

## Short Communication

**Nobiletin, a Polymethoxy Flavonoid, Suppresses Bone Resorption by Inhibiting NF $\kappa$ B-Dependent Prostaglandin E Synthesis in Osteoblasts and Prevents Bone Loss Due to Estrogen Deficiency**Suguru Harada<sup>1</sup>, Tsukasa Tominari<sup>1</sup>, Chiho Matsumoto<sup>1</sup>, Michiko Hirata<sup>1</sup>, Morichika Takita<sup>1</sup>, Masaki Inada<sup>1</sup>, and Chisato Miyaura<sup>1,\*</sup><sup>1</sup>Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2-24-16 Nakamachi, Koganei, Tokyo 184-8588, Japan

Received July 20, 2010; Accepted November 4, 2010

**Abstract.** Nobiletin, a polymethoxy flavonoid, prevents cancer and inflammation, but the roles of nobiletin in bone are unclear. We examined the effects of nobiletin on bone resorption in vitro and on bone mass in ovariectomized (OVX) mice in vivo. In vitro, nobiletin suppressed osteoclast formation and bone resorption induced by interleukin (IL)-1. Nobiletin suppressed the expression of cyclooxygenase-2, NF $\kappa$ B-dependent transcription, and prostaglandin E (PGE) production induced by IL-1 in osteoblasts. OVX mice showed severe bone loss in the femur by increased bone resorption due to estrogen deficiency, and nobiletin significantly restored the bone mass. Nobiletin could be beneficial to bone health in postmenopausal women.

**Keywords:** polymethoxy flavonoid, bone resorption, osteoporosis

Bone remodeling is regulated by osteoclastic bone resorption and new bone formation by osteoblasts. In bone resorption associated with inflammation, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is mainly produced by osteoblasts and acts as a potent stimulator of bone resorption (1). Interleukin (IL)-1 is known to induce PGE<sub>2</sub> production by osteoblasts and the receptor activator of NF $\kappa$ B ligand (RANKL) expression on their surface. For PGE<sub>2</sub> synthesis, two-types of cyclooxygenase (COX), COX-1 and COX-2, are expressed in osteoblasts, and COX-2 is markedly induced by inflammatory stimulants such as IL-1. RANKL-dependent osteoclast formation induced by IL-1 is dependent on PGE<sub>2</sub> production by osteoblasts, and the blockage of PGE<sub>2</sub> synthesis suppresses osteoclastic bone resorption induced by IL-1 (2).

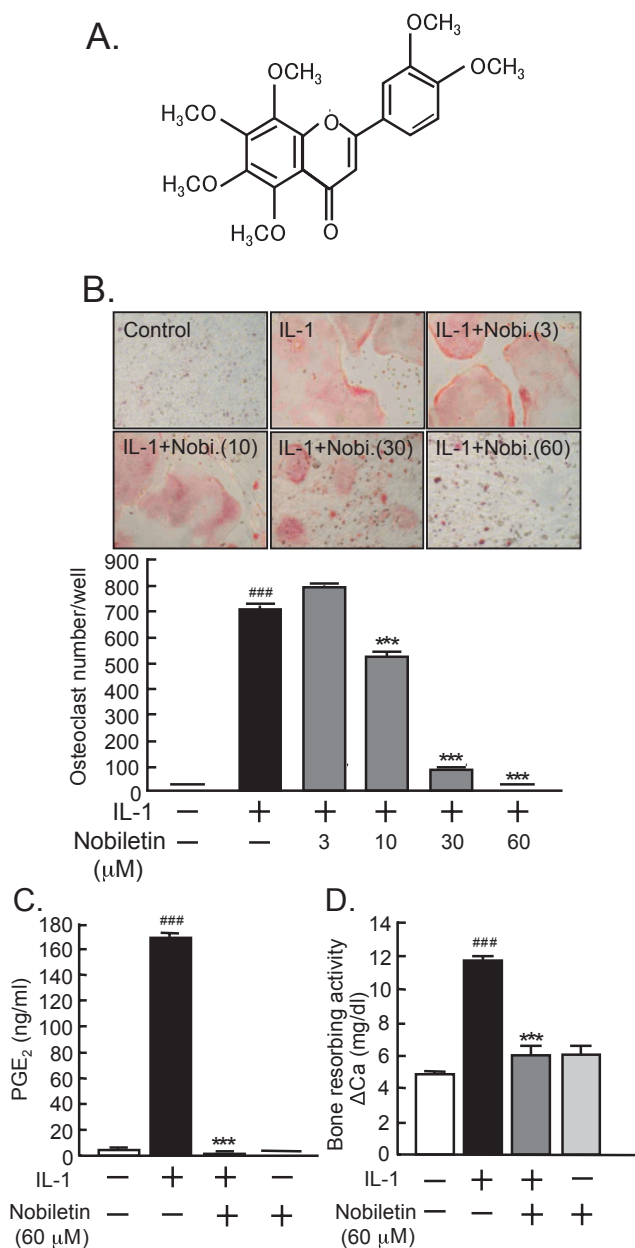
Nobiletin (5,6,7,8,3',4'-hexamethoxy flavone), a citrus polymethoxy flavonoid, is abundantly present in *Citrus depressa* and has several biological activities. Previous studies have shown that nobiletin exhibits anti-proliferative and apoptotic effects on various cancer cells (3) and inhibits inflammation pathways (4). Nobiletin attenuated

