

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 (ER α) in mediating estrogen-dependent bone maintenance in female mice. We selectively ablated ER α 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 ER α 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;

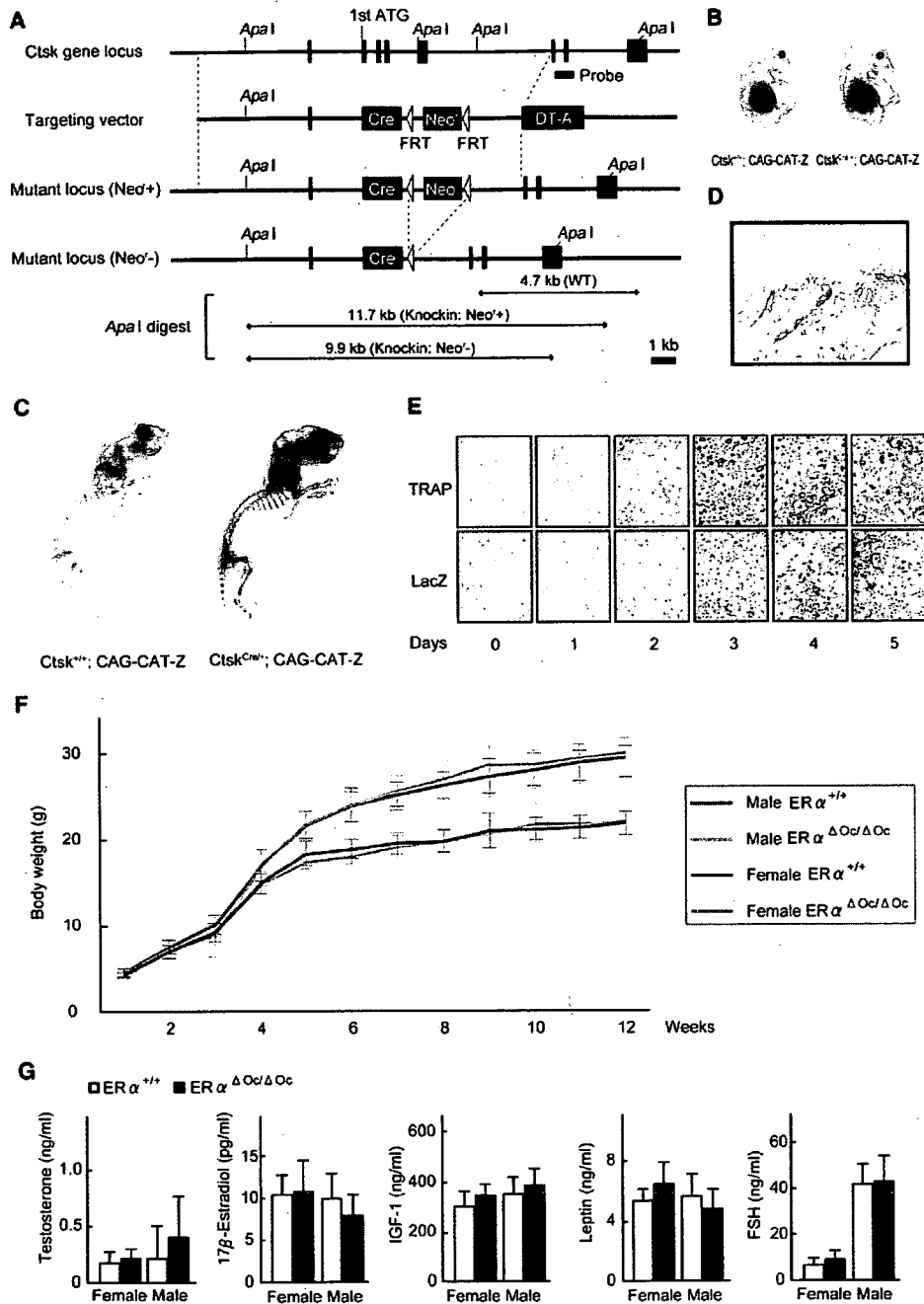


Figure 1. Generation of Knockin Mice Selectively Expressing Cre in Mature Osteoclasts

(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 *Neoc* 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 lumbar 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 (ER α , ER β) (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 ER α (ER $\alpha^{-/-}$) and ER β (ER $\beta^{-/-}$) provide insights into ER function (Mueller and Korach, 2001; Windahl et al., 2002). In mice, though ER α 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 ER α single or ER α /ER β 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 ER α (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 ER α in mature osteoclasts (ER $\alpha^{AOC/\Delta Oc}$). Selective ablation of ER α in differentiated osteoclasts (ER $\alpha^{AOC/\Delta Oc}$) was accomplished by crossing a *Cathepsin K-Cre* knockin mouse with a floxed ER α 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 $\alpha^{AOC/\Delta Oc}$ females. Induction of FasL and apoptosis by estrogen as well as a SERM also required ER α in cultured osteoclasts. Thus, we propose that the osteoprotective actions of estrogen and SERMs are mediated at least in part through osteoclastic ER α in trabecular bone, and the life span of mature osteoclasts is regulated through the activation of the FasL signaling.

