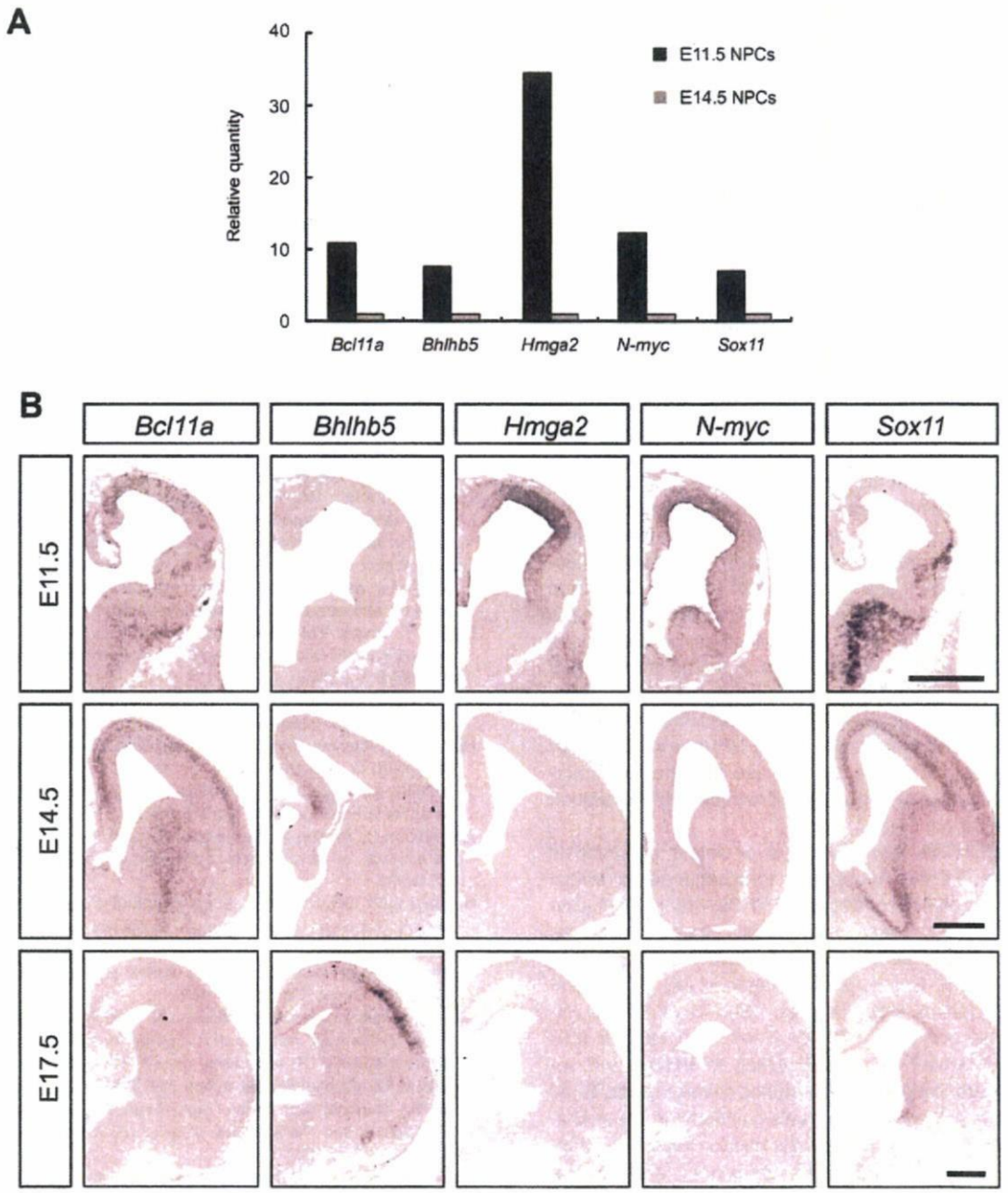

Fig. 2. *N-myc* and *Hmga2* are highly expressed in the VZ of E11.5 mouse brain. (A) Gene-specific real-time RT-PCR was performed to validate GeneChip analysis data. (B) *In situ* hybridization was performed for E11.5, E14.5 and E17.5 mouse brain sections. No signal was detected when sense-probes for each gene were used (data not shown). Scale bar=500 μm.

DISCUSSION

In this study, we compared NPC gene expression profiles at different developmental stages using Affymetrix GeneChips and the Percellome method, and then analyzed by *in situ* hybridization the spatio-temporal expression patterns of genes which were highly expressed in E11.5 NPCs. We found that *N-myc* and *Hmga2* were specifically expressed in E11.5 NPC both *in vivo* and *in vitro* and, furthermore, that the transduction of these genes into NPCs suppressed LIF-induced astrocytic differentiation without affecting DNA demethylation of the astrocyte-specific *gfap* gene promoter.

The basic HLH leucine zipper transcription factor N-myc,a member of the myc family of oncogenes, is a nuclear phosphoprotein exhibiting site-specific DNA-binding activity (Ramsay et al., 1986; Alex et al., 1992), and has

been reported to be expressed in a wide range of vertebrate tissues, primarily during embryogenesis (Schreiber-Agus et al., 1993). The mice deficient for functional *N-myc* are embryonic lethal (Stanton et al., 1992). Since N-myc has been shown to be a transcriptional activator, it may inhibit astrocyte differentiation via induction of neurogenic bHLH factors such as *Ngn1* (Sun et al., 2001), which have already been suggested to inhibit astrocyte differentiation in midgestational NPCs. However, this scenario seems unlikely because *N-myc* expression in NPCs did not affect neuronal differentiation, as assessed by monitoring expression of the neuronal marker βIII-tubulin (Fig. 3C, D). On the other hand, Hmga2 possesses an acidic C-terminal tail and three individual DNA-binding domains which bind short stretches of AT-rich DNA with high affinity (Reeves, 2001). *Hmga2* is expressed in pluripotent embryonic stem (ES) cells and in most tissues and organs during embryogenesis, but at very low levels or not at all in adult tissues (Zhou et al., 1995). Its function appears to be critical for cell growth, because mice lacking functional *Hmga2* exhibit a pygmy phenotype (Zhou et al., 1995). Recently, it was reported that Hmga2 specifically accumulates on senescent cell chromatin and that it functions as a structural component of senescence-associated heterochromatin foci and as a repressor of proliferation-associated genes (Narita et al., 2006). We therefore expected that Hmga2 would maintain the hypermethylation status of the astrocyte-specific *gfap* promoter via transcription-repressive heterochromatin formation in E11.5 NPCs. However, our results indicate that this is not the case. The mechanism(s) whereby N-myc and Hmga2 inhibit astrocyte differentiation must await further investigation.

Although DNA methylation is a critical cell-intrinsic determinant for the neurogenic-to-astrogliogenic switch and/or astrocyte differentiation of NPCs, many other spatio-temporally expressed extracellular factors such as CT-1, Notch and Wnt1 (Barnabe-Heider et al., 2005; Hirabayashi and Gotoh, 2005; Nagao et al., 2007) and intracellular factors including Ngn (Sun et al., 2001), N-CoR (Hermanson et al., 2002), N-myc and Hmga2 (this study) complement DNA methylation to ensure the sequential differentiation of NPCs during development. Thus, to better understand the mechanism underlying these processes, this study emphasizes the need to take cell-extrinsic cues, cell-intrinsic programs and factors, and their interaction into consideration.

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Estrogen Prevents Bone Loss via Estrogen Receptor α and Induction of Fas Ligand in Osteoclasts

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SUMMARY

Estrogen prevents osteoporotic bone loss by attenuating bone resorption; however, the molecular basis for this is unknown. Here, we report a critical role for the osteoclastic estrogen receptor α (ERα) in mediating estrogen-dependent bone maintenance in female mice. We selectively ablated ERα in differentiated osteoclasts ($ER\alpha^{\Delta Oc/\Delta Oc}$) and found that $ER\alpha^{\Delta Oc/\Delta Oc}$ females, but not males, exhibited trabecular bone loss, similar to the osteoporotic bone phenotype in postmenopausal women. Further, we show that estrogen induced apoptosis and upregulation of Fas ligand (FasL) expression in osteoclasts of the trabecular bones of WT but not $ER\alpha^{\Delta Oc/\Delta Oc}$ mice. The expression of ERα was also required for the induction of apoptosis by tamoxifen and estrogen in cultured osteoclasts. Our results support a model in which estrogen regulates the life span of mature osteoclasts via the induction of the Fas/FasL system, thereby providing an explanation for the osteoprotective function of estrogen as well as SERMs.

