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ORIGINAL PAPER

Involvement of receptor-interacting protein 140 in estrogen-mediated osteoclasts differentiation, apoptosis, and bone resorption

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Abstract Estrogen withdrawal following menopause results in an increase of osteoclasts formation and bone resorption, which is one of the most important mechanisms of postmenopausal osteoporosis. Recently, growing evidence has suggested that receptor-interacting protein 140 was implicated in estrogen-regulated metabolic disease, including fat metabolism and lipid metabolism. However, little is known regarding the role of receptor-interacting protein 140 in the regulation of bone metabolic by estrogen. In the present study, Western blotting disclosed that estrogen brings a significant increasing expression of receptor-interacting protein 140 in osteoclasts, but not in osteoblasts and bone marrow mesenchymal stem cells. Furthermore, analysis of TRAP staining and bone resorption assay showed that depletion of receptor-interacting protein 140 could significantly alleviate the inhibitory effects of estrogen on osteoclasts formation and bone resorption activity. Moreover, estrogen could induce osteoclasts apoptosis by increasing receptor-interacting protein 140 expression through the Fas/FasL pathway. Taken together, receptor-interacting protein 140 might be a critical player in estrogen-mediated osteoclastogenesis and bone resorption.

Keywords RIP140 · Estrogen · Osteoporosis · Osteoclast

Introduction

Bone metabolism is regulated by continuous remodeling through the maintenance in the reciprocal cycles of resorption and formation [1]. Bone remodeling is maintained by a well-organized balance between the destruction of old bone by osteoclasts and building by osteoblasts [2, 3]. However, the balance between bone formation and bone resorption is not preserved in pathological conditions. Thus, a greater rate of bone resorption than bone formation will result in osteoporosis, which is the most common metabolic bone disease. Loss of ovarian function at menopause is the leading cause of the development of osteoporosis in women [4, 5]. Therefore, acknowledgement mechanisms for bone turnover imbalance induced by estrogen depletion play a crucial role in research on bone metabolism.

Estrogen deficiency has been found to be associated with high bone turnover, characterized by increased osteoclasts activity leading to enhanced bone resorption [6]. The osteoclasts derive from hematopoietic cells of the monocyte-macrophage lineage, exposed to macrophage-colony stimulating factor (MSC-F) and receptor activator of nuclear factor B (RANK) ligand (RANKL), and undergo fusion and differentiation, resulting in large multinucleated cells [7]. The inhibition of osteoclast formation and bone resorption and promotion of osteoclasts apoptosis by

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estrogen plays a critical role in estrogen protecting from bone loss [8, 9]. However, it is not fully understood how estrogen regulates osteoclasts formation, apoptosis, and bone resorption.

The transcription cofactor receptor-interacting protein 140 (RIP140) is widely expressed and regulates several physiological responses, including metabolism, inflammation, and cell fate determination. The alternation of RIP140 expression and function will result in several related diseases [10–13]. RIP140 has been shown to interact with many proteins, including most nuclear receptors (NRs), transcription factors, and histone modifying enzymes. RIP140 is remarkable in its ability to function either as a coactivator or a corepressor following its recruitment to target genes [14]. Previous studies showed that RIP140 was involved in the transcriptional regulation of estrogen signaling. RIP140 could positively regulate estrogen-related receptor (ERR) transactivation when the receptors are recruited to target promoters through interaction with the Sp1 transcription factor and this effect could involve titration of histone deacetylases [15]. Furthermore, RIP140 was also involved in estrogen-regulated metabolic disease, including fat metabolism and lipid metabolism [16, 17]. Additionally, RIP140 plays a crucial role in estrogen-mediated cells differentiation, such as adipogenesis and N2a cell neuronal differentiation [17]. However, it is unclear whether RIP140 may play an important role in the regulation of bone metabolism and osteoclastogenesis by estrogen.

Here, we demonstrated that RIP140 was required for estrogen-mediated osteoclasts formation, apoptosis, and bone resorption. Our results showed that the expression of RIP140 was significantly down-regulated in ovariectomized (OVX) mice. Furthermore, estrogen greatly increased the expression of RIP140 in osteoclastogenesis. Analysis of tartrate-resistant acid phosphatase staining (TRAP) staining and pit formation assay suggested that blocking of RIP140 significantly alleviated the inhibitory effects of estrogen on osteoclast formation and bone resorption activity. Furthermore, estrogen could induce osteoclast apoptosis by increasing receptor-interacting protein 140 expression through the Fas/FasL pathway. Taken together, we speculated that RIP 140 might play a central role during estrogen-controlled osteoclastogenesis, osteoclasts apoptosis, and bone resorption.

Materials and methods

Generation of osteoclasts, osteoblasts (OB), and bone marrow mesenchymal stem cells (BMSCs)

Mice preosteoclasts and osteoclasts were generated as previous reports [18]. Briefly, the femur and tibia were removed from mice. The cells derived from the bone marrow of the femur and tibias were cultured in alpha-minimum essential medium (α-MEM) (Invitrogen, Paisley, UK) containing 10 % fetal bovine serum and 10 ng/ml M-CSF. The following day, non-adherent cells were collected and seeded in six-well plates and treated with M-CSF (50 ng/ml). After 2 days, the non-adherent cells were washed out and the adherent cells were used as bone marrow-derived monocytes (BMMs). After 3 days, 10 ng/ml recombinant M-CSF and 50 ng/ml recombinant RANKL were added for 4 days to generate mature osteoclasts. Cytochemical staining of TRAP-positive cells was with the leukocyte acid phosphate assay kit (Sigma-Aldrich, MO, USA), in accordance with the manufacturer's protocol. Mature osteoclasts were treated with 10^{-8} M 17β-estradiol for 12 h to measure apoptosis.

