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ATP6v0d2 deficiency increases bone mass, but does not influence ovariectomy-induced bone loss

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ABSTRACT

Bone homeostasis is maintained through the balanced action of bone-forming osteoblasts and bone-resorbing osteoclasts. Under pathological conditions or with age, excessive bone loss is often observed due to increased bone resorption. Since osteoclasts are the primary cells in the body that can resorb bone, molecular understanding of osteoclast fate has important clinical implications. Over the past 20 years, many molecular players that govern osteoclast differentiation during normal development have been identified. However, whether the same molecules regulate bone loss occurring under pathological conditions remains largely unknown. We report here that although ATP6v0d2-deficient (ATP6v0d2 KO) mice exhibit an osteopetrotic phenotype due to inefficient osteoclast maturation, this deficiency fails to protect mice from ovariectomy (OVX)-induced bone loss, a model for post-menopause-associated osteoporosis. Moreover, we show that an OVX-induced increase in the number of colony forming unit-granulocyte/macrophage (CFU-GM) in bone marrow cells and subsequent osteoclast formation *in vitro* was not affected in the absence of ATP6v0d2. However, even after OVX, formation of large osteoclasts (>100 μm in diameter) with actin rings was still reduced in the absence of ATP6v0d2. Taken together, these findings suggest that the critical role of ATP6v0d2 may be limited to the control of bone homeostasis under normal development, and that OVX-induced bone loss is likely to be governed mostly by the increase in osteoclast precursors rather than increased efficiency of osteoclast maturation.

1. Introduction

Bone remodeling controls homeostasis of the skeletal systems and is maintained by a balance between bone matrix-producing osteoblasts and matrix-resorbing osteoclasts [1,2]. Mature osteoclasts are the only cells that efficiently resorb bone. They differentiate from hematopoietic precursors, which also comprise colony forming unit-granulocyte/macrophage (CFU-GM) cells [3,4]. Bone marrow precursors differentiate to $TRAP^+$ preosteoclasts, which are mononuclear, via receptor activator of the NF-κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) stimulation. These mononuclear preosteoclasts undergo cell–cell fusion to form multinucleated osteoclasts. Such multinucleated giant cell formation is critical for osteoclast maturation and efficient bone resorption [5,6]. In addition, the activity or viability of mature multinuclear osteoclasts can be further regulated by various stimulators including inflammatory cytokines [3,7]. Mature osteoclasts can resorb bone by secreting hydrogen ions and various acidic proteases, including TRAP and cathepsin K, into the resorption lacunae between the osteoclasts and the bone surface to facilitate the removal of inorganic and organic bone matrix, respectively [4]. Acidification of the lacunae is primarily mediated by vacuolar H^+-ATPase (v-ATPase), which is predominantly located in the ruffled border of osteoclasts [8,9].

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Previous studies have identified multiple isoforms of v-ATPase subunits, with distinct cell type- and tissue-specific expressions. These isoforms are proposed to account for the diverse physiological properties of v-ATPases, which are ubiquitous in all cell types [10,11]. It was previously reported that gene-targeted mice that are deficient in the a3 isoform of the v-ATPase (Atp6i) exhibited severe osteopetrosis due to defects in osteoclast-mediated extracellular acidification [12]. Furthermore, mutations in the a3 subunit in humans (known as TCIRG1) were responsible for infantile malignant osteopetrosis [13,14].

We previously reported [6], that mice lacking the d2 isoform of the v-ATPase V0 domain (ATP6v0d2) had an increased bone mass due to defective maturation of osteoclasts and enhanced bone formation by osteoblasts. However, ATP6v0d2 deficiency did not affect either the early differentiation or the v-ATPase activity of osteoclasts. Despite an increase in bone formation observed in ATP6v0d2 deficient mice *in vivo*, ATP6v0d2 mRNA was not detected in osteoblasts, and *ex vivo* osteoblast differentiation and mineralization did not seem to be affected in the absence of ATP6v0d2. Therefore, it seemed that increased bone formation was probably due to osteoblasts-extrinsic factors.

Estrogen deficiency, which induces marked increases in osteoclastic bone resorption, produces rapid bone loss in humans and experimental animals. Ovariectomized (OVX) animal models have been extensively used as an experimental model of enhanced bone resorption [15,16]. Previously, it was found that various cytokines, growth factors and free radicals, which were locally produced, stimulated osteoclastic bone resorption in an estrogen-deficient environment [17–19]. However, it is still largely unknown how estrogen deficiency causes increased number of osteoclasts and subsequent bone resorption *in vivo*.

