Received: 23 March 2022 | Accepted: 3 July 2022

DOI: 10.1002/biof.1878

RESEARCH COMMUNICATION

Biofactors WILEY

Suffruticosol A elevates osteoblast differentiation targeting BMP2-Smad/1/5/8-RUNX2 in pre-osteoblasts

Hyung-Mun Yun[1] | **Bomi Kim**[2] | **Yun Hee Jeong**[2] | **Jin Tae Hong**[3] | **Kyung-Ran Park**[4]

[1]Department of Oral and Maxillofacial Pathology, School of Dentistry, Kyung Hee University, Seoul, Republic of Korea

[2]National Development Institute of Korean Medicine, Gyeongsan, Republic of Korea

[3]College of Pharmacy and Medical Research Center, Chungbuk National University, Chungbuk, Republic of Korea

[4]Gwangju Center, Korea Basic Science Institute (KBSI), Gwangju, Republic of Korea

Correspondence
Jin Tae Hong, College of Pharmacy and Medical Research Center, Chungbuk National University, Osongsaengmyeong 1-ro 194-31, Osong-eup, Heungduk-gu, Cheongju, Chungbuk 28160, Republic of Korea.
Email: jinthong@chungbuk.ac.kr

Kyung-Ran Park, Gwanju Center, Korea Basic Science Institute (KBSI), 49, Dosicheomdansanup-ro, Nam-gu, Gwangju, 61751, Republic of Korea.
Tel:+82-62-712-4412,
Fax:+82-62-372-4102,
Email: krpark83@kbsi.re.kr

Funding information
National Research Foundation of Korea (NRF), Korea government (MSIP), Grant/Award Numbers: MRC 2017R1A5A2015541, NRF-2022R1C1C1003491

Abstract

The *Paeonia suffruticosa* ANDR. (*P. suffruticosa*) is commonly used in traditional medicine for various purposes. Suffruticosol A (Suf-A), isolated from *P. suffruticosa*, is a beneficial compound with antibiofilm, antivirulence, and anti-inflammatory properties. The aim of the present study was to investigate the biological effects of Suf-A on osteogenic processes in pre-osteoblasts. It was determined here in that Suf-A (>98.02%), isolated from *P. suffruticosa*, showed no cytotoxicity at 0.1–30 μM; however, it induced cytotoxicity at 50–100 μM in pre-osteoblasts. Suf-A increased osteogenic alkaline phosphatase activity and expression levels of noncollagenous proteins. Adhesion and trans-migration on the extracellular matrix were potentiated by Suf-A, but not by wound-healing migration. Suf-A did not affect autophagy or necroptosis during osteoblast differentiation. We found that Suf-A increased runt-related transcription factor 2 (RUNX2) levels and mineralized matrix formation. RUNX2 expression was mediated by Suf-A-induced BMP2-Smad1/5/8 and mitogen-activated protein kinase signaling, as demonstrated by Noggin, a BMP2 inhibitor. These results suggest that Suf-A is a potential natural osteogenic compound.

KEYWORDS

P. suffruticosa, pre-osteoblasts, RUNX2, Suffruticosol A

Abbreviations: ALP, alkaline phosphatase; ARS, Alizarin Red S; BSP, bone sialoprotein; CC, column chromatography; DW, distilled water; ECM, extracellular matrix; L-AA, L-ascorbic acid; MAPKs, mitogen-activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; OCN, osteocalcin; OPN, osteopontin; OS, osteogenic supplement; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; RUNX2, runt-related transcription factor 2; Suf-A, suffruticosol A; TBS, Tris-buffered saline; TNF-α, tumor necrosis factor-α.

1 | INTRODUCTION

Paeonia suffruticosa ANDR. (*P. suffruticosa*) has been found to yield various compounds, including monoterpenes, paeonol, and phenolic compounds.[1–4] Suffruticosol A (Suf-A), a methanolic extract from the *P. suffruticosa* fruit, has been shown to exert substantial antieffects on stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) free radicals.[5] Recently, Suf-A from *Paeonia lactiflora* seed was shown to suppress pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and IL-1β, and harmful inflammation in cigarette smoke- and LPS-treated mice.[6] Natural stilbenes and their derivatives have been demonstrated to impart beneficial properties, including antioxidative, anticancer, anti-inflammatory, and anti-osteoporosis activities.[7–10] To date, the osteogenic properties of Suf-A, purified from the *P. suffruticosa* fruit, have not been demonstrated effectively.

Skeletal tissues undergo ongoing renewal to maintain healthy bone. This is accomplished by physiological processes of bone formation, which are minutely controlled by multiple cellular pathways.[11,12] Cellular events are mainly activated by key proteins, including BMPs and Wnts, in mesenchymal stem cells and pre-osteoblasts, allowing them to differentiate into osteoblasts.[12–15] Osteoblasts are specialized bone-forming cells that play essential roles in osteogenic protein synthesis and bone matrix mineralization; conversely, abnormal osteogenic processes cause aberrant bone formation, which is linked to skeletal fragility and disorders, such as periodontal bone loss and osteoporosis.[16,17] Therefore, anabolic drugs against skeletal disorders are of interest for regulating osteoblast differentiation and function.[18,19] Consequently, it is critical to investigate and discover prospective compounds to prevent and cure skeletal disorders.

In the present study, Suf-A was extracted from the *P. suffruticosa* fruit (>98.02% purity), and its effects on osteoblast differentiation, behavior, and bone-forming biological activities were demonstrated.

2 | EXPERIMENTAL PROCEDURES

2.1 | Plant material and extraction

Dried fruits of *P. suffruticosa* were purchased from a commercial herbal medicine market. The P836 voucher specimen was deposited at the Natural Products Bank of the National Institute for Korean Medicine Development. Dried fruits (130.0 g) were extracted using MeOH (2 L, 1×) at 50°C for 8 h in a water bath. Crude extracts (40.0 g) were suspended in distilled water and organic solvents (methylene chloride, ethyl acetate, and *n*-butanol). The EtOAc-soluble fractions (3.0 g) were subjected to Sephadex LH-20 column chromatography (CC) and eluted with a gradient of MeOH 100%. Fraction 2 (1.0 g) was further purified by ODS-A CC and eluted with a MeOH-H_2O gradient solvent system (45:55 to 0:100, v/v). Fraction 21 (350 mg) was further purified on a Lobar-A Lichroprep Si-60 CC and eluted with a $CHCl_3$-MeOH-H_2O gradient solvent system (40:10:1, v/v) to obtain 70 mg Suf-A (brown powder; molecular formula: $C_{42}H_{32}O_9$).

2.2 | Pre-osteoblast MC3T3-E1 cells

Pre-osteoblast MC3T3-E1 cells (ATCC, Manassas, VA, USA) were cultured in α-Minimum essential medium (*α*-MEM) (without L-ascorbic acid [L-AA]) containing 1 × Gibco® antibiotic–antimycotic (Thermo Fisher Scientific, Waltham, MA, USA) and 10% FBS at 37°C, 95% air, and 5% CO_2. Differentiation of the pre-osteoblasts was generated in osteogenic supplement (OS) medium containing 10% FBS, 1 × Gibco® antibiotic–antimycotic, L-AA (50 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA), and β-glycerophosphate (β-GP, 10 mM) (Sigma-Aldrich), as previously described.[20]

2.3 | Cell viability

Cell viability was assessed using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich), as previously described.[21] Briefly, cells were seeded in 96-well plates and treated with Suf-A for 24 h, and then formazan formation was measured spectrophotometrically (540 nm) using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific) after dissolution in 100% Dimethyl sulfoxide (DMSO).

2.4 | Alkaline phosphatase and Alizarin Red S

Osteoblast differentiation was induced in OS with Suf-A, and alkaline phosphatase (ALP) staining intensity and activity were measured at 7 days using a substrate solution for the ALP reaction (Takara Bio Inc) and an ALP activity kit (Biovision, Milpitas, CA, USA), respectively, as previously described.[22] Osteoblast differentiation was induced in OS with Suf-A for 21 days, and Alizarin Red S (ARS) staining was measured using 2% ARS (pH 4.2; Sigma-Aldrich) to detect matrix mineralization, as previously described.[20]

2.5 | Reverse transcription polymerase chain reaction

Osteoblast differentiation was induced in OS with Suf-A for 7 days, and total RNA was extracted using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). cDNA was synthesized and amplified using RT PreMix (AccuPower, Bioneer Corporation, Daejeon, South Korea) and polymerase chain reaction (PCR) PreMix (AccuPower, Bioneer Corporation). Reverse transcription PCR (RT-PCR) was performed using an AllinOneCycler™ Thermal Block (Bioneer Corporation), and the PCR products were detected under ultraviolet exposure (ProteinSimple Inc., Santa Clara, CA, USA).

