ARTICLE
Journal of Cellular Biochemistry 115:651–658 (2014)

Journal of Cellular Biochemistry

Estrogen Inhibits RANKL-Induced Osteoclastic Differentiation by Increasing the Expression of TRPV5 Channel

Fangjing Chen, Yueping OuYang, Tianwen Ye,* Bin Ni,* and Aimin Chen

Department of Orthopaedic Surgery, Shanghai Changzheng Hospital, Second Military Medical University, Shanghai 200003, China

ABSTRACT

The inhibitor effect of estrogen on osteoclasts differentiation is very important in the etiology of estrogen protecting the adult skeleton against bone loss. However, the precise molecular events underlying the effect of estrogen on osteoclasts differentiation are not known. Recent studies implicated an important role of transient receptor potential vanilloid 5 (TRPV5) in osteoclast differentiation and bone resorption. Furthermore, some studies have confirmed that estrogen is involved in the regulation of calcium ion (Ca^{2+}) influx in many cells via TRPV5 channel. Therefore, we hypothesize that TRPV5 channel may be implicated in the process of estrogen-inhibited osteoclastogenesis and bone resorption. Western blot, quantitative real-time PCR, tartrate-resistant acid phosphatase (TRAP) staining, and pit formation assay were employed to investigate the role of TRPV5 in estrogen decreasing osteoclast differentiation and bone resorption. We found that the expression of TRPV5 is significantly down-regulated during estrogen deficiency-induced osteoclastogenesis. Furthermore, TRAP staining and pit formation assay showed that the depletion of TRPV5 significantly blocks the inhibitor effects of estrogen on osteoclasts differentiation and bone resorption activity. Further studies confirmed that estrogen regulates the expression of TRPV5 channel via estrogen receptor. Based on these results above, we can draw conclusion that TRPV5 may contribute to the process of estrogen-inhibited osteoclastogenesis and bone resorption activity. J. Cell. Biochem. 115: 651–658, 2014.

KEY WORDS: OSTEOPOROSIS; TRPV5; ESTROGEN; OSTEOCLASTOGENESIS

Bone remodeling is a dynamic metabolic process. The destruction or "resorption" of pre-existing bone by mature osteoclasts accompanies with the formation of new bone by osteoblasts. Bone resorption and deposition are tightly coupled, and their balance is involved in bone mass as well as quality [Rodan and Martin, 2000; Karsenty and Wagner, 2002; Teitelbaum and Ross, 2003; Karsenty, 2006; Nakamura et al., 2007]. The imbalance of bone turnover in which resorption outpaces formation will cause osteoporosis. The most common type of osteoporosis is postmenopausal, due to a lack of estrogen [Chien and Karsenty, 2005; Raisz, 2005]. The mechanism underlying the imbalance of bone turnover after estrogen depletion has been one of the major issues in research on bone metabolism.

Osteoclasts, which are the sole bone-resorbing cell, play a central role in estrogen deficient-induced osteoporosis. Osteoclasts arise from hematopoietic stem cells that, in the presence of receptor activator of nuclear factor B (RANK) ligand (RANKL) and macrophage-colony stimulating factor (MSC-F), undergo differentiation and fusion resulting in large multinucleated cells [Li et al., 2000; Garcia Palacios et al., 2005]. Previous studies suggest that estrogen exerts direct inhibitory effects on RANKL-induced osteoclast differentiation both in primary murine bone marrow cells and in the murine macrophagic cell line RAW264.7 [Garcia Palacios et al., 2005]. However, the precise molecular events underlying the effect of estrogen on osteoclasts differentiation are not known.

Calcium ion (Ca^{2+}) signals are required for osteoclast differentiation. RANKL signaling induces oscillatory changes in intracellular Ca^{2+} concentrations, resulting in Ca^{2+}/calcineurin-dependent dephosphorylation and activation of nuclear factor of activated T cells c1 (NFATc1), which translocates to the nucleus and induces osteoclast-specific gene transcription to allow differentiation of

Grant sponsor: Science and Technology Commission of Shanghai Municipality (STCSM); Grant numbers: 10411963500, 12140903902.

* Correspondence to: Drs. Tianwen Ye and Bin Ni, Department of Orthopaedic Surgery, Shanghai Changzheng Hospital, Second Military Medical University, 415 Fengyang Road, Shanghai 200003, China.
E-mail: yetianwenvip@126.com, binni2000@163.com

Manuscript Received: 16 July 2013; Manuscript Accepted: 16 October 2013
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 21 October 2013
DOI 10.1002/jcb.24700 • © 2013 Wiley Periodicals, Inc.

osteoclasts [Kim et al., 2010; Kajiya, 2012; Kuroda et al., 2012]. Osteoclast differentiation is dependent not only on intracellular Ca^{2+} release but also on extracellular Ca^{2+} influx. Previous studies showed that Transient Receptor Potential Vanilloid 5 (TRPV5) of TRP superfamily has clarified the nature of the calcium entry channels [Vanoevelen et al., 2011]. Chamoux et al. confirmed that the TRPV5 Ca^{2+} channel as a mediator of RANKL-induced increases in the cytosolic level of Ca^{2+}, which may contribute to long-term inhibition of bone resorption [Chamoux et al., 2010]. Furthermore, analysis of femoral bone sections from TRPV5 knockout ($TRPV5^{-/-}$) mice revealed increased osteoclast numbers and osteoclast area [van der Eerden et al., 2005]. Therefore, TRPV5 was essential for osteoclast differentiation and bone resorption. TRPV5 also have been confirmed to involve in the estrogen-mediated Ca^{2+} influx in many cells. It has been demonstrated that TRPV5 protein expression is highly modulated by estrogen in kidney [Van Abel et al., 2002]. Furthermore, estrogen has the ability to regulate renal Ca^{2+} re-absorption through rapid effects on TRPV5 channel activity [Nijenhuis et al., 2003]. However, no previous studies have reported whether the TRPV5 is involved in the process of estrogen-regulated osteoclasts differentiation and bone resorption activity.

