Osteogenic Effects of Triterpene Saponin Soyasapogenol B on Differentiation, Mineralization, Autophagy, and Necroptosis

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Research

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Abstract

**Background:** Triterpenoid saponins are a diverse group of natural compounds in plants. A triterpene saponin, Soyasapogenol B (SoyB), from *Arachis hypogaea* (peanut) has various pharmacological properties. This study aimed to elucidate pharmacological properties and mechanisms of SoyB on bone-forming cells.

**Methods:** Cell viability adhesion, and migration were analyzed using MTT assay, cell adhesion assay, and Boyden chamber assay. Osteogenic activity and osteogenicity were analyzed using alkaline phosphatase (ALP) staining and activity, and Alizarin Red S (ARS) staining. Cell signaling, protein expression, and autophagy were analyzed using Western blot analysis, immunofluorescence assay, and DAPGreen autophagy detection assay.

**Results and Conclusion:** In the present study, SoyB (> 99.99% purity), triterpene saponin, was isolated from the fruit of *A. hypogaea*. At concentrations ranging from 1 to 20 mM, SoyB showed no cell proliferation effects, whereas 30 - 100 mM SoyB increased cell proliferation in MC3T3-E1 cells. Next, osteoblast differentiation was analyzed and found that SoyB enhanced ALP staining and activity and bone mineralization as evidence for early and late osteoblast differentiation. SoyB also induced RUNX2 expression in nucleus with the increased phosphorylation of Smad1/5/8 and JNK2 during osteoblast differentiation. In addition, SoyB-mediated osteoblast differentiation was not associated with autophagy and necroptosis. Furthermore, SoyB increased cell migration and adhesion with the upregulation of MMP13 levels during osteoblast differentiation. The findings of this study provide new evidence that SoyB possesses biological effects on osteogenic activity and osteogenicity in bone-forming cells, and suggest a potentially beneficial role for peanuts foods and drugs containing SoyB in the treatment and prevention of bone diseases.

1. **Background**

Bone development, formation, and regeneration are regulated by complex process involving the differentiation of osteoblast derived from mesenchymal stem cells (MSCs) [1]. Osteoblast differentiation and migration are required for forming new bone and remodeling old bone through the synthesis and secretion of the organic component of the bone matrix and the bone matrix mineralization comprising hydroxyapatite [2–3]. The multiple signaling pathways associated with BMP2 and Wnt3a proteins tightly regulate osteoblast differentiation by upregulating the expression and activity of RUNX2 which is a core transcription factor in osteoblast differentiation [4–5]. Pathologically, the impairment and dysregulation of osteoblast differentiation lead to the pathogenesis in bone diseases such as osteoporosis and periodontitis [6–7]. Accordingly, the compounds targeting osteoblast differentiation are attracting attention as a promising strategy for the prevention and treatment of bone diseases.

Natural compounds obtained from plants have been widely used as traditional medicine in diseases, including osteoporosis [8]. A member of the legume family (*Fabaceae*), *Arachis hypogaea*, commonly
known as peanut, is widely cultivated as a major oilseed and food for human consumption worldwide [9]. *A. hypogaea* is also used as a traditional medicine with neuroprotective, anti-oxidative, and anti-obesity properties, and extracts of *A. hypogaea* contain bioactive compounds including triterpenoid saponins, isoflavones, resveratrol, and polyphenols [9–11]. Soyasapogenol B (SoyB), a triterpene saponin, has been reported for biological effects and mechanisms including anti-viral, anti-cancer, anti-inflammatory, and hepatoprotective activities [12–14]. Recently, it was reported that germinated soy germ with increased soyasaponin Ab protects osteoporosis [15]. However, the biological activity and mechanism of SoyB have not been reported in bone-forming cells yet.

In the present study, we isolated a pure triterpene saponin compound, SoyB (> 99.9% purity) from *A. hypogaea* fruits, and initially examined its pharmacological properties on cell proliferation in pre-osteoblast MC3T3-E1 cells. Subsequently, we investigated its effects on osteogenic activity, osteogenicity, autophagy, and necroptosis to demonstrate the underlying biological mechanisms.

