Reciprocal Regulation of Adipocyte and Osteoblast Differentiation of Mesenchymal Stem Cells by *Eupatorium japonicum* Prevents Bone Loss and Adiposity Increase in Osteoporotic Rats

Min-Ji Kim,^{1,*} Woo-Seok Jang,^{1,*} In-Kyoung Lee,² Jong-Keun Kim,^{1,3} Ki-Seung Seong,⁴ Cho-Rong Seo,¹ No-Joon Song,¹ Min-Hyuk Bang,¹ Young Min Lee,⁵ Haeng Ran Kim,⁵ Ki-Moon Park,¹ and Kye Won Park¹

¹Department of Food Science and Biotechnology, Sungkyunkwan University, Suwon, Korea.

²Division of Biotechnology, Advanced Institute of Environment and Bioscience, College of Environmental and Bioresource Sciences, Chonbuk National University, Iksan, Korea.

³Sungkyun Biotech Co., Ltd, Ansan, Korea.

⁴Food Function Research Group, Korea Food Research Institute, Seongnam, Korea.

⁵Department of Agrofood Resources, National Academy of Agricultural Science, Rural Development Administration, Suwon, Korea.

ABSTRACT Pathological increases in adipogenic potential with decreases in osteogenic differentiation occur in osteoporotic bone marrow cells. Previous studies have shown that bioactive materials isolated from natural products can reciprocally regulate adipogenic and osteogenic fates of bone marrow cells. In this study, we showed that *Eupatorium japonicum* stem extracts (EJE) suppressed lipid accumulation and inhibited the expression of adipocyte markers in multipotent C3H10T1/2 and primary bone marrow cells. Conversely, EJE stimulated alkaline phosphatase activity and induced the expression of osteoblast markers in C3H10T1/2 and primary bone marrow cells. Daily oral administration of 50 mg/kg of EJE for 6 weeks to ovariectomized rats prevented body weight increase and bone mineral density decrease. Finally, activity-guided fractionation led to the identification of coumaric acid and coumaric acid methyl ester as bioactive anti-adipogenic and pro-osteogenic components in EJE. Taken together, our data indicate a promising possibility of *E. japonicum* as a functional food and as a therapeutic intervention for preventing osteoporosis and bone fractures.

KEY WORDS: • bone marrow • C3H10T1/2 • coumaric acid • *Eupatorium japonicum* • osteoporosis • ovariectomized rat

INTRODUCTION

AN IMBALANCE BETWEEN BONE FORMATION by osteoblasts and resorption by osteoclasts results in osteoporosis, characterized by low bone mineral density (BMD), reduced bone strength, and an increased risk of fracture.¹ Osteoporosis is more common with increasing age and is associated with metabolic diseases such as diabetes and obesity.² Thus, the prevalence of osteoporosis is forecasted to increase worldwide due to the increase in the aging population and in the incidence of metabolic diseases.³

Estrogen deficiency, particularly in elderly women, often leads to rapid bone loss. Therefore, estrogen replacement

therapy has been used to prevent osteoporosis in postmenopausal women. However, recent studies of estrogen use as part of postmenopausal hormone replacement therapy have revealed unwanted side effects such as endometrial cancer and weight gain.^{4,5} Another strategy using bisphosphonate is also a leading treatment for osteoporosis but is associated with unpleasant side effects such as gastric disturbances.⁶ Other anabolic approaches confer increases in the number and activity of osteoblasts but also carry the risk of various side effects.⁷ New effective strategies are desired to ameliorate osteoporotic conditions without unwanted side effects.

Bone marrow cells from osteoporotic mice and aged humans are characterized by increased adipogenic activity but with decreased osteogenic potentials.^{8–11} Both osteoblasts and adipocytes are derived from bone marrow mesenchymal stem cells (MSCs),^{12,13} and generation of these cells has been reported as reciprocal processes.^{14,15} Similarly, age-related osteoporosis is closely associated with reduced bone formation and increased fat accumulation in the bone

*The first two authors contributed equally to this article.

Manuscript received 3 September 2013. Revision accepted 2 April 2014.

Address correspondence to: Kye Won Park, PhD, Department of Food Science and Biotechnology, Sungkyunkwan University, Suwon 440-746, Korea, E-mail: kwpark@skku.edu

marrow. Subsequent studies have shown that MSCs from osteoporotic bone marrow have less potential to differentiate into osteoblasts, suggesting that MSCs lose their ability to differentiate into osteoblasts, which contributes to the loss of bone formation.^{16,17} Previous studies have shown that numerous herbal extracts are potentially useful for inducing bone-promoting effects, mostly by suppressing bone resorbing actions.¹⁸ Similarly, *in vivo* studies have shown that several herbal extracts possess beneficial effects in ovariectomized (OVX) rats and menopausal women.^{19,20} Furthermore, bioactive compounds found in herbal extracts, including genistein, resveratrol, and kaempferol, are known to enhance osteogenesis and repress adipogenesis of bone marrow cells. Therefore, we investigated the possibility that such bioactive compounds containing natural products could affect adipogenic and osteogenic fates and thus be potentially useful for the treatment of osteoporosis and for preventing bone fractures.

To identify herbal extracts that reciprocally regulate osteoblast differentiation and adipocyte differentiation, various herbal extracts were tested in multipotent C3H10T1/2 cells, and *Eupatorium japonicum* was selected for its pro-effects in osteoblasts and its anti-effects in adipocytes. In this study, the effects of *E. japonicum* stem extracts (EJE) on osteoblast and adipocyte differentiation in mesenchymal stem cells and a possible new strategy for osteoporosis treatment in ovariectomized rats were investigated.

