Ethanol extract of *Lithospermum erythrorhizon* Sieb. et Zucc. promotes osteoblastogenesis through the regulation of Runx2 and Osterix

YOU HEE CHOI[1*], GEUM SOOG KIM[2*], JAE HO CHOI[3], SUN WOO JIN[3], HYUNG GYUN KIM[3], YOUNHO HAN[1], DAE YOUNG LEE[2], SOO IM CHOI[4], SEUNG YU KIM[2], YOUNG SUP AHN[2], KWANG YOUL LEE[1] and HYE GWANG JEONG[3]

[1]College of Pharmacy, Chonnam National University, Gwangju 500-757; [2]Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, RDA, Eumseong 369-873; [3]College of Pharmacy, Chungnam National University, Daejeon 305-764; [4]YD Global Life Science Co., Ltd., Seongnam 462-807, Republic of Korea

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Abstract. Bone remodeling and homeostasis are largely the result of the coordinated action of osteoblasts and osteoclasts. Osteoblasts are responsible for bone formation. The differentiation of osteoblasts is regulated by the transcription factors, Runx2 and Osterix. Natural products of plant origin are still a major part of traditional medicinal systems in Korea. The root of *Lithospermum erythrorhizon* Sieb. et Zucc. (LR), the purple gromwell, is an herbal medicine used for inflammatory and infectious diseases. LR is an anti-inflammatory and exerts anticancer effects by inducing the apoptosis of cancer cells. However, the precise molecular signaling mechanisms of osteoblastogenesis as regards LR and osteoblast transcription are not yet known. In this study, we investigated the effects of ethanol (EtOH) extract of LR (LES) on the osteoblast differentiation of C2C12 myoblasts induced by bone morphogenetic protein 4 (BMP4) and the potential involvement of Runx2 and Osterix in these effects. We found that the LES exhibited an ability to induce osteoblast differentiation. LES increased the expression of the osteoblast marker, alkaline phosphatase (ALP), as well as its activity, as shown by ALP staining and ALP activity assay. LES also increased mineralization, as shown by Alizarin Red S staining. Treatment with LES increased the protein levels (as shown by immunoblotting), as well as the transcriptional activity of Runx2 and Osterix and enhanced osteogenic activity. These results suggest that LES modulates osteoblast differentiation at least in part through Runx2 and Osterix.

Correspondence to: Professor Hye Gwang Jeong, College of Pharmacy, Chungnam National University, 220 Gung-dong, Daejeon 305-764, Republic of Korea
E-mail: hgjeong@cnu.ac.kr

Professor Kwang Youl Lee, College of Pharmacy, Chonnam National University, 300 Yongbong-dong, Gwangju 500-757, Republic of Korea
E-mail: kwanglee@jnu.ac.kr

*Contributed equally

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Introduction

Bone is actively maintained by the coordinated balance between osteoblasts and osteoclasts (1-3). Osteoblasts mediate bone formation, while osteoclasts are responsible for bone resorption (1,2). The activities of osteoblasts can be regulated at the level of differentiation by various regulatory signals. The differentiation and activity of osteoblasts are regulated by various anabolic factors, such as insulin, members of the transforming growth factor (TGF)-β family [including bone morphogenetic proteins (BMPs)] and Wnt proteins, and by intracellular kinases, such as Akt (4). In particular, BMPs initiate osteoblast differentiation through the induction of the expression and post-translational modification of various osteogenic transcription factors, including Runx2 (Cbfa1), Osterix and several homeodomain-containing Dlx proteins (5-10). Subsequently, these osteogenic transcription factors regulate the differentiation of osteoblasts (10-16).

The Runt domain transcription factors are composed of a larger DNA-binding α subunit and a smaller non-DNA-binding β subunit. There are three mammalian genes (Runx1, Runx2, and Runx3) encoding the α subunit. Runx2 and Runx3 are essential for chondrocyte maturation, a prerequisite for endochondral ossification. In addition, Runx2 is essential for the commitment of multipotent mesenchymal cells into the osteoblastic lineage, and it inhibits adipocyte differentiation (17). Runx2 DNA binding sequences have been identified in the enhancer/promoter regions of many osteoblast specific genes, and Runx2 can bind to the osteoblast-specific *cis*-acting element (*OSE*) present in the promoter regions of collagen type I,

α1 (*COL1A1*), osteocalcin (*OC*), alkaline phosphatase (*ALP*), bone sialoprotein (*BSP*) and osteopontin (*OPN)* (18,19). The function of Runx2 is regulated by osteogenic signaling factors, such as BMP2, a member of the TGF-β superfamily (20). BMP2 induces Runx2 expression through Smad1/5/8, and BMP2-activated Smads physically interact with Runx2 to induce osteoblast differentiation (7,21-23).