In this study, we examined the role of TRPV5 in estrogen-inhibited osteoclasts differentiation and activity. We found that the expression of TRPV5 is significantly up-regulated during estrogen deficiency-induced osteoclasts differentiation. Furthermore, TRAP staining and pit formation assay showed that TRPV5 depletion significantly blocked the inhibitor effects of estrogen on osteoclastogenesis and bone resorption activity. Moreover, estrogen decreased the expression of TRPV5 through estrogen receptor (ER). Based on these results above, we believed that TRPV5 may contribute to the process of estrogen-inhibited osteoclasts differentiation and bone resorption activity.

MATERIALS AND METHODS

ANIMAL MODEL

All procedures involving mice were approved by The Second Military Medical University Animal Study Committee and were carried out in accordance with the guide for the humane use and care of laboratory animals. Eight-week-old C57BL/6J female mice were randomly assigned in equal numbers to SHAM and ovariectomy operation (OVX) groups. The animals were housed six per cage and were maintained under a strict 12 h light and 12 h darkness cycle at 22°C with standard mice food pellets and had free access to tap water. After anesthetization, mice were ovariectomized, or SHAM-operated. After 6 weeks, mice were anesthetized, then intact tibias and femurs were removed to place on the flat plate, and blood was taken for estrogen measurement.

MEASUREMENTS OF PLASMA ESTROGEN

Plasma estrogen concentrations were determined using a Third Generation Estradiol kit (DSL-39100; Diagnostic System Laboratories, Dallas, TX). The assay has insensitivity of 0.6 pg/ml with the lowest standard concentration being 1.5 pg/ml. The intraassay coefficient of variation was 3.5%, and the interassay coefficient of variation was 4.1%.

IN VITRO OSTEOCLASTOGENESIS

Mice preosteoclasts, and osteoclasts were generated as described [Takuma et al., 2003]. Briefly, isolated bone marrow-derived monocytes (BMMs) from mice were cultured in a-MEM containing 10% FBS added 50 ng/ml recombinant M-CSF for 3 days to generate preosteoclasts. After 3 days, 20 ng/mL recombinant M-CSF and 40 ng/ml recombinant RANKL were added for 72–96 h to generate mature osteoclasts. The cells were stained for tartrate-resistant acid phosphatase (TRAP) according to the manufacturer's suggestions (Sigma, St. Louis, MO). TRAP-positive cells containing more than three nuclei were counted as osteoclasts under microscopic examination.

SKELETAL PHENOTYPING

Micro-CT (GE Locus SP) was used to assess bone mass, density, and trabecular microarchitecture of femurs. Parameters computed from these data include bone mineral density and trabecular number (Tb.N) Serial tomographs, reconstructed from raw data using the cone-beam reconstruction software (NRecon, v.1.4.4.0; Skyscan, Kontich, Belgium), were used to compute trabecular and cortical parameters, respectively from the metaphyseal and mid-diaphyseal area.

TRAP STAINING

TRAP staining was used as a marker for mature osteoclasts. Cells were fixed and stained for TRAP activity using a Leukocyte acid phosphatase kit (Sigma). Pre-osteoclasts and mature multinucleated osteoclasts (more than three nuclei) appeared dark red and were counted by light microscopy. The experiment was performed in duplicate on five independent occasions.

PIT FORMATION ASSAY

Bone resorption activity was assessed by pit formation assay according to previous reports [Feng et al., 2009] with slight modification. BMMs were cultured on bovine cortical bone slices in 24-well plates and induced by RANKL and MCSF. After 7–9 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 3 min. The resorption pits appeared dark blue and were viewed by light microscopy. The surfaces of resorption pits on the slices were measured by Image-Pro Plus 6.0 software. The percentage of pit area to a "random field of view" was counted.

SELECTION OF SIRNA AND PREPARATION OF LENTIVIRUS

The sequence of small interference RNA (siRNA) specifically targeting mRNA of TRPV5 is 5′-ATGTAGCTGATCCACGTGC-3′. The negative control siRNA targeting LacZ (si-LacZ) is 5′-CTCGGCGT TTCATCTGTGG-3′. The short hairpin RNA (shRNA) oligos were annealed and ligated into the *Bgl*II/*Hind*III-site of pSUPER. The H1 promoter shRNA expression cassettes were subcloned into the lentivirus transfer vector pLB. This was co-transfected with the packaging plasmids, pCMV-Dr8.2 and pCMV-VSV-G, into HEK293T cells using a calcium phosphate co-precipitation method. The medium was replaced with fresh DMEM after co-transfection for 8 h. The lentiviral supernatant was harvested after 48–72 h and titers were determined by infecting HEK293T cells with serial dilutions of concentrated lentivirus in the presence of 4 μg/ml polybrene (Sigma). For depletion of TRPV5 in osteoclasts, BMMs were induced with

M-CSF for 3 days and then transduced with lentiviral supernatant for 8 h. The medium was replaced with fresh α-MEM containing 20 ng/ml RANKL and 40 ng/ml M-CSF with or without estrogen for primary culture.

ISOLATION OF RNA AND QUANTITATIVE REAL TIME-PCR (QRT-PCR)

To assess Trpv5 mRNA expression, total RNA was extracted with Trizol (Invitrogen, Carlsbad) followed by phenol/chloroform purification. RNA was isolated using the RNeasy minikit (QIAGEN, Hilden, Germany). cDNA was synthesized using reverse transcriptase SuperScript II RT (Invitrogen). qRT-PCR was performed on the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), using specific forward and reverse oligonucleotide primers for Trpv5 (forward: AAAATGCCTCGTTTCCTG; reverse: GGTGGTGTTCAACCCGTA) and GAPDH (forward: GACAGCCGCATCTTCTTG; reverse: TTACCACTTCCAGCCACA). Gene expression of Trpv5 was normalized for the expression of GAPDH.