MATERIALS AND METHODS

Preparation of E. japonicum stem extracts

E. japonicum plants were collected between May and September from Gam-ag Mountain in Gyeonggi, Korea. *E. japonicum* was identified by Dr. Young Jae Chung (Department of Life Science and Biotechnology, Shingyeong University, Hwaseong, Korea). Stems of *E. japonicum* were dried and powdered, and then 20 g was extracted with 99% ethanol (400 mL) in a shaking incubator (VS-8480 SF; Vision, Daejeon, Korea) at 40°C at 120 rpm for 48 h. The extracts were filtered through filter paper (Whatman No. 1), and concentrated with a rotary vacuum evaporator at 45°C before being freeze-dried at -50°C. The ethanol extract from the stems was further fractionated in methanol, n-hexane, dichloromethane (DCM), ethyl acetate, butanol, and H₂O, successively. Finally, the extracts were resolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL, and stored at -20°C before use.

Cell culture

Murine C3H10T1/2 multipotent cells, 3T3-L1, and MC3T3-E1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA).²¹ C3H10T1/2 cells were cultured at 37°C in a 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS). MC3T3-E1 cells were maintained in α -Minimum Essential Media (α -MEM; Thermo Scientific, Waltham, MA, USA)

supplemented with 10% FBS. A subculture of mouse 3T3-L1 preadipocytes was supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA). For osteoblast differentiation of C3H10T1/2 cells, the cells were seeded at a density of 2.5×10^4 /mL in 6-well plates and cultured to confluence, and the medium was changed to osteoblast differentiation medium, consisting of DMEM, 5% FBS, 50 μ g/mL ascorbic acid, and 10 mM β -glycerophosphate (Sigma, St. Louis, MO, USA). MC3T3-E1 cells were induced to osteoblast in α -MEM, 10% FBS, supplemented with 50 μ g/mL ascorbic acid and 10 mM β -glycerophosphate. The medium was changed every 3 days in the presence of 5, 10, 20, and 40 μ g/mL of the extract from each part of the plant for a total of 10 days.²² For adipocyte differentiation of C3H10T1/2 and 3T3-L1 cells, the medium was changed to adipocyte differentiation medium containing DMEM, 10% FBS, 1 μ M dexamethasone, 1 μ M troglitazone, and 5 μ g/mL insulin (Sigma) for 2 days, followed by insulin and troglitazone only.²³ The medium was changed every 2 days in the presence of 5, 10, 20, and 40 μ g/mL of the extract.

Rat bone marrow-derived primary cells

Primary bone marrow cells were isolated using a modified procedure described previously.²⁴ The tibia and femur were harvested from 6–8-week-old Sprague–Dawley female rats (Orient. Co. Ltd., Young-In, Korea). Bone marrow cells were obtained by inserting syringes with 27-gauge needles into the end of the bone and flushing strongly with DMEM containing 15% FBS and $1 \times$ penicillin/streptomycin. The cells were then cultured at 37°C in a 5% CO₂ atmosphere, and the medium was changed twice weekly. The cells were seeded in 6-well plates until reaching 70–80% confluence. After the cells reached confluence, differentiation of osteoblasts and adipocytes was initiated.

Alkaline phosphatase and Oil Red O staining

At 10 days of osteoblast differentiation, the cells were stained to measure alkaline phosphatase (ALP) with 0.2 mg/mL of 5-bromo-4-chloro-3-indolyl phosphate and 0.4 mg/mL of nitro blue tetrazolium (Sigma). For adipocyte differentiation staining, cells at 7 days were fixed with 4% formaldehyde (Sigma) in PBS for 1 h and stained with 0.5% Oil Red O (Sigma) in isopropanol for 1 h. To quantify triglyceride content, stained cells were resolved with isopropanol, and absorbance was measured at 520 nm.

Real-time quantitative polymerase chain reaction analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA and 300 pmol of random primers were mixed and heated at 65°C for 5 min. This RNA mixture was then reverse transcribed using 4 μ L 5 $\times$ RTase M-MLV buffer, 10 mM dNTP mixture, RTase M-MLV (2640A; Takara, Ohtsu, Japan) at 37°C for 50 min, followed by heat inactivation at 70°C for 10 min. The cDNA was analyzed using a thermal cycler (Takara) using the Power SYBR Premix Ex Taq (RP041A, Takara).

