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Soy peptide-induced stem cell proliferation: involvement of ERK and TGF-β1☆

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Abstract

This study was conducted to investigate the proliferative effect of vegetable soy peptides on adult stem cells (ASCs) in the absence of serum and their possible mechanisms of action. The proliferation of human adipose tissue-derived mesenchymal stem cells (ADSCs) and cord blood-derived mesenchymal stem cells (CB-MSCs) treated with soy peptides was found to increase significantly upon 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and Click-iT 5-ethynyl-2′-deoxyuridine flow cytometry assay. In addition, soy peptides led to stepwise phosphorylation of the p44/42 MAPK (ERK), mammalian target of rapamycin (mTOR), p70 S6 kinase, S6 ribosomal protein (S6RP) and eukaryotic initiation factor 4E (eIF4E) in ADSCs. Furthermore, quantitative analysis of the cytokines revealed that the production of transforming growth factor-beta1 (TGF-β1), vascular endothelial growth factor and interleukin-6 increased significantly in response to treatment with soy peptides in both ADSCs and CB-MSCs. Similarly, soy peptide-induced phosphorylation of the ERK/mTOR/S6RP/eIF4E pathway was blocked in response to pretreatment with PD98059, a specific ERK inhibitor. Moreover, inhibition of TGF-β1 through PD98059 pretreatment and a consecutive decrease in ADSC proliferation revealed that TGF-β1 induces the phosphorylation of mTOR/S6RP/eIF4E. Collectively, the results of this study indicate that ERK-dependent production of TGF-β1 plays a crucial role in the soy peptide-induced proliferation of ADSCs under serum-free conditions.

Keywords: Adult stem cells; Soy peptide; Growth factor; mTOR pathway; TGF-β1; ERK

1. Introduction

A hallmark of stem cell biology is the capacity for self-renewal and differentiation. Regulation of cell cycling is a key process involved in the fate of stem cells, including renewal and differentiation. Adult stem cells (ASCs) have restricted life spans *in vitro*, similar to other somatic cells, and can therefore only be expanded for a limited number of cell divisions before entering a senescent state and unequivocally stopping proliferation [1–3]. Cell proliferation is an extensively coordinated process that is regulated in both time and space. When nutrients and other appropriate growth stimuli are present, cells up-regulate their macromolecular synthesis, which results in their size and mass increasing. These processes are regulated by multiple extracellular growth and differentiation factors that include soluble or membrane-bound factors and extracellular matrix components.

As a result, when growth conditions are favorable, TOR is active and yeast cells maintain a robust rate of ribosome biogenesis, translation initiation and nutrient import. Accordingly, when growth conditions permit, rapamycin-sensitive TOR signaling promotes anabolic processes and antagonizes catabolic processes. Many of these rapamycin-sensitive readouts of TOR are conserved in mammals [4]. The mammalian target of rapamycin (mTOR) kinase phosphorylates p70 S6 kinase (p70S6K), which regulates the phosphorylation of S6 ribosomal protein (S6RP), the functional site of the 40S ribosomal subunit. Thus, mTOR/p70S6K is required for cell growth and cell cycle progression [5,6].

Soy proteins are an important source of nitrogen and essential amino acids in adult humans in specific segments of the population [7]. Recently, isoflavones have attracted increased attention owing to their health-related beneficial aspects [8]. Moreover, several studies conducted using soy-derived extracts or isoflavones have reported that they have proapoptotic activity against cancer [9]. Peptones are small polypeptides that are intermediate products involved in the hydrolysis of proteins. Depending on the degree of hydrolysis, peptones may supply nutrients, adhesion components or growth factor analogues [10]. The use of peptones in a serum-free medium is not new; however, the clear deficiency of most of these peptones is their animal origin. As a result, most currently available serum-free media contain animal-derived peptones and hydrolysates [11,12] or animal-derived purified proteins [13,14]. Utilization of serum-free medium facilitates the analysis of interactions between growth factors and cytokines during the proliferation and differentiation of ASCs without the complexity of exogenous serum. Therefore, vegetable peptones may be safer supplements for animal protein-free medium. However, no studies have been conducted to evaluate the influence of soy peptides on the proliferative aspects of ASCs under serum-free conditions to date.

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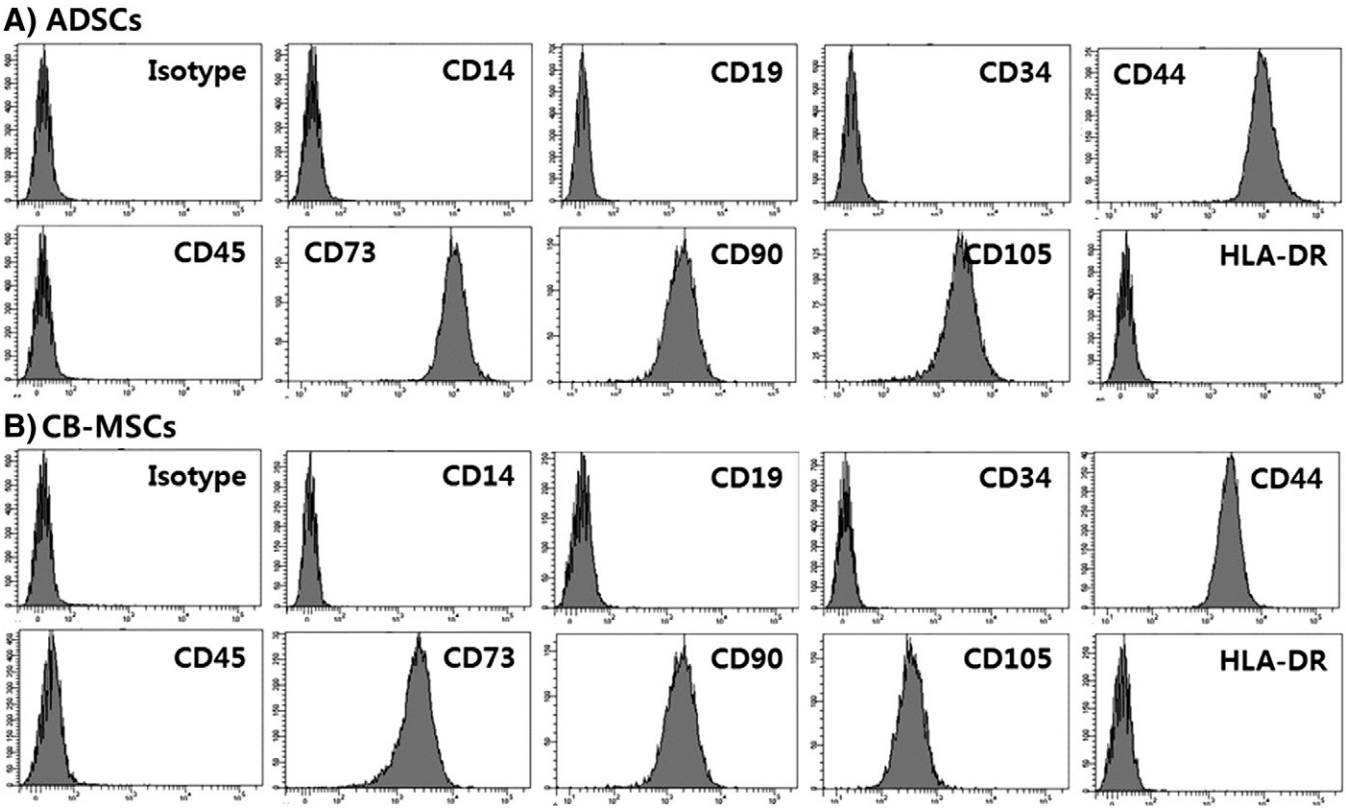


Fig. 1. Cell surface characterization of ADSCs and CB-MSCs. The cell surface protein profile of ADSCs (A) and CB-MSCs (B) was analyzed by flow cytometry at P2 (A) and P5 (B), and the following criteria was user to characterize the ASCs: positive markers — CD44, CD73, CD90, CD105 (>95%); negative markers — CD14, CD19, CD34, CD45, HLA-DR (<2%).