INTRODUCTION

Bone remodeling is a dynamic metabolic process. The destruction or "resorption" of pre-existing bone by mature osteoclasts is followed by the formation of new bone by osteoblasts. Osteoblasts are derived from pleiotropic mesenchymal stem cells in the bone marrow. Mature osteoclasts are multinuclear, macrophage-like cells, derived from hematopoietic stem cells also in the bone marrow. Bone resorption and deposition are tightly coupled, and their balance defines both bone mass as well as quality. The regulation of bone remodeling is complex. A number of systemic hormones and transcription factors directly regulate the proliferation and differentiation of osteoblasts and osteoclasts (Karsenty, 2006; Karsenty and Wagner, 2002; Rodan and Martin, 2000; Teitelbaum and Ross, 2003). Additionally, the indirect cellular communication among groups of bone cells is also physiologically critical for bone growth and remodeling (Martin and Sims, 2005; Mundy and Elefteriou, 2006). The molecular and genetic mechanisms governing bone cell fate have been intensively studied; however, how the life span of bone cells is determined on a molecular level remains elusive.

Estrogen is a key hormone in bone remodeling in several species. The osteoprotective action of estrogen is demonstrable in rodents and is clinically important in humans, particularly older women (Chien and Karsenty, 2005;

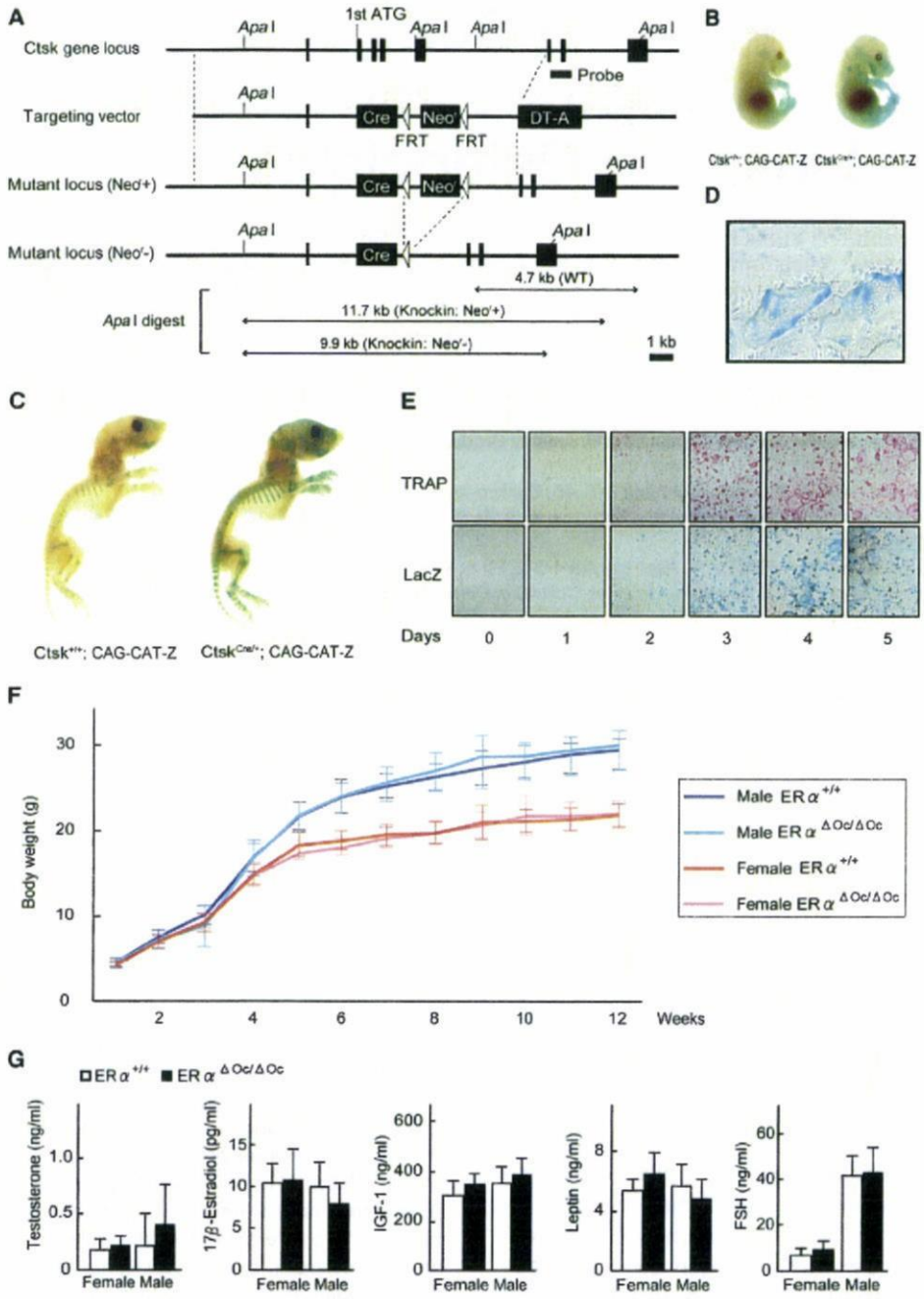

Figure 1. Generation of Knockin Mice Selectively Expressing Cre in Mature Osteoclasts

(A) Illustration of the targeting strategy for insertion of the *Cre* gene into the mouse *Cathepsin K* (*Ctsk*) gene. A targeting vector was generated to contain the *Cre* cDNA at the endogenous ATG start site, followed by a *FRT* (Flp-recombinase target)-flanked *Neo*^{r} cassette. The *DT-A* (diphtheria toxin-A) gene was also inserted to avoid random integrations.

(B and C) *Ctsk-Cre* mice were then crossed with *CAG-CAT-Z* mice. β-galactosidase activity derived from the activated *LacZ* reporter gene was monitored to test if expressed Cre excised the *loxP* sites in mature osteoclasts. LacZ expression patterns reflected the localization patterns of mature osteoclasts in the developing bone at 16.5 days post coitum embryos and in the skeletal tissues of 7-day-old pups.

(D) The LacZ expression induced by Cre-mediated excision was also seen in osteoclasts attached to trabecular bone in the lumber vertebrae of 12-week-old mice.

(E) LacZ expression was induced during osteoclastogenesis. Osteoclast-like cells that differentiated from bone-marrow macrophages following culture in the presence of M-CSF and RANKL were stained with TRAP (tartrate-resistant acid phosphatase), a mature osteoclast marker.

Delmas, 2002; Raisz, 2005; Rodan and Martin, 2000). Estrogen deficiency in postmenopausal women frequently leads to osteoporosis, the most common skeletal disorder. Similarly, ovariectomy clearly produces an osteoporotic bone phenotype in mice. Osteoporotic bone loss is the result of high bone turnover in which bone resorption outpaces bone deposition (Rodan and Martin, 2000; Teitelbaum, 2007). This imbalance in bone turnover that is induced by estrogen deficiency in women and female rodents can be ameliorated with bio-available estrogens including selective estrogen receptor modulators (SERMs) (Riggs and Hartmann, 2003).

Estrogen and SERMs primarily act by regulating gene transcription via estrogen receptors (ERα, ERβ) (Couse and Korach, 1999; Shang and Brown, 2002). ERs belong to the nuclear receptor gene superfamily and act as ligand-inducible transcriptional factors (Mangelsdorf et al., 1995). ER dimers directly or indirectly associate with specific DNA elements in the target gene promoter (Shang and Brown, 2002) and control transcription through reorganizing chromatin structure and histone modifications (Belandia and Parker, 2003). Genetic mouse models (KO mice) lacking ERα ($ER\alpha^{-/-}$) and ERβ ($ER\beta^{-/-}$) provide insights into ER function (Mueller and Korach, 2001; Windahl et al., 2002). In mice, though ERα appears to be the major receptor in most estrogen target tissues including bone (Sims et al., 2003), neither clear bone loss nor high bone turnover is detectable in ERα single or ERα/ERβ double-KO females (Syed and Khosla, 2005; Windahl et al., 2002). This unexpected maintenance of bone mass in female mutants is presumed to be due to unphysiologically elevated levels of other osteoprotective hormones, like androgens. Systemic defects in the hypothalamus caused by ER inactivation appear to impair the negative feedback system of hormone production (Syed and Khosla, 2005). This leads to an excess in estrogen precursors, notably androgens. In fact, the anabolic effects of androgens mediated by the androgen receptor (AR) are evident in female mice (Kawano et al., 2003; Sims et al., 2003). In males, estrogen is also osteoprotective, as is evident by the development of osteopenia in male patients genetically deficient in ERα (Smith et al., 1994) or aromatase activity (Simpson and Davis, 2001). Thus, irrespective of the accumulating clinical and basic research data on the osteoprotective actions of estrogen and SERMs, the molecular basis of this osteoprotection in females remains elusive.