Primer	Sequences
Alp	*F*: 5′-ACACCTTGACTGTGGTTACTG-3′
	R: 5′-CCATATAGGATGGCCGTGAAG-3′
Bsp	*F*: 5′-TGTTTGTAGTGGGCTTCTTCTT-3′
	R: 5′-TCCATCTAGTCCCAGCTCATAG-3′
Ocn	*F*: 5′-ACACCATGAGGACCATCTTTC-3′
	R: 5′-CGGAGTCTGTTCACTACCTTATT-3′
Opn	*F*: 5′-GAGGTGATAGCTTGGCTTATGG-3′
	R: 5′-TCCTTAGACTCACCGCTCTT-3′
β-Actin	*F*: 5′-AATGTGGCTGAGGACTTTG-3′
	R: 5′-GGGACTTCCTGTAACCACTTATT-3′

2.6 | Adhesion and trans-migration on the extracellular matrix, and wound healing

Osteoblast differentiation was induced in OS with Suf-A for 48 h, and adhesion and trans-migration were performed using Matrigel solution (Corning Life Sciences, Tewksbury, MA, USA), as previously described.[20,23] Briefly, adherent cells were fixed in 10% formalin for 15 min and stained with 0.5% crystal violet for 10 min. The absorbance was measured at a wavelength of 540 nm using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific). After incubation in the Boyden chamber for 4 h, the cells were fixed with 10% formalin for 15 min and stained with 0.5% crystal violet for 10 min. Migration was counted under a light microscope.

Wound healing was performed as previously described.[20] Briefly, monolayers were scratched with a 200-μl tip, cell debris was removed with 1× phosphate buffered saline (PBS) , and osteoblast differentiation was induced in OS with Suf-A for 48 h. Wound healing was observed under a light microscope.

2.7 | Western blotting

Western blotting was performed as previously described.[24] Briefly, 20 μg of protein, measured using Bradford reagent (Bio-Rad, Hercules, CA, USA), was resolved by sodium dodecyl-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 1× Tris-buffered saline (TBS) for 1 h at room temperature. The membranes were incubated overnight at 4°C with primary antibodies, washed with 1× TBS with Tween 20 (TBST), and incubated with secondary antibodies (1:10,000, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. After the membranes were washed with 1 × TBST, protein bands were detected using an enhanced chemiluminescence kit (Millipore). The antibodies used are as follows: p-ERK1/2 (1:1000, #9101S, Cell Signaling Technology, Beverly, MA, USA), ERK1/2 (1:2000, #9102, Cell Signaling Technology), p-JNK (1:1000, #9251, Cell Signaling Technology), JNK (1:1000, #9252, Cell Signaling Technology), p-p38 (1:1000, #9211, Cell Signaling Technology), p38 (1:1000, #9212, Cell Signaling Technology), RUNX2 (1:1000, #12556, Cell Signaling Technology), BMP2 (1:500, #CSB-PAO9419AORb; CUSABIO, Houston, TX, USA), β-catenin (1:1000, #8480, Cell Signaling Technology), p-GSK3β (1:1000, #9336, Cell Signaling Technology), GSK3β (1:1000, #12456, Cell Signaling Technology), p-Smad1/5/8 (1:2000, #13820, Cell Signaling Technology), Wnt3a (1:1000, #2721; Cell Signaling Technology), β-actin (1:1000, #sc-47778, Santa Cruz Biotechnology), Beclin1 (1:1000, #3495, Cell Signaling Technology), LC3A/B (1:1000, #12741, Cell Signaling Technology), p62 (1:1000, #5114, Cell Signaling Technology), p-MLKL (1:1000, #91689, Cell Signaling Technology), MLKL (1:1000, #14993, Cell Signaling Technology), p-RIP3(1:1000, #93654, Cell Signaling Technology), and RIP (1:1000, #13526, Cell Signaling Technology).

2.8 | Autophagic and necroptotic signaling detection

Osteoblast differentiation was induced in OS with Suf-A for 24 h, and the total protein concentration was determined using Bradford reagent (Bio-Rad). Autophagic signaling proteins (Beclin-1, LC3A/B, and p62) and necroptotic signaling proteins (p-MLKL, MLKL, p-RIP3, RIP3) were detected using western blotting.

2.9 | Immunocytochemistry

Immunocytochemistry was performed as previously described.[20] Briefly, osteoblast differentiation was induced in OS with Suf-A for 3 days, fixed, permeabilized, and blocked with 3% bovine serum albumin (BSA) diluted in 1× PBS for 1 h. Anti-RUNX2 antibody (1:200 dilution, Cell Signaling Technology) and Alexa Fluor 568-secondary antibody (1:500 dilution, Invitrogen, Carlsbad, CA, USA) were used to detect RUNX2 levels. 4′,6-diamidino-2-phenylindole (DAPI) solution (Sigma-Aldrich) was used for the nuclear staining.

2.10 | Statistical analysis

GraphPad Software (Version 5) was used to investigate the data (San Diego, CA, USA). One-way analysis of variance was performed to determine statistical significance ($p < 0.05$). Data were provided as the mean ± SEM.

3 | RESULTS

3.1 | Purification of Suf-A and its activities on the viability and differentiation in pre-osteoblasts

The chemical structure, high-performance liquid chromatography (HPLC), ^{1}H-NMR, and ^{13}C-NMR spectra of Suf-A (brown powder, $C_{20}H_{24}O_5$, >98.02% purity), purified from the dried fruit of *P. suffruticosa*, are shown in Figure 1A–D. The procedure for purifying Suf-A from 130 g *P. suffruticosa* is shown in Figure 1E. First, the effects of Suf-A on cell viability were investigated in pre-osteoblasts. The MTT assay showed that Suf-A did not affect cytotoxic activities at 0.1–30 μM, but not 50 and 100 μM, against pre-osteoblasts (Figure 2A). Next, to investigate the osteogenic activities of Suf-A, the staining pattern and activity of ALP were analyzed 7 days after the early differentiation induction; the obtained results showed that Suf-A significantly stimulated early osteoblast differentiation, compared with OS (Figure 2B,C). It was further demonstrated that Suf-A-stimulated differentiation enhanced the expression of osteogenic genes, including bone sialoprotein (*Bsp*), osteocalcin (*Ocn*), osteopontin (*Opn*), and *Alp*, compared with the corresponding expression levels after treatment with OS (Figure 2D).

3.2 | Suf-A induced adhesion and trans-migration during differentiation of pre-osteoblasts

Adhesion, wound-healing, and trans-migration assays were performed to analyze whether Suf-A affects osteoblast-mediated bone formation. First, adhesion experiments were performed on Matrigel-coated plates, and the results revealed that Suf-A facilitated cell adhesion to the extracellular matrix (ECM), compared with OS (Figure 3A,B). Second, wound-healing experiments were performed on the wounded monolayers. As shown in Figures 3A,B, Suf-A did not affect wound-healing migration toward the wound region compared with OS (Figure 3C,D). Trans-migration experiments revealed that Suf-A enhanced trans-migration across Matrigel-coated membranes compared with OS, suggesting that Suf-A stimulates trans-migration due to increased adhesion on the ECM.

3.3 | Suf-A enhanced RUNX2 expression and bone matrix mineralization

To examine the biological activities of Suf-A on the main RUNX2 transcription factor involved in osteoblast differentiation, RUNX2 expression was investigated using western blotting. Suf-A-stimulated osteoblast differentiation increased the RUNX2 protein level compared with the corresponding protein level after treatment with OS (Figure 4A). Consistent with these results, immunocytochemical experiments showed that Suf-A enhanced RUNX2 accumulation in the nucleus (Figure 4B). To validate the effects of Suf-A on osteoblast differentiation, matrix mineralization generated by osteoblast maturation was assessed using ARS experiments. The results revealed that Suf-A significantly stimulated mineralized formation compared with OS (Figure 4C). These observations were also confirmed by mineralization quantification, compared with OS (Figure 4D).

