

## Original Article

# 17 $\beta$ -estradiol attenuates homocysteine-induced oxidative stress and inflammatory response as well as MAPKs cascade via activating PI3-K/Akt signal transduction pathway in Raw 264.7 cells

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## Abstract

Oxidative stress, inflammatory response, and mitogen-activated protein kinases (MAPKs) cascade are significant pathogenic factors of osteoporosis. It has been reported that elevated homocysteine (Hcy) may activate oxidative stress and reduce bone mineral density in post-menopausal osteoporosis. Moreover, hormone replacement therapy has been widely used in clinic to prevent and treat post-menopausal women with osteoporosis and osteoporotic fracture, but the molecular mechanisms and relevant signal transduction pathways underlying the action of Hcy remain unclear. In this study, we investigated the effects of 17 $\beta$ -estradiol (17 $\beta$ -E<sub>2</sub>) on the Hcy-induced oxidative stress, inflammatory response and MAPKs cascade, as well as the underlying signal transduction pathway in murine Raw 264.7 cells. The reactive oxygen species (ROS) was assessed by fluorospectrophotometry. The proinflammatory cytokines tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin (IL)-1 $\beta$  were analyzed by double-immunofluorescence labeling and reverse transcriptase polymerase chain reaction assay, respectively. Furthermore, phosphorylation levels of MAPKs cascade were measured by western blot analysis. A specific phosphatidylinositol 3-kinase (PI3-K) inhibitor, Wortmannin (1  $\mu$ M) was employed to determine whether PI3-K/Akt signaling pathway mediated the 17 $\beta$ -E<sub>2</sub>'s effect on Raw 264.7 cells. 17 $\beta$ -E<sub>2</sub> markedly decreased the ROS production induced by Hcy, the expression of TNF- $\alpha$  and IL-1 $\beta$  at protein and mRNA levels, and down-regulated the phosphorylation of MAPKs (ERK1/2, JNK and p38). These suppressing effects of 17 $\beta$ -E<sub>2</sub> on Hcy-induced changes were reversed by pretreatment with PI3-K inhibitor Wortmannin. The results indicate that 17 $\beta$ -estradiol may attenuate Hcy-induced oxidative stress, inflammatory response and up-regulation of MAPKs in Raw 264.7 cells via PI3-K/Akt signal transduction pathway.

**Key words:** 17 $\beta$ -estradiol, homocysteine, reactive oxygen species, MAPKs, PI3-K/Akt

## Introduction

With an increasingly aging population, osteoporosis and resultant bone fracture have emerged as a major public health problem across the world, placing a huge economic burden on healthcare service resources [1–3]. There are many risk factors responsible for osteoporosis and osteoporotic fracture. It has been reported that an elevated level of homocysteine (Hcy) is one of the risk factors relevant to osteoporotic fracture [4,5]. Hcy may contribute to oxidative stress and reduce bone mineral density (BMD) in post-menopausal osteoporosis [6]. Moreover, it has also been demonstrated that Hcy-lowering therapy reduced the bone loss and occurrence rate of hip fracture [7].

Mitogen-activated protein kinases (MAPKs) family has been implicated in the production of proinflammatory mediators and cytokines [8]. Furthermore, a multitude of studies have reported that the increased production of reactive oxygen species (ROS) and, proinflammatory cytokine, as well as the activation of MAPKs may lead to bone loss and osteoporosis *in vitro* or *in vivo* [9–11].

Estrogen plays an important role in maintaining the bone mass and structure through regulating the balance between bone-resorbing osteoclasts and bone-forming osteoblasts in adult [12]. A recent study showed that estrogen deficiency promoted osteoclast activation and subsequent bone loss in ovariectomized mice [13]. Moreover, in clinic, it has also been shown that hormone replacement therapy (HRT) significantly increases BMD and reduces the bone turnover markers (BTMs) [14,15]. However, the underlying molecular mechanisms of how estrogen inhibits the activation of osteoclasts induced by Hcy and the relevant signal transduction pathway remain largely unknown.

The Raw 264.7 cell line derived from murine macrophage has been widely used as an osteoclast-like cell model *in vitro*. In our previous study, we have found that lipopolysaccharide (LPS) treatment increases the production of NO, the expression of tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$ , and up-regulates the phosphorylation of MAPKs in Raw 264.7 cells, which is dependent on the activation of PI3-K/Akt pathway [16]. Additional studies have also demonstrated that ROS formation and inflammation in response to LPS or other stimuli may be regulated via MAPKs or PI3-K/Akt signal pathway in Raw 264.7 cells [17–19]. In this study, we proposed that Hcy may stimulate Raw 264.7 cells by activating inflammatory response and MAPKs activation, thereby increasing the generation of intracellular ROS, and that 17 $\beta$ -E<sub>2</sub> may prevent macrophages from Hcy-induced activation via PI3-K/Akt pathway. To test this hypothesis, we examined the direct effects of 17 $\beta$ -E<sub>2</sub> on Hcy-stimulated oxidative stress and inflammatory responses, as well as activation of MAPKs activation in Raw 264.7 cells, and the potential role of PI3-K signaling in this process.

## Materials and Methods

### Cell culture and treatments

Raw 264.7 cell line obtained from American Type Culture Collection (Manassas, USA) was cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, USA) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, USA), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Sigma, St Louis, USA) at a density not exceeding  $1 \times 10^5$  cells/ml at 37°C in a 5% CO<sub>2</sub> incubator. Cells ( $\sim 1 \times 10^6$  cells/ml) were seeded in six-well plates before exposure to treatments. Raw 264.7 cells were pretreated with 17 $\beta$ -E<sub>2</sub> (Sigma) at 1 nM, 10 nM, and 1  $\mu$ M for 1 h, followed by stimulation with Hcy (5 mM; Sigma) for 1 h. Raw 264.7 cells were pretreated with Wortmannin (1  $\mu$ M) (Sigma) for 1 h, followed by 17 $\beta$ -E<sub>2</sub> and Hcy stimulation.

### Cell viability assay

Raw 264.7 cells ( $100 \mu$ l;  $1 \times 10^3$  cells/ml) were seeded in a 96-well dish for 24 h, followed by stimulation with 17 $\beta$ -E<sub>2</sub> (1 nM, 10 nM and 1  $\mu$ M), 17 $\beta$ -E<sub>2</sub> with Hcy (5 mM), 17 $\beta$ -E<sub>2</sub> with Wortmannin (1  $\mu$ M), or 17 $\beta$ -E<sub>2</sub> with Hcy and Wortmannin for 24 h. 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (20  $\mu$ l; 0.5 mg/ml) was then added to each well, and the cells were incubated for 4 h at 37°C and in 5% CO<sub>2</sub>. Subsequently, the supernatant was removed, and 150  $\mu$ l of DMSO was added to each well. The value of optical density was measured at 540 nm with a microplate reader until formazan was completely solubilized.

