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# Upregulation of osteoblastic differentiation marker mRNA expression in osteoblast-like UMR106 cells by puerarin and phytoestrogens from *Pueraria mirifica*

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ABSTRACT

Phytoestrogens have attracted attention for their potential in the prevention of postmenopausal osteoporosis. Recently, phytoestrogen-rich herb *Pueraria mirifica* has been demonstrated to possess an osteogenic effect on bone in ovariectomized rats, but its underlying cellular mechanism was not known. Here, we investigated the effects of *P. mirifica* extract and its major isoflavone compound, puerarin, on cell viability, cell proliferation and the expression of differentiation markers in rat osteoblast-like UMR106 cells. After exposure to 17β-estradiol (E2), genistein, *P. mirifica* extract and puerarin, proliferation but not viability of UMR106 cells was markedly decreased. Quantitative real-time PCR revealed that *P. mirifica* extract and puerarin significantly increased the mRNA expression of alkaline phosphatase (ALP) and osteoprotegerin, but not Runx2, osterix or osteocalcin. Puerarin also decreased the mRNA expression of receptor activator of nuclear factor-κB ligand, an osteoclastogenic factor, suggesting that it could induce bone gain by enhancing osteoblast differentiation and suppressing osteoclast function. Furthermore, after an exposure to high affinity estrogen receptor (ER) antagonist (ICI182780), the E2-, genistein-, *P. mirifica* extract- and puerarin-induced upregulation of ALP expressions were completely abolished. It could be concluded that *P. mirifica* extract and puerarin induced osteoblast differentiation rather than osteoblast proliferation in an ER-dependent manner. The present findings, therefore, corroborated the potential benefit of *P. mirifica* extract and puerarin in the prevention and treatment of postmenopausal osteoporosis.



## Introduction

Osteoporosis, a metabolic bone disease characterized by low bone density and microarchitectural deterioration, progressively compromises bone strength, predisposing patients to the vertebral fractures, fragility fractures of the neck of the femur, and Colles fracture of the wrist (Cole et al. 2008; Lane 2006). The economic burden of osteoporosis is markedly increased paralleling the expansion of aging world population (Lane 2006). Approximately 9 million patients with osteoporotic fractures were reported worldwide in 2000, and the incidence has been predicted to reach 6.26 million by 2050 (Cole et al. 2008). Therefore, development of effective preventions, early interventions and treatments is needed to mitigate clinical complications of osteoporosis and the accompanying economic impact.

Since 17β-estradiol (E2) is an important hormone for maintaining bone mass, postmenopausal women with estrogen deficiency thus are prone to develop osteoporosis (Lane 2006). Although estrogen replacement therapy is an effective regimen for postmenopausal bone loss, long-term exposure to synthetic estrogens markedly increases the prevalence of malignant neoplasia in several tissues, such as mammary gland, endometrium, and ovary (Manolagas et al. 2002). Phytoestrogens—estrogen-like compounds of plant origin—are capable of binding to estrogen receptor (ER) with minimal cancer risk (Tham et al. 1998), and have become a widely accepted alternative treatment of choice (Setchell and Lydeking-Olsen 2003; Tham et al. 1998). Recently, the tuberous root extract of phytoestrogen-rich herb *Pueraria mirifica* has

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been shown to effectively prevent osteoporosis in ovariectomized rats by increasing bone mineral density (BMD) and bone mineral content (BMC) (Urasopon et al. 2008). However, the exact anti-osteoporotic mechanism of *P. mirifica* extract and its major constituent, puerarin, in osteoblasts (bone-forming cells) remained elusive.

*P. mirifica* belongs to the family Leguminosae endemic to Thailand (Malaivijitnond 2012; Chansakaow et al. 2000). Its tuberous root contains a number of isoflavones, such as puerarin, daidzin, genistin, daidzein and genistein (Cherdshewasart et al. 2007a,b; Cherdshewasart and Sriwatcharakul 2007). Some isoflavone derivatives are phytoestrogens that exert osteogenic effects on bone like estrogen (Urasopon et al. 2008; Marini et al. 2007; Morabito et al. 2002). For instance, a randomized double-blind placebo-controlled study in postmenopausal women showed that genistein administration significantly increased BMD and the circulating levels of bone formation markers, e.g., bone-specific alkaline phosphatase (ALP), osteocalcin and insulin-like growth factor (IGF)-1 (Marini et al. 2007; Morabito et al. 2002). It was shown that genistein exerted its action by binding to α- and β-isoforms of ER (Kuiper et al. 1998). Since the chemical structures and actions of isoflavones resemble those of E2, it is possible that *P. mirifica* extract and its major isoflavone compound, puerarin, may also induce positive osteogenic effect on osteoblasts.

It was noteworthy that although Thai *P. mirifica* contained some isoflavones similar to that found in Chinese and Korean *Pueraria lobata* (Cherdshewasart et al. 2007b), Kim et al. (2003) showed that Puerariae radix from different geographical regions exhibited different constituents and estrogenic activity as determined by uterotrophic assay. Specifically, the estrogenic activity of *P. mirifica* extract was stronger than that of *P. thunbergiana* (*lobata*). The *P. lobata* extract also contained kudzusapogenols and soyasaponin, both of which were not reported in *P. mirifica* extract (Malaivijitnond 2012). Up till now, the effect of *P. mirifica* extract on osteoblasts has never been investigated.

Although it was possible that *P. mirifica* extract prevented bone loss by promoting bone formation, whether it affected proliferation or differentiation stage of osteoblasts was not known. Under normal conditions, to induce bone formation, stromal cells or osteoprogenitor cells undergo proliferation, followed by differentiation with a slowdown of proliferation (Zaidi 2007; Owen et al. 1990). When osteoblasts differentiate, they sequentially express different markers specific to each stage of maturation, i.e., *runt*-related transcription factor 2 (Runx2), osterix, ALP, and osteocalcin in this order (Komori 2006; Stein et al. 2004). Moreover, differentiated osteoblasts also express the receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG), both of which are commonly used as markers for the assessment of osteoblast-regulated osteoclast function and bone resorption (Lin et al. 2007; Abdallah et al. 2005).

Therefore, the objectives of the present study were (i) to investigate the effect of *P. mirifica* extract and its major isoflavone, puerarin, on proliferation and viability of osteoblasts, (ii) to determine the direct effect of *P. mirifica* extract and puerarin on the expression of differentiation markers in osteoblasts, and (iii) to investigate whether ER mediated the osteoregulatory actions of *P. mirifica* extract and puerarin.