In this study, we report the novel observation that soy peptides promote ASC proliferation via activation of the mTOR/p70S6K/S6RP/eukaryotic initiation factor 4E (eIF4E) signaling pathway through p44/42 MAPK (ERK)-dependent production of transforming growth factor-beta1 (TGF-β1) and subsequent production of cytokines such as vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) under serum-free conditions. Specifically, this study was conducted to evaluate the proliferative effects of soy peptides on ASCs and the possible mechanisms underlying their operation.

2. Materials and methods

2.1. Cells, cell characterization and reagents

Human adipose tissue-derived mesenchymal stem cells (ADSCs) were purchased from Invitrogen (Invitrogen, CA, USA). The cryopreserved cells were thawed at 37°C and then immediately cultured in MesenPRO RS medium (Gibco, CA, USA) at 37°C under 5% CO_2 incubator. The cells were then expanded using MesenPRO RS medium. We obtained the two sources of human cord blood-derived stem cells (CB-MSCs) from Cha University (Seongnam, Gyunggi, Korea). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing low glucose levels that were supplemented with 15% fetal bovine serum (FBS), 1% penicillin and streptomycin at 37°C under 5% CO_2 incubator. The medium was changed every 3 days until the cells were confluent, at which time they were passaged. ADSCs and CB-MSCS were characterized using ASC markers by flow cytometry analysis. Cells were detached by 0.05% trypsin and stained with antibodies against CD14, CD19, CD34, CD44, CD45, CD73, CD90, CD105 and HLA-DR (all BD Pharmingen, Heidelberg, Germany) directly. Following incubation, the samples were analyzed using a FACS Aria flow cytometer (BD Biosciences, San Jose, CA, USA). We used ADSCs and CB-MSCs before they lost their ability to grow or differentiate into all potential phenotypes.

2.2. Preparation of soy peptides

Soy beans (*Glycine max* var.) were used for the experiment. Defatted soy bean flour was made by grinding soy beans in a ball mill and then immersing the flour in hexane at room temperature (RT) for at least 6 h. After being defatted, soy flour was obtained by filtration and drying at RT. Defatted soy flour (100 g) was then dispersed in distilled water at RT (10%) and incubated at 90°C for 30 min. The samples were then cooled to RT, after which the insoluble solid was separated out by centrifugation (RC Legend RT, Sorvall Products, Newtown, CT, USA) at 10,000*g* for 30 min. The aqueous extract (crude protein extract) was hydrolyzed with 0.1% Protamex (Novozymes, Bagsvaerd, Denmark) at pH 8.0 and 50°C. The mixture was then continuously stirred at 300 rpm for 24 h while controlling the temperature and pH by the pH-stat method (TIM 854, Radiometer Analytical, SAS, France) and by the addition of 1 M NaOH. Hydrolysis was stopped by heating at 80°C for 15 min. After cooling at RT, the hydrolysate was centrifuged at 20,000*g* for 30 min, and the supernatant was then freeze-dried (46 g) and stored at −20°C. Ultrafiltration was conducted using a Pellicon 2 ultrafiltration system (Millipore, Bedford, MA, USA) equipped with a 10-kDa Ultracel PLC-composite regenerated cellulose membrane of 0.5 m^2 in the form of a cassette filter (Millipore). Thirty liters of 5% hydrolysate was then reduced to 3 L at RT by ultrafiltration. The retentate was subsequently freeze-dried and stored at −20°C as a soy peptidic powder (39 g). Next, soy peptides were used to prepare 1% medium in DMEM without serum by filtration through a 0.2-μm membrane filter. Under serum-free conditions, the cell culture medium was replaced with serum-free DMEM (catalog no. 11885, Invitrogen).

Fig. 2. Effects of soy peptides on the proliferation of ADSCs (A, C, E, G) or CB-MSCs (B, D, F, H). ASCs were grown in a combination culture medium that contained soy peptides for 3 days. Under serum-free conditions, cell culture medium was replaced with serum-free DMEM. For a positive control, cells were cultured in control medium (DMEM with 15% FBS). The cell proliferation was then determined by an MTT assay (A, B) and a Click-iT EdU flow cytometry assay (C, D) as described in the Materials and Methods section. Moreover, cells were stained with PI solution and subjected to FACS analysis based on the DNA content for cell cycle analysis (E, F). Analysis of the samples was conducted using a FACS Vantage flow cytometer. Cell density of ASC cultured in different media (G, H). ASCs were seeded at the same cell density and cultured in different media for 3 days. Values are expressed as a percentage of the control, which was defined as 100%. Mean values for each group were obtained from four samples (n=4). *P<.05. Abbreviations: CM, complete medium (+); S/F, serum-free (−); SP, soy peptide in serum-free medium.

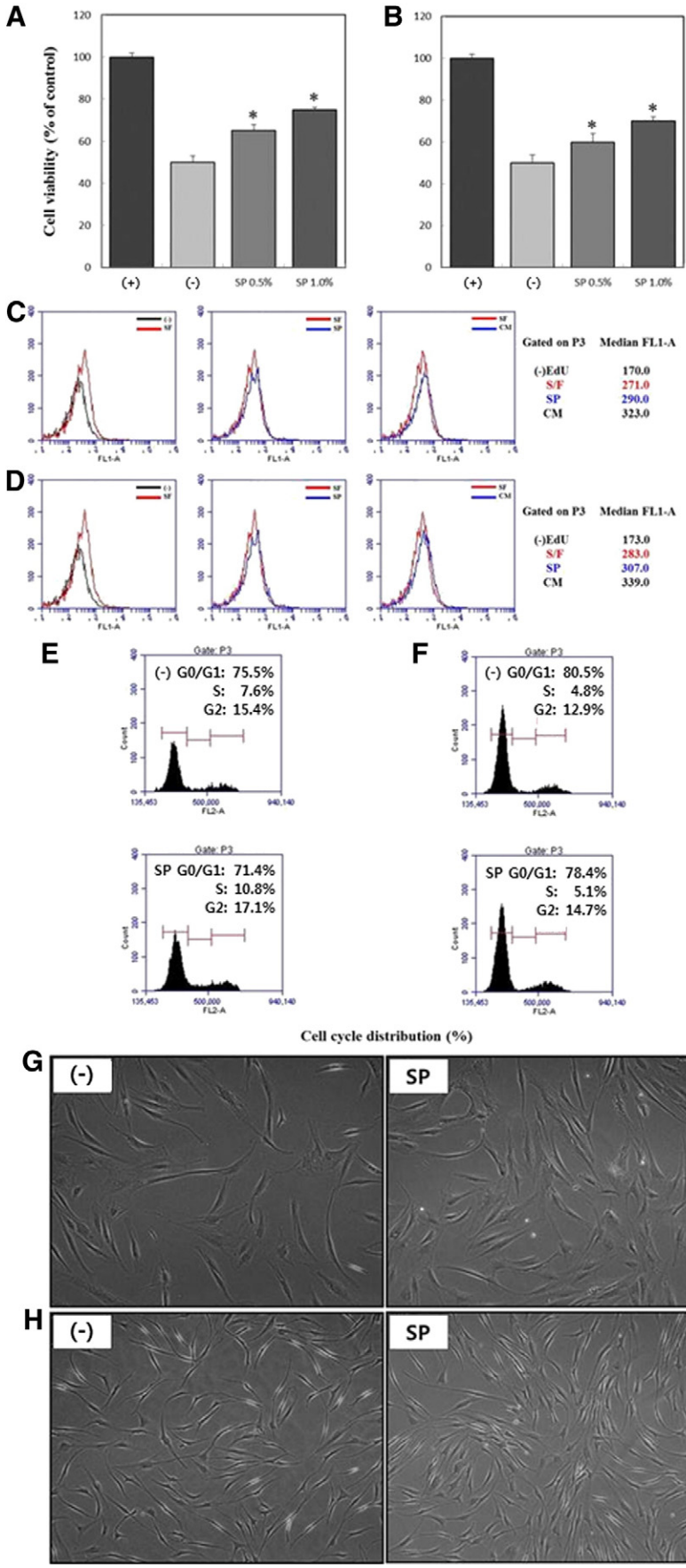

A
Cell viability (% of control)
120
100
80
60
40
20
0
(+)
(-)
SP 0.5%
SP 1.0%
B
C
Gated on P3 Median FL1-A
(-)EdU 170.0
S/F 271.0
SP 290.0
CM 323.0
D
Gated on P3 Median FL1-A
(-)EdU 173.0
S/F 283.0
SP 307.0
CM 339.0
E
(-) G0/G1: 75.5%
S: 7.6%
G2: 15.4%
SP G0/G1: 71.4%
S: 10.8%
G2: 17.1%
F
(-) G0/G1: 80.5%
S: 4.8%
G2: 12.9%
SP G0/G1: 78.4%
S: 5.1%
G2: 14.7%
Cell cycle distribution (%)
G
(-)
SP
H
(-)
SP