The following specific oligonucleotide primers were used: mouse peroxisome proliferator-activated receptor γ (*PPAR* γ), (forward primer, 5'-CCA TTC TGG CCC ACC AAC-3'; reverse primer, 5'-AAT GCG AGT GGT CTT CCA TCA-3'), mouse *aP2* (forward primer, 5'-CAC CGC AGA CGA CAG GAA G-3'; reverse primer, 5'-GCA CCT GCA CCA GGG C-3'), mouse *CD36* (forward primer, 5'-GGC CAA GCT ATT GCG ACA T-3'; reverse primer, 5'-CAG ATC CGA ACA CAG CGT AGA-3'), mouse *LPL* (forward primer, 5'-GTG GCC GAG AGC GAG AAC-3'; reverse primer, 5'-AAG AAG GAG TAG GTT TTA TTT GTG GAA-3'), mouse *adiponectin* (forward primer, 5'-CCG GAA CCC CTG GCA G-3'; reverse primer, 5'-CTG AAC GCT GAG CGA TAC ACA-3'), mouse *36B4* (forward primer, 5'-AGA TGC AGC AGA TCC GCA T-3'; reverse primer, 5'-GTT CTT GCC CAT CAG CAC C-3'), mouse *ALP* (forward primer, 5'-AAA CCC AGA ACA CAA GCA TTC C-3'; reverse primer, 5'-TCC ACC AGC AAG AAG AAG CC-3'), mouse *osterix* (forward primer, 5'-GCT AGA GAT CTG AGC CGG GTA-3'; reverse primer, 5'-AAG AGA CCT GGC AAG AGG-3'), mouse osteocalcin (forward primer, 5'-TCT CTC TGA CCT CAC AGA TGC C-3'; reverse primer, 5'-TAC CTT ATT GCC CTC CTG CTT G-3'), mouse *collagen I* (forward primer, 5'-ACC TAA GGG TAC CGC TGG A-3'; reverse primer, 5'-TCC AGC TTC TCC ATC TTT GC-3'), mouse Runx II (forward primer, 5'-GCC CAG GCG TAT TTC AGA-3'; reverse primer, 5'-TGC CTG GCT CTT CTT ACT GAG-3'), rat *ALP* (forward primer, 5'-GCA CAA CAT CAA GGA CAT CG-3'; reverse primer, 5'-TCA GTT CTG TTC TTG GGG TAC-3'), rat *osterix* (forward primer, 5'-TTC TGA CTG CCT GCC TAG TGT-3'; reverse primer, 5'-TGG ATG CCC GCC TTG TA-3'), rat *collagen I* (forward primer, 5'-CAT GTT CAG CTT TGT GGA CCT-3'; reverse primer, 5'-GCA GCT GAC TTC AGG GAT GT), rat RUNX II (forward primer, 5'-CCA CAG AGC TAT TAA AGT GAC-3'; reverse primer, 5'-AAC AAA CTA GGT TTA GAG TCA-3'), rat *PPAR* γ (forward primer, 5'-CCC AAT GGT TGC TTA CA-3'; reverse primer, 5'-GGA CGC AGG CTC TAC TTT GA-3'), rat *ap2* (forward primer, 5'-AAT GTG CGA CGC CTT TGT-3'; reverse primer, 5'-TGA TGA TCA AGT TGG GCT TG-3'), rat *LPL* (forward primer, 5'-CAG AGA AGG GGC TTG GAG A-3'; reverse primer, 5'-TTC ATT CAG GGA GTC AA-3'), rat *adiponectin* (forward primer, 5'-TGG TCA CAA TGG GAT ACC G-3'; reverse primer, 5'-CCC TTA GGA CCA AGA ACA CCT-3'), rat *36B4* (forward primer, 5'-GAT GCC CAG GGA AGA CAG-3'; reverse primer, 5'-CAC AAT GAA GCA TTT TGG GTA). All animal studies were carried out in accordance with the guidelines of the Animal Research Committee of Sungkyunkwan University. Fifteen-week-old female Sprague–Dawley rats were purchased (Orient. Co. Ltd.) and housed in controlled temperature ($24 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$) conditions under a 12 h light–dark cycle. The rats were individually housed and allowed free access to water and food (Solid feed 5L79; Orient. Co. Ltd.). After an adaptation period of 1 week, 16-week-old female rats were randomized into two groups: five rats were sham-operated, and 10 rats were bilaterally ovari-

ectomized (OVX). For ovariectomy, rats were anesthetized with Rompun and Zoletil (4:6), and both sides of the back were shaved bilaterally. Skin and muscles on both sides were incised about 1.5 cm, the ovaries were removed, and the uterine tubes were ligated. OVX rats were further divided into two groups. The control OVX group was administrated by oral gavage once daily with vehicle (10% corn oil and 90% water) for 6 weeks. The EJE OVX group was administrated with 50 mg/kg of EJE by oral gavage for 6 weeks once a day. To prepare the suspension, 50 mg of EJE was suspended in 1 mL of corn oil, sonicated for 10 min, and diluted in 9 mL of water. Body weight was measured every day, and food intake was determined three times per week.

Animal studies

At the end of the 6 week treatment, rats fasted for 16 h and were anesthetized by inhalation of ether, followed by cardiac puncture, and collection of the liver, uterus, kidneys, blood, femur, and tibia. Serum was collected by centrifugation (VS-5500N; Vision, Daejeon, Korea) at 3000 rpm for 10 min. The uterus was weighed after removing fat and draining the intra fluid; the trimmed right femur was analyzed using dual energy X-ray absorptiometry to determine BMD (Forearm: Norland Bone Densitometer; CooperSurgical, Inc., Trumbull, CT, USA). BMD was measured from the right femur and calculated from the same cross-section. For histological studies, the harvested left tibiae were trimmed to remove any visible soft tissues and fixed in 10% formaldehyde for 2 days, decalcified in 0.5 M EDTA (pH 8.0) for 3 days, and embedded in paraffin. Paraffin blocks were sectioned at a thickness of 5 μm and were subjected to hematoxylin and eosin (H&E) staining. Lipid vacuole area was assessed using a light microscope with a digitalized camera and image J software. The area of lipid vacuoles was calculated from three different fields per rat (three rats per group).