3.4 | Suf-A-facilitated RUNX2 expression was mediated by BMP2 signaling, regardless of Wnt3a-, autophagic- and necroptotic signaling

To further examine the biological mechanism of RUNX2 expression, osteoblast differentiation was induced in OS with Suf-A for 48 h, and upstream signaling molecules associated with RUNX2 expression were analyzed using western blotting. The obtained results showed that Suf-A stimulated BMP2 levels and Smad1/5/8 phosphorylation, but not GSK3β phosphorylation and β-catenin levels, compared with OS (Figure 5A). It was investigated the mitogen-activated protein kinases (MAPKs) that is the non-canonical BMP2 pathway. Western blotting revealed that Suf-A stimulated p38, JNK, and ERK phosphorylation (Figure 5B). However, Suf-A did not affect autophagic or necroptotic signaling (Figure S1A,B). This was

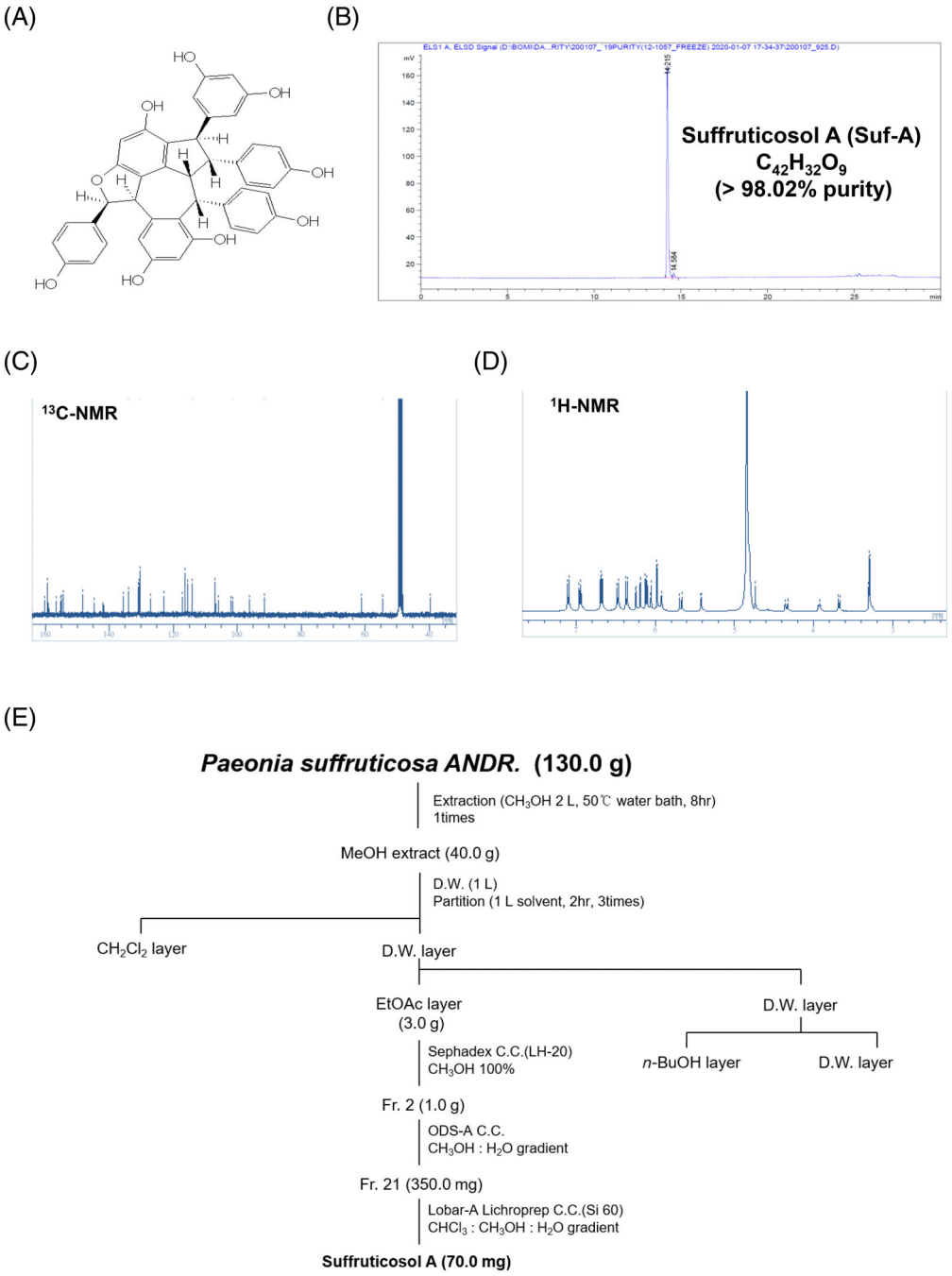

FIGURE 1 Purification of Suffruticosol A (Suf-A) from the dried fruit of *Paeonia suffruticosa*. (A) Chemical structure of Suf-A. (B) HPLC chromatogram. Purity: >98.02%. (C,D) ^{13}C NMR (C) and ^{1}H NMR spectra (D). (E) Procedure for extracting Suf-A from *P. suffruticosa*

further examined using a BMP2 inhibitor (Noggin) and MAPKs inhibitors (U0126, SP600125, and SB 203580). Inhibitor treatments validated that Suf-A promotes RUNX2 expression through BMP2 signaling during the differentiation of pre-osteoblasts (Figure 6A,B).

4 | DISCUSSION

Osteogenic processes require osteoblast differentiation, adhesion, and migration, together with bone matrix mineralization, for bone development, formation, remodeling, and repair, right from the time of conception until death.[25–29] Pathologically, the etiology of bone disorders, including osteoporosis and periodontitis, is directly linked to osteoblast-mediated bone formation.[16–18,30] Therefore, it is crucial to focus on osteoblasts for drug development to treat bone disorders. Various natural compounds have been reported to stimulate osteoblast differentiation and bone formation.[18,19,31,32] Herein, we extracted Suf-A from the *P. suffruticosa* fruit and examined its osteogenic activities.