Table 1
Amino acid distribution (mg/g) of soy peptides

Soy peptide	Total amino acids (T)	Free amino acids (F)	(F/T)×100		T	F	(F/T)×100
Aspartic acid	79.80	0.43	0.54	Methionine	10.38	1.56	15.03
Threonine	29.48	6.36	21.57	Isoleucine	31.63	7.95	25.13
Serine	42.08	2.73	6.49	Leucine	55.61	14.32	25.75
Glutamic acid	148.67	1.19	0.80	Tyrosine	24.40	4.00	16.40
Proline	43.94	0.40	0.91	Phenylalanine	40.27	9.89	24.56
Glycine	33.64	0.95	2.82	Histidine	24.13	3.78	15.67
Alanine	26.62	1.81	6.80	Lysine	45.39	5.89	12.98
Cysteine	23.54	1.46	6.20	Arginine	57.93	15.55	26.84
Valine	33.69	8.26	24.52	Tryptophan	60.30	9.49	15.74

For positive/negative controls, cells were cultured in DMEM medium (with/without 15% FBS).

2.3. Cell proliferation and cycle

Cell proliferation was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and 5-ethynyl-2′-deoxyuridine (EdU) assay. Cells were exposed to MTT (0.1 mg/ml) for 3 h at 37°C under 5% CO_2 incubator. The medium was then removed, and the cells were solubilized with dimethyl sulfoxide (1 ml). After complete solubilization, the presence of blue formazan was evaluated spectrophotometrically by measuring the absorbance at a wavelength of 570 nm. The Click-iT EdU flow cytometry assay kit is a novel alternative to the BrdU assay (Molecular Probes, Eugene, OR, USA). Cells were incubated with EdU for 1 h. For a negative control, cells from the same population were not treated with EdU (10 μM). Following incubation, the samples were analyzed using a FACS Aria flow cytometer (BD Biosciences, San Jose, CA, USA).

The cells were also stained with propidium iodide (PI) and subjected to FACS analysis based on the DNA content for cell cycle analysis. Briefly, the cells were suspended in 1 ml of PBS and washed twice. The centrifuged pellets were then suspended in 70% ethanol and fixed for 2 h, after which they were vortexed and centrifuged. The ethanol was then discarded, and the pellets were stained with PI solution containing RNase A. Following incubation at RT, the samples were analyzed using a FACS Aria flow cytometer in conjunction with the ModFit software.

2.4. Differentiation of ADSCs

ADSCs were grown in a combination culture medium that contained 1% soy peptides for 3 days under serum-free conditions. Next, ADSCs were induced to undergo adipogenic differentiation. To induce adipogenesis, cells were plated at 4.0×10^4 cells/cm^2 and then incubated in the presence of adipocyte differentiation medium (MesenPRO RS medium, Gibco). The medium was changed every 3 days for 3 weeks. Adipogenesis was detected at day 21 based on the presence of Oil-Red-O-stained cytoplasmic lipid droplets.

2.5. RNA preparation and real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was extracted from ADSCs grown in a combination culture medium that contained 1% soy peptides for 3 days under serum-free conditions with TRIzol reagent according to the manufacturer's instructions (Invitrogen). Real-time RT-PCR analysis was conducted using an ABI7900HT machine (Applied Biosystems, Darmstadt, Germany). All Taqman RT-PCR reagents, including primers and probes, were purchased from Applied Biosystems (Reference sequences/Assay ID: Oct-4; NM_002701[Hs03005111_g1], Nanog; NM_024865[Hs02387400_g1], Rex-1; NM_174900[Hs01938187_s1], Sox-2; NM_003106.2 [Hs01053049_s1], GAPDH; NM_002046[Hs99999905_m1]). In the present study, the GAPDH gene was selected as an internal control, and this was validated using the NormFinder software. Relative quantification was conducted as previously described using GAPDH as a control gene. The $2^{-\Delta CT}$ method described by Livak and Schmittgen [15] was employed to normalize gene expression values prior to statistical analysis.

2.6. Cytokine profile

For quantitative analysis of the cytokines, the supernatants of the ASCs that were cultured with soy peptides in serum-free medium were analyzed using a MILLIPLEX human cytokine kit (Millipore, St. Charles, MO, USA) according to the manufacturer's recommended protocols. The cell culture supernatant may require dilution with an appropriate control medium prior to assay. Briefly, 200 μl of assay buffer was added to each well of the microtiter plate. After 10 min, 25 μl of sample was added to the appropriate wells with matrix and beads. After 2 h at RT with shaking, 25 μl of detection antibody was added to each well, and the samples were then incubated at RT for 1 h. Next, 25 μl of streptavidin phycoerythrin was added to each well, and the samples were incubated for 30 min at RT. Finally, 150 μl of sheath fluid was added to each well, and the samples were read using Luminex (Hitachi America, Brisbane, CA, USA).

2.7. Western blot analysis

Cells were seeded in a 100-mm culture dish at a density of 5×10^5 cells. The next day, the medium was replaced with serum-free medium, and the cells were incubated for 24 h. The medium was then changed to serum-free medium containing 1% soy peptides. Next, the cells were harvested and lysed with lysis buffer and protease inhibitor cocktail kit (Roche, Germany), after which the lysates were centrifuged at 12,000 rpm for 10 min and the supernatants were collected. The proteins were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Uppsala, Sweden) at 250 mA in transfer buffer (20 mM Tris base, 150 mM glycine, 20% MeOH in D.W. 1 L). PVDF membranes were blocked at RT for 1 h with 5% bovine serum albumin (BSA)/tris-buffered saline (TBS)-tween 20 (T) buffer. The membranes were then washed and incubated with primary phospho-p44/42 MAPK (phospho-ERK1/2), phospho-mTOR, phospho-raptor, phospho-p70 S6 kinase (phospho-p70S6K), phospho-S6RP, phospho-eIF4E and β-actin antibodies overnight (Cell Signaling Technology, Danvers, MA, USA) at 4°C and then washed and incubated with horseradish-peroxidase (HRP)-conjugated IgG secondary antibody (Cell Signaling Technology) in 5% BSA/TBS-T buffer at RT for 1 h. After washing, the immunoreactive proteins were detected by chemiluminescence (ECL kit, GE Healthcare). The detected proteins were normalized to β-actin or the respective total protein as appropriate.