## Materials and methods

### *Preparation of P. mirifica extract and high-performance liquid chromatography (HPLC)*

The tuberous roots of *P. mirifica* cultivar SARDI 190 (lot no. 0070317) were purchased from Dr. Sompoch Tubcharoen, Kasetsart University, Kamphaeng Saen Campus, Thailand, and was authenticated as *P. mirifica* by comparing with the voucher specimens (nos. BCU010250 and BCU011045) kept at the Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand. The specimens were washed, sliced, dried in a hot-air oven at 70 °C, and ground. One gram of tuberous powder was extracted twice by mixing with 4 ml of 70% ethanol (Urasopon et al. 2008). The supernatants collected from two extractions were mixed together and dried for 4 h in a centrifugal concentrator at room temperature. Dry sample was kept at 4 °C until used in cell culture, and some portion was used for HPLC analysis.

The concentrations of isoflavones in *P. mirifica* extract (i.e., puerarin, daidzin, genistin, daidzein and genistein) were determined by HPLC, as described previously (Malaivijitnond et al. 2004). Briefly, 10 μl of extracted solution was injected through a sensory guard column [model Hyperclone ODS(C18); Phenomenex, Torrance, CA, USA] into a HPLC system (model Agilent 1000; Agilent, Waldbronn, Germany) equipped with a reverse phase Symmetry C18 column (250 mm × 4.6 mm, 5 μm; Phenomenex). Mobile phase consisted of 0.1% vol/vol phosphoric acid and acetonitrile with gradient elution (flow rate 1 ml/min). Ultraviolet detection was performed at a wavelength corresponding to the most intense absorption maximum at 255 nm. The isoflavone concentrations in each sample were analyzed in duplicate by comparing the retention times, and the amounts were quantified using the peak area of the standard curves. The standard compounds of daidzin, genistin, daidzein, and genistein (catalog nos. 30408, 48756, D7802 and G6649, respectively) were purchased from Sigma (St. Louis, MO, USA), and puerarin (catalog no. P5555) was purchased from Fluka (Buchs, Switzerland).

### *Cell culture*

Rat osteoblast-like UMR106 cells [American Type Culture Collection (ATCC) no. CRL-1661] were cultured in 100-mm petri-dish with Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; PAA, Pasching, Austria), and 100 U/ml penicillin-streptomycin (Gibco, Grand Island, NY, USA). Cells were maintained at 37 °C in 5% $CO_2$ incubator, and subcultured according to the ATCC's protocol.

### *Experimental design*

Prior to determination of osteoblast proliferation and differentiation marker expression, UMR106 cells were first investigated for the mRNA expression of ER-α and ER-β by quantitative real-time PCR (qRT-PCR). To investigate the time-dependent effect of E2 on osteoblast mRNA expression, UMR106 cells were incubated with 10 nmol/l E2 (Sigma) for 24, 48 and 72 h before determining the mRNA expression of ALP, an osteoblast differentiation marker. Thus, the incubating time (i.e., 48 or 72 h) was used in the subsequent experiments. To determine the dose-dependent effects of *P. mirifica* extract and related isoflavones on osteoblasts, confluent UMR106 cells were incubated for 48 or 72 h with vehicle [0.3% vol/vol dimethyl sulfoxide (DMSO); control group], or various concentrations of genistein (positive control; 0.1, 10 and 1000 nmol/l), *P. mirifica* extract (1, 10 and 100 μg/ml) or puerarin (major component of *P. mirifica* extract; 0.1, 10 and 1000 nmol/l). Thereafter, cell proliferation and viability were determined by 5-bromo-2′-deoxyuridine (BrdU) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, respectively, while the mRNA expressions of osteoblast differentiation markers (i.e., Runx2, osterix, ALP, osteocalcin, RANKL and OPG) were quantified by qRT-PCR. Finally, to demonstrate whether *P. mirifica* extract exerted its osteogenic effect through ER in the same manner

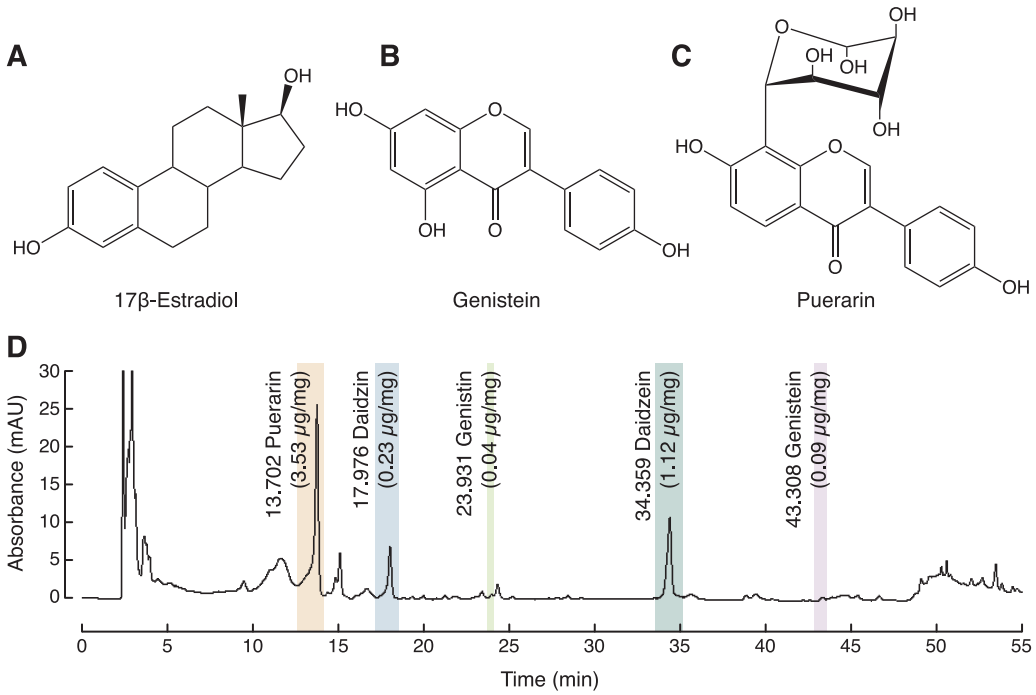


**Fig. 1.** (A–C) Chemical structures of 17β-estradiol (E2) and two isoflavones, genistein and puerarin. (D) A chromatogram of *P. mirifica* extract reveals its components, i.e., puerarin, daidzin, genistin, daidzein and genistein. The total retention time and concentration of each component as determined by HPLC are also presented.

as E2 and genistein, UMR106 cells were pre-incubated for 2 h with ER antagonist (10 nmol/l ICI182780; half maximal inhibitory concentration of ~0.3 nmol/l, Sigma), and then incubated for 48 h with 10 nmol/l ICI182780 plus 10 nmol/l E2, 1000 nmol/l genistein, 100 μg/ml *P. mirifica* extract, or 1000 nmol/l puerarin before determining the ALP expression. Chemical structures of E2, genistein and puerarin are shown in Fig. 1A–C.

