Reactive oxygen species induce the association of SHP-1 with c-Src and the oxidation of both to enhance osteoclast survival

Ke Ke,1 Ok-Joo Sul,1 Eun-Kyung Choi,1 Ali M. Safdar,1 Eun-Sook Kim,2 and Hye-Seon Choi1

1Department of Biological Sciences, University of Ulsan, Ulsan, South Korea; and 2Department of Endocrinology, Ulsan University Hospital, Ulsan, South Korea

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Ke K, Sul O, Choi E, Safdar AM, Kim E, Choi H. Reactive oxygen species induce the association of SHP-1 with c-Src and the oxidation of both to enhance osteoclast survival. Am J Physiol Endocrinol Metab 307: E61–E70, 2014. First published May 13, 2014; doi:10.1152/ajpendo.00044.2014.—Loss of ovarian function causes oxidative stress as well as bone loss. We hypothesized that reactive oxygen species (ROS) induced by the failure of ovarian function are responsible for the bone loss by increasing the number of osteoclasts (OC). We found that ROS enhanced OC survival via Src homology 2 domain-containing phosphatase-1 (SHP-1), c-Src, Akt, and ERK. ROS induced the association of SHP-1 with c-Src as well as the oxidation of c-Src and SHP-1. This resulted in inactivation of SHP-1 and activation of c-Src via phosphorylation of Tyr416. Knockdown of c-Src or SHP-1 abolished the effect of ROS on OC survival. Moreover, downregulation of SHP-1 upregulated activation of c-Src, Akt, and ERK in the absence of any stimulus, suggesting that inactivation of SHP-1 is required for OC survival. We demonstrated that the association and oxidation of c-Src and SHP-1 by ROS are key steps in enhancing OC survival, which are responsible for increased bone loss when ovarian function ceases.

POSTMENOPAUSAL OSTEOPOROSIS IS CAUSED by abnormally high bone turnover with enhanced bone resorption. Loss of ovarian function induces an increase in the number of active osteoclasts (OC), which is responsible for postmenopausal bone loss (31). OC are multinucleated cells derived from hematopoietic cells, and the pool size of active OC determines the extent of bone resorption. A variety of cytokines, hormones, and growth factors control physiological bone resorption by increasing the formation of new OC or the lifespan of mature OC through antiapoptotic activity (32). Several epidemiological studies have demonstrated a decrease in plasma antioxidants in osteoporotic women (28), an association of antioxidant intake with increased bone mineral density in postmenopausal women (17), and a risk of hip fracture in smokers (29), suggesting that reactive oxygen species (ROS) play a critical role in the pathology of bone loss. Upon cytokine stimulation, OC express NADPH oxidase, which generates ROS (42). Receptor activator of nuclear factor-kB ligand (RANKL), an essential cytokine for OC differentiation, stimulates to produce ROS, which serve to activate their own signal transduction pathway (20, 25). Homocysteine, an intracellular ROS generator, increases the number and activity of OC formed upon RANKL stimulation (21), supporting a role of ROS in increasing OC levels. In addition, H2O2 alone stimulates osteoclastic bone resorption and cell motility in osteoclast cultures (5). However, it is not clear which molecule is the direct target of ROS in OC.

ROS play a role in tuning the balance between the activity of protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs) (14, 44). PTPs are reported to be redox sensitive, with the oxidation-sensitive active site cysteine (Cys) (44). Oxidative inactivation of PTPs is a critical step in the transmission of the PTK signals, resulting in favoring the balance toward phosphorylation. ROS also elevate protein tyrosine (Tyr) phosphorylation by specific oxidation of the Cys residue, which results in a conformational change leading to activation of PTK. ROS produced when integrin is activated has been demonstrated to stimulate the nonreceptor tyrosine kinase c-Src (14). Based on the ability of loss-of-function mutations, c-Src is required for normal OC function. The lack of c-Src produces an osteopetrotic skeletal phenotype due to impaired OC, suggesting a critical role of c-Src in OC function (7). Once recruited to activated integrins in OC, c-Src activates Cbl and phosphoinositide 3-kinase (PI3K) (18). ROS generated upon integrin stimulation lead to phosphorylation of Tyr616 and dephosphorylation of Tyr527 as a consequence of the oxidation of Cys245 and Cys487 of c-Src (14).

