

were cytodifferentiated for 1 week in the presence of M-CSF (macrophage colony stimulating factor) and RANKL (receptor activator of NF κ B ligand) (Koga et al., 2004). TRAP-positive osteoclasts emerged after 3 days of culture (Figure 1E). The number of TRAP-positive osteoclasts and the number of LacZ-expressing cells simultaneously increased. In the contrast, the LacZ expression was not detected in primary cultured osteoblasts derived from the calvaria (Figure S1F). In view of both our *in vivo* and *in vitro* observations, we conclude that the *Ctsk^{Cre/+}* mouse line expresses Cre in differentiated osteoclasts. Moreover, estrogen response in bone mass control was not distinguishable in between *Ctsk^{Cre/+}* and *Ctsk^{+/+}* mice (Figure S2A).

We then crossed floxed *ER α* mice (Dupont et al., 2000) with *Ctsk^{Cre/+}* mice to disrupt *ER α* in differentiated osteoclasts (*ER α ^{ΔOc/ΔOc}*). Excision of the *ER α* gene (Figure S1G) was confirmed by Southern blotting of DNA from adult female and male (data not shown) bone as well as in cultured mature osteoclasts (Figure S1H). No overt differences were observed in the growth curve, reproduction, or tissues for up to 12 weeks of age (Figure 1F) between the *Ctsk^{Cre/+}; ER α ^{+/+}* (*ER α ^{+/+}*) and the *Ctsk^{Cre/+}; ER α ^{lox/lox}* (*ER α ^{ΔOc/ΔOc}*) mice, with the exception of the female bones. Serum levels of sex hormones and bone remodeling regulators such as IGF-I, leptin, and follicle-stimulating hormone (Sun et al., 2006; Takeda et al., 2002) appeared unchanged in both male and female *ER α ^{ΔOc/ΔOc}* mice at 12 weeks (Figure 1G).

Osteopenia Occurred in Osteoclast-Specific *ER α* KO Females But Not Males

The 12-week-old *ER α ^{ΔOc/ΔOc}* females exhibited a clear reduction in bone mineral density (BMD) in the femurs (Figures 2A–2C) and tibiae (data not shown) when compared with *ER α ^{+/+}* mice. Though cortical bone appeared unaffected, trabecular bone loss (Figure 2A) with significant reduction of trabecular bone volume (BV/TV) (Figure 2F) was clearly seen. This is similar to the osteoporotic abnormalities observed in women during natural menopause or following ovariectomy (Delmas, 2002; Tolar et al., 2004). However, unlike men deficient in aromatase or *ER α* activity (Simpson and Davis, 2001; Smith et al., 1994), *ER α ^{ΔOc/ΔOc}* males unexpectedly exhibited no clear bone loss even in the trabecular areas (Figures 2A–2C). In *ER α ^{ΔOc/ΔOc}* females, both the bone-formation rate, estimated by double-calcein labeling (Figure 2D), as well as the bone-resorption rate, estimated from TRAP-positive differentiated osteoclast numbers (Figure 2E), were increased, indicating high bone turnover. Histomorphometric analyses of *ER α ^{ΔOc/ΔOc}* females supported the observation of accelerated bone resorption, as increased numbers of osteoclasts (Oc.S/BS and N.Oc/BS) were observed together with more eroded bone surface (ES/BS in Figure 2F). Bone formation was also enhanced as the rates of mineral apposition (MAR) and bone formation (BFR/BS) were both upregulated without an increase in osteoblast numbers (Ob.S/BS) (Figure 2F). Thus, considering all of these find-

ings, it is conceivable that the increased number of differentiated osteoclasts following *ER α* ablation accelerates bone resorption over formation, leading to bone loss in the trabecular areas.

No Further Bone Loss Results from Estrogen Deficiency in *ER α ^{ΔOc/ΔOc}* Females

To verify whether osteoclastic *ER α* indeed mediates osteoprotective estrogen actions, estrogen action was investigated by ovariectomy (OVX) of 12-week-old female mice. As expected, OVX in *ER α ^{+/+}* females resulted in significantly reduced BMD particularly in the trabecular bone (Figures 3A and 3B) but not in the cortical bone (Figure 3C). Consistent with previous reports, (Kimble et al., 1995; Teitelbaum and Ross, 2003), estrogen deficiency following OVX upregulated the serum levels of cytokines like TNF α and IL-1 α (Figure 3D). These cytokines enhance bone resorption through stimulation of osteoclastogenesis, leading to the loss of bone mass (Teitelbaum and Ross, 2003). OVX did not further reduce BMD or trabecular bone volume of the femurs of *ER α ^{ΔOc/ΔOc}* females (Figure 3B) nor affect increased number of TRAP-positive osteoclasts (see lower panel in Figure 3A) despite upregulation of serum cytokines. This suggests that the expression of cytokines known to regulate bone resorption is not under the control of osteoclastic *ER α* .

Estrogen Treatment Failed to Rescue the Osteoporotic Bone Phenotype of *ER α ^{ΔOc/ΔOc}* Mice

Estrogen treatment by estrogen pellet implantation (OVX + E2) for 2 weeks after OVX in *ER α ^{+/+}* mice elicited a dramatic increase in bone mass in both the trabecular and cortical areas of the femurs (data not shown) and lumbar vertebral bodies (Figure 4A). Estrogen action during E2 treatment in female mutants (*ER α ^{ΔOc/ΔOc}*) was not as pronounced as in the *ER α ^{+/+}* females (Figures 4A and 4B), and the increase in the trabecular portions of the distal femurs was slight (data not shown). Histomorphometric analysis of the lumbar vertebral bodies (Figure 4B) supported the idea that E2 treatment in the female mutants was not sufficient to suppress accelerated bone resorption. These *in vivo* findings in the *ER α ^{ΔOc/ΔOc}* females suggest that in at least the trabecular areas of the long bones and lumbar vertebral bodies, the osteoprotective estrogen action is primarily mediated via osteoclastic *ER α* inhibiting bone resorption.