WESTERN BLOT ANALYSIS

Western blotting experiments were carried out by standard technique with modifications. Briefly, cells were gently washed twice with PBS and scraped into SDS sample buffer. Equal amounts of total protein were resolved on 8% SDS–PAGE gels and transferred onto polyvinyl difluoride membranes using the semi-dry transfer technique. The primary antibodies against TRPV-1, TRPV-2, TRPV-4, TRPV-5, and TRPV-6 were used and GAPDH was used as an internal control.

STATISTICS

The composite data are expressed as mean ± SEM. Statistical analysis was performed with one-way ANOVA. Differences were considered to be significant at $P < 0.05$.

RESULTS

REGULATION OF OSTEOCLASTS DIFFERENTIATION BY ESTROGEN IN VIVO

Ovariectomized mice were applied to observe the effects of endogenous estrogen deficiency on osteoclasts differentiation. Firstly, plasma estrogen concentrations in SHAM mice and OVX mice were determined (Fig. 1A). Estrogen deficiency by ovariectomy resulted in noticeable decline in Tb.N (Fig. 1B and C). Subsequently,

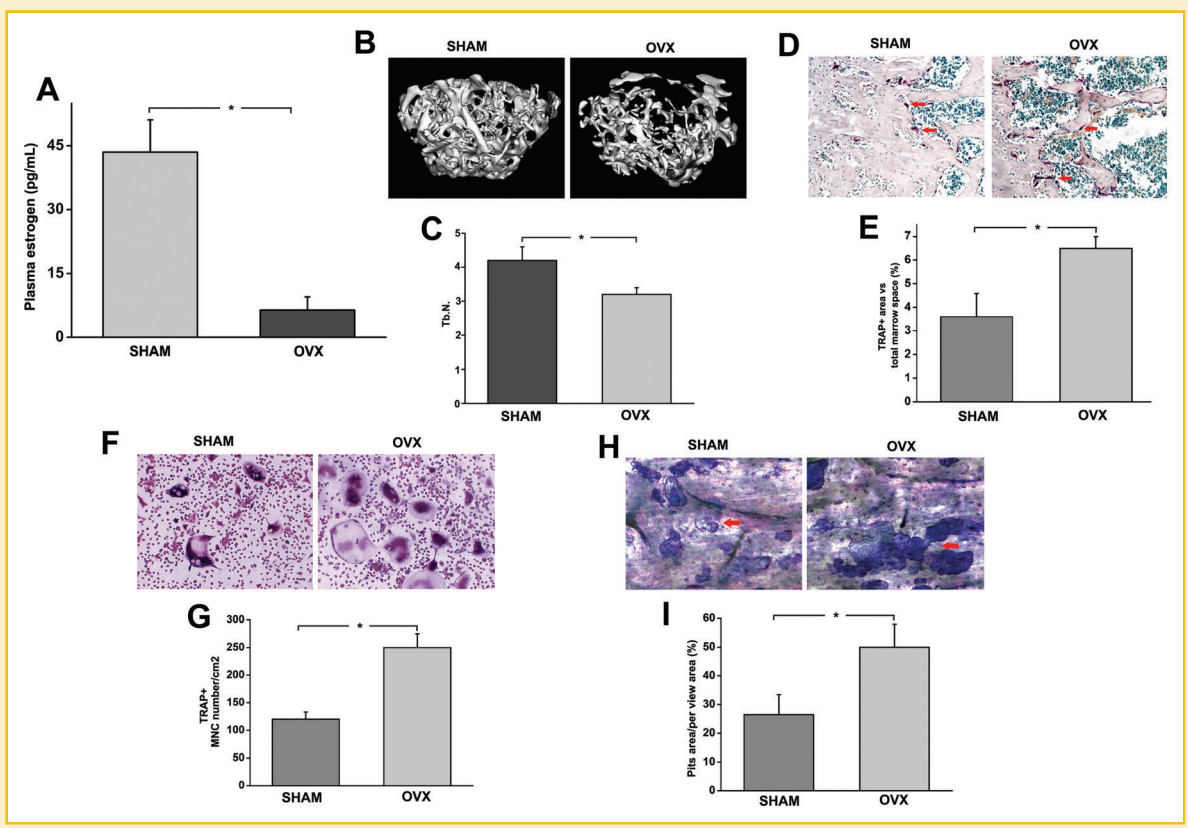

Fig. 1. Regulation of osteoclasts differentiation by estrogen in vivo. A: Plasma estrogen concentrations in SHAM mice and OVX mice were determined. n = 4, $^{*}P < 0.05$. B: Representative figures of micro-CT analysis of the distal end of intact femurs of mice after OVX or SHAM surgery. C: Tb.N. in the distal end of intact femurs of each experimental group. n = 4, $^{*}P < 0.05$. D: Histologic sections of femurs were stained for TRAP activity. The results showed increased osteoclasts number and strong TRAP activity in OVX mice (arrows). E: Quantitative analysis of $TRAP^{+}$-stained area in SHAM and very strong $TRAP^{+}$-stained area in OVX femurs sections expressed as the percentage of $TRAP^{+}$-stained area versus total marrow space. n = 4, $^{*}P < 0.05$. F and G: BMMs from SHAM and OVX mice were incubated with RANKL/M-CSF as described in Materials and Methods Section. $TRAP^{+}$ multinucleated cells (MNCs) could be detected at 8 days in the SHAM cell culture by TRAP staining analysis, while much more any were detected in OVX cells. n = 3, $^{*}P < 0.05$. H: Patterns of resorption pits on bovine cortical bone slices. I: Quantification of resorption area per view area. n = 3, $^{*}P < 0.05$.

we examined whether differentiation morphology properties of osteoclasts were changed in OVX mice. Histochemical stains of OVX mouse femurs for the osteoclast enzyme, TRAP, showed that the number of TRAP positive cells was increased in the OVX mice compared with that in SHAM mice. Quantification of the TRAP staining showed a significantly increased osteoclast number (Fig. 1D and E) in the metaphysis of OVX femurs. In addition, we further assessed the involvement of estrogen in osteoclast formation and function by culturing bone marrow cells from SHAM mice and OVX mice with osteoclast-inducing cytokines M-CSF and RANKL. We found that estrogen deficiency produced a twofold increase in the number of osteoclasts formed and bone resorbing activity in vitro (Fig. 1F–I). Our data confirmed previous studies that estrogen is an important regulator of osteoclasts differentiation.