### Assay of ROS production

Raw 264.7 cells ( $1 \sim 2 \times 10^4$  cells/ml) were seeded and incubated in a 25 cm<sup>2</sup> culture bottle at 80% confluency, and then treated with Hcy (0, 1, 3, 5, and 8 mM) for 1 h or pretreated with 17 $\beta$ -E<sub>2</sub> (0, 1 nM, 10 nM, and 1  $\mu$ M) for 1 h before 5 mM Hcy stimulation for 1 h. Collected cells were suspended in a serum free medium with 10  $\mu$ M H<sub>2</sub>DCFDA and incubated for 2 h at 37°C. The suspension was mixed up and down every 3–5 min to make H<sub>2</sub>DCFDA penetrate to cells and convert to a fluorescent H<sub>2</sub>DCF when ROS existed. After being washed twice with phosphate buffered saline (PBS), the cells were resuspended in 1 ml PBS, and the fluorescence intensity was monitored by a fluorospectrophotometry at 488 nm excitation wavelength and 525 nm emission wavelength.

### Double-immunofluorescence labeling

The coverslips with adherent Raw 264.7 cells derived from different treatments were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min, rinsed twice with PBS, and then used for double immunofluorescence labeling. Raw 264.7 cells were incubated with DAPI (1 : 50,000; Sigma) plus goat anti-rabbit TNF- $\alpha$  monoclonal antibody (1 : 500; Cell Signaling Technology, Charlottesville, USA) or anti-IL-1 $\beta$  monoclonal antibody (1 : 500; Cell Signaling Technology) overnight at 4°C. Subsequently, the cells were incubated with TRITC-conjugated secondary antibody (Santa Cruz, Santa Cruz, USA) for 1 h at room temperature. For negative controls, a set of culture slides was incubated under similar conditions without the primary antibodies. All images were captured with a fluorescence microscope (80i; Nikon, Tokyo, Japan).

### Reverse transcription-polymerase chain reaction analysis

Total RNA was extracted from Raw 264.7 cells using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Total RNA was reverse transcribed by using the Superscript<sup>TM</sup>-III kit (Invitrogen) with 2.5  $\mu$ g total RNA and oligo dT. The sequences of primers were as follows:

TNF- $\alpha$  primers, 5'-CGTCAGCCGATTGCTATCT-3' (sense) and 5'-CGGACTCCGCAAAGTCTAAG-3' (antisense).

IL-1 $\beta$  primers, 5'-GCCCATCCTCTGTGACTCAT-3' (sense) and 5'-AGGCCACAGGTATTTGTCTG-3' (antisense).

$\beta$ -actin primers, 5'-AGCCATGTACGTAGCCATCC-3' (sense) and 5'-GCTGTGGTGGTGAAGCTGTA-3' (antisense). Polymerase chain reaction amplifications were performed for 45 cycles under the following conditions: initial denaturation at 95°C for 15 min, denaturation at 95°C for 15 s, annealing at 60°C for 25 s, and extension at 72°C for 20 s. Amplified fragment sizes for TNF- $\alpha$ , IL-1 $\beta$ , and

$\beta$ -actin were 205, 229, and 222 bp, respectively. Images were captured with a Gel Doc 2000 image analyzer (Bio-Rad, Richmond, USA).

### Western blot analysis

Raw 264.7 cells were seeded overnight in 6-well plates at a density of  $1 \times 10^6$  cells per plate. The cells were further incubated in DMEM medium with 10% FBS free for at least 1 h before treatments. Treated cells were collected and incubated with ice-cold PBS and centrifuged at 16,000 g for 5 min at 4°C. Cells were lysed in ice-cold lysis buffer, and the whole cell lysate was centrifuged at 16,000 g for 5 min at 4°C, and the supernatants were collected. Protein content was determined by the BCA protein assay kit (Pierce, Rockford, USA). Proteins (~50  $\mu$ g per lane) from whole cell lysate were loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred onto an immunoblot polyvinylidene difluoride membrane (Chemicon, Billerica, USA). The membranes were blocked with 5% non-fat milk in 0.1% Tween 20 in Tris-buffered saline (TBST) for 2 h at room temperature and incubated separately with goat anti-rabbit antibodies against ERK1/2, phospho-ERK1/2, JNK, phospho-JNK (1 : 1000; Cell Signaling Technology), p38 MAPK and phospho-p38 MAPK (1 : 1000; Santa Cruz) overnight at 4°C. The membranes were then washed three times for 15 min with TBST, and incubated with horseradish peroxidase conjugated secondary antibodies (1 : 2000; Santa Cruz) for 2 h at room temperature. The membranes were washed with TBST three times for 5 min each and developed by the ECL detection system (Santa Cruz). The bands were exposed to Fuji Medical X-Ray Film (Fuji Photo Film Co., Ltd, Karagawa, Japan), and the band density was determined by Image J software (NIH, Bethesda, USA).

### Statistical analysis

The values were expressed as the mean  $\pm$  standard error of the mean (SEM) from three independent experiments. The statistical analysis was performed by one-way analysis of variance, followed by Student–Newman–Keuls test for various groups.  $P < 0.05$  was considered to be significant. The SPSS 13.0 statistical software package was used in the analysis.

