 has increased in various atrophy conditions, including immobilization, denervation, hindlimb unloading, DEX treatment, and interleukin-1-induced cachexia.^{33–38} MuRF1 regulates muscle atrophy and attenuates muscle loss when deleted.³⁹

Mechanism associated with myogenic differentiation by SF in human myotubes has not been well documented. We have assessed if SF could promote myogenic differentiation and which pathway it exploits in human skeletal muscle cells (HSkMCs).

According to our study, SF treatment increased protein synthesis through upregulation of mTOR/p-4E-BP1/p-P70S6K, while it did not reduce MuRF1 in human myotubes. Nevertheless, SF enhanced myogenic differentiation. In addition, SF attenuated atrophy caused by DEX through an increased protein synthesis. SF induced muscle protein synthesis but did not inhibit protein degradation in human myotubes.

Materials and methods

Materials

SF extract was obtained from Research Center for Anti-Aging Technology Development (Busan Technopark, Busan, Korea). Extraction method of SF was indicated by previous research.^{15,21,22} SF was dissolved in dimethyl sulfoxide as a 20 mg/mL stock solution and diluted with medium prior to use. The following antibodies were purchased from the individual providers: mTOR (sc-136269), p-mTOR (#2971), 4E-BP1 (#9452), p-4E-BP1 (#2855), P70S6K (#2708), p-P70S6K (#9234), myosin heavy chain 3 (MYH3, sc-53091), MuRF1 (ab172479), p-FOXO1 (#9461), and GAPDH (MB001) antibodies were purchased from Cell Signaling (Danvers, MA, USA), Santa Cruz Biotechnology Inc. (Dallas, TX, USA), Bioworld (St Louis Park, MN, USA), and Abcam (Cambridge, UK). Secondary antibodies of antimouse (ADI-SAB-300-J) and antirabbit (ADI-SAB-100-J) were bought from Enzo Life Sciences (Farmingdale, NY, USA). Collagenase, dispase II, basic fibroblast growth factor, and DEX were bought from Sigma-Aldrich Co. (St Louis, MO, USA).

Primary culture of HSkMCs

We used HSkMCs cultured primarily from donated human muscle pieces. All donors gave their written informed consent and agreed to muscle sampling during their surgical procedures. This experiment was approved by Institutional Review

Board of Pusan National University Yangsan Hospital. The satellite cells were isolated from the muscle piece by collagenase/dispase digestion and were grown in Ham's F10 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 20% fetal bovine serum, antibiotics (penicillin 50 U/mL and streptomycin 50 mg/mL) and basic fibroblast growth factor (2.5 ng/mL).^{40,41} Muscle tissue was washed in Hank's balanced salt solution. Muscle (~250 mg) was then minced and placed in 1 mL of collagenase (0.2%)/dispase II (2.4 U/mL) solution containing 8.3 mM CaCl₂. Until completely digested, it was incubated at 37°C for 1 hour, being vortexed every 15 minutes. Digested muscle solution was filtered by a 100 µm nylon cell strainer and diluted with complete medium. Strained solution was centrifuged for 5 minutes at 150× g, and the pellet was washed in 2 mL of complete Ham's F10 medium. Then, the pellets were resuspended and incubated in complete medium. Cells were seeded on gelatin-coated dish. Quality of human myoblast sample was checked with PAX3 (green, upper panel) and PAX7 (red, lower panel) stains (Figure S1). Growth medium was changed every 3 days. At confluence, HSkMCs were allowed to differentiate into myotubes. Differentiation of HSkMCs was induced by HSkMC differentiation medium (DM) (C-23061; PromoCell, Heidelberg, Germany).