ovalbumin-induced eosinophilic airway inflammation in asthmatic rats (5) and Type II collagen-induced arthritis (6), but the roles of nobiletin in bone metabolism are unclear.

Osteoporosis is the most common bone disease. Postmenopausal women lose bone due to a decrease in ovarian estrogen and an increase in bone resorption. Previous studies have shown that inflammatory cytokines such as IL-1 and PGE<sub>2</sub> may be involved in the mechanism of bone loss due to estrogen deficiency (7, 8). In the present study, we examined the effects of nobiletin on bone resorption in vitro and on bone mass in ovariectomized (OVX) mice.

Nobiletin (Fig. 1A) was obtained from Wako Co., Ltd., Tokyo. Eight-week-old female mice of *ddy* strains were either sham-operated or OVX, and some of the mice were administered daily with nobiletin (2 mg/day/mouse) intraperitoneally. The mice were fed laboratory chow containing 1.12% calcium and 1.07% phosphorus (Nippon Clea, Tokyo). All procedures were performed in accordance with the institutional guidelines and permission for animal research. To examine bone mass, the bone mineral density (BMD) of the femurs was measured by dual X-ray absorptiometry (model DCS-600R; Aloka, Tokyo). Three-dimensional (3D) reconstruction images

\*Corresponding author. miyaura@cc.tuat.ac.jp  
Published online in J-STAGE  
doi: 10.1254/jphs.10193SC



**Fig. 1.** Effects of nobiletin on IL-1-induced osteoclast formation and PGE<sub>2</sub> production in cocultures of osteoblasts and bone marrow cells and on the bone-resorbing activity induced by IL-1 in mouse calvarial organ cultures. A) Chemical structure of nobiletin. B) Mouse bone marrow cells and osteoblastic cells were cocultured for 7 days with 3, 10, 30, and 60 μM nobiletin in the presence of IL-1 (2 ng/ml). The cells were stained for tartrate-resistant acid phosphatase (TRAP), a specific marker for osteoclasts, and the number of TRAP-positive multinucleated cells containing 3 or more nuclei was counted. Data are expressed as the mean ± S.E.M. of 3 independent wells. C) The level of PGE<sub>2</sub> was measured by EIA using the conditioned medium of the cocultures. D) Mouse calvariae were dissected in half and cultured with or without nobiletin (60 μM) in the presence of IL-1 (2 ng/ml) for 5 days. The concentration of calcium in the medium was measured to calculate bone-resorbing activity. Data are expressed as the mean ± S.E.M. of 4–6 independent wells. A significant difference between the two groups is indicated, <sup>###</sup>*P* < 0.001 vs. control, <sup>\*\*\*</sup>*P* < 0.001 vs. IL-1.