To study the molecular interactions behind the antibone resorptive actions of estrogen in women and female animals, we genetically ablated ERα in mature osteoclasts ($ER\alpha^{\Delta Oc/\Delta Oc}$). Selective ablation of ERα in differentiated osteoclasts ($ER\alpha^{\Delta Oc/\Delta Oc}$) was accomplished by crossing a *Cathepsin K-Cre* knockin mouse with a floxed *ERα* mouse. This resulted in clear trabecular bone loss and high bone turnover associated with increased osteoclast numbers in females but not in males. In the female mutants, further bone loss following ovariectomy was not significant and recovery by estrogen was ineffective in the trabecular areas of long bones and lumbar vertebral bodies. Upregulated expression of *Fas ligand* (*FasL*) gene, and increased apoptosis in differentiated osteoclasts by estrogen was found in the intact bone of wild-type females but undetectable in $ER\alpha^{\Delta Oc/\Delta Oc}$ females. Induction of FasL and apoptosis by estrogen as well as a SERM also required ERα in cultured osteoclasts. Thus, we propose that the osteoprotective actions of estrogen and SERMs are mediated at least in part through osteoclastic ERα in trabecular bone, and the life span of mature osteoclasts is regulated through the activation of the FasL signaling.

RESULTS

Generation of Osteoclast-Specific *ERα* Gene Disruption by Knocked-In *Cre* in the *Cathepsin K* Gene

To specifically disrupt *ERα* gene in mature osteoclasts, we knocked in *Cre* into the gene locus of *Cathepsin K* ($Ctsk^{Cre/+}$) (Figures 1A, S1A, and S1B), a gene known to be expressed in differentiated osteoclastic cells arising from hematopoietic stem cells. This gene is functionally indispensable for mature osteoclasts (Saftig et al., 1998). Only one copy appears enough to support normal bone formation and bone turnover, since heterozygous mutant mice of *Cathepsin K* ($Ctsk^{+/-}$) have no obvious bone phenotype (Gowen et al., 1999; Li et al., 2006; Saftig et al., 1998) . Clear, bone-specific expression of the *Cre* transcript in the adult $Ctsk^{Cre/+}$ mice was observed in the tested tissues (Figure S1C). To confirm Cre protein expression, the $Ctsk^{Cre/+}$ mice were crossed with tester mice (*CAG-CAT-Z*). These mice were genetically engineered to express β-galactosidase by excision of the transcribed stop sequence in front of the *β-galactosidase* gene (*LacZ*) in cells expressing Cre (Sakai and Miyazaki, 1997). β-galactosidase expression visualized by LacZ staining was observed in the bones of 16.5 dpc embryos and 7-day-old pups of $Ctsk^{Cre/+}$; *CAG-CAT-Z* mice. Expression patterns were consistent with the appearance and skeletal localization of functionally mature osteoclasts (Figures 1B and 1C). Histochemical staining of LacZ in the lumbar vertebrae of 12-week-old mice was localized in multinuclear osteoclasts (Figure 1D) but not seen in osteoblasts and osteocytes (Figure S1D) and the hypothalamus (Figure S1E). Since *Cathepsin K* gene expression is evident in differentiated osteoclasts (Saftig et al., 1998), we used an in vitro culture cell system to test whether Cre expression was driven by the endogenous promoter that is induced at the time of osteoclast differentiation. Osteoclast-precursor cells derived from bone marrow

(F) The growth curve of $ER\alpha^{\Delta Oc/\Delta Oc}$ mice was indistinguishable from that of the control mice. Data are represented as mean ± SEM.
(G) Serum hormone levels were normal in 12-week-old $ER\alpha^{\Delta Oc/\Delta Oc}$ (filled column) versus $ER\alpha^{+/+}$ (open column) mice (n = 10–11 animals per genotype). Data are represented as mean ± SEM.

were cytodifferentiated for 1 week in the presence of M-CSF (macrophage colony stimulating factor) and RANKL (receptor activator of NFκB 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 $ER\alpha$ mice (Dupont et al., 2000) with $Ctsk^{Cre/+}$ mice to disrupt ERα in differentiated osteoclasts ($ER\alpha^{\Delta Oc/\Delta Oc}$). Excision of the $ER\alpha$ 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 $Ctsk^{Cre/+}$; $ER\alpha^{+/+}$ ($ER\alpha^{+/+}$) and the $Ctsk^{Cre/+}$; $ER\alpha^{flox/flox}$ ($ER\alpha^{\Delta Oc/\Delta Oc}$) 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 $ER\alpha^{\Delta Oc/\Delta Oc}$ mice at 12 weeks (Figure 1G).

Osteopenia Occurred in Osteoclast-Specific ERα KO Females But Not Males

The 12-week-old $ER\alpha^{\Delta Oc/\Delta Oc}$ females exhibited a clear reduction in bone mineral density (BMD) in the femurs (Figures 2A–2C) and tibiae (data not shown) when compared with $ER\alpha^{+/+}$ 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 ERα activity (Simpson and Davis, 2001; Smith et al., 1994), $ER\alpha^{\Delta Oc/\Delta Oc}$ males unexpectedly exhibited no clear bone loss even in the trabecular areas (Figures 2A–2C). In $ER\alpha^{\Delta Oc/\Delta Oc}$ 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 $ER\alpha^{\Delta Oc/\Delta Oc}$ 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 findings, it is conceivable that the increased number of differentiated osteoclasts following ERα ablation accelerates bone resorption over formation, leading to bone loss in the trabecular areas.

No Further Bone Loss Results from Estrogen Deficiency in $ER\alpha^{\Delta Oc/\Delta Oc}$ Females

To verify whether osteoclastic ERα indeed mediates osteoprotective estrogen actions, estrogen action was investigated by ovariectomy (OVX) of 12-week-old female mice. As expected, OVX in $ER\alpha^{+/+}$ 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 TNFα and IL-1α (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 $ER\alpha^{\Delta Oc/\Delta Oc}$ 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 ERα.

Estrogen Treatment Failed to Rescue the Osteoporotic Bone Phenotype of $ER\alpha^{\Delta Oc/\Delta Oc}$ Mice

Estrogen treatment by estrogen pellet implantation (OVX + E2) for 2 weeks after OVX in $ER\alpha^{+/+}$ 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 ($ER\alpha^{\Delta Oc/\Delta Oc}$) was not as pronounced as in the $ER\alpha^{+/+}$ 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 $ER\alpha^{\Delta Oc/\Delta Oc}$ 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 ERα inhibiting bone resorption.

To further test this hypothesis, we investigated ERα protein expression in mature osteoclasts from trabecular bone. Few reports document osteoclastic expression of ERα 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 ERα protein expression is drastically decreased during culture (Kato et al., 1989). Using highly sensitive immunohistochemistry, we investigated whether