Osterix (also known as Sp7) is a zinc finger-containing osteoblast-specific transcription factor and it is essential for the differentiation and proliferation of osteoblasts (24-27). The DNA-binding domain of Osterix is located at the C-terminus and it contains three C2H2-type zinc finger domains that share a high degree of identity with similar motives in Sp1, Sp3 and Sp4. N-terminal proline-rich region (PRR) mediates the protein-protein interaction. Osterix acts downstream of Runx2 and regulates the expression of many osteoblast differentiation markers including ALP, OC, osteonectin, OPN and Runx2 (15,24,28).

Bone mass in adult humans decreases with age, leading to an increased risk of fractures. Bone mineral density (BMD) and bone metabolism are affected by genetic, endocrine, mechanical and nutritional factors, with interactions among the different factors (29). Nutritional factors are particularly important for bone health as they are modifiable (30). Natural products of plant origin are still a major part of traditional medicinal systems in Korea.

The root of *Lithospermum erythrorhizon* Sieb. et Zucc. (LR), an herbal medicine, is known to possess various antiviral and biological activities, including the inhibition of human immunodeficiency virus type 1 (HIV-1), and it is extensively used in traditional medicine due to the known functions of herbs described in the literature of traditional Korean and Chinese medicines (31,32). Ethanol (EtOH) extract from LR (LES) has long been used in traditional Asian medicine for the treatment of skin cancer. It has been reported that the extracts from LR attenuates immunosuppression induced by cyclophosphamide, an antitumor agent (33,34). The majority of studies on the bioactivities of LR have been carried out mainly with naphthoquinone pigments, including shikonin and its derivatives, which are extracted using non-polar solvents, such as hexane or by supercritical extraction (35,36). Shikonin, a major active component of LR, possesses numerous pharmacological properties, including anti-inflammatory properties. Shikonin plays a dual role in the regulation of the early and late stages of collagen type II arthritis (37). Shikonin exerts protective effects on cartilage in rheumatoid arthritis (38), but also induces osteoclast differentiation *in vitro* (39). However, the function of LR in osteoblast differentiation remains unknown. Moreover, to the best of our knowledge, there are only a few studies available to date on the bioactivities of water-soluble and EtOH extracts of LR, even though they are more suitable materials for the development of health functional food from LR.

In this study, we examined the effects of LES on osteoblastogenesis and we aimed to determine whether the osteoblast transcription factors, Runx2 and Osterix, play a role in these effects. We demonstrated that treatment with LES promoted osteoblast differentiation induced by BMP4 and enhanced the osteogenic functions of Runx2 and Osterix by increasing their protein levels and transcriptional activity.

Materials and methods

Plant materials and extraction. Dried 1-year-old Lithospermi radix (LR) was purchased from Jacheon, Chungbuk, South Korea. A voucher specimen (MPS000071) has been deposited at the Herbarium of the Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, RDA, Eumseong, Korea. The powder of LR (1.5 kg) was extracted with 70% EtOH (15 l) at 80˚C for 2.5 h using a natural substance extractor (EG-BE1; TIPBio, Siheung, Korea) to obtain 70% EtOH extract. The EtOH extract was concentrated under a vacuum using a rotary evaporator (N-1200B; Eyela, Tokyo, Japan) and dried in a freeze dryer (LP20; ilShinBioBase Co., Ltd., Dongducheon, Korea) to yield the final test samples (LES, 424 g).

Plasmids, antibodies and reagents. Plasmids for Myc-tagged Osterix and Myc-tagged Runx2 were constructed in a CMV promoter-derived mammalian expression vector (pCS4-3Myc; obtained from Dr C.Y. Yeo, Ehwa University, Korea). Anti-Myc (no. 9E10, diluted 1:1,000) was purchased from Roche Applied Science (Seokyung Bldg. Seoul, Korea). Anti-α-tubulin antibody (no. B-5-1-2, diluted 1:5,000) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-Osterix (no. A-13, diluted 1:1,000) and Dlx5 antibodies (no. C-20, diluted 1:1,000) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-Runx2 antibody (no. ab76956, diluted 1:1,000) was purchased from Abcam (Boston, MA, USA). Recombinant human BMP4 was purchased from R&D Systems (Minneapolis, MN, USA).

Cell culture and transient transfection. The cells (293 cells and C2C12 mouse myoblasts) were cultured at 37˚C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (for 293 cells) or 10% (for C2C12 cells) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 g/ml streptomycin. All cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The C2C12 cells were treated with BMP4 (30 ng/ml) and the EtOH extract from *Lithospermum erythrorhizon* Sieb. et Zicao (LES) (each 30 or 60 mg/ml) for 3 days. DMEM, FBS, and the antibiotics were purchased from Life Technologies (Grand Island, NY, USA). Transient transfection was performed using a polyethylenimine (PEI) (Polysciences, Inc., Warrington, PA, USA)-mediated method. Total amounts of transfected plasmids in each group were equalized by adding the empty vector (pCS4+; obtained from Dr Yeo CY).