2.8. Statistical analysis

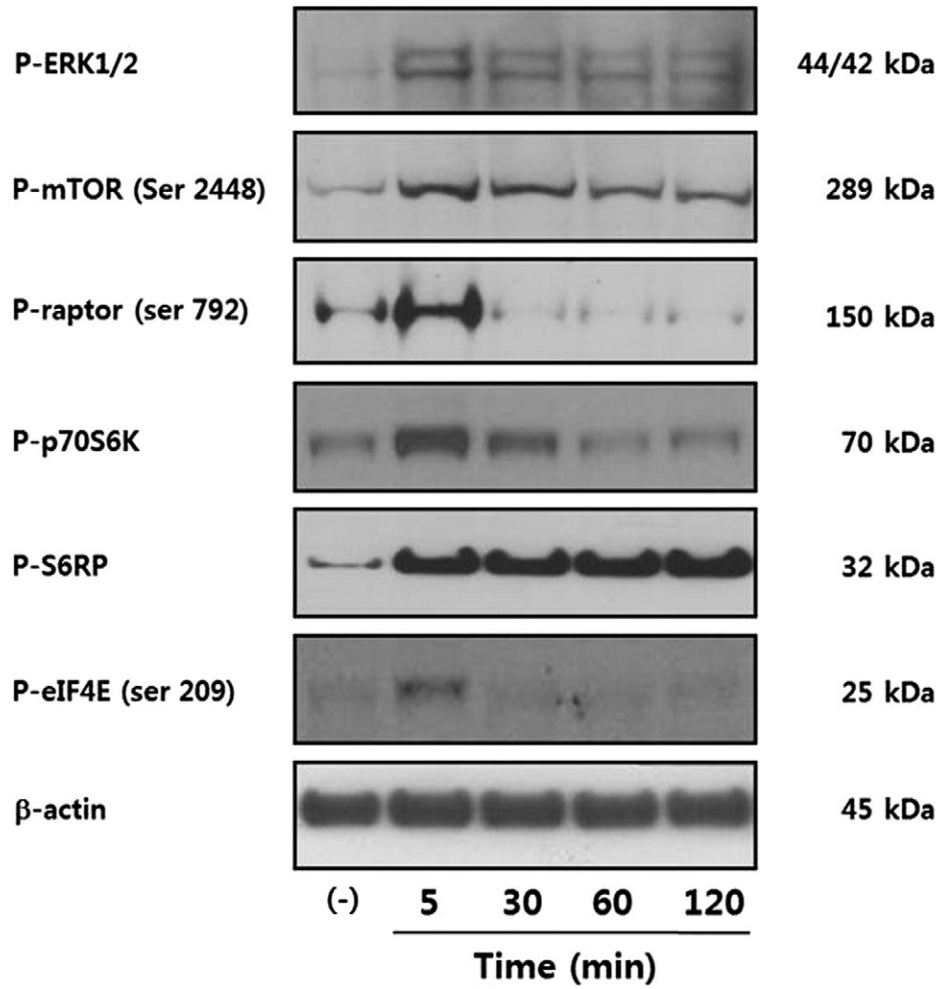
All experiments were conducted at least three times, and the data were expressed as the mean±S.D. Data were analyzed by a Student's t test. A value of $P<.05$ was considered to be statistically significant.

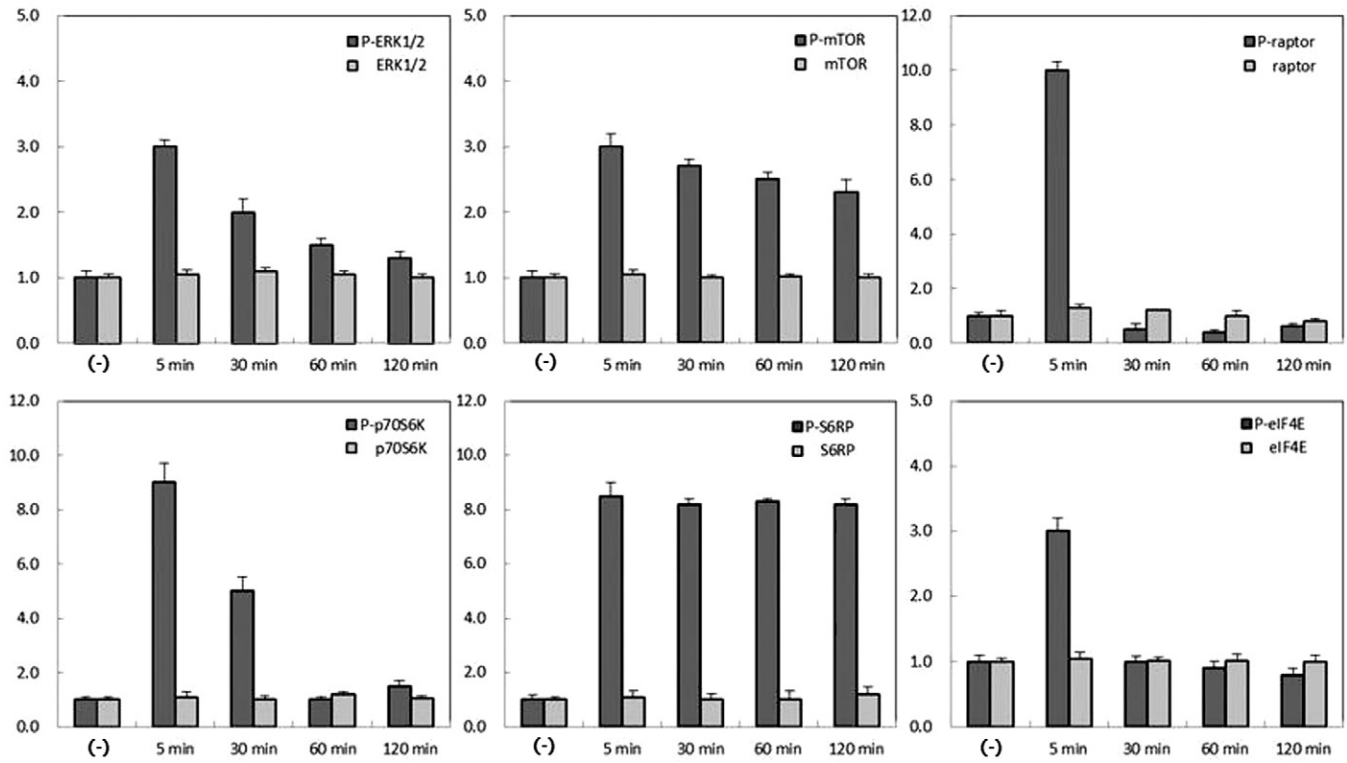
3. Results

3.1. Soy peptides promote the proliferation of ASCs

In this study, we first verified the characteristics of the ASCs (Fig. 1). Next, we examined the involvement of soy peptides in the proliferation of ASCs using an MTT assay under serum-free conditions. The results of this study revealed that soy peptides induced the proliferation of ASCs such as human ADSCs and CB-MSCs under serum-free conditions. Specifically, soy peptides induced a 25% and 20% increase in the cell growth rate of ADSCs and CB-MSCs when compared with the serum-free group, respectively (Fig. 2A, B). These effects of soy peptides were also confirmed by the results of the Click-iT EdU flow cytometry assay of ASCs. As shown in Fig. 2C and D, the EdU/fluorescein isothiocyanate (FITC)-positive portion of the group treated with soy peptides increased when compared with the untreated control group in the Click-iT EdU flow cytometry assay. Moreover, to characterize the proliferative

Fig. 3. Western blot analysis of extracts from ADSCs. Cells were harvested and lysed with lysis buffer and protease inhibitor cocktail. Proteins were separated by SDS-PAGE and transferred to the membranes that were subsequently incubated with primary phospho-ERK1/2, phospho-mTOR, phospho-raptor, phospho-p70S6K, phospho-S6RP, phospho-eIF4E and β-actin antibodies overnight at 4°C and then washed and incubated with HRP-conjugated IgG secondary antibody. After washing, the immunoreactive proteins were detected by chemiluminescence. The detected proteins were then normalized against β-actin or the respective total protein as appropriate. Protein bands were quantified by densitometry, and the level of the phosphorylated band was calculated for each time point after normalization in the same sample. Unstimulated basal expression (S/F) was set as unity.