### *Cell proliferation assay*

UMR106 cells were seeded in 96-well culture plate (1000 cells/well). After a 48-h incubation with 10 nmol/l E2, 1000 nmol/l genistein, 100 μg/ml *P. mirifica* extract, or 1000 nmol/l puerarin, cell proliferation was determined by BrdU enzyme-linked immunosorbent assay kit (catalog no. 11647229001; Roche, Mannheim, Germany), according to the manufacturer's instruction. Since BrdU incorporated into the newly synthesized DNA of proliferating cells, the amount of BrdU in each well represented cell proliferation. The absorbance of each well was measured at 370 nm with the reference wavelength of 490 nm by a microplate reader (model 1420; Wallac, Turku, Finland). The BrdU assay was performed in triplicate with 6 independent samples ($n = 6$).

### *Cell viability assay*

UMR106 cells were seeded in 96-well culture plate (1000 cells/well). After a 48-h incubation with 10 nmol/l E2, 1000 nmol/l genistein, 100 μg/ml *P. mirifica* extract, or 1000 nmol/l puerarin, cell viability was determined by MTT assay. In viable cells, MTT was converted by a mitochondrial reductase to purple formazan crystal. Briefly, 40 μl of MTT (catalog no. M2128; Sigma) was pipetted into each well to obtain a final concentration of 1 mg/ml, and incubated at 37 °C for 3.5 h prior to removing culture medium. Thereafter, 150 μl of solvent containing 4 mmol/l HCl and 0.1% vol/vol Nonidet P-40 in isopropanol was added to dissolve the formazan crystal. The absorbance of each well was determined at 590 nm with the reference wavelength of 620 nm. The MTT assay was performed in triplicate with 6 independent samples ($n = 6$).

### *Total RNA preparation*

Total RNA samples were extracted from UMR106 cells by using TRIzol reagent (invitrogen, Carlsbad, CA, USA). Purity of the total RNA was determined by the ratio of absorbance reading at 260 and 280 nm, the ratio of which was between 1.8 and 2.0. One microgram of total RNA was then reverse-transcribed with iScript cDNA synthesis kit and oligo-$dT_{20}$ (Bio-Rad, Hercules, CA, USA) to cDNA by Bio-Rad MyCycler. Rat β-actin served as a control gene to check the consistency of the reverse transcription (% coefficient of variation <5%, $n = 6$).

### *Quantitative real-time PCR (qRT-PCR)*

Primers used in the present study are listed in Table 1. All primers were first verified by conventional PCR followed by amplicon sequencing. Conventional PCR was performed by Bio-Rad MyCycler with GoTaq Green Master Mix (Promega, Madison, WI, USA). Thereafter, PCR products were visualized on 1.5% agarose gel stained with 1 μg/ml ethidium bromide (Sigma) under UV transilluminator (Alpha Innotech, San Leandro, USA). qRT-PCR and melting curve analysis were performed in triplicate by Bio-Rad MiniOpticon real-time PCR system with iQ SYBR Green SuperMix (Bio-Rad) for 40 cycles at 95 °C for 60 s, 53–60 °C annealing temperature for 30 s, and 72 °C for 30 s.

### *Statistical analysis*

Unless otherwise specified, the results are expressed as means ± SE. Two-group comparisons were analyzed by Mann–Whitney test. Multiple comparisons were performed by one-way analysis of variance (ANOVA) followed by Dunnett's post-test. The level of significance was $p < 0.05$. All data were analyzed by GraphPad Prism 5 (Graphpad, San Diego, CA, USA).

**Table 1**
*Rattus norvegicus* primers used in the qRT-PCR experiments.

| Gene name (abbreviated name) | Accession no. | Primer (forward/reverse) | Product length (bp) |
|---|---|---|---|
| *Estrogen receptors (ER)* | | | |
| Estrogen receptor-α | NM_012689 | 5′-CCAAGTCCACTTGTGATCAAGC-3′<br>5′-TTGAGGCTTCACTGAAGGGTC-3′ | 148 |
| Estrogen receptor-β | NM_012754 | 5′-AAAGCCAAGAGAAACGGTGGGCAT-3′<br>5′-GCCAATCATGTGCACCAGTTCCTT-3′ | 204 |
| *Osteoblast-related genes* | | | |
| *Runt*-related transcription factor 2 (Runx2) | XM_001066909 | 5′-TAACGGTCTTCACAAATCCTC-3′<br>5′-GGCGGTCAGAGAACAAACTA-3′ | 135 |
| Osterix | AY177399 | 5′-GCCTACTTACCCGTCTGA-3′<br>5′-CTCCAGTTGCCCACTATT-3′ | 139 |
| Alkaline phosphatase (ALP) | NM_013059 | 5′-GCAGGATCGGAACGTCAAT-3′<br>5′-ATGAGTTGGTAAGGCAGGGTC-3′ | 144 |
| Osteocalcin | J04500 | 5′-CACAGGGAGGTGTGTGAG-3′<br>5′-TGTGCCGTCCATACTTTC-3′ | 203 |
| Receptor activator of nuclear factor-κB ligand (RANKL) | NM_057149 | 5′-TCGCTCTGTTCCTGTACT-3′<br>5′-AGTGCTTCTGTGTCTTCG-3′ | 145 |
| Osteoprotegerin (OPG) | NM_012870 | 5′-ATTGGCTGAGTGTTCTGGT-3′<br>5′-CTGGTCTCTGTTTTGATGC-3′ | 140 |
| *Housekeeping gene* | | | |
| β-actin | NM_031144 | 5′-CAGAGCAAGAGAGGCATCCT-3′<br>5′-GTCATCTTTTCACGGTTGGC-3′ | 185 |

## Results

### *Puerarin was the major component of P. mirifica extract*

As depicted in Fig. 1D, five isoflavones, namely puerarin, daidzin, genistin, daidzein and genistein were identified in *P. mirifica* extract. Puerarin with a concentration of 3.53 μg/mg was the major component of the extract. The concentrations of daidzin, genistin, daidzein and genistein were lower than that of puerarin by 15-, 90-, 3-, and 40-fold, respectively.