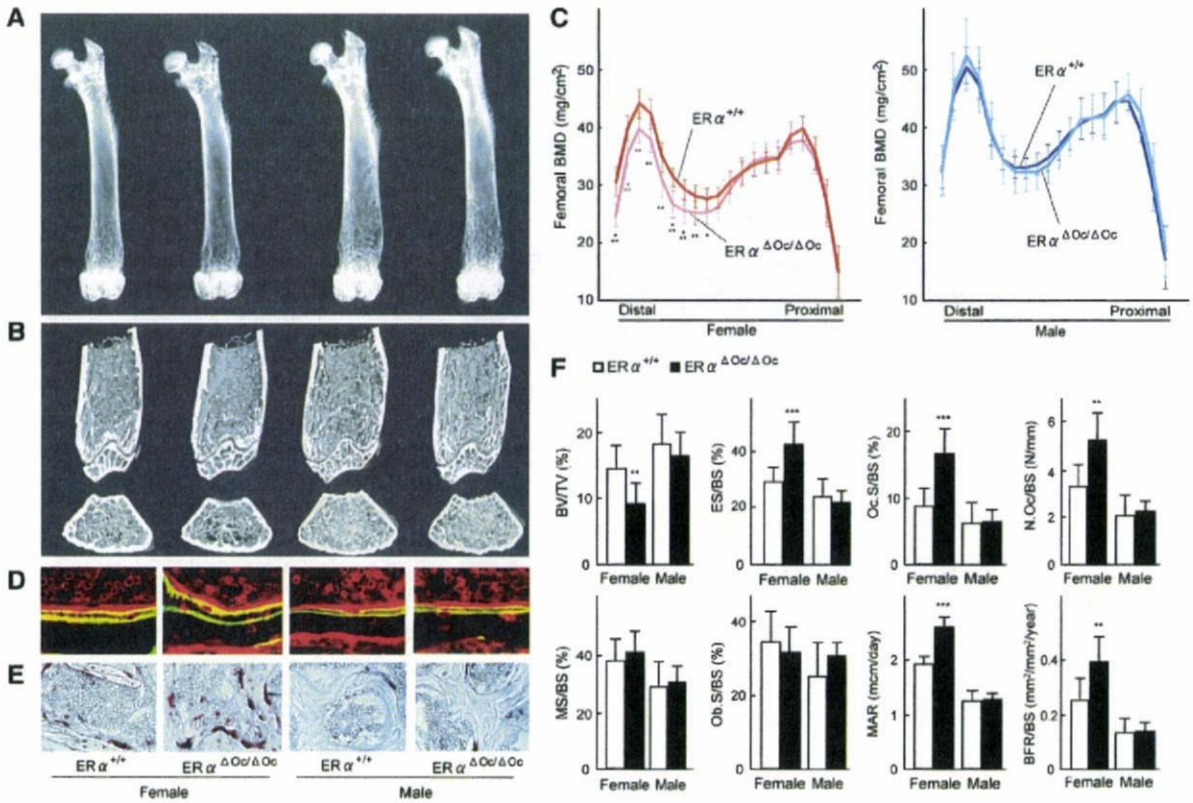

Figure 2. High Bone Turnover Osteopenia Was Observed in $ER\alpha^{\Delta Oc/\Delta Oc}$ Females But Not Males

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

(C) BMD of each of 20 equal longitudinal divisions of femurs from 12-week-old $ER\alpha^{+/+}$ and $ER\alpha^{\Delta Oc/\Delta Oc}$ 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 ± SEM.

(D) Bone formation was also accelerated in $ER\alpha^{\Delta Oc/\Delta Oc}$ 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 ERα in osteoclasts, indicating enhanced bone resorption.

(F) Bone turnover parameters as measured by dynamic bone histomorphometry after calcein labeling indicated high bone turnover in $ER\alpha^{\Delta Oc/\Delta Oc}$ females. Parameters are measured in the proximal tibia of 12-week-old $ER\alpha^{+/+}$ (open column) and $ER\alpha^{\Delta Oc/\Delta Oc}$ (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 ± SEM.

ERα protein expresses in differentiated osteoclasts in the bone tissues of femur sections from 12-week-old mice. ERα 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 ERα in primary cultured osteoblasts derived from calvaria of $ER\alpha^{\Delta Oc/\Delta Oc}$ females appeared unaffected (Figure S2C). In contrast, in differentiated osteoclasts of the same femur sections, ERα expression was definitely detectable but very low in the $ER\alpha^{+/+}$ but undetectable in $ER\alpha^{\Delta Oc/\Delta Oc}$ females (Figure 4C).

Signaling by Osteoclastogenic Factors and Osteoclastogenesis Is Intact in Osteoclasts Deficient in ERα

It is possible that the osteoprotective function of osteoclastic ERα inhibits osteoclastogenesis. To address this issue, osteoclastogenesis was tested in cultured osteoclasts derived from bone-marrow cells of $ER\alpha^{\Delta Oc/\Delta Oc}$ 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).

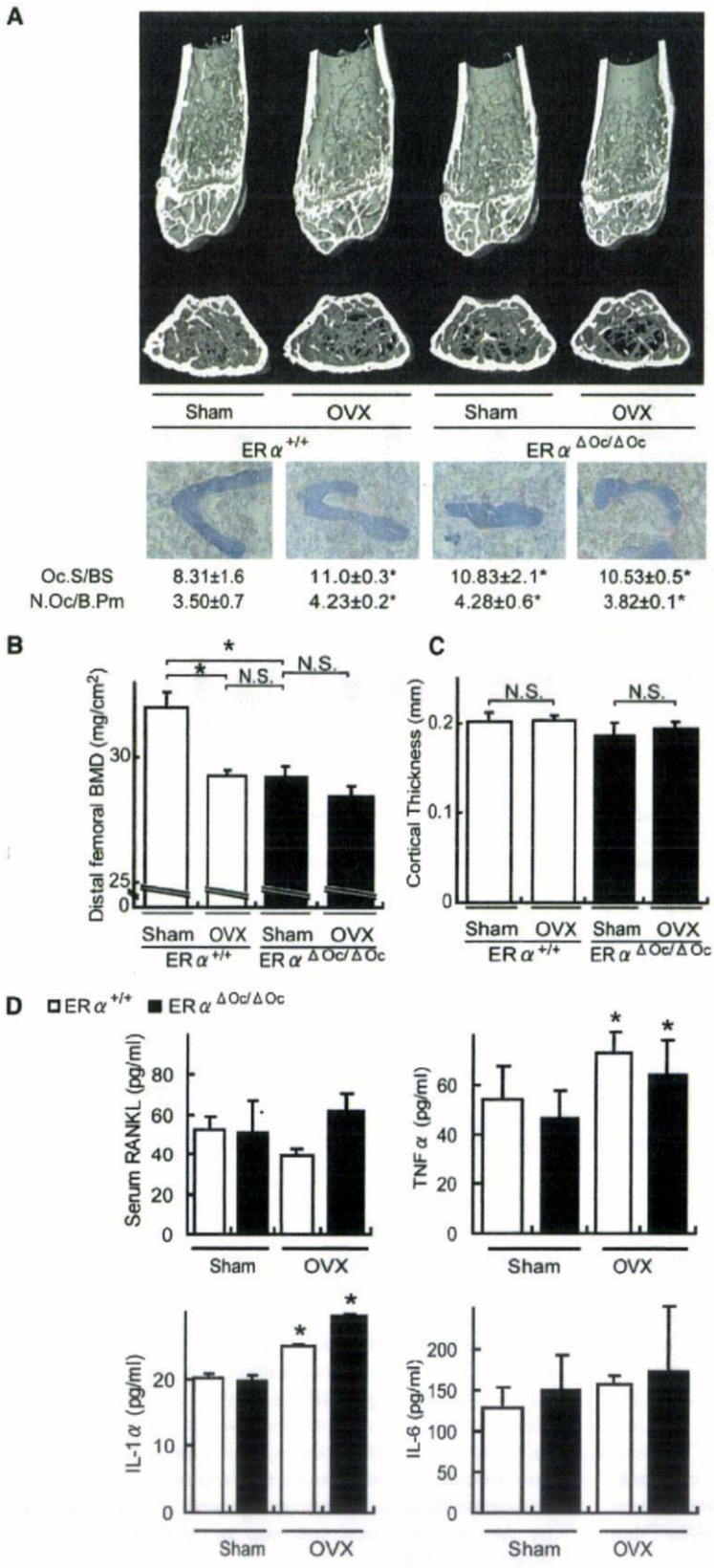

Figure 3. No Further Bone Loss of $ER\alpha^{\Delta Oc/\Delta Oc}$ Females by Ovariectomy

(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 ± SEM.

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

(D) The levels of TNFα, IL-1α, 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 ± SEM.