2. Materials And Methods

2.1. General experimental procedures of Plant material

Organic solvents used for extraction and partition such as ethanol (EtOH), methanol (MeOH), *n*-hexane, ethyl acetate (EtOAc), and dichloromethane (CH₂Cl₂), were obtained from Duksan Chemical (Anseong, Korea). For determination of chemical structure, ¹H- and ¹³C-NMR instrumental analyses were performed. The spectra were recorded on a Jeol ECA-500 spectrometer (Jeol, Akishima, Japan) at 500 and 125 MHz for ¹H- and ¹³C-NMR, respectively. The chemical shifts were given in δ(parts permillion) from the internal standard substance, tetramethylsilane (TMS). The determination of high performance liquid chromatography (HPLC) spectrum were recorded on a Agilent 1200series (Agilent Technologis, USA) at photodiode array detector (PDA) and evaporative light scattering detector (ELSD).

2.2. Cell culture and osteoblast differentiation

Pre-osteoblast MC3T3E-1 cells (#CRL-2593, American type culture collection, Manassas, VA, USA) were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air using α-minimum essential medium (α-MEM) (WELGEME, Inc., Republic of Korea) without L-ascorbic acid (L-AA) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and 1 X Gibco® Antibiotic-Antimycotic (Thermo Fisher Scientific, Waltham, MA, USA). The osteoblast differentiation of MC3T3E-1 cells was induced by changing to osteogenic supplement medium (OS) containing 50 µg/ml L-AA and 10 mM β-glycerophosphate (β-GP). The OS was replaced every 2 days during the incubation period. 100% DMSO was used for dissolution of SoyB, and the vehicle control was used at a final concentration of 0.1% DMSO.

2.3. Cell proliferation assay
3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure cell viability as previously described [16]. Briefly, the cells were treated with MTT solution, incubated for 2 h, and then formazan was solubilized using 100% DMSO. Absorbance was detected at 540 nm using the Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Early and late osteogenic activity analyses

For early osteogenic activity analysis, the osteoblast differentiation of MC3T3E-1 cells was induced for 7 days, and alkaline phosphatase (ALP) staining and activity were performed as previously described [17]. Briefly, for ALP staining assay, the cells were incubated for 1 h at 37°C with ALP reaction solution (Takara Bio Inc., Tokyo, Japan) and the level of ALP staining was observed using a scanner and light microscope. For ALP activity assay, an alkaline phosphatase activity colorimetric assay kit was used and the ALP activity was quantitatively detected at 405 nm using the Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific).

For late osteogenic activity analysis, the differentiation was induced for 21 days, and Alizarin red S (ARS) staining assay was performed as previously described [17]. Briefly, cells were stained with 2% Alizarin red S (pH 4.2) (Sigma-Aldrich) for 10 min, ARS staining was observed using a scanner and light microscope, and then the staining level was quantitatively detected at 590 nm using the Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific).

2.5. Western blot analysis

Osteogenic-, autophagic-, and necroptotic- regulatory protein levels, and phosphorylation levels were analyzed using Western blot analysis as previously described [18–19]. Briefly, protein concentration was detected using Bradford reagent (Bio-Rad, Hercules, CA, USA). Equal lysates (20 µg) were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, polyvinylidene fluoride membrane (Millipore, Bedford, MA), 1 × TBS containing 0.05% Tween 20 (TBST), 5% skim milk. The specific primary antibodies were incubated overnight at 4°C and horseradish peroxidase-conjugated secondary antibodies (1:10,000, Jackson ImmunoResearch, West Grove, PA, USA) were incubated for 2 h at room temperature. Protein signals were detected in the ProteinSimple detection system (ProteinSimple Inc., Santa Clara, CA, USA).

2.6. Immunofluorescence

Immunofluorescence assay was performed as previously described [17]. Briefly, anti-RUNX2 antibodies (1:200, Cell Signaling Technology) were incubated overnight at 4°C and Alexa-Fluor 488-conjugated secondary antibodies (1:500, Invitrogen, Carlsbad, CA, USA) were incubated for 2 h at room temperature. Nuclei are stained using DAPI solution (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at room temperature. 8-well chamber slides (Thermo Fisher Scientific) were mounted using Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich).

2.7. Autophagosome formation assay
The formation of autophagosome was detected using the DAPGreen Autophagy Detection Kit (Dojindo, Japan) according to the manufacturer's instructions. Briefly, cells were incubated with 0.1 µM DAPGreen solution, washed using culture medium, and treated with SoyB. The slides were mounted and autophagosomes were observed under a fluorescence microscope.