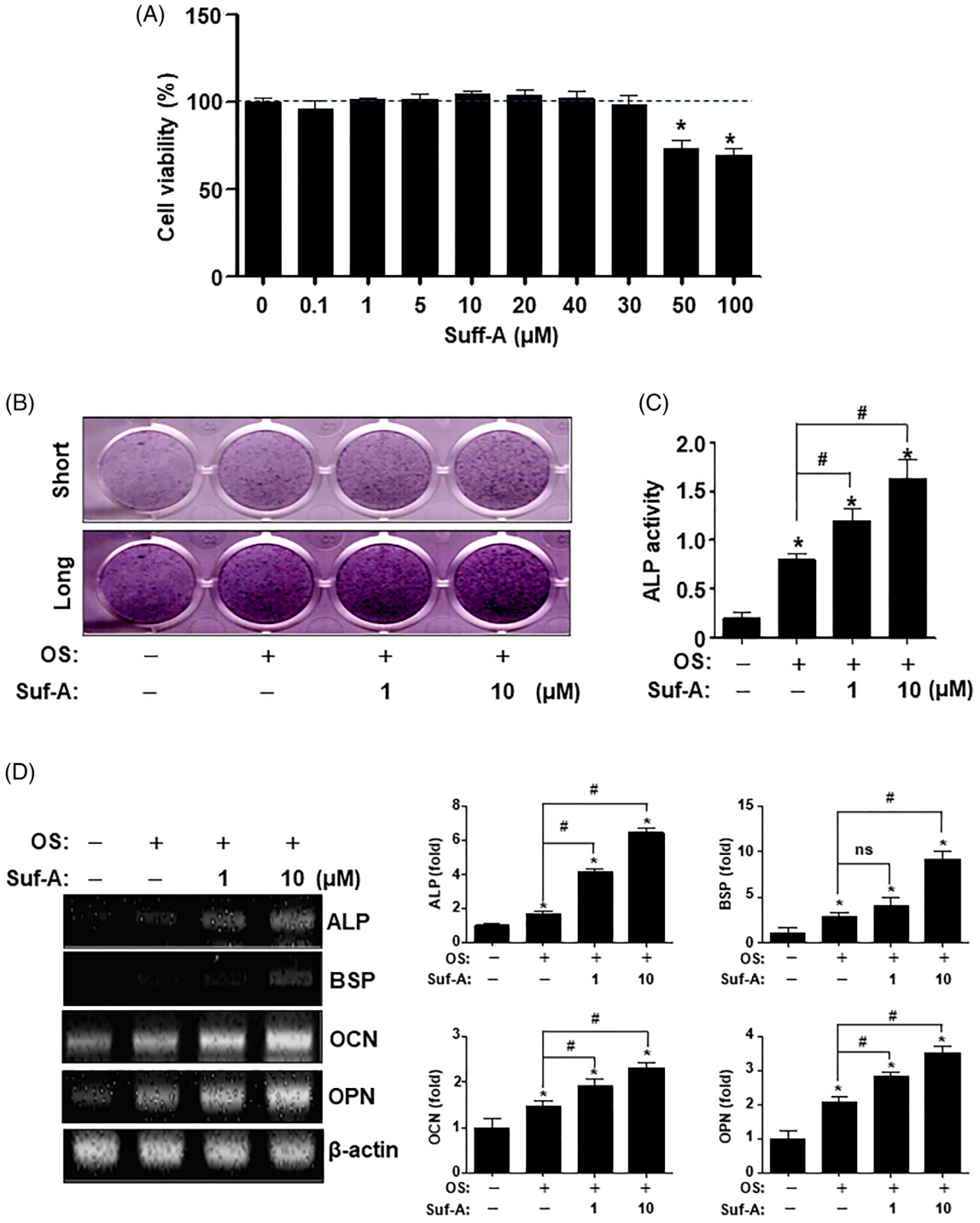

FIGURE 2 Activities of Suffruticosol A (Suf-A) on the viability and differentiation. (A) Pre-osteoblasts were incubated for 24 h with Suf-A (0.1–100 μM). Cell viability was determined using an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. (B,C) After osteoblast differentiation was induced by osteogenic supplement (OS) medium for 7 days with Suf-A (1 and 10 μM), the differentiation was visualized using alkaline phosphatase (ALP) reaction solution and captured using a scanner. Short: Short exposure time of detection. Long: Long exposure time of detection (B). ALP activity was detected using a spectrophotometer (C). (D) After the cells were seeded onto six-well plates and differentiated for 7 days, alkaline phosphatase (*Alp*), bone sialoprotein (*Bsp*), osteocalcin (*Ocn*), and osteopontin (*Opn*), mRNA levels were analyzed using reverse transcription polymerase chain reaction (RT-PCR). Data are presented as the mean ± SEM. Statistical significance: $*p < 0.05$ was compared to the control and $\#p < 0.05$ was compared to OS medium.

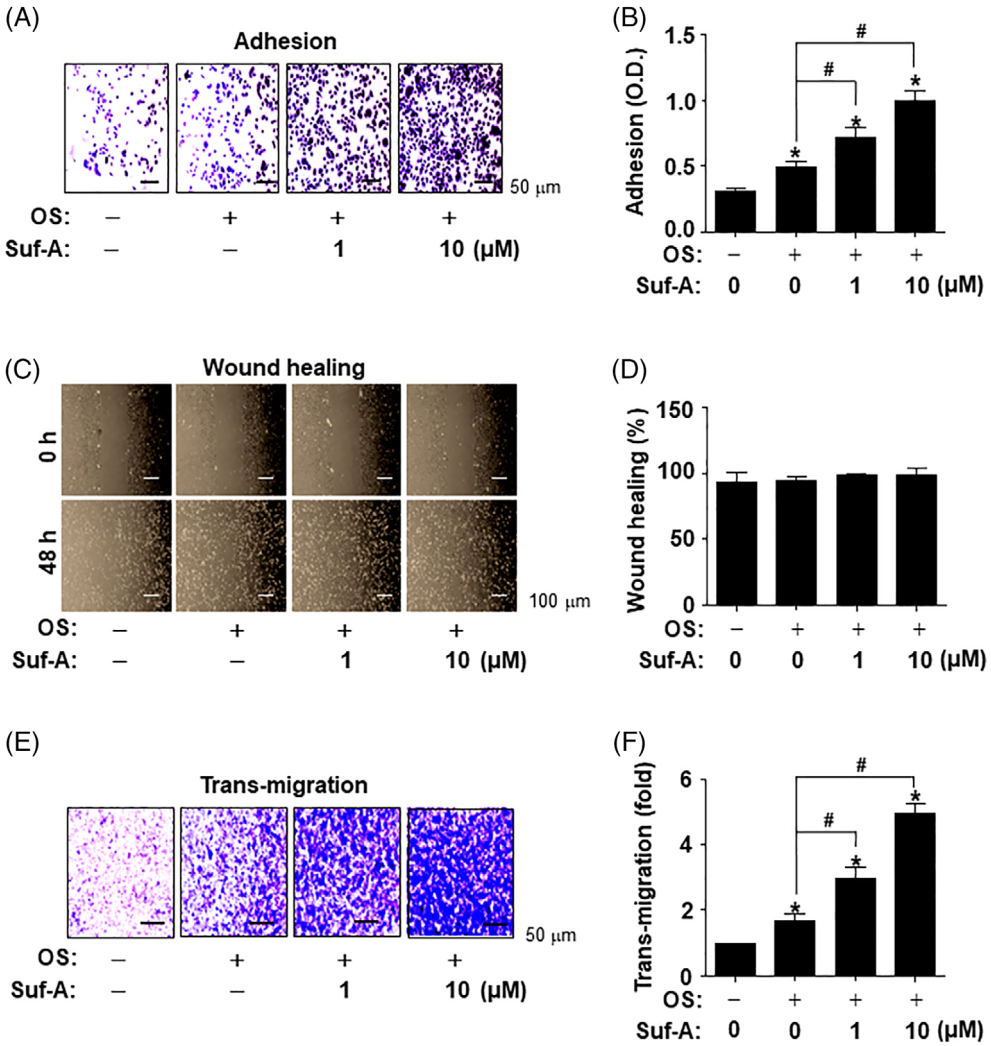

FIGURE 3 Suffruticosol A (Suf-A) induces adhesion and trans-migration during the differentiation of pre-osteoblasts. (A,B) Adhesion was observed using a light microscope (A) and depicted as a bar graph (B). (C,D) Wound-healing migration was observed using a light microscope (C) and depicted as a bar graph (D). (E,F) Trans-migration was detected using the Boyden chamber, observed using a light microscope (E), and depicted as a bar graph (F). Data are presented as the mean ± SEM. Statistical significance: $^{*}p < 0.05$ was compared to the control and $\#p < 0.05$ was compared to osteogenic supplement (OS) medium.

ALP is a well-known early osteoblast differentiation marker; furthermore, non-collagenous proteins OCN, BSP, and OPN are components of mineralized tissues responsible for calcium metabolism, mineral deposition, and hydroxyapatite crystallization on the ECM, leading to mineralization and skeletal tissue formation.[33,34] In the present study, our data showed that Suf-A stimulates ALP activity and expression, with increased expression of osteoblast genes, including *Ocn*, *Bsp*, and *Opn*. Therefore, our findings suggest that Suf-A promotes osteoblast differentiation by stimulating ALP activity and upregulating bone tissue components.

Osteoblast-induced bone formation involves migration and adhesion, leading to the formation of osteoid and mineralized tissues.[35,36] Pre-osteoblasts migrate into specific niches of bone formation and attach to the site, leading to further osteogenic activities, differentiation, and bone formation via bone matrix mineralization.[37–39] Zn induces the migration and recruitment of osteoblasts to participate in bone formation.[40] Smriti et al. found that Nck, an adaptor molecule that links the cytoskeleton and cell motility, causes osteoblast migration and bone formation.[41] Jia et al. reported that graphene accelerates osteoblast adhesion and mineralization.[42] It was also reported that the downregulated levels of RPTOR-independent companion of mammalian target of rapamycin (mTOR) complex 2 in osteoblasts result in decreased adhesion, survival, and mineralization.[43,44] In the present study, we found that Suf-A promotes adhesion and migration in the ECM, suggesting that Suf-A exerts osteogenic activities by stimulating osteoblast adhesion and migration.