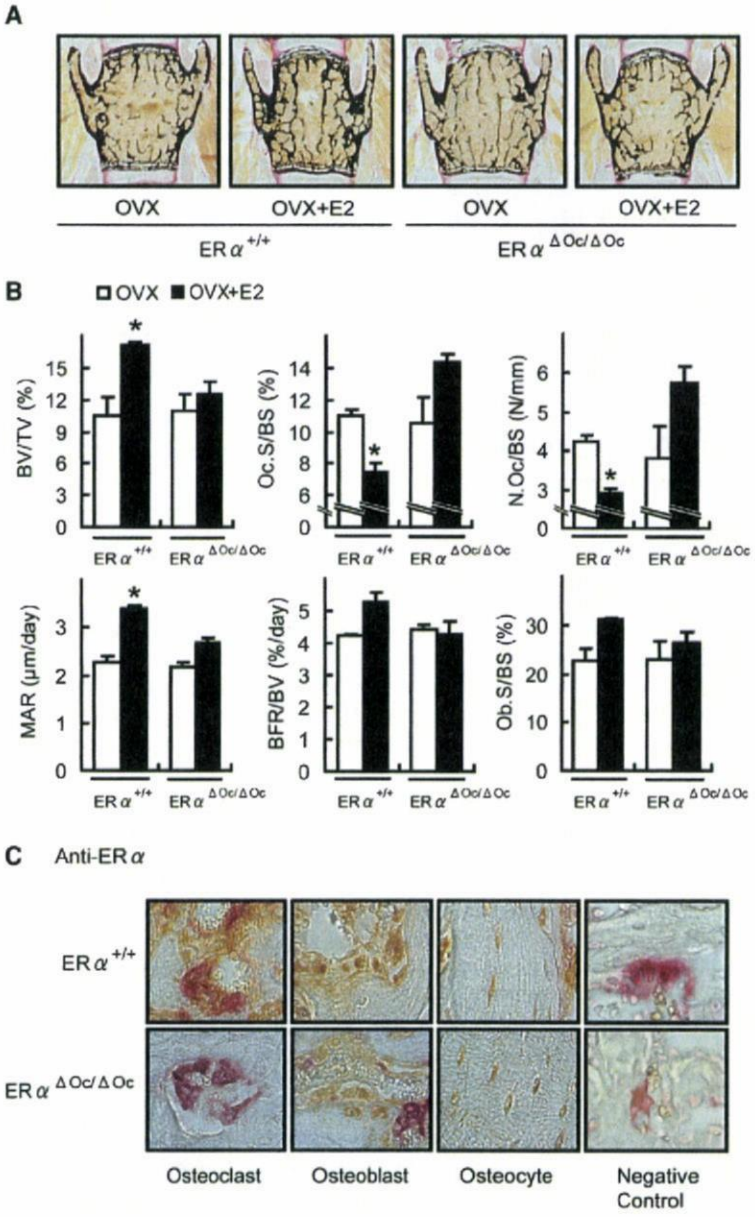

Figure 4. Estrogen treatment failed to reverse trabecular bone loss of ovariectomized $ER\alpha^{\Delta Oc/\Delta Oc}$ females

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

(B) Bone histomorphometrical analyses of the lumber vertebral bodies of 12-week-old ovariectomized $ER\alpha^{+/+}$ (left columns) and $ER\alpha^{\Delta Oc/\Delta Oc}$ (right columns) mice with (filled columns) or without (open columns) E2 treatment for 2 weeks (*$p < 0.05$ compared with E2-treated ovariectomized $ER\alpha^{\Delta Oc/\Delta Oc}$ 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 ± SEM.

(C) Immunochemical identification of ERα (brown) in TRAP-positive (red) differentiated osteoclasts. The femurs of 12 week-old mice were used for the immunodetection of ERα 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 $ER\alpha^{\Delta Oc/\Delta Oc}$ females was almost the same as that from $ER\alpha^{+/+}$ females (Figure 5A) and males (data not shown). The differentiated $ER\alpha^{\Delta Oc/\Delta Oc}$ 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 ERα deficiency in differentiated osteoclasts (Figure 5C). Furthermore, responses to RANKL in intracellular signaling, as represented by phosphorylation of p38 and IκB, were unaffected in $ER\alpha^{\Delta Oc/\Delta Oc}$ osteoclasts from females (Figure 5D) as well as males (data not shown). In light of these findings, it is unlikely that activated ERα in osteoclastic cells attenuates osteoclastogenesis.

Activation of the Fas/FasL System by Estrogen in Intact Bone Is Impaired by Osteoclastic ERα Deficiency

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

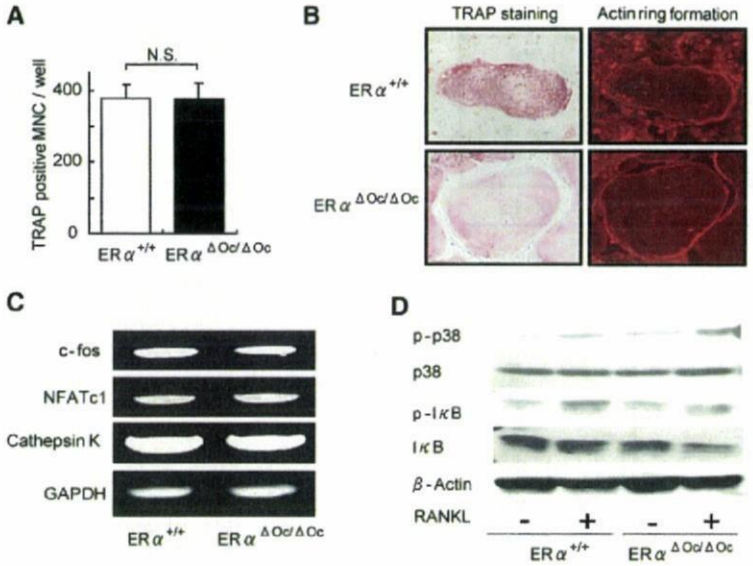

Figure 5. ERα Deficiency Did Not Affect Osteoclastogenesis

(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 ± SEM.

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

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

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

the search for candidate ERα 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 $ERα^{+/+}$ females but dysregulated in $ERα^{ΔOc/ΔOc}$ 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 $ERα^{+/+}$, but this induction was not obvious in ovariectomized $ERα^{ΔOc/ΔOc}$ 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 $ERα^{+/+}$ mice was detected, but this E2 response was abolished in the $ERα^{ΔOc/ΔOc}$ 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 ERα Mediates Estrogen-Induced apoptosis by FasL

The expression level of ERα 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 $ERα^{+/+}$ females as well as males (Figure 7A). However, this E2 response was impaired in cultured osteoclasts from $ERα^{ΔOc/ΔOc}$ 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). ERα overexpression augmented *FasL* gene expression in response to estrogen in cultured osteoclasts from $ERα^{ΔOc/ΔOc}$ 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 $ERα^{+/+}$ females, but E2-induced apoptosis was undetectable in $ERα^{ΔOc/ΔOc}$ 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 ERα function (Figures S4B and S4C). Expression levels of *Fas* and *ERα* as well as E2 response in apoptosis appeared to fluctuate during osteoclast differentiation (Figures S4B–S4D); however, in FasL mutant ($FasL^{gld/gld}$) females, the E2-induced apoptosis was abolished (Figure S4E). These findings suggest that activated ERα 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 ERα in mature osteoclasts in female mice shows that the osteoprotective effect of estrogen is mediated by osteoclastic ERα, at least in the trabecular regions of the tibiae, femur, and lumbar vertebrae of female mice. Activated ERα 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.