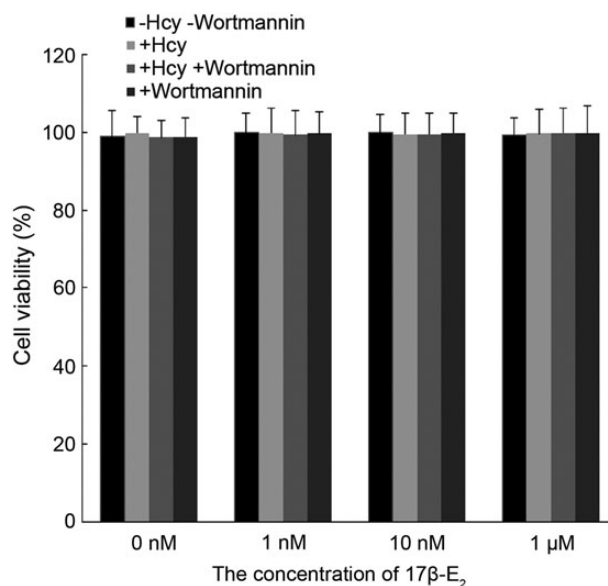
## Results

### 17 $\beta$ -E<sub>2</sub> and Wortmannin had no toxic effect on the viability of Raw 264.7 cells

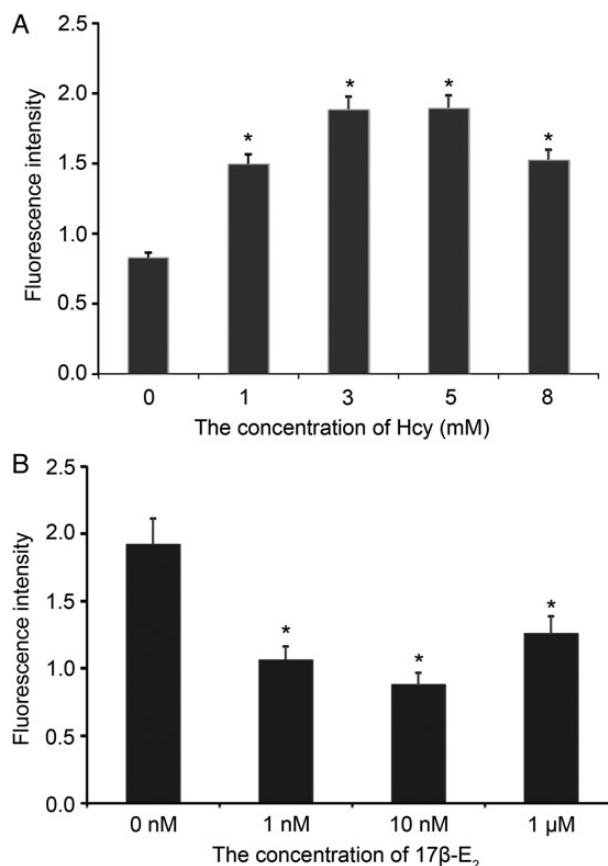
To exclude the possibility that any change may result from direct toxicity of 17 $\beta$ -E<sub>2</sub> and the PI3-K inhibitor Wortmannin on the cells, the cytotoxicity of 17 $\beta$ -E<sub>2</sub> (0, 1 nM, 10 nM, 1  $\mu$ M) and Wortmannin (1  $\mu$ M) was assessed in the presence or absence of 5 mM Hcy by MTT assay. The results showed that 17 $\beta$ -E<sub>2</sub>, Hcy, or Wortmannin alone and together exerted no significant cytotoxicity on Raw 264.7 macrophage cells (Fig. 1).

### 17 $\beta$ -E<sub>2</sub> decreased Hcy-induced intracellular ROS production in Raw 264.7 cells

To determine the effect of Hcy on intracellular ROS generation in Raw 264.7 cells, the cells were treated with Hcy (1, 3, 5, and 8 mM) for 1 h. As shown in Fig. 2A, treatment with Hcy caused a significant increase in ROS production, especially at 3 and 5 mM Hcy treatment (Fig. 2A). However, pretreatment with 17 $\beta$ -E<sub>2</sub> (1 nM, 10 nM, and 1  $\mu$ M) significantly decreased the ROS generation induced by Hcy (5 mM) in Raw 264.7 cells, especially at 10 nM 17 $\beta$ -E<sub>2</sub> (Fig. 2B). These data suggest that 17 $\beta$ -E<sub>2</sub> suppresses intracellular ROS production in Hcy-stimulated Raw 264.7 cells.



**Figure 1. Cytotoxic assessment of 17 $\beta$ -E<sub>2</sub> and Wortmannin in Raw 264.7 macrophage cells** Raw 264.7 cells were treated with 1 nM, 10 nM, and 1  $\mu$ M 17 $\beta$ -E<sub>2</sub> for 1 h with or without 5 mM Hcy or with 1  $\mu$ M Wortmannin treatments for 24 h. Raw 264.7 cell viability was measured.



**Figure 2. Hcy increased the ROS production in Raw 264.7 cells while 17 $\beta$ -E<sub>2</sub> reduced the ROS generation** (A) Different concentrations of Hcy induced the ROS production in Raw 264.7 cells. \* $P < 0.05$  vs. 0 mM Hcy. (B) 17 $\beta$ -E<sub>2</sub> reduced the ROS generation in Hcy (5 mM)-induced Raw 264.7 cells. \* $P < 0.05$  vs. 0 nM 17 $\beta$ -E<sub>2</sub>.

17β-E<sub>2</sub> inhibited Hcy-induced inflammatory cytokines expression via PI3-K/Akt signaling pathway in Raw 264.7 cells

Cells were stimulated with 5 mM Hcy for 1 h with or without 17β-E<sub>2</sub> pretreatment (1 nM, 10 nM, and 1 μM) for 1 h. The results showed that Hcy treatment resulted in the significant increases of TNF-α and IL-1β at both protein and mRNA levels. However, pretreatment with 17β-E<sub>2</sub> (1 nM, 10 nM, and 1 μM) for 1 h markedly reduced the expression of TNF-α and IL-1β at both protein and mRNA levels compared with Hcy treatment alone (Figs. 3 and 4). To further investigate whether this inhibitory effect of 17β-E<sub>2</sub> was associated with the PI3-K/Akt pathway, a specific PI3-K inhibitor Wortmannin was used. The results showed that pretreating Raw 264.7 cells with 1 μM Wortmannin for 1 h significantly rescued the down-regulation of TNF-α and IL-1β induced by 17β-E<sub>2</sub> (Figs. 3 and 4). These results suggest that 17β-E<sub>2</sub> inhibits Hcy-induced inflammatory cytokines TNF-α and IL-1β at protein and mRNA levels via activation of the PI3-K signaling pathway.