Cell lysate preparation and immunoblot analysis. The cells were lysed in ice-cold lysis buffer [25 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 1 mM EDTA] containing phosphatase inhibitors (25 mM NaF, 1 mM Na_3VO_4) and protease inhibitors (250 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin A). Lysates were cleared by centrifugation at 16,000 x g for 15 min at 4˚C, and the supernatants were used as cell lysates. Cell lysates containing 30 μg of total proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein content was estimated using a bovine serum albumin protein assay. Proteins were mixed with sample buffer containing

β-mercaptoethanol and heated at 100˚C for 3 min. A total of 30 μg of each cell lysate was fractionated by SDS-PAGE on a 10% polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skim milk in Tris-buffered saline (TBS) containing 0.02% Tween-20 at room temperature for 40 min, the proteins were visualized with appropriate primary antibodies (diluted 1:1,000) at room temperature for 1 h. Tubulin (diluted 1:5,000) was used as a loading control. This was followed by the addition of anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies diluted 1:25,000 in TBS/Tween-20. The blots were visualized by enhanced ECL-chemiluminescence reagent (GE Healthcare Life Sciences, Logan, UT, USA). Signals were detected and analyzed using the LAS-4000 luminescent image analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Luciferase reporter assay. The C2C12 cells were seeded on 24-well plates 1 day prior to transfection. ALP-Luc (obtained from H.M. Ryoo, Seoul University, Korea) and BSP-Luc (obtained from J.T. Kho, Chonnam National University, Korea) luciferase reporters contained the regulatory sequence of the osteoblast differentiation markers, ALP or BSP. The cells were transfected with a CMV promoter-driven β-galactosidase reporter (pCMV-β-gal), luciferase reporter and the indicated combinations of the expression plasmids. Thirty-six hours later, luciferase activities were measured using the Luciferase Reporter Assay kit (E1501; Promega, Madison, WI, USA) and a luminometer, and normalized with the corresponding β-galactosidase activities for transfection efficiency. Experiments were performed in triplicate and repeated at least 3 times. The averages and standard deviations (SD) of representative experiments are shown.

RNA preparation and semi-quantitative RT-PCR. Total cellular RNA was prepared using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Random hexamer-primed cDNA was synthesized from 1 μg of total RNA using the SuperScript III First-Strand Synthesis System (Life Technologies). The following conditions were used for the amplification by PCR: initial denaturation at 94˚C for 1 min; followed by 23-30 cycles of denaturation at 94˚C for 30 sec, annealing at a temperature optimized for each primer pair for 30 sec, and extension at 72˚C for 30 sec; final extension at 72˚C for 5 min. The following PCR primers were used: *ALP* forward, 5'-GGGTGGACTACCTCTTAGGTC-3' and reverse, 5'-ATGATGTCCGTGGTCAATCCTG-3' (30 cycles); *BSP* forward, 5'-CAGAAGTGGATGAAAACGAG-3' and reverse, 5'-CGGTGGCGAGGTGGTCCCAT-3' (25 cycles); *COL1A1* forward, 5'-TCTCCACTCTTCTAGGTTCCT-3' and reverse, 5'-TTGGGTCATTTCCACATGC-3' (23 cycles); *Runx2* forward, 5'-AGCAACAGCAACAGCAG-3' and reverse, 5'-GTAATCTGACTCTGTCCTTG-3' (35 cycles); *Osterix* forward, 5'-GGGTTAAGGGGAGCAAAGTCAGAT-3' and reverse, 5'-CTGGGGAAAGGAGGCACAAAGAAG-3' (35 cycles); *GAPDH* forward, 5'-ACCACAGTCCATGCCATCAC-3' and reverse, 5'-TCCACCACCCTGTTGCTGTA-3' (25 cycles).

ALP staining and activity assay. The C2C12 cells were treated with BMP4 (30 ng/ml) for 3 days, fixed in 4% paraformaldehyde for 15 min at room temperature, rinsed with phosphate-buffered saline (PBS), and stained with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution (Sigma-Aldrich) for 15 min at room temperature. For ALP assay, the cells were washed with PBS and lysed in 0.5% Triton X-100. ALP enzymatic activity was measured using the SensoLyte pNPP ALP assay kit (AnaSpec, Inc., Fremont, CA, USA) according to the manufacturer's instructions.

Alizarin Red S staining. The C2C12 cells in 24-well plates were transfected using the PEI-mediated method. The C2C12 cells were stimulated with BMP4. The cells were pre-treated with BMP4 for 10 days. These cells were cultured at 5% CO_2, 37˚C. The transfected C2C12 cells were then fixed in 4% paraformaldehyde for 15 min at room temperature and washed with PBS. They were then exposed to Alizarin Red S solution (no. A5533; Sigma-Aldrich) adjusted to pH 4.1-4.3 using 0.5% ammonium hydroxide for 30 min at room temperature. The mineralization-positive cells were stained red.