Fusion index and myotube area

To observe differentiation efficiency, HSkMCs were seeded on 12-well plates (2×10⁴ cells/well), and after 1 day, HSkMCs were treated with SF of 0.1 µg/mL, 0.5 µg/mL, 1 µg/mL, 5 µg/mL, and 10 µg/mL in DM. Medium was changed to fresh DM with SF every 2 days. HSkMCs were photographed three times per group every 2 days for 8 days. Differentiation of HSkMCs was observed and photographed by phase-contrast microscopy (MCXI 600; MICROS, Vienna, Austria). Fusion index and myotube area were manually analyzed using ImageJ software. Fusion index is calculated as the ratio of the number of nuclei in fused myotubes per entire field. Myotube area is measured as the area covered by myotube in each photo.^{42,43}

Induction of atrophy

To observe differentiation efficiency, HSkMCs were seeded on 6-well plates (1×10⁵ cells/well), and after 1 day, HSkMCs were treated with SF of 0.5 µg/mL in DM. Medium was changed to fresh DM with SF every 2 days. Two days after induction of differentiation, cells were incubated with 100 µM DEX for 4 days to induce atrophy.^{44–47} Six days after the induction of differentiation, we took photos at three random spots in each group. For observing atrophy, we measured fusion index and myotube area. Cells were harvested with a Lipa buffer for protein extraction.

Immunofluorescent staining

DM containing 0.5 µg/mL of SF was applied to the cells. After 6 days, cells were washed with phosphate buffered saline (PBS) and fixed by 4% paraformaldehyde. Then, cells were permeabilized with PBS containing 0.25% Triton X-100 (PBST) for 25 minutes. These cells were incubated in 2% BSA for 30 minutes and then with MYH3 antibodies for 2 hours. After washing with PBS, cells were incubated with fluorescent anti-mouse IgG antibody (Alexa 594; Thermo Fisher Scientific) for 1 hour. Nuclei were stained by Hoechst 33342 (DAPI, 1 µg/mL) for 3 minutes. All antibodies were diluted in PBST containing 1% BSA. These cells were mounted on a glass slide. The results were recorded using fluorescence microscopy (Eclipse 80i; Nikon Corporation, Tokyo, Japan).

Western blot

The cells were lysed on ice using a Lipa buffer (GenDEPOT, Barker, TX, USA) for 30 minutes. Cell lysates were centrifuged at 13,000 rpm for 20 minutes at 4°C. The supernatant was collected, and the protein concentration was determined by a bicinchoninic acid protein assay kit. Equal amounts of protein (40 µg) were separated using 4%–15% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk and incubated with p-mTOR, total mTOR, p-P70S6K (Thr389), P70S6K, p-4E-BP1, 4E-BP1, MuRF1, MYH, and GAPDH primary antibodies overnight at 4°C. Those membranes were washed with PBS containing 0.1% Tween 20 and incubated with a horseradish peroxidase-conjugated mouse or rabbit secondary antibodies for 1 hour at room temperature. The immunoblots were visualized by chemiluminescence horseradish peroxidase substrate (RPN 2235; GE Healthcare UK Ltd, Little Chalfont, UK).

Statistical analysis

All the experiments in this study were performed in triplicate. All data were shown as the mean ± standard error of the mean. Statistical analysis was performed using analysis of variance followed by Tukey's honest significant difference post hoc test except for the Western blot results. Protein expression levels were analyzed using Student's *t*-test. The significance was taken as $P < 0.05$.

Results

SF induced differentiation in HSkMCs

Proliferation effects of SF prior to differentiation test were investigated in HSkMCs. Various concentrations (0.1 µg/mL,

0.5 µg/mL, 1 µg/mL, 5 µg/mL, and 10 µg/mL) of SF in growth medium were treated in a 12-well plate 1 day after the seeding of 4×10^4 cells per well. At 2 days after SF treatment, HSkMCs were trypsinized to be counted. Proliferation efficiency has not shown significant difference compared with control (Figure 1A). HSkMCs were treated with SF of 0.1 µg/mL, 0.5 µg/mL, 1 µg/mL, 5 µg/mL, and 10 µg/mL in DM. Differentiation of HSkMCs was observed and photographed by phase-contrast microscopy (MCXI 600; MICROS) every 2 days. For observing differentiation efficiency, fusion index and myotube area were analyzed. As shown in Figure 1B and C, overall fusion index and myotube area were the highest at 6 days. SF treatment of 0.5 µg/mL resulted in significantly higher fusion index at 6 days after differentiation, which tended to remain high until day 8 (Figure 1B). Myotube area was 1.6-fold larger than that of control with SF treatment of 0.1 µg/mL and 0.5 µg/mL at 8 days after differentiation (Figure 1C and D). Concentrations > 1 µg/mL caused rather reduced differentiation effects at day 8, supposedly by toxic effect. We chose SF concentration of 0.5 µg/mL, which showed the best result on differentiation, to be used on the subsequent experiment of HSkMCs.