2.8. Transmigration Assay

Transmigration Assay was performed using Boyden chamber as previously described [17]. Briefly, cells were incubated in the Boyden chamber with Matrigel (Corning Life Sciences, Tewksbury, MA, USA)-coated nuclear pore filters, fixed with 10% formalin, and stained with crystal violet. Transmigration was monitored using a light microscope.

2.9. Cell adhesion assay

Cell adhesion assay was performed as previously described [19]. Briefly, cells were seeded onto Matrigel (Corning Life Sciences)-coated 96 well culture plates and adherent cells were fixed with 10% formalin, and stained with crystal violet for 10 min. Cell adhesion was monitored using a light microscope.

2.10. Statistical analysis

The statistical significance was analyzed using a Student’s unpaired t test in Prism Version 5 program (GraphPad Software, Inc., San Diego, CA, USA). P < 0.05 was considered statistically significant. All data are presented as the mean ± standard error of the mean (S.E.M.).

3. Results

3.1. Isolation and identification of SoyB purified from A. hypogaea

The fruit of *Arachis hypogaea* (4 kg) was extracted MeOH (18 L, 3 times) at room temperature for 3 days. The crude extract (1,000 g) was suspended in distilled water (DW), and then solvent partitioned with CH₂Cl₂, EtOAc. The EtOAc soluble fraction was chromatographed on a silica gel column (Hexane : Acetone = 200 :1) eluted with a stepwise gradient. Fr. 12 was rechromatographed on a silica gel column (CH₂Cl₂: EtOAc = 10 : 1) to obtain 4 fraction (Fr. 12-1 ~ 12-4). Fraction 12-1 was recrystallized from MeOH to afford compound (140 mg). The isolation roadmap is summarized in Fig. 1A. The chemical structure was expected to by NMR analysis and identified as SoyB by comparison of the spectroscopic data with the previous literature [20]. El-MS m/z = 458.72 [M]+. ¹³C-NMR (125 MHz, CDCl₃) δ : 38.3 (C-1), 27.6 (C-2), 80.8 (C-3), 42.7 (C-4), 55.8 (C-5), 18.4 (C-6), 33.0 (C-7), 39.6 (C-8), 47.7 (C-9), 36.6 (C-10), 23.7 (C-11), 122.3 (C-12), 143.9 (C-13), 42.0 (C-14), 25.8 (C-15), 28.1 (C-16), 37.3 (C-17), 44.7 (C-18), 46.1 (C-19), 30.5 (C-20), 41.4 (C-21), 76.6 (C-22), 22.3 (C-23), 64.5 (C-24), 16.1 (C-25), 16.8 (C-26), 25.4 (C-27), 28.1 (C-28), 32.7 (C-29), 20.0 (C-30) (Fig. 1B). ¹H-NMR (500 MHz, CDCl₃) δ : 5.28 (1H, m, J = 3.7 Hz, H-12), 4.24 (1H, d, J = 11.2 Hz, H-22α), 3.50 (1H, overlap, H-3a), 3.48 (1H, overlap, H-24a), 3.39 (1H, t, J = 11.0 Hz, H-24b), 2.79 (1H, d, J = 7.5 Hz, H-18β), 0.91–1.29 (each 3H, s, 7×CH₃) (Fig. 1C). The HPLC chromatogram and
chemical structure of SoyB (white amorphous powder, molecular formula: $\text{C}_{30}\text{H}_{50}\text{O}_{3}$, purity: > 99.99%) are shown in Fig. 1D and E. To investigate cell proliferation effects of SoyB in MC3T3-E1 cells, the cells were treated with 1, 5, 10, 20, 30, 50, and 100 µM for 24, after which cell viability was analyzed using an MTT assay and found that 1-20 µM SoyB did not affect cell viability, but high concentrations (30 – 100 µM) significantly enhanced cell viability (1 µM: 106.7 ± 4.08; 5 µM: 107.2 ± 1.49; 10 µM: 111.2 ± 9.02; 20 µM: 115.1 ± 4.90; 30 µM: 120.9 ± 4.56; 50 µM: 123.3 ± 2.77; 100 µM: 116.2 ± 3.69) (Fig. 1F). We further investigated the osteogenic effect of SoyB at 1-10 µM with no effect on cell viability in MC3T3-E1 cells.