Statistical analysis. All experiments were performed with triplicate independent samples and were repeated at least 3 times yielding qualitatively identical results. The results are expressed as the means ± standard error of the mean. Data were analyzed using the Student's t-test (SPSS version 17.0 software; SPSS, Inc., Chicago, IL, USA). A value of $p<0.05$ was considered to indicate a statistically significant difference.

Results

LES enhances osteoblast differentiation induced by BMP4. Recently, it was reported that LES regulates osteoclastogenesis *in vitro* (39). However, the signaling mechanisms of LES are less well known in osteoblastogenesis. The precise molecular signaling mechanisms of action of LES as regards osteoblast differentiation are not yet fully understood. Therefore, in this study, we examined the effects of LES on the osteoblast differentiation of C2C12 cells. We examined whether LES affects BMP4-induced osteoblast differentiation. Stimulation with BMP4 induced the osteoblast differentiation of the C2C12 myoblasts. The C2C12 cells were cultured and treated with low and high concentrations (30 or 60 μg/ml) of LES for 3 days. LES increased the expression of ALP, an osteoblast marker, in the cells stimulated with BMP4, as shown by ALP staining (Fig. 1A, top panel), and also increased ALP activity (Fig. 1B). Similarly, LES increased mineralization, as observed by Alizarin Red S staining (Fig. 1A, bottom panel). These results suggest that LES enhances osteoblast differentiation induced by BMP4.

LES affects the expression levels and transcriptional activity of osteoblast markers in BMP4-induced osteoblast differentiation. Previous studies have demonstrated that BMP4 gene transfer into rodent muscle promotes bone formation (40-43). In this study, we confirmed that LES enhances BMP4-induced osteoblast differentiation (Fig. 1). Treatment with LES increased the expression levels of the osteoblast transcription factors, Runx2, Osterix and Dlx5 (Fig. 2A), and those of the specific markers, *ALP*, *BSP* and *COL1A1*, in the cells also treated with BMP4 (Fig. 2B). Moreover, we examined whether LES can modulate the transcriptional activity using the

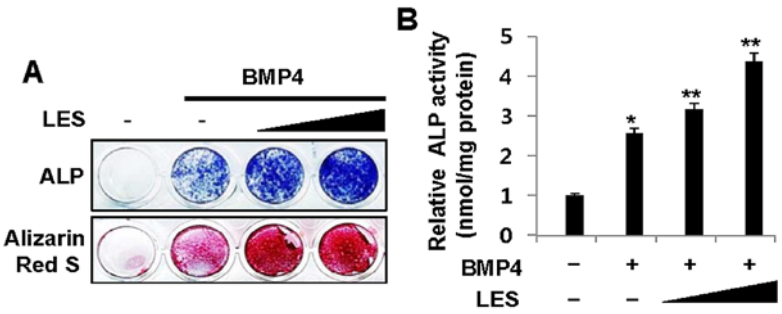

Figure 1. LES promotes bone morphogenetic protein 4 (BMP4)-induced osteoblast differentiation. C2C12 myoblasts were treated with BMP4 (30 ng/ml) and LES (each 30 or 60 μg/ml for A and B) for 3 days for alkaline phosphatase (ALP) staining, and for 14 days for (Alizarin Red S staining). (A) Osteoblast differentiation and mineralization evaluated by ALP staining and Alizarin Red S staining. The ALP-positive cells were stained blue/purple. (B) ALP activity was measured by ALP assay. *p<0.05 compared to the BMP4-untreated cells; **p<0.05 compared to the BMP4-treated cells not transfected with DNA. All experiments were repeated at least 3 times. The averages and standard deviations (SD) of representative experiments are shown. LES, ethanol (EtOH) extract from *Lithospermum erythrorhizon* Sieb. et Zucc.

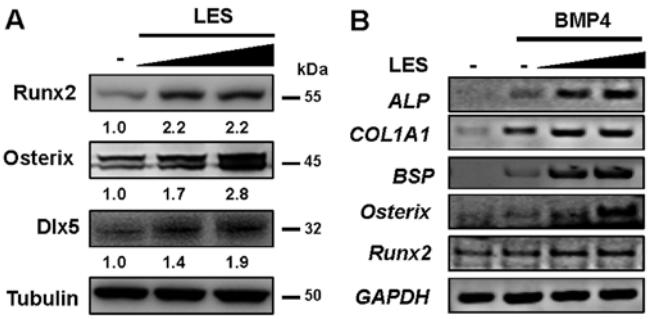

Figure 2. LES increases the expression of osteoblast target genes during osteoblast differentiation. C2C12 cells were treated with bone morphogenetic protein 4 (BMP4) (30 ng/ml) and increasing amounts of LES (30 or 60 μg/ml) for 3 days. (A) The endogenous expression levels of osteoblast target genes (Runx2, Osterix and Dlx5) in cell lysates compared by immunoblotting using antibody against Runx2, Osterix or Dlx5, respectively. Tubulin was used as a loading control. (B) The expression levels of the osteoblast-specific markers, alkaline phosphatase (*ALP*), bone sialoprotein (*BSP*), collagen type I, α1 (*COL1A1*), *Osterix*, *Runx2* compared by RT-PCR. GAPDH was used as a loading control. LES, ethanol (EtOH) extract from *Lithospermum erythrorhizon* Sieb. et Zucc.