To further test this hypothesis, we investigated *ER α* protein expression in mature osteoclasts from trabecular bone. Few reports document osteoclastic expression of *ER α* protein and an estrogen response in both intact animals and in *in vitro* cultured osteoclasts (Bland, 2000). We therefore reasoned that *ER* expression ceases during differentiation into mature cells from primary cultures of osteoclast precursors, similar to that observed in other primary culture cell systems such as avian oviduct cells, in which *ER α* protein expression is drastically decreased during culture (Kato et al., 1989). Using highly sensitive immunohistochemistry, we investigated whether

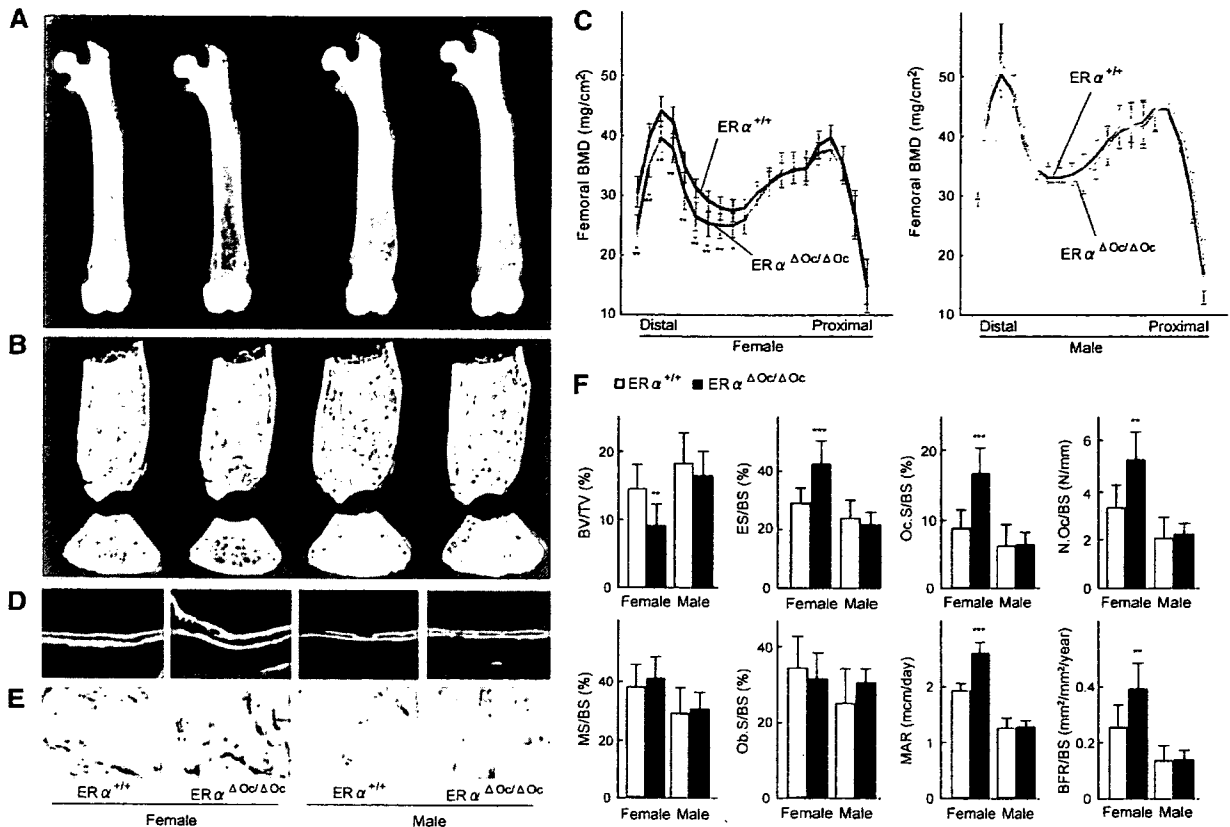


Figure 2. High Bone Turnover Osteopenia Was Observed in $ER\alpha^{\Delta Ocl/\Delta Ocl}$ Females But Not Males

(A) Soft X-ray images of femurs from 12-week-old $Ctsk^{Cre/+}; ER\alpha^{lox/lox}$ ($ER\alpha^{\Delta Ocl/\Delta Ocl}$) mice.

(B) Three-dimensional computed tomography images of the distal femurs and axial sections of distal metaphysis from representative 12-week-old $Ctsk^{Cre/+}; ER\alpha^{+/+}$ ($ER\alpha^{+/+}$) and $ER\alpha^{\Delta Ocl/\Delta Ocl}$ mice.

(C) BMD of each of 20 equal longitudinal divisions of femurs from 12-week-old $ER\alpha^{+/+}$ and $ER\alpha^{\Delta Ocl/\Delta Ocl}$ mice. ($n = 10-11$ animals per genotype; Student's t test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Data are represented as mean \pm SEM.

(D) Bone formation was also accelerated in $ER\alpha^{\Delta Ocl/\Delta Ocl}$ females when two calcein-labeled mineralized fronts visualized by fluorescent micrography were measured in the proximal tibia of 12-week-old mice.

(E) The number of TRAP-positive osteoclasts in the lumbar spine of female mice was increased by selective disruption of $ER\alpha$ in osteoclasts, indicating enhanced bone resorption.

(F) Bone turnover parameters as measured by dynamic bone histomorphometry after calcein labeling indicated high bone turnover in $ER\alpha^{\Delta Ocl/\Delta Ocl}$ females. Parameters are measured in the proximal tibia of 12-week-old $ER\alpha^{+/+}$ (open column) and $ER\alpha^{\Delta Ocl/\Delta Ocl}$ (filled column) mice. BV/TV: bone volume per tissue volume. ES/BS: eroded surface per bone surface. Oc.S/BS: osteoclast surface per bone surface. N.Oc/BS: osteoclast number per bone surface. MS/BS: mineralizing surface per bone surface. Ob.S/BS: osteoblast surface per bone surface. MAR: mineral apposition rate. BFR/BS: bone formation rate per bone surface ($n = 10-11$ animals per genotype; Student's t test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Data are represented as mean \pm SEM.

$ER\alpha$ protein expresses in differentiated osteoclasts in the bone tissues of femur sections from 12-week-old mice. $ER\alpha$ protein expression appeared abundant in osteoblasts and osteocytes of femur sections (Figure 4C) as well as hypothalamus (Figure S2B) from 12-week-old mice, in agreement with a previous report (Zaman et al., 2006). Likewise, expression levels of $ER\alpha$ in primary cultured osteoblasts derived from calvaria of $ER\alpha^{\Delta Ocl/\Delta Ocl}$ females appeared unaffected (Figure S2C). In contrast, in differentiated osteoclasts of the same femur sections, $ER\alpha$ expression was definitely detectable but very low in the $ER\alpha^{+/+}$ but undetectable in $ER\alpha^{\Delta Ocl/\Delta Ocl}$ females (Figure 4C).