Activity-guided fractionation and compound isolation

The ethanol extracts from the stems of *E. japonicum* was fractionated in methanol, n-hexane, dichloromethane, ethyl acetate, butanol, and H_2O . Active fractions, dichloromethane fractionated extracts were further separated by Sephadex LH-20 column chromatography to produce six subfractions, and each subfraction at 10 $\mu\text{g/mL}$ was tested for biological activity. The selected subfraction was then separated by preparative reverse phase high performance liquid chromatography (HPLC) using HPLC systems equipped with an ultraviolet detector and Agilent Zorbax Eclipse Plus C18 column (4.6 mm $\times$ 150 mm, 5 μm) with two solvent systems, 0.04% TFA in 10% MeOH (A) and 100% MeOH (B) at a flow rate of 1.0 mL/min. Two major compounds in the DCM fraction were obtained at retention times of 14 min and 18 min. The structures of the compounds were elucidated based on ^1H -nuclear magnetic resonance (NMR), ^{13}C NMR spectra, and ESI-MS.

Statistical analysis

Statistical analyses were conducted using PASW Statistics 17 (SPSS, Inc., Chicago, IL, USA). Data are presented

as means and standard error of the mean (SEM). Analysis of variance followed by Student–Newman–Keuls tests was used to analyze the differences in gene expression and lipid accumulation using statistical analysis using SPSS software program (PASW Statistics 17, SPSS Inc., Chicago, IL, USA). Differences in animal studies and gene expression for coumaric acids treated samples were analyzed with an unpaired Student's *t*-test. Statistical significance was defined as $P < .05$.

RESULTS

EJE inhibit adipocyte differentiation but promote osteoblast differentiation in C3H10T1/2 and primary bone marrow cells

Although various compounds were identified as having either pro-bone or anti-fat activities, a few herbal extracts have been demonstrated to inhibit adipogenesis and promote osteogenesis simultaneously.^{25,26} To show anti-adipogenic effects of EJE, C3H10T1/2 cells were differentiated into adipocytes and treated with various doses of EJE. EJE significantly suppressed morphological differentiation and lipid accumulation in a dose-dependent manner (Fig. 1A). Consistent with the effects on lipid accumulation, *PPAR* γ and its target genes including *aP2*, *C/EBP* α , and *LPL* decreased after 7 days of treatment with EJE, further demonstrating its inhibitory effects on adipogenic differentiation in C3H10T1/2 cells (Fig. 1B).

We also investigated whether EJE would indeed act as an osteoinductive material. C3H10T1/2 cells were treated with various concentrations of EJE and were induced for osteoblast differentiation. ALP activity was induced by EJE

treatment (Fig. 2A). Expression of *ALP*, *osterix*, and *Runx2* in C3H10T1/2 cells was consistently induced following 10 days of treatment with EJE (Fig. 2B). Together, these data indicate anti-adipogenic and pro-osteogenic activities of *E. japonicum* in C3H10T1/2 cells.

The degree of anti-adipogenic and pro-osteogenic effects by 20 $\mu\text{g/mL}$ of the stem extracts was shown to be similar, if not stronger, than the effects of treatment with 10 μM of resveratrol (Supplementary Fig. S1; Supplementary Data are available online at www.liebert.com/jmf). Furthermore, the anti-fat and pro-bone activities of the stem extracts were not restricted to C3H10T1/2 cells but were common to all multipotent cells. The dose-dependent inhibitory effects during adipocyte differentiation were consistently observed in primary bone marrow cells that were freshly isolated from rat tibiae (Fig. 1C). By contrast, the stem extracts increased expression of *ALP*, *Col I*, and *Runx2* in primary bone marrow cells (Fig. 2C). Thus, the stem extracts of *E. japonicum* demonstrated the strongest pro-bone and anti-fat effects in C3H10T1/2 cells and primary bone marrow cells.

Effects of the stem extracts harvested during various periods on pro-bone and anti-fat activities

The harvesting period can directly affect the profiles of bioactive compounds and thus influence their biological activities.²⁷ As such, the anti-adipogenic and pro-osteogenic effects of EJE harvested during May–September were determined. The anti-adipogenic effects increased gradually in the stems harvested from May, June, July, to August and September (Supplementary Fig. S2). Consistent with this

FIG. 1. *Eupatorium japonicum* stem extracts (EJE) inhibit adipocyte differentiation in C3H10T1/2 and primary bone marrow cells. (A, B) C3H10T1/2 cells were treated with 5, 20, or 40 $\mu\text{g/mL}$ of *E. japonicum* stem extracts and were induced to differentiate into adipocytes for 7 days. (A) Lipid accumulation was assessed by Oil Red O staining. (B) mRNA expression of adipogenic genes encoding *PPAR* γ , *aP2*, *CD36*, *adiponectin*, *C/EBP* α , and *LPL* was measured by real-time polymerase chain reaction (PCR). (C) Rat primary bone marrow cells isolated from tibiae were differentiated into adipocytes in the presence of various concentrations of *E. japonicum* stem extracts for 7 days, and the relative mRNA levels were measured. Relative mRNA expression of adipogenic genes encoding *PPAR* γ , *aP2*, and *adiponectin* was measured by real-time PCR. Data are means $\pm$ standard error of the mean (SEM) from three independent experiments. ^{abcd}Means with different letters for each sample are significantly different ($P < .05$) by Student–Newman–Keuls test. Color images available online at www.liebertpub.com/jmf