Primary OB cells were isolated from calvaria of 1-day-old mice according to previous reports [19]. For experiments, primary OB cells were cultured in osteogenic media containing 50 μg/ml ascorbic acid (Sigma-Aldrich, MO, USA) and 10 mM β-glycerophosphate (Sigma-Aldrich, MO, USA) in addition to α-MEM/10 % fetal bovine serum to stimulate OB differentiation. BMSCs of mice were acquired from Cyagen Biosciences Inc (Santa Clara, CA). BMSCs of mice were cultured in α-MEM adding 10 % FBS and 100 μg/ml penicillin/streptomycin.

Animals and treatments

The generation of ovariectomized (OVX) mice was performed as previously described [19]. Briefly, 7-week-old female C57BL/6 mice were randomly divided into three groups: a SHAM group (in these mice, the ovaries were exposed and not removed), the OVX group, and OVX added estrogen group (after 1 week of recovery from surgery, the OVX mice were given daily sc injections of 17β-estradiol (20 μg/kg). The animals were given standard laboratory chow and water, and kept in an ambient room with the following conditions: temperature (22 ± 4 °C), humidity 60–65 %, and light (7:00–19:00). After anesthetization, mice were ovariectomized, or SHAM-operated. At 8 weeks after ovariectomy, intact tibias and femurs were removed from mice. All procedures involving mice were approved by Daqing Oilfield General Hospital Animal Study Committee.

Analysis of bone microarchitecture

Bone microarchitecture of the femur from SHAM, OVX, and OVX added estrogen was measured by a three-dimensional Micro-CT (μCT system, SkyScan 1076, Aartselaar, Belgium). The morphometric index of the bone region was determined from the microtomographic data

using a 3D image (NRecon, v.1.4.4.0; SkyScan). Hematoxylin-eosin staining was performed to observe the structure of bone trabecular as described elsewhere [20].

Lentiviral transduction of pre-osteoclasts cells and mature osteoclasts

The BMMs were seeded at a density of 2×10^5 into a six-well tissue culture plate in 3 ml of α-MEM supplemented with 10 % FBS and 100 μg/ml penicillin/streptomycin per well. When BMMs were grown to 80 % confluence, cells were infected by adding the RIP140 shRNA lentiviral particles (Santa Cruz, CA, USA) to the culture medium for 24 h. Furthermore, negative control (NC) cells were transduced with control shRNA lentiviral particles (Santa Cruz, CA, USA). Subsequently, the medium was replaced with fresh α-MEM containing 10 ng/ml M-CSF and 50 ng/ml RANKL with or without 10^{-8} M estrogen for primary culture.

Mature osteoclasts were infected with the FasL shRNA lentiviral particles (Santa Cruz, CA, USA) for 24 h. Furthermore, NC cells were transduced with control shRNA lentiviral particles (Santa Cruz, CA, USA). Subsequently, the medium was replaced with fresh α-MEM containing with 10^{-8} M estrogen for 12 h to determine the expression of RIP140.

Bone resorption assay

Bone resorptive activity was measured by directly measuring the number and size of pits formed by osteoclasts on bone slices. BMMs were cultured on bovine cortical bone slices in 24-well plates. After 6 days, the slices were placed for 10 min in 1 M NH_4OH and were sonicated to remove the cells. The cell-free slices were stained in 1 % toluidine blue in 1 % sodium borate for 1 min. The resorption pits appeared dark blue and were viewed by light microscopy. The percentage of pits area to a "random field of views" was counted.

Furthermore, mature osteoclast bone resorption activity was also measured with the OsteoLyse assay kit (Lonza, Allendale, NJ) according to the manufacturer's protocol. Briefly, BMMs infected with lentiviral shRNA-targeting RIP140 or control shRNA lentiviral particles were cultured in 96-well OsteoLyse plates at 50,000 cells/well in complete a-MEM. Then, BMMs were incubated with fresh α-MEM containing 10 ng/ml M-CSF and 50 ng/ml soluble RANKL with or without 10^{-8} M estrogen for primary culture. Six days later, culture medium was renewed and samples were collected after an additional 24 h of culture, and calcium release was measured by fluorescence.

Measurement of caspase-3 activity

Caspase-3 activity was measured by cleavage of chromogenic caspase substrates, Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Aspp-nitroanilide). The absorbance of the substrate was measured at 405 nm after cleavage by caspase-3. The optical density value at 405 nm was thus used as an indication for the amount of caspase-3. The protein samples were prepared as indicated in Western-blot analysis. Then, 50 mg of total proteins were added to the reaction buffer containing Ac-DEVD-pNA (2 mM), incubated for 2 h at 37 °C, and the absorbance of yellow pNA cleaved from its corresponding precursors was measured using a spectrometer at 405 nm. The specific caspase-3 activity, normalized for total proteins of cell, was then expressed as fold of the baseline caspase activity of control cell.