RUNX2 is a master regulator of gene transcription: it facilitates the transcription of various mRNAs, including *Bsp*, *Ocn*, *Opn*, and *Alp*, leading to osteoblast differentiation, maturation, and mineralization.[45–47] Our data revealed that Suf-A augmented RUNX2 expression in the nucleus, together with increased expression of *Alp*, *Ocn*, *Opn*, and *Bsp*. Furthermore, Suf-A facilitates calcium deposition and mineralization generated by mature osteoblasts. RUNX2 expression and osteoblast differentiation are tightly regulated by various extracellular proteins and intracellular signaling pathways.[47] In particular, BMP2

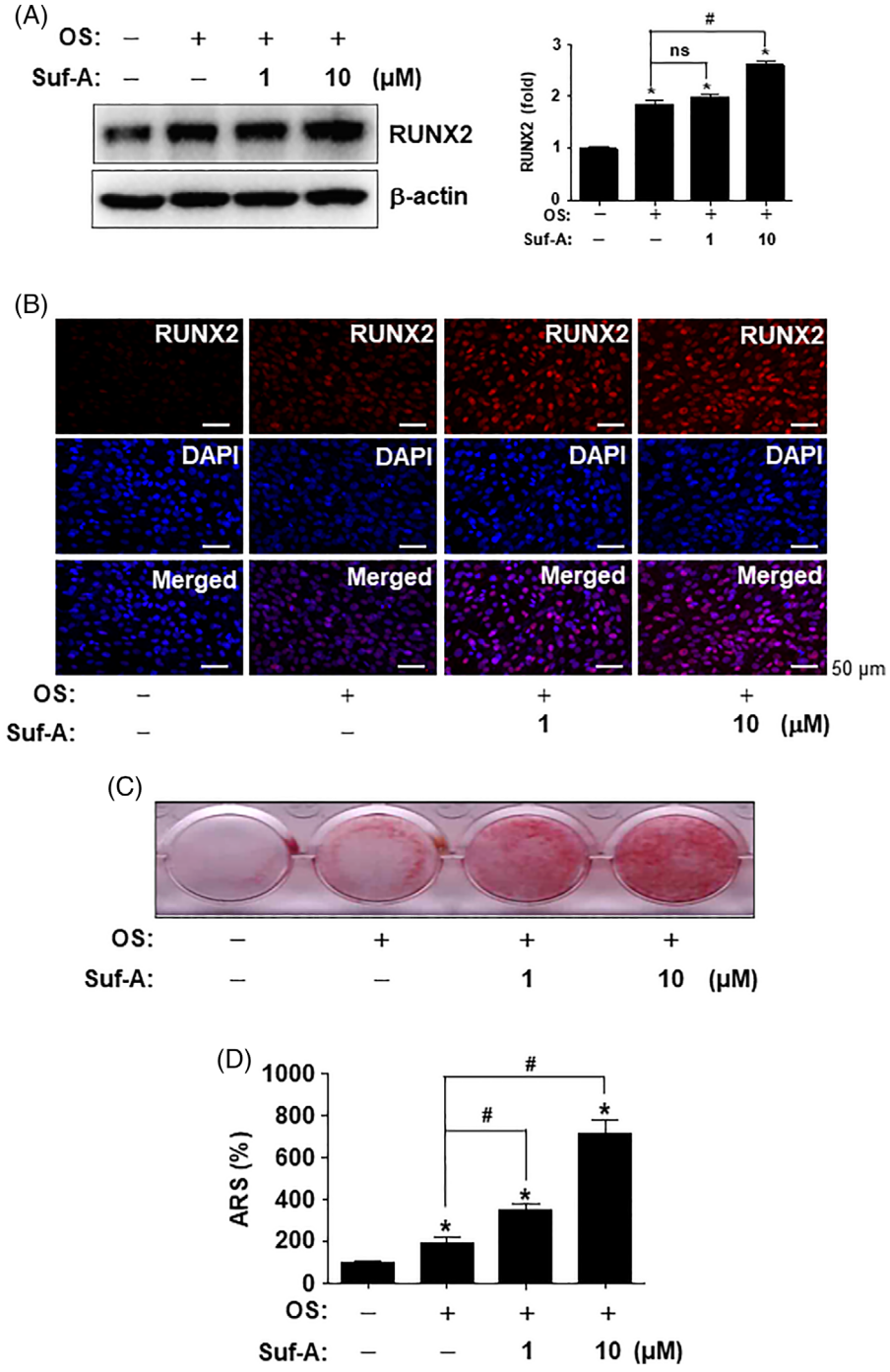

FIGURE 4 Suffruticosol A (Suf-A) enhances RUNX2 expression and bone matrix mineralization. (A) After differentiation for 3 days, western blotting was used to detect RUNX2 and β-actin (a loading control) proteins in whole-cell lysates. (B) After differentiation for 3 days, immunocytochemistry was used to detect RUNX2 protein with DAPI (a nuclear marker) in the nucleus. (C,D) After differentiation for 21 days, bone matrix mineralization was stained by Alizarin Red S (ARS) and captured using a scanner (C). The mineralization levels were detected using an ELISA reader (D). Data are presented as the mean ± SEM. Statistical significance: $*p < 0.05$ was compared to the control and $\#p < 0.05$ was compared to osteogenic supplement (OS) medium.

and Wnt3a extracellular proteins primarily activate intracellular proteins, such as Smad1/5/8 and β-catenin, respectively, which induce RUNX2 expression.[48,49] Our results demonstrated that Suf-A enhances RUNX2 expression via BMP2-mediated Smad1/5/8 and MAPKs (ERK1/2, p38, and JNK) signaling, whereas Suf-A has no influence on Wnt3a-mediated GSK3β/β-catenin signaling. *BMP2* knockout mice were demonstrated to have impaired maturation and formation of mineralized tissues, including bone, enamel, and periodontium.[50–52] *RUNX2* knockout mice demonstrate ossification failure, and reduced RUNX2 expression has been linked to aberrant bone formation.[53] Therefore, our findings suggest that Suf-A has biological mechanisms to enhance osteoblast differentiation and mineralization by stimulating RUNX2 via BMP2 signaling cascades.

Recently, necroptosis and autophagy have been reported to play critical roles in osteogenic activity and bone formation under physiological and pathological conditions.[54–57] Iron overload induces osteoblast necroptosis via the RIPK1/RIPK3/Mixed Lineage Kinase Domain Like Pseudokinase (MLKL) pathway.[56] Chronic