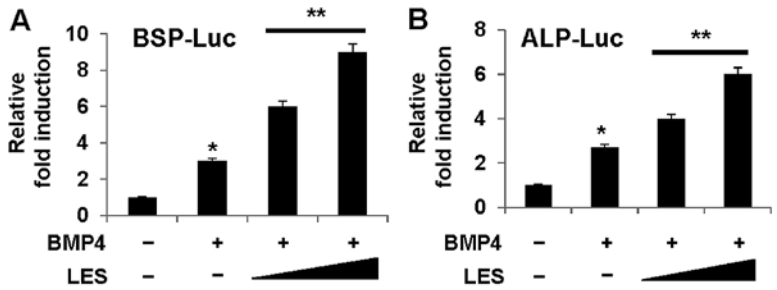

Figure 3. LES enhances the transcriptional activity of osteoblast markers during osteoblast differentiation. C2C12 cells stimulated with bone morphogenetic protein 4 (BMP4) were transfected with CMV promoter-driven β-galactosidase reporter (pCMV-β-gal) (0.05 μg), and with (A) a luciferase reporter bone sialoprotein (BSP)-Luc or (B) alkaline phosphatase (ALP)-Luc (0.2 μg), and treated with increasing amounts of LES (30 or 60 μg/ml). Luciferase activity was measured. *p<0.05 compared to cells not treated with BMP4; **p<0.05 compared to BMP4-treated cells and not transfected with any DNA plasmid. All experiments were repeated at least 3 times. The averages and standard deviations (SD) of representative experiments are shown. LES, ethanol (EtOH) extract from *Lithospermum erythrorhizon* Sieb. et Zucc.

osteoblast-specific luciferase reporters, BSP-Luc (Fig. 3A) and ALP-Luc (Fig. 3B). LES significantly enhanced the expression of the reporters. These results indicate that LES regulates the transcriptional activity of osteoblast transcription factors and is critical for the osteoblast transcription factor-induced expression, of at least a subset of osteoblast markers.

LES regulates the functions of the osteoblast-specific transcription factor, Runx2, in BMP4-induced osteoblast differentiation. The runt-related transcription factor, Runx2, regulates the expression of bone and cartilage-related genes and is required for bone formation. The regulatory mechanisms control both the activation and repression of Runx2 gene transcription during osteoblast differentiation and skeletal development. Runx2 is essential for the proper execution of the osteogenic program (7,19,44). In this study, in order to elucidate the mechanisms of action of the major transcription factor, Runx2, in cells treated with LES, we examined whether

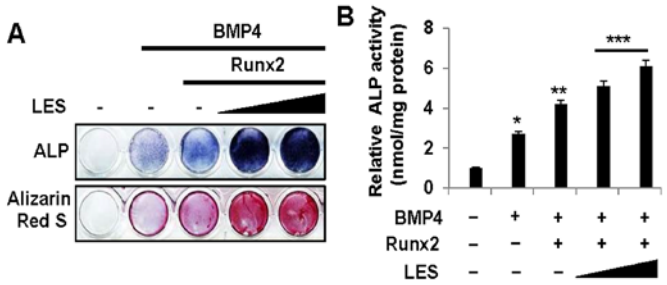

Figure 4. LES promotes osteoblast differentiation through Runx2 activation. C2C12 cells were transfected with Myc-Runx2 (1 μg) and then stimulated with bone morphogenetic protein 4 (BMP4) (30 ng/ml) or increasing amounts of LES (30 or 60 μg/ml). (A) The extent of osteoblast differentiation and mineralization was evaluated by alkaline phosphatase (ALP) staining (at 3 days) or Alizarin Red S staining (at 14 days). (B) ALP activity was measured by ALP assay. *p<0.05 compared to BMP4-untreated cells; **p<0.05 compared to BMP4-treated cells not transfected with any DNA plasmid; ***p<0.05 compared to cells transfected with Runx2 expression plasmid and not treated with LES. All experiments were repeated at least 3 times. The averages and standard deviations (SD) of representative experiments are shown. LES, ethanol (EtOH) extract from *Lithospermum erythrorhizon* Sieb. et Zucc.