In the present study, we investigated the effect of ATP6v0d2 on OVX-induced bone loss by measuring trabecular bone mass, osteoclast differentiation and CFU-GM formation in wild-type and ATP6v0d2 KO mice.

2. Materials and methods

2.1. Experimental animals

All experiments were performed on WT and ATP6v0d2 KO mice in a C57BL/6J background. The WT controls and homozygous ATP6v0d2 KO mice were generated by mating of heterozygous ATP6v0d2 KO littermates. Initially, ATP6v0d2 KO mice were produced in a mixed background of C57BL/6 and 129 SV. Later, these mice were backcrossed into C57BL/6 for at least 10 generations prior to the experiments. The WT and ATP6v0d2 KO mice were either sham-operated (SHAM) or OVX, and were euthanized 4 weeks later. OVX status was confirmed by measurement of uterine weight at the time of death. All mouse work was performed under veterinary supervision in an accredited facility using protocols approved by the Animal Care and Use Committee of the University of Pennsylvania School of Medicine and the Wonkwang University School of Dentistry.

2.2. Reagents

All cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA, USA). Soluble recombinant mouse RANKL was purified from insect cells as previously described [20], and recombinant human M-CSF was the kind gift of David H. Fremont (Washington University, St. Louis, MO). GM-CSF was purchased from R&D Systems (Minneapolis, MN, USA) and other reagents were purchased from Sigma (St. Louis, MO, USA).

2.3. Bone analysis by μCT

Trabecular morphometry within the metaphyseal region of the distal femur was quantified using micro-CT (μCT40, Scanco Medical AG, Bassersdorf, Switzerland) as described [21]. μCT analysis was performed in the Center for Bone Histomorphometry at the University of Connecticut Health Center. Bones from 5 to 6 mice per group were examined. Three-dimensional images were reconstructed using standard convolution back-projection algorithms with Shepp and Logan filtering, and were rendered at a discrete density of 578,704 voxels/mm^3 (isometric 12-μm voxels). A threshold segmentation of bone from marrow and soft tissue was performed in conjunction with a constrained Gaussian filter to reduce noise. Volumetric regions for trabecular analysis were selected within endosteal borders to include the central 80% of vertebral height, as well as the secondary spongiosa of femoral metaphyses located 960 μm (about 6% of length) from the growth plate. Trabecular morphometry was characterized by measuring bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular spacing (Tb.Sp). Cortical morphometry was analyzed within a 600 μm-long section at the mid-diaphysis of the femur and included measurements of average thickness and cross-sectional area.

2.4. In vitro formation of osteoclast-like cells

Bone marrow cells from WT and ATP6v0d2 mice were isolated and allowed to differentiate into osteoclast-like cells (OCLs) as described previously [21,22]. Briefly, bone marrow cells from the femur and tibia were collected and washed with α-MEM. After washing, bone marrow cells were cultured (1.5×10^5 cells/well in 96-well culture plate) in α-MEM containing 10% FBS with RANKL (100 ng/ml) and M-CSF (50 ng/ml) for up to 4 days. Every 3 days, the medium was replaced with fresh medium containing RANKL and M-CSF. Cells were fixed with 10% formalin after 4 days, followed by TRAP staining. The F-actin ring was stained with rhodamine phalloidin (Molecular Probes) before TRAP staining. Any TRAP$^+$-multinuclear cells (MNCs) that measured more than 100 μm in diameter and contained more than three nuclei and actin ring were considered to be OLCs. In some experiments, total TRAP activity was measured at an absorbance of 405 nm after treatment with substrate (ρ-nitrophenyl phosphate) as described previously [6].

2.5. Colony forming unit-granulocyte/macrophage (CFU-GM) assay

Bone marrow cells from WT and ATP6v0d2 mice were prepared as described above. The CFU-GM assay was performed as previously described [22] with slight modification. Briefly, bone marrow cells (1×10^5 cells/ml/dish) were plated on a 35-mm tissue culture dish in 1 ml of 1.3% methylcellulose (MethoCultTMH4100; Stem Cell Technologies Inc.) supplemented with 30% FBS, 2% BSA (Sigma), 2 mM L-glutamine (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma) and 5 ng/ml GM-CSF (R&D Systems), as a source of colony-stimulating activity. Cultures were maintained at 37 °C for 6 days and colonies (>40 cells) were scored at the end of the incubation.