Quantitative real-time PCR (qRT-PCR)

Total cellular RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's recommended procedure. Total RNA (1 μg) was reverse-transcribed using oligo-(dT) primers and avian myeloblastosis virus (AMV) reverse transcriptase (Invitrogen). qRT-PCR was accomplished with the 7500 Fast Real-Time PCR System (Applied Biosystems). The primers of *RIP140, Fas, FasL* and *α-actin* as follows: *RIP140* (forward: 5′-GGTGCGCAGTT GACTGAGGAG-3′; reverse: 5′-CGGGCAGAGATGCACT GTTAAC -3′), *Fas* (forward: 5′- TATCAAGGAGGCCCATTTTGC-3′; reverse: 5′-TGTTTCCACTTCTAAACCATGCT-3′), *FasL* (forward: 5′- TCCGTGAGTTCACCAACCAAA-3′; reverse: 5′- GGGGGTTCCCTGTTAAATGGG-3′) and *α-actin* (forward: 5′-TGGCCTT AGGGTGCAGGG-3′; reverse: 5′-GTGGGCCGCTCTAGGCA CCA-3′). Gene expression of *RIP140*, *Fas*, and *FasL* were normalized for the expression of *α-actin*.

Western-blot analysis

Between 60 and 100 μg of protein from the nuclear extract, cytoplasmic lysate, or whole cell pellets was subjected to SDS-PAGE (6–12 % polyacrylamide gels). Separated proteins were blot transferred onto a nitrocellulose membrane. After blocking with 0.1 % Tween 20 and 5 % nonfat dry milk in Tris-buffered saline at room temperature for 1 h, the membrane was incubated overnight at 4 °C in one of the following primary antibodies: RIP140 (Santa Cruz, CA, USA), Bcl2 (Abcam, MA, USA), Bax (Abcam), and α-actin (Santa Cruz, CA, USA) as an internal control. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:2000) for 1 h and detected using the Enhanced Chemiluminescence Western blot System (Amersham Biosciences).

Statistics

Data are expressed as the mean ± SD for each group. SPSS statistical software was used for data analysis. Statistical analysis was performed with one-way ANOVA followed by Dunnett's test where appropriate. Differences were considered to be significant at $p \leq 0.05$.

Results

The upregulation of RIP140 expression by estrogen in osteoclasts but not OB and BMSCs

To examine whether RIP140 is implicated in the process of estrogen-regulated bone metabolic, we firstly observed the effects of estrogen on RIP140 expression in OB, BMSCs, and osteoclasts. No significant alternation of RIP140 protein and mRNA expression were presented in OB and BMSCs exposed to 10^{-8} M estrogen for 2 days (Fig. 1a–d). However, estrogen could remarkably increase RIP140 protein and mRNA expression in mature osteoclasts (Fig. 1e, f). Subsequently, we further observed the effect of estrogen on RIP140 expression in osteoclastogenesis. Our results showed that the protein and mRNA expression of RIP140 were decreased during osteoclasts differentiation process in a time-dependent manner, but the inhibition of RIP140 expression were obviously attenuated by estrogen (Fig. 1g, h). Taken together, these results suggested that RIP140 might be involved in estrogen-mediated osteoclastogenesis.

Effect of estrogen on RIP140 expression during osteoclast differentiation in vivo

OVX mice were applied to observe the effect of estrogen on RIP140 expression during osteoclasts differentiation in vivo. Micro-CT analysis revealed that bone mineral density (BMD) and bone volume/tissue volume (BV/TV) were decreased and trabecular separation/spacing (Tb.Sp) was increased in femurs from OVX mice compared to SHAM control, suggesting that estrogen deficiency greatly induced bone loss. However, bone loss was significantly alleviated in OVX mice injected with estrogen (Fig. 2a–d). Similar results were observed by hematoxylin-eosin staining (Fig. 2e). Subsequently, BMMs isolated from the SHAM, OVX, and OVX plus estrogen groups were cultured in osteoclasts differentiation medium containing 10 ng/ml M-CSF and 50 ng/ml RANKL for different time course to induce osteoclast differentiation. We found that RIP140 protein and mRNA expression were inhibited in a time-dependent manner during the osteoclast differentiation process in the OVX mice group compared with the SHAM control group. However, inhibition of RIP140 protein and mRNA expression were significantly attenuated in the OVX plus estrogen group (Fig. 2f, g).

Involvement of RIP140 in estrogen-inhibited osteoclast differentiation and bone resorption

To identify whether RIP140 was implicated in estrogen-inhibited osteoclast differentiation and bone resorption, we firstly silenced RIP140 expression using lentiviral constructs encoding shRNA-targeting RIP140 in osteoclast precursors. An obvious reduction of RIP140 protein and mRNA expression were presented in osteoclast precursors, demonstrating that shRNA-targeting RIP140 was effective (Fig. 3a, b). Subsequently, we found that RIP140 depletion could promote osteoclasts formation by TRAP staining. In addition, RIP140 silencing also markedly alleviated the inhibitory effects of estrogen on osteoclast formation (Fig. 3c, d). Osteoclast bone resorption in vitro was measured by release of labeled collagen and pit formation assay. The results showed that estrogen produced a significant decrease of bone resorption in osteoclasts, which was greatly alleviated by RIP140 deletion (Fig. 3e–g). Then we observed that effects of RIP140 depletion on the expression of osteoclasts marker gene including NFATc1, TRAP, and c-Fos in estrogen-inhibited osteoclastogenesis. We found that estrogen could decrease the expressions of NFATc1, TRAP, and c-Fos, while blocking of RIP140 could protect from downregulation of osteoclasts marker gene by estrogen (Fig. 3h–j). Taken together, these results showed that RIP140 might be required for estrogen-inhibited osteoclasts formation and bone resorption.