Signaling by Osteoclastogenic Factors and Osteoclastogenesis Is Intact in Osteoclasts Deficient in $ER\alpha$

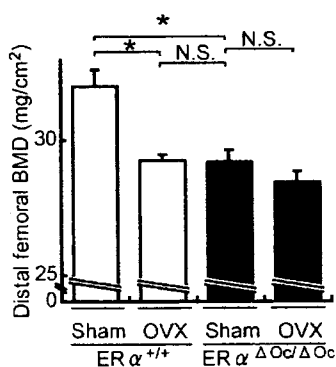
It is possible that the osteoprotective function of osteoclastic $ER\alpha$ inhibits osteoclastogenesis. To address this issue, osteoclastogenesis was tested in cultured osteoclasts derived from bone-marrow cells of $ER\alpha^{\Delta Ocl/\Delta Ocl}$ mutants. In this cell culture system, a possible contribution of contaminated immune cells and stromal cells could be excluded, since osteoclastogenesis is only inducible by M-CSF treatment followed by M-CSF + RANKL (Koga et al., 2004).

A

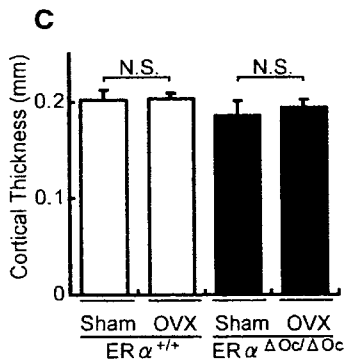


	Sham	OVX	Sham	OVX
	$ER\alpha^{+/+}$		$ER\alpha^{\Delta Oc/\Delta Oc}$	
Oc.S/BS	8.31±1.6	11.0±0.3*	10.83±2.1*	10.53±0.5*
N.Oc/B.Pm	3.50±0.7	4.23±0.2*	4.28±0.6*	3.82±0.1*

B



C



D

□ $ER\alpha^{+/+}$ ■ $ER\alpha^{\Delta Oc/\Delta Oc}$

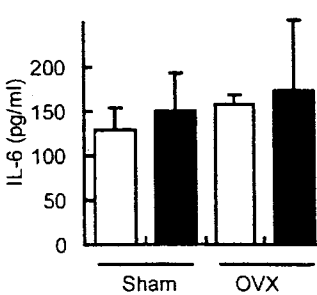
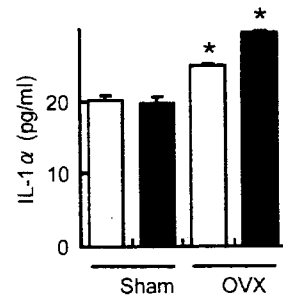
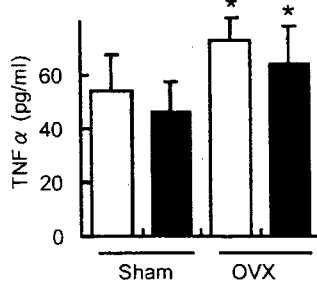
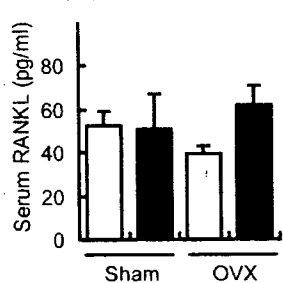


Figure 3. No Further Bone Loss of $ER\alpha^{\Delta Oc/\Delta Oc}$ Females by Ovariectomy

(A) Distal femoral micro CT analysis and lumbar vertebral bone histomorphometrical analysis of sham-operated or ovariectomized (OVX) 12-week-old $ER\alpha^{+/+}$ and $ER\alpha^{\Delta Oc/\Delta Oc}$ mice (* $p < 0.05$ compared to $ER\alpha^{+/+}$ sham group). Two weeks after OVX, the bone phenotype was analyzed.

(B) BMD of the distal femurs within each group are described in Figure 3A (* $p < 0.05$; N.S., not significant). Data are represented as mean \pm SEM.

(C) Cortical thickness evaluation from micro CT analysis of femurs within each group described in Figure 3A. Data are represented as mean \pm SEM.

(D) The levels of TNF α , IL-1 α , and IL-6 in the bone-marrow cells culture media and serum RANKL (* $p < 0.05$ compared to each sham group). Data are represented as mean \pm SEM.

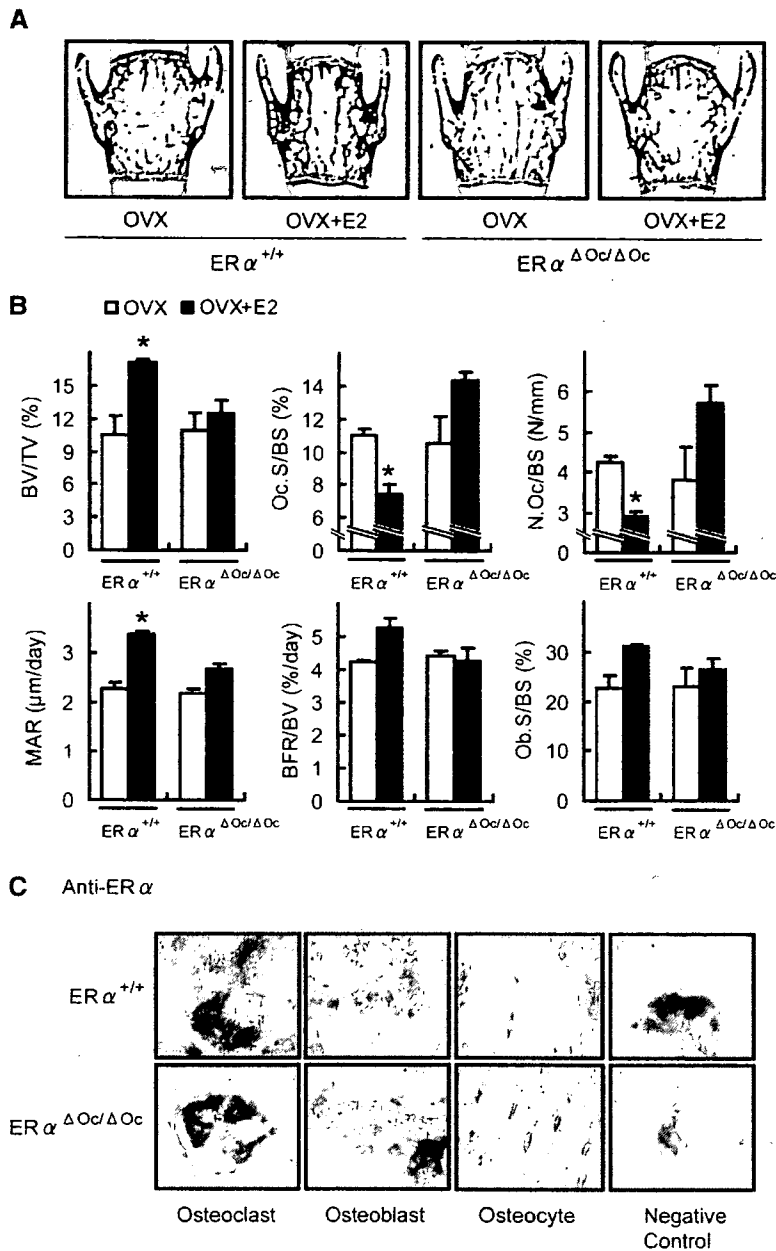


Figure 4. Estrogen treatment failed to reverse trabecular bone loss of ovariectomized $ER\alpha^{\Delta Ocl/\Delta Ocl}$ females