2.6. Statistical analysis

Statistical analysis for mouse bone was performed by one-way ANOVA and the Bonferroni posthoc test when ANOVA showed significant differences. Student's *t*-test was used when the others were compared. All experiments were repeated at least twice, and representative data are shown.

3. Results

3.1. Ovariectomy (OVX) in WT and ATP6v0d2 KO mice

To assess the role of ATP6v0d2 in estrogen deficiency-induced bone loss, we examined WT and ATP6v0d2 mice that were operated (i.e., SHAM or OVX) at 8 weeks of age. After 4 weeks, mice were euthanized and all analyses were performed. The body and uterine weights of operated WT and ATP6v0d2 KO mice (i.e., both SHAM and OVX) were measured at the end of experiment (Fig. 1). The WT- and ATP6v0d2 KO-SHAM mice did not significantly differ in mean body and uterine weights. Four weeks after operation, the mean body weight increased slightly in both WT- and ATP6v0d2 KO-OVX mice (Fig. 1A). OVX seemed to be effective in all experiments because the mean uterine weights decreased by 7.8- and 8.4-folds in WT- and ATP6v0d2 KO-OVX mice, respectively, compared to the corresponding SHAM mice (Fig. 1B).

3.2. Effects of ATP6v0d2 deficiency on OVX-induced bone loss

To assess the role of ATP6v0d2 on estrogen withdrawal-induced bone loss, we examined WT and ATP6v0d2 KO mice that were subjected to SHAM or OVX. The trabecular and cortical bone mass of femurs from WT and ATP6v0d2 KO mice were measured by μCT. As shown in Fig. 2, femoral trabecular bone volume (BV/TV) was 75% higher in ATP6v0d2 KO-SHAM mice than in WT-SHAM controls. In addition, trabecular number (Tb.N) was 32% higher and trabecular spacing (Tb.Sp) was 27% lower in ATP6v0d2 KO-SHAM than in WT-SHAM bones. However, the trabecular thickness (Tb.Th) of the bones from WT-SHAM and ATP6v0d2 KO-SHAM mice did not differ significantly. Three-dimensional images and bone parameters indicate that ATP6v0d2-deficiency induce an osteopetrotic phenotype in mice. With regards to OVX, trabecular bone volume (BV/TV) was decreased by 44% and 52% in WT- and ATP6v0d2 KO-OVX mice, respectively, compared with their corresponding SHAM mice. Trabecular thickness was 10% and 18% lower in WT- and ATP6v0d2 KO-OVX mice, respectively, than in SHAM controls. WT- and ATP6v0d2 KO-OVX mice had 23% and 18% lower trabecular number, and 31% and 27% higher trabecular spacing compared with SHAM mice, respectively. In addition, bones from ATP6v0d2 KO-OVX mice exhibited increased trabecular bone volume and trabecular number, and lower trabecular spacing than did WT-OVX mice, similar to the findings in SHAM mice. In contrast, μCT analysis revealed no differences in cortical bone mass between SHAM and OVX mice (data not shown). Together, these results suggest that ATP6v0d2 KO mice are susceptible to estrogen withdrawal-induced bone loss, and that inactivation of ATP6v0d2 does not affect this phenomenon.

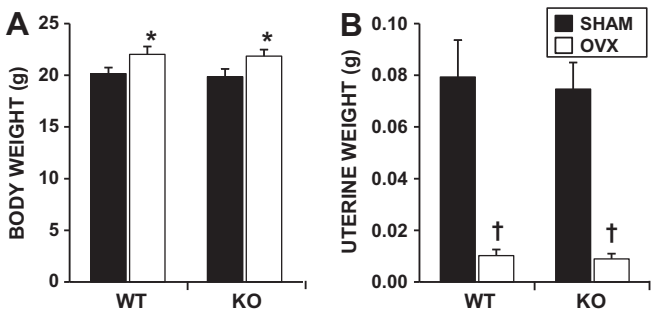

Fig. 1. Assessment of ovariectomy (OVX) with WT and ATP6v0d2 KO mice. (A) Body weight, (B) uterine weight was measured at 4 weeks after sham-operated (SHAM) or ovariectomized (OVX) with WT and ATP6v0d2 mice. Data are expressed as the mean ± SD (n = 6/group). *P < 0.05 versus each respective SHAM controls. †P < 0.01 versus each respective SHAM controls.