Estrogen induced osteoclasts apoptosis by increasing RIP140 expression through the Fas/FasL pathway

Caspase-3 activity assay was firstly used to examine the effect of estrogen on osteoclast apoptosis. Our results showed that caspase-3 activity was significantly increased in mature osteoclasts treated with 10^{-8} M estrogen for 12 h (Fig. 4a). Due to the fact that the Bcl-2/Bax ratio determines whether a cell will undergo apoptosis, the levels of Bcl-2 and Bax protein from osteoclasts were analyzed by Western blot. Estrogen caused a decrease in the levels of Bcl-2 protein and a significant increase in Bax protein. The Bcl-2/Bax ratio was decreased in osteoclasts treated with 10^{-8} M estrogen for 12 h (Fig. 4b). These results showed that estrogen could induce osteoclasts apoptosis. Subsequently, we found that RIP140 expression was

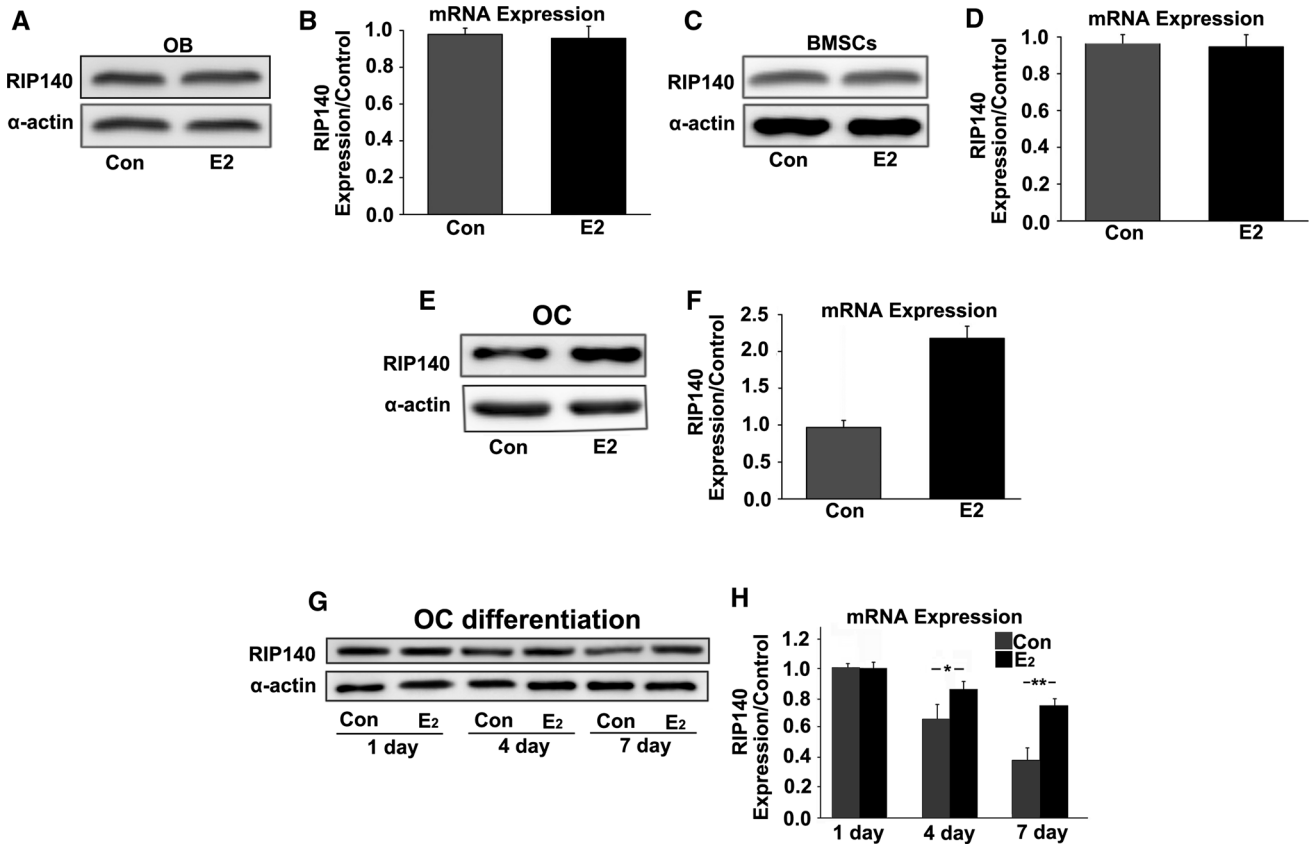

Fig. 1 The effects of estrogen on RIP140 expression in OB, BMSCs, and osteoclasts. **a**, **b** RIP140 protein and *mRNA* expression were respectively measured by Western blot and qRT-PCR in OB exposed to 10^{-8} M estrogen for 2 days. $n = 3$. **c**, **d** RIP140 protein and *mRNA* expression were respectively measured by Western blot and qRT-PCR in BMSCs exposed to 10^{-8} M estrogen for 2 days. $n = 3$. **e**, **f** RIP140 protein and *mRNA* expression were respectively measured by Western blot and qRT-PCR in mature osteoclasts exposed to 10^{-8} M estrogen for 2 days. $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$. **g**, **h** The effects of 10^{-8} M estrogen on RIP140 protein and *mRNA* expression were respectively measured by Western blot and qRT-PCR during osteoclasts differentiation process at different time course. $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$. *OB* osteoblasts, *BMSCs* bone marrow mesenchymal stem cells, *Con* control, *E2* 17β-estradiol