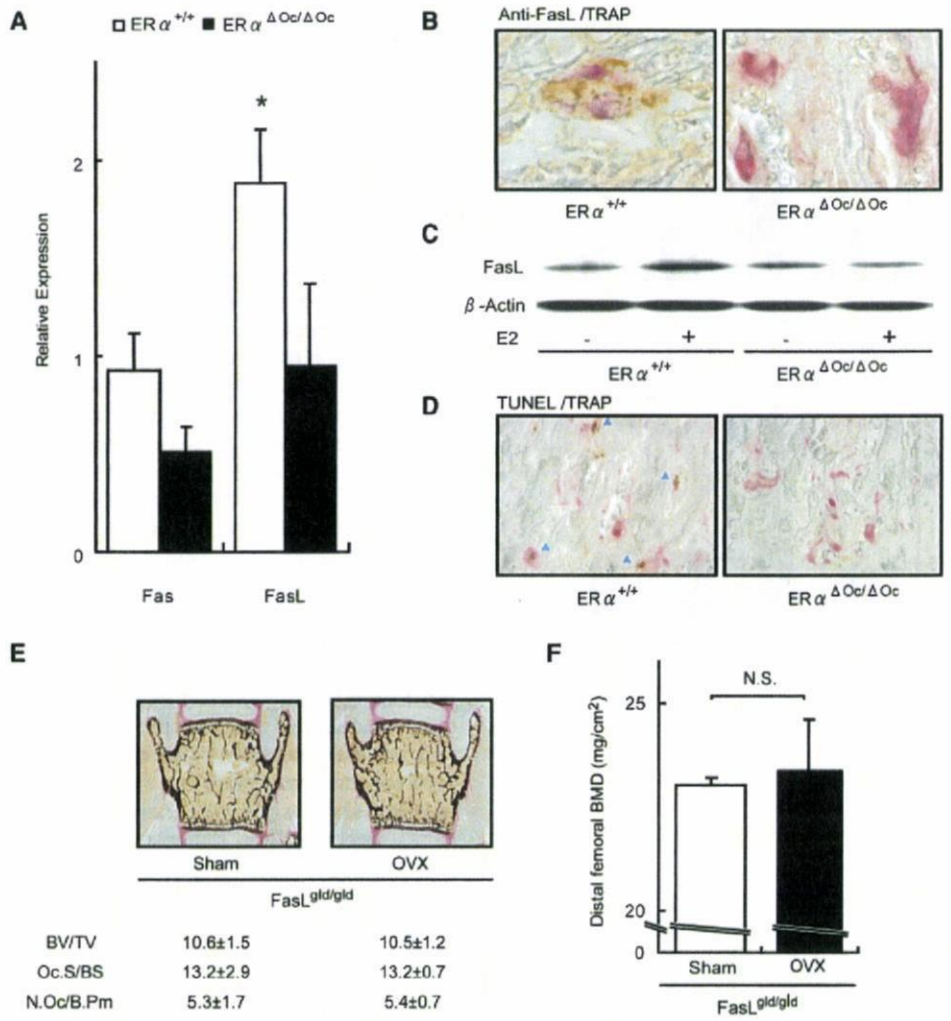

Figure 6. Activated ERα Induced Fas Ligand Expression and Apoptosis in Differentiated Osteoclasts of Intact Bone

(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 ± 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 ± 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 ERα-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 *ERα* gene suffer from osteoporosis (Smith et al., 1994). Considering that the

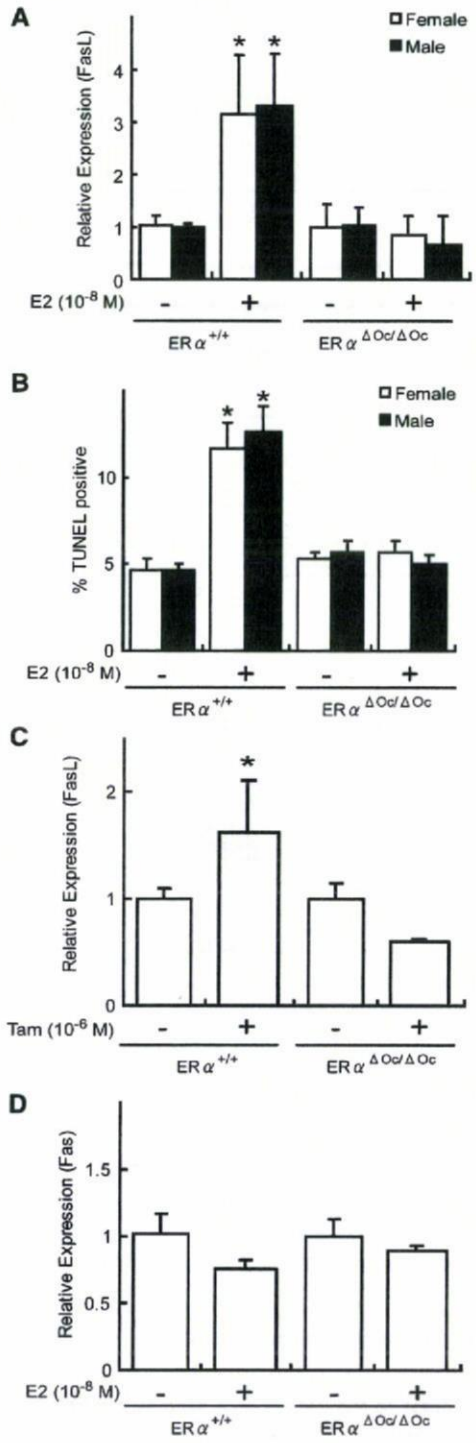

Figure 7. Estrogen-Induced *FasL* Expression and Apoptosis Required ERα in Cultured Osteoclasts

(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.

(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.

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 Elefteriou, 2006; Teitelbaum and Ross, 2003). Indeed, upregulation of osteoclastogenic cytokines by ovariectomy was unaffected in $ER\alpha^{\Delta Oc/\Delta Oc}$ females. Considering the observation that cortical bone mass is increased in ovariectomized $ER\alpha^{\Delta Oc/\Delta Oc}$ 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 in osteoblasts may contribute to the osteoprotective estrogen action, and *FasL* gene 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.

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-Kan^r/Neo^r-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-Kan^r/Neo^r-FRT* cassette in the pSK2/3-*FRT-Neo* plasmid. The *nlsCre-FRT-Kan^r/Neo^r-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 TT2 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 *Kan^r/Neo^r* 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 17β-estradiol (E2) (Sigma-Aldrich Co.) or 10^{-6} M 4-hydroxytamoxifen (Tam) (Sigma-Aldrich Co.) in phenol-red free medium.

Generation of Osteoclast-Specific ERα KO Mice

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

Radiological Analysis

Bone radiographs of the femurs of 12-week-old *Ctsk$^{Cre/+}$; ERα$^{flox/flox}$* (*ERα$^{ΔOc/ΔOc}$*) and *Ctsk$^{Cre/+}$; ERα$^{+/+}$* (*ERα$^{+/+}$*) 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: Nittetsu ELEX Co.) (Kawano et al., 2003). Tomograms were obtained with a slice thickness of 10 μm and reconstructed at 12 × 12 pixels into a 3D image by the volume-rending method (VIP-Station; Teijin System Technology) using a computer.

Analysis of Skeletal Morphology

Twelve-week-old *Ctsk$^{Cre/+}$; ERα$^{flox/flox}$* (*ERα$^{ΔOc/ΔOc}$*) and *Ctsk$^{Cre/+}$; ERα$^{+/+}$* (*ERα$^{+/+}$*) 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-μm thick) of the proximal tibia were cut using a Microtome (LEICA). The cancellous bone was measured in the secondary spongiosa located 500 μm from the epiphyseal growth plate and 160 μm 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 Histomophometry Nomenclature Committee.

Ovariectomy and Hormone Replacement

Female *Ctsk$^{Cre/+}$; ERα$^{flox/flox}$* (*ERα$^{ΔOc/ΔOc}$*) and *Ctsk$^{Cre/+}$; ERα$^{+/+}$* (*ERα$^{+/+}$*) 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 μg/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$^{Cre/+}$; ERα$^{flox/flox}$* (*ERα$^{ΔOc/ΔOc}$*) and *Ctsk$^{Cre/+}$; ERα$^{+/+}$* (*ERα$^{+/+}$*) littermates were fixed with 4% PFA by perfusion. Serial sections of the brain (20 μm thick) were divided into two groups and used for single labeling for the ERα 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% H_2O_2 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-ERα (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′-diaminobendizine tetrahydrochloride substrate (Sigma), counterstained with TRAP, dehydrated through an ethanol series and xylene, before mounting (Sato et al., 2004).

ERα Overexpression

Two days after RANKL stimulation, an expression vector of mouse ERα was tranfected into immature osteoclastic cells from *ERα$^{ΔOc/ΔOc}$* mice using Superfect (QIAGEN) as manufacture'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 TNFα, IL-1α, 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 IκB 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 ± 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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Chapel Hill bisphenol A expert panel consensus statement: Integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure

Keywords: Bisphenol A; *In vitro*; *In vivo*; Rat; Mouse; Aquatic animal; Cancer: Low dose; Non-monotonic dose–response curves; Developmental programming

1. Introduction

This document is a summary statement of the outcome from the meeting: "*Bisphenol A: An Examination of the Relevance of Ecological, In vitro and Laboratory Animal Studies for Assessing Risks to Human Health*" sponsored by both the NIEHS and NIDCR at NIH/DHHS, as well as the US-EPA and Commonweal on the estrogenic environmental chemical bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl)propane; CAS# 80-05-7). The meeting was held in Chapel Hill, NC, 28–30 November 2006 due to concerns about the potential for a relationship between BPA and negative trends in human health that have occurred in recent decades. Examples include increases in abnormal penile/urethra development in males, early sexual maturation in females, an increase in neurobehavioral problems such as attention deficit hyperactivity disorder (ADHD) and autism, an increase in childhood and adult obesity and type 2 diabetes, a regional decrease in sperm count, and an increase in hormonally mediated cancers, such as prostate and breast cancers. Concern has been elevated by published studies reporting a relationship between treatment with "low doses" of BPA and many of theses negative health outcomes in experimental studies in laboratory animals as well as *in vitro* studies identifying plausible molecular mechanisms that could mediate such effects. Importantly, much evidence suggests that these adverse effects are occurring in animals within the range of exposure to BPA of the typical human living in a developed country, where virtually everyone has measurable blood, tissue and urine levels of BPA that exceed the levels produced by doses used in the "low dose" animal experiments.