3.3. Effect of OVX on osteoclast-like cells (OCL) formation derived from bone marrows of WT and ATP6v0d2 KO mice

To further examine the role of ATP6v0d2 in osteoclast formation *in vitro*, we isolated bone marrow cells from SHAM and OVX of WT and ATP6v0d2 mice. Bone marrow cells were cultured with or without M-CSF (50 ng/ml) and RANKL (100 ng/ml) for 4 days to stimulate osteoclast formation. Formation of large TRAP$^+$ osteoclasts (>100 μm in diameter) containing more than three nuclei and having an actin ring was measured, because we previously showed that formation of large osteoclasts in ATP6v0d2 KO is markedly reduced compared to WT controls [6]. As shown Fig. 3, formation of large TRAP$^+$ osteoclasts was dramatically reduced in ATP6v0d2 KO-SHAM mice compared to WT-SHAM mice. However, total TRAP activity (as measured by a TRAP solution assay) was not significantly different in bone marrow cultures from ATP6v0d2 KO-SHAM and WT-SHAM mice (Fig. 3C). With regards to OVX, the extent of formation of large TRAP$^+$ osteoclasts was 18% higher in cultures from WT-OVX mice than in those of SHAM controls. In contrast, OVX did not enhance formation of large TRAP$^+$ osteoclast with acting ring in similar cultures from ATP6v0d2 KO mice (Fig. 3A and B). However, OVX increased total TRAP activity by 35% and 46% in cultures from WT and ATP6v0d2 KO mice compared with the respective SHAM controls (Fig. 3C). Total TRAP activity was similar in cultures from WT-OVX and ATP6v0d2 KO-OVX mice. These results demonstrate that, although OVX does not restore formation of large TRAP$^+$ osteoclasts with actin ring in cultures from ATP6v0d2 mice, OVX does increase osteoclast differentiation in ATP6v0d2 KO mice in a manner similar to that seen in WT controls.

3.4. Effect of ATP6v0d2 deficiency on CFU-GM formation

Fig. 3 shows that OVX increases *in vitro* osteoclastogenesis in bone marrow cells from both WT and ATP6v0d2 KO mice. Thus, we postulated that OVX is able to increase the number of osteoclast precursor cells in bone marrow from both ATP6v0d2 KO mice and WT controls. To assess the role of ATP6v0d2 in the formation of osteoclast precursor cells under OVX conditions, we measured formation of colony forming unit-granulocyte/macrophage (CFU-GM) using bone marrow cells from SHAM or OVX mice with WT and ATP6v0d2 KO backgrounds. As shown in Fig. 4, OVX increased the number of CFU-GM by 19% in WT and 12% in ATP6v0d2 KO mice compared with the respective SHAM controls. In addition, the number of CFU-GM did not differ in the bone marrows between WT-OVX and ATP6v0d2 KO-OVX mice as well as in their respective SHAM mice. These data indicate that ATP6v0d2 KO and WT mice produce similar numbers of osteoclast precursors following OVX, although ATP6v0d2 KO mice appear to be defective in the formation of large osteoclasts. Therefore, ATP6v0d2 does not affect CFU-GM formation in bone marrow cells after OVX.

4. Discussion

In the present study, we examined the effect of ATP6v0d2 on ovariectomy-induced bone loss in ATP6v0d2 KO mice. Bone samples from WT and ATP6v0d2 KO mice were analyzed by μCT. As shown in Fig. 2, the trabecular bone volume of ATP6v0d2 KO-SHAM mice was significantly higher than that of WT-SHAM controls. We found that OVX mice in the ATP6v0d2 KO and WT backgrounds exhibited 52% and 44% lower trabecular bone volume, respectively, compared with their SHAM controls. Although the