SF increased phosphorylation of P70S6K and 4E-BP1 through mTOR signaling

To identify signaling pathways that were involved in SF-enhanced differentiation, HSkMCs were seeded on a $\phi 100$ mm dish (1×10^6 cells/plate), and the next day, they were treated with SF of 0.5 µg/mL in DM. At 1 day, 2 days, and 4 days after differentiation, the cells were harvested and lysed. We observed that SF increased phosphorylation of mTOR compared with control on the first day (Figure 2A and B). Levels of p-mTOR were normalized to the levels of GAPDH, as it has been suggested that myogenesis is affected by mTOR expression in kinase-independent way.⁴⁸ This increase significantly upregulated the phosphorylation of P70S6K and 4E-BP1 (Figure 2C and D). p-P70S6K exhibited upregulation also in the late phase (day 4) of differentiation (Figure 2C). We concluded that SF induces muscle hypertrophy by protein synthesis through mTOR/P70S6K and 4E-BP1 signaling in HSkMCs.

SF inhibited muscle atrophy through increasing protein synthesis

DEX is a glucocorticoid that mediates muscle proteolysis.^{19,21,35,46} Atrophy of myotubes was induced by 100 µM DEX. HSkMCs were treated with SF of 0.5 µg/mL in DM and changed to fresh DM and SF every 2 days. At 2 days after induction of differentiation, cells were incubated with