(A) von kossa staining of lumbar vertebral bodies of ovariectomized $ER\alpha^{+/+}$ and $ER\alpha^{\Delta Ocl/\Delta Ocl}$ mice treated with or without 17 β -estradiol (0.83 $\mu\text{g}/\text{day}$) for 2 weeks (+E2) groups.

(B) Bone histomorphometrical analyses of the lumbar vertebral bodies of 12-week-old ovariectomized $ER\alpha^{+/+}$ (left columns) and $ER\alpha^{\Delta Ocl/\Delta Ocl}$ (right columns) mice with (filled columns) or without (open columns) E2 treatment for 2 weeks (* $p < 0.05$ compared with E2-treated ovariectomized $ER\alpha^{\Delta Ocl/\Delta Ocl}$ mice). BV/TV: bone volume per tissue volume. ES/BS: eroded surface per bone surface. Oc.S/BS: osteoclast surface per bone surface. N.Oc/BS: osteoclast number per bone surface. MS/BS: mineralizing surface per bone surface. Ob.S/BS: osteoblast surface per bone surface. MAR: mineral apposition rate. BFR/BS: bone formation rate per bone surface. Data are represented as mean \pm SEM.

(C) Immunohistochemical identification of $ER\alpha$ (brown) in TRAP-positive (red) differentiated osteoclasts. The femurs of 12 week-old mice were used for the immunodetection of $ER\alpha$ in bone cells. All labels were abolished when the primary antibody was preadsorbed with the immunizing peptide (negative control).

The number of TRAP-positive osteoclasts differentiated from the bone-marrow cells of $ER\alpha^{\Delta Ocl/\Delta Ocl}$ females was almost the same as that from $ER\alpha^{+/+}$ females (Figure 5A) and males (data not shown). The differentiated $ER\alpha^{\Delta Ocl/\Delta Ocl}$ osteoclasts had typical osteoclastic features, including the characteristic cell shape, TRAP-positive, multiple nuclei, and actin-ring formation, and were indistinguishable from the $ER\alpha^{+/+}$ osteoclasts (Figure 5B).

The expression levels of the prime osteoclastogenic transcription factors, *c-fos* and *NFATc1*, were unaltered by $ER\alpha$ deficiency in differentiated osteoclasts (Figure 5C). Furthermore, responses to RANKL in intracellular signaling, as represented by phosphorylation of p38

and $I\kappa B$, were unaffected in $ER\alpha^{\Delta Ocl/\Delta Ocl}$ osteoclasts from females (Figure 5D) as well as males (data not shown). In light of these findings, it is unlikely that activated $ER\alpha$ in osteoclastic cells attenuates osteoclastogenesis.

Activation of the Fas/FasL System by Estrogen in Intact Bone Is Impaired by Osteoclastic $ER\alpha$ Deficiency

To examine osteoclastic $ER\alpha$ function in intact bone, DNA microarray analysis following real-time RT-PCR of RNA from the femurs of ovariectomized $ER\alpha^{\Delta Ocl/\Delta Ocl}$ females treated with or without estrogen, was performed. During

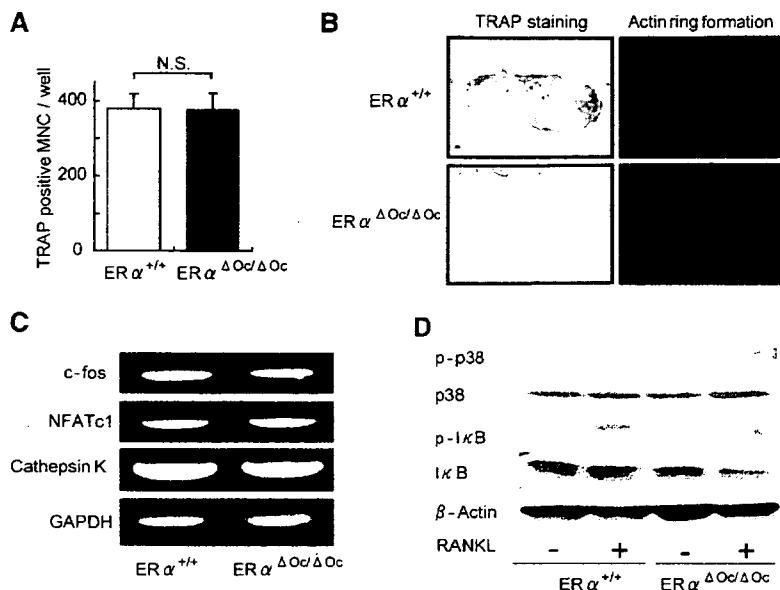


Figure 5. ER α Deficiency Did Not Affect Osteoclastogenesis

(A) TRAP-positive multinucleated cell count at 3 days after RANKL stimulation, cultured in 24-well plates ($n = 6$, N.S., not significant). Data are represented as mean \pm SEM.

(B) TRAP staining and actin ring formation of RANKL induced primary cultured osteoclasts from bone-marrow cells of ER $\alpha^{+/+}$ and ER $\alpha^{\Delta Oc/\Delta Oc}$ mice.

(C) RT-PCR analysis of genes related to osteoclastogenesis.