THE EFFECT OF ESTROGEN ON TRPV5 EXPRESSION IN OSTEOCLASTS

In our study, bone marrow cells were isolated from the tibiae and femurs of SHAM and OVX mice, which were induced by M-CSF (50 ng/ml) for 3 days to acquire osteoclast precursors. After 3 days, 20 ng/ml recombinant M-CSF and 40 ng/ml recombinant RANKL were added for 96 h to generate mature osteoclasts. We found that the expressions of TRPV5 at mRNA and protein levels were greatly down-regulated in osteoclast from OVX mice compared with SHAM mice (Fig. 2A–C). To further verify the effect of estrogen on TRPV5 expression, osteoclast precursors from normal mice were incubated with 10^{-8} M estrogen at different time points. We found that the TRPV5 channel protein expression started increasing in 24 h exposure to estrogen, and the increase was more obvious in 48 h. Similarly, the mRNA levels of TRPV5 showed time-dependent increases in 24 and 48 h exposure to estrogen (Fig. 2D–F).

DEPLETION OF TRPV5 DISTURBS THE INHIBITORY EFFECT OF ESTROGEN ON OSTEOCLAST DIFFERENTIATION AND BONE RESORPTION ACTIVITY

To confirm that estrogen regulates osteoclasts differentiation and bone resorption through TRPV5, we silenced TRPV5 expression using lentiviral constructs encoding siRNA targeting TRPV5 to infect osteoclast precursors and deplete TRPV5 expression. Western blotting confirmed that the depletion of TRPV5 is effective (Fig. 3A and B). Sequentially, TRAP staining and pit formation assay were employed

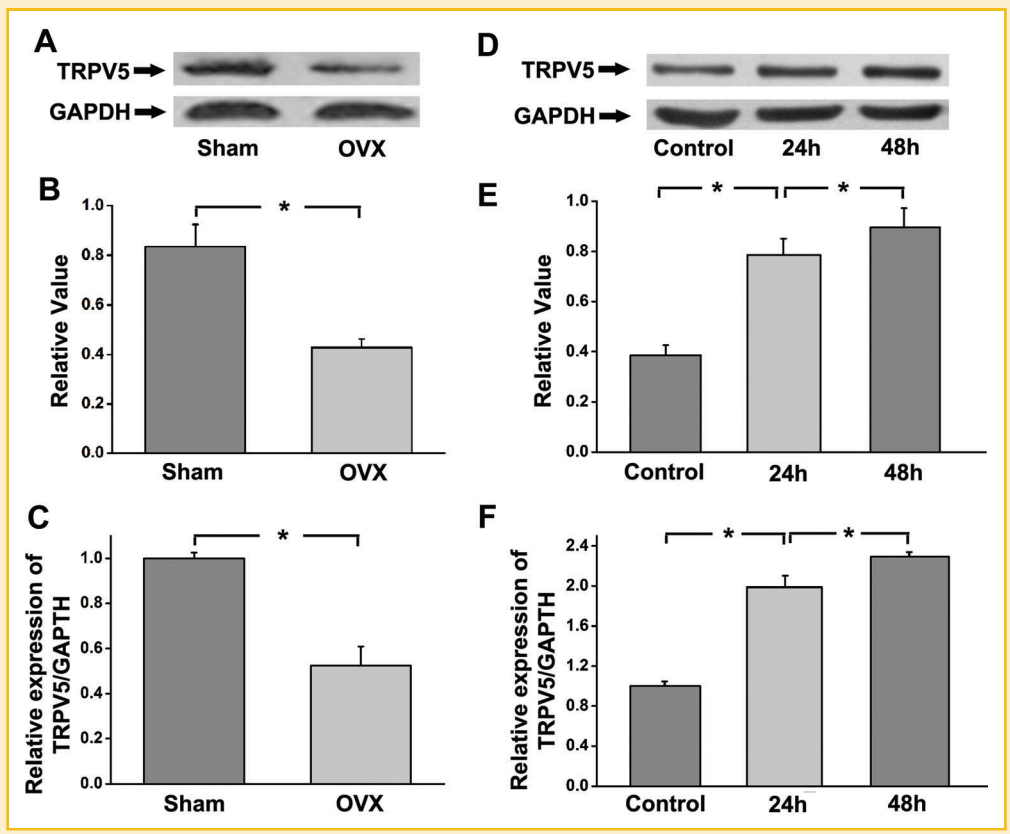

Fig. 2. The effect of estrogen on TRPV5 expression in osteoclasts. A and B: Western blot analysis of TRPV5 expression in osteoclasts from SHAM mice and OVX mice. Summarized data showed that TRPV5 expression was significantly decreased by estrogen deficiency in osteoclasts. n = 3, $^{*}P < 0.05$. C: Real-time RT-PCR analysis of TRPV5 mRNA expression in osteoclast from SHAM mice and OVX mice. Summarized data showed that TRPV5 expression was significantly decreased by estrogen deficiency in osteoclasts. n = 3, $^{*}P < 0.05$. D and E: Western blot analysis of TRPV5 expression in osteoclasts precursors exposure to 17β-estradiol in different time point. Summarized data showed that TRPV5 protein expression started increasing in 24 h exposure to estrogen, and the increase was more obvious in 48 h. n = 3, $^{*}P < 0.05$. F: Real-time RT-PCR analysis of TRPV5 mRNA expression in osteoclasts precursors exposure to 17β-estradiol in different time point. Summarized data suggested that the mRNA levels of TRPV5 showed time-dependent increases in 24 and 48 h exposure to estrogen. n = 3, $^{*}P < 0.05$.