greatly increased in estrogen-induced osteoclasts apoptosis (Fig. 4c, d). To further examine whether RIP140 was involved in estrogen-induced osteoclasts apoptosis, we silenced RIP140 expression using lentiviral constructs encoding shRNA-targeting RIP140 in osteoclast. The results showed that depletion of RIP140 was remarkably alleviated osteoclasts apoptosis induced by estrogen, revealing that RIP-140 was involved in estrogen-mediated osteoclasts apoptosis (Fig. 4e, f). Moreover, qRT-PCR results showed that induction of FasL expression was detected in osteoclasts treated with 10^{-8} M estrogen for 12 h (Fig. 4g, h), whereas depletion of FasL could attenuate the increasing expression of RIP140 by estrogen (Fig. 4i, j). Taken together, these results suggested that estrogen induced osteoclasts apoptosis by increasing RIP140 expression through the Fas/FasL pathway. A illustrate schematic diagram of RIP140 involved pathways on estrogen-regulated osteoclastogenesis, osteoclasts apoptosis and bone resorption was added in Fig. 4k.

Discussion

It is well known that bone homeostasis is deregulated by estrogen deficiency, leading to increased bone turnover with enhanced bone formation and an even greater rate of bone resorption. However, the exact cellular and molecular mechanism of estrogen deficiency-induced osteoporosis remains elusive. In the present study, we demonstrated that RIP140 was a critical player in estrogen-mediated osteoclasts formation, apoptosis, and bone resorption. This is the study linking an RIP140 with estrogen-regulated osteoclasts, and provides a better understanding of the role of RIP140 during osteoclastogenesis mediated by estrogen.

RIP140 is a transcriptional coregulator that finely tunes the activity of various transcription factors, and plays very important physiological roles [21]. RIP140 could implicate in the control of energy expenditure, metabolism, cognition, mammary gland development, and intestinal homeostasis [21, 22]. Furthermore, RIP140 was also involved in