FIG. 2. *E. japonicum* stem extracts stimulate osteoblast differentiation in C3H10T1/2 cells and in primary bone marrow cells. (A) C3H10T1/2 cells were treated with 5, 20, or 40 $\mu\text{g/mL}$ of *E. japonicum* stem extracts and were induced to differentiate for 10 days followed by staining for ALP activity. (B) Relative levels of *ALP*, *osterix*, *Col1*, and *Runx2* mRNA were determined by real-time PCR in C3H10T1/2 cells. (C) Rat primary bone marrow cells isolated from tibia were differentiated into osteoblasts in the presence of various concentrations of stem extracts for 10 days and expression of *ALP*, *Col1*, and *Runx2* was determined by real-time PCR. Data are means $\pm$ SEM from three independent experiments. ^{a-d}Means with different letters for each sample are significantly different ($P < .05$) by the Student–Newman–Keuls test. Color images available online at www.liebertpub.com/jmf

finding, the expression of *PPAR γ* , *aP2*, *CD36*, and *adiponectin* was strongly suppressed by the stem extracts harvested from August and September compared to that of earlier harvested stems. In contrast, pro-osteogenic activities were more markedly induced by late-harvested stems and less by earlier-harvested stems (Supplementary Fig. S2). The expression of the osteoblast marker *ALP* was also strongly induced by *E. japonicum* harvested between July and September. Notably, *E. japonicum* harvested in May failed to induce *ALP* expression. Therefore, the strongest pro-bone and anti-fat effects of *E. japonicum* were observed in the samples harvested in August or September.

Stem extracts prevent bone loss in OVX rats

Our data show that the stem extracts of *E. japonicum* harvested in September contained strong pro-bone and anti-fat effects *in vitro*. Thus, the stem extract was further tested for its effects on osteoporosis. To test its *in vivo* effects, 3-month-old female Sprague–Dawley rats were sham-operated or ovariectomized (OVX), and then OVX rats were orally administrated with vehicle (control OVX group) or 50 mg/kg/day of the stem extract (EJE OVX group) for 6 weeks. After 6 weeks, body weight increased to about 20% in the control OVX group, whereas the EJE OVX groups gained about 10% compared to weight of the sham group (Fig. 3A). Serum triglycerides (36.4 vs. 48.4 mg/dL) and free fatty acid levels (1.60 vs. 1.87 mEq/L) were lower in the EJE OVX group compared to those in the control OVX group. The mean uterine weight was three times higher in the

stem extract-fed OVX group than that in the vehicle-treated OVX control group (Fig. 3B). However, no differences were observed in other organs such as the liver, kidneys, and spleen across the three groups (Fig. 3B). Food intake, serum total cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), uric acid, creatine, and high-density lipoprotein-cholesterol levels were also not different between the control and EJE OVX groups.

To investigate the effects of preventing bone loss, BMD was measured using dual energy X-ray absorptiometry. As expected, OVX induced significant changes in the BMD of the right femur (0.116 g/cm²) compared to that of the sham group (0.127 g/cm²; Fig. 4). OVX-induced bone loss was significantly prevented by daily consumption of the stem extract for 6 weeks (0.123 g/cm², a 5.4% increase compared to that in the control OVX groups; Fig. 4A). To test directly whether EJE reversed the enhanced adipogenic potential of bone marrow cells found in osteoporotic bone, decalcified left tibiae of the OVX rats were sectioned at a thickness of 5 μm and stained with H&E. Lipid droplets were detected as vacuole areas. Increased adiposity in the bone marrow of tibiae in the control OVX group was compared to that in the sham control group (arrow in Fig. 4B). Remarkably, increased adiposity in the bone marrow of EJE OVX rats was inhibited and was close to the levels seen in the sham group following EJE supplementation, further supporting the anti-fat and pro-bone effects of *E. japonicum* in bone marrow (Fig. 4C). Altogether, these data show the potential preventive effects of *E. japonicum* against bone loss and osteoporosis.

FIG. 3. Body weight and wet uterine weight changes in sham control, ovariectomized (OVX), and OVX rats orally administered with 50 mg/kg *E. japonicum* stem extracts for 6 weeks. **(A)** Body weights of sham-operated group, OVX rats orally administered with vehicle for 6 weeks (control OVX group), and OVX rats orally administered with 50 mg/kg of stem extracts for 6 weeks (EJE OVX group). **(B)** Organ weights of sham, control OVX, and EJE OVX groups. Data are means $\pm$ SEM ($n=5$). Statistical significance of differences between the EJE OVX group and the control OVX group was determined using Student's *t*-test. * $P<.05$.

FIG. 4. Stem extracts of *E. japonicum* prevent bone loss in ovariectomized (OVX) rats. **(A)** Bone mineral density (BMD) of the right femur was measured using dual energy X-ray absorptiometry (DEXA). OVX induced a significant reduction in BMD of the right femur (0.116 g/cm²) compared to that in the sham group (0.127 g/cm²). BMD of the OVX rats orally administered with 50 mg/kg of EJE (EJE OVX group; 0.123 g/cm²) was significantly higher than that of the OVX rats orally administered with vehicle (control OVX group). Data are means $\pm$ SEM ($n=5$). Statistical significance of differences between the EJE OVX group and the control OVX group was determined using Student's *t*-test. * $P<.05$). **(B)** Hematoxylin and eosin (H&E) staining of tibial sections from the sham, control OVX, and EJE OVX groups showed increased bone marrow adiposity of the tibiae in OVX rats. Increased adiposity in the control OVX group decreased significantly in the EJE OVX group. Arrows point to bone marrow adiposity. Two independent photomicrographs at 40 $\times$ magnification per group are shown. **(C)** Quantification of adipocyte vacuole area of tibial sections from the sham, control OVX, and EJE OVX groups. The relative area of vacuoles was calculated from three different fields per rat ($n=3$ /group). Statistical significance of EJE OVX group was determined by comparison with the control OVX group using Student's *t*-test. * $P<.05$. Color images available online at www.liebertpub.com/jmf