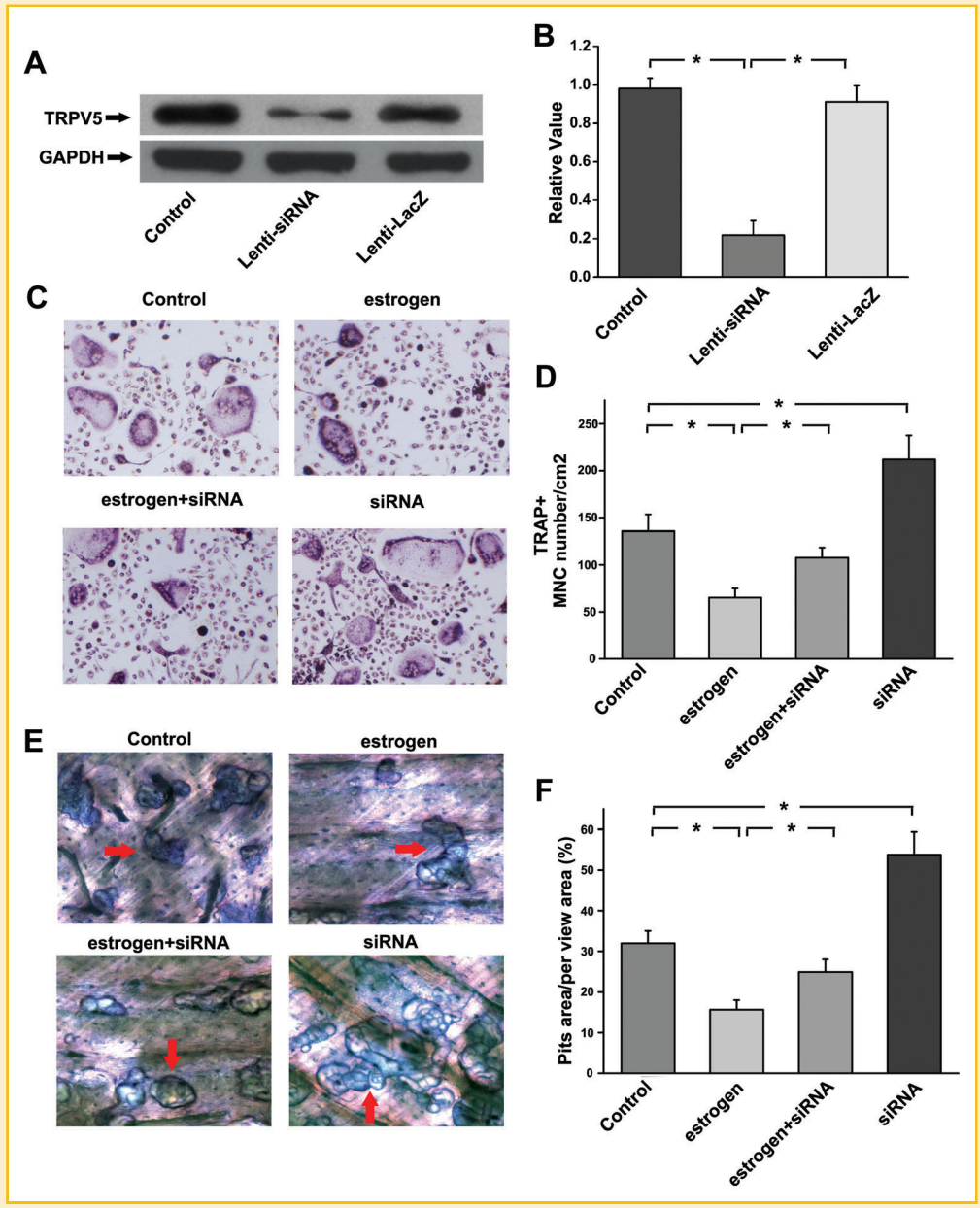

Fig. 3. Depletion of TRPV5 disturbs the inhibitory effect of estrogen on osteoclast differentiation and bone resorption activity. A and B: Verified TRPV5 knockdown effect by lentivirus-mediated transduction of primary culture osteoclasts precursors. Quantification of expressions of TRPV5 normalized to the GAPDH level. n = 3, $^{*}P < 0.05$. C and D: TRAP stain of osteoclasts precursors with or without estrogen and transfection with lentivirus-siRNA targeting TRPV5. Summarized data showed that the depletion of TRPV5 attenuated the inhibitor effect of estrogen on osteoclastogenesis. n = 5, $^{*}P < 0.05$. E and F: Pit formation assay of osteoclasts precursors with or without estrogen and transfection with lentivirus-siRNA targeting TRPV5. Summarized data showed that the depletion of TRPV5 attenuated the inhibitor effect of estrogen on osteoclasts bone resorption activity. n = 5, $^{*}P < 0.05$.

to observe the effects of TRPV5 depletion on inhibitory role of estrogen in osteoclast differentiation and bone resorption. We found that the depletion of TRPV5 resulted in the increasing of osteoclasts formed and bone resorption. Furthermore, TRPV5 depletion also significantly attenuated the inhibitor effects of estrogen on osteoclastogenesis and bone resorption (Fig. 3C–F). These results showed that TRPV5 might be implicated in the process of estrogen-inhibited osteoclasts differentiation and bone resorption activity.