We hypothesized that ROS induced by loss of ovarian function might result in bone loss by increasing numbers of OC via redox-mediated modulation of PTK and PTP. We reported that ROS alone induced the association of PTK, c-Src and PTP, Src homology 2 domain-containing phosphatase-1 (SHP-1), and the oxidation of both molecules, leading to enhanced OC survival.

MATERIALS AND METHODS

Subjects. This study was performed with a group of 36 Korean women recruited at the Department of Endocrinology, Ulsan University Hospital, Ulsan, South Korea, from October 2012 to March 2013. The study was approved by the Institutional Review Board of the University Hospital (UUH-IRB-11-048), and all subjects provided signed informed consent. Subjects in this study were divided into two age groups, one of 20–30 yr (n = 15) and another of subjects >60 yr of age (n = 21) who were postmenopausal women. All age >60 subjects were in the presence of chronic disease, but they had been stable for ≥3 mo with no change in medication. All the subjects reported that their body weights had been stable for ≥3 mo, and none were taking any antiosteoporotic medication. Inclusion criteria were absence of systemic disease and the absence of clinical symptoms and signs of infection in the previous month. Peripheral blood was collected in 5-ml serum separation tubes. Serum was separated by centrifugation of whole blood at 1,400 g for 10 min. The aliquots of serum were stored at −80°C.

Reagents and antibodies. Recombinant mouse macrophage colony-stimulating factor (M-CSF) and RANKL were obtained from R & D Systems (Minneapolis, MN). N-(biotinoyl)-N’-(iodoacetyl)ethylenediamine (BIAM) was from Molecular Probes (Carlsbad, CA), and...
hormone radish peroxidase (HRP)-streptavidin was from Santa Cruz Biotechnology (Santa Cruz, CA).

**Animals and OC formation.** Ten-week-old female C57BL/6J mice were subjected to sham (n = 6) or ovariectomy (OVX) surgery (n = 6) for 8 wk. The mice were housed in the specific-pathogen-free animal facility of the Immunomodulation Research Center. Animal experimentation protocols were approved by the Institutional Animal Care and Use Committee of the University of Ulsan Immunomodulation Research Center (nos. 2011-006 and 2012-002). For three-dimensional visualization of the architecture of the long bone, femurs were scanned in a high-resolution microcomputed tomography imaging system using the SkyScan 1072 system (SkyScan) with the setting at 6.9 μm effective detector pixel size and a threshold of 77–255 mg/cc. A 1.5-mm-long region of the trabecular bone 0.2 mm below the distal growth plate of the femur was analyzed. Two-hundred fifty to 300 tomographic slices were acquired, and three-dimensional analyses were performed with CT volume software (version 1.11; SkyScan). The following serum markers were measured according to the manufacturer’s recommendations (Immunodiagnostic Systems, Jena, Germany): collagen type I fragments (CTX-1) by RatLaps EIA (mouse) and Serum CrossLaps ELISA (human) by competitive EIA. Serum H2O2 was determined with an Amplex Red hydrogen peroxide/H2O2 or M-CSF (30 ng/ml) for 15 min. The medium was removed, and the cells were frozen rapidly in liquid nitrogen. The frozen cells were transferred to 100 μM BIAM-containing lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 10 μg/ml aprotinin, and 10 μg/ml leupeptin; the buffer was rendered free of oxygen by bubbling nitrogen gas at a low flow rate for 20 min). After sonication in a bath sonicator for three periods of 1 min each, the lysate was then clarified by centrifugation and subjected to immunoprecipitation with 2 μg of c-Src-specific antibody (Ab). Immunocomplexes labeled with BIAM were detected with HRP-conjugated streptavidin and developed with an enhanced chemiluminescence kit.

**Detection of oxidized c-Src and SHP-1 by carboxymethylation.** Mature OC cells were cytokine-starved for 6 h and then exposed to H2O2 or M-CSF (30 ng/ml) for 15 min. The medium was removed, and the cells were frozen rapidly in liquid nitrogen. The frozen cells were transferred to 100 μM BIAM-containing lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 10 μg/ml aprotinin, and 10 μg/ml leupeptin; the buffer was rendered free of oxygen by bubbling nitrogen gas at a low flow rate for 20 min). After sonication in a bath sonicator for three periods of 1 min each, the lysate was then clarified by centrifugation and subjected to immunoprecipitation with 2 μg of c-Src-specific antibody (Ab). Immunocomplexes labeled with BIAM were detected with HRP-conjugated streptavidin and developed with an enhanced chemiluminescence kit.