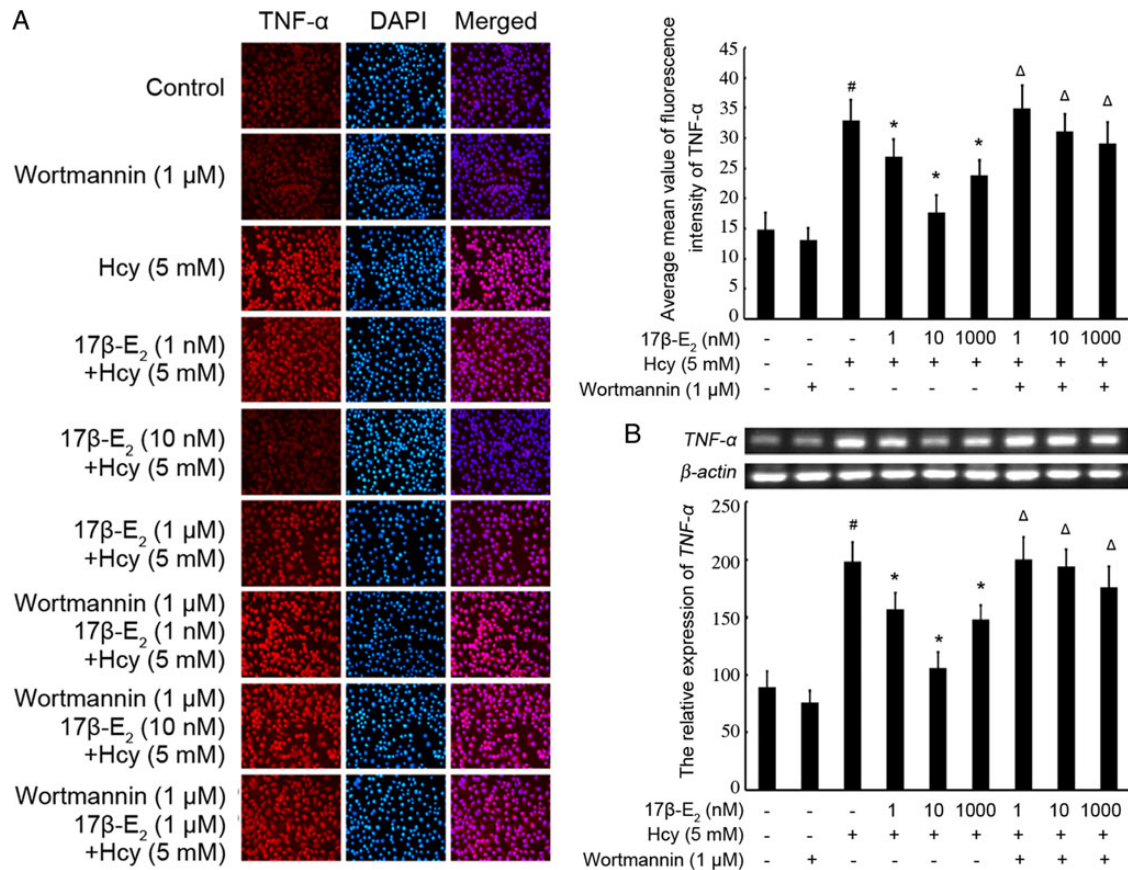
**Wortmannin reversed the inhibition of 17β-E<sub>2</sub> on the Hcy-induced MAPKs phosphorylation in Raw 264.7 cells**  
To further investigate the upstream signaling molecules involved in inflammatory responses which were induced by Hcy and suppressed by 17β-E<sub>2</sub> in Raw 264.7 cells, cells were stimulated with 5 mM Hcy for

1 h with or without 17β-E<sub>2</sub> pretreatment (1 nM, 10 nM, and 1 μM) for 1 h. Western blot analysis was carried out to measure the expression of phospholated or total forms of three MAPKs such as ERK1/2, JNK, and p38. The results showed that Hcy treatment alone led to a dramatic increase in phosphorylation of ERK1/2, JNK, and p38, while no any effect on the total forms (Fig. 5). Pretreatment with 17β-E<sub>2</sub> (1 nM, 10 nM, and 1 μM) for 1 h significantly down-regulated the phosphorylation of ERK1/2, JNK, and p38 compared with Hcy treatment alone (Fig. 5). Pretreatment of Raw 264.7 cells with 1 μM Wortmannin for 1 h rescued the inhibition of 17β-E<sub>2</sub> on the phosphorylation of ERK1/2, JNK and p38 induced by Hcy (Fig. 5).

Discussion

Postmenopausal osteoporosis and resulting fractures are becoming one of major public health issues. Bone strength is determined by bone mass as well as bone quality. Low bone mass and microarchitectural deterioration of bone tissue lead to fragile bone and subsequent fracture risk.

Major risk factors of osteoporosis and osteoporotic fracture include aging, endocrine disorder, malnutrition, heredity, immunity, and so on. As one of the possible risk factors, Hcy was firstly linked to osteoporosis caused by hyperhomocystinemia (HHcy), a rare genetic disorder characterized as serious homocystinuria and complicated atherosclerosis and osteoporosis [20]. Hcy is a non-protein α-amino



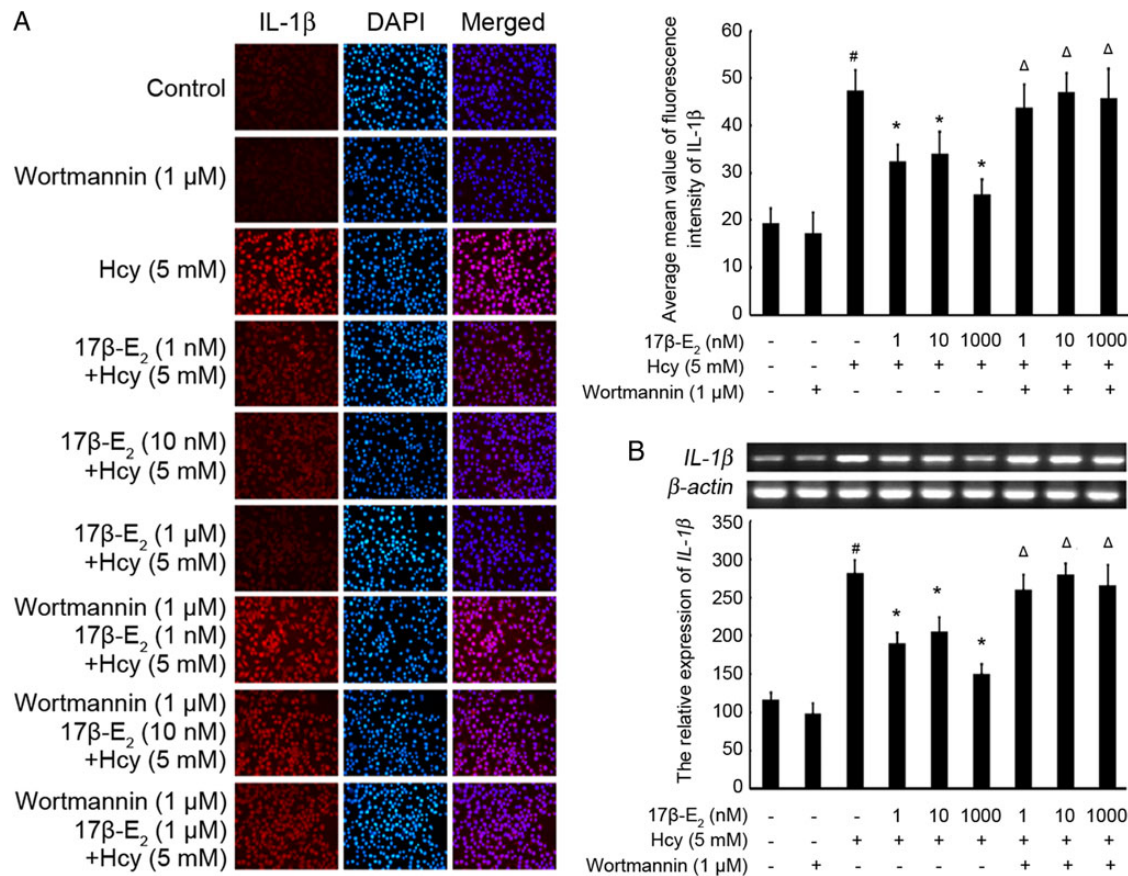
**Figure 3. 17β-E<sub>2</sub> inhibited the expression of TNF-α at protein and mRNA levels induced by Hcy, which was dependent on PI3-K signaling pathway in Raw 264.7 macrophage cells** (A) The immunofluorescence images of TNF-α and (B) the corresponding mRNA levels of TNF-α under different conditions. <sup>#</sup>*P* < 0.05 vs. control alone. <sup>\*</sup>*P* < 0.05 vs. Hcy alone. <sup>Δ</sup>*P* < 0.05 vs. Hcy + 17β-E<sub>2</sub>.