P-ERK1/2
44/42 kDa
P-mTOR (Ser 2448)
289 kDa
P-raptor (ser 792)
150 kDa
P-p70S6K
70 kDa
P-S6RP
32 kDa
P-eIF4E (ser 209)
25 kDa
β-actin
45 kDa
(-) 5 30 60 120
Time (min)


P-ERK1/2
ERK1/2
P-mTOR
mTOR
P-raptor
raptor
P-p70S6K
p70S6K
P-S6RP
S6RP
P-eIF4E
eIF4E
(-)
5 min
30 min
60 min
120 min

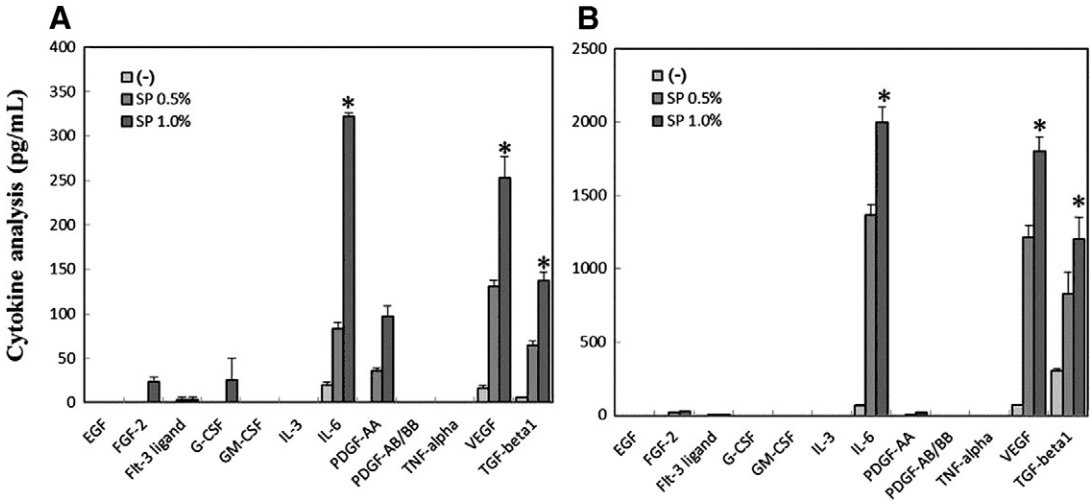


Fig. 4. Cytokine profile analysis of conditioned medium of ADSCs (A) or CB-MSCs (B) treated with soy peptides. ASCs were grown in a combination culture medium containing soy peptides for 3 days. For quantitative analysis of the cytokines, the supernatants of ASCs that were cultured with soy peptide in serum-free medium were analyzed using a MILLIPLEX Human cytokine kit according to the manufacturer's recommended protocols. The mean values for each group were obtained from four samples (n=4). *P<.05.

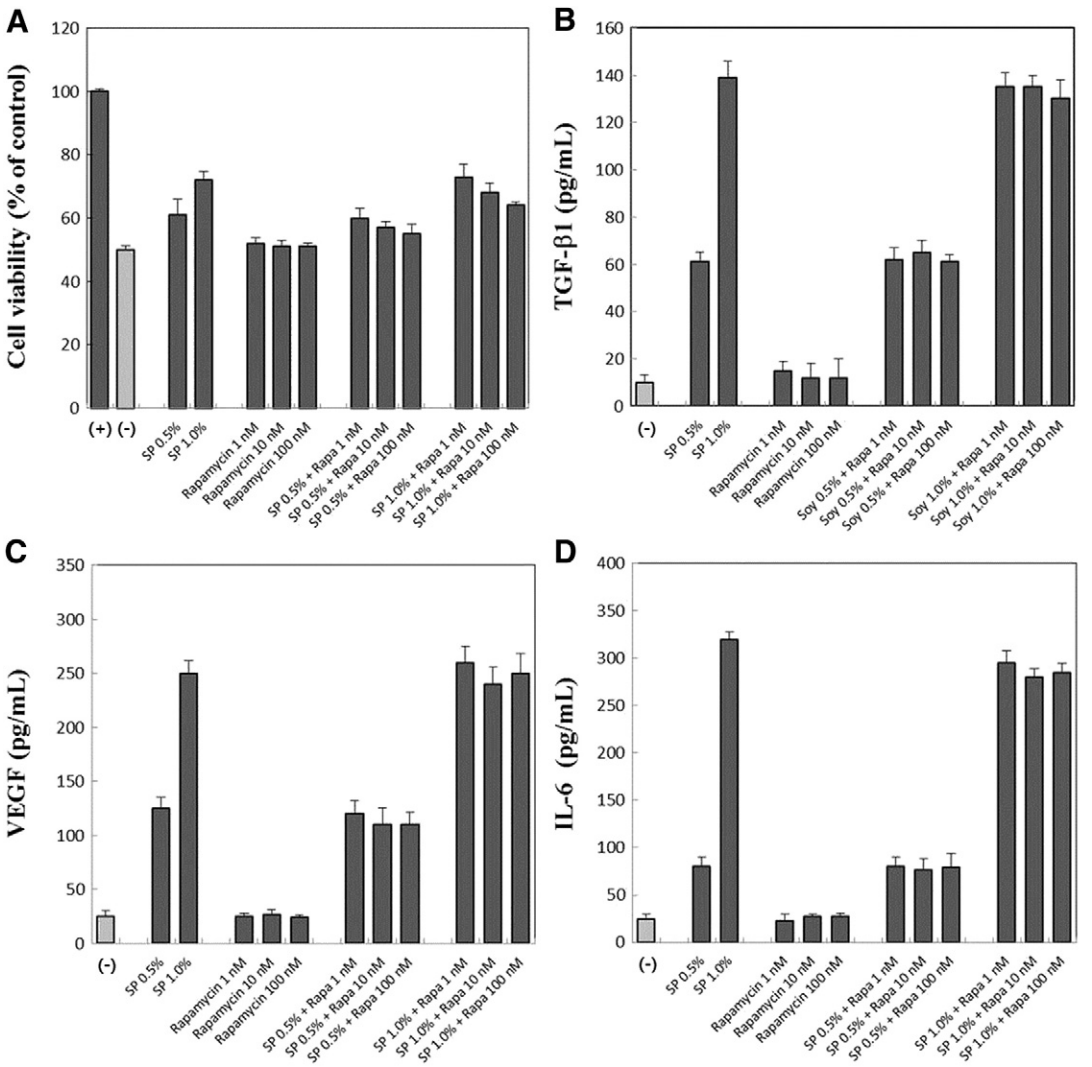


Fig. 5. Effect of rapamycin (mTOR inhibitor) on the proliferation and cytokine secretion of ADSCs treated with soy peptides. ADSCs were grown in a combination culture medium that contained soy peptides (1%) or soy peptides (1%) with treated rapamycin (100 nM) for 3 days under serum-free conditions. The cell proliferation and cytokine analysis were then determined by MTT assay and using a MILLIPLEX Human cytokine kit, respectively. Mean values for each group were obtained from four samples (n=4). *P<.05.