Identification of active compounds from *E. japonicum* stem extracts

Activity-guided fractionation showed that the DCM fractions of stem extracts of *E. japonicum* are enriched with pro-osteogenic and anti-adipogenic bioactive compounds (Supplementary Fig. S3). To identify bioactive compounds, DCM fractions were further separated using Sephadex LH-20 and HPLC, followed by ^1H NMR, ^{13}C NMR spectra, and ESI-MS analysis (Fig. 5A). Two major peaks were separated by HPLC with a fragmentation ion of SF3-1 at m/z 119.0 and 163.0 ($\text{M}-\text{H}$) $^-$ and m/z 145.0 and SF3-2 at 177.0 [$\text{M}-\text{H}$] $^-$ in ESI-MS (Fig. 5B and C). These were further identified as *p*-coumaric acid and *p*-coumaric acid methyl ester by NMR studies. In addition, *p*-coumaric acid was further confirmed by comparison with commercial *p*-coumaric acid. To our knowledge, coumaric acid and its derivative coumaric acid methyl ester were detected in *E. japonicum* for the first time.

Identification of coumaric acid and coumaric acid methyl ester as osteogenic and anti-adipogenic compounds

Previous studies have shown that coumaric acid can protect against bone loss and obesity in mice.^{28,29} Those studies are consistent with our assumption that coumaric acid and its

derivatives would mediate pro-bone and anti-fat effects of *E. japonicum*. To test this possibility directly, we treated cells with coumaric acid or coumaric acid methyl ester and examined their ability to inhibit adipocyte differentiation and promote osteoblast differentiation (Fig. 6A). Coumaric acid dose dependently inhibited adipocyte differentiation in 3T3-L1 and C3H10T1/2 cells (Fig. 6B). Similarly, coumaric acid methyl ester also inhibited lipid accumulation of 3T3-L1 cells (Fig. 6C). Furthermore, coumaric acid inhibited the expression of *PPAR* γ , further demonstrating its inhibitory effects on adipogenic differentiation (Fig. 6D). Consistently, coumaric acid and coumaric acid methyl ester also increased osteoblast differentiation in C3H10T1/2 and MC3T3-E1 cells (Fig. 6E, F). The expression of osteoblast markers such as *ALP* and *osterix* was also induced following 10 days of treatment with coumaric acid (Fig. 6G). Taken together, our data indicate that coumaric acid and coumaric acid methyl ester could mediate, at least in part, the pro-bone and anti-fat effects of *E. japonicum*.

DISCUSSION

Osteoporosis is a degenerative skeletal disease marked by reduced bone mass, resulting in an increased risk for bone

FIG. 5. Identification of *p*-coumaric acid and *p*-coumaric acid methyl ester as bioactive compounds from stem extracts of *E. japonicum*. **(A)** A scheme for isolation of bioactive compounds from DCM subfractions of *E. japonicum*. **(B)** HPLC chromatographic separation of compounds SF3-1 and SF3-2 from DCM fractionated extracts of *E. japonicum* stems. The mobile phase comprised 0.04% TFA in 10 % MeOH and 100 % MeOH. The flow rate was 1.0 mL/min and detector wavelength was 245 nm. The injection volume was 20 μL . **(C)** ESI-MS spectrum of SF3-1 and SF3-2. Two major peaks were separated by HPLC with a fragmentation ion at m/z 119.0 and 163.0 [$\text{M}-\text{H}$] $^-$ for SF3-1 and m/z 145.0 and 177.0 [$\text{M}-\text{H}$] $^-$ for SF3-2 in ESI-MS. These were identified as *p*-coumaric acid (SF3-1) and *p*-coumaric acid methyl ester (SF3-2).

FIG. 6. Coumaric acid and coumaric acid methyl ester inhibit adipocyte differentiation but promote osteoblast differentiation. **(A)** Structures of coumaric acid and coumaric acid methyl ester isolated from DCM-fractionated extracts of *E. japonicum*. **(B)** 3T3-L1 cells (upper) and C3H10T1/2 cells (lower) were treated with coumaric acid (commercially available) and were induced to differentiate into adipocytes for 7 days. **(C)** 3T3-L1 cells were treated with coumaric acid methyl ester (isolated from EJE) and were induced to differentiate into adipocytes for 7 days. Lipid accumulation was assessed by Oil Red O staining. **(D)** C3H10T1/2 cells were treated with coumaric acid (500 μ M) under adipogenic condition and relative levels of *PPAR γ* mRNA were determined by real-time PCR. **(E)** C3H10T1/2 cells (upper) and MC3T3-E1 cells (lower) were treated with coumaric acid and were stimulated to differentiate into osteoblasts for 10 days and ALP-positive cells were stained. **(F)** C3H10T1/2 cells were treated with coumaric acid methyl ester and were stimulated to differentiate into osteoblasts for 10 days and ALP-positive cells were stained. **(G)** C3H10T1/2 cells were treated with coumaric acid (500 μ M) under osteogenic conditions for 10 days, and relative levels of *ALP* and *osterix* mRNA were determined by real-time PCR. Data are means $\pm$ SEM from three independent experiments. Statistical significance was determined by comparison to the control using Student's *t*-test. **P* < .05; ***P* < .005; ****P* < .0005. Color images available online at www.liebertpub.com/jmf