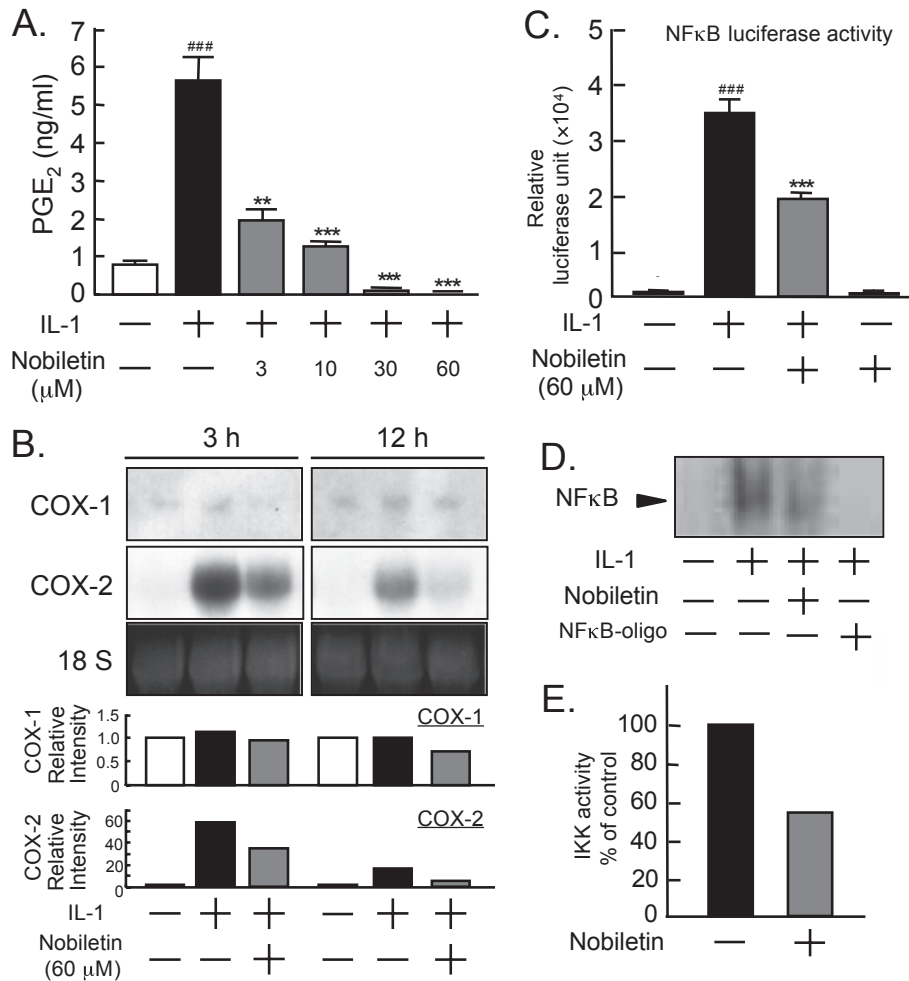
of distal femurs were obtained by micro-computed tomography (μCT) (inspeXio SMX-90CT; Shimadzu, Kyoto).

Mouse bone marrow cells were cocultured with primary osteoblasts for 7 days, and the cells were stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive multinucleated cells were counted as osteoclasts. To detect bone-resorbing activity, mouse calvariae were collected from 5-day-old mice and cultured for 5 days.

Primary osteoblastic cells were isolated from newborn mouse calvariae. Northern blot analysis was performed using total RNA and <sup>32</sup>P-labeled DNA probes of mouse COX-1 and COX-2. PGE<sub>2</sub> level was determined using an enzyme immunoassay (Amersham, Aylesbury, UK). For the luciferase NFκB reporter assay, plasmid pNFκB-TA-Luc (0.2 μg) with the firefly luciferase reporter gene (Clontech Laboratories, Inc., Mountain View, CA, USA) and pGL4.74[hLuc/TK] (20 ng) containing the renilla luciferase reporter gene (Promega Co., Ltd., Madison, WI, USA) were transfected into mouse primary osteoblasts. Luciferase activity was measured with the dual-luciferase reporter assay system (Promega Co., Ltd.). DNA binding of NFκB was analyzed by electrophoretic mobility shift assay (EMSA) using <sup>32</sup>P-labeled NFκB oligonucleotide and nuclear extracts collected from osteoblasts. DNA-protein complexes were separated by a polyacrylamide gel, and the gel was then exposed to X-ray film. IKK activity was measured by an IKKβ assay kit (CycLex Co., Ltd., Nagano) using IKKβ, IκBα, and anti-phospho-IκBα antibody.