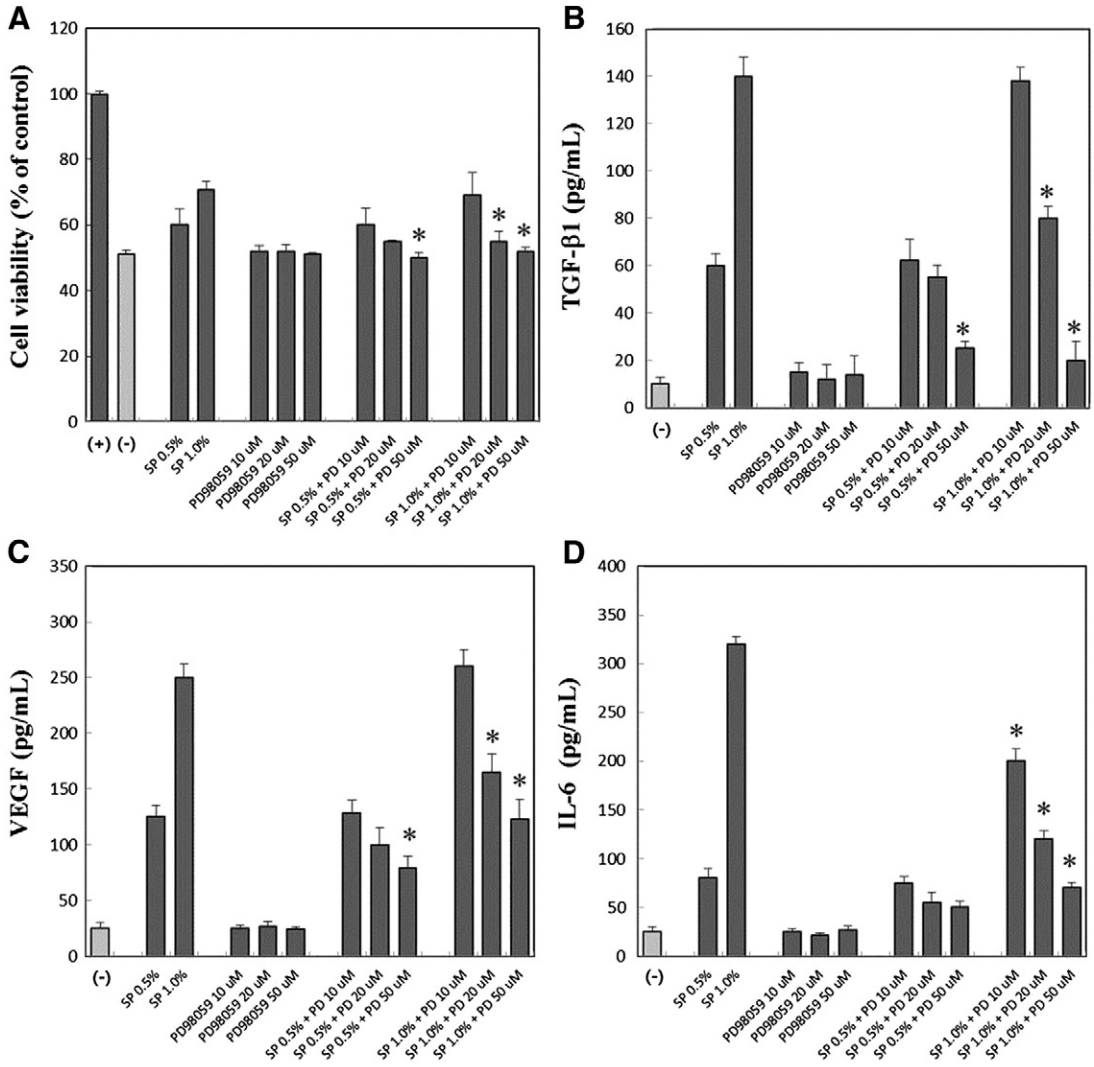


Fig. 6. Effect of PD98059 (ERK inhibitor) on the proliferation and cytokine secretion of ADSCs treated with soy peptides. ADSCs were grown in a combination culture medium that contained soy peptides (1%) or soy peptides (1%) with treated PD98059 (50 μM) for 3 days under serum-free conditions. The cell proliferation and cytokine analysis were then determined by MTT assay and using a MILLIPLEX Human cytokine kit, respectively. Mean values for each group were obtained from four samples (n=4). *P<.05.

properties of ASCs, we examined their cell cycle distribution. As shown in Fig. 2E and F, the DNA content in the S phase of the group treated with soy peptides increased when compared with the serum-free group (Fig. 2). Microscopic images also showed that the number of soy peptides-treated ASCs increased when compared to the untreated control (Fig. 2G, H). Broad bean-, kidney bean-, and papaic bean-derived peptides did not affect the stem cell proliferative effect under serum-free conditions (data not shown). These findings suggest that soy peptides could effectively contribute to the proliferation of ASCs under serum-free conditions. Table 1 represents the amino acid distribution (mg/g) of the soy peptides.

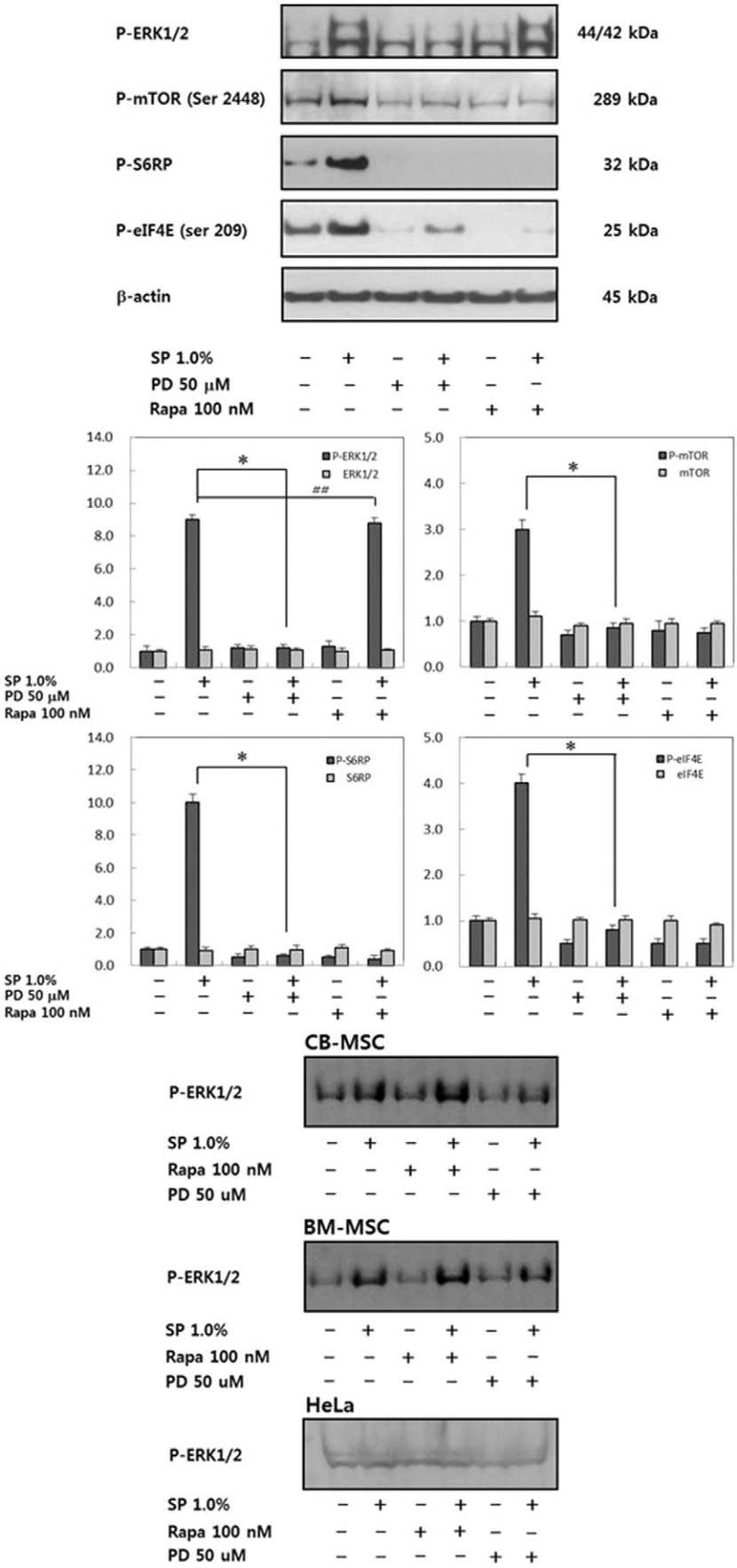
3.2. Soy peptides activate the mTOR signaling pathway and ERK

As previously mentioned, soy peptides significantly increased the proliferation of ASCs under serum-free condition. Next, to investigate the molecular mechanisms of soy peptides, we conducted immunoblot analysis using phospho-mTOR antibody in soy peptide-treated cell extracts. The mTOR is a central regulator of ribosome biogenesis, protein synthesis, cell growth and neurite plasticity. The mTOR kinase controls the translation machinery in response to amino acids and growth factors via activation of p70S6K and inhibition of eIF4E binding protein (4E-BP1) [16]. We found that soy peptides phosphorylate mTOR greatly at 5 min in ADSCs. Moreover, to further confirm the involvement of soy peptides in the mTOR signaling pathway, the phosphorylation of p70S6K and eIF4E was examined. Consistently, we found that soy peptides induced the phosphorylation of p70S6K, S6RP and eIF4E. ERK was also highly phosphorylated at 5 min in ADSCs (Fig. 3).

Raptor (regulatory associated protein of mTOR) binds mTOR substrates including 4E-BP1 and p70S6K through their TOR signaling motif and is required for mTOR-mediated phosphorylation of these substrates [17,18]. Raptor-containing mTOR complex 1 kinase complex is a critical component involved in the regulation of cell growth. Its activity is modulated by energy levels, growth factors and amino acids [19]. In this study, soy peptides phosphorylated raptor (Fig. 3), suggesting that raptor is a critical switch that correlates cell cycle progression with energy status.