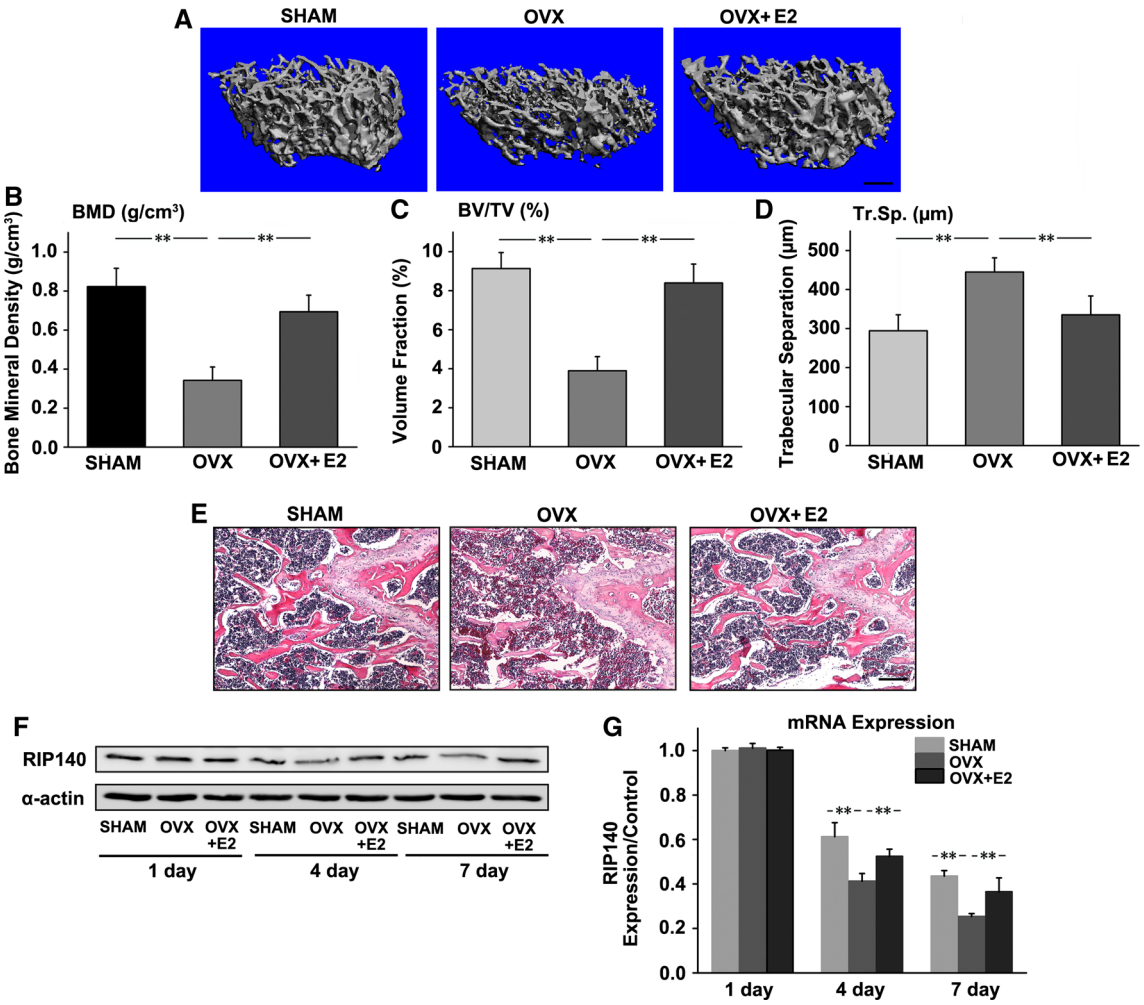

Fig. 2 The effect of estrogen on RIP140 expression during osteoclast differentiation in vivo. **a** Representative figures of micro-CT analysis of the distal end of intact femurs of mice in different group. *Scale bar* 10 μm. **b** BMD in the distal end of intact femurs was showed in different groups. $n = 4$, $^{**}p < 0.01$. **c** BV/TV in the distal end of intact femurs was showed in different groups. $n = 4$, $^{**}p < 0.01$. **d** Tr.Sp in the distal end of intact femurs was showed in different groups. $n = 4$, $^{**}p < 0.01$. **e** Representative figures of hematoxylin-eosin staining of the distal end of intact femurs of mice in different group. *Scale bar* 25 μm. **f** Western-blot analysis of RIP140 protein expression during osteoclast differentiation process in a different group. **g** qRT-PCR analysis of *RIP140 mRNA* expression during osteoclasts differentiation process in a different group. $n = 4$, $^{**}p < 0.01$. *E2* 17β-estradiol, *BMD* bone mineral density, *BV/TV* bone volume/tissue volume, *Tr.Sp* trabecular separation/spacing

the regulation of various oncogenic signaling pathways and participates in the development and progression of solid tumors [23, 24]. Recently, studies have shown that RIP140 was a critical player in estrogen-related disease. Rosell et al. found that RIP140 was implicated in ERα-mediated transcriptional regulation in breast cancer and response to tamoxifen treatment [25]. Liu et al. showed that RIP140 was involved in the fat and lipid metabolic regulation of estrogen [16]. However, this study reports, for the first time, that RIP140 was required for estrogen-mediated bone metabolism. We know that normal bone remodeling maintains constant bone mass by the balance between the resorption of old bone by osteoclasts and formation of new bone by OB. However, in this study, we found that estrogen only regulated RIP140 expression in osteoclasts but not OB or BMSCs, suggesting that RIP140 is involved in estrogen-mediated bone resorption but not bone formation.

Previous studies have shown that RIP140 is involved in several cell differentiation systems. Feng et al. found that RIP140 overexpression promoted N2a cell neuronal differentiation by activating the ERK1/2 pathway [26]. Pawan et al. confirmed that PKC epsilon stimulated arginine methylation of RIP140 for its nuclear-cytoplasmic export in adipocyte differentiation [27]. Furthermore, RIP140 expression was stimulated by estrogen-related receptor alpha during adipogenesis [17]. To our knowledge, our study is the first to observe the effect RIP140 on