(D) Western blot analysis of phosphorylated p38, JNK, and I κ B of primary cultured bone-marrow cells stimulated with or without 100 ng/ml of RANKL for 15 min.

the search for candidate ER α target genes in bone by DNA microarray analysis (Figure S3), we found that a number of apoptosis-related factors were regulated by estrogen in the intact bone of ER $\alpha^{+/+}$ females but dysregulated in ER $\alpha^{\Delta Oc/\Delta Oc}$ females. This observation is consistent with a previous report of estrogen-induced apoptosis of mature osteoclasts (Kameda et al., 1997). Real-time RT-PCR to validate the estrogen regulations of the candidate genes revealed that gene expression of *FasL*, an apoptotic factor, was responsive to E2 (Figure 6A). Estrogen treatment (+E2) indeed induced expression of *FasL* protein in bone of ovariectomized ER $\alpha^{+/+}$, but this induction was not obvious in ovariectomized ER $\alpha^{\Delta Oc/\Delta Oc}$ mice (Figures 6B and 6C). Reflecting *FasL* induction by estrogen, estrogen-induced apoptosis (as observed by the TUNEL assay) in TRAP-positive mature trabecular osteoclasts in the distal femurs of the ER $\alpha^{+/+}$ mice was detected, but this E2 response was abolished in the ER $\alpha^{\Delta Oc/\Delta Oc}$ mice (Figure 6D). Furthermore, in mice lacking functional *FasL* (*FasL^{gld/gld}*), neither enhanced bone resorption nor bone mass loss was induced by ovariectomy (Figures 6E and 6F).

Osteoclastic ER α Mediates Estrogen-Induced apoptosis by *FasL*

The expression level of ER α protein in differentiated osteoclasts derived from bone marrow cells was very low, but induction of *FasL* gene expression was also detectable in the cultured osteoclasts of ER $\alpha^{+/+}$ females as well as males (Figure 7A). However, this E2 response was impaired in cultured osteoclasts from ER $\alpha^{\Delta Oc/\Delta Oc}$ females (Figure 7A). It is notable that such responses are also induced by tamoxifen (Figure 7C), which is an osteoprotective SERM (Harada and Rodan, 2003). ER α overexpression augmented *FasL* gene expression in response to estrogen in cultured osteoclasts from ER $\alpha^{\Delta Oc/\Delta Oc}$ females

(Figure S4A). In primary cultured calvarial osteoblasts from females as well as males (Suzawa et al., 2003), *FasL* gene induction by E2 and tamoxifen was also seen; however, it was not accompanied by increased apoptosis (data not shown). Thus, it appears that estrogen-induced apoptosis in osteoclasts is mediated by *FasL* expression in osteoclasts in the trabecular bone areas, presumably as well as in osteoblasts in cortical bone areas. As expected, the cell number of TUNEL-positive osteoclasts was increased by E2 in the cultured osteoclasts from ER $\alpha^{+/+}$ females, but E2-induced apoptosis was undetectable in ER $\alpha^{\Delta Oc/\Delta Oc}$ osteoclasts (Figure 7B). Consistent with *FasL*-induced apoptosis, *Fas* gene expression was observed (Figure 7D), but it was likely that *Fas* expression did not require ER α function (Figures S4B and S4C). Expression levels of *Fas* and ER α as well as E2 response in apoptosis appeared to fluctuate during osteoclast differentiation (Figures S4B–S4D); however, in *FasL^{gld/gld}* females, the E2-induced apoptosis was abolished (Figure S4E). These findings suggest that activated ER α in differentiated osteoclasts induces apoptosis through activating *FasL*/*Fas* signaling. This leads to suppression of bone resorption through truncating the already short life span of differentiated osteoclasts (Teitelbaum, 2006).

DISCUSSION

Selective ablation of ER α in mature osteoclasts in female mice shows that the osteoprotective effect of estrogen is mediated by osteoclastic ER α , at least in the trabecular regions of the tibiae, femur, and lumbar vertebrae of female mice. Activated ER α by estrogen as well as SERMs appears to truncate the already short life span (estimated at 2 weeks) of differentiated osteoclasts by inducing apoptosis through activation of the *Fas*/*FasL* system.

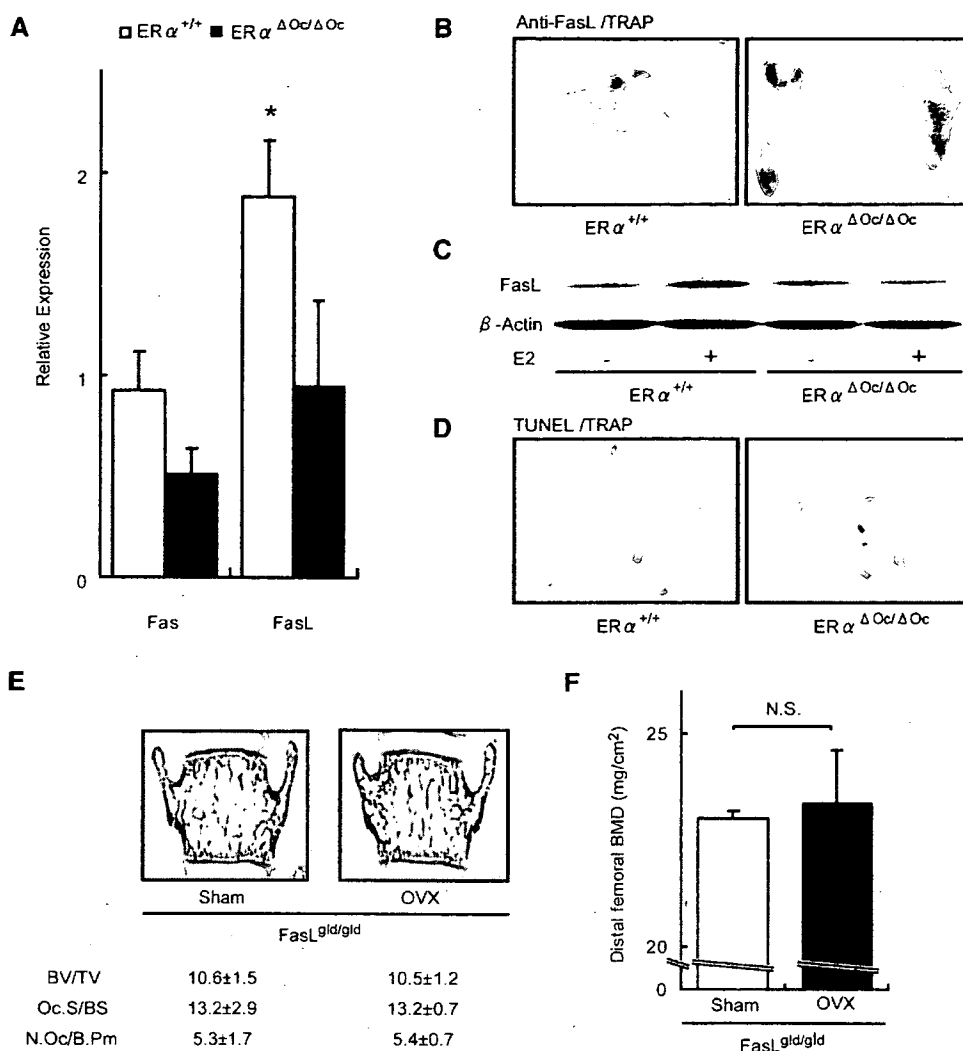


Figure 6. Activated ER α Induced Fas Ligand Expression and Apoptosis in Differentiated Osteoclasts of Intact Bone

(A) Real-time RT-PCR analysis of *Fas* and *FasL*. Expression levels in bones from E2-treated ovariectomized $ER\alpha^{+/+}$ (open column) and $ER\alpha^{\Delta Oc/\Delta Oc}$ (filled column) were compared with the ovariectomized groups of each genotype without E2 administration (* $p < 0.05$ compared to $ER\alpha^{+/+}$). Data are represented as mean \pm SEM.