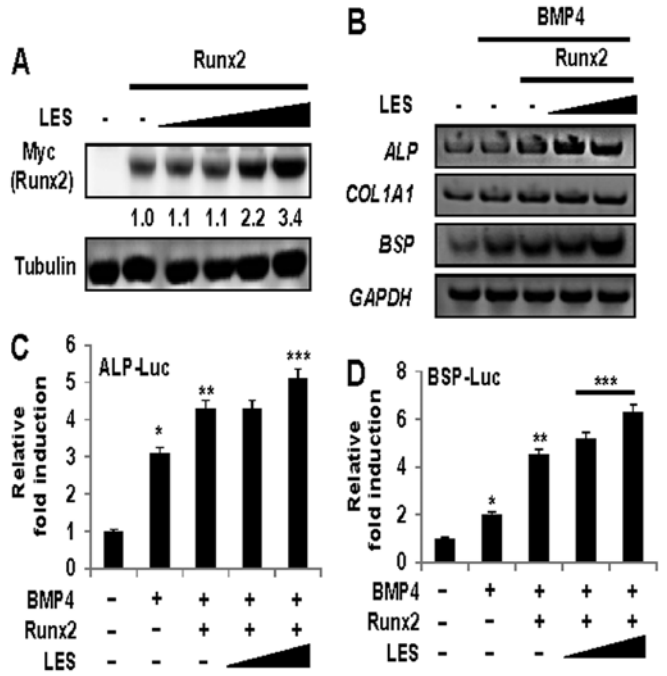

Figure 5. LES promotes osteoblast differentiation induced by bone morphogenetic protein 4 (BMP4) by ncreasing the expression and transcriptional activity of Runx2. (A) Cells (293) were transfected with Myc-Runx2 (1 μg) and then treated with increasing amounts of LES (5, 15, 30 and 60 μg/ml). The levels of over-expressed Runx2 were compared by immunoblotting using anti-Myc. Tubulin was used as a loading control. (B) The expression levels of the osteoblast-specific markers, alkaline phosphatase (*ALP*), bone sialoprotein (*BSP*) and collagen type I, α1 (*COL1A1*), were compared by RT-PCR. GAPDH was used as a loading control. (C and D) C2C12 cells were transfected with CMV promoter-driven β-galactosidase reporter (pCMV-β-gal) (0.05 μg), Myc-tagged Runx2 (0.5 μg), and with luciferase reporter (C) ALP-Luc or (D) BSP-Luc (0.2 μg) and treated with increasing amounts of LES (30 or 60 μg/ml). Luciferase activity was measured. *p<0.05 compared to BMP4-untreated cells; **p<0.05 compared to BMP4-treated cells not transfected with any DNA plasmid; ***p<0.05 compared to cells transfected with Runx2 expression plasmid and not treated with LES. The averages and standard deviations (SD) of representative experiments are shown. LES, ethanol (EtOH) extract from *Lithospermum erythrorhizon* Sieb. et Zucc.

LES affects Runx2 in cells stimulated with BMP4 to induce osteoblast differentiation. The cells stimulated with BMP and treated with LES were transfected with a Runx2 expression plasmid. First, we found that LES increased ALP activity, as shown by ALP staining (Fig. 4A, top panel), and also increased ALP activity (Fig. 4B) in the cells overexpressing Runx2 in the presence of BMP4. Similarly, LES increased mineralization, as shown by Alizarin Red S staining (Fig. 4A, bottom panel). We then investigated whether LES affects the protein level of Runx2. The protein levels of Runx2 were increased in the cells treated with LES (Fig. 5A). In addition, LES increased the expression of osteoblast specific markers in the cells stimulated with BMP4 (Fig. 2B). We then examined the effects of LES on the BMP4- and Runx2-induced expression of osteoblast marker genes (*ALP*, *BSP* and *COL1A1*). As shown in Fig. 5B, not only *ALP* and *COL1A1*, the early-stage osteogenic differentiation markers, but also *BSP*, a late-stage osteogenic differentiation marker, was positively affected by LES. The expression of osteoblast markers in the cells stimulated with BMP4 to induce osteoblast differentiation was increased in the presence of Runx2 and LES. Finally, to examine whether LES can modulate the transcriptional activity of Runx2, we examined the effects of LES on the transcriptional activity of Runx2 using the osteoblast-specific luciferase reporters,

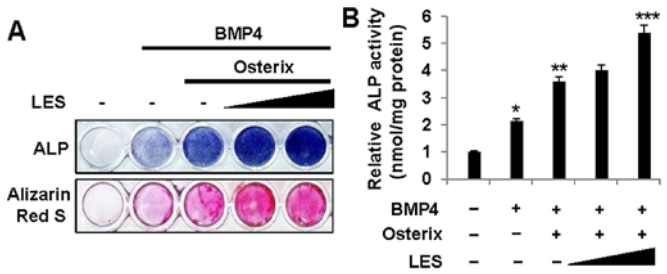

Figure 6. LES promotes osteoblast differentiation through Osterix activation. C2C12 cells were transfected with Myc-Osterix (0.5 μg) and then treated with bone morphogenetic protein 4 (BMP4) (30 ng/ml) or increasing amounts of LES (30 or 60 μg/ml). (A) The extent of osteoblast differentiation and mineralization was evaluated by alkaline phosphatase (ALP) staining (at 3 days) or Alizarin Red S staining (at 14 days). (B) ALP activity was measured by ALP assay. *p<0.05 compared to BMP4-untreated cells; **p<0.05 compared to BMP4-treated cells not transfected with any DNA plasmid; ***p<0.05 compared to cells transfected wtih Osterix expression plasmid and not treated with LES. All experiments were repeated at least 3 times. The averages and standard deviations (SD) of representative experiments are shown. LES, ethanol (EtOH) extract from *Lithospermum erythrorhizon* Sieb. et Zucc.