RESULTS

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

To specifically disrupt ER α 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 $\alpha^{AOC/\Delta Oc}$ mice was indistinguishable from that of the control mice. Data are represented as mean \pm SEM.

(G) Serum hormone levels were normal in 12-week-old ER $\alpha^{AOC/\Delta Oc}$ (filled column) versus ER $\alpha^{+/+}$ (open column) mice (n = 10–11 animals per genotype). Data are represented as mean \pm SEM.

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* ^{*Δ*Oc/*Δ*Oc}). 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* ^{flx/flx} (*ER α* ^{*Δ*Oc/*Δ*Oc}) mice, with the exception of the female bones. Serum levels of sex hormones and bone remodeling regulators such as IGF-1, leptin, and follicle-stimulating hormone (Sun et al., 2006; Takeda et al., 2002) appeared unchanged in both male and female *ER α* ^{*Δ*Oc/*Δ*Oc} mice at 12 weeks (Figure 1G).

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

The 12-week-old *ER α* ^{*Δ*Oc/*Δ*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 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* ^{*Δ*Oc/*Δ*Oc} males unexpectedly exhibited no clear bone loss even in the trabecular areas (Figures 2A–2C). In *ER α* ^{*Δ*Oc/*Δ*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 α* ^{*Δ*Oc/*Δ*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 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* ^{*Δ*Oc/*Δ*Oc} 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* ^{*Δ*Oc/*Δ*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 *ER α* .

Estrogen Treatment Failed to Rescue the Osteoporotic Bone Phenotype of *ER α* ^{*Δ*Oc/*Δ*Oc} 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* ^{*Δ*Oc/*Δ*Oc}) 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* ^{*Δ*Oc/*Δ*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 *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