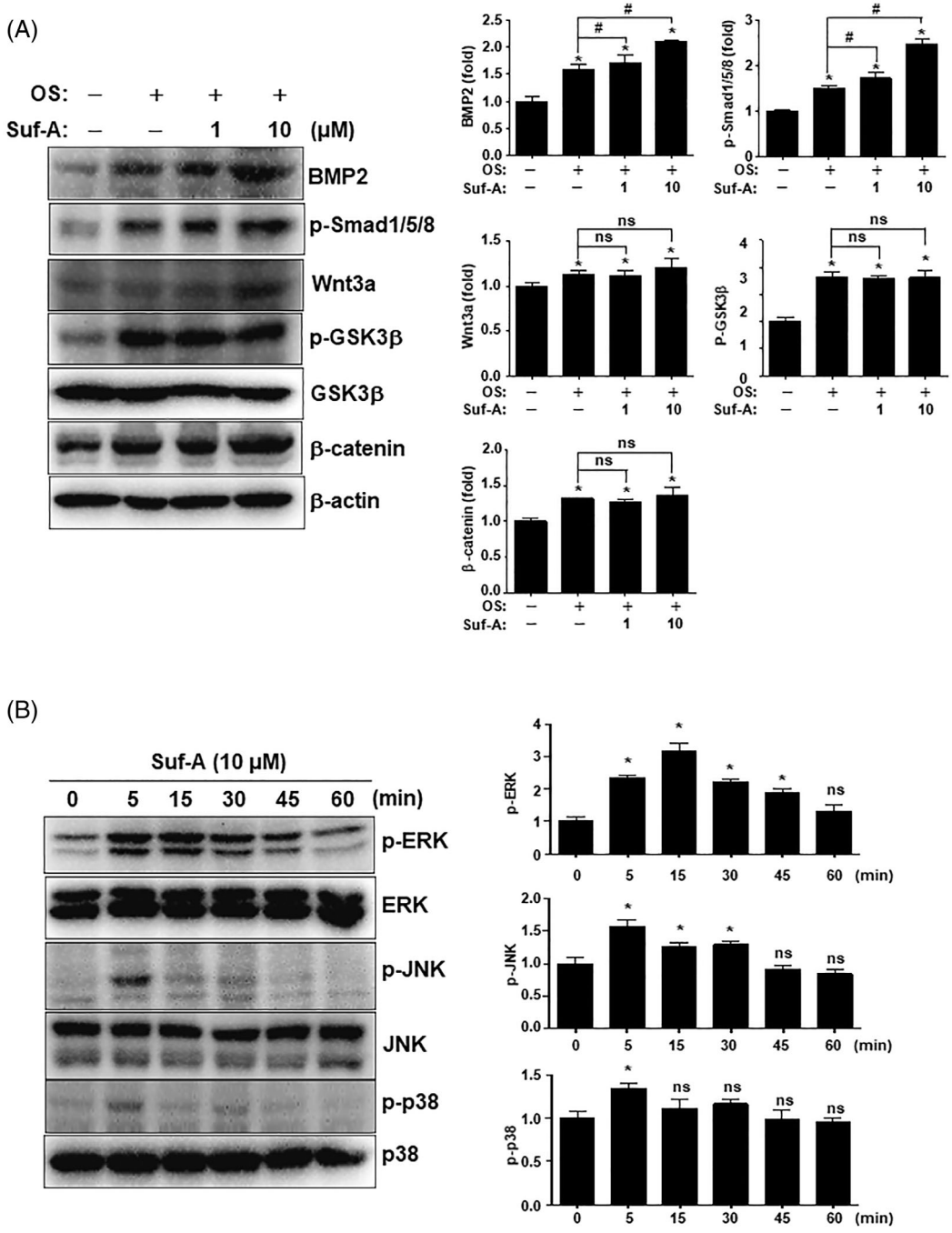

FIGURE 5 Suffruticosol A (Suf-A) stimulates BMP2 signaling during the differentiation of pre-osteoblasts. (A) BMP2, p-Smad1/5/8, Wnt3a, p-GSK3β, GSKβ, and β-catenin, as well as β-Actin (a loading control), proteins in whole-cell lysates were detected using western blotting. (B) p-ERK, ERK, p-JNK, JNK, p-p38, and p38 proteins in whole-cell lysates were detected using western blotting. Data are presented as the mean ± SEM. Statistical significance: $^*P < 0.05$ was compared to the control and $\#p < 0.05$ was compared to osteogenic supplement (OS) medium.

alcohol consumption also causes osteoblast necroptosis, which leads to osteopenia.[57] Necrostatin-1, a necroptosis inhibitor, enhances the levels of bone-formation markers and stimulates bone formation in glucocorticoid-induced osteoporosis in rats.[58] Necrostatin-1 also protects pre-osteoblast MC3T3-E1 cells against dexamethasone-induced cell death.[59] Recent evidence shows that differentiation and bone disease are affected by autophagy.[60–62] Kaempferol activates autophagy-related molecules, such as Beclin-1, microtubule-associated protein light chain 3 (LC3), and p62, and kaempferol-induced autophagy stimulates osteoblast differentiation of pre-osteoblast MC3T3-E1 cells.[63] Vitamin K2 stimulates differentiation and mineralization by inducing

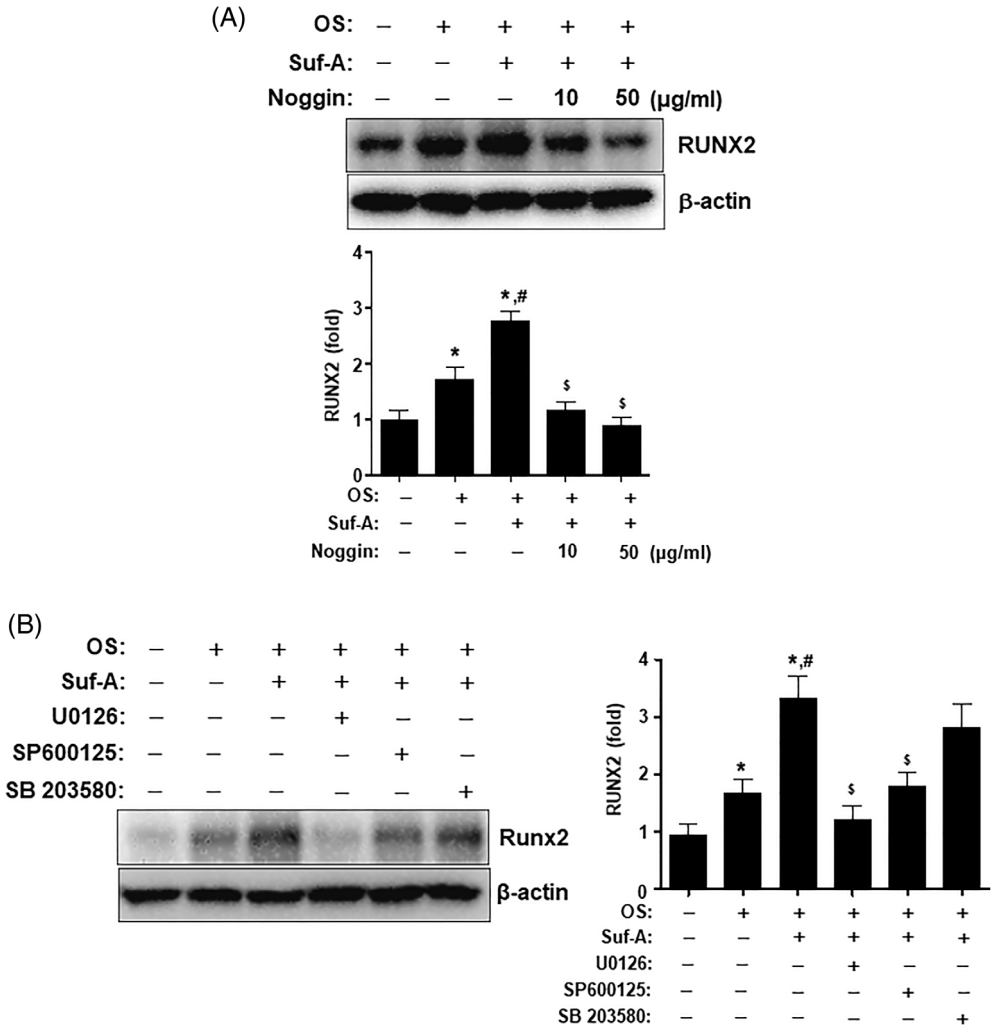

FIGURE 6 Suffruticosol A (Suf-A)-facilitated RUNX2 expression is mediated by BMP2 signaling during the differentiation of pre-osteoblasts. (A) After Suf-A were treated with or without Noggin (10 and 50 μg/ml) for 3 days, RUNX2 and β-actin proteins were detected using western blotting. (B) After Suf-A were treated with or without U0126 (1 μM), SP600125 (1 μM), and SB 203580 (1 μM) for 3 days, RUNX2 and β-actin proteins were detected using western blotting. Data are presented as the mean ± SEM. Statistical significance: *$p < 0.05$ was compared to the control, #$p < 0.05$ was compared to osteogenic supplement (OS) medium, and \$$p < 0.05$ was compared to OS + Suf-A.

autophagy in pre-osteoblast MC3T3-E1 cells.[64] TNF-α inhibits osteoblast differentiation and induces apoptotic cell death, whereas autophagy prevents TNF-α-mediated effects.[65,66] In the present study, we found that Suf-A does not affect autophagic and necroptotic proteins, such as Beclin-1, LC3, ubiquitin-binding protein p62 (p62), MLKL, and receptor-interacting protein kinase 3 (RIP3), suggesting that the osteogenic effects of Suf-A are unrelated to autophagy or necroptosis.

In conclusion, anabolic drugs enhance osteoblast differentiation and bone formation in skeletal disorders.[19,67] Natural compounds derived from plants are increasingly being sought after as the treatment of choice for skeletal disorders because they have fewer side effects and are more efficacious.[18,31,68] The results obtained herein demonstrate that Suf-A, isolated from the *P. suffruticosa* fruit, possesses osteogenic properties by enhancing osteoblast differentiation, adhesion, migration, and calcification based on biological mechanisms via BMP2 signaling-induced RUNX2 expression. Therefore, our findings suggest that Suf-A may serve as a promising supplement or anabolic medicine for treating skeletal disorders.