In cocultures of mouse bone marrow cells and osteoblasts, IL-1 markedly induced osteoclast differentiation on day 7, while 3–60 μM nobiletin dose-dependently suppressed the osteoclast formation induced by IL-1 (Fig. 1B). The level of PGE<sub>2</sub> in the conditioned medium treated with IL-1 was higher than that of the control and was completely suppressed by adding 60 μM nobiletin (Fig. 1C). Next, we examined the effects of nobiletin on bone resorption in mouse calvarial organ cultures. Bone-resorbing activity was measured by the increase in calcium in the conditioned medium. IL-1 markedly induced bone-resorbing activity in calvarial cultures. Adding nobiletin at 60 μM completely suppressed the bone-resorbing activity induced by IL-1 (Fig. 1D), indicating that nobiletin clearly suppresses the bone resorption associated with inflammatory cytokine in bone tissues.

In bone tissues, PGE<sub>2</sub> is mainly produced by osteoblasts. In mouse primary osteoblast culture, nobiletin dose-dependently suppressed IL-1-induced PGE<sub>2</sub> production (Fig. 2A). In northern blot analysis, the expression of COX-2 mRNA was markedly induced by IL-1 in osteoblasts at 3 and 12 h, and simultaneous addition of nobiletin clearly suppressed the COX-2 expression (Fig.



**Fig. 2.** Effects of nobiletin on the production of PGE<sub>2</sub>, the expression of COXs mRNAs, and the IKK-dependent NFκB activity in mouse primary osteoblasts. **A)** Osteoblasts were treated with 3, 10, 30, and 60 μM nobiletin in the presence of IL-1 (2 ng/ml) for 24 h. The levels of PGE<sub>2</sub> were measured using the conditioned medium. A significant difference between the two groups is indicated, <sup>###</sup>*P* < 0.001 vs. control; <sup>\*\*</sup>*P* < 0.01, <sup>\*\*\*</sup>*P* < 0.001, vs. IL-1. **B)** Mouse osteoblasts were pretreated with 60 μM nobiletin for 3 h and then cultured with IL-1 (2 ng/ml) for 3 or 12 h, with or without nobiletin (60 μM). Total RNA was extracted, and the mRNA expression of COX-1 and COX-2 was detected using Northern blotting. The relative intensity of mRNA expression was calculated. **C)** Mouse osteoblasts were transfected with pNFκB-TA-Luc and pGL4.74[hLuc/TK] vectors. NFκB activation was measured by luciferase assay. Mouse osteoblasts were pretreated with 60 μM nobiletin for 3 h and then cultured for 12 h with IL-1 (2 ng/ml) and nobiletin (60 μM). Data are expressed as the mean ± S.E.M. of 3 independent wells. A significant difference between the two groups is indicated, <sup>###</sup>*P* < 0.001 vs. control, <sup>\*\*\*</sup>*P* < 0.001 vs. IL-1. **D)** Mouse osteoblasts were treated with IL-1 (2 ng/ml) for 60 min with or without nobiletin (60 μM). Nuclear extracts were collected from osteoblasts, and DNA binding of NFκB was analyzed by EMSA using <sup>32</sup>P-labeled NFκB oligonucleotide. The specificity of binding was confirmed by competition with excess amount of unlabeled oligonucleotide (NFκB-oligo). **E)** IKK activity was measured in the presence or absence of nobiletin (3 mM) by the IKKβ assay kit using IKKβ, IκBα, and anti-phospho-IκBα antibody. IKK activity was expressed as % of the control without nobiletin.

2B). Low level COX-1 mRNA expression was detected in osteoblasts, and this expression was not influenced by IL-1 or nobiletin. Activation of NFκB is critical for the expression of various IL-1-induced inflammatory genes, including COX-2 (9). Then, we examined the effects of nobiletin on NFκB activation in mouse osteoblasts using a luciferase assay and EMSA. When osteoblasts were treated for 12 h with IL-1, a marked activation of NFκB-

dependent transcription was detected, and nobiletin significantly suppressed the NFκB transcription (Fig. 2C). In mouse osteoblasts, treatment for 60 min with IL-1 showed the increase in NFκB-DNA binding in EMSA, and nobiletin suppressed the NFκB-DNA binding (Fig. 2D). In addition, nobiletin suppressed the enzyme activity of IKK (Fig. 2E). Since the mouse COX-2 genes promoter is known to possess a functional regulatory ele-