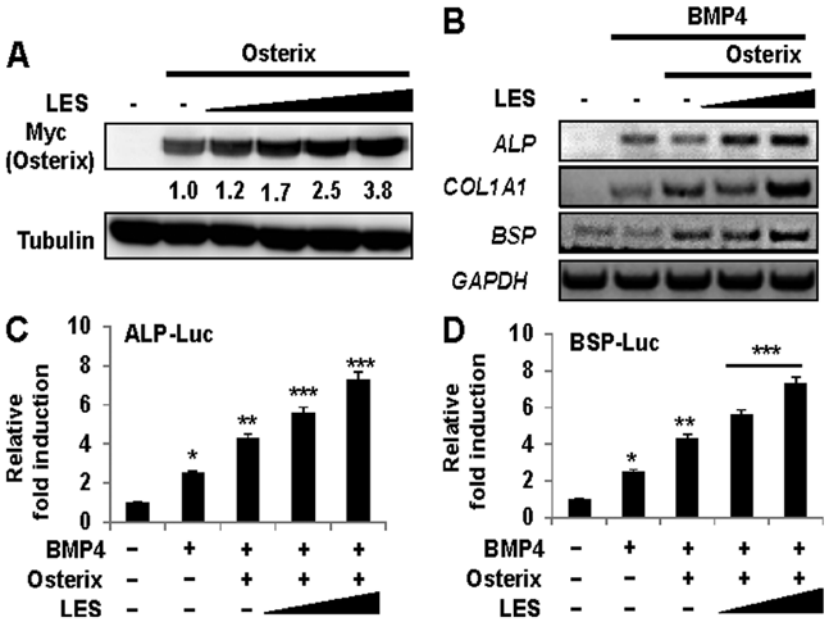

Figure 7. The expression and transcriptional activity of Osterix is markedly increased by LES during osteoblast differentiation. (A) Cells (293) were transfected with Myc-Osterix (0.5 μg) and then treated with increasing amounts of LES (5, 15, 30 and 60 μg/ml). The levels of overexpressed Osterix were compared by immunoblotting using anti-Myc. Tubulin was used as a loading control. (B) The expression levels of the osteoblast-specific markers, alkaline phosphatase (*ALP*), bone sialoprotein (*BSP*) and collagen type I, α1 (*COL1A1*), were compared by RT-PCR. GAPDH was used as a loading control. (C and D) C2C12 cells were transfected with CMV promoter-driven β-galactosidase reporter (pCMV-β-gal) (0.05 μg), Myc-tagged Osterix (0.5 μg), and with luciferase reporter (C) ALP-Luc or (D) BSP-Luc (0.2 μg) and treated increasing amounts of LES (30 or 60 μg/ml). Luciferase activity was measured. *p<0.05 compared to BMP4-untreated cells; **p<0.05 compared to BMP4-treated cells not transfected with any DNA plasmid; ***p<0.05 compared to cells transfected with Osterix expression plasmid and not treated with LEs. All experiments were repeated at least 3 times. The averages and standard deviations (SD) of representative experiments are shown. LES, ethanol (EtOH) extract from *Lithospermum erythrorhizon* Sieb. et Zucc.

ALP-Luc (Fig. 5C) and BSP-Luc (Fig. 5D). LES significantly enhanced the transcriptional activity of the Runx2-induced expression of the reporters. Taken together, these results suggest that LES regulates the expression levels and transcriptional activity of Runx2 during osteoblast differentiation.