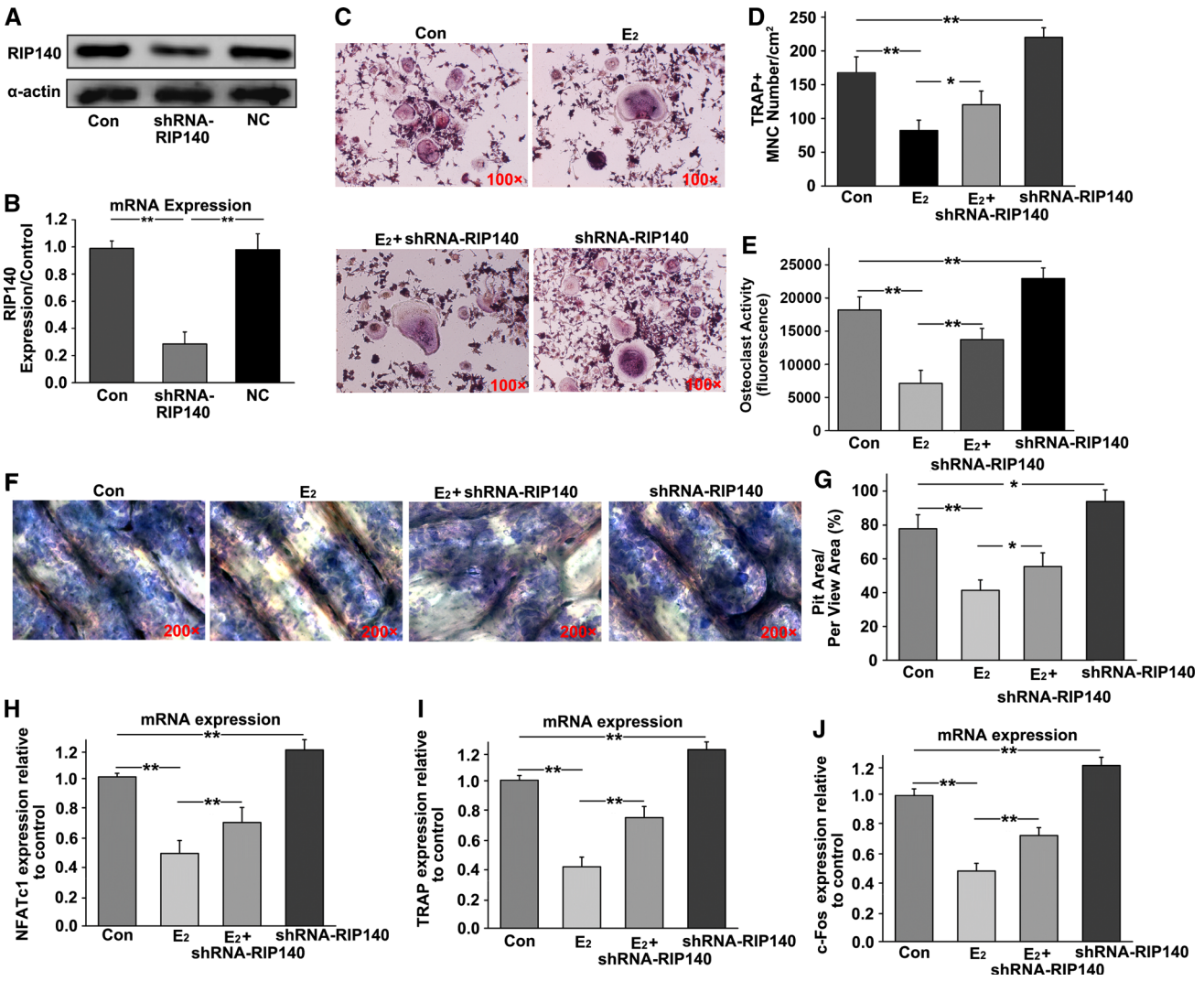

Fig. 3 Involvement of RIP140 in estrogen-inhibited osteoclast differentiation and bone resorption. **a** Verified RIP140 knockdown effect by Western blot in lentivirus-mediated transduction of primary culture osteoclasts precursors. **b** Verified RIP140 knockdown effect by qRT-PCR in lentivirus-mediated transduction of primary culture osteoclasts precursors. $n = 3$, $^{**}p < 0.01$. **c** TRAP staining of osteoclasts precursors with or without 10^{-8} M estrogen and transfected with lentivirus-shRNA-targeting RIP140. **d** Summarized data showed that the depletion of RIP140 significantly attenuated the inhibitor effect of estrogen on osteoclastogenesis. $n = 3$, $^{*}p < 0.05$. $^{**}p < 0.01$. **e** Osteoclast activity was measured by release of europium-labeled collagen measured by florescence. $n = 3$, $^{*}p < 0.05$. $^{**}p < 0.01$. **f** Pit formation assay of osteoclasts precursors with or without 10^{-8} M estrogen and transfection with Lentivirus-shRNA-targeting RIP140. **g** Summarized data showed that the depletion of RIP140 attenuated the inhibitor effect of estrogen on osteoclasts bone resorption activity. $n = 3$, $^{*}p < 0.05$. $^{**}p < 0.01$. **h–j** qRT-PCR analysis of osteoclasts marker gene in osteoclasts precursors with or without estrogen and transfection with Lentivirus-shRNA-targeting RIP140. *Con* control, *NC* negative control, *E2* 17β-estradiol

osteoclastogenesis. However, it is unclear how RIP140 regulates osteoclast differentiation. Future studies will be required to elucidate the mechanisms of RIP140-mediated osteoclast formation.

The expression of RIP140 could be regulated by estrogen in many cells, such as breast cancer cells, ovarian cancer cells, and kidney cells [23, 25, 28]. This was the first report that estrogen might regulate RIP140 expression in osteoclasts. It is well known that estrogen, which regulates diverse physiological effects, has established both classical and nonclassical estrogen signaling pathway involving estrogen receptor α (ERα), ERβ, and the newly described G protein-coupled estrogen receptor (GPER). In the classical estrogen signaling pathway, ER conformational alternation caused by estrogen increase the ability of affinity for DNA. ER binding to estrogen response elements (ERE) in the nucleus regulates their transcription, leading to de novo protein synthesis. In contrast to this classical estrogen signaling pathway, the nonclassical estrogen signaling pathway operates independently of ER-