fractures. There are fewer bone-forming cells in the bone marrow of patients with osteoporosis, which can be attributed to a reduced potential of osteoblast differentiation with a reciprocally enhanced ability for adipocyte differentiation from MSCs.^{10–12} Bone mass from osteoblasts increases during childhood and decreases with ageing, showing that bone homeostasis is not constant throughout life.⁸ These observations led to formulation of our hypothesis that MSCs in bone marrow can be reoriented toward bone formation in the presence of certain stimuli, and thus, MSCs are considered important tools as preventive and therapeutic interventions against osteoporosis.

In an effort to identify herbal extracts that can potentially be used to shift bone fate from away from bone mineral loss and osteoporosis, C3H10T1/2 cells were differentiated into osteoblasts and adipocytes in the presence

of natural products. In the present study, we showed that EJE inhibited lipid accumulation and *PPAR γ* expression in C3H10T1/2 cells. Conversely, EJE stimulated ALP activity and induced the expression of osteoblast markers in C3H10T1/2 cells. Importantly, these activities were detected in primary bone marrow cells isolated from rat tibiae, further validating the effects of EJE. Stem extracts harvested in May–September showed significantly variable dual effects with the highest activity from stems harvested in August and September. Finally, *in vivo* administration of the stem extract prevented increases in body weight, triglyceride levels, adiposity in bone marrow, and a decrease in bone density in OVX rats. Therefore, our data show that *E. japonicum* possesses anti-fat and pro-bone effects and its application in the potential treatment of osteoporosis.

A few bioactive compounds including rutin, pyrrolizidine alkaloids, and essential oils such as thymol have been identified in *Eupatorium*.^{30,31} Rutin affects lipid accumulation, whereas pyrrolizidine alkaloids may potentially affect tumor cells. Thymol inhibits bone resorption by suppressing osteoclast activity. Activity-guided fractionation combined with ESI-MS and NMR led to the identification of coumaric acid and coumaric acid methyl ester as the bioactive compounds. Interestingly, coumaric acid was shown to prevent high-fat diet induced obesity in mice as well as bone loss in OVX rats.^{28,29} In addition, our data also show that both coumaric acid and coumaric acid methyl ester inhibited lipid accumulation but conversely stimulated ALP activity. Although we cannot exclude the possibility of additive or synergistic effects by combination with other compounds such as thymol and rutin, these data support the idea that coumaric acid and coumaric acid methyl ester can at least in part mediate the anti-fat and pro-bone effects of *E. japonicum*. Our data warrant further investigation on the underlying mechanisms of pro-osteogenic and anti-adipogenic actions of *E. japonicum* and its bioactive compound, coumaric acid. Furthermore, extensive studies with larger numbers of animals should also be conducted in the future.

Osteoporosis can potentially be treated by preventing bone resorption or promoting bone formation.³ Bisphosphonates are the most commonly prescribed antiresorptive drugs for the treatment of osteoporosis. However, they are associated with unpleasant gastric side effects and other possible adverse outcomes such as esophageal cancer and osteonecrosis of the jaw.³² Furthermore, a study of women with osteoporosis treated with bisphosphonates beyond 5 years showed that very little new bone formed, suggesting that a combinatorial strategy for new bone formation is required for treating osteoporosis.³³ Therefore, the current study may also provide a new complementary approach to antiresorptive bisphosphonates for the treatment of age-related osteoporosis and other bone diseases in humans.

In summary, our study suggests the possibility that coumaric acid, coumaric acid methyl ester, and natural products harboring these compounds such as *E. japonicum* may prevent bone loss by modulating adipocyte and osteoblast differentiation of bone marrow cells in patients with osteoporosis.

ACKNOWLEDGMENTS

This study was supported by a grant from the AGENDA program (project No. PJ008447), Rural Development Administration, Republic of Korea, and also supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology (NRF-2013R1A1A2060447).