**RESULTS**

Loss of ovarian function induces bone loss and increased oxidative stress. To investigate whether loss of ovarian function is associated with oxidative stress, we measured serum ROS in mice 8 wk after OVX or sham surgery. As shown in Fig. 1A, serum H2O2 level was increased by OVX. As an evaluation of bone loss, serum CTX-1, a marker of in vivo bone resorption, was increased (Fig. 1B), and an elevated number of TRAP-positive MNC was found in bone marrow cells of OVX mice upon M-CSF and RANKL stimulation compared with that of sham mice (Fig. 1C). In addition, the OC from OVX mice was larger with more nuclei, which are responsible for bone resorption, compared with that from sham mice (Fig. 1, D and E). These data suggested that oxidative stress may be associated with the modulation of number and activity of OC. To evaluate the relationship between oxidative stress and bone loss in humans, serum ROS and CTX-1 were determined in two groups of women, one between the ages of 20 and 30 yr and the other age >60 yr. As shown in Fig. 2A, serum levels of H2O2 and CTX-1 in these women were higher than in younger women (20–30 yr). In addition, serum levels for...
of H₂O₂ and CTX-1 were positively correlated (Fig. 2B). These results indicated that the bone loss following cessation of ovarian function is associated with induced oxidative stress and is at least partly due to an increased number of OC.

**ROS increase OC survival.** Differentiated OC undergo an apoptosis upon removal of survival factors such as cytokines (22). To investigate whether ROS act as a survival factor for mature OC, we examined the effect of ROS on OC survival by counting TRAP-positive MNC. As shown in Fig. 3A, the number of OC surviving after removal of cytokines for 6 h was increased by exogenous H₂O₂, although it was lower than that obtained with the survival cytokine M-CSF. OC survival was maximal at 50 μM H₂O₂ and decreased at higher doses, indicative of a cytotoxic effect. The increased OC survival was abolished by addition of a general antioxidant, N-acetyl-l-cysteine (NAC), whereas homocysteine, an intracellular ROS generator, like ROS, increased OC survival (Fig. 3B). To see whether the enhanced OC survival was due to an antiapoptotic effect, we measured the incorporation of annexin V by mature OCs. The fraction of annexin V-positive cells was decreased dramatically by treatment of H₂O₂, whereas addition of NAC reversed the effect of H₂O₂ (Fig. 3C). Next, we examined whether ROS affects the resorptive capacity of OC. To test this idea, we assessed the ability of mature OC to form pits in dentine slices. As shown in Fig. 3D, mature OC treated with H₂O₂ in the presence of M-CSF and RANKL had significantly larger pit areas than those without H₂O₂. Cotreatment of NAC dramatically decreased bone resorption enhanced by H₂O₂. However, cells exposed to H₂O₂ alone formed few pits.

**ROS stimulate the association of SHP-1 with c-Src and the oxidation of both molecules.** Since activation of ERK and Akt is critical for the survival of OC (26), and c-Src mediates Akt activation (18), we determined the effect of pharmacological inhibitors of these enzymes on the increased OC survival induced by ROS. As shown in Fig. 4A, inhibition of Akt, ERK, or c-Src significantly attenuated the stimulatory effect of ROS.
on OC survival, suggesting that activation of these enzymes is required for OC survival in response to ROS.

Next, we examined whether ROS activated ERK and Akt. As shown in Fig. 4B, phosphorylation of ERK was detectable 5 and 15 min after M-CSF stimulation as a positive control. ROS alone also induced phosphorylation of ERK and Akt, but with a delay compared with M-CSF.