THE EFFECTS OF ESTROGEN ON TRPV1/2, TRPV4, AND TRPV6 EXPRESSION IN OSTEOCLASTS PRECURSORS

It has been demonstrated that osteoclasts express different families of TRPV channels, including TRPV1/2, TRPV4, and TRPV5/6. To further explore whether these TRPV channels are also involved in the process of estrogen inhibited osteoclasts differentiation and activity, we observed the expression of TRPV1/2 (Fig. 4A and B), TRPV4 (Fig. 4C), and TRPV6 (Fig. 4D) in osteoclasts precursors treated with 17β-

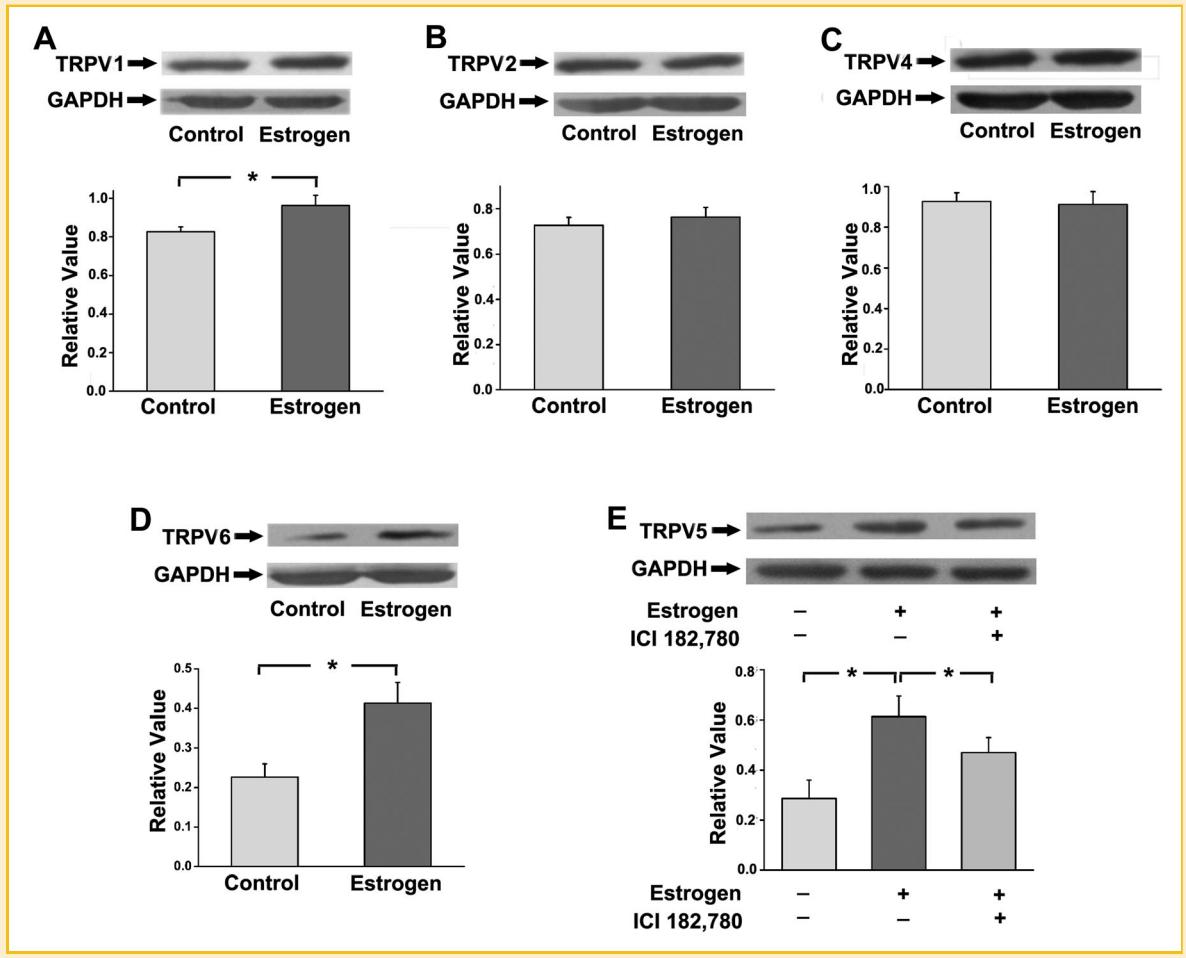

Fig. 4. The effects of estrogen on TRPV1/2, TRPV4, and TRPV6 expression in osteoclasts precursors. A: Western blot analysis of TRPV1 expression in osteoclasts precursors exposure to 17β-estradiol for 24 h. Summarized data showed that TRPV1 expression was increased by 17β-estradiol. n = 3, $^{*}P < 0.05$. B: Western blot analysis of TRPV2 expression in osteoclasts precursors exposure to 17β-estradiol for 24 h. Summarized data showed that TRPV2 expression was not affected by 17β-estradiol. n = 3, $^{*}P < 0.05$. C: Western blot analysis of TRPV4 expression in osteoclasts precursors exposure to 17β-estradiol for 24 h. Summarized data showed that TRPV4 expression was not affected by 17β-estradiol. n = 3, $^{*}P < 0.05$. D: Western blot analysis of TRPV6 expression in osteoclasts precursors exposure to 17β-estradiol for 24 h. Summarized data showed that TRPV6 expression was significantly increased by 17β-estradiol. n = 3, $^{*}P < 0.05$. E: Western blot analysis of TRPV5 expression in osteoclasts precursors exposure to 17β-estradiol with or without ICI 182,780 for 24 h. Summarized data showed that ICI 182,780 significantly attenuated the promoter effect of estrogen on TRPV5 expression. n = 3, $^{*}P < 0.05$.

estradiol (10^{-8} M) for 24 h. We found that estrogen increased TRPV1 and TRPV6 expression, but the expressions of TRPV2 and TRPV4 were not significantly regulated by estrogen.

ESTROGEN REGULATES THE EXPRESSION OF TRPV5 CHANNEL VIA ER

To further explore whether ER is implicated in the process of estrogen-increased TRPV5 expression, we observed the effect of estrogen on TRPV5 expression in osteoclasts precursors treated with ER blocker. Bone marrow-derived osteoclasts precursors were incubated in ICI 182,780 (10^{-7} M) and 17β-estradiol (10^{-8} M) in phenol red-free DMEM with charcoal-stripped serum for 24 h. We found that 10^{-7}M ICI 182,780 significantly attenuated the promoter effect of estrogen on TRPV5 expression (Fig. 4E). Therefore, we believed that estrogen regulates the expression of TRPV5 channel via ER.