3.2. SoyB enhances osteogenic activity and maturation

To determine the osteogenic effect of SoyB, osteoblast differentiation was induced for 7 days in the absence and presence of SoyB, which was detected using ALP staining and found that SoyB increased ALP stains (Fig. 2A). The ALP-positively stained cells were also observed by light microscopy (Fig. 2B). Consistent with the results, it was validated that SoyB significantly increased ALP enzyme activity in a dose-dependent manner (Fig. 2C). Next, we determine whether SoyB influences the mineralization of the bone matrix from mature osteoblasts. Osteoblast differentiation was induced for 21 days to generate mature osteoblast in the absence and presence of SoyB. ARS staining assay was performed and the results revealed that SoyB significantly increased ARS stains (Fig. 2D and E). Light microscopy observation also demonstrated that SoyB increased the mineralized nodule formation from mature osteoblasts (Fig. 2F). These data suggest that SoyB promotes the early and late osteoblast differentiation.

3.3. SoyB enhances the nuclear expression of RUNX2 and phosphorylation of Smad1/5/8 and JNK

To determine the molecular mechanism underlying the osteogenic effects of SoyB, RUNX2 expression was investigated since RUNX2 is a core transcriptional factor for osteoblast differentiation. Western blot analysis revealed that SoyB enhanced the expression of RUNX2 (Fig. 3A). It was further observed the expression of RUNX2 in the nucleus after the treatment with SoyB using Immunofluorescence assay. The results demonstrated that SoyB increased the RUNX2-positively stained cells in the nucleus (Fig. 3B). SoyB treatment had no effects on bone morphogenetic protein 2 (BMP2), Wnt3a, and β-catenin, whereas SoyB increased the phosphorylation of Smad1/5/8 (Fig. 3C). Next, we examined whether SoyB affects Mitogen-activated protein kinases (MAPKs) signaling. SoyB did not affect the phosphorylation of ERK1/2 and p38, whereas SoyB increased the phosphorylation of JNK. These data suggest that SoyB enhances osteoblast differentiation through RUNX2 expression with the activation of Smad1/5/8 and JNK.

3.4. SoyB does not influences autophagy and necroptosis during osteoblast differentiation
To determine whether SoyB affects autophagy during osteoblast differentiation, the expression and conversion of microtubule-associated protein light chain 3 (LC3) were detected since LC3 is a widely used marker to monitor autophagy. Western blot analysis revealed that SoyB had no discernible effect on LC3A/B expression and LC3A/BII conversion (Fig. 4A). DAPGreen was used to observe the autophagosome formation using immunofluorescence assay. As shown in Fig. 4B and C, SoyB also had no significant effect on the autophagosome formation. In addition, molecular machinery of necroptosis was monitored and the results revealed that SoyB did not discernibly affect the phosphorylation of receptor-interacting serine/threonine-protein kinase (RIP) and mixed lineage kinase domain like pseudokinase (MLKL) (Fig. 4D). These data indicate that the osteogenic effects of SoyB are unrelated to autophagic flux and necroptosis.

3.5. SoyB enhances adhesion and cell transmigration during osteoblast differentiation

We subsequently investigated whether SoyB affect cell adhesion during osteoblast differentiation on Matrigel-coated 96-well culture plates. We found that SoyB increased the number of adherent cells with morphological stabilization, compared to the control and OS alone (Fig. 5A). Next, transmigration assay was performed using the Matrigel-coated polycarbonate filter in the Boyden chamber, and the results revealed that SoyB significantly increased cell penetration, compared to the control and OS alone (Fig. 5B and C). In addition, SoyB increased the expression of matrix metalloproteinase 13 (MMP13), which is an important role in the degradation of components of the extracellular matrix and is required for bone remodeling and repair (Fig. 5D). Overall, these data suggest that SoyB is an enhancer for osteoblast differentiation, osteogenic activity, and maturation.