### *Osteoblast-like UMR106 cells expressed ER, and responded to E2 and genistein*

Prior to investigating the effects of isoflavones on osteoblasts, we first determined whether osteoblast-like UMR106 cells were responsive to estrogens. qRT-PCR revealed that UMR106 cells strongly expressed both ER-α and ER-β mRNAs (Fig. 2A), suggesting that UMR106 cells could respond to phytoestrogens through ERs. After exposure to 10 nmol/l E2, cell viability was not changed (Fig. 2B), but the proliferation rate was significantly decreased

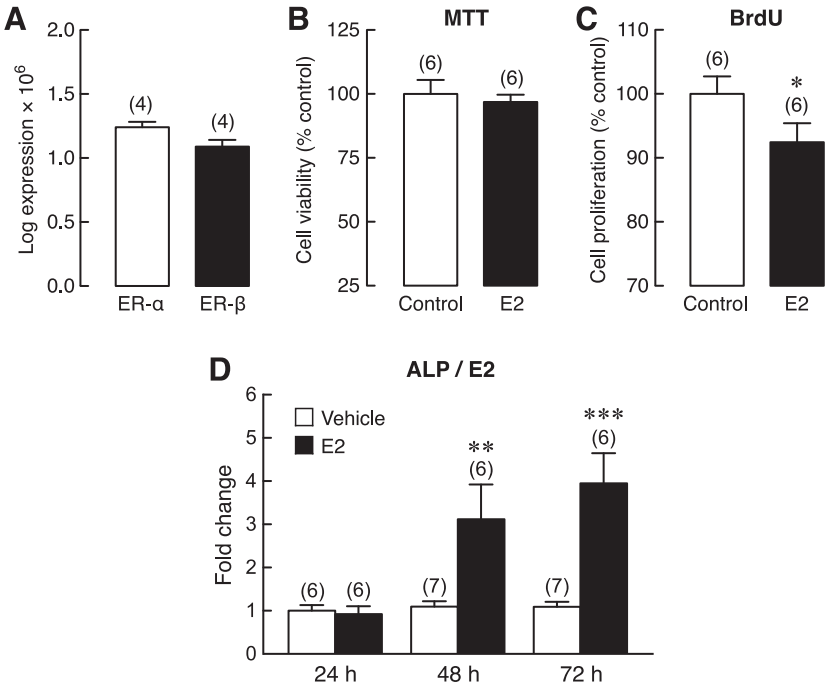


**Fig. 2.** (A) The mRNA expression of ER-α and ER-β mRNA in UMR106 cells, as determined by qRT-PCR. (B) Cell viability and (C) proliferation of UMR106 cells upon direct exposure for 48 h to vehicle (control) or 10 nmol/l E2, as determined by MTT and BrdU assays, respectively. The value of each control group was normalized to 100%. (D) The mRNA expression of ALP in UMR106 cells treated for 24, 48, or 72 h with 10 nmol/l E2. The expression level of the control group was normalized to 1. Numbers in parentheses represent the numbers of independent samples. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control group.

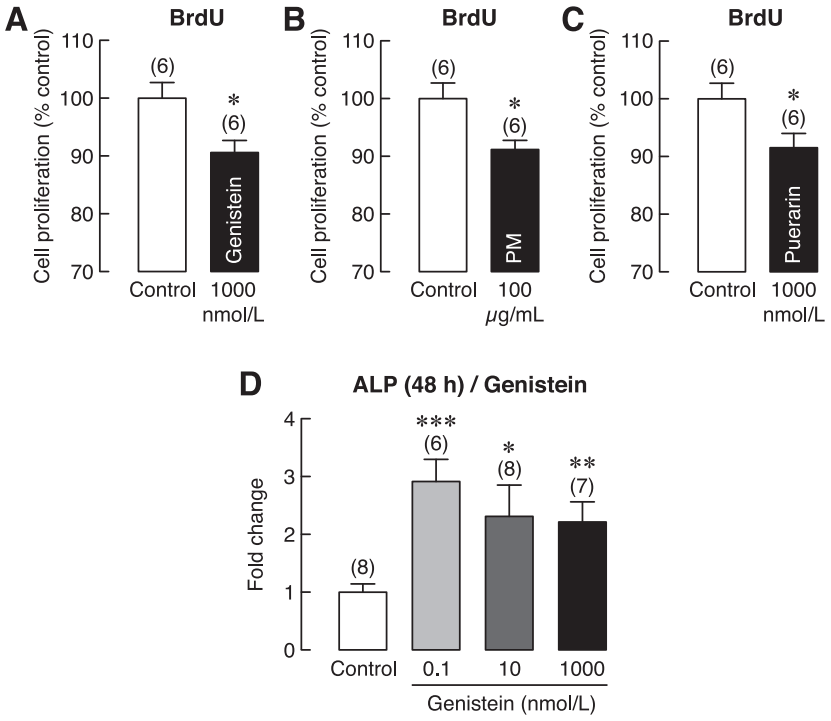


**Fig. 3.** (A–C) Proliferation of UMR106 cells after a 48-h exposure to vehicle (control), 1000 nmol/l genistein, 100 μg/ml *P. mirifica* extract (PM), or 1000 nmol/l puerarin, as determined by BrdU assay. (D) The mRNA expression of ALP in UMR106 cells after a 48-h direct exposure to vehicle or 0.1, 10 or 1000 nmol/l genistein. The expression level of the control group was normalized to 1. Numbers in parentheses represent the numbers of independent samples. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control group.

(Fig. 2C), as normally observed when osteoblasts underwent differentiation (Stein et al. 2004; Owen et al. 1990). Further time-dependent study showed that UMR106 cells upregulated the mRNA expression of an osteoblast differentiation marker ALP after 48- and 72-h exposure to E2 as compared to the corresponding vehicle-treated groups (Fig. 2D). Similar to E2 action, the phytoestrogen genistein decreased UMR106 cell proliferation (Fig. 3A), without effects on cell viability (data not shown). Moreover, 0.1, 10 and 1000 nmol/l genistein also increased the expression of ALP transcript in UMR106 cells after 48-h incubation (Fig. 3D).