(B) Immunohistochemical analysis of anti-FasL with TRAP staining of the sections from the distal femurs of E2-treated ovariectomized $ER\alpha^{+/+}$ and $ER\alpha^{\Delta Oc/\Delta Oc}$ mice. Brawny stained cells are anti-FasL positive.

(C) Anti-FasL western blot analysis of proteins obtained from femurs of ovariectomized $ER\alpha^{+/+}$ and $ER\alpha^{\Delta Oc/\Delta Oc}$ mice treated with or without E2, using anti- β -actin as internal control.

(D) TUNEL staining with TRAP staining of the sections from the distal femurs of E2-treated ovariectomized $ER\alpha^{+/+}$ and $ER\alpha^{\Delta Oc/\Delta Oc}$ mice. Arrowheads indicate both TUNEL (brown)- and TRAP-positive staining cells.

(E) Bone histomorphometrical analysis of sham-operated or ovariectomized $FasL^{gld/gld}$ mice.

(F) BMD of the distal femurs of sham operated or ovariectomized $FasL^{gld/gld}$ mice. Data are represented as mean \pm SEM.

This attenuates bone resorption. This idea is supported by previous observations that estrogen deficiency following menopause or ovariectomy leads to high bone turnover, particularly in the trabecular areas, as bone is rapidly lost through enhanced resorption (Delmas, 2002; Tolar et al., 2004). Thus, estrogen treatment leads to recovery from osteopenia by reducing resorption (Delmas, 2002; Rodan and Martin, 2000), partly by the induction of osteoclast cell death.

In contrast to the osteopenia seen in the $ER\alpha^{\Delta Oc/\Delta Oc}$ females, the $ER\alpha^{\Delta Oc/\Delta Oc}$ male mice unexpectedly had no bone loss. The male mice still demonstrated an ER α -mediated induction of FasL in response to estrogen with subsequent apoptosis of osteoclasts (Figure 7). Both male mice with a deficiency of aromatase that are unable to locally produce estrogen from testosterone and men with a genetic mutation in the *ER α* gene suffer from osteoporosis (Smith et al., 1994). Considering that the

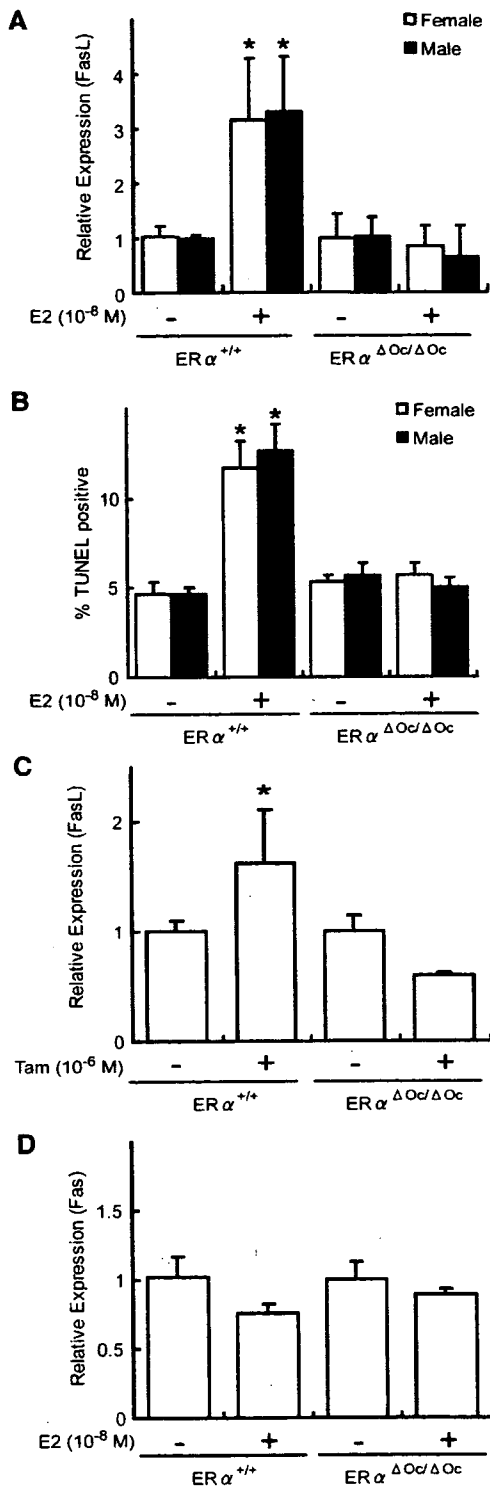


Figure 7. Estrogen-Induced FasL Expression and Apoptosis Required ERα in Cultured Osteoclasts

(A) Real-time RT-PCR analysis of *FasL* expression using total RNA obtained from in vitro primary cultured osteoclasts of each genotype at 3 days after RANKL stimulation, treated with or without E2 (10⁻⁸ M) for 4 hr (*p < 0.05 compared to the group treated without E2). Data are represented as mean ± SEM.

markedly elevated levels of testosterone in ERα KO females may be potent enough to maintain normal bone turnover (Syed and Khosla, 2005), it is likely that the activated AR might be functionally sufficient in male mice to compensate for the ERα deficiency in bone (Kawano et al., 2003). However, species differences in the osteoprotective action of sex steroid hormones still need to be carefully addressed.