4. Discussion

*A. hypogaea* is widely used in foods and nutritional support. Most of the bioactive compounds (catechin, anthocyanidins, oleic acid, procyanidins, and epicatechin) from *A. hypogaea* have been associated with reduced risk of cardiovascular diseases and cancers [21]. The present study is the first to demonstrate that SoyB purified from *A. hypogaea* enhances osteoblast differentiation by inducing osteogenic activity and bone matrix mineralization without apparent autophagy and necroptosis. Osteoblast differentiation and its maturation are required for bone formation and remodeling as well as for bone repair process. ALP which is a marker for early osteoblast differentiation is a critical enzyme in osteogenic activity [22–24]. In the present study, we found that SoyB increased ALP enzyme activity and ALP staining level for early osteoblast differentiation. Moreover, SoyB promotes bone matrix mineralization during late osteoblast differentiation. As well established, inorganic pyrophosphate and organic phosphomonoesters are hydrolyzed by ALP enzyme activity leading to the synthesis of hydroxyapatite which is provided for bone matrix mineralization [22, 25]. It was reported that ALP knockout mice show spontaneous fractures, skeletal deformations, and areas of hypomineralization [25], thereby indicating that SoyB increases osteogenic activities to promote bone matrix mineralization from pre-osteoblasts.
RUNX2 is a core transcription factor that regulates gene expressions including ALP for the osteoblast differentiation and bone matrix mineralization [26–27]. Based on the role of RUNX2 on ALP expression and osteoblast differentiation, we investigated RUNX2 expression and demonstrated that SoyB increases RUNX2 expression in the nucleus. It was well known that RUNX2 expression is controlled by BMP2/Smad1/5/8 and Wnt3a/β-catenin signals. In the present study, we demonstrated that SoyB has no effects on BMP2 and Wnt3a/β-catenin. However, we found that SoyB stimulates the phosphorylation of Smad1/5/8 that is the downstream molecules of BMP2, and also the phosphorylation of JNK but not ERK1/2 and p38. As well established, MAPKs play important roles in the regulation of RUNX2 protein and also the major pathway to induce ALP expression is the Smad1/5/8 and RUNX2 signaling [28–31]. These findings indicate that SoyB induces RUNX2 expression through the activation of Smad1/5/8 and JNK to promote osteoblast differentiation and maturation.

Gradually, osteogenic studies appeared that autophagy is involved in osteoblast differentiation. It was reported that rapamycin, an autophagic inducer, promotes osteoblast differentiation [32]. Deletion of focal adhesion kinase family interacting protein of 200 kDa, an essential component of autophagy shows a significant decrease in bone mass and osteoblast differentiation [33]. Kaempferol, a flavonoid, induces autophagy to promote osteoblast differentiation and mineralization [34]. Vitamin K2-induced autophagy stimulates osteoblast differentiation and mineralization [35]. Previously, it was reported that SoyB regulates autophagy in colorectal cancer [36]. In the present study, we investigated the role of SoyB on autophagy and demonstrated that SoyB is not involved in the autophagic system in osteoblast differentiation. Next, we investigated a possible role of SoyB on necroptosis. Studies have revealed that tumor necrosis factor-a and ROS stimulates necroptosis in osteoblasts, and also chronic ethanol consumption activates the necroptotic signaling to stimulate osteoblast necroptosis, resulting in decreases in osteoblast differentiation and bone formation [37–39]. In the present study, we found that SoyB is not involved in the necroptotic system in osteoblast differentiation. Therefore, our data suggest that SoyB induces osteogenic effects through the activation of Smad/15/8 and JNK and the expression of RUNX2, regardless of autophagy and necroptosis.

The migration and adhesion are required for bone formation, and bone repair. It was reported that MMP13 production in osteoblasts is involved in degradation and remodeling of extracellular matrix (ECM) during bone repair and also is considered to play an important role in bone repair [40–41]. Toriseva et al reported that the activity of MMP-13 may affect cell adhesion on matrix and on adjacent cells [42]. Cell adhesion is closely involved in the initiation and progression of cell proliferation and differentiation [43–47]. In the present study, we demonstrated that SoyB stimulates cell adhesion on ECM as well as cell migration across ECM with the induction of MMP13. Thus, our findings provide convincing evidence that SoyB is a regulator of osteogenic activities by enhancing osteoblast adhesion, migration, and subsequent differentiation.

5. Conclusion
In conclusion, to the best of our knowledge, this is the first study to report that SoyB promotes the adhesion and migration of pre-osteoblasts and subsequently stimulates osteoblast differentiation through the osteogenic Smad1/5/8 and JNK pathway and RUNX2 expression, regardless of autophagy and necroptosis. Our findings provide convincing evidences that SoyB has the potential to be useful for drug development that modulates osteoblast differentiation and osteogenic activities in bone diseases.