Since c-Src is a major target of ROS (14), we supposed that redox-regulated modification of c-Src might be associated with the ROS-mediated OC survival. To detect any oxidation of c-Src stimulated by ROS, we labeled OC with BIAM, a sulfhydryl-modifying reagent that selectively targets the thiolic form of reduced Cys residues. Mature OC were exposed to sulfhydryl-modifying reagent that selectively targets the thiolate form of reduced Cys residues. Mature OC were exposed to exogenous H2O2, lysed, and labeled with BIAM. c-Src was immunoprecipitated, and the biotinylated and reduced fractions of c-Src were reacted with HRP-streptavidin. As shown in Fig. 4C, c-Src was oxidized upon ROS stimulation, as it was by M-CSF, whereas the addition of NAC prevented the effect. To assess the effect of c-Src oxidation on its enzymatic activity, we measured ROS-dependent phosphorylation of Tyr416 of c-Src, which is believed to activate the enzyme (14). As shown in Fig. 4D, H2O2 induced phosphorylation of Tyr416 of c-Src, whereas NAC treatment decreased it dramatically. We also observed degradation of Bim along with the activation of c-Src kinase (Fig. 4D). Together, these findings imply that oxidant-enhanced activation of c-Src Tyr kinase results in elevated OC survival by decreasing the level of the proapoptotic protein Bim. The effect of c-Src on OC survival was confirmed by knockdown; downregulation of c-Src by si-cSrc completely eliminated the stimulatory effect of ROS on OC survival (Fig. 5A).

Sustained activation of protein kinase by ROS is dependent on oxidation of the corresponding phosphatase (19). SHP-1 is shown to negatively regulate c-Src kinase activity in thymocytes (27) and bind to the Tyr-phosphorylated protein via its SH2 domain (34). These findings led us to investigate the potential role of SHP-1 in ROS-induced OC survival. We examined the effect of ROS on OC survival with a selective pharmacological inhibitor of SHP-1, sodium stibogluconate. Inhibition of SHP-1 upregulated the basal level of OC survival but reduced the effect of ROS on OC survival (Fig. 4A). We also investigated whether ROS act directly to oxidize SHP-1. ROS induced the oxidation of SHP-1, as did M-CSF, whereas the addition of NAC attenuated it (Fig. 4C, bottom), implying that oxidation of SHP-1 is required for OC survival. Next, we showed that c-Src became associated with SHP-1 upon ROS stimulation, as shown by coimmunoprecipitation with SHP-1, followed by binding of c-Src, whereas no association of these proteins was detected in vehicle-treated OCs (Fig. 4E, top 2 blots). The association of c-Src and SHP-1 was confirmed by coimmunoprecipitation with c-Src, followed by binding of SHP-1 (Fig. 4E, bottom 2 blots). The association between SHP-1 and c-Src was reduced by the addition of NAC together with ROS, suggesting a direct role of ROS in the complex formation. A similar pattern was observed with M-CSF treatment. To confirm the effect of SHP-1 on ROS-induced OC survival, SHP-1 was downregulated using siSHP-1. As shown in Fig. 5B, knockdown of SHP-1 elevated OC survival even without any stimulus, indicating the negative role of SHP-1 on OC survival. In addition, enhanced OC survival by ROS was abolished completely compared with scRNA. Next, we examined whether SHP-1 affected the activation of c-Src, ERK, and Akt using siSHP-1. Downregulation of SHP-1 elevated the phosphorylation of c-Src at Tyr416 before stimulation with ROS, and no further increase was observed upon ROS stimulation (Fig. 5C). A similar pattern was observed for the activation of Akt and ERK (Fig. 5C).

**DISCUSSION**

The current study contributes to the view that ROS are key factors promoting OC survival. How do ROS signal to increase OC survival? It is known that RANKL-receptor-mediated ROS generation acts as a survival signaling for activation of downstream molecules (16). ROS have been shown to stimulate kinase-mediated phosphorylation as well as to inactivate phosphatase activities (14, 15, 38, 44). Therefore, a potential mechanism for ROS-mediated cell signaling is through the
Fig. 3. ROS increase OC survival and elevate OC activity induced by M-CSF and RANKL. A–C: mature OC were stimulated with H$_2$O$_2$ (0, 20, 50, and 100 μM) with or without N-acetyl-L-cysteine (NAC; 3 mM), M-CSF (30 ng/ml), or homocysteine (HC; 100 μM) for 6 h to measure TRAP-positive MNC (A and B) and annexin V-positive cells (C). A significant difference was observed after treatment with NAC in the presence of H$_2$O$_2$ (50 μM; B and C). D: mature OC were incubated on dentine slices with V and H$_2$O$_2$ (50 μM) in the absence or presence of M-CSF and RANKL for 1 day and stained with Mayer’s hematoxylin. Representative photos of resorption pits were taken (scale bar, 50 μm). Total area of resorption pits was determined with the ImageJ 1.37v program. Treatment of H$_2$O$_2$ and co-treatment of NAC significantly increased and decreased resorption compared with no treatment and H$_2$O$_2$ treatment, respectively, in the presence of M-CSF and RANKL. Similar results were obtained in 3 independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with vehicle (V)-treated cells.
oxidation of susceptible Cys residues of target proteins to sulfonic acid derivatives or disulfides (38, 44).