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

1. Henry MJ, Pasco JA, Seeman E, *et al.*: Assessment of fracture risk: value of random population-based samples—the Geelong Osteoporosis Study. *J Clin Densitom* 2001;4:283–289.
2. Montagnani A, Gonnelli S, Alessandri M, Nuti R: Osteoporosis and risk of fracture in patients with diabetes: an update. *Aging Clin Exp Res* 2011;23:84–90.
3. Silva BC, Bilezikian JP: New approaches to the treatment of osteoporosis. *Annu Rev Med* 2010;62:307–322.
4. Turgeon JL, McDonnell DP, Martin KA, Wise PM: Hormone therapy: physiological complexity belies therapeutic simplicity. *Science* 2004;304:1269–1273.
5. Karas RH: Current controversies regarding the cardiovascular effects of hormone therapy. *Clin Obstet Gynecol* 2004;47:489–499.
6. Fulfaro F, Casuccio A, Ticozzi C, Ripamonti C: The role of bisphosphonates in the treatment of painful metastatic bone disease: a review of phase III trials. *Pain* 1998;78:157–169.
7. Salari Sharif P, Abdollahi M, Larijani B: Current, new and future treatments of osteoporosis. *Rheumatol Int* 2010;31:289–300.
8. Moerman EJ, Teng K, Lipschitz DA, Lecka-Czernik B: Aging activates adipogenic and suppresses osteogenic programs in mesenchymal marrow stroma/stem cells: the role of PPAR-gamma2 transcription factor and TGF-beta/BMP signaling pathways. *Aging Cell* 2004;3:379–389.
9. Beresford JN, Bennett JH, Devlin C, Leboy PS, Owen ME: Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. *J Cell Sci* 1992;102:341–351.
10. Beresford JN: Osteogenic stem cells and the stromal system of bone and marrow. *Clin Orthop Relat Res* 1989;270–280.
11. Rosen CJ, Bouxsein ML: Mechanisms of disease: is osteoporosis the obesity of bone? *Nat Clin Pract Rheumatol* 2006;2:35–43.
12. Bianco P, Riminucci M, Gronthos S, Robey PG: Bone marrow stromal stem cells: nature, biology, and potential applications. *Stem Cells* 2001;19:180–192.
13. Park KW, Halperin DS, Tontonoz P: Before they were fat: adipocyte progenitors. *Cell Metab* 2008;8:454–457.
14. Tontonoz P, Spiegelman BM: Fat and beyond: the diverse biology of PPARgamma. *Annu Rev Biochem* 2008;77:289–312.
15. Hong JW, Park KW: Further understanding of fat biology: lessons from a fat fly. *Exp Mol Med* 2010;42:12–20.
16. Halade GV, Rahman MM, Williams PJ, Fernandes G: High fat diet-induced animal model of age-associated obesity and osteoporosis. *J Nutr Biochem* 2010;21:1162–1169.
17. Botolin S, McCabe LR: Bone loss and increased bone adiposity in spontaneous and pharmacologically induced diabetic mice. *Endocrinology* 2007;148:198–205.
18. Putnam SE, Scutt AM, Bicknell K, Priestley CM, Williamson EM: Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. *Phytother Res* 2007;21:99–112.
19. Shim JG, Yeom SH, Kim HJ, *et al.*: Bone loss preventing effect of Sophorae Fructus on ovariectomized rats. *Arch Pharm Res* 2005;28:106–110.
20. Lee J, Kim KW, Kim HK, *et al.*: The effect of Rexflavone (Sophorae Fructus extract) on menopausal symptoms in postmenopausal women: a randomized double-blind placebo controlled clinical trial. *Arch Pharm Res* 2010;33:523–530.

21. Jung SR, Song NJ, Hwang HS, *et al.*: Silk peptides inhibit adipocyte differentiation through modulation of the Notch pathway in C3H10T1/2 cells. *Nutr Res* 2011;31:723–730.
22. Park KW, Waki H, Kim WK, *et al.*: The small molecule phenamil induces osteoblast differentiation and mineralization. *Mol Cell Biol* 2009;29:3905–3914.
23. Jung SR, Kim YJ, Gwon AR, *et al.*: Genistein mediates the anti-adipogenic actions of *Sophora japonica* L. extracts. *J Med Food* 2011;14:360–368.
24. Soleimani S, Van Geel PJ, Isgor OB, Mostafa MB: Modeling of biological clogging in unsaturated porous media. *J Contam Hydrol* 2009;106:39–50.
25. Lim SH, Ha TY, Kim SR, *et al.*: Ethanol extract of *Psoralea corylifolia* L. and its main constituent, bakuchiol, reduce bone loss in ovariectomized Sprague–Dawley rats. *Br J Nutr* 2009;101:1031–1039.
26. Palumbo C, Ferretti M, Bertoni L, *et al.*: Influence of ferutinin on bone metabolism in ovariectomized rats. I: role in preventing osteoporosis. *J Bone Miner Metab* 2009;27:538–545.
27. Aires A, Fernandes C, Carvalho R, *et al.*: Seasonal effects on bioactive compounds and antioxidant capacity of six economically important brassica vegetables. *Molecules* 2011;16:6816–6832.
28. Folwarczna J, Zych M, Burczyk J, Trzeciak H, Trzeciak HI: Effects of natural phenolic acids on the skeletal system of ovariectomized rats. *Planta Med* 2009;75:1567–1572.
29. Hsu CL, Wu CH, Huang SL, Yen GC: Phenolic compounds rutin and o-coumaric acid ameliorate obesity induced by high-fat diet in rats. *J Agric Food Chem* 2009;57:425–431.
30. Edgar JA, Lin HJ, Kumana CR, Ng MM: Pyrrolizidine alkaloid composition of three Chinese medicinal herbs, *Eupatorium cannabinum*, *E. japonicum* and *Crotalaria assamica*. *Am J Chin Med* 1992;20:281–288.
31. Hendriks H, Malingre TM, Elema ET: Pyrrolizidine alkaloids, flavonoids and volatile compounds in the genus *Eupatorium*. *Eupatorium cannabinum* L., an ancient drug with new perspectives. *Pharm Weekbl Sci* 1983;5:281–286.
32. Carey JJ: What is a “failure” of bisphosphonate therapy for osteoporosis? *Cleve Clin J Med* 2005;72:1033–1039.
33. Ott SM: What is the optimal duration of bisphosphonate therapy? *Cleve Clin J Med* 2011;78:619–630.