### *P. mirifica extract and puerarin upregulated the expression of osteoblast differentiation markers*

Consistent with the results of E2- and genistein-treated cells, *P. mirifica* extract and puerarin significantly decreased osteoblast proliferation (Fig. 3B and C), but not cell viability (data not shown). Since we found that UMR106 cells responded to E2 and genistein by upregulating ALP expression, the osteoblast differentiation marker expression was investigated in UMR106 cells treated for 48 or 72 h with *P. mirifica* extract and puerarin. As shown in Fig. 4, *P. mirifica* extract significantly increased the mRNA expression of ALP, RANKL and OPG, but not Runx2, osterix or osteocalcin. Consistent with the action of *P. mirifica* extract, puerarin similarly upregulated the mRNA expression of ALP, RANKL and OPG in UMR106 cells (Fig. 5), suggesting that puerarin was the major active component of *P. mirifica* extract acting on bone remodeling.

### *ER mediated the osteoregulatory effect of P. mirifica extract on osteoblast-like UMR106 cells*

To confirm that ER mediated the osteoregulatory actions of E2, genistein, *P. mirifica* extract and puerarin, UMR106 cells were exposed to high affinity ER antagonist (10 nmol/l ICI182780). As shown in Fig. 6, UMR106 cells treated with E2, genistein, *P. mirifica* extract or puerarin significantly increased the mRNA expression of ALP in the absence of ICI182780 by 3-, 2-, 2- and 2-fold, respectively, as compared to the respective vehicle-treated group. On the other hand, in the presence of ICI182780, none of the four treatments showed the stimulatory effect on ALP expression (Fig. 6), suggesting that ER mediated the upregulation of ALP mRNA expression.

## Discussion

Phytoestrogen has attracted attention for its potential in the prevention of bone loss (Setchell and Lydeking-Olsen 2003; Tham et al. 1998). Thai phytoestrogen-rich herb *P. mirifica* has also shown osteogenic effect on bone by increasing BMD and BMC in both ovariectomized and orchidectomized rats (Urasopon et al. 2008, 2007). In the present study, puerarin was identified as the major component of *P. mirifica* extract, which upregulated the expression of osteoblast differentiation markers, especially ALP, in an ER-dependent manner.

Osteoblast-like UMR106 cells used in the present study constitutively expressed ER-α and -β transcripts, and were responsive to E2 and phytoestrogens by upregulating ALP expression (Li et al. 2005). However, *P. mirifica* extract and two phytoestrogens (genistein and puerarin) were found to modestly decrease cell proliferation rate, suggesting that they probably induced bone formation by enhancing osteoblast differentiation rather than proliferation. Since cell viability as determined by MTT assay was not altered, consistent with that observed in *P. lobata* extract-treated osteoblast-like SaOS-2 cells (Huh et al. 2006), *P. mirifica* extract and puerarin might be protective against apoptosis. In diabetic rats, it was evident that the expression of caspase-3, a marker of apoptosis, was decreased after administration of puerarin (Liang et al. 2012). Further investigation is, therefore, required to demonstrate whether puerarin induces bone formation, in part, by inhibiting the caspase-3-mediated osteoblast apoptosis.

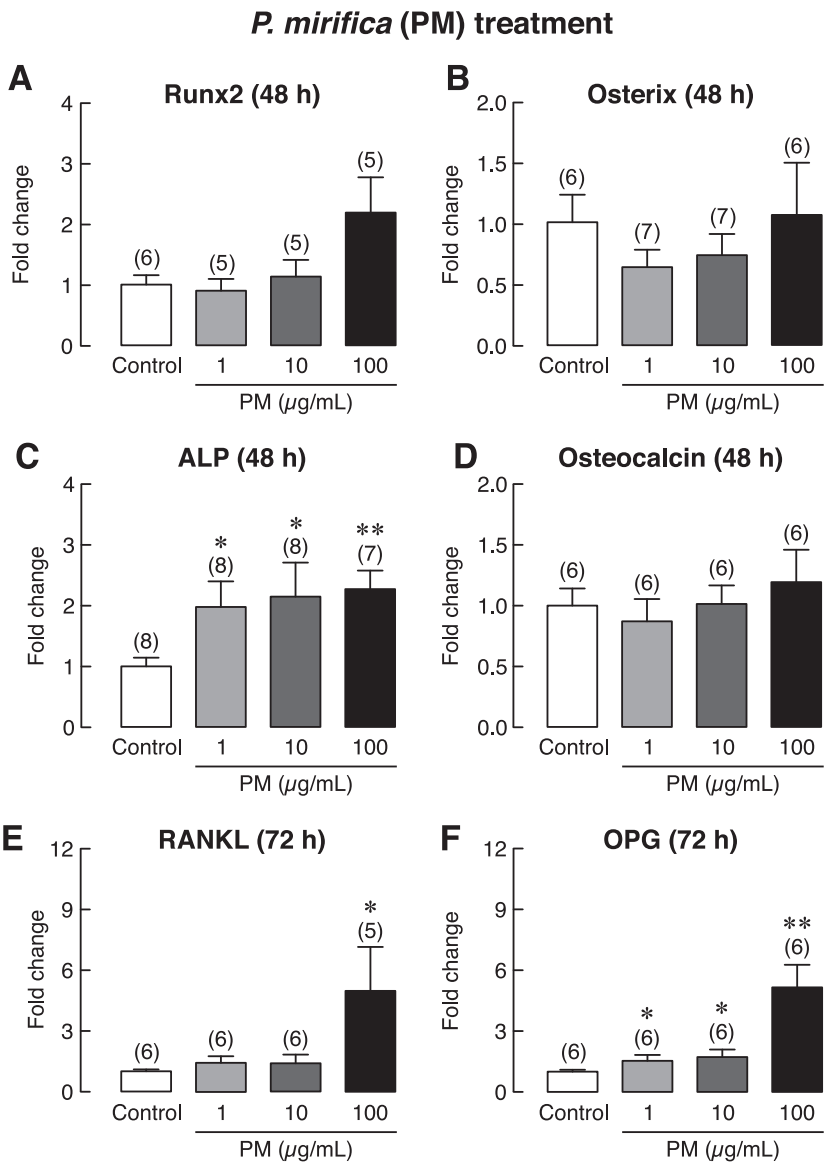


**Fig. 4.** The mRNA expression of (A) Runx2, (B) osterix, (C) ALP, (D) osteocalcin, (E) RANKL, and (F) OPG in UMR106 cells treated for 48 h or 72 h with vehicle (control), or 1, 10 or 100 μg/ml *P. mirifica* extract (PM). The mRNA expression was quantified by qRT-PCR. The expression level of the control group was normalized to 1. Numbers in parentheses represent the numbers of independent samples. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control group.