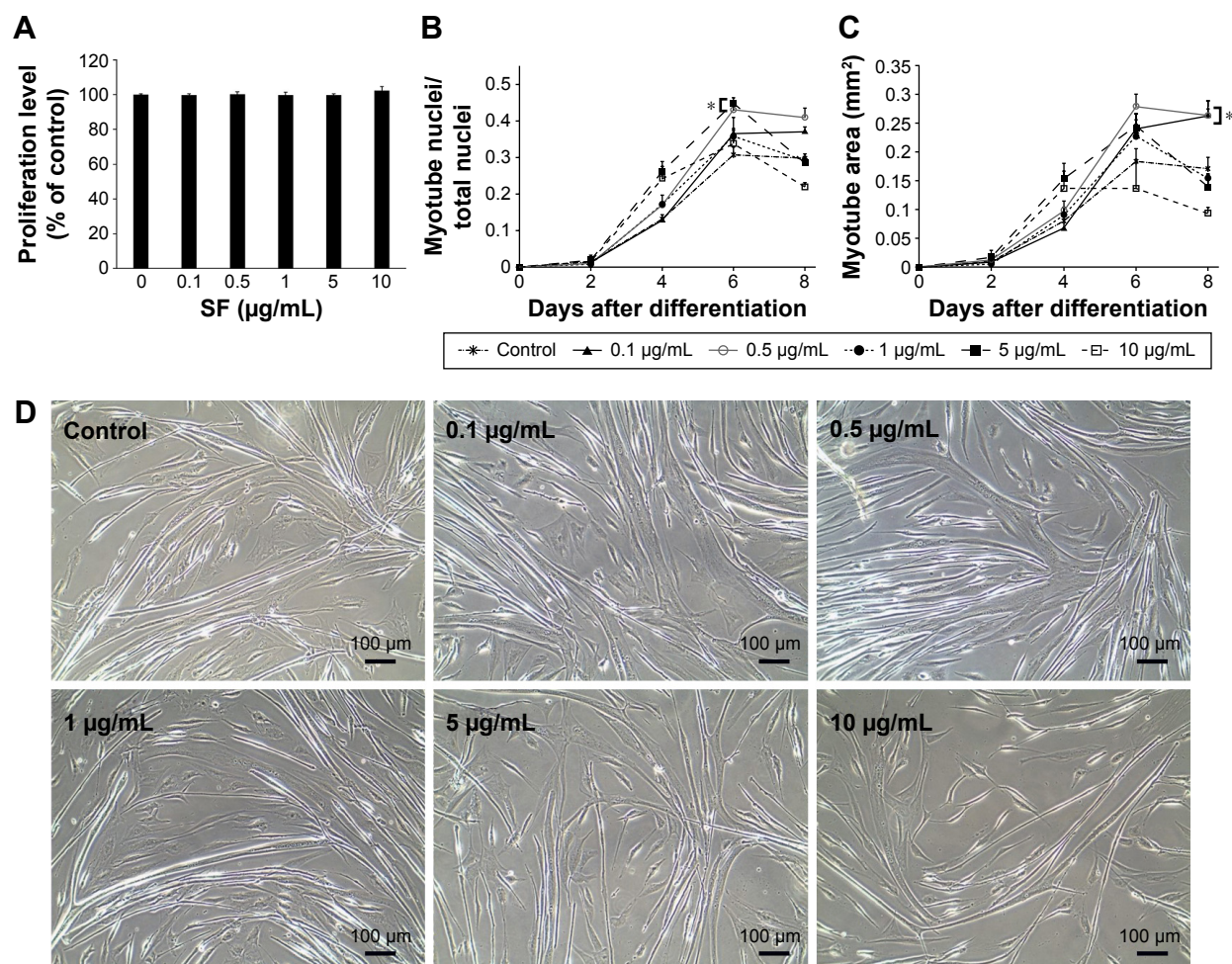


Figure 1 SF-induced differentiation in HSkMCs.

Notes: (A) SF was treated with various concentrations (0.1 µg/mL, 0.5 µg/mL, 1 µg/mL, 5 µg/mL, and 10 µg/mL) in growth medium. Two days after treatment, HSkMCs were counted. As a result, SF did not induce proliferation. (B and C) SF (0.1 µg/mL, 0.5 µg/mL, 1 µg/mL, 5 µg/mL, and 10 µg/mL) was treated with DM and changed with fresh DM every 2 days. HSkMCs were photographed three times every 2 days. For observing differentiation efficiency, fusion index and myotube area were analyzed. (D) Eight days after differentiation induction with SF, HSkMCs were photographed. A total of 0.5 µg/mL of SF significantly induced more differentiation. All data represented mean $\pm$ SEM (n=3). *Symbol indicates $P < 0.05$ compared to control.

Abbreviations: DM, differentiation medium; HSkMCs, human skeletal muscle cells; SF, Schisandrae fructus.

100 µM DEX for 4 days to induce atrophy. In the last 6 days after induction of differentiation, we could see that DEX treatment inhibits differentiation by fusion index and myotube area (Figure 3A–C) and MYH3 expression (Figure 3D and E). Those cells that induced atrophy were attenuated by SF treatment in concert with the upregulation of p-4E-BP1 and p-P70S6K (Figure 3E). However, SF did not inhibit the increase in MuRF1, which is responsible for muscle protein degradation through ubiquitin–proteasome pathway, induced by DEX (Figure 3F). Finally, differentiation enhancement by SF was revealed by MYH expression, a late-phase marker of myotube differentiation (Figure 3D and E). By these data, we could see that SF suppresses muscle atrophy by inducing protein synthesis in human myotubes.

Discussion

Muscle mass is frequently compromised by aging.^{1–5} Search for the dietary supplements to ameliorate and maintain skeletal muscle function is becoming an important topic in antiaging research field.^{49–55}

Muscle growth starts by the activation of quiescent myoblasts (proliferation). These cells get committed to myogenic differentiation and fuse with existing muscle fibers.^{56–61} In vivo skeletal muscle growth is identified by hypertrophy (increase in fiber size) and hyperplasia (increase in fiber number),^{62–64} while in vitro myogenic differentiation can be quantified by fusion index (ratio of fused nucleus) and area (hypertrophy) of myotubes.^{42,43} In addition, maintenance of myotubes is influenced by signals of skeletal muscle protein