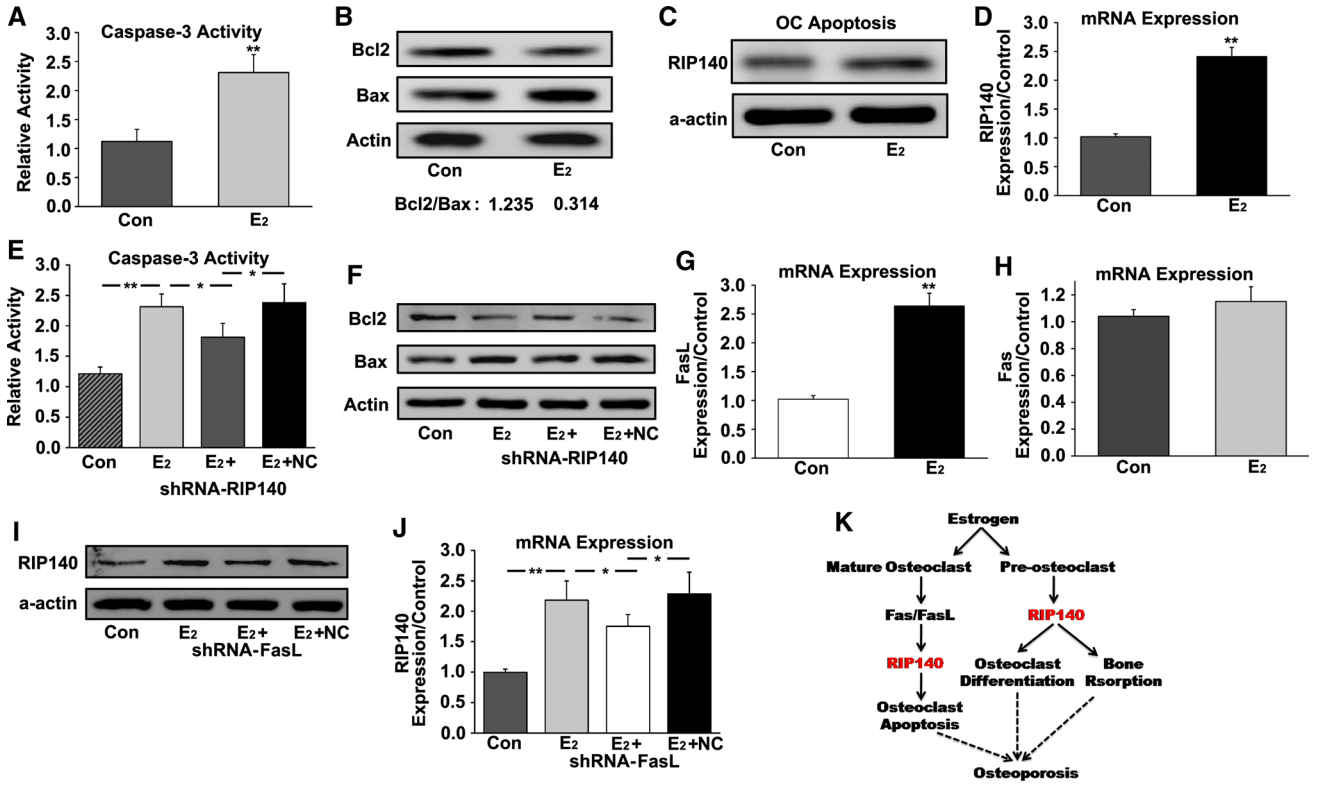

Fig. 4 Estrogen induced osteoclast apoptosis by increasing RIP140 expression through the Fas/FasL pathway. **a** Regulation of caspase-3 activity by estrogen. Osteoclasts were treated for 12 h with 10^{-8} M estrogen. $n = 3$, $^{**}p < 0.01$. **b** The levels of Bcl-2 and Bax protein from osteoclasts treated with estrogen were analyzed by Western blot. **c** Western-blot analysis of RIP140 protein expression in osteoclasts treated with 10^{-8} M estrogen for 12 h. **d** qRT-PCR analysis of *RIP140 mRNA* expression in osteoclasts treated with 10^{-8} M estrogen for 12 h. $n = 3$, $^{**}p < 0.01$, $^{*}p < 0.05$. **e** The effects of estrogen and Lentivirus-shRNA-targeting RIP140 on caspase-3 activity in osteoclasts. $n = 3$, $^{**}p < 0.01$, $^{*}p < 0.05$. **f** The levels of Bcl-2 and Bax protein from osteoclasts treated with estrogen and Lentivirus-shRNA-targeting RIP140 were analyzed by Western blot. **g** qRT-PCR analysis of *FasL mRNA* expression in osteoclasts treated with 10^{-8} M estrogen for 12 h. $n = 3$, $^{**}p < 0.01$. **h** qRT-PCR analysis of *Fas mRNA* expression in osteoclasts treated with 10^{-8} M estrogen for 12 h. $n = 3$. **i** Western-blot analysis of RIP140 protein expression in osteoclasts treated with estrogen and Lentivirus-shRNA-targeting FasL. **j** qRT-PCR analysis of *RIP140 mRNA* expression in osteoclasts treated with estrogen and Lentivirus-shRNA-targeting FasL. $n = 3$, $^{**}p < 0.01$, $^{*}p < 0.05$. *Con* control, *NC* negative control, *E2* 17β-estradiol. **k** A schematic diagram of RIP140-involved pathways on estrogen-regulated osteoclastogenesis, osteoclast apoptosis, and bone resorption

ERE binding and involves protein–protein interactions that elicit rapid responses [29]. Previous studies have identified that estrogen stimulates RIP140 transcription through two EREs ~ 100 and 200 kb upstream of the coding region in breast cancer [30]. Furthermore, Donnal et al. found that in adipocytes, ERR was capable of activating RIP140 gene transcription by two mechanisms, directly by binding to an estrogen receptor element/ERR element at -650/-633 and indirectly through Sp1 binding sites in the proximal promoter [17]. In our study, we only confirmed that estrogen greatly increased RIP140 expression in osteoclastogenesis. Further studies are required to further uncover the regulation mechanisms of estrogen on RIP140 expression in osteoclasts.

Takashi et al. [31] showed that estrogen prevented bone loss via estrogen receptor alpha and induction of fas ligand in osteoclasts. In the present study, we also found that estrogen could induce FasL expression and promote differentiated osteoclasts apoptosis, which consistent with Takashi et al. reports. Furthermore, we demonstrated that estrogen could induce osteoclast apoptosis by upregulation of RIP140 expression through the Fas/FasL pathway. The relationship between RIP and the Fas/FasL pathway has been confirmed in many cells [32]. Mohd et al. [32] confirmed that FasL played a prominent role in this lung cell death pathway and might work in part through activation of the RIP2. Furthermore, Holler et al. [33] showed that Fas could initiate cell death by two alternative pathways, one relying on caspase-8 and the other dependent on the kinase RIP. In the present study, we confirmed, for the first time, that RIP140 as downstream of Fas/FasL pathway was involved in estrogen-induced osteoclasts apoptosis.

In conclusion, our data provide new evidence that RIP140 plays a dominant effect in estrogen-mediated osteoclast formation, apoptosis, and bone resorption. This study is an effort to establish a mechanism of estrogen

protecting the adult skeleton against bone loss, and to provide insights into the potential contribution of RIP140 in the regulation of osteoclast physiology.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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