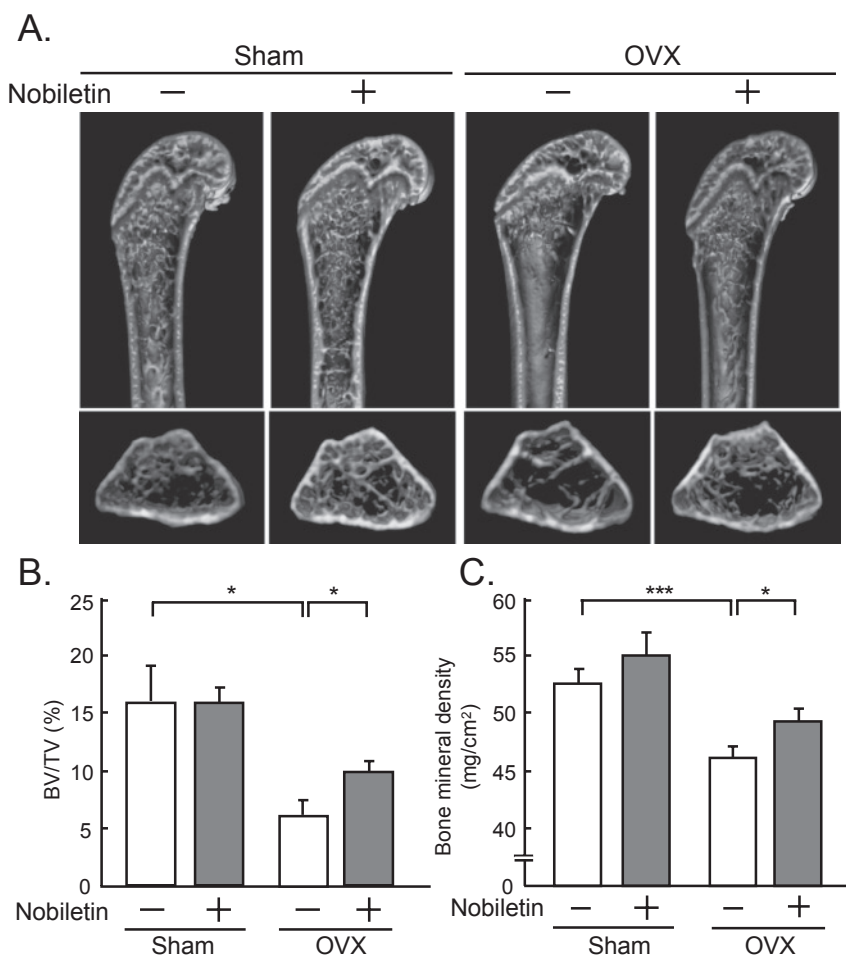
ment for NF $\kappa$ B (9), nobiletin may suppress COX-2 expression and PGE<sub>2</sub> synthesis by interfering with IKK-dependent NF $\kappa$ B transcription.

To determine the effects of nobiletin on bone mass in vivo, OVX mice were treated intraperitoneally with nobiletin (2 mg/day/mouse) and femurs were subjected to radiographic analysis. Using  $\mu$ CT, a 3D reconstruction image was obtained at the trabecular-rich region of the distal femurs. Trabecular bone mass, bone volume / tissue volume (BV/TV), was markedly reduced by OVX, and nobiletin significantly restored the trabecular bone loss in OVX mice (Fig. 3: A and B). BMD of the femurs was measured by dual X-ray absorptiometry to detect bone mass of cortical and trabecular bone. The BMD was significantly reduced by OVX at the distal metaphysis of the femur, and nobiletin significantly recovered the reduced bone mass in OVX mice (Fig. 3C). The uterine weight decreased markedly in OVX mice due to estrogen deficiency, and nobiletin did not recover the uterine weight in OVX mice (data not shown). Nobiletin did not exhibit binding to ER $\alpha$  and ER $\beta$  and transcriptional activity via SRC-1 in a receptor cofactor assay system (data

not shown). These results indicate that nobiletin is beneficial to bone tissues to prevent trabecular bone loss in OVX mice, without estrogenic action in the uterus.