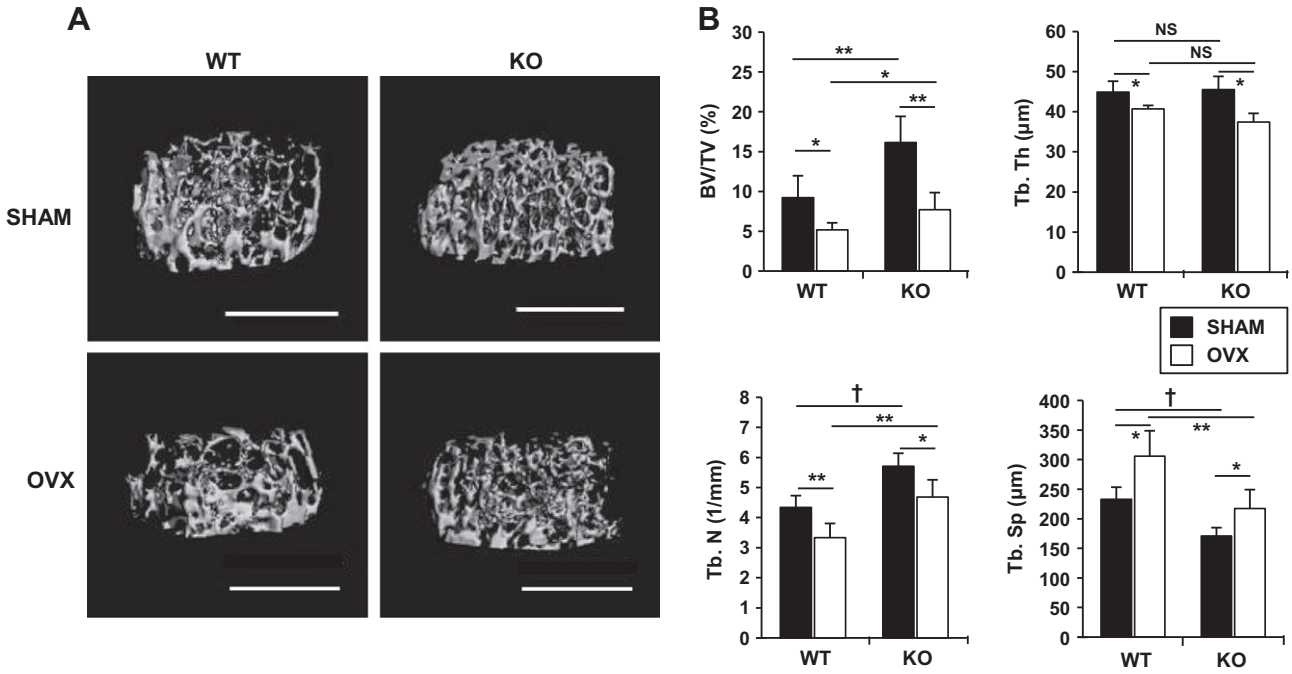

Fig. 2. Effects of estrogen deficiency on bone mass of WT and ATP6v0d2 mice. Distal femurs were collected from WT and ATP6v0d2 KO mice that were either SHAM or OVX at end of experiment and were examined by μCT. (A) Three-dimensional reconstruction of femurs from SHAM and OVX mice with WT or ATP6v0d2 KO backgrounds. Scale bar = 1.0 mm. (B) Histograms represent three-dimensional trabecular structural parameters in the secondary spongiosa of the distal femur: bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular spacing (Tb.Sp). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\dagger}P < 0.001$. NS, not significant. Data represent mean ± SD $n = 6$/group.

rate of OVX-induced bone loss was somewhat higher in ATP6v0d2 KO-OVX mice than in WT-OVX mice, ATP6v0d2 KO-OVX mice still exhibited higher trabecular bone volume than did WT-OVX mice. In addition, ATP6v0d2 KO-OVX mice had a higher trabecular number and lower trabecular spacing than did WT-OVX mice, which was a finding that was also seen in SHAM mice. In contrast, OVX did not induce a rapid change in cortical bone mass in either group of mice (data not shown). It has been suggested that assays of cortical bone mass are less sensitive to change after ovariectomy than is trabecular bone, which responds more rapidly to estrogen withdrawal [23]. Our finding, that WT and ATP6v0d2 KO mice lose similar amounts of trabecular bone mass after OVX, indicates that ATP6v0d2 is not required for OVX-induced bone loss.