AUTHOR CONTRIBUTIONS

Planning and designing the experiments: Kyung-Ran Park, Jin Tae Hong, and Hyung-Mun Yun. *Performing the experiments*: Kyung-Ran Park and Hyung-Mun Yun. *Analyzing the data*: Kyung-Ran Park, Yun Hee Jeong, Bomi Kim, Jin Tae Hong, and Hyung-Mun Yun. *Writing the manuscript*: Kyung-Ran Park, Jin Tae Hong, and Hyung-Mun Yun. The final manuscript has been read and approved by all the authors.

ACKNOWLEDGMENT

The present study was supported by National Research Foundation of Korea (NRF) grants funded by the Korean government (MSIP) (MRC, 2017R1A5A2015541; NRF-2022R1C1C1003491).

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Hyung-Mun Yun https://orcid.org/0000-0001-6504-1610

REFERENCES

1. Yoshikawa M, Ohta T, Kawaguchi A, Matsuda H. Bioactive constituents of Chinese natural medicines. V. Radical scavenging effect of Moutan cortex. (1): absolute stereostructures of two monoterpenes, paeonisuffrone and paeonisuffral. Chem Pharm Bull. 2000;48:1327–31.
2. Ryu G, Park EK, Joo JH, Lee BH, Choi BW, Jung DS, et al. A new antioxidant monoterpene glycoside, alpha-benzoyloxypaeoniflorin from *Paeonia suffruticosa*. Arch Pharm Res. 2001;24:105–8.
3. Li G, Seo CS, Lee KS, Kim HJ, Chang HW, Jung JS, et al. Protective constituents against sepsis in mice from the root cortex of *Paeonia suffruticosa*. Arch Pharm Res. 2004;27:1123–6.
4. Wang X, Cheng C, Sun QL, Li FW, Liu JH, Zheng CC. Isolation and purification of four flavonoid constituents from the flowers of *Paeonia suffruticosa* by high-speed counter-current chromatography. J Chromatogr A. 2005;1075:127–31.
5. Sarker SD, Whiting P, Dinan L, Sik V, Rees HH. Identification and ecdysteroid antagonist activity of three resveratrol trimers (suffruticosols A, B and C) from *Paeonia suffruticosa*. Tetrahedron. 1999;55:513–24.
6. Ryu HW, Song HH, Shin IS, Cho BO, Jeong SH, Kim DY, et al. Suffruticosol A isolated from *Paeonia lactiflora* seedcases attenuates airway inflammation in mice induced by cigarette smoke and LPS exposure. J Funct Foods. 2015;17:774–84.
7. Simoni D, Invidiata FP, Eleopra M, Marchetti P, Rondanin R, Baruchello R, et al. Design, synthesis and biological evaluation of novel stilbene-based antitumor agents. Bioorg Med Chem. 2009;17:512–22.
8. Cai T, Cai Y. Cis-Ampelopsin E, a stilbene isolated from the seeds of *Paeonia suffruticosa*, inhibits lipopolysaccharide-stimulated nitric oxide production in RAW 264.7 macrophages via blockade of nuclear factor-kappa B signaling pathway. Biol Pharm Bull. 2011;34:1501–7.
9. Almosnid NM, Gao Y, He C, Park HS, Altman E. In vitro antitumor effects of two novel oligostilbenes, cis- and trans-suffruticosol D, isolated from *Paeonia suffruticosa* seeds. Int J Oncol. 2016;48:646–56.
10. Zhang J, Chen X, Chen B, Tong L, Zhang Y. Tetrahydroxy stilbene glucoside protected against diabetes-induced osteoporosis in mice with streptozotocin-induced hyperglycemia. Phytother Res. 2019;33:442–51.
11. Donsante S, Palmisano B, Serafini M, Robey PG, Corsi A, Riminucci M. From stem cells to bone-forming cells. Int J Mol Sci. 2021;22:3989.
12. Thomas S, Jaganathan BG. Signaling network regulating osteogenesis in mesenchymal stem cells. J Cell Commun Signal. 2022;16:47–61.
13. Karsenty G, Wagner EF. Reaching a genetic and molecular understanding of skeletal development. Dev Cell. 2002;2:389–406.
14. Fakhry M, Hamade E, Badran B, Buchet R, Magne D. Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts. World J Stem Cells. 2013;5:136–48.
15. Islam R, Yoon WJ, Ryoo HM. Pin1, the master orchestrator of bone cell differentiation. J Cell Physiol. 2017;232:2339–47.
16. Jiang Y, Zhang P, Zhang X, Lv L, Zhou Y. Advances in mesenchymal stem cell transplantation for the treatment of osteoporosis. Cell Prolif. 2021;54:e12956.
17. Li Y, Ling J, Jiang Q. Inflammasomes in alveolar bone loss. Front Immunol. 2021;12:691013.
18. An J, Yang H, Zhang Q, Liu C, Zhao J, Zhang L, et al. Natural products for treatment of osteoporosis: the effects and mechanisms on promoting osteoblast-mediated bone formation. Life Sci. 2016;147:46–58.
19. Russow G, Jahn D, Appelt J, Mardian S, Tsitsilonis S, Keller J. Anabolic therapies in osteoporosis and bone regeneration. Int J Mol Sci. 2018;20:83.
20. Park KR, Lee JY, Cho M, Yun HM. Ziyuglycoside I upregulates RUNX2 through ERK1/2 in promoting osteoblast differentiation and bone mineralization. Am J Chin Med. 2021;49:883–900.
21. Park KR, Yun HM. RANKL-induced osteoclastogenesis in bone marrow-derived macrophages is suppressed by cisapride. Toxicology. 2019;422:95–101.
22. Park KR, Lee H, Cho M, Yun HM. A phytochemical constituent, (E)-methyl-cinnamate isolated from *Alpinia katsumadai* Hayata suppresses cell survival, migration, and differentiation in pre-osteoblasts. Int J Mol Sci. 2020;21:3700.
23. Park KR, Leem HH, Cho M, Kang SW, Yun HM. Effects of the amide alkaloid piperyline on apoptosis, autophagy, and differentiation of pre-osteoblasts. Phytomedicine. 2020;79:153347.
24. Park KR, Kim EC, Hong JT, Yun HM. Dysregulation of 5-hydroxytryptamine 6 receptor accelerates maturation of bone-resorbing osteoclasts and induces bone loss. Theranostics. 2018;8:3087–98.
25. Zayzafoon M. Calcium/calmodulin signaling controls osteoblast growth and differentiation. J Cell Biochem. 2006;97:56–70.
26. Tome M, Lopez-Romero P, Albo C, Sepulveda JC, Fernandez-Gutierrez B, Dopazo A, et al. miR-335 orchestrates cell proliferation, migration and differentiation in human mesenchymal stem cells. Cell Death Differ. 2011;18:985–95.
27. Histing T, Stenger D, Kuntz S, Scheuer C, Tami A, Garcia P, et al. Increased osteoblast and osteoclast activity in female senescence-accelerated, osteoporotic SAMP6 mice during fracture healing. J Surg Res. 2012;175:271–7.
28. Broz A, Ukraintsev E, Kromka A, Rezek B, Hubalek Kalbacova M. Osteoblast adhesion, migration, and proliferation variations on chemically patterned nanocrystalline diamond films evaluated by live-cell imaging. J Biomed Mater Res A. 2017;105:1469–78.
29. Yang Y, Zhang T, Jiang M, Yin X, Luo X, Sun H. Effect of the immune responses induced by implants in a integrated three-dimensional micro-nano topography on osseointegration. J Biomed Mater Res A. 2021;109:1429–40.
30. Guntur AR, Rosen CJ. The skeleton: a multi-functional complex organ: new insights into osteoblasts and their role in bone formation: the central role of PI3Kinase. J Endocrinol. 2011;211:123–30.
31. I. N. Soelaiman, S. Das, A. N. Shuid, H. Mo, N. Mohamed (2013) Use of medicinal plants and natural products for treatment of osteoporosis and its complications. Evid Based Complement Alternat Med 2013:764701.