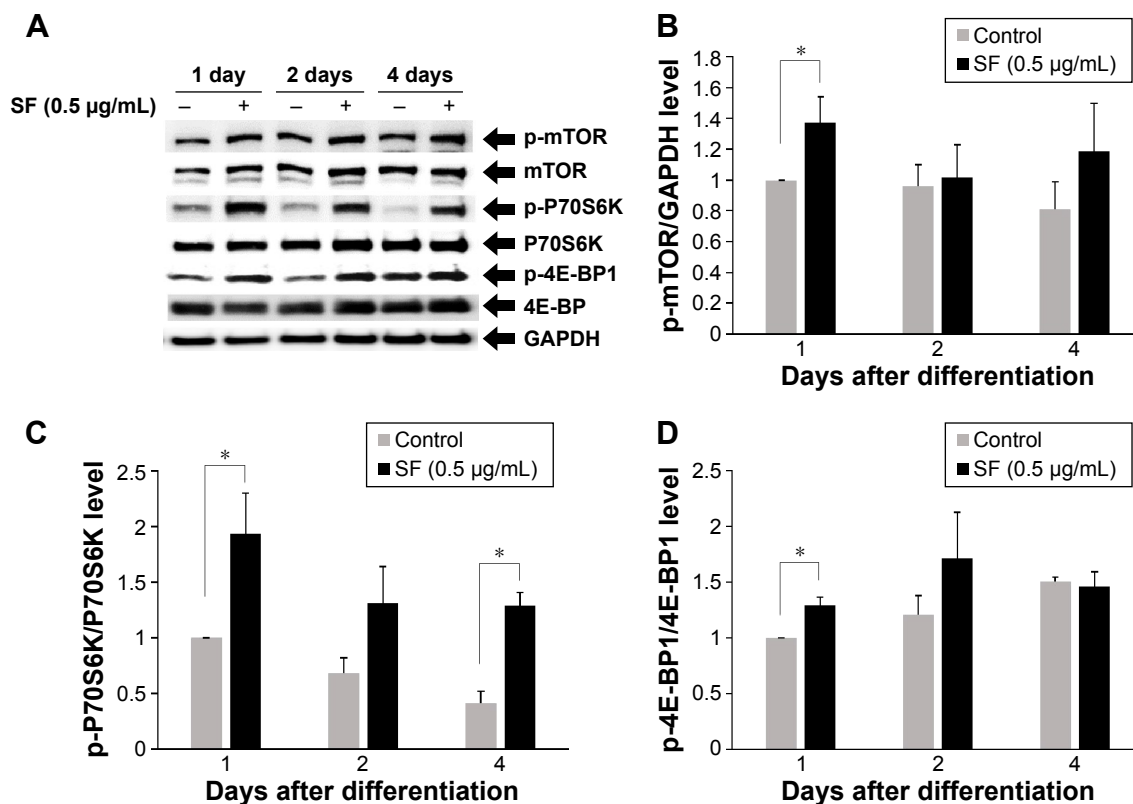


Figure 2 SF increased protein synthesis through mTOR/P70S6K and 4E-BP1 signaling.

Notes: (A) SF (0.5 µg/mL) was treated with differentiation medium. HSkMCs' differentiation was induced for 1 day, 2 days, and 4 days. Phosphorylation and expression of mTOR, P70S6K, and 4E-BP1 were observed for Western blot. GAPDH expression was analyzed to identify equal loading. (B–D) Levels of mTOR activation (phosphorylation) were normalized to the levels of GAPDH.⁴⁶ Phosphorylation of P70S6K and 4E-BP1 was normalized to the levels of each total protein. All data represented mean ± SEM (n=3). *Symbol indicates $P < 0.05$.

Abbreviations: 4E-BP1, 4E-binding protein I; HSkMCs, human skeletal muscle cells; mTOR, mammalian target of rapamycin; p-4E-BP1, phosphorylated 4E-BP1; p-P70S6K, phosphorylated P70S6K; P70S6K, 70 kDa ribosomal protein S6 kinase; SF, Schisandrae fructus; SEM, standard error of mean; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

synthesis and degradation.²⁵ So we investigated which signals regulate differentiation by SF treatment. Various concentrations of SF were treated with HSkMCs with growth medium and DM, while SF did not significantly affect HSkMCs under proliferation (Figure 1A), but 0.5 µg/mL of SF could remarkably increase differentiation at 6 days by fusion index and at 8 days by myotube area after differentiation (Figure 1B–D).