DISCUSSION

The inhibitor effects of estrogen on osteoclasts differentiation and bone resorption are very important in the etiology of estrogen protecting the adult skeleton against bone loss. However, the cellular and molecular mechanism remains elusive. In the present study, we identified TRPV5 as a positive regulator of estrogen inhibited osteoclasts differentiation and activity. This is the study linking a TRPV5 with estrogen inhibited osteoclastogenesis and function, and provides key insights into the mechanisms underlying TRPV5-dependent differentiation and bone resorption in osteoclasts exposed to estrogen.

The transient receptor potential (TRP) family is a large protein family consisting of several subfamilies, of which the TRPV is an example. The TRPV family can be divided into four groups: TRPV1/2,

TRPV3, TRPV4, and TRPV5/6. Osteoclasts express different families of TRPV channels, including TRPV1/2, TRPV4, and TRPV5/6 [Vanoevelen et al., 2011; Lieben and Carmeliet, 2012]. Previous studies showed that TRPV1/2 are likely involved in the Ca^{2+}-permeable pathway, which mediates osteoclastic Ca^{2+} oscillations [Valdes et al., 2011]. Furthermore, Ca^{2+} oscillations disappear during osteoclast differentiation and are replaced by a sustained Ca^{2+} influx via members of the TRP family, including TRPV4 and TRPV5 [Hasegawa et al., 2010]. The Ca^{2+} oscillations followed by the sustained Ca^{2+} influx are both needed for NFATc1 activation and proper osteoclast differentiation [Takayanagi et al., 2002]. In the present study, we confirmed that TRPV5 is involved in the regulation of estrogen on osteoclasts differentiation and activity. To our knowledge, there have been no reports on whether estrogen can affects $[Ca^{2+}]_i$ oscillation via the regulation of TRPV5 expression to decrease osteoclasts differentiation and function. Our study is the first effort to observe the effect of estrogen on TRPV5 expression during osteoclastogenesis.

In exploring whether other TRPV channels are also involved in the process of estrogen inhibited osteoclasts differentiation and activity, we found that estradiol increased TRPV1 and TRPV6 expression levels, whereas the expression of TRPV2 and TRPV4 was not obviously regulated by estrogen. Since many studies have suggested that TRPV6 is not critical for bone metabolism [van der Eerden et al., 2012], and we found here that the expression of TRPV5 was more significantly induced by estrogen compared with that of TRPV1, we believed that TRPV5 may play a dominant role in estrogen-regulated osteoclastogenesis.

Previous studies showed that Ca^{2+} signaling controls bone cells proliferation, differentiation, transcription, activation, and apoptosis [Adams et al., 2001; Wang et al., 2008]. The consequences of Ca^{2+} signals can be classified according to whether short-term or long-term functions are affected, which are known to affect different physiological functions. According to reports, short-term functions are generally influenced within minutes and are independent of new gene expression, including the regulation of cell motility and the inhibition of bone resorption in mature osteoclasts. The long-term functions downstream of Ca^{2+} signaling include Ca^{2+} dependent phosphatase (calcineurin), transcription factors, and the differentiation of osteoclast precursors into mature osteoclasts [Kajiya, 2012]. In our study, the expression of TRPV5 was not changed in preosteoclasts exposure to estrogen within minutes (data were not showed). However, the TRPV5 expression started increasing in 24 h exposure to estrogen, and the increase was more obvious in 48 h. Therefore, we speculated that estrogen may affect the Ca^{2+} signaling long-term function through increasing TRPV5 expression, which induce a sustained oscillatory Ca^{2+}changes to regulate the osteoclasts differentiation.

Estrogen, which regulates diverse physiological effects, has established both genomic and non-genomic mechanisms involving ERα, ERβ. The estrogen causes the ER to undergo a conformational change that increases its affinity for DNA. In this conformation, it binds to specific genes in the nucleus, regulating their transcription, resulting in de novo protein synthesis [Condliffe et al., 2001]. In contrast to this genomic response, estrogen also elicits rapid responses, which are observed within seconds to minutes and occur independently of genome interaction and protein synthesis, such as the regulation of cAMP-dependent protein kinase (PKA), protein kinase C, and K^+ channel activity [Condliffe et al., 2001]. Previous studies showed that the human and mouse TRPV5 promoter contains AP-1 and Sp1 sites, which can mediates the transcriptional activation of estrogen-liganded ER [Van Abel et al., 2002]. In our study, we only confirmed that ER is involved in the regulation of estrogen on TRPV5 expression.

In summary, TRPV5 is essential for the effects estrogen on osteoclast differentiation and bone resorption activity. 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 TRPV5 in the regulation of osteoclats differentiation.

ACKNOWLEDGMENTS

We would like to thank Lei Guo, Ph.D., Shanghai Ruijin Hospital, Shanghai, China, for his help in research techniques. We also thank Erin L Hsu, Ph.D., Department of Orthopaedic surgery, Northwestern University, Chicago, IL 60611, USA, for her English editing extensively.

REFERENCES

Adams CS, Mansfield K, Perlot RL, Shapiro IM. 2001. Matrix regulation of skeletal cell apoptosis. Role of calcium and phosphate ions. J Biol Chem 276:20316–20322.

Chamoux E, Bisson M, Payet MD, Roux S. 2010. TRPV-5 mediates a receptor activator of NF-kappaB (RANK) ligand-induced increase in cytosolic Ca2+ in human osteoclasts and down-regulates bone resorption. J Biol Chem 285: 25354–25362.

Chien KR, Karsenty G. 2005. Longevity and lineages: Toward the integrative biology of degenerative diseases in heart, muscle, and bone. Cell 120:533–544.

Condliffe SB, Doolan CM, Harvey BJ. 2001. 17beta-oestradiol acutely regulates Cl^- secretion in rat distal colonic epithelium. J Physiol 530:47–54.