Loss of estrogen induced the production of bone-resorbing cytokines such as IL-1, IL-6, and tumor necrosis factor (TNF)  $\alpha$  by osteoblasts, bone marrow stromal cells, and T-lymphocytes (7, 10, 11). All cytokines such as IL-1, IL-6, and TNF $\alpha$  can induce PGE<sub>2</sub> production in osteoblasts. We have shown an increased level of PGE<sub>2</sub> in bone marrow in OVX mice compared with sham mice (7). Raisz's group reported that the expression of COX-2 was elevated in bone tissue in OVX mice and that osteoclast formation and bone loss were attenuated in COX-2-deficient mice (8, 12). In addition, COX-2 inhibitors preserved bone architecture in OVX rats (13). Therefore, the suppressive effects of nobiletin on PGE<sub>2</sub> production may be involved in nobiletin's mechanism of action in OVX mice.

In osteoblasts, nobiletin suppresses COX-2 expression and PGE<sub>2</sub> synthesis by interfering with IKK activity and NF $\kappa$ B transcription. Nobiletin did not influence RANKL expression induced by IL-1 in osteoblasts (data not



**Fig. 3.** Effects of nobiletin on femoral bone mass in OVX mice. Female mice were ovariectomized and sham-operated at 8 weeks of age, and mice were injected daily with nobiletin (2 mg/day/mouse) or a vehicle solution intraperitoneally. At 4 weeks after the operation, femurs were excised for  $\mu$ CT analysis and measurement of Bone mineral density (BMD). A) Three-dimensional (3D)  $\mu$ CT reconstruction image at the trabecular-rich region of the distal femurs. B) Bone volume density, bone volume / tissue volume (BV/TV), was calculated by 3D analysis of  $\mu$ CT, as shown in A. C) BMD of the distal metaphysis of the femurs was measured by dual X-ray absorptiometry. Data are expressed as the means  $\pm$  S.E.M. of 6–8 mice. A significant difference between the two groups is indicated, \* $P$  < 0.05, \*\*\* $P$  < 0.001.

shown). The IKK–NF $\kappa$ B pathway may be a main molecular target of nobiletin in osteoblasts. On the other hand, nobiletin suppressed RANKL-dependent differentiation of macrophages into osteoclasts in RAW264.7 cells (6) and in bone marrow macrophages (data not shown), suggesting that the macrophage is another target cell for nobiletin. Kim et al. (14) have shown that plant flavonoids exhibit anti-inflammatory action by suppressing NO production in various cells. Further studies are needed to define the molecular mechanism of nobiletin.

Previous studies have shown that natural ingredients may act as a beneficial factor for bone mass. Ishimi et al. (15) reported that genistein, a typical soybean isoflavone, prevents bone loss due to estrogen deficiency. Hesperidin, a citrus flavonoid, also attenuated bone loss in a model of osteoporosis (16). *Citrus depressa* is mostly cultivated in the northern area of Okinawa prefecture in Japan, and the concentration of nobiletin is higher in *Citrus depressa* than in other citrus fruits. Norimatsu et al. (17) examined the incidence of femoral neck fractures in ten cities in Okinawa and found the lowest incidence of fractures in Nago city, the most northern city of the main island in Okinawa. Therefore, it is possible that an increased intake of nobiletin-rich *Citrus depressa* contributes to the low incidence of fractures in this area. Further epidemiology regarding osteoporosis is necessary to define the possible contribution of *Citrus depressa* to the low incidence of fractures.

In conclusion, nobiletin suppressed IL-1-induced osteoclast formation by inhibiting NF $\kappa$ B-dependent PGE<sub>2</sub> production in osteoblasts. Since nobiletin restored bone loss due to estrogen deficiency, the intake of nobiletin-rich citrus fruits or a nutritional supplement may be beneficial for maintaining bone mass in postmenopausal women.