LES regulates the functions of the osteoblast-specific transcription factor, Osterix, in BMP4-induced osteoblast differentiation. Osterix has been identified as a zinc finger-containing transcription factor. It is required for osteoblast differentiation and bone formation (15). BMP4 induces osteoblast differentiation and promotes bone formation (40-43). In the present study, we examined whether LES affects the BMP4-induced osteoblast differentiation of C2C12 cells. Osteoblast differentiation and mineralization were measured by ALP staining and Alizarin Red S. LES increased ALP activity, as shown by ALP staining (Fig. 6A, top panel) and increased ALP activity (Fig. 6B) in the presence of Osterix and BMP4. Similarly, LES increased mineralization, as shown by Alizarin Red S staining (Fig. 6A, bottom panel). We also examined whether LES regulates the level of ectopically expressed Osterix protein in non-osteogenic 293 cells. LES significantly enhanced the level of overexpressed Osterix protein in a dose-dependent manner (Fig. 7A). In addition, we examined whether LES affects the transcriptional activity of Osterix using the osteoblast-specific luciferase reporters, ALP-Luc (Fig. 7C) and BSP-Luc (Fig. 7D). LES significantly enhanced the transcriptional activity of the Osterix-induced

expression of the reporters. LES increased the expression of the osteoblast-specific marker genes *ALP*, *BSP*, and *COL1A1*, in the cells stimulated with BMP4 (Fig. 7B). Taken together, these results suggest that LES regulates the expression levels and transcriptional activity of Osterix during osteoblast differentiation.

Discussion

In this study, we examined the effects of LES, as a novel osteogenic material, on osteoblast differentiation. In previous studies, shikonin, from the root of *Lithospermum erythrorhizon*, has been shown to protect cartilage in rheumatoid arthritis, but also to induce osteoclastogenesis *in vitro* (38,39). However, hydrophobic shikonin and its derivatives are not extracted in the water-soluble EtOH extract, LES.

In our results, we found that LES promoted osteoblast differentiation and increased the expression levels and transcriptional activity of Runx2 and Osterix, the master genes of osteoblast differentiation. Even though the functions of LES in osteoblastogenesis are not yet well understood, our results indicate that the water-soluble components of LR could be the main active components which enhance osteoblastogenesis, instead of shikonin and its derivative. In other studies, lithospermic acid, a water-soluble phenolic compound, which is a standardized major bioactive component at a concentration of 2 mg/g (45), and polysaccharides such as lithospermans A, B and C (46) have also been identified in the EtOH extracts of LR. Therefore, a strong osteoblast differentiation effect by LES may be induced directly or indirectly by lithospermic acid and lithospermans A, B and C. However, further studies are required in order to evaluate the effects of the hydrophilic compounds from LES on osteoblastogenesis. Lithospermic acid is known to have antioxidant activity (47) and hypouricemic activity, and to exert anti-inflammatory effects (48), and anti-diabetic effects (49). However, there has been no study to date on the effects of lithospermic acid on osteoblast differentiation, at least to the best of our knowledge. In other studies, the 95% EtOH extract of LR was shown to exert a moisturizing effect (50) and the 70% EtOH extract of LR enhanced the epidermal level of ceramides (51).

The specific master gene of osteoblast differentiation, Runx2, plays a key role in bone formation. A loss-of-function mutation of Runx2 in mice results in no mineralized bone and the lack of mature osteoblasts and osteogenic differentiation markers (12,52,53). The function of Runx2 is regulated at several levels, such as transcription, translation, post-translational modification and protein-protein interactions by multiple signal transduction pathways. It is known that various kinases phosphorylate Runx2. The phosphorylation of Runx2 is an important mechanism that regulates its activity during osteoblastogenesis. Bone formation is stimulated by the phosphorylation of Runx2 via the MAPK/ERK signaling pathway (54,55) or protein kinase C (PKC)-δ (56). Thus, the regulation of Runx2 activity by the alteration of its phosphorylation status is important in bone formation.

Osterix is a novel zinc finger-containing transcription factor that is essential for the differentiation of pre-osteoblasts into functional osteoblasts (15). The function of Osterix can be regulated via post-translational modification by protein kinase-mediated osteogenesis. In a previous study, p38 was shown to regulate the expression of osteoblast-specific genes by the phosphorylation of Osterix (57). It has also been suggested that Akt induces the phosphorylation of threonine residue(s) on Osterix during osteoblast differentiation (58). Tyrosine kinase Src enhances osteogenic differentiation through the phosphorylation of Osterix (59).

Previous studies have revealed that shikonin, a major component of LES is involved in the regulation of ERK1/2 and Akt phosphorylation (60,61). Therefore, studies determining the phosphorylation by several protein kinases are warranted in order to understand the significance of the regulation of LES and the osteoblast-specific master genes, Runx2 and Osterix. The identification of protein kinase mechanisms underlying the effects of LES on osteoblast differentiation will enhance our understanding of the regulatory mechanisms of Osterix and Runx2 osteogenic function. Our study provides a basis for understanding the effects of LES on osteoblast differentiation.

In conclusion, in this study, we demonstrate that LES enhances osteoblast differentiation. LES modulates the function of the master genes, Runx2 and Osterix, through the regulation of their protein expression and transcriptional activity. Thus, LES may be a potential therapeutic agent for bone diseases, including osteoporosis. In addition, LR has been approved as a food source by the Ministry of Food and Drug safety, South Korea (MFDS), and the EtOH extract of LR (LES) is recognized as a safe food material for humans. Our data indicate that LES has potential for development as a potential candidate material for health functional food for bone health.

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