Feng S, Deng L, Chen W, Shao J, Xu G, Li YP. 2009. Atp6v1c1 is an essential component of the osteoclast proton pump and in F-actin ring formation in osteoclasts. Biochem J 417:195–203.

Garcia Palacios V, Robinson LJ, Borysenko CW, Lehmann T, Kalla SE, Blair HC. 2005. Negative regulation of RANKL-induced osteoclastic differentiation in RAW264.7 cells by estrogen and phytoestrogens. J Biol Chem 280:13720–13727.

Hasegawa H, Kido S, Tomomura M, Fujimoto K, Ohi M, Kiyomura M, Kanegae H, Inaba A, Sakagami H, Tomomura A. 2010. Serum calcium-decreasing factor, caldecrin, inhibits osteoclast differentiation by suppression of NFATc1 activity. J Biol Chem 285:25448–25457.

Kajiya H. 2012. Calcium signaling in osteoclast differentiation and bone resorption. Adv Exp Med Biol 740:917–932.

Karsenty G. 2006. Convergence between bone and energy homeostases: Leptin regulation of bone mass. Cell Metab 4:341–348.

Karsenty G, Wagner EF. 2002. Reaching a genetic and molecular understanding of skeletal development. Dev Cell 2:389–406.

Kim MS, Yang YM, Son A, Tian YS, Lee SI, Kang SW, Muallem S, Shin DM. 2010. RANKL-mediated reactive oxygen species pathway that induces long lasting Ca^{2+} oscillations essential for osteoclastogenesis. J Biol Chem 285: 6913–6921.

Kuroda Y, Hisatsune C, Mizutani A, Ogawa N, Matsuo K, Mikoshiba K. 2012. Cot kinase promotes Ca^{2+} oscillation/calcineurin-independent osteoclastogenesis by stabilizing NFATc1 protein. Mol Cell Biol 32:2954–2963.

Li J, Sarosi I, Yan XQ, Morony S, Capparelli C, Tan HL, McCabe S, Elliott R, Scully S, Van G, Kaufman S, Juan SC, Sun Y, Tarpley J, Martin L, Christensen K, McCabe J, Kostenuik P, Hsu H, Fletcher F, Dunstan CR, Lacey DL, Boyle WJ. 2000. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. Proc Natl Acad Sci USA 97:1566–1571.

Lieben L, Carmeliet G. 2012. The involvement of TRP channels in bone homeostasis. Front Endocrinol (Lausanne) 3:99.

Nakamura T, Imai Y, Matsumoto T, Sato S, Takeuchi K, Igarashi K, Harada Y, Azuma Y, Krust A, Yamamoto Y, Nishina H, Takeda S, Takayanagi H, Metzger D, Kanno J, Takaoka K, Martin TJ, Chambon P, Kato S. 2007. Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. Cell 130:811–823.

Nijenhuis T, Hoenderop JG, van der Kemp AW, Bindels RJ. 2003. Localization and regulation of the epithelial Ca^{2+} channel TRPV6 in the kidney. J Am Soc Nephrol 14:2731–2740.

Raisz LG. 2005. Pathogenesis of osteoporosis: Concepts, conflicts, and prospects. J Clin Invest 115:3318–3325.

Rodan GA, Martin TJ. 2000. Therapeutic approaches to bone diseases. Science 289:1508–1514.

Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, Saiura A, Isobe M, Yokochi T, Inoue J, Wagner EF, Mak TW, Kodama T, Taniguchi T. 2002. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cell 3:889–901.

Takuma A, Kaneda T, Sato T, Ninomiya S, Kumegawa M, Hakeda Y. 2003. Dexamethasone enhances osteoclast formation synergistically with transforming growth factor-beta by stimulating the priming of osteoclast progenitors for differentiation into osteoclasts. J Biol Chem 278:44667–44674.

Teitelbaum SL, Ross FP. 2003. Genetic regulation of osteoclast development and function. Nat Rev Genet 4:638–649.

Valdes AM, De Wilde G, Doherty SA, Lories RJ, Vaughn FL, Laslett LL, Maciewicz RA, Soni A, Hart DJ, Zhang W, Muir KR, Dennison EM, Wheeler M, Leaverton P, Cooper C, Spector TD, Cicuttini FM, Chapman V, Jones G, Arden NK, Doherty M. 2011. The Ile585Val TRPV1 variant is involved in risk of painful knee osteoarthritis. Ann Rheum Dis 70:1556–1561.

Van Abel M, Hoenderop JG, Dardenne O, St Arnaud R, Van Os CH, Van Leeuwen HJ, Bindels RJ. 2002. 1,25-dihydroxyvitamin D(3)-independent stimulatory effect of estrogen on the expression of ECaC1 in the kidney. J Am Soc Nephrol 13:2102–2109.

van der Eerden BC, Hoenderop JG, de Vries TJ, Schoenmaker T, Buurman CJ, Uitterlinden AG, Pols HA, Bindels RJ, van Leeuwen JP. 2005. The epithelial Ca^{2+} channel TRPV5 is essential for proper osteoclastic bone resorption. Proc Natl Acad Sci USA 102:17507–17512.

van der Eerden BC, Weissgerber P, Fratzl-Zelman N, Olausson J, Hoenderop JG, Schreuders-Koedam M, Eijken M, Roschger P, de Vries TJ, Chiba H, Klaushofer K, Flockerzi V, Bindels RJ, Freichel M, van Leeuwen JP. 2012. The transient receptor potential channel TRPV6 is dynamically expressed in bone cells but is not crucial for bone mineralization in mice. J Cell Physiol 227:1951–1959.

Vanoevelen J, Janssens A, Huitema LF, Hammond CL, Metz JR, Flik G, Voets T, Schulte-Merker S. 2011. Trpv5/6 is vital for epithelial calcium uptake and bone formation. FASEB J 25:3197–3207.

Wang J, de Boer J, de Groot K. 2008. Proliferation and differentiation of MC3T3-E1 cells on calcium phosphate/chitosan coatings. J Dent Res 87:650–654.