After the proliferation stage, osteoblasts normally undergo differentiation through a two-step sequential event, i.e., matrix maturation (early differentiation) and mineralization (late differentiation) (Ducy et al. 2000; Owen et al. 1990), which are controlled by different regulators. For example, early development from mesenchymal cells to osteoblastic lineage is determined by the key transcription factors, Runx2 and osterix (Komori 2006). Runx2 induces mesenchymal stem cell proliferation and initiates their differentiation into pre-osteoblasts (Komori 2006; Ducy et al. 2000). Osterix is downstream from Runx2, and is also essential for osteoblast differentiation (Komori 2006). Pre-osteoblasts also proliferate under the stimulation of various humoral factors, e.g., IGF-1, fibroblast growth factor-2, and bone morphogenetic proteins (Ou et al. 2010; Zaidi 2007). Once osteoblast proliferation declines, expression of a marker of matrix maturation phase, ALP, is gradually increased (Zaidi 2007; Stein et al. 2004; Owen et al. 1990). Finally, bone matrix is mineralized, concurrently with an increase in osteocalcin expression (Stein et al. 2004; Owen et al. 1990).

Herein, after a 48-h exposure to *P. mirifica* extract or puerarin, ALP transcript, but not Runx2, osterix and osteocalcin transcripts, was markedly increased, suggesting that both treatments promoted bone formation by enhancing early osteoblast differentiation into matrix maturation stage. Since ICI182780 completely abolished the *P. mirifica* extract- and puerarin-induced upregulation of ALP transcript, their osteoregulatory actions were mediated by ER, similar to the actions of E2 and genistein. Indeed, the observed increase in ALP expression after exposure to E2 and phytoestrogens was consistent with the previous reports of in vitro studies in primary osteoblasts and osteoblast cell lines (Zhang et al. 2007; Li et al. 2005). Zhang et al. (2007) also demonstrated that 10–100 μmol/l puerarin increased ALP activity and mineralizing nodules in rat primary osteoblasts via the phosphoinositide-3-kinase/Akt pathway.

In addition, differentiated osteoblasts, in turn, control bone remodeling process by producing RANKL and OPG (Teitelbaum 2000). After being released from osteoblasts, RANKL binds to its

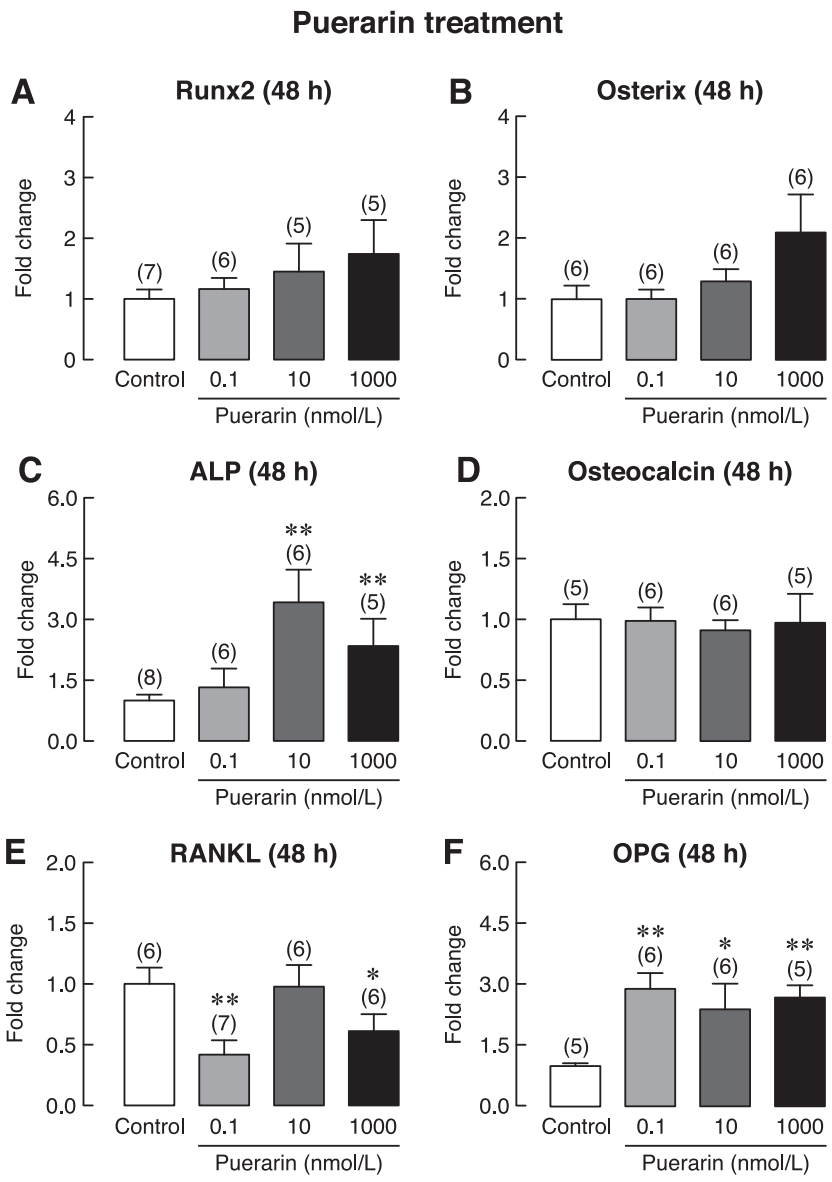


**Fig. 5.** The mRNA expression of (A) Runx2, (B) osterix, (C) ALP, (D) osteocalcin, (E) RANKL, and (F) OPG in UMR106 cells treated for 48 h with vehicle (control), or 0.1, 10 or 1000 nmol/l puerarin. The mRNA expression was quantified by qRT-PCR. The expression level of the control group was normalized to 1. Numbers in parentheses represent the numbers of independent samples. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control group.