**Abbreviations**

ALP  
Alkaline phosphatase  
ARS  
Alizarin Red S  
\( \beta \)-GP  
\( \beta \)-glycerophosphate  
ECM  
Extracellular matrix  
L-AA  
L-ascorbic acid  
LC3  
Microtubuleassociated protein light chain 3  
MAPKs  
Mitogen-activated protein kinases  
MMP  
Matrix metalloproteinase  
MSCs  
Mesenchymal stem cells  
MTT  
3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide  
OS  
Osteogenic supplement medium  
RUNX2  
Runt-related transcription factor 2  
SoyB  
Soyasapogenol B

**Declarations**

- **Ethical Approval and Consent to participate**

Not applicable
• **Consent for publication**

Not applicable

• **Availability of supporting data**

Not applicable

• **Competing interests**

The authors declare that they have no competing interests

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• **Authors’ contributions**

Conceptualization, K.-R.P. and H.-M.Y.; methodology, K.-R.P. and J.Y.L.; software, K.-R.P.; validation, K.-R.P.; formal analysis, K.-R.P.; investigation, K.-R.P.; resources, J.Y.L., S.H.K., and I.K.K.; data curation, K.-R.P.; writing—original draft preparation, K.-R.P.; writing—review and editing, K.-R.P., I.K.K., and H.-M.Y.; visualization, K.-R.P.; supervision, H.-M.Y.; project administration, H.-M.Y.; funding acquisition, I.K.K. and H.-M.Y. All authors read and approved the final manuscript.

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**Figures**
Isolation of SoyB from fruits of *A. hypogaea*, and the effect of SoyB on cell proliferation. (A - C) Isolation roadmap of SoyB from fruits of *A. hypogaea* (A), and $^{13}$C NMR (B) and $^1$H NMR (C) spectra (D, E) HPLC chromatogram, 99.9% purity, (D) and chemical structure (E) of SoyB. (F) After MC3T3-E1 cells were seeded and treated with SoyB at concentrations of 1, 5, 10, 20, 30, 50, and 100 mM for 24 h, cell viability
Effects of SoyB on early and late osteogenic activities. (A - C) After MC3T3-E1 cells were cultured in OS with SoyB at concentrations of 0, 1, or 10 mM for 7 days, early osteogenic activities were detected using
ALP staining assay (A) and ALP enzyme activity (C) assays, and the individual ALP-stained cells were visualized under a light microscope (B) Scale bar: 50 mm. (D - F) Late osteogenic activities were detected using ALP staining assay at 21 days (D), the ALP staining was quantitatively measured using a spectrophotometer (E), and the mineralization visualized under a light microscope (F). Data are presented as the mean ± S.E.M. from three independent experiments. *\( p < 0.05 \) was considered statistically significant compared to the control. #\( p < 0.05 \) was considered statistically significant compared to OS.
Figure 3

Osteogenic effects of SoyB on the RUNX2 expression and upstream molecules. (A, B) Total RUNX2 expression levels were assessed by Western blot analysis (A), and nuclear RUNX2 expression and localization were determined using RUNX2 (green) and DAPI (blue) staining and were observed under a confocal microscope (B). Scale bar: 50 mm. (C, D) Equal amounts were probed using antibodies against Wnt3a, BMP2, b-catenin, phospho-Smad1/5/8 (p-Smad1/5/8), b-actin (C), phospho-ERK1/2 (p-ERK1/2), ERK1/2, phospho-JNK (p-JNK), JNK, phospho-p38 (p-p38), and p38 (D). Data represent the results of three independent experiments.
Figure 4

Osteogenic effects of SoyB on autophagic flux and necroptosis. (A - C) LC3A/Bi and LC3A/BII levels were assessed by Western blot analysis (A), autophasome formation was analyzed using DAPGreen Autophagy detection assay (B), and the intensity (fold) was shown as a bar graph (C). (D) Phospho-MLKL (p-MLKL), MLKL, phospho-RIP (p-RIP), RIP, and b-actin levels were assessed by Western blot analysis. Data are presented as the mean ± S.E.M. from three independent experiments. NS: not significant.
Figure 5

Osteogenic effects of SoyB on cell adhesion and transmigration. (A) SoyB-treated cells were seeded onto Matrigel-coated plates, fixed, and stained at 120 min. Cell adhesion was visualized under a light microscope. Magnified regions were indicated by red arrow. Scale bar: 50 mm. (B, C) Transmigration of SoyB-treated cells was observed using a light microscope (B), and the numeric values (%) were shown as a bar graph. Scale bar: 50 mm. (D) MMP-13 levels were assessed by Western blot analysis. *, p < 0.05
was considered statistically significant compared to the control. #, $p < 0.05$ was considered statistically significant compared to OS.