32. Park KR, Kim S, Cho M, Yun HM. Limonoid triterpene, Obacunone increases runt-related transcription factor 2 to promote osteoblast differentiation and function. Int J Mol Sci. 2021;22: 2483.
33. Ogata Y. Bone sialoprotein and its transcriptional regulatory mechanism. J Periodontal Res. 2008;43:127–35.
34. Lin X, Patil S, Gao YG, Qian A. The bone extracellular matrix in bone formation and regeneration. Front Pharmacol. 2020; 11:757.
35. Granero-Molto F, Weis JA, Miga MI, Landis B, Myers TJ, O'Rear L, et al. Regenerative effects of transplanted mesenchymal stem cells in fracture healing. Stem Cells. 2009;27:1887–98.
36. Infante A, Rodriguez CI. Osteogenesis and aging: lessons from mesenchymal stem cells. Stem Cell Res Ther. 2018;9:244.
37. Kalbacova M, Broz A, Kong J, Kalbac M. Graphene substrates promote adherence of human osteoblasts and mesenchymal stromal cells. Carbon. 2010;48:4323–9.
38. Aryaei A, Jayatissa AH, Jayasuriya AC. The effect of graphene substrate on osteoblast cell adhesion and proliferation. J Biomed Mater Res A. 2014;102:3282–90.
39. Delaisse JM. The reversal phase of the bone-remodeling cycle: cellular prerequisites for coupling resorption and formation. Bonekey Rep. 2014;3:561.
40. Kanno S, Anuradha CD, Hirano S. Chemotactic responses of osteoblastic MC3T3-E1 cells toward zinc chloride. Biol Trace Elem Res. 2001;83:49–55.
41. Aryal ACS, Miyai K, Izu Y, Hayata T, Notomi T, Noda M, et al. Nck influences preosteoblastic/osteoblastic migration and bone mass. Proc Natl Acad Sci USA. 2015;112:15432–7.
42. Ren J, Zhang XG, Chen Y. Graphene accelerates osteoblast attachment and biomineralization. Carbon Lett. 2017;22:42–7.
43. Sen B, Xie Z, Case N, Thompson WR, Uzer G, Styner M, et al. mTORC2 regulates mechanically induced cytoskeletal reorganization and lineage selection in marrow-derived mesenchymal stem cells. J Bone Miner Res. 2014;29:78–89.
44. Lai P, Song Q, Yang C, Li Z, Liu S, Liu B, et al. Loss of Rictor with aging in osteoblasts promotes age-related bone loss. Cell Death Dis. 2016;7:e2408.
45. Schroeder TM, Jensen ED, Westendorf JJ. Runx2: a master organizer of gene transcription in developing and maturing osteoblasts. Birth Defects Res C Embryo Today. 2005;75: 213–25.
46. Komori T. Regulation of bone development and extracellular matrix protein genes by RUNX2. Cell Tissue Res. 2010;339: 189–95.
47. Amarasekara DS, Kim S, Rho J. Regulation of osteoblast differentiation by cytokine networks. Int J Mol Sci. 2021;22:2851.
48. Chen G, Deng C, Li YP. TGF-beta and BMP signaling in osteoblast differentiation and bone formation. Int J Biol Sci. 2012;8: 27288.
49. Artigas N, Urena C, Rodriguez-Carballo E, Rosa JL, Ventura F. Mitogen-activated protein kinase (MAPK)-regulated interactions between Osterix and Runx2 are critical for the transcriptional osteogenic program. J Biol Chem. 2014;289:27105–17.
50. Feng J, Yang G, Yuan G, Gluhak-Heinrich J, Yang W, Wang L, et al. Abnormalities in the enamel in bmp2-deficient mice. Cells Tissues Organs. 2011;194:216–21.
51. McBride SH, McKenzie JA, Bedrick BS, Kuhlmann P, Pasteris JD, Rosen V, et al. Long bone structure and strength depend on BMP2 from osteoblasts and osteocytes, but not vascular endothelial cells. PLoS One. 2014;9:e96862.
52. Yu S, Guo J, Sun Z, Lin C, Tao H, Zhang Q, et al. BMP2-dependent gene regulatory network analysis reveals Klf4 as a novel transcription factor of osteoblast differentiation. Cell Death Dis. 2021;12:197.
53. Liu TM, Lee EH. Transcriptional regulatory cascades in Runx2-dependent bone development. Tissue Eng Part B Rev. 2013;19:254–63.
54. Liu F, Fang F, Yuan H, Yang D, Chen Y, Williams L, et al. Suppression of autophagy by FIP200 deletion leads to osteopenia in mice through the inhibition of osteoblast terminal differentiation. J Bone Miner Res. 2013;28:2414–30.
55. Shi G, Jia P, Chen H, Bao L, Feng F, Tang H. Necroptosis occurs in osteoblasts during tumor necrosis factor-alpha stimulation and caspase-8 inhibition. Braz J Med Biol Res. 2018;52: e7844.
56. Tian Q, Qin B, Gu Y, Zhou L, Chen S, Zhang S, et al. ROS-mediated necroptosis is involved in iron overload-induced osteoblastic cell death. Oxid Med Cell Longev. 2020;2020:1295382.
57. Guo M, Huang YL, Wu Q, Chai L, Jiang ZZ, Zeng Y, et al. Chronic ethanol consumption induces osteopenia via activation of osteoblast necroptosis. Oxid Med Cell Longev. 2021; 2021:3027954.
58. Feng M, Zhang R, Gong F, Yang P, Fan L, Ni J, et al. Protective effects of necrostatin-1 on glucocorticoid-induced osteoporosis in rats. J Steroid Biochem Mol Biol. 2014;144:455–62.
59. Feng M, Qiang H, Zhang RR, Wang KZ, Wang CS, Yang P. Necrostatin-1 inhibits the cell death of osteoblasts induced by glucocorticoid. Int J Clin Exp Pathol. 2018;11:675–84.
60. Zhang LS, Guo YF, Liu YZ, Liu YJ, Xiong DH, Liu XG, et al. Pathway-based genome-wide association analysis identified the importance of regulation-of-autophagy pathway for ultradistal radius BMD. J Bone Miner Res. 2010;25:1572–80.
61. Darcy A, Meltzer M, Miller J, Lee S, Chappell S, Donck KV, et al. A novel library screen identifies immunosuppressors that promote osteoblast differentiation. Bone. 2012;50:1294–303.
62. Zhao X, Huang B, Wang H, Ni N, He F, Liu Q, et al. A functional autophagy pathway is essential for BMP9-induced osteogenic differentiation of mesenchymal stem cells (MSCs). Am J Transl Res. 2021;13:4233–50.
63. Kim IR, Kim SE, Baek HS, Kim BJ, Kim CH, Chung IK, et al. The role of kaempferol-induced autophagy on differentiation and mineralization of osteoblastic MC3T3-E1 cells. BMC Complement Altern Med. 2016;16:333.
64. Li WW, Zhang SK, Liu J, Liu YY, Liang QW. Vitamin K2 stimulates MC3T3-E1 osteoblast differentiation and mineralization through autophagy induction. Mol Med Rep. 2019;19:3676–84.
65. Zheng LW, Wang WC, Ni JD, Mao XZ, Song DY, Liu T, et al. Role of autophagy in tumor necrosis factor-alpha-induced apoptosis of osteoblast cells. J Invest Med. 2017;65:1014–20.
66. Chen LL, Bao JQ, Yang YT, Wang ZX, Xia MJ, Tan JY, et al. Autophagy was involved in tumor necrosis factor-alpha-inhibited osteogenic differentiation of murine calvarial osteoblasts through Wnt/beta-catenin pathway. Tissue Cell. 2020;67: 101401.
67. Lee WC, Guntur AR, Long F, Rosen CJ. Energy metabolism of the osteoblast: implications for osteoporosis. Endocr Rev. 2017; 38:255–66.

68. Liu Y, Liu JP, Xia Y. Chinese herbal medicines for treating osteoporosis. Cochrane Database Syst Rev. 2014;6:CD005467.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Yun H-M, Kim B, Jeong YH, Hong JT, Park K-R. Suffruticosol A elevates osteoblast differentiation targeting BMP2-Smad/1/5/8-RUNX2 in pre-osteoblasts. BioFactors. 2023;49(1):127–39. https://doi.org/10.1002/biof.1878