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Macrophage Migration Inhibitory Factor-Deficient Mice Are Resistant to Ovariectomy-Induced Bone Loss

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ABSTRACT: A link between macrophage migration inhibitory factor (MIF) and estrogen has recently emerged. We examined the involvement of MIF in osteoporotic changes in bone after ovariectomy (OVX), and revealed that MIF- deficient mice (MIF-KO) were completely protected from this phenomenon. The increase in osteoclast number per bone surface and serum IL-1 β levels, which were observed in wild-type mice after OVX, did not occur in MIF-KO. Our data suggest that MIF plays an important role in the pathogenesis of postmenopausal osteoporosis, and could be a novel target for the treatment of this disease.

key words; macrophage migration inhibitory factor; deficient mice; osteoporosis; ovariectomy; cytokine

Abbreviations: MIF, macrophage migration inhibitory factor; OVX, ovariectomy; WT, wild type; IL, interleukin; TNF, tumor necrosis factor; KO, knock out; TRAP, tartrate-resistant acid phosphatase; ELISA, enzyme-linked immunosorbent assay

1. Introduction

Postmenopausal osteoporosis is one of the major health problems affecting elderly women in developed countries. Estrogen deficiency results in an increase in bone turnover, leading to bone resorption and an increased risk of fracture [1].

There is now a large body of evidence suggesting that estrogen deficiency after natural or surgical menopause increases proinflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)- α [2-5]. These proinflammatory cytokines enhance osteoclast formation and activate mature osteoclasts, resulting in increased bone resorption [6,7]. In addition, animal models have shown that the deficiency or the functional block of one of these cytokines prevents the bone loss induced by ovariectomy (OVX) [8].

Macrophage Migration Inhibitory Factor (MIF) was initially identified as a soluble factor in culture medium of activated T cells [9,10]. Following the cloning of MIF cDNA [11], the novel biological functions of MIF have been reported. MIF is released as a proinflammatory cytokine and a glucocorticoid-induced immunomodulator in response to a variety of inflammatory stimuli [12-14]. Recently, a link between MIF and estrogen has emerged. It was reported that MIF was up-regulated in the wound healing process of estrogen-deficient mice, and that estrogen decreased MIF production by murine macrophages [15].

OVX-induced osteoporosis is a model for postmenopausal osteoporosis. The purpose of this study is to examine the role of MIF in this process by comparing the effect of OVX between wild-type mice (WT) and MIF knock-out mice (MIF KO).

2. Materials and Methods

2.1. Animals

In the present study, we used MIF-deficient mutant mice with generation numbers of F11 to F13 from a mouse strain (bred onto a Balb/c background) deficient in the MIF gene, which was established by Honma et al. [16]. In brief, they generated a gene targeting vector containing the endogenous MIF gene in which a 201-bp SacI fragment consisting of the 3' region of exon 1 and the 5' region of intron 1 was replaced with a pMCI-neo poly (A) cassette in a forward orientation relative to MIF gene transcription. R1 embryonic stem (ES) cells were transected and subjected to positive selection. Subsequently, they generated germ line chimeras with targeted disruption of the MIF gene by the aggregation method, and they established a mouse strain deficient in MIF gene. To achieve a pure strain of the Balb/c background on MIF KO mice, backcrossing was performed more than 10 times. These mice developed normally and appeared healthy. Macroscopic and microscopic examinations of ovary and uterus from homozygous mutants revealed no significant abnormalities (data not shown). Balb/c wild-type

(WT) mice were purchased from Sankyo Lab (Japan) and maintained under specific pathogen-free conditions. Eight-week-old female WT and MIF KO mice were used in all experiment. All mice were maintained under specific pathogen-free conditions. All animal procedures were conducted according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee, using an approved protocol. Bilateral OVX or sham surgeries (n = 5-8) were performed under anesthesia. Four weeks after surgery, all the mice were sacrificed by means of an overdose of anesthesia.

2.2. Microcomputed Tomography (μ CT):

Right femora were fixed in 10% formaldehyde after the removal of soft tissues and were analyzed by a μ CT system (Hitachi Medical Corporation, Tokyo, Japan). The two-dimensional image of the trabecular bone was quantified as follows. Axial bone slices were made with a thickness of 16 μ m in the area of 0.7mm-1.0mm-1.3mm proximal from the distal growth plate of the femora. The mean volume (bone volume/ tissue volume; BV/TV), number, and separation of the trabecular bone at the areas of the three slices mentioned above were quantified by an image analyzer (KGT, Tokyo, Japan).