receptor, RANK, on osteoclast precursors, thereby inducing differentiation of these cells into osteoclasts. The osteoclastogenesis is inhibited by OPG, a soluble decoy receptor of RANKL. Thus, the balance between the expression of osteoclastogenic stimulator (RANKL) and inhibitor (OPG) maintains equal bone formation and resorption in bone remodeling (Teitelbaum 2000). However, osteoblasts are targets of several osteoclastogenic agents, such as parathyroid hormone (PTH) and prolactin, that shift the RANKL/OPG ratio toward bone resorption, which eventually leads to bone loss and osteoporosis (Charoenphandhu et al. 2010; Zaidi 2007; Teitelbaum 2000). In the present study, *P. mirifica* extract and puerarin significantly upregulated the OPG mRNA expression although RANKL expression was upregulated by the high-dose *P. mirifica* extract (100 μg/ml). Puerarin might have greater anti-osteoporotic potency than *P. mirifica* extract since it could reduce the RANKL mRNA expression, thereby decreasing the RANKL/OPG ratio by ~80%. *P. mirifica* extract and puerarin thus exerted a positive effect on bone not only by increasing osteoblast differentiation, but also by suppressing the osteoclast-mediated bone resorption. Consistent with our findings, Li et al. (2004) reported that 0.01–1 μmol/l puerarin could suppress osteoclast activity in osteoblast/osteoclast co-culture with bovine bone slices by reducing the number and surface area of osteoclast absorption lacunae. The puerarin-induced decrease in RANKL/OPG ratio could explain the suppressed osteoclast activity in this co-culture system.

In conclusion, the present study provided corroborative evidence for the first time that *P. mirifica* extract and its major isoflavone, puerarin, were likely to enhance bone formation by promoting osteoblast differentiation, as indicated by the upregulation of ALP mRNA expression. Moreover, both *P. mirifica* extract and puerarin may suppress osteoclast activity since they predominantly upregulated OPG expression, thereby decreasing the RANKL/OPG ratio. The present mRNA expression study also suggested that the effect of *P. mirifica* extract on osteoblasts was mainly due to the action of puerarin because they similarly increased the mRNA levels of ALP and OPG, but not Runx2, osterix or osteocalcin. However, puerarin might have greater anti-osteoporotic

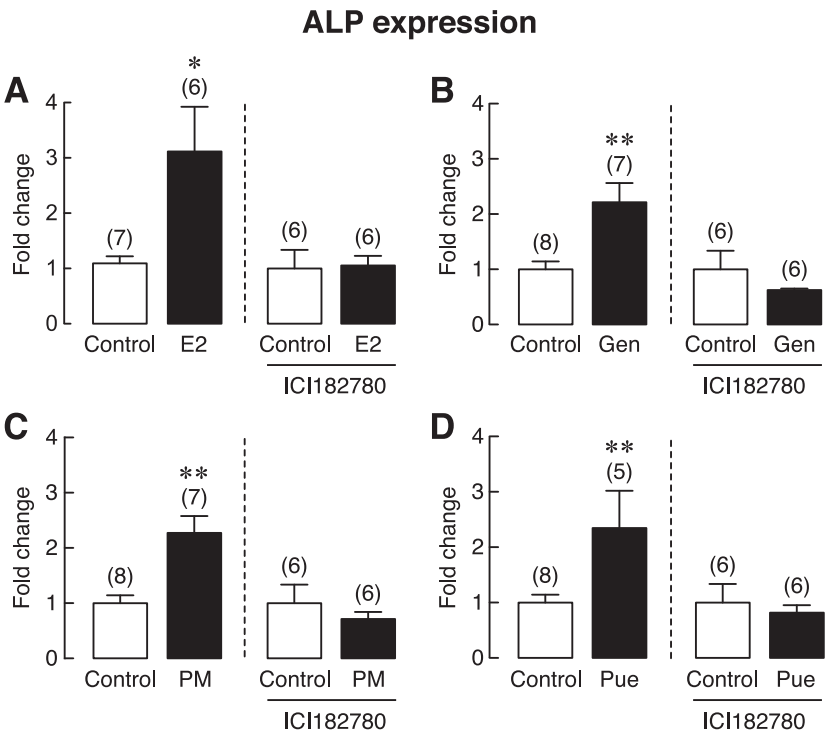


**Fig. 6.** The ALP mRNA expression in UMR106 cells treated for 48 h with (A) 10 nmol/l E2, (B) 1000 nmol/l genistein (Gen), (C) 100 μg/ml *P. mirifica* extract (PM), or (D) 1000 nmol/l puerarin (Pue) in the absence or presence of 10 nmol/l ICI182780 (ER antagonist). The expression level of the control group was normalized to 1. Numbers in parentheses represent the numbers of independent samples. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the respective vehicle-treated group (control).

potency than *P. mirifica* extract as puerarin directly decreased the mRNA level of RANKL, which was the major osteoclastogenic factor. Although our study focused on changes at the transcriptional level, the present findings have underlined the potential of *P. mirifica* extract and puerarin in prevention of bone loss, and the use of puerarin as an anti-osteoporotic drug would be an avenue worth exploring.

## Conflict of interest

No conflict to disclose.

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## References

Abdallah, B.M., Stilgren, L.S., Nissen, N., Kassem, M., Jørgensen, H.R., Abrahamsen, B., 2005. Increased RANKL/OPG mRNA ratio in iliac bone biopsies from women with hip fractures. Calcified Tissue International 76, 90–97.

Chansakaow, S., Ishikawa, T., Seki, H., Sekine, K., Okada, M., Chaichantipyuth, C., 2000. Identification of deoxymiroestrol as the actual rejuvenating principle of "Kwao Keur", *Pueraria mirifica*. The known miroestrol may be an artifact. Journal of Natural Products 63, 173–175.

Charoenphandhu, N., Wongdee, K., Krishnamra, N., 2010. Is prolactin the cardinal calciotropic maternal hormone? Trends in Endocrinology and Metabolism 21, 395–401.

Cherdshewasart, W., Kitsamai, Y., Malaivijitnond, S., 2007a. Evaluation of the estrogenic activity of the wild *Pueraria mirifica* by vaginal cornification assay. Journal of Reproduction and Development 53, 385–393.

Cherdshewasart, W., Sriwatcharakul, S., 2007. Major isoflavonoid contents of the 1-year-cultivated phytoestrogen-rich herb *Pueraria mirifica*. Bioscience, Biotechnology, and Biochemistry 71, 2527–2533.

Cherdshewasart, W., Subtang, S., Dahlan, W., 2007b. Major isoflavonoid contents of the phytoestrogen-rich herb *Pueraria mirifica* in comparison with *Pueraria lobata*. Journal of Pharmaceutical and Biomedical Analysis 43, 428–434.