Issues relating to BPA were extensively discussed by five panels of experts prior to and during the meeting, and are summarized in five reports included in this issue: (1) human exposure to bisphenol A (BPA) [1]; (2) *in vitro* molecular mechanisms of bisphenol A action [2]; (3) *in vivo* effects of bisphenol A in laboratory animals [3]; (4) an ecological assessment of bisphenol A: evidence from comparative biology [4]; (5) an evaluation of evidence for the carcinogenic activity of bisphenol A [5]. Further discussion occurred at the meeting where participants from the panels were reorganized into four breakout groups. The consensus statements from the meeting are presented below.

The definition of "low dose" of BPA at this meeting used the same two criteria established at a prior NIH meeting concerning the low dose endocrine disruptor issue [6]: (1) for laboratory animal studies "low doses" involved administration of doses below those used in traditional toxicological studies conducted for risk assessment purposes. For BPA the lowest dose previously examined for risk assessment purposes was 50 mg (kg^{-1} day^{-1}) in studies with rats and mice. The 50 mg (kg^{-1} day^{-1}) dose is the currently accepted lowest adverse effect level (LOAEL) that was used to calculate the current US-EPA reference dose (the daily dose that EPA calculates is safe for humans over the lifetime) of 50 μg (kg^{-1} day^{-1}). The current reference dose is thus based on "high dose" experiments conducted in the 1980s [7]. (2) "Low dose" also refers to doses within the range of typical human exposure (excluding occupational exposures). For purposes of this meeting, the published literature that was reviewed met both of these criteria for being considered within the "low dose" range.

Hundreds of *in vitro* and *in vivo* studies regarding the mechanisms and effects of low doses of BPA, as well as studies of biomonitoring and sources of exposure, have been published in peer reviewed journals over the last 10 years, since the first "low dose" BPA *in vivo* studies were published [8–10]. The meeting was convened specifically to integrate this relatively new information. This task required the combined expertise of scientists from many different disciplines, and care was taken to ensure that participants covered these diverse areas.

BPA is a high-volume (>6 billion pounds per year) production chemical used to make resins and polycarbonate plastic [11]. Of particular concern is the use of BPA in food and beverage plastic storage and heating containers and to line metal cans. In addition, potential environmental sources of BPA contamination are due to use in dental fillings and sealants [12], losses at the production site [13], leaching from landfill [14,15], and presence in indoors air [16].

BPA has become a chemical of "high concern" only in recent years, even though BPA was shown to stimulate the reproductive

Abbreviations: ADHD, attention deficit hyperactivity disorder; BADGE, bisphenol A diglycidyl ether; BIS-DMA, bisphenol A dimethacrylate; BIS-GMA, bisphenol A glycerolate dimethacrylate; BPA, bisphenol A; ER, estrogen receptor

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system in female rats and thus to be an "environmental estrogen" in 1936 [17], long before it was used as the monomer to synthesize polycarbonate plastic and resins in the early 1950s. However, more recent evidence has shown that BPA also exhibits other modes of endocrine disruption in addition to binding to estrogen receptors, such as alterations in endogenous hormone synthesis, hormone metabolism and hormone concentrations in blood. BPA also results in changes in tissue enzymes and hormone receptors, and interacts with other hormone-response systems, such as the androgen and thyroid hormone receptor signaling systems. While BPA was initially considered to be a "weak" estrogen based on a lower affinity for estrogen receptor alpha relative to estradiol [18], research shows that BPA is equipotent with estradiol in its ability to activate responses via recently discovered estrogen receptors associated with the cell membrane [19–22]. It is through these receptors that BPA stimulates rapid physiological responses at low picogram per ml (parts per trillion) concentrations.

2. Purpose and organization of the BPA meeting

2.1. Topic-focused expert panels

To address the strength of the evidence regarding the published BPA research, an organizing committee was formed, and five panels of experts from different disciplines were established. Each panel had a chair or co-chairs and included a scientist who agreed to be primarily responsible, along with the chair, for preparing a preliminary draft of the panel's report. A web site was established on which all of the available electronic files of articles concerning BPA were posted, along with other pertinent information relating to the meeting. Prior to the meeting, the panel members began working on draft reports and communicated via electronic media and telephone conference calls. The resulting preliminary report from each panel was posted on the web site and distributed at the meeting for all participants to read. After the meeting, each panel completed a manuscript that is a part of this meeting report. These five panel reports were peer reviewed using the normal manuscript submission process to *Reproductive Toxicology*.The following specific concerns about BPA led to the five expert panels being established:

(1) Leaching of BPA occurs from the resin lining of metal cans and from plastic food and beverage containers under conditions of normal use. BPA is also detected in water and air samples.
(2) Parts per billion (ppb) levels of BPA that are unconjugated (not metabolized and thus biologically active) are detected in human blood and tissues in different countries, and these levels appear to be higher than blood levels that would be present in animals exposed to the US-EPA reference dose.
(3) BPA causes a wide range of adverse effects at "low doses" that are below the US-EPA reference dose in animals, both terrestrial and aquatic.
(4) There is evidence from *in vitro* mechanistic studies that indicates the potential for disruption of human and animal cell function at concentrations of BPA far below unconjugated levels typically found in human blood and tissues.
(5) There is evidence that at very low doses, BPA may be carcinogenic or increase susceptibility to cancer in animals.

The five panels each addressed a different topic related to their specific area of expertise with BPA and prepared a panel report that included documentation of the relevant published studies:

Panel (1) Sources and amounts of human exposure to BPA as well as pharmacokinetics.
Panel (2) *In vitro* studies related to the molecular mechanisms that mediate responses to BPA with an emphasis on studies using low doses.
Panel (3) *In vivo* studies of BPA at "low doses" in laboratory animals.
Panel (4) *In vivo* studies of BPA in aquatic wildlife and laboratory animals.
Panel (5) Relationship of BPA to cancers.

The purpose of the 3-day meeting was to provide an opportunity for members of the different panels to interact with each other to integrate information from different disciplines concerning low dose effects of BPA after each panel of experts had prepared a report in its specific area. The agenda of the meeting was designed to allow the members of the five panels to have time to discuss the information in their panel reports and finalize statements about the strength of the evidence for the literature that the panel had reviewed.

2.2. Integration of information by breakout groups

For the second part of the meeting the focus was on integrating the information from each of the panel reports. This was accomplished by assigning panel members to one of four breakout groups. The four replicate breakout groups were established using the following criteria, such that each breakout group should have

(1) At least two members from each of the five panels.
(2) A person from each panel who had published on BPA.
(3) A person with general knowledge of endocrine disruption research or endocrinology, but who had not necessarily published on BPA.
(4) A person with experience in the process of reaching consensus.
(5) A mixture of junior and senior investigators.

The charge to the replicate breakout groups was to individually integrate the information relating to the following four issues:

Issue (1) Determine the degree to which the findings on BPA mechanisms of action identify mechanisms and bioactive doses that explain results of the studies reported by the panel on *in vivo* laboratory animal studies. Determine the strength of the evidence for plausible mechanisms mediating *in vivo* effects at low doses. In

addition, identify any *in vivo* findings that are unexpected based on the *in vitro* literature.

Issue (2) Assess the degree to which ecological studies with wildlife are consistent with laboratory studies in similar and different species. For example, determine the similarity of exposure levels and types of responses seen in wildlife and laboratory animals.

Issue (3) Discuss the degree to which the low doses of BPA used in laboratory animal studies relate to the levels detected in human serum and tissues (including urine).

Issue (4) Assess the importance of life stage in the pharmacokinetics of BPA, levels of exposure to BPA, and the health effects of BPA in animals and humans.