Fas/FasL system-mediated apoptotic induction of osteoclasts by estrogen may well be a part of the mechanism for the antiresorptive action of estrogen and SERMs in trabecular bone areas (Delmas, 2002; Rodan and Martin, 2000; Simpson and Davis, 2001; Syed and Khosla, 2005; Tolar et al., 2004). Regulation of osteoclast differentiation is tightly coupled to osteoblastic function in terms of cytokine production and cell-cell contact (Karsenty and Wagner, 2002; Martin and Sims, 2005; Mundy and Eleftheriou, 2006; Teitelbaum and Ross, 2003). Indeed, upregulation of osteoclastogenic cytokines by ovariectomy was unaffected in ERα^{ΔOc/ΔOc} females. Considering the observation that cortical bone mass is increased in ovariectomized ERα^{ΔOc/ΔOc} females during estrogen treatment, it is conceivable that the antiresorptive estrogen action in cortical bone is also mediated by osteoblastic ERα. In this regard, FasL induction by estrogen was in fact detected in primary cultured osteoblasts from female calvaria by us as well as another group (S. Krum and M. Brown, personal communication). Thus, similar experiments in which ERα is selectively ablated in osteoblasts are needed to define the role of ERα in these cells.

In osteoclastic cells, expression of the *FasL* gene, which leads to apoptosis, appears to be positive controlled by activated ERα. Not surprisingly, a direct binding site for ERα has been mapped in the *FasL* gene locus (S. Krum and M. Brown, personal communication). An osteoclast- and cell-differentiation stage-specific mechanism may underlie this gene induction in the *FasL* gene promoter. A recent study demonstrated that ERα recruitment to specific promoter sites of given ERα target genes was cell-type specific (Carroll et al., 2005). Thus, there is significant impetus to identify the osteoclastic factor that associates with ERα in the *FasL* gene promoter. Such identification will lead to a better understanding of the molecular basis of the osteoprotective estrogen action and provide a target against which to develop SERMs of greater effectiveness.

(B) Apoptotic cells were defined as those with TUNEL-positive nuclei among TRAP-positive multinucleated primary cultured osteoclasts treated with or without E2 (10⁻⁸ M) for 12 hr in 96-well plates (*p < 0.05 compared to the group treated without E2). Data are represented as mean ± SEM.

(C) *FasL* expression in each genotypic female osteoclastic cells treated with or without Tam (10⁻⁶ M) (*p < 0.05 compared to the group treated without Tam). Data are represented as mean ± SEM.

(D) Expression of *Fas* was measured as described in the legend of Figure 7A. Data are represented as mean ± SEM.

EXPERIMENTAL PROCEDURES

Ctsk-Cre Construction and Generation of the Knockin Mouse Lines

An RP23-422n18 BAC clone containing the mouse *Ctsk* gene was purchased from Invitrogen (Carlsbad, CA). The *FRT-Kar1/Neo⁻-FRT* and *nlsCre* fragments were obtained from plasmids pSK2/3-*FRT-Neo* and pIC-*Cre*. Two homologous arms of 500 bp from the *Ctsk* gene were inserted into both sides of the *nlsCre-FRT-Kar1/Neo⁻-FRT* cassette in the pSK2/3-*FRT-Neo* plasmid. The *nlsCre-FRT-Kar1/Neo⁻-FRT* cassette was introduced into the endogenous ATG start site of the *Ctsk* gene by recombineering approaches (Copeland et al., 2001). Targeted BAC was reduced in size from 189 kb to 26 kb and subcloned into the pMC1-DTPA vector by the gap-repair method. The targeted T12 ES clones were selected after positive-negative selection with G418 and DT-A with Southern analysis, then aggregated with single eight-cell embryos from CD-1 mice (Yoshizawa et al., 1997). Chimeric mice were then crossed with a general deleter mouse line, *ACTB-Flpe* (Jackson Laboratory), to remove the *Kar1/Neo⁻* cassette. The *Ctsk-Cre* mice (*Ctsk^{Cre/+}*), originally on a hybrid C57BL/6 and CBA genetic background, were backcrossed for four generations into a C57BL/6J background. *FasL^{gld/gld}* mice were also purchased from Jackson Laboratory.

Analysis of Cre Recombinase Activities

Expression of the Cre transcript was detected by RT-PCR. Southern analysis using a Cre cDNA probe was performed with total RNA extracted from 12-week-old mice. To evaluate the specificity and efficiency of Cre-mediated recombination, we mated the *Ctsk^{Cre/+}* mice to CAG-CAT-Z reporter mice (kindly provided by J. Miyazaki) (Sakai and Miyazaki, 1997) and genotyped their offspring with Cre-specific primers. β -galactosidase activity of the expressed LacZ gene driven by the CAG promoter was expected to be detected in the given cells expressing functional Cre recombinase.

In Vitro Osteoclastogenesis and Ligand Application

Bone-marrow cells derived from 8-week-old mice were plated in culture dishes containing α -MEM (GIBCO-BRL) with 10% FBS (JRH) and 10 ng/ml M-CSF (Genzyme). After incubation for 48 hr, adherent cells were used as osteoclast precursor cells after washing out the nonadherent cells. Cells were cultured in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL (Peprotech) to generate osteoclast-like cells (Koga et al., 2004) for 3 days, so the total culture time was 5 days. Three days after RANKL stimulation, primary cultured osteoclasts were treated with 10^{-8} M of 17 β -estradiol (E2) (Sigma-Aldrich Co.) or 10^{-6} M 4-hydroxytamoxifen (Tam) (Sigma-Aldrich Co.) in phenol-red free medium.

Generation of Osteoclast-Specific ER α KO Mice

The ER α conditional (*ER $\alpha^{lox/lox}$*) (Dupont et al., 2000) and null alleles with a C57BL/6J background have been previously described. *ER $\alpha^{lox/lox}$* mice were crossed with *Ctsk^{Cre/+}* mice to generate *Ctsk^{Cre/+}; ER $\alpha^{lox/+}$* mice. *Ctsk^{Cre/+}; ER $\alpha^{+/+}$* (*ER $\alpha^{+/+}$*) and *Ctsk^{Cre/+}; ER $\alpha^{lox/lox}$* (*ER $\alpha^{ΔOc/ΔOc}$*) mice were obtained by crossing *Ctsk^{Cre/+}; ER $\alpha^{lox/+}$* with *ER $\alpha^{lox/+}$* mouse lines.

Radiological Analysis

Bone radiographs of the femurs of 12-week-old *Ctsk^{Cre/+}; ER $\alpha^{lox/lox}$* (*ER $\alpha^{ΔOc/ΔOc}$*) and *Ctsk^{Cre/+}; ER $\alpha^{+/+}$* (*ER $\alpha^{+/+}$*) littermates were visualized with a soft X-ray apparatus (TRS-1005; SOFTRON). BMD was measured by DXA using a bone mineral analyzer (DCS-600EX; ALOKA). Micro Computed Tomography scanning of the femurs was performed using a composite X-ray analyzer (NX-CP-C80H-IL; Nitetsu ELEX Co.) (Kawano et al., 2003). Tomograms were obtained with a slice thickness of 10 μ m and reconstructed at 12 \times 12 pixels into a 3D image by the volume-rendering method (VIP-Station; Teijin System Technology) using a computer.