ATP6v0d2 is a d2 isoform of v-ATPase subunit d and significantly induced during osteoclast differentiation. Gene-targeted deletion of ATP6v0d2 significantly increased bone mass in mice without affecting v-ATPase activity, because it caused inefficient osteoclast maturation [6]. Although the formation of a large (>100 μm in diameter) TRAP^{+} osteoclasts containing actin ring from ATP6v0d2 KO mice was dramatically diminished compared with that seen in WT controls, once formed TRAP^{+} osteoclasts from ATP6v0d2 KO mice were able to form resorption pits on dentin slices and showed a resorption rate equivalent to that of WT controls [6].

In the present study, we consistently observed increased osteoclast formation in cultured bone marrow cells from WT and ATP6v0d2 KO mice, for up to 4 weeks after OVX (Fig. 3). When bone marrow cells from both WT and ATP6v0d2 KO mice were directly differentiated to osteoclast by treatment with M-CSF and RANKL, ATP6v0d2 deficient bone marrow cells formed large (>100 μm in diameter) TRAP^{+} osteoclasts containing actin rings. However, the number of these cells was significantly lower than that formed by WT controls (Fig. 3A and B), consistent with a previous result [6]. In WT mice, OVX increased the formation of large TRAP^{+} osteoclasts containing actin rings. In addition, OVX increased total TRAP activity, which was measured by TRAP^{+} mono-, bi-, and multi-nuclear osteoclasts (Fig. 3). In contrast, although the formation of a large TRAP^{+} osteoclasts containing an actin ring was not increased in cultures of cells from ATP6v0d2 KO mice, total TRAP activity was increased and comparable to that of WT controls. It seems that TRAP^{+} osteoclasts produced from bone marrow cells of ATP6v0d2 KO-OVX mice were mostly mono-, di- and multinuclear cells without actin ring, although large TRAP^{+} osteoclasts with actin ring were infrequently formed. These results indicate that estrogen withdrawal is able to increase *in vitro* osteoclastogenesis of bone marrow cells from ATP6v0d2 KO mice, even though the number of large TRAP^{+} osteoclasts is lower than in WT controls.

Previously, it was shown that a similar number of TRAP^{+} mononuclear preosteoclasts were formed by co-culture of WT- or ATP6v0d2 KO-bone marrow cells with osteoblast [6]. In addition, several previous reports explained that increased osteoclastogenesis via OVX was probably due to increased numbers of osteoclast precursor cells in bone marrow [17,21]. As shown in Fig. 4, we found that OVX increased the numbers of CFU-GM (osteoclast precursor cells) in bone marrow cells from both WT and ATP6v0d2 KO mice, to a similar extent, and there were no significant differences in SHAM or OVX mice with WT and ATP6v0d2 KO backgrounds, respectively. These findings imply that ATP6v0d2 is not associated with the formation of osteoclast precursor cells in either model.

In summary, our results show that ATP6v0d2 regulates the late stage of osteoclast maturation, but not the early differentiation of osteoclasts, nor the generation of osteoclast precursors *in vivo* following estrogen withdrawal. Our findings suggest that, although ATP6v0d2 has a critical role in bone remodeling under normal conditions, it does not appear to control bone loss induced by pathological conditions, such as OVX. Similar to our findings, it has previously been reported that immunoreceptor tyrosine-based activation motif (ITAM) adaptor molecules (DAP12 and FcRγ) do not affect bone loss induced by OVX, despite their critical roles for osteoclast differentiation and maturation under normal