3.3. Soy peptides secrete cytokines such as TGF-β1, VEGF and IL-6

Comparative studies have demonstrated that ASCs from different sources have comparable characteristics, even though their


P-ERK1/2
44/42 kDa
P-mTOR (Ser 2448)
289 kDa
P-S6RP
32 kDa
P-eIF4E (ser 209)
25 kDa
β-actin
45 kDa
SP 1.0%
PD 50 μM
Rapa 100 nM
P-ERK1/2
ERK1/2
P-mTOR
mTOR
P-S6RP
S6RP
P-eIF4E
eIF4E
CB-MSC
BM-MSC
HeLa
P-ERK1/2
SP 1.0%
Rapa 100 nM
PD 50 uM

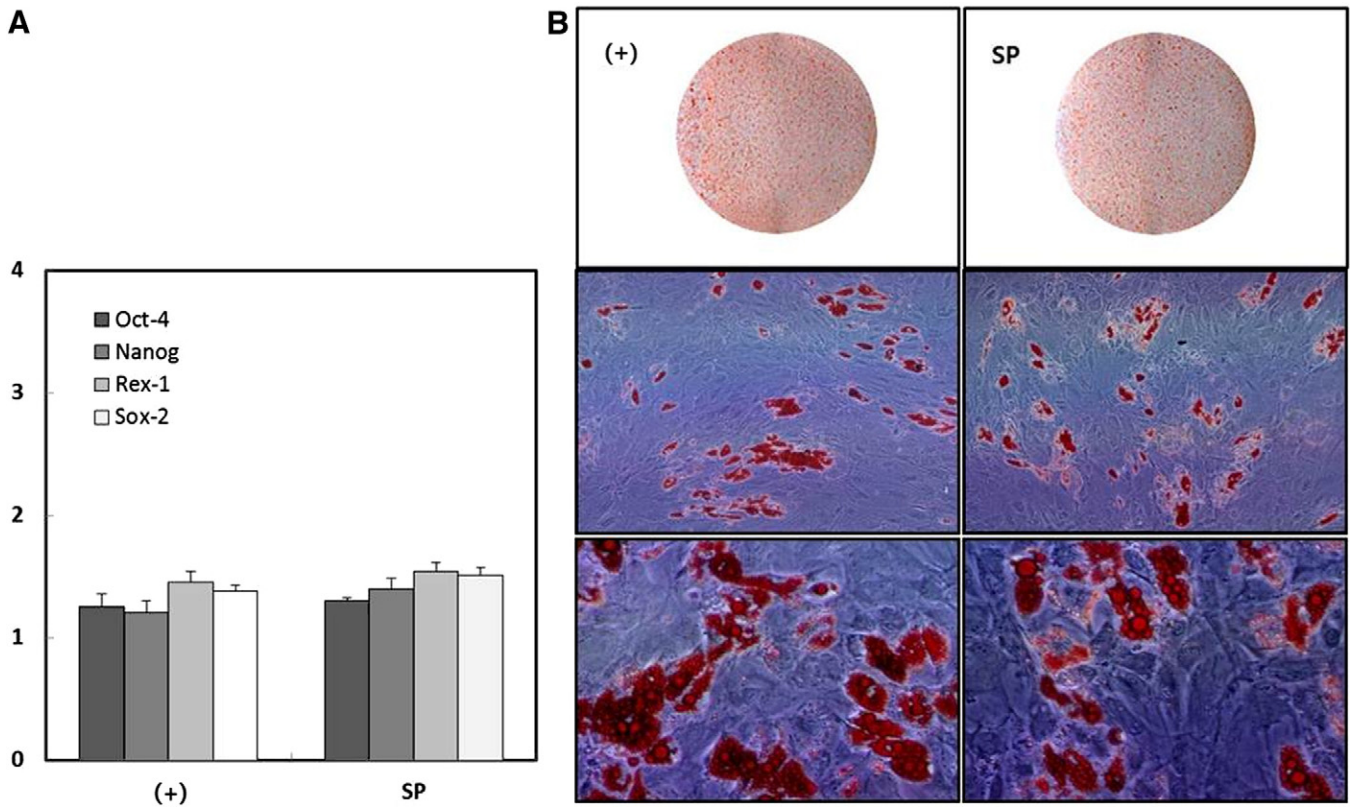


Fig. 8. Effect of soy peptides on the stem cell-like properties. ADSCs were grown in a combination culture medium containing soy peptides for 3 days. Total cellular RNA was extracted from the ADSCs with TRIzol reagent according to the manufacturer's instructions (Invitrogen). Real-time RT-PCR analysis was conducted using an ABI7900HT machine. Our data showed expression levels of the ADSC-related transcription factors Oct-4, Nanog, Rex-1 and Sox-2, which are all associated with stemness (A). Next, ADSCs that were grown in a combination culture medium containing soy peptides for 3 days were induced to undergo adipogenic differentiation. To induce adipogenesis, cells were plated at 4.0×10^4 cells/cm^2 and then incubated in the presence of adipocyte differentiation medium. The medium was changed every 3 days for 3 weeks. Adipogenesis was detected at day 21 based on the presence of Oil-red-O-stained cytoplasmic lipid droplets (B).

expression profiles differ for a large number of genes [20]. However, the regulatory mechanisms underlying the self-renewal and differentiation of ASCs are not well understood, which has limited the potential use of ASCs in practical applications such as tissue engineering and gene therapy [21]. Therefore, the involvement of cytokines in the soy peptide-induced proliferation of ASCs was investigated. Specifically, we evaluated changes in the cytokine expression profiles of ASCs in response to treatment with soy peptides using a MILLIPLEX Human cytokine kit.

We measured the levels of EGF, FGF-2, Flt-3 ligand, G-CSF, GM-CSF, IL-3, IL-6, PDGF-AA, PDGF-AB/BB, TNF-α, VEGF and TGF-β1 in ADSCs and CB-MSCs treated with soy peptides and found that the production of TGF-β1, VEGF, IL-6 and PDGF-AA was significantly up-regulated in response to treatment with soy peptides when compared to the untreated control in ASCs. However, treatment with soy peptides induced the production of 137 pg/ml (an increase of 22.95± 1.53-fold), 252 pg/ml (an increase of 15.70±1.49-fold), 321 pg/ml (an increase of 15.94±0.21-fold) and 97 pg/ml (an increase of 97.22± 11.62-fold) of TGF-β1, VEGF, IL-6 and PDGF-AA, respectively, in ADSCs (Fig. 4A). Moreover, soy peptides led to a significant increase in the production of TGF-β1, VEGF and IL-6 to 1210 pg/ml (an increase of 3.95±0.49-fold), 1822 pg/ml (an increase of 25.65±1.43-fold) and 1988 pg/ml (an increase of 29.38±1.47-fold), respectively, in CB-MSCs. However, the production of PDGF-AA by CB-MSCs was not affected by treatment with soy peptides (Fig. 4B). Moreover, no significant changes in other cytokines were observed in response to treatment with soy peptides in ADSCs or CB-MSCs. Taken together, these findings suggest that the stimulatory effect of soy peptides on the proliferation of ASCs may be mediated by TGF-β_1, VEGF, and IL-6.

3.4. Soy peptides activate the mTOR signaling pathway through ERK

In the present study, we found that mTOR signaling and cytokines (TGF-β_1, VEGF, and IL-6) are involved in the soy peptide-induced proliferation of ASCs. Therefore, the correlation between them was investigated. Using rapamycin (mTOR specific inhibitor), we found that soy peptides-induced cell proliferation of ASC was affected by rapamycin and that the production of TGF-β_1, VEGF, and IL-6 was not (Fig. 5A–D). These results suggest that the expression of these cytokines is independent of mTOR signaling or regulated by molecules upstream of mTOR signaling. It is well known that TGF-β_1 regulates the expression of VEGF and IL-6 and that TGF-β_1 expression is

Fig. 7. Effect of PD98059 (ERK inhibitor) on the protein expression of ADSCs treated with soy peptides. Cells were harvested and lysed with lysis buffer and protease inhibitor cocktail. Proteins were separated by SDS-PAGE and transferred to membranes. The membranes were incubated with primary phospho-ERK1/2, phospho-mTOR, phospho-S6RP and phospho-eIF4E antibodies overnight at 4°C and then washed and incubated with HRP-conjugated IgG secondary antibody. After washing, the immunoreactive proteins were detected by chemiluminescence. *Significant difference between SP and SP/PD conditions ($P<.05$). ##Expression of ERK1/2 is independent of mTOR signaling or regulated by molecules upstream of mTOR signaling. Protein bands were quantified by densitometry, and the level of the phosphorylated band was calculated for each time point after normalization in the same sample (A). To verify the specificity of these responses, Western blot for phospho-ERK1/2 was conducted in CB-MSCs, BM-MSCs and HeLa cervical cancer cell (B). Unstimulated basal expression (S/F) was set as unity.

dependent on ERK expression. Therefore, we examined the involvement of ERK in both cell proliferation and the expression of cytokines.