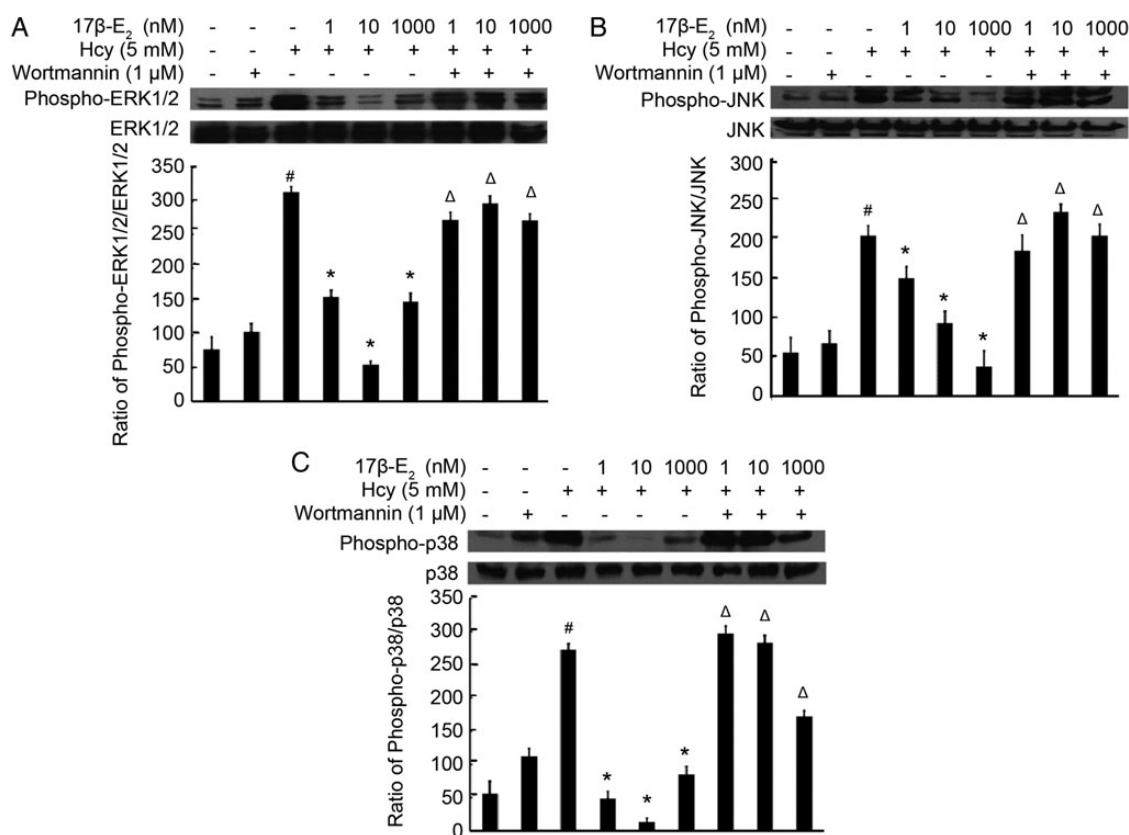
acid, which is biosynthesized during methionine metabolism via a multi-step process [21]. Furthermore, recent studies demonstrated that HHcy could reduce BMD, increase bone loss and turnover, leading to osteoporotic fractures, especially in elderly persons [22,23]. Moreover, it was shown that HHcy compromised fracture repair in mice [24]. It has also been demonstrated that Hcy-lowering therapy attenuated the bone loss and occurrence rate of hip fracture [7]. Collectively, these findings strongly suggest that elevated Hcy is a risk factor for osteoporosis and osteoporotic fractures. With respect to the pathogenesis and mechanism of Hcy-induced bone tissue injury, it has been reported that Hcy directly activated osteoclasts through increasing the production of intracellular ROS [25]. Yilmaz and Eren showed that mildly elevated homocysteinemia may contribute to increasing total peroxide levels and reducing total antioxidant status in the low BMD group [6]. Hcy is also thought to interfere with collagen cross-linking, resulting in bone disorder and increased susceptibility to fracture [26,27]. On the other hand, several investigations reported that no or weak associations between Hcy and decreased BMD or fracture occurrence in postmenopausal women [28,29].

In fact, the most basic mechanism in all risk factors leading to osteoporosis is the imbalance between bone resorption and formation, while hormonal factors strongly influence the rate of bone resorption in adult. Postmenopausal osteoporosis and osteoporotic fracture is an epidemic across the world due to decreased estrogen level in elder females. Up to date, the active effects of estrogen have been found to

be relative to osteoblast proliferation and increased production of alkaline phosphate in cells treated with estrogen [30], and decreased differentiation of bone marrow mononuclear cells into mature osteoclasts in postmenopausal women *in vivo* [31]. 17β-E<sub>2</sub> could improve bone mass and suppress bone resorption by stimulating IL-10 and TGF-β1 secretion [32]. In addition, HRT has been developed into the selective estrogen receptor modulators (SERMs) in phase-3 clinical trials. It has been revealed that SERMs significantly reduced the risk of vertebral fracture and BTMs, and increased BMD in post-menopausal women without stimulating the breast or endometrium [33,34]. These researches have in part provided concrete evidence for the protective and therapeutic mechanism of estrogen to osteoporosis and osteoporotic fracture. A study from Vijayan *et al.* [35] confirmed that high-methionine diet-induced hyperhomocysteinemia increased the synthesis of inflammatory cytokines such as IL-1α, IL-1β, and TNF-α, while such up-regulation could be exacerbated by ovariectomy, which is consistent with our results. In this study, we showed that 17β-E<sub>2</sub> (1 nM, 10 nM, 1 μM) significantly reduced the production of ROS in 5 mM Hcy-treated Raw 264.7 cells. This study also demonstrated that 17β-E<sub>2</sub> attenuated the expression of TNF-α and IL-1β at protein and mRNA levels in Hcy-stimulated Raw 264.7 macrophage cells. As a result, the data are consistent with previous findings that estrogen inhibits oxidative stress and down-regulates proinflammatory mediators and proinflammatory factors, suggesting the potential beneficial effects of estrogen on osteoclast-like cells by suppressing the inflammatory response and subsequent ROS generation.