Cole, Z.A., Dennison, E.M., Cooper, C., 2008. Osteoporosis epidemiology update. Current Rheumatology Reports 10, 92–96.

Ducy, P., Schinke, T., Karsenty, G., 2000. The osteoblast: a sophisticated fibroblast under central surveillance. Science 289, 1501–1504.

Huh, J.E., Yang, H.R., Park, D.S., Choi, D.Y., Baek, Y.H., Cho, E.M., Cho, Y.J., Kang-Il, K., Kim, D.Y., Lee, J.D., 2006. Puerariae radix promotes differentiation and mineralization in human osteoblast-like SaOS-2 cells. Journal of Ethnopharmacology 104, 345–350.

Kim, H.Y., Hong, J.H., Kim, D.S., Kang, K.J., Han, S.B., Lee, E.J., Chung, R.W., Song, K.H., Sho, K.A., Kwack, S.J., Kim, S.S., Park, K.L., Lee, S.K., Kim, M.C., Kim, C.M., Song, I.S., 2003. Isoflavone content and estrogen activity in arrowroot Puerariae radix. Food Science and Biotechnology 12, 29–35.

Komori, T., 2006. Regulation of osteoblast differentiation by transcription factors. Journal of Cellular Biochemistry 99, 1233–1239.

Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., Gustafsson, J.A., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 139, 4252–4263.

Lane, N.E., 2006. Epidemiology, etiology, and diagnosis of osteoporosis. American Journal of Obstetrics and Gynecology 194, S3–S11.

Li, L., Liu, Q., Jiang, M., Chen, H., Wang, S., 2004. Inhibitory effect of puerarin on osteoclastic bone resorption in vitro. Acta Academiae Medicinae Militaris Tertiae 26, 1830–1833.

Li, L., Liu, Q., Zhang, Y., Cui, Y., Gong, H., 2005. Effects of puerarin on the proliferation and alkaline phosphatase activity of UMR-106 cells. Chinese Journal of New Drugs 14, 1291–1294.

Liang, J., Chen, H., Pan, W., Xu, C., 2012. Puerarin inhibits caspase-3 expression in osteoblasts of diabetic rats. Molecular Medicine Reports 5, 1419–1422.

Lin, J.M., Callon, K.E., Lin, C.Q., Bava, U., Zheng, M.H., Reid, I.R., Cornish, J., 2007. Alteration of bone cell function by RANKL and OPG in different in vitro models. European Journal of Clinical Investigation 37, 407–415.

Malaivijitnond, S., 2012. Medical applications of phytoestrogens from the Thai herb *Pueraria mirifica*. Frontiers of Medicine 6, 8–21.

Malaivijitnond, S., Kiatthaipipat, P., Cherdshewasart, W., Watanabe, G., Taya, K., 2004. Different effects of *Pueraria mirifica*, a herb containing phytoestrogens, on LH and FSH secretion in gonadectomized female and male rats. Journal of Pharmacological Sciences 96, 428–435.

Manolagas, S.C., Kousteni, S., Jilka, R.L., 2002. Sex steroids and bone. Recent Progress in Hormone Research 57, 385–409.

Marini, H., Minutoli, L., Polito, F., Bitto, A., Altavilla, D., Atteritano, M., Gaudio, A., Mazzaferro, S., Frisina, A., Frisina, N., Lubrano, C., Bonaiuto, M., D'Anna, R., Cannata, M.L., Corrado, F., Adamo, E.B., Wilson, S., Squadrito, F., 2007. Effects of the phytoestrogen genistein on bone metabolism in osteopenic postmenopausal women: a randomized trial. Annals of Internal Medicine 146, 839–847.

Morabito, N., Crisafulli, A., Vergara, C., Gaudio, A., Lasco, A., Frisina, N., D'Anna, R., Corrado, F., Pizzoleo, M.A., Cincotta, M., Altavilla, D., Ientile, R., Squadrito, F., 2002. Effects of genistein and hormone-replacement therapy on bone loss in early postmenopausal women: a randomized double-blind placebo-controlled study. Journal of Bone and Mineral Research 17, 1904–1912.

Ou, G., Charles, L., Matton, S., Rodner, C., Hurley, M., Kuhn, L., Gronowicz, G., 2010. Fibroblast growth factor-2 stimulates the proliferation of mesenchyme-derived progenitor cells from aging mouse and human bone. Journals of Gerontology. Series A, Biological Sciences and Medical Sciences 65, 1051–1059.

Owen, T.A., Aronow, M., Shalhoub, V., Barone, L.M., Wilming, L., Tassinari, M.S., Kennedy, M.B., Pockwinse, S., Lian, J.B., Stein, G.S., 1990. Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. Journal of Cellular Physiology 143, 420–430.

Setchell, K.D., Lydeking-Olsen, E., 2003. Dietary phytoestrogens and their effect on bone: evidence from in vitro and in vivo, human observational, and dietary intervention studies. American Journal of Clinical Nutrition 78, 593S–609S.

Stein, G.S., Lian, J.B., van Wijnen, A.J., Stein, J.L., Montecino, M., Javed, A., Zaidi, S.K., Young, D.W., Choi, J.Y., Pockwinse, S.M., 2004. Runx2 control of organization, assembly and activity of the regulatory machinery for skeletal gene expression. Oncogene 23, 4315–4329.

Teitelbaum, S.L., 2000. Bone resorption by osteoclasts. Science 289, 1504–1508.

Tham, D.M., Gardner, C.D., Haskell, W.L., 1998. Clinical review 97: potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. Journal of Clinical Endocrinology and Metabolism 83, 2223–2235.

Urasopon, N., Hamada, Y., Asaoka, K., Cherdshewasart, W., Malaivijitnond, S., 2007. *Pueraria mirifica*, a phytoestrogen-rich herb, prevents bone loss in orchidectomized rats. Maturitas 56, 322–331.

Urasopon, N., Hamada, Y., Cherdshewasart, W., Malaivijitnond, S., 2008. Preventive effects of *Pueraria mirifica* on bone loss in ovariectomized rats. Maturitas 59, 137–148.

Zaidi, M., 2007. Skeletal remodeling in health and disease. Nature Medicine 13, 791–801.

Zhang, Y., Zeng, X., Zhang, L., Zheng, X., 2007. Stimulatory effect of puerarin on bone formation through activation of PI3K/Akt pathway in rat calvaria osteoblasts. Planta Medica 73, 341–347.