In this study, PD98059 (ERK specific inhibitor) blocked soy peptides-induced ASC proliferation and the secretion of major cytokines such as TGF-β1, VEGF, and IL-6 (Fig. 6A–D). The results of the present study suggest that ASC proliferation by treatment with soy peptides is linked to activation of the ERK. In addition, we found that PD98059 blocked the phosphorylation of ERK, p70S6K, S6RP and eIF4E that occurred in response to treatment with soy peptides. However, rapamycin did not affect ERK phosphorylation in response to treatment with soy peptides (Fig. 7A). Based on the results of this study, ERK might be important for soy peptide-induced proliferation of ASCs. To verify the specificity of these responses, the same experiments were conducted in CB-MSCs, BM-MSCs and HeLa cervical cancer cells (Fig. 7B). As shown in Fig. 7B, we found that similar events occurred in the CB-MSCs and BM-MSCs as in the ASCs, but not in HeLa cells, suggesting that soy peptide-induced responses are specific for mesenchymal stem cells. Therefore, the results presented here indicate that ERK-dependent production of TGF-β1 plays a crucial role in the soy peptide-induced proliferation of ASCs under serum-free conditions.

3.5. Effect of soy peptides on the stem cell-like properties

The experiments described to this point revealed that soy peptides induce the proliferation of mesenchymal stem cells. However, stem cell characteristics such as stemness and differentiation potential were not examined. Therefore, we examined the stemness and differentiation potential of soy peptide-treated stem cells using real-time PCR and Oil Red O staining assays.

As shown in Fig. 8A, the expression levels of stemness-related genes such as Oct-4, Nanog, Rex-1 and Sox-2 were not changed when compared to the untreated stem cells, suggesting that there are no significant changes in the stemness of soy peptide-treated stem cells. In addition, the adipogenic potential of soy peptide-treated stem cells was examined. As shown in Fig. 8B, similar to untreated stem cells, soy peptide-treated cells differentiated into adipocytes, suggesting that the adipogenic potential of soy peptide-treated stem cells is not affected by treatment with soy peptides. Collectively, our data suggest that soy peptides have the ability to induce proliferation of stem cells and maintain their differentiation potential.

4. Discussion

The mTOR pathway is emerging as an integrator of many signals that control cell growth and proliferation. Two multimeric serine/threonine kinase complexes, mTOR1 and mTOR2, are the central components of this pathway. Each of these complexes regulates a collection of distinct but complementary processes. mTOR1 is a sensor of the availability of nutrients such as glucose and amino acids, mitochondrial activity and growth factors. The complex regulates cell growth and metabolism by modulating processes such as protein synthesis, ribosome biogenesis and autophagy. mTOR2 responds to mitogenic and growth factor signals, which, in turn, influence cell survival and proliferation. Many of the downstream consequences of mTOR signaling are mediated by the AGC family members S6 kinase and Akt, which are phosphorylated and regulated by mTOR1 and mTOR2, respectively. Defects in both branches of mTOR signaling are increasingly associated with a variety of diseases, including cancer and diabetes [22]. The results presented here indicate that soy peptides are effective at inducing the proliferation of ASCs under serum-free conditions. Given the above findings, we analyzed the molecular mechanisms by which soy peptides exert their cell cycle-promoting effect and activate mTOR during the cell cycle orchestration of ASCs. Furthermore, we demonstrated that mTOR plays a pivotal role as an igniter in the control of cell cycling by the soy peptides in ASCs.

The raptor-mTOR complex positively regulates cell growth, and its inhibition causes a large decrease in cell size. In addition, S6K1 is a famous protein in the TOR field. The raptor-mTOR activates S6K1 and likely the related S6K2 by phosphorylating them within the hydrophobic motif conserved in the AGC family of kinases. Raptor has been identified as an mTOR binding partner that mediates mTOR signaling to downstream targets. mTOR controls cell growth, in part by regulating p70S6K and eukaryotic initiation factor 4E binding protein 1 (4EBP1). Raptor binds 4EBP1 and p70S6K. In addition, the binding of raptor to mTOR is necessary for the mTOR-catalyzed phosphorylation of 4EBP1 *in vitro* and strongly enhances the mTOR kinase activity toward p70S6K [23,24]. p70S6K is a mitogen-activated Ser/Thr protein kinase that is required for cell growth and G1 cell cycle progression [5,6]. Based on previous studies indicating the essential role of raptor-mTOR complex under serum-free conditions, our study was conducted to examine soy peptides for their ability to proliferate in ASCs of serum-free conditions. The results revealed that soy peptides phosphorylate raptor-mTOR and p70S6K. In addition, the DNA contents in the S phase of the group treated with soy peptides were up-regulated when compared with the serum-free group.

eIF4E binds to the mRNA cap structure, thereby mediating the initiation of translation. eIF4E interacts with eIF4G, which serves as a scaffold protein for the assembly of eIF4E and eIF4A to form the eIF4F complex [25]. eIF4B is thought to assist the eIF4F complex during the initiation of translation. Mnk1 has been shown to phosphorylate eIF4E at Ser209 *in vivo* upon activation by mitogenic or stress stimuli mediated by ERK and p38 MAPK [26]. Our results indicate that soy peptides phosphorylate ERK and eIF4E.

Among the various pathways that are known to be involved in proliferation, we suggest that soy peptides induce ASC proliferation by the mTOR signaling pathway through ERK-dependent production of TGF-β1 and subsequent production of VEGF and IL-6.

Of these proteins, VEGF is known to exert its proliferation-promoting functions via two tyrosine kinase receptors, VEGF-1 (Flt-1) and VEGF-2 (Flk-1/KDR) [27–30]. It has also been shown that VEGF is involved in the proliferation of endothelial progenitor cells and human stromal-vascular fraction cells [31–33], and VEGF has been shown to regulate endothelial cell proliferation and migration [34]. Furthermore, Gerber et al. [35] reported that VEGF regulates hematopoietic stem cell survival via an apparent autocrine loop mechanism. ADSCs release angiogenic factors, and it has been suggested that these factors contribute to their regenerative capability in ischemic injury models [36].

IL-6, which was also induced in cells that were treated with soy peptides, is reportedly involved in cell proliferation via activation of Janus-activated kinases and of signal transducers and activators of transcription (STAT) [37]. Activated STAT3 and MAPK induced by IL-6 are known to play a crucial role in cell proliferation [38,39]. Collectively, these findings indicate that VEGF and IL-6 are involved in the proliferation of cells induced by soy peptides. TGF-β1 is also involved in cell proliferation, although its effects are controversial and have been reported to be dose dependent [40]. However, recent studies have revealed that production of VEGF and IL-6 can be induced by TGF-β, which regulates a variety of cellular responses. For example, Jeon et al. [41] reported that TGF-β induces VEGF at the transcriptional and protein level in mouse macrophages. TGF-β1 is ubiquitous, and information regarding its indirect promotion of angiogenesis in different tissues has been described [42,43], although its effects may be influenced by the local environment [40]. Several studies have shown that TGF-β is involved in angiogenesis through the induction of VEGF in human tumor cells [44], mouse fibroblasts [45] and epithelial cells [46]. Furthermore, Pelaia et al. [47]

demonstrated that the increase in IL-6 secretion elicited by TGF-β1 may play an important role in its proliferative effect. In light of these reports, our results suggest that the stimulation of proliferation induced by soy peptides is mediated through the induction of TGF-β1.

In this study, we evaluated the proliferation-inducing effect and the possible mechanisms by which soy peptides elicit their activating effects on the proliferation of human ASCs. Collectively, the results of this study indicate that ERK-dependent production of TGF-β1 plays a crucial role in the soy peptide-induced proliferation of ASCs under serum-free conditions.

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