According to the previous studies, many kinds of materials, such as ursolic acid and leucine,^{26,32,37,51} as well as exercise that have been known to induce skeletal muscle hypertrophy are associated with mTOR and transforming growth factor-beta signaling.^{21,26,27} SF also activated signaling pathway of mTOR/p-P70S6K and p-4E-BP1 in early phases of differentiation (Figure 2). This activated signaling increased the synthesis of skeletal muscle proteins (Figure 3D and E). However, SF did not reduce MuRF1, which is responsible for muscle protein degradation through ubiquitin–proteasome pathway (Figure 3F). Nevertheless, we could observe

substantial myotube hypertrophy by SF treatment showing increase in fusion index and myotube area and MYH expression in human myotubes (Figures 1 and 3).

Much research has utilized DEX-treated mice and C2C12 to generate muscle atrophy.^{19,21,47} In this study, DEX provoked atrophic changes accompanied by increase in MuRF1 expression.

The upstream p-FOXO1 increased on the application of SF, which links to the inhibition of the protein degradation (Figure S2). However, contrary to the previous report with DEX-treated mice,²¹ SF treatment did not attenuate MuRF1 expression but rather raised it (Figure 3F). We speculate that MuRF1 expression by SF might be associated with FOXO3 levels or activity. Nevertheless, MYH expression and P70S6K activation were restored by SF inducing differentiation (Figure 3D and E). We suppose that this SF-induced muscle hypertrophy is influenced by the activation of mTOR signaling and the resultant increased protein synthesis than the inhibition of MuRF1 (Figure 4). It is still

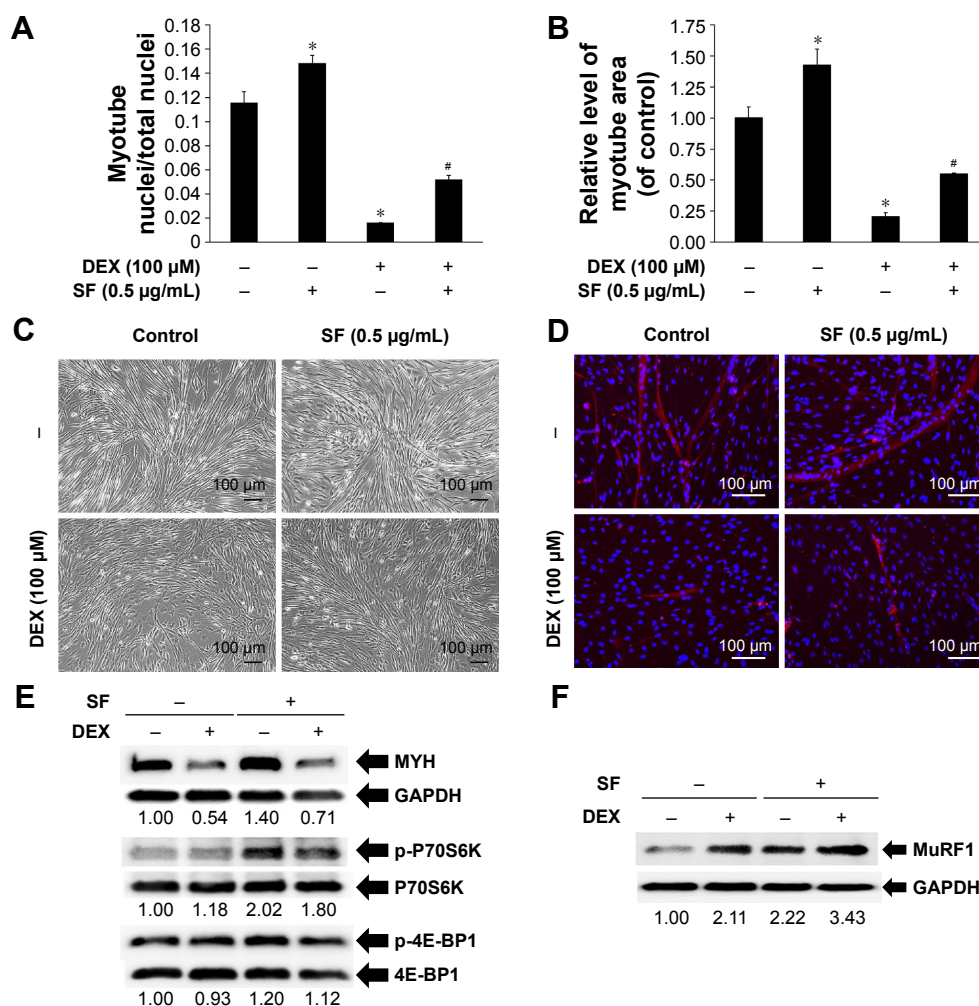


Figure 3 SF inhibited muscle atrophy through increasing protein synthesis.