We demonstrated that the PTK c-Src and the PTP SHP-1 underwent redox regulation during OC survival, leading to increased numbers of OC. In our data, the oxidation of c-Src in response to ROS resulted in enhanced activation of c-Src, as indicated by the phosphorylation of Tyr416 in OC. A similar pattern has been demonstrated in the case of ROS generated by stimulation of integrin during fibroblast adhesion and spreading. ROS-mediated oxidation of c-Src occurs together with phosphorylation of c-Src at Tyr416, which is the catalytically active form, suggesting a role of c-Src oxidation in kinase activation (14).

The oncogenic activity of constitutively active mutants suggests c-Src as a regulator for cell growth and proliferation. However, c-Src is highly expressed in differentiated cells such as OC, implying that c-Src plays physiological roles that are not positively related to cell growth. The role of c-Src in OC survival is controversial. The absence of c-Src reduces bone resorption, but the number of OC in bones of c-Src-deficient mice is much higher than in normal mice (7), implying that OC differentiation and survival is elevated in the lack of c-Src in vivo. However, our results demonstrated that knockdown of c-Src by siRNA or inhibition of c-Src by a chemical inhibitor reduced the effect of ROS on OC survival, suggesting that activated c-Src plays a role in OC survival. c-Src plays key roles as a PTK that phosphorylates some components of the signaling complex as well as an adaptor protein that links other signaling proteins. It is plausible that catalytically active c-Src promotes the dissociation of the ubiquitin-containing enzyme from Cbl (45), resulting in binding of another ubiquitin-charged protein to facilitate the process. Actually, deficiency of Cbl significantly increases the level of proapoptotic Bim, resulting in impaired OC survival (37). In agreement with these, the kinase-inactive c-Src mutant substantially rescues OC-mediated bone resorption in c-Src-deficient mutants but aggravates osteopetrosis by inducing OC to undergo apoptosis (40). Since the removal of ROS by the antioxidant NAC reduced activation of c-Src, we propose that redox regulation is a central feature of c-Src function in response to ROS.

In the present study, ROS induced the oxidation of SHP-1 as well as the association of SHP-1 with c-Src, concurrent with c-Src activation. SHP-1 is a cytosolic PTP that is expressed predominantly and recruited upon RANKL stimulation in mac-
Since SHP-1 is not associated with any receptor, SHP-1 is not likely to interact constitutively with c-Src. Instead, it may facilitate to be associated with c-Src upon stimulation. In contrast to c-Src, it is likely that oxidation of SHP-1 decreases its activity. Knockdown of SHP-1 by siRNA resulted in elevated OC survival without any stimulus with enhanced phosphorylation of Tyr416, indicative of activation of c-Src kinase. The effect of exogenous H2O2 on OC survival was decreased by downregulation of SHP-1 by siRNA or a pharmacological inhibitor. Those findings suggested that the stimulatory effect of ROS could be due to oxidative inactivation of SHP-1. Genetic mutations of SHP-1, as shown in mev mice, abolish the phosphatase activity of SHP-1, which is equivalent to the outcome of oxidative stress and correlates with osteopenic phenotypes (2), suggesting that bone loss is associated with inactivation of SHP-1. Indeed, SHP-1 is a negative regulator in various signaling systems. It is sensitive to oxidation due to the two Cys residues in its catalytic residue (1). Since reversible redox regulation of Cys-based PTPs also plays an important role in setting appropriate levels of Tyr phosphorylation (34), ROS may turn on intracellular protein kinases by the transient inactivation of PTP due to reversible oxidation of the active site Cys of PTP (39). In agreement with our results, SHP-1 deficiency led to hyperactivation of c-Src in thymocytes (27), supporting the idea that PTP inactivates the PTK c-Src. Taken together, the evidence suggests that ROS contribute to inactivation of SHP-1 by oxidation, resulting in activation of c-Src and increased OC survival.