**Figure 4.** 17β-E<sub>2</sub> inhibited the expression of IL-1β at protein and mRNA levels induced by Hcy, which was dependent on PI3-K signaling pathway in Raw 264.7 macrophage cells (A) The immunofluorescence images of IL-1β and (B) The corresponding mRNA levels of IL-1β under different conditions. <sup>#</sup>*P* < 0.05 vs. control alone. <sup>\*</sup>*P* < 0.05 vs. Hcy alone <sup>Δ</sup>*P* < 0.05 vs. Hcy + 17β-E<sub>2</sub>.



**Figure 5. 17 $\beta$ -E<sub>2</sub> inhibited the phosphorylation of MAPKs induced by Hcy, which involved PI3-K in Raw 264.7 macrophage cells** (A) The western blot images and analysis of phospho- or total forms of ERK1/2. (B) The western blot images and analysis of phospho- or total forms of JNK and (C) The western blot images and analysis of phospho- or total forms of p38. Approximately  $1 \times 10^6$  cells/ml were seeded in six-well plates and incubated until 80% confluency. Cells were pre-treated with 17 $\beta$ -E<sub>2</sub> (1 nM, 10 nM, and 1  $\mu$ M) for 1 h with or without Wortmannin (1  $\mu$ M), then exposure to Hcy (5 mM) for 1 h. Whole cell lysate was prepared, and western blot analysis was performed by using specific antibodies against phospho- or total forms of ERK1/2, JNK, and p38 MAPK as mentioned above. The relative protein levels were quantified by scanning densitometry and normalized to total ERK1/2, JNK, or p38 MAPK. The values shown are the mean  $\pm$  SEM of data from three independent experiments. # $P < 0.05$  vs. control alone. \* $P < 0.05$  vs. Hcy alone.  $\Delta P < 0.05$  vs. Hcy +17 $\beta$ -E<sub>2</sub>.

PI3-Ks are a family of enzymes involved in cell signaling pathways that regulate many cellular functions such as cell growth, proliferation, differentiation, survival, and motility, as well as many pathological conditions [36]. Recently, evidence has suggested that PI3-K/Akt signaling pathway plays an important role in osteoclast differentiation and osteoclastogenesis [37–39]. Likewise, it is generally accepted that Hcy-induced activation of macrophages and endothelial cells is involved in the PI3K/Akt signaling pathway, ERK and oxidative stress [40–42].

Hence, based on the involvement of PI3-K associated signal cascades in the Hcy-induced inflammatory responses, we first investigated whether 17 $\beta$ -E<sub>2</sub> attenuated Hcy-induced inflammatory cytokines via PI3-K/Akt signaling pathway. We found that Wortmannin (1  $\mu$ M) pre-treatment significantly rescued the inhibition of 17 $\beta$ -E<sub>2</sub> on TNF- $\alpha$  and IL-1 $\beta$ . The results demonstrate that the down-regulation of 17 $\beta$ -E<sub>2</sub> on inflammatory cytokines is in part dependent on PI3-K/Akt activation, and TNF- $\alpha$  and IL-1 $\beta$  are the downstream targets of PI3-K/Akt signal pathway. Additionally, inhibition of PI3-K can induce the expression of proinflammatory mediators and cytokines production in LPS or Hcy-stimulated microphages and cardiomyocytes and glomerular mesangial cells [16,43,44]. These reports further strengthen our findings, and demonstrate a pivotal role of PI3-K/Akt signaling in the regulation of proinflammatory cytokines in various cell types.

It has become clear that MAPKs are ubiquitous in many organisms. The proinflammatory responses involved in MAPK signal transduction pathways have been extensively investigated [8]. MAPKs are implicated in the responses to a host of stimulus, such as proinflammatory cytokines, osmotic stress, heat shock, and mitogens, which regulate inflammation, proliferation, differentiation, mitosis, cell survival, and apoptosis [45]. It has also been shown that MAPKs play important roles in up-regulating the expression of classic proinflammatory enzymes iNOS, COX-2, and cytokines TNF- $\alpha$  and IL-1 $\beta$  in various types of cells treated with LPS [46–48]. It has long been recognized that inflammation significantly impacts bone turnover, and inflammatory cytokines such as IL-1, TNF- $\alpha$ , and M-CSF that are associated with osteoclastic bone loss [49]. Several studies reported that increased Hcy stimulated these inflammatory parameters in serum, heart, and brain tissue *in vivo* or *in vitro* [50–52], suggesting that Hcy might be associated with inflammatory response. At present, it is clear that MAPK and PI3-K/Akt signaling pathways can cross-talk with each other [53,54]. To our knowledge, there have been few studies investigating whether or not estrogen improves Hcy-induced inflammatory response and the activation of MAPKs is dependent on PI3-K/Akt signaling pathway in Raw 264.7 cells. To investigate the molecular signaling pathway by which 17 $\beta$ -E<sub>2</sub> attenuates Hcy-induced phosphorylation of MAPKs and whether PI3-K/Akt is involved in

this process in Raw 264.7 cells, we also examined the effect of 17 $\beta$ -E<sub>2</sub> on phosphorylation (activate form) of three MAPKs in Hcy-induced Raw 264.7 cells. As expected, we found that Hcy treatment up-regulated the levels of phosphorylated MAPKs including ERK1/2, JNK, and p38 MAPK. However, 17 $\beta$ -E<sub>2</sub> pretreatment down-regulated the Hcy-induced phosphorylation of MAPKs and decreased ROS production and TNF- $\alpha$  and IL-1 $\beta$  expression. Furthermore, to further confirm whether the inhibition of 17 $\beta$ -E<sub>2</sub> on inflammatory response was mediated through PI3-K/Akt pathway, Wortmannin, a specific PI3-K inhibitor was employed. Pretreatment of Raw 264.7 cells with 1  $\mu$ M Wortmannin for 1 h prior to Hcy and 17 $\beta$ -E<sub>2</sub> treatment prevented the down-regulation of 17 $\beta$ -E<sub>2</sub> on the phosphorylation of ERK1/2, JNK, and p38 and the expressions of proinflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$ . These results altogether suggest that the ability of 17 $\beta$ -E<sub>2</sub> to inhibit inflammatory response and phosphorylation of MAPKs family is mediated by PI3-K signaling pathway.

In conclusion, this study showed that Hcy could increase intracellular ROS generation in Raw 264.7 cells, which could be attenuated by 17 $\beta$ -E<sub>2</sub>. In addition, we provided the first evidence that 17 $\beta$ -E<sub>2</sub> could inhibit the MAPKs activation and reduce proinflammatory cytokines production, in response to Hcy in Raw 264.7 cells, which is dependent on PI3-K/Akt pathway. Thus, 17 $\beta$ -E<sub>2</sub> exerts its cytoprotective effects on osteoclast-like Raw 264.7 cells via PI3-K/Akt pathway.