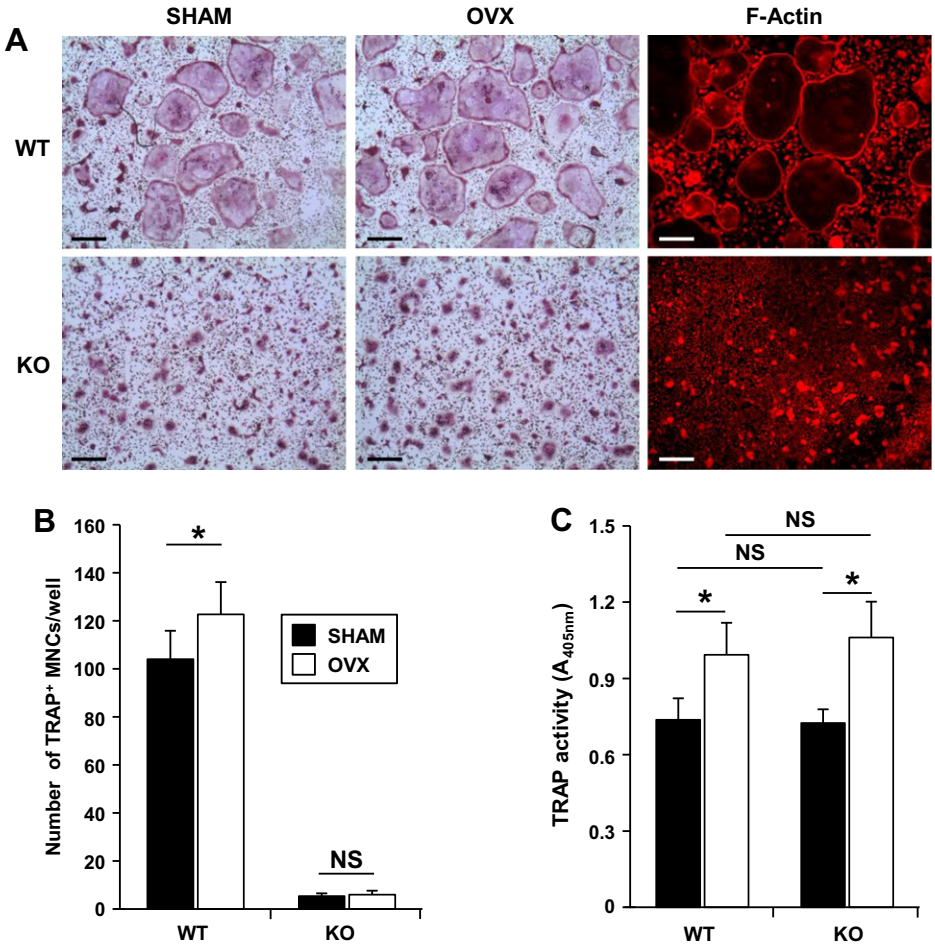

Fig. 3. Effects of ovariectomy on osteoclasts formation. The bone marrow cells from WT and ATP6v0d2 KO mice that were either SHAM or OVX, were cultured with or without M-CSF (50 ng/ml) and RANKL (100 ng/ml) for 4 days. (A) TRAP and F-actin was stained after 4 days of stimulation. (B) The number of TRAP$^+$ MNCs (>100 μm in diameter) with actin ring was counted at 4 days after stimulation with M-CSF and RANKL. (C) TRAP solution assay was performed to measure total TRAP activity at 4 days after stimulation with M-CSF and RANKL. A_{405}, absorbance at 405 nm. Data represent mean ± SD and are representative of at least three experiments. NS, not significant. $^*P < 0.05$. Scale bar = 200 μm.

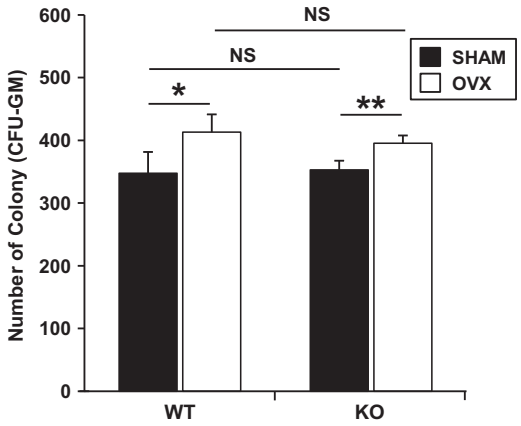

Fig. 4. Assay of CFU-GM formation. Bone marrow cells from WT and ATP6v0d2 KO mice that were either SHAM or OVX were cultured in semisolid methylcellulose to examine the number of osteoclast precursor cells. Data represent mean ± SD and are representative of at least three experiments. $^*P < 0.02$, $^{**}P < 0.001$. NS, not significant.

physiological conditions [24]. Hence, bone remodeling under pathological conditions may be regulated by distinct mechanisms than those governing normal development/homeostasis. Alternatively, under stressed conditions such as OVX, pathological factors may override deficiencies induced by the recessive mutation of genes such as ATP6v0d2 or DAP12/FcRγ. As such, future studies will be required to determine how distinct molecular players operate during bone remodeling under different physiological conditions.

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