Analysis of Skeletal Morphology

Twelve-week-old *Ctsk^{Cre/+}; ER $\alpha^{lox/lox}$* (*ER $\alpha^{ΔOc/ΔOc}$*) and *Ctsk^{Cre/+}; ER $\alpha^{+/+}$* (*ER $\alpha^{+/+}$*) littermates were double labeled with subcutaneous injections of 16 mg/kg of calcein (Sigma) at 4 and 2 days before sacrifice. Tibiae were removed from each mouse and fixed with 70% ethanol. They were stained with Villanueva bone stain for 7 days and embedded in methyl-methacrylate (Wako) (Yoshizawa et al., 1997). Frontal plane sections (5- μ m thick) of the proximal tibia were cut using a Microtome (LEICA). The cancellous bone was measured in the secondary spongiosa located 500 μ m from the epiphyseal growth plate and 160 μ m from the endocortical surface (Kawano et al., 2003; Nakamichi et al., 2003). Bone histomorphometric measurements of the tibia were made using a semiautomatic image analyzing system (System Supply) and a fluorescent microscope (Optiphot; Nikon). Similar measurements of the lumbar vertebral bodies were done as previously reported (Takeda et al., 2002). Standard bone histomorphometrical nomenclatures, symbols, and units were used as described in the report of the ASBMR Histomorphometry Nomenclature Committee.

Ovariectomy and Hormone Replacement

Female *Ctsk^{Cre/+}; ER $\alpha^{lox/lox}$* (*ER $\alpha^{ΔOc/ΔOc}$*) and *Ctsk^{Cre/+}; ER $\alpha^{+/+}$* (*ER $\alpha^{+/+}$*) littermates were ovariectomized or sham operated at 8–12 weeks of age for 2 weeks for all experiments, and slow releasing pellets of E2 (0.83 μ g/day) or placebo (Innovative Research, Sarasota, FL) were implanted subcutaneously in the scapular region behind the neck (Sato et al., 2004; Shiina et al., 2006).

Immunohistochemistry

Twelve-week-old *Ctsk^{Cre/+}; ER $\alpha^{lox/lox}$* (*ER $\alpha^{ΔOc/ΔOc}$*) and *Ctsk^{Cre/+}; ER $\alpha^{+/+}$* (*ER $\alpha^{+/+}$*) littermates were fixed with 4% PFA by perfusion. Serial sections of the brain (20 μ m thick) were divided into two groups and used for single labeling for the ER α or thionin to allow determination of the areas to be measured. Tibiae and femurs were decalcified in 10% EDTA for 2–4 weeks after fixation and then embedded in paraffin sections. Sections were incubated in L.A.B. solution (Polysciences) for 30 min to retrieve antigen. The cooled sections were incubated in 1% H₂O₂ for 30 min to quench endogenous peroxidase and then washed with 1% Triton X-100 in PBS for 10 min. To block nonspecific antibody binding, sections were incubated in blocking solution (DAKO) for 5 min. Sections were then incubated with anti-ER α (Santa Cruz, CA) and anti-FasL (Santa Cruz, CA) in blocking solution overnight at 4°C. Staining was then performed using the EnVision+ HRP System (Dako) and 3, 3'-diaminobenzidine tetrahydrochloride substrate (Sigma), counterstained with TRAP, dehydrated through an ethanol series and xylene, before mounting (Sato et al., 2004).

ER α Overexpression

Two days after RANKL stimulation, an expression vector of mouse ER α was transfected into immature osteoclastic cells from *ER $\alpha^{ΔOc/ΔOc}$* mice using Superfect (QIAGEN) as manufacturer's instruction.

Real-Time RT-PCR

One microgram of total RNA from each sample was reverse transcribed into first-strand cDNA with random hexamers using Superscript III reverse transcriptase (Invitrogen). Primer sets for all genes were purchased from Takara Bio. Inc. (Tokyo, Japan). Real-time RT-PCR was performed using SYBR Premix Ex Taq (Takara) with the ABI PRISM 7900HT (Applied Biosystems) according to the manufacturer's instructions. Experimental samples were matched to a standard curve generated by amplifying serially diluted products using the same PCR protocol. To correct for variability in RNA recovery and efficiency of reverse transcription, *Gapdh* cDNA was amplified and quantified in each cDNA preparation. Normalization and calculation steps were performed as reported previously (Takezawa et al., 2007).

TUNEL/TRAP Staining

The TUNEL method was performed using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (CHEMICON international) according to the manufacturer's instructions with a slight modification. This was followed by TRAP staining as previously reported (Kobayashi et al., 2000).

Cytokine Assays

Bone marrow and blood were collected at 2 weeks after sham operation or ovariectomy. Bone-marrow cells were cultured for 3 days in DMEM. The levels of TNF α , IL-1 α , and IL-6 in the culture media and serum RANKL were determined by ELISA (R&D Systems).

Western Blot

Osteoclast precursor cells were treated with or without 100 ng/ml of soluble RANKL. After 15 minutes, cell extracts were harvested from the cells using lysis buffer containing 100 mM Tris-HCl (pH 7.8), 150 mM NaCl, 0.1% Triton X-100, 5% protease inhibitor cocktail (Sigma), and 5% phosphatase inhibitor cocktail (Sigma). An equivalent amount of protein from each of the cell extracts and proteins of femoral bone extracted using ISOGEN was loaded for SDS-PAGE and transferred to PVDF membranes (Amersham Biosciences). The membranes were developed with enhanced chemiluminescence reagent (Amersham Biosciences) (Ohtake et al., 2003). Phosphorylation of p38 MAPK and I κ B were evaluated using antibodies purchased from Cell Signaling Technology (Koga et al., 2004) and anti-FasL antibody was purchased from Santa Cruz Biotechnology (sc-834).

Actin-Ring Formation

Cells were fixed for 15 min in warm 4% paraformaldehyde (PFA). After fixation, cells were washed three times with PBS with 0.1% Triton X-100 (PBST) and incubated with 0.2 U/ml rhodamine phalloidin (Molecular Probes) for 30 min and washed again three times in PBST.

Statistical Analysis

Data were analyzed by two-tailed student's t test. For all graphs, data are represented as mean \pm SEM.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and four figures and can be found with this article online at <http://www.cell.com/cgi/content/full/130/5/811/DC1/>.

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Accession Numbers

Microarray can be seen in Gene Expression Omnibus under accession number GSE7798.