## References

- 1 Raisz LG, Vanderhoek JY, Simmons HA, Kream BE, Nicolaou KC. Prostaglandin synthesis by fetal rat bone in vitro: evidence for a role of prostacyclin. *Prostaglandins*. 1979;17:905–914.
- 2 Miyaura C, Inada M, Suzawa T, Sugimoto Y, Ushikubi F, Ichikawa A, et al. Impaired bone resorption to prostaglandin E<sub>2</sub> in prostaglandin E receptor EP4-knockout mice. *J Biol Chem*. 2000;275:19819–19823.
- 3 Akao Y, Itoh T, Ohguchi K, Iinuma M, Nozawa Y. Interactive effects of polymethoxy flavones from Citrus on cell growth inhibition in human neuroblastoma SH-SY5Y cells. *Bioorg Med Chem*. 2008;16:2803–2810.
- 4 Murakami A, Shigemori T, Ohigashi H. Zingiberaceous and citrus constituents, 1'-acetoxychavicol acetate, zerumbone, auranone, and nobiletin, suppress lipopolysaccharide-induced cyclooxygenase-2 expression in RAW264.7 murine macrophages through different modes of action. *J Nutr*. 2005;135:2987S–2992S.
- 5 Wu YQ, Zhou CH, Tao J, Li SN. Antagonistic effects of nobiletin, a polymethoxy flavonoid, on eosinophilic airway inflammation of asthmatic rats and relevant mechanisms. *Life Sci*. 2006;78:2689–2696.
- 6 Murakami A, Song M, Katsumata S, Uehara M, Suzuki K, Ohigashi H. Citrus nobiletin suppresses bone loss in ovariectomized ddY mice and collagen-induced arthritis in DBA/1J mice: possible involvement of receptor activator of NF- $\kappa$ B ligand (RANKL)-induced osteoclastogenesis regulation. *Biofactors*. 2007;30:179–192.
- 7 Miyaura C, Kusano K, Masuzawa T, Chaki O, Onoe Y, Aoyagi M, et al. Endogenous bone-resorbing factors in estrogen deficiency: cooperative effects of IL-1 and IL-6. *J Bone Miner Res*. 1995;10:1365–1373.
- 8 Kawaguchi H, Pilbeam CC, Vargas SJ, Morse EE, Lorenzo JA, Raisz LG. Ovariectomy enhances and estrogen replacement inhibits the activity of bone marrow factors that stimulate prostaglandin production in cultured mouse calvariae. *J Clin Invest*. 1995;96:539–548.
- 9 Yamamoto K, Arakawa T, Ueda N. Transcriptional roles of nuclear factor  $\kappa$  B and nuclear factor-interleukin-6 in the tumor necrosis factor  $\alpha$ -dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. *J Biol Chem*. 1995;270:31315–31320.
- 10 Jilka RL, Hangoc G, Girasole G, Passeri G, Williams DC, Abrams JS, et al. Increased osteoclast development after estrogen loss: mediation by interleukin-6. *Science*. 1992;247:88–91.
- 11 Kimble RB, Bain S, Pacifici R. The functional block of TNF but not of IL-6 prevents bone loss in ovariectomized mice. *J Bone Miner Res*. 1997;12:935–941.
- 12 Okada Y, Lorenzo JA, Freeman AM, Tomita M, Morham SG, Raisz LG, et al. Prostaglandin G/H synthase-2 is required for maximal formation of osteoclast-like cells in culture. *J Clin Invest*. 2000;105:823–832.
- 13 Gregory LS, Kelly WL, Reid RC, Fairlie DP, Forwood MR. Inhibitors of cyclo-oxygenase-2 and secretory phospholipase A<sub>2</sub> preserve bone architecture following ovariectomy in adult rats. *Bone*. 2006;39:134–142.
- 14 Kim HP, Son KH, Chang HW, Kang SS. Anti-inflammatory plant flavonoids and cellular action mechanisms. *J Pharmacol Sci*. 2004;96:229–245.
- 15 Ishimi Y, Miyaura C, Ohmura M, Onoe Y, Sato T, Uchiyama Y, et al. Selective effects of genistein, a soybean isoflavone, on B-lymphopoiesis and bone loss caused by estrogen deficiency. *Endocrinology*. 1999;140:1893–1900.
- 16 Chiba H, Uehara M, Wu J, Wang X, Masuyama R, Suzuki K, et al. Hesperidin, a citrus flavonoid, inhibits bone loss and decreases serum and hepatic lipids in ovariectomized mice. *J Nutr*. 2003;133:1892–1897.
- 17 Norimatsu H, Mori S, Uesato T, Yoshikawa T, Katsuyama N. Bone mineral density of the spine and proximal femur in normal and osteoporotic subjects in Japan. *Bone Miner*. 1989;5:213–222.