We also demonstrated that the activation of ERK and Akt is involved in ROS-stimulated OC survival. Activation of ERK
and Akt has also been associated with cytokine-induced OC survival (26). Our data showed that knockdown of SHP-1 impaired subsequent further activation of Akt and ERK by ROS. Since SHP-1 is reported to regulate phosphorylation of PI3K and its activity in T cells (10), the effect of SHP-1 on Akt, a serine/threonine kinase, could be mediated by PI3K. In response to RANKL, SHP-1 activates PI3K, leading to Akt phosphorylation during osteoclastogenesis (46). An inverse association between ERK and SHP-1 has been reported in podocytes (11) and mast cells (33). It is likely that upstream PTK activation by SHP-1 is required for ERK activation. Knockdown of SHP-1 resulted in activation of Akt and ERK without any stimuli, suggesting that SHP-1 is an upstream stimulatory effector of ROS in the activation axis of ERK and Akt in OC.

Our data also showed that exogenous ROS enhanced OC activity by increasing resorption pits induced by M-CSF or RANKL, possibly via fortifying signaling pathways of resorptive cytokines (13, 16, 21, 23). ROS have been implicated in bone loss in humans and rodents. Parathyroid hormone-derived ROS stimulate bone resorption in vivo and in vitro (13), and a strong correlation has been observed between oxidative stress and bone mineral density (BMD) in women (4). In mice, OVX causes a significant fall of glutathione, whereas antioxidants such as ascorbic acid and NAC protect against the OVX-induced bone loss (23, 47), suggesting a role of ROS in bone loss. Consistent with these findings, we demonstrated that OVX induces oxidative stress along with bone loss by increasing the number and activity of OC in mice. In addition, a positive correlation between serum ROS and CTX-1 was observed in humans, supporting the findings in mice. In addition to H2O2, peroxynitrite and hydroxyl ions derived from nitric oxide (6) that is generated in OC have been reported to affect bone, although that is not clear. A nitric oxide-derived anion, peroxynitrite has been inversely associated with BMD in idiopathic hypercalciurea patients (8), but it inhibits bone resorption in vitro (30). Heated natural bone has a higher hydroxyl ion with a lower resorption activity of OC compared with untreated bone, implying a protective effect of hydroxyl ions on bone (36). Although we focused the effect of ROS on OC to explain bone loss, it is worth mentioning the role of ROS on osteoblasts. Increased intracellular ROS stimulate RANKL production (3), induce apoptosis of osteoblast (41), and reduce the viability of osteoblasts (35), resulting in decreased bone formation. Controversially, H2O2 stimulates proliferation of periodontal ligament fibroblasts and increases their differentiation into osteoblasts (9).

Taken together, our findings indicate that loss of ovarian function-induced bone loss is associated with increased ROS and elevated numbers and activity of OC in vivo. ROS induced the association of SHP-1 with c-Src and oxidized both molecules, resulting in inactivation of SHP-1 and activation of c-Src, which resulted in elevated OC survival. Impaired SHP-1 induced activation of c-Src, ERK, and Akt, leading to increased OC survival in vitro (Fig. 6). We propose that an in vivo redox switch acts to enhance OC survival.

Loss of ovarian function-induced bone loss is associated with increased ROS and elevated numbers and activity of OC in vivo. ROS induced the association of SHP-1 with c-Src and oxidized both molecules, resulting in inactivation of SHP-1 and activation of c-Src, resulting in enhanced OC survival. Impaired SHP-1 and c-Src attenuated the effect of ROS on OC survival. Downregulation of SHP-1 induced activation of ERK, Akt, and c-Src, implying SHP-1 as an upstream molecule of these. Our observations open new avenues for antioxidant-based pharmaceutical intervention to prevent bone loss, suggesting that an in vivo redox switch acts to enhance OC survival and organize the cytoskeleton for OC function.
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