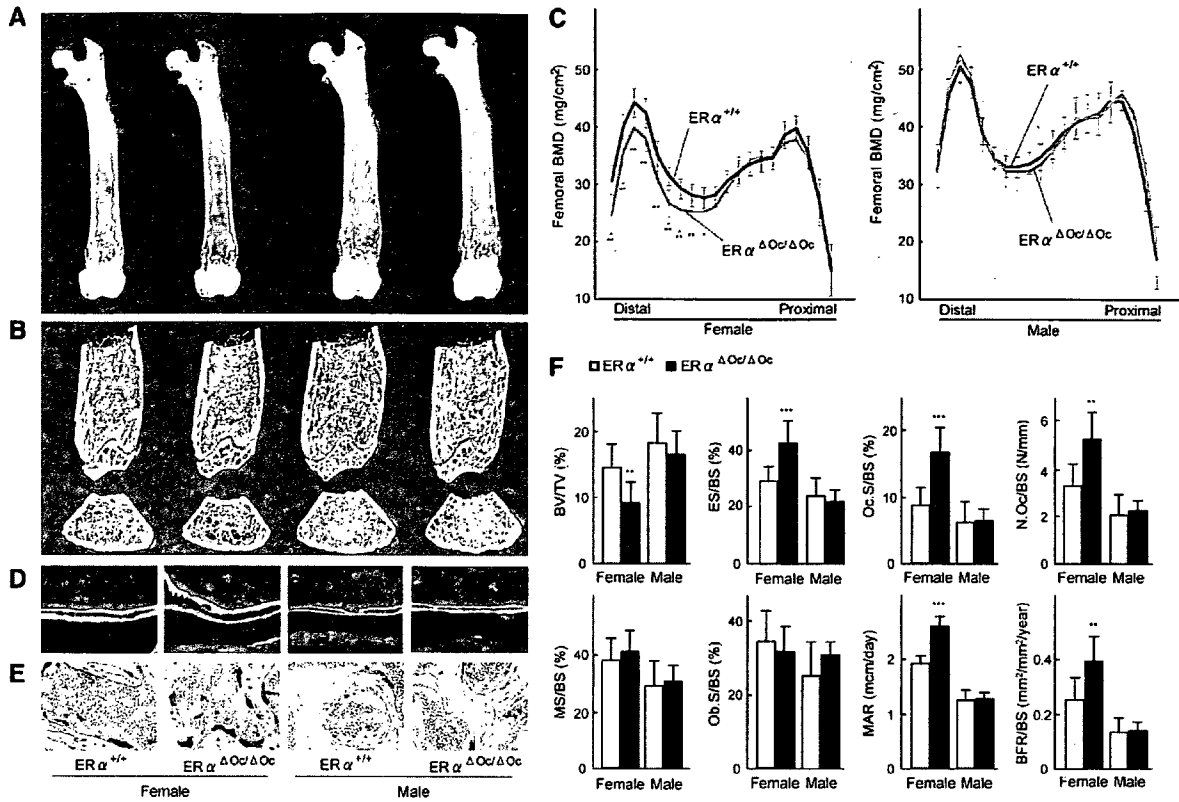


Figure 2. High Bone Turnover Osteopenia Was Observed in $ER\alpha^{\Delta Ocl/\Delta Ocl}$ Females But Not Males
 (A) Soft X-ray images of femurs from 12-week-old $Ctsk^{Cre/+}; ER\alpha^{lox/lox}$ ($ER\alpha^{\Delta Ocl/\Delta Ocl}$) mice.
 (B) Three-dimensional computed tomography images of the distal femur 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 microscopy 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 osteoclasts 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).

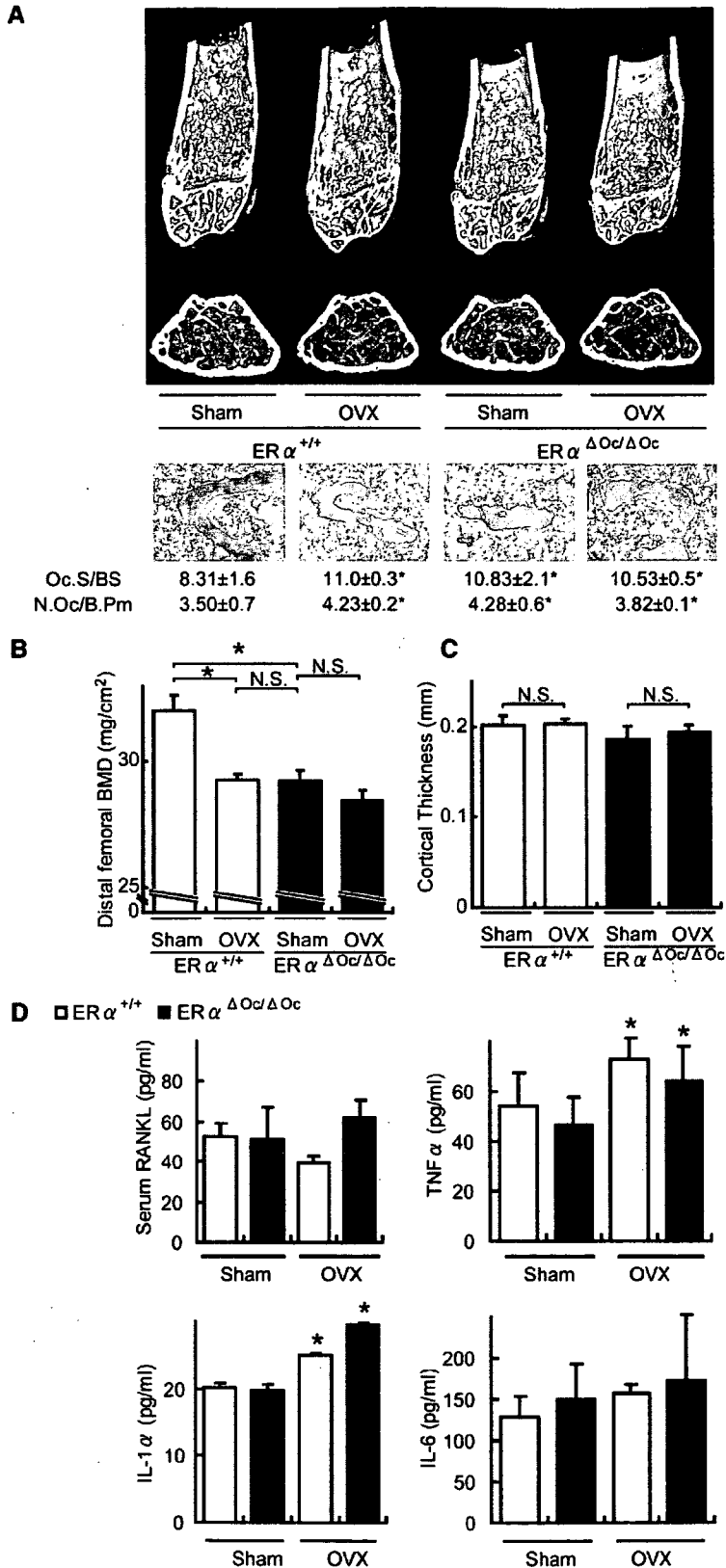


Figure 3. No Further Bone Loss of ER $\alpha^{\Delta Oc/\Delta Oc}$ Females by Ovariectomy

(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.