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## References

- Looker AC, Orwoll ES, Johnston CC Jr, Lindsay RL, Wahner HW, Dunn WL, Calvo MS, *et al.* Prevalence of low femoral bone density in older U.S. adults from NHANES III. *J Bone Miner Res* 1997, 12: 1761–1768.
- Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005–2025. *J Bone Miner Res* 2007, 22: 465–475.
- Hernlund E, Svedbom A, Ivergård M, Compston J, Cooper C, Stenmark J, McCloskey EV, *et al.* Osteoporosis in the European Union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). *Arch Osteoporos* 2013, 8: 136.
- Yang J, Hu X, Zhang Q, Cao H, Wang J, Liu B. Homocysteine level and risk of fracture: a meta-analysis and systematic review. *Bone* 2012, 51: 376–382.
- Kuroda T, Tanaka S, Saito M, Shiraki Y, Shiraki M. Plasma level of homocysteine associated with severe vertebral fracture in postmenopausal women. *Calcif Tissue Int* 2013, 93: 269–275.
- Yilmaz N, Eren E. Homocysteine oxidative stress and relation to bone mineral density in post-menopausal osteoporosis. *Aging Clin Exp Res* 2009, 21: 353–357.
- Lee SH, Kim MJ, Kim BJ, Kim SR, Chun S, Ryu JS, Kim GS, *et al.* Homocysteine-lowering therapy or antioxidant therapy for bone loss in Parkinson's disease. *Mov Disord* 2010, 25: 332–340.
- Kyriakis JM, Avruch J. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. *Physiol Rev* 2012, 92: 689–737.
- Lee YS, Lee SJ, Seo KW, Bae JU, Park SY, Kim CD. Homocysteine induces COX-2 expression in macrophages through ROS generated by NMDA receptor-calcium signaling pathways. *Free Radic Res* 2013, 47: 422–431.
- Manolagas SC. From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. *Endocr Rev* 2010, 31: 266–300.
- Yin H, Shi ZG, Yu YS, Hu J, Wang R, Luan ZP, Guo DH. Protection against osteoporosis by statins is linked to a reduction of oxidative stress and restoration of nitric oxide formation in aged and ovariectomized rats. *Eur J Pharmacol* 2012, 674: 200–206.
- Syed F, Khosla S. Mechanisms of sex steroid effects on bone. *Biochem Biophys Res Commun* 2005, 328: 688–696.
- Miyauchi Y, Sato Y, Kobayashi T, Yoshida S, Mori T, Kanagawa H, Katsuyama E, *et al.* HIF1 $\alpha$  is required for osteoclast activation by estrogen deficiency in postmenopausal osteoporosis. *Proc Natl Acad Sci USA* 2013, 110: 16568–16573.
- Cauley JA, Robbins J, Chen Z, Cummings SR, Jackson RD, LaCroix AZ, LeBoff M, *et al.* Effects of estrogen plus progestin on risk of fracture and bone mineral density: the Women's Health Initiative randomized trial. *JAMA* 2002, 287: 1729–1738.
- Gallagher JC, Rapuri PB, Haynatzki G, Detter JR. Effect of discontinuation of estrogen, calcitriol, and the combination of both on bone density and bone markers. *J Clin Endocrinol Metab* 2002, 87: 4914–4923.
- Zong Y, Sun L, Liu B, Deng YS, Zhan D, Chen YL, He Y, *et al.* Resveratrol inhibits LPS-induced MAPKs activation via activation of the phosphatidylinositol 3-kinase pathway in murine Raw 264.7 macrophage cells. *PLoS One* 2012, 7: e44107.
- Feng D, Zhou Y, Xia M, Ma J. Folic acid inhibits lipopolysaccharide-induced inflammatory response in RAW264.7 macrophages by suppressing MAPKs and NF- $\kappa$ B activation. *Inflamm Res* 2011, 60: 817–822.
- Hou GQ, Guo C, Song GH, Fang N, Fan WJ, Chen XD, Yuan L, *et al.* Lipopolysaccharide (LPS) promotes osteoclast differentiation and activation by enhancing the MAPK pathway and COX-2 expression in RAW264.7 cells. *Int J Mol Med* 2013, 32: 503–510.
- Li DZ, Zhag QX, Dong XX, Li HD, Ma X. Treatment with hydrogen molecules prevents RANKL-induced osteoclast differentiation associated with inhibition of ROS formation and inactivation of MAPK, AKT and NF-kappa B pathways in murine RAW264.7 cells. *J Bone Miner Metab* 2014, 32: 494–504.
- Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE, Andria G, *et al.* The natural history of homocystinuria due to cystathionine beta-synthase deficiency. *Am J Hum Genet* 1985, 37: 1–31.
- Selhub J. Homocysteine metabolism. *Annu Rev Nutr* 1999, 19: 217–246.
- Gerdhem P, Ivaska KK, Isaksson A, Pettersson K, Väänänen HK, Obrant KJ, Akesson K. Associations between homocysteine, bone turnover, BMD, mortality, and fracture risk in elderly women. *J Bone Miner Res* 2007, 22: 127–134.
- Leboff MS, Narwekar R, LaCroix A, Wu L, Jackson R, Lee J, Bauer DC, *et al.* Homocysteine levels and risk of hip fracture in postmenopausal women. *J Clin Endocrinol Metab* 2009, 94: 1207–1213.
- Claes L, Schmalenbach J, Herrmann M, Olkú I, Garcia P, Histing T, Obeid R, *et al.* Hyperhomocysteinemia is associated with impaired fracture healing in mice. *Calcif Tissue Int* 2009, 85: 17–21.
- Koh JM, Lee YS, Kim YS, Kim DJ, Kim HH, Park JY, Lee KU, *et al.* Homocysteine enhances bone resorption by stimulation of osteoclast formation and activity through increased intracellular ROS generation. *J Bone Miner Res* 2006, 21: 1003–1011.
- Herrmann M, Tami A, Wildemann B, Wolny M, Wagner A, Schorr H, Taban-Shomal O, *et al.* Hyperhomocysteinemia induces a tissue specific accumulation of homocysteine in bone by collagen binding and adversely affects bone. *Bone* 2009, 44: 467–475.
- Thaler R, Agsten M, Spitzer S, Paschalis EP, Karlic H, Klaushofer K, Varga F. Homocysteine suppresses the expression of the collagen cross-linker lysyl oxidase involving IL-6, Fli1, and epigenetic DNA methylation. *J Biol Chem* 2011, 286: 5578–5588.
- Cagnacci A, Baldassari F, Rivolta G, Arangino S, Volpe A. Relation of homocysteine, folate, and vitamin B12 to bone mineral density of postmenopausal women. *Bone* 2003, 34: 956–959.
- El Maghraoui A, Ghazali I, Mounach A, Rezaei A, Oumghar K, Achemlal L, Bezza A, *et al.* Homocysteine, folate, and vitamin B12 levels and vertebral fracture risk in postmenopausal women. *J Clin Densitom* 2012, 15: 328–333.