Notes: At 2 days of differentiation, atrophy of myotubes was induced by 100 µM DEX for 4 days, and DM was changed to fresh DM with SF every 2 days. (A–C) In the last 6 days after induction of differentiation, HSkMCs were photographed three times per group. For observing differentiation efficiency, fusion index and myotube area were analyzed. All data represented mean ± SEM (n=3). *Symbol indicates $P < 0.05$ compared to control. #Symbol represents $P < 0.05$ compared to DEX treatment alone. (D) Myotubes were fluorescence stained with anti-MYH (red) and DAPI (blue), which was observed as a marker of late differentiation. (E and F) Examples of representative Western blot were shown for MYH, p-P70S6K, P70S6K p-4E-BP1, 4E-BP1, GAPDH, and MuRF1. Levels of MYH, MuRF1, p-P70S6K, and p-4E-BP1 were normalized to the levels of GAPDH or total protein.

Abbreviations: DEX, dexamethasone; DM, differentiation medium; 4E-BP1, 4E-binding protein I; HSkMCs, human skeletal muscle cells; MuRF1, muscle RING finger 1; MYH, myosin heavy chain; p-4E-BP1, phosphorylated eukaryotic translation initiation factor 4E-binding protein I; p-P70S6K, phosphorylated P70S6K; P70S6K, 70 kDa ribosomal protein S6 kinase; SF, Schisandrae fructus; SEM, standard error of mean; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

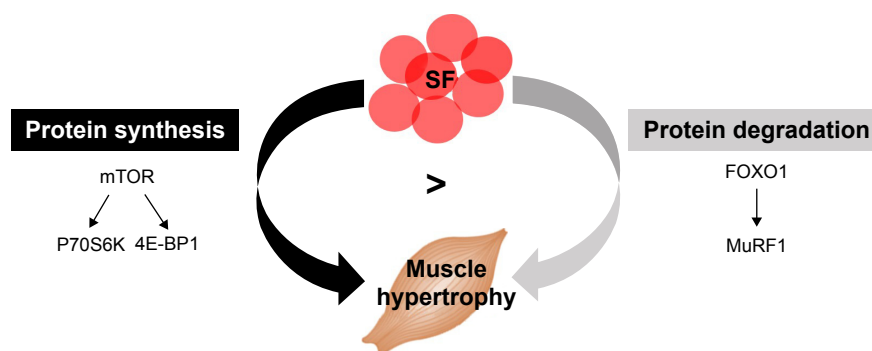


Figure 4 The diagram describes the mechanism of SF that increases muscle hypertrophy.

Notes: It is suggested that SF increased protein degradation through increasing MuRF1 expression; nevertheless, it could also increase muscle hypertrophy by inducing myogenic differentiation and attenuating atrophy by protein synthesis through mTOR/P70S6K and 4E-BP1 signaling pathway.

Abbreviations: 4E-BP1, 4E-binding protein I; mTOR, mammalian target of rapamycin; MuRF1, muscle RING finger 1; P70S6K, 70 kDa ribosomal protein S6 kinase; SF, Schisandrae fructus.

probable that application of DEX early in the differentiation posed antidifferentiation effect, which was alleviated by SF extract to result in myotube hypertrophy.

Conclusion

SF could enhance myogenic differentiation and attenuate atrophy in human myotubes, supporting previous reports from mouse models. We confirmed that these effects are mediated by increased protein synthesis through mTOR/P70S6K/4E-BP1 signaling pathway (Figure 4). We find that the human myotube culture is a good testbed to screen materials to benefit human skeletal muscle, which may reveal aspects different from those by mice experiment. It will eventually help the people with sarcopenia or myopathy to maintain and strengthen their skeletal muscle.