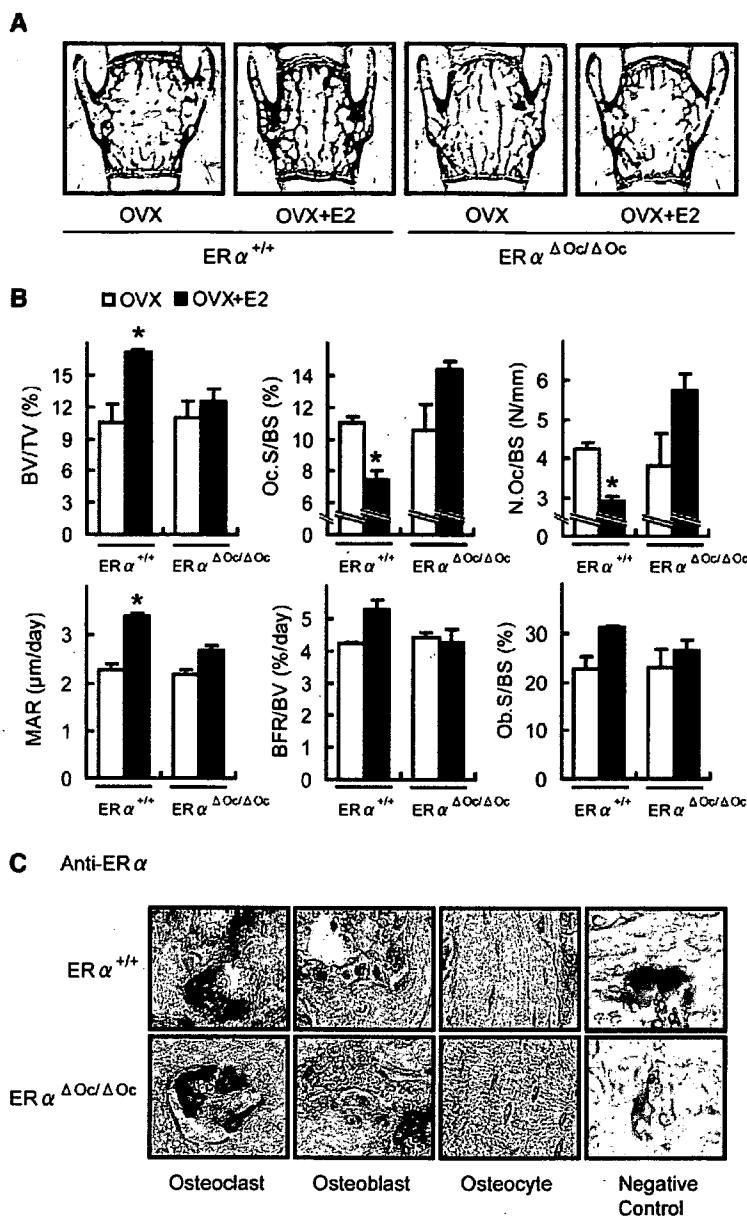


Figure 4. Estrogen treatment failed to reverse trabecular bone loss of ovariectomized $ER\alpha^{\Delta Ocl/\Delta Ocl}$ females

(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 κ 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

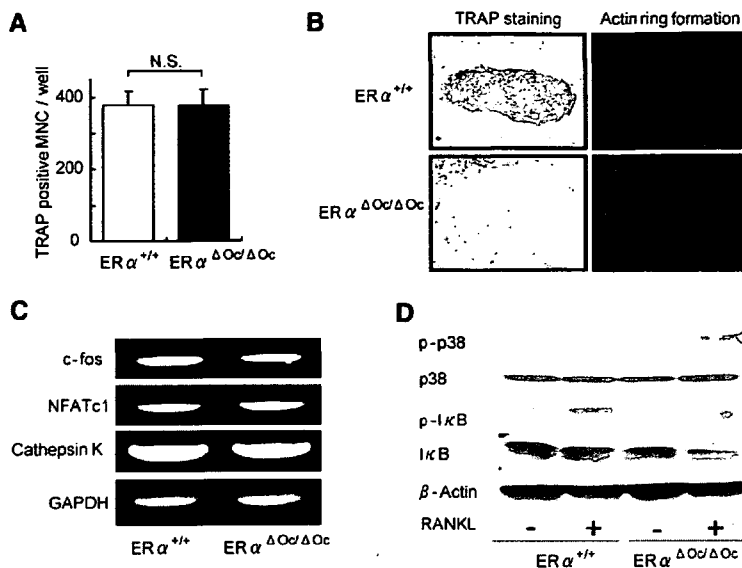


Figure 5. ER α Deficiency Did Not Affect Osteoclastogenesis

(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 mutant (*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.