30. White J, Morton V, Whittington K, Wilson G, Tucci M, Benghuzzi H. The effects of dematerialized bone matrix proteins, estrogen, and an antagonist to neuropeptide  $\gamma$  on osteoblast and osteoclast viability and function related to osteoporosis. *Biomed Sci Instrum* 2012, 48: 478–484.
31. Clowes JA, Eghbali-Fatourehchi GZ, McCready L, Oursler MJ, Khosla S, Riggs BL. Estrogen action on bone marrow osteoclast lineage cells of postmenopausal women *in vivo*. *Osteoporos Int* 2009, 20: 761–769.
32. Luo CY, Wang L, Sun C, Li DJ. Estrogen enhances the functions of CD4(+) CD25(+)Foxp3(+) regulatory T cells that suppress osteoclast differentiation and bone resorption *in vitro*. *Cell Mol Immunol* 2011, 8: 50–58.
33. Silverman SL, Christiansen C, Genant HK, Vukicevic S, Zanchetta JR, de Villiers TJ, Constantine GD, *et al*. Efficacy of bazedoxifene in reducing new vertebral fracture risk in postmenopausal women with osteoporosis: results from a 3-year, randomized, placebo-, and active-controlled clinical trial. *J Bone Miner Res* 2008, 23: 1923–1934.
34. Silverman SL, Chines AA, Kendler DL, Kung AW, Tegljbærg CS, Felsenberg D, Mairon N. Sustained efficacy and safety of bazedoxifene in preventing fractures in postmenopausal women with osteoporosis: results of a 5-year, randomized, placebo-controlled study. *Osteoporos Int* 2012, 23: 351–363.
35. Vijayan V, Khandelwal M, Mangani K, Singh RR, Gupta S, Suroli A. Homocysteine alters the osteoprotegerin/RANKL system in the osteoblast to promote bone loss: pivotal role of the redox regulator forkhead O1. *Free Radic Biol Med* 2013, 61: 72–84.
36. Katso R, Okkenhaug K, Ahmadi K, White S, Waterfield MD. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu Rev Cell Dev Biol* 2001, 17: 615–675.
37. Kinjo M, Minami A, Iwasaki N. Siglec-15 regulates osteoclast differentiation by modulating RANKL-induced phosphatidylinositol 3-kinase/AKT and Erk pathways in association with signaling adaptor DAP12. *J Bone Miner Res* 2013, 28: 2463–2475.
38. Moon JB, Kim JH, Kim K, Youn BU, Ko A, Lee SY, Kim N. AKT induces osteoclast differentiation through regulating the GSK3 $\beta$ /NFATc1 signaling cascade. *J Immunol* 2012, 188: 163–169.
39. Cicek M, Vrabel A, Sturchio C, Pederson L, Hawse JR, Subramaniam M, Spelsberg TC, *et al*. TGF- $\beta$  inducible early gene 1 regulates osteoclast differentiation and survival by mediating the NFATc1, AKT, and MEK/ERK signaling pathways. *PLoS One* 2011, 6: e17522.
40. Xiao-Hong D, Chang-Qin X, Jian-Hua H, Wen-Jiang Z, Bing S. Icaritin delays homocysteine-induced endothelial cellular senescence involving activation of the PI3K/AKT-eNOS signaling pathway. *Pharm Biol* 2013, 51: 433–440.
41. Lee SJ, Lee YS, Seo KW, Bae JU, Kim GH, Park SY, Kim CD. Homocysteine enhances MMP-9 production in murine macrophages via ERK and AKT signaling pathways. *Toxicol Appl Pharmacol* 2012, 260: 89–94.
42. Yan TT, Li Q, Zhang XH, Wu WK, Sun J, Li L, Zhang Q, *et al*. Homocysteine impaired endothelial function through compromised vascular endothelial growth factor/AKT/endothelial nitric oxide synthase signaling. *Clin Exp Pharmacol Physiol* 2010, 37: 1071–1077.
43. Li XQ, Cao W, Li T, Zeng AG, Hao LL, Zhang XN, Mei QB. Amlodipine inhibits TNF- $\alpha$  production and attenuates cardiac dysfunction induced by lipopolysaccharide involving PI3K/AKT pathway. *Int Immunopharmacol* 2009, 9: 1032–1041.
44. Shastry S, James LR. Homocysteine-induced macrophage inflammatory protein-2 production by glomerular mesangial cells is mediated by PI3 Kinase and p38 MAPK. *J Inflamm (Lond)* 2009, 6: 27.
45. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 2001, 22: 153–183.
46. Chaves de Souza JA, Nogueira AV, Chaves de Souza PP, Kim YJ, Silva Lobo C, Pimentel Lopes de Oliveira GJ, Cirelli JA, *et al*. SOCS3 expression correlates with severity of inflammation, expression of proinflammatory cytokines, and activation of STAT3 and p38 MAPK in LPS-induced inflammation *in vivo*. *Mediators Inflamm* 2013, 2013: 650812.
47. Lin MC, Pan CY, Hui CF, Chen JY, Wu JL. Shrimp anti-lipopolysaccharide factor (SALF), an antimicrobial peptide, inhibits proinflammatory cytokine expressions through the MAPK and NF- $\kappa$ B pathways in LPS-induced HeLa cells. *Peptides* 2013, 40: 42–48.
48. Dai JN, Zong Y, Zhong LM, Li YM, Zhang W, Bian LG, Ai QL, *et al*. Gastrodin inhibits expression of inducible NO synthase, cyclooxygenase-2 and proinflammatory cytokines in cultured LPS-stimulated microglia via MAPK pathways. *PLoS One* 2011, 6: e21891.
49. Weitzmann MN. The role of inflammatory cytokines, the RANKL/OPG Axis, and the immunoskeletal interface in physiological bone turnover and osteoporosis. *Scientifica (Cairo)* 2013, 2013: 125705.
50. Lee BJ, Huang YC, Chen SJ, Lin PT. Effects of coenzyme Q10 supplementation on inflammatory markers (high-sensitivity C-reactive protein, interleukin-6, and homocysteine) in patients with coronary artery disease. *Nutrition* 2012, 28: 767–772.
51. Wang D, Wang H, Luo P, Hwang A, Sun D, Wang Y, Zhang Z, *et al*. Effects of ghrelin on homocysteine-induced dysfunction and inflammatory response in rat cardiac microvascular endothelial cells. *Cell Biol Int* 2012, 36: 511–517.
52. da Cunha AA, Ferreira AG, Wyse AT. Increased inflammatory markers in brain and blood of rats subjected to acute homocysteine administration. *Metab Brain Dis* 2010, 25: 199–206.
53. Aksamitiene E, Kiyatkin A, Kholodenko BN. Cross-talk between mitogenicRas/MAPK and survival PI3K/AKT pathways: a fine balance. *Biochem Soc Trans* 2012, 40: 139–146.
54. Dent P. Crosstalk between ERK, AKT, and cell survival. *Cancer Biol Ther* 2014, 15: 245–246.