Acknowledgment

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Immunofluorescent staining

Primary cultured human skeletal muscle cells (2×10^4 cells/well) were seeded on a cover glass in a 12-well plate, which was coated with 1% gelatin. After 2 days, cells were washed with phosphate buffered saline (PBS) and fixed by 4% paraformaldehyde. Then, cells were permeabilized with PBS containing 0.25% Triton X-100 (PBST) for 25 minutes. These cells were incubated in 2% BSA for 30 minutes and then with PAX3

(ab180754; Abcam) or PAX7 (ab34360; Abcam) antibodies overnight at 4°C. After washing with PBS, cells were incubated with fluorescent antirabbit IgG antibodies (Alexa 594 or Alexa 488; Thermo Fisher Scientific, Waltham, MA, USA) for 1 hour. Nuclei were stained by Hoechst 33342 (DAPI, 1 µg/mL) for 3 minutes. All antibodies were diluted in PBST containing 1% BSA. These cells were mounted on a glass slide. These results were recorded using fluorescence microscopy (Eclipse 80i; Nikon Corporation, Tokyo, Japan).

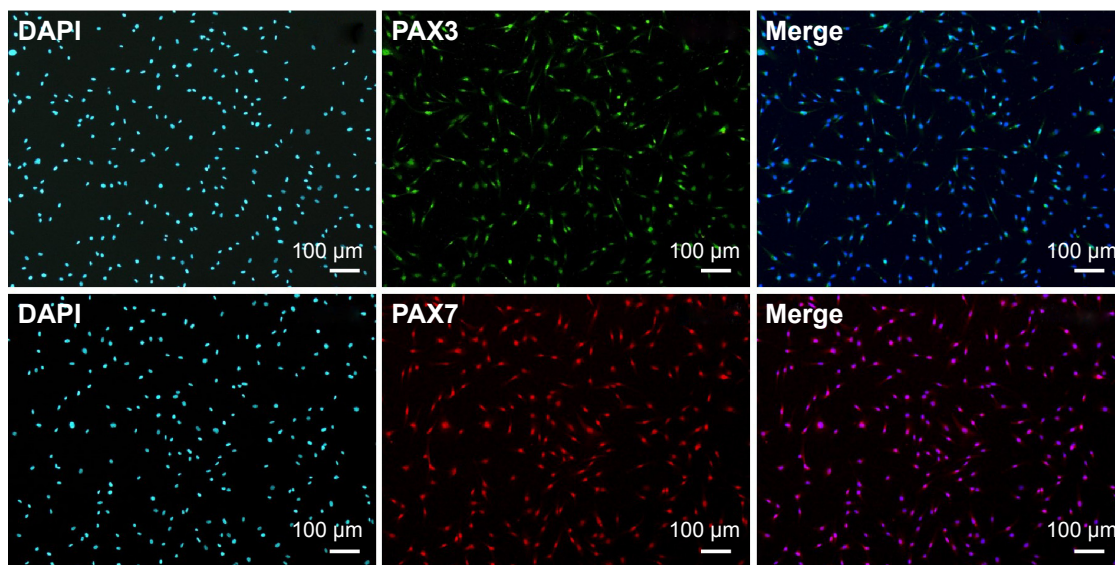


Figure S1 Human skeletal muscle cells were identified by the expression of PAX3 and PAX7.

Notes: Primary cultured human skeletal muscle cells have exhibited the expression of PAX3 (green, upper panel) and PAX7 (red, lower panel), which have been known as human myoblast marker. Each of the stained cells was merged with nuclei.

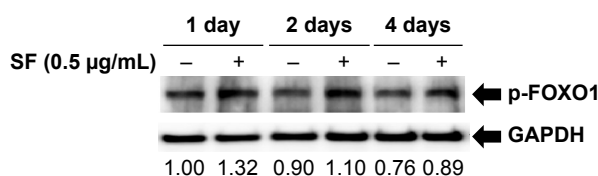


Figure S2 SF increased phosphorylation of FOXO1.

Notes: SF (0.5 µg/mL) was treated with differentiation medium. HSkMCs' differentiation was induced for 1 day, 2 days, and 4 days. Phosphorylation of FOXO1 was observed by Western blot. p-FOXO1 levels were normalized to the levels of GAPDH.

Abbreviations: HSkMCs, human skeletal muscle cells; SF, Schisandrae fructus; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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