2.3. Histomorphometric analysis of bone

For histological analysis, serial 5 μ m sections of proximal tibiae were made and stained for hematoxylin/eosin (H-E). For bone histomorphometric analysis, mice were injected

subcutaneously with 8 mg/kg body weight of calcein at 4 and 1 day before sacrifice. The right tibia removed from each mouse was fixed with 70% ethanol and embedded in methyl methacrylate (MMA) without decalcification. The fixed tibia was sectioned (5 μ m) serially using the microtome, and the sections were then stained by Villanueva-Goldner's trichrome method for discrimination between mineralized and unmineralized bones and also for identification of cellular components. Stained bone sections were analyzed using an image analyzer (System Supply Co.). The measurements were done in an area 1.2 mm in length from 0.3 mm below the growth plate at the proximal metaphysis of the tibiae, and histomorphometry was conducted as previously defined [17]. The parameters measured for bone structures were the total bone volume per tissue volume (BV/TV, %), trabecular number (Tb.N, μ m), and trabecular separation (Tb.Sp, μ m). The parameters obtained for the bone formation were the osteoid surface per bone surface (OS/BS, %), the osteoblast surface per bone surface (Ob.S/BS %), mineral apposition rate (MAR, /day), and bone formation rate per bone surface (BFR/BS, mm³/cm²/y). The parameters measured for bone resorption were the eroded surface per bone surface (ES/BS, %), the osteoclast surface per bone surface (Oc.S/BS, %), and number of osteoclasts per the bone perimeter (N.Oc/B.Pm, /100mm)

2.4. Serum cytokine levels

The serum contents of IL-1 β , IL-6, and TNF- α were measured using enzyme-linked

immunosorbent assay (ELISA) kits (BioSource International, California, USA), and the serum contents of MIF using an ELISA kit (Sapporo Immuno Diagnostic Laboratory, Sapporo, Japan), according to the manufacturer's instructions.

The data were evaluated for statistical significance by one-way ANOVA using Fisher's test as a post hoc test. A p-value of < 0.05 was considered to be statistically significant.

3. Results

Trabecular bone loss in distal femur: μ CT examination of femoral bones revealed the effect of OVX in WT mice. As expected, compared with the sham-operated controls, the distal femoral trabecular bones of OVX mice were significantly reduced (Fig. 1A, B) in each corresponding axial slice. On the other hand, in MIF KO, the decrease in trabecular bones induced by OVX was completely avoided (Fig. 1C, D). Quantification of trabecular bones showed that BV/TV of the WT and MIF KO after sham operation were not different, and that of WT after OVX was significantly reduced compared with the sham-operated WT (47% reduction, $P < 0.05$, Fig. 2A). No significant BV/TV reduction was seen in MIF KO after OVX (Figure 2A). Trabecular bone numbers were also decreased in WT after OVX, (30% reduction, $P < 0.01$, Fig. 2B), and trabecular separation was also increased about 1.6-fold over that of the sham-operated mice ($P < 0.05$, Fig. 2C). In MIF KO, these changes were not observed between OVX and sham-operated

ones.

Bone histomorphometry in WT and MIF KO: It was apparent from the histological observations that the cancellous bone volume in the proximal region of the tibiae was reduced in WT after OVX (Fig. 3A, B), whereas this reduction did not occur in MIF KO-OVX compared with MIF KO-sham (Fig. 3C, D). These losses of reduction of trabecular bone in MIF KO-OVX were further confirmed by bone histomorphometry (Table 1). The parameters for bone resorption, i.e., Oc.S/BS and N.Oc/B.Pm, were significantly increased by OVX ($P < 0.01$ and $p < 0.05$, respectively), whereas these increase did not occur in the absence of MIF. Accordingly, the parameters for bone resorption after OVX were significantly lower in MIF KO than in WT ($p < 0.05$). On the other hand, the parameters for bone formation and bone turnover were similar or slightly increased to some extent, though not significant, in WT and MIF KO after OVX. These suggested that in MIF KO, the capacity for osteoblastogenesis or bone formation was not harmed.

Changes of serum proinflammatory cytokines after OVX: The levels of serum IL-1 β , IL-6, and TNF- α were also measured at 4 weeks after OVX by ELISA. In WT after OVX, serum IL-1 β levels were significantly increased, compared with the levels in sham-operated mice (96% increase, $P < 0.05$; Fig. 4), while in MIF KO there was no significant difference between the OVX and control groups. The changes of serum TNF- α or IL-6 were not observed in this series

of experiments (data not shown).

Effect of estrogen depletion on MIF production: Next, we investigated whether OVX induced an increase in the serum MIF level in WT. The serum MIF was significantly increased at 24 hours after OVX compared with sham-operated mice, while at 7 days after OVX, this increase was not observed (Fig. 5).

4. Discussion

In this study, we demonstrated for the first time that MIF is required for the bone loss induced by estrogen depletion after OVX, a model for postmenopausal osteoporosis, using MIF KO. As shown in our data, OVX did not affect indices related to bone structure or bone turnover. It is widely accepted that after menopause, the depletion of estrogen causes a high bone-turnover state that leads the bone metabolic balance to resorption, which results in osteoporosis [18,19]. Histomorphometry of the tibiae revealed that in WT, OVX-induced bone loss was associated with the increase in osteoclastic bone resorption. In contrast, in MIF KO, resistance to bone loss after OVX was associated with the absence of the increase in osteoclastic bone resorption, as shown by the absence of an increase in N.Oc/B.Pm and Oc.S/BS after OVX.

We observed rapid enhancement of serum MIF levels following OVX. Several lines of evidence show very rapid increase of MIF production after various stimuli. Matsuda *et al*

reported the immunohistochemical localization of MIF in the cornea, and the very rapid increase in MIF expression after injury in a rat corneal wound-healing model [20,21]. Similar observations showing rapid up-regulation of MIF expression after various stimuli have also been reported in *in-vivo* and *in-vitro* experiments [12,22]. Our results showed that OVX is another stimulus that rapidly induces the secretion of MIF. Estrogen deficiency increases bone-resorbing molecules such as IL-1, IL-6, and TNF- α , which causes increased bone resorption after OVX [4-7]. On the other hand, there have been no reports so far concerning the direct action of MIF on bone resorption. Several lines of evidence suggest that MIF is located upstream of these bone-resorbing molecules and regulates their production. *In-vitro* and *in-vitro* studies have shown that MIF has the properties of inducing and regulating the production of TNF, IL-1, IL-6, and NO [14, 23-26]. An emerging body of data indicates that the role of MIF within the cytokine cascade is to control the 'set point' and magnitude of the immune and inflammatory response [27]. Therefore, we may be allowed to suppose that the up-regulation of bone-resorbing molecules after OVX is, at least in part, triggered by the rapid increase in serum MIF.

The molecular mechanism of the interaction between estrogen and the transcription of bone-resorbing cytokines has been extensively studied. Translocation of nuclear factor-kappa B (NF- κ B) to the nucleus can up-regulate the expression of proinflammatory cytokines by binding

of NF- κ B to specific NF- κ B binding sites in the promoter regions of their genes [28]. The inhibitory action of the activated estrogen receptor on the binding of NF- κ B to the IL-6 gene is one of the best-examined examples of cross-talk between estrogen receptors and proinflammatory transcription factors [4]. Recent studies revealed that estrogen also down-regulated the production of MIF in murine macrophages [15]. Sequence analysis of MIF revealed that the 5'-flanking region of the MIF gene also contains an NF- κ B binding site, and the transcription of MIF was inhibited by an inhibitor of NF- κ B activation [29,30]. Therefore, it is possible that MIF, like other proinflammatory cytokines, could be induced by the activation of NF- κ B, and negatively regulated by estrogen via the repression of NF- κ B binding. Further investigations are under way to determine the molecular mechanisms underlying the regulation of MIF production by estrogen.

The exact role of MIF in the pathogenesis of postmenopausal osteoporosis remains unelucidated; however, the results of this study suggest that MIF could be a novel mediator between estrogen and osteoclasts, and could be a candidate as a target for a novel osteoporosis therapy.

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Figure legends

Fig. 1.

Micro CT analysis of the right femora of mice at 4 weeks after ovariectomy or sham operation.

(A) Wild-type (WT), sham-operated. (B) WT, ovariectomised (OVX). (C) MIF KO, sham-operated. (D) MIF KO, OVX. Axial bone slices of femur were made as described in “Materials and Methods”. The slices made 1.0mm proximal to the distal growth plate of the femora are shown.

Fig. 2.

Quantification of two-dimensional μ CT-based values. (A) The OVX-induced reductions in BV/TV were prevented in MIF KO. (B) MIF KO were also protected from the decrease in the trabecular number following OVX. (C) The OVX-induced increase in trabecular separation was also prevented in MIF KO. Values are mean \pm SD. $n = 8$ in all groups; * $P < 0.05$ vs. WT-sh, MIF KO-sh, MIF KO-sh.

Fig. 3.

Sections of trabecular bone of proximal tibiae stained for hematoxylin/eosin (H-E). (A) WT, sham-operated. (B) WT, OVX. (C) MIF KO, sham-operated. (D) MIF KO, OVX.

Fig. 4.

Effect of OVX on serum IL-1 β levels at 4 weeks after OVX or sham operation. Values are mean \pm SD. $n = 5$ in all groups; * $P < 0.05$ vs. WT-sh and MIF KO-sh, * $P < 0.01$ vs. MIF KO-OVX.

Fig. 5.

MIF concentrations in serum collected at 24 hours and 7 days after OVX. MIF levels were significantly increased in WT-OVX at 24 hours compared with WT-sh . Values are mean \pm SD. $n=5$ in all groups at 24 hours and at 7 days. * $P < 0.05$ vs. WT-sh at 24 hours.

Table 1. Changes in proximal tibial bone histomorphometric parameters in WT and MIF KO mice

Bone parameters	WT-sham	WT-OVX	MIF KO-sham	MIF KO-OVX
Static parameters				
BV/TV (%)	6.95±2.06	3.66±1.75 [†]	4.91±1.86	5.65±0.61
Tb.N (µm)	2.38±0.93	1.32±0.19 [†]	1.84±0.46	2.03±0.16
Tb.Sp (µm)	456±171	906±429 [†]	546±120	469±38.2
Formation parameters				
OS/BS (%)	3.46±0.49	7.42±4.25	4.70±3.30	5.11±1.99
Ob.S/BS (%)	7.29±1.55	8.79±3.91	7.76±2.84	11.12±3.14
MAR (/day)	1.77±0.19	1.95±0.16	1.42±0.20 ^{††}	1.54±0.23 [‡]
BFR/BS (mm ³ /cm ² /y)	13.76±3.86	13.38±5.64	7.72±3.23	11.85±2.13
Resorption parameters				
ES/BS (µm)	7.57±3.33	11.00±2.46	4.45±2.99 [‡]	4.77±2.50 [‡]
Oc.S/BS (%)	1.61±0.80	5.10±1.62 ^{††}	2.72±1.91	2.22±0.61 [‡]
N.Oc/B.Pm (/100mm)	138±44.4	352±147 [†]	223±107	122±17.5 [‡]

Values are shown as mean±SD (n=5 per group).

[†]Significantly different from WT-sham (p<0.05)

^{††}Significantly different from WT-sham (p<0.01)

[‡]Significantly different from WT-ovx (p<0.05)

^{‡‡}Significantly different from WT-ovx (p<0.01)

Fig. 1

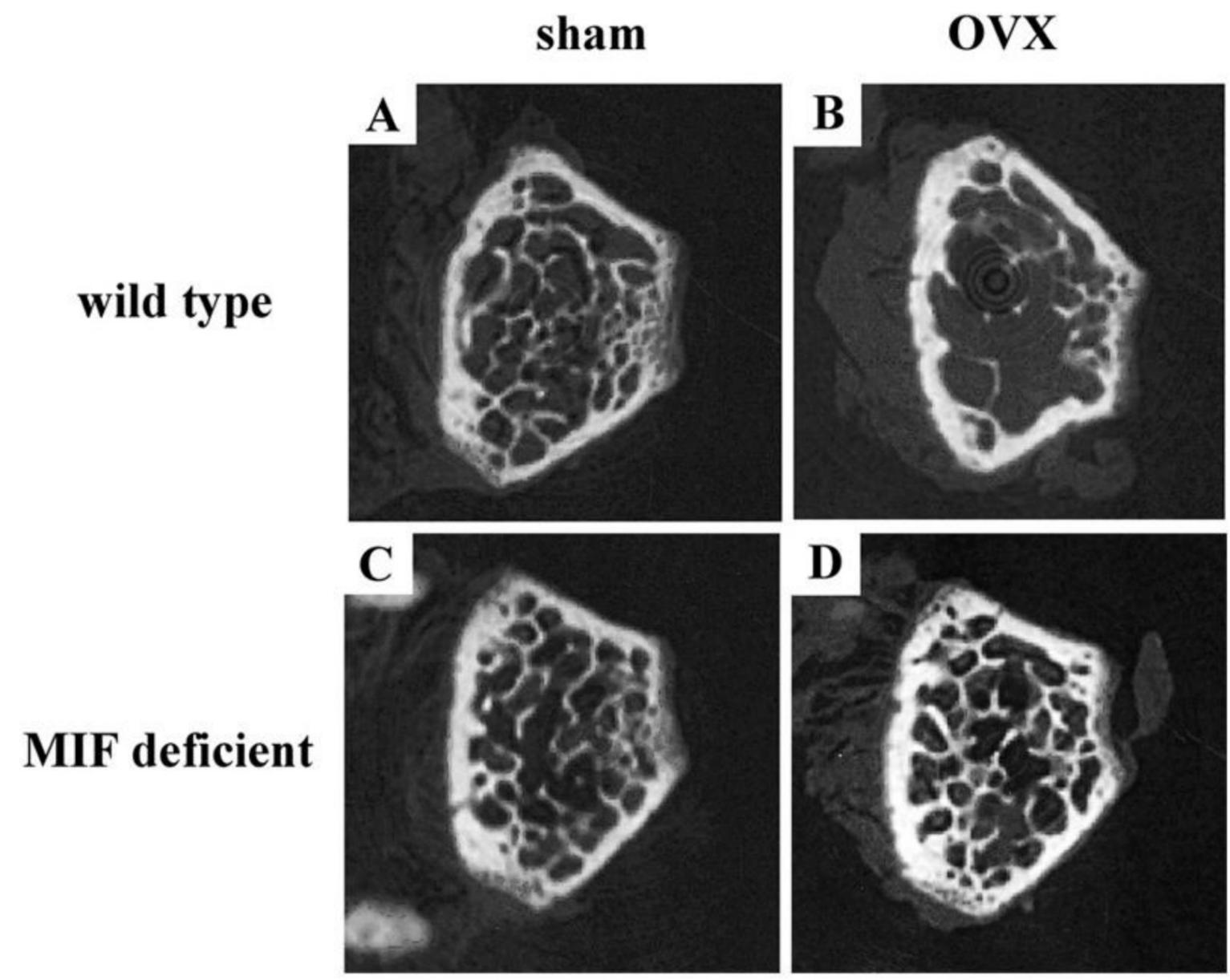
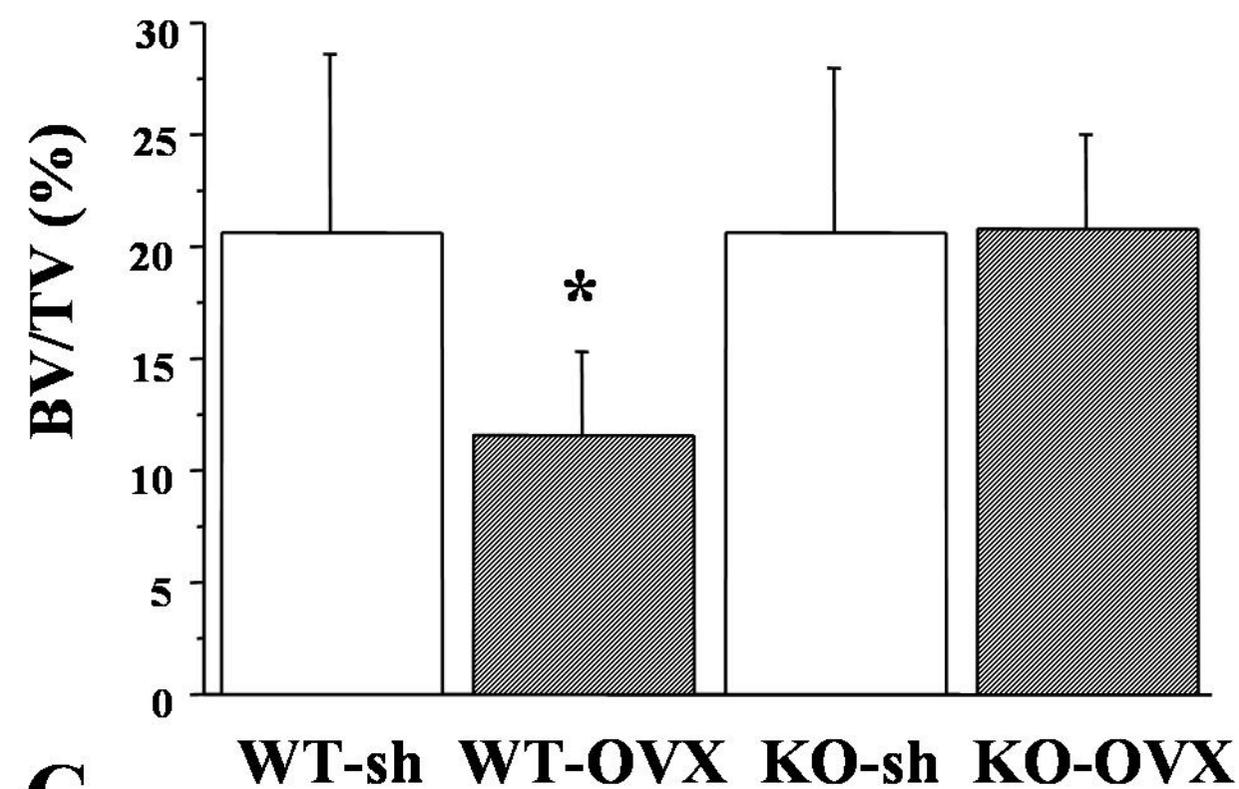
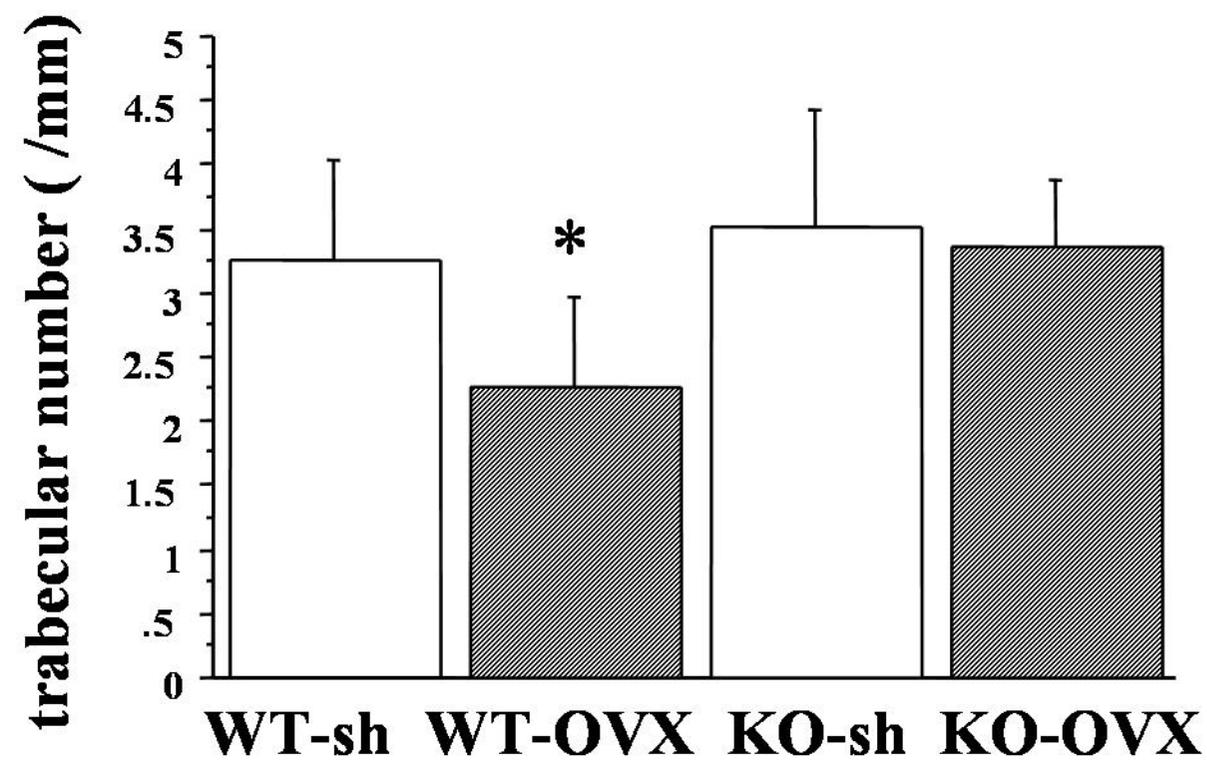


Fig. 2

A



B



C

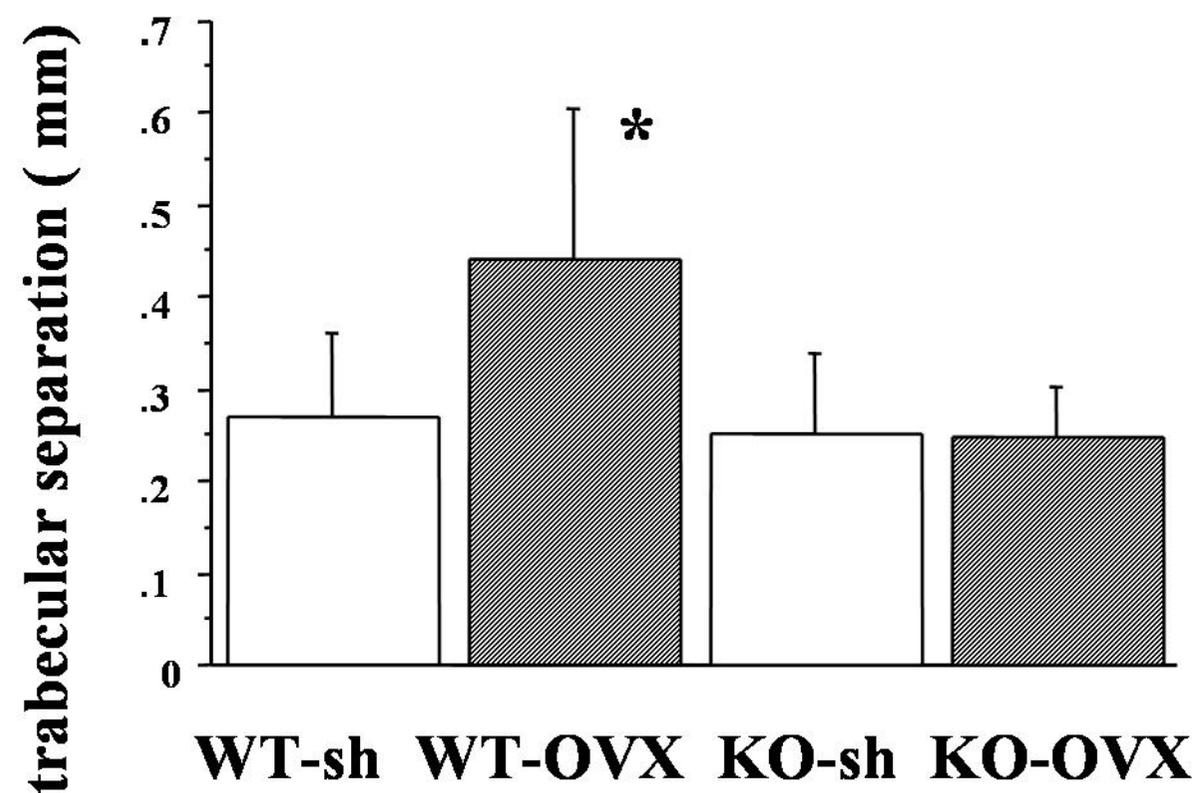


Fig. 3

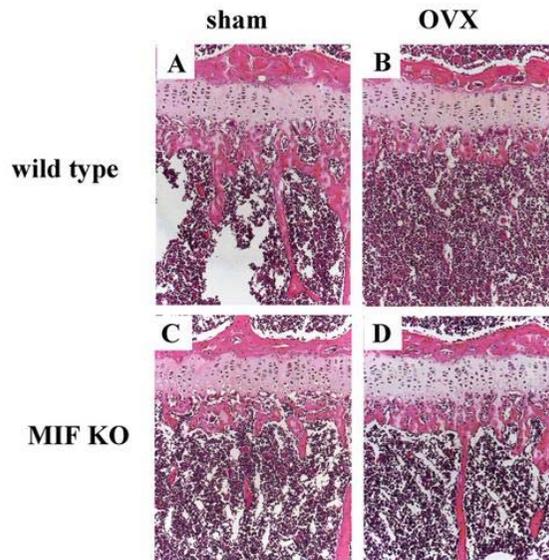


Fig. 4

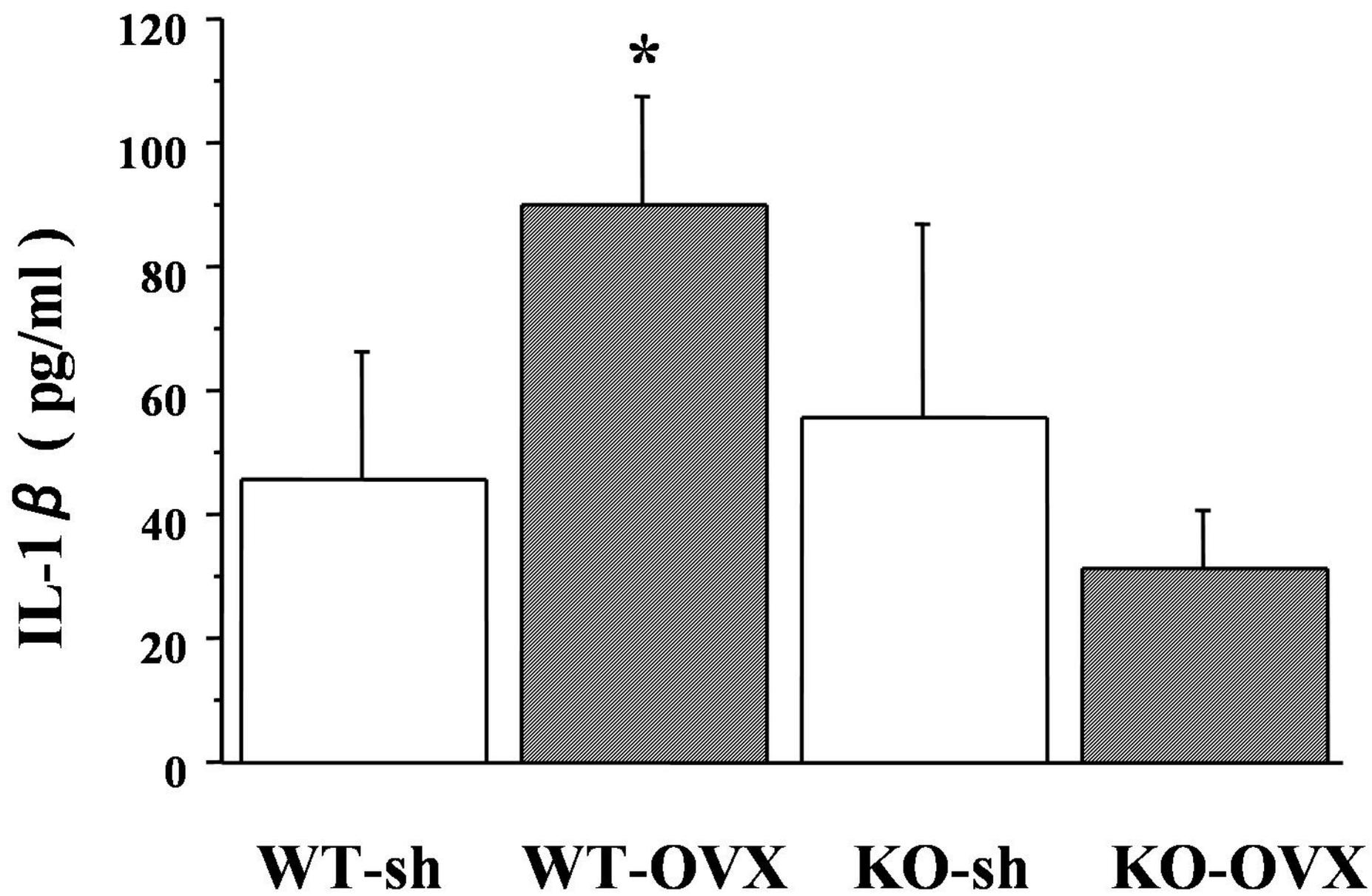


Fig. 5