3. Findings submitted by the four breakout groups

The reports from the breakout groups are presented below. The four breakout groups conducted a critical examination of the published research on BPA in relation to the four topics described above. Each of the breakout groups identified areas of knowledge and research gaps and made suggestions for future directions of research. In addition, each group identified which of the following two categories applied to specific outcomes:

- "We are confident of the following": this category applied when there were findings reported in multiple papers from multiple labs that were in agreement. There should have been no papers reporting conflicting findings, unless there were flaws in those papers, in which case the flaw(s) should have been identified.
- "We believe the following to be likely but requiring confirmation": This category applied when there were multiple consistent findings from one lab, or there may have been some conflicting reports along with reports of significant findings.

4. Levels of confidence for published BPA findings

The responses from the four different breakout groups were integrated together and organized based on levels of confidence. The criterion for a statement being included in a category was that there had to be consensus among all four of the breakout groups about the statement.

4.1. Based on existing data we are confident of the following

4.1.1. Issue 1: In vitro mechanistic research—laboratory animal research connection

1. *In vitro* studies have provided two routes of plausibility for low dose *in vivo* effects of BPA. These include binding to nuclear estrogen receptors that regulate transcription as well as estrogen receptors associated with the cell membrane that promote calcium mobilization and intracellular signaling. Receptors associated with the cell membrane are more sensitive to BPA than the nuclear receptors. Actions mediated by membrane associated receptor signaling may underlie much of the low dose BPA phenomena (effects have been reported at doses as low as 1 pM or 0.23 ppt). This increases the plausibility of effects at low doses, which are within the range of environmentally relevant doses (human and wildlife levels of exposure).
2. *In vitro* mechanistic information has informed us that exposing tissues to only an extremely narrow range of doses of BPA may lead to erroneous conclusions. Non-monotonic dose–response curves are encountered frequently in basic endocrinological research, and numerous examples have been reported for BPA reviewed in Refs. [18,23,24]. Because of this animal experiments on unstudied systems must avoid narrow dose ranges, especially the use of only a few very high doses. Thus, testing one or two doses and concluding that there are no effects is inappropriate. At somewhat higher doses than are required for estrogen receptor (ER)-mediated responses, BPA also interacts with androgen and thyroid hormone receptors, making predictions of effects at different doses very complex.
3. *In vitro* studies can dissect mechanisms of complicated effects observed *in vivo*. The proposed potential mechanisms acting *in vitro* and *in vivo* are the same, involving estrogen receptor mediated (nuclear- and membrane-associated) actions. However, specific effects are dose and cell/tissue specific. In addition, there are *in vivo* processes that are not reflective of currently known mechanisms that have been identified *in vitro*. This is due to previously unknown mechanisms as well as the complexity (due to interactions among cell and tissue types) of *in vivo* systems.

4.1.2. Issue 2: Wildlife—laboratory animal research connection

1. BPA is found in the environment: aquatic, terrestrial and air.
2. Studies of wildlife demonstrate estrogenic responses that are similar to responses seen in laboratory animals. Specifically, reductions in spermatogenesis are seen in wildlife at ecological concentrations of BPA, and these effects are also seen in controlled laboratory studies with BPA. In addition, vitellogenin response is a common biomarker in non-mammalian wildlife and laboratory species for BPA-induced estrogen receptor activation as well as activation by other estrogens.
3. BPA exposure induces similar effects in reproductive systems in wildlife and experimental animal model systems, but concentrations used in experiments involving wildlife species are often higher than environmental exposures. There are conditions in the environment, such as landfill leachates and effluent outflow that cause episodic exposure of field populations to elevated doses of BPA.
4. Responses in a variety of vertebrate wildlife species are qualitatively consistent with controlled laboratory studies with BPA. Thus, animals in the wild show evidence of harm, and controlled laboratory studies with model aquatic animals (i.e., medaka, zebrafish, and fathead minnows) are consistent with observations made in wildlife species. Low dose effects of BPA (low ppb range) have been observed in many of these animals.

5. The similar effects observed in wildlife and laboratory animals exposed to BPA predict that similar effects are also occurring in humans.

4.1.3. Issue 3: Laboratory animal research—human exposure connection

1. Human exposure to BPA is widespread.
2. Human exposure to BPA is variable, and exposure levels cover a broad range [central tendency for unconjugated BPA: 0.3–4.4 ng ml^{-1} (ppb)] in tissues and fluids in fetuses, children and adults.
3. Because the current published literature states that there is a linear relationship between administered dose and circulating levels of BPA in animal studies, this allows circulating levels at lower administered doses to be predicted in experimental animals based on the results from studies in which higher doses were administered.
4. All of the currently published metabolic studies in rats predict circulating BPA levels after acute low dose oral exposures at blood levels less than or equal to 2 ng ml^{-1} (ppb), which is the approximate median and mean unconjugated circulating BPA level in humans. Therefore, the commonly reported circulating levels in humans exceed the circulating levels extrapolated from acute exposure studies in laboratory animals.
5. BPA levels in the fetal mouse exposed to BPA by maternal delivery of 25 μg kg^{-1}, a dose that has produced adverse effects in multiple experiments, are well within the range of unconjugated BPA levels observed in human fetal blood.

4.1.4. Issue 4: Life stage—relationship to exposure pharmacokinetics and health effects

1. Sensitivity to endocrine disruptors, including BPA, varies extensively with life stage, indicating that there are specific windows of increased sensitivity at multiple life stages. Therefore, it is essential to assess the impact of life stage on the response to BPA in studies involving wildlife, laboratory animals, and humans.
2. Developmental windows of susceptibility are comparable in vertebrate wildlife species and laboratory animals.
3. BPA alters "epigenetic programming" of genes in experimental animals and wildlife that results in persistent effects that are expressed later in life [25]. These organizational effects (functional and structural) in response to exposure to low doses of BPA during organogenesis persist into adulthood, long after the period of exposure has ended. Specifically, prenatal and/or neonatal exposure to low doses of BPA results in organizational changes in the prostate, breast, testis, mammary glands, body size, brain structure and chemistry, and behavior of laboratory animals.
4. There are effects due to exposure in adulthood that occurs at low doses of BPA. Substantial neurobehavioral effects and reproductive effects in both males and females have been observed during adult exposures in laboratory animals.
5. Adult exposure studies cannot be presumed to predict the results of exposure during development.
6. Life stage impacts the pharmacokinetics of BPA.

4.2. We believe the following to be likely but require confirmation

4.2.1. Issue 1: In vitro mechanistic research—laboratory animal research connection

1. BPA metabolism occurs in cell culture systems, and although there are differences between cell types, there is less variability than in the entire animal. Metabolism is an important issue for humans and wildlife field populations with large genetic variability. Individual differences in BPA pharmacokinetics allow for underlying variability within a population, and may allow for the identification of sensitive and insensitive subpopulations.
2. The activity of various enzymes involved in drug, chemical, and hormone metabolism, as well as protection against oxidative stress, are programmed by hormone levels during sensitive periods in development. Developmental alterations in hormonal programming (activation or inhibition) may thus affect metabolism of BPA and other hormones and chemicals. Direct interaction of BPA with enzymes in cells has only been reported at higher doses than expected for human exposures.
3. The set of genes regulated by BPA is expected to differ among doses. Therefore, different doses of BPA do not produce different effects only due to a quantitative difference in the expression of the same set of genes.
4. Differential expression of estrogen receptor subtypes (α/ß; variant isoforms), and protein–protein interactions (estrogen receptor homo- and hetero-dimer formation, co-regulators, etc) modulate the cellular response to BPA. Direct actions of BPA on intracellular signal transduction modulate some cellular responses, which are similarly dependent on differential expression and protein–protein interactions.
5. Bioactive doses can be mathematically modeled, but further model refinement and experimental confirmation is required.
6. Other mechanisms (androgen receptors, thyroid hormone receptors) may be relevant for BPA action, but at higher doses than for estrogen responsive mechanisms.

4.2.2. Issue 2: Wildlife—laboratory animal research connection

1. The effects observed in laboratory animals could be present in wildlife, because the low doses being studied in laboratory animals are now relevant to environmental exposure levels of wildlife. The similarities in mechanisms that have been observed between different species suggest that field populations will respond to the same low levels.
2. Measurements of vitellogenin production in fish have established that there are exogenous estrogenic signals in the their environment. BPA may be contributing to this phenomenon as it enters natural water systems after leaching from landfills and due to plastic debris in water.
3. Delayed spawning is seen in male and female fish, which may relate to observed changes in estrous cyclicity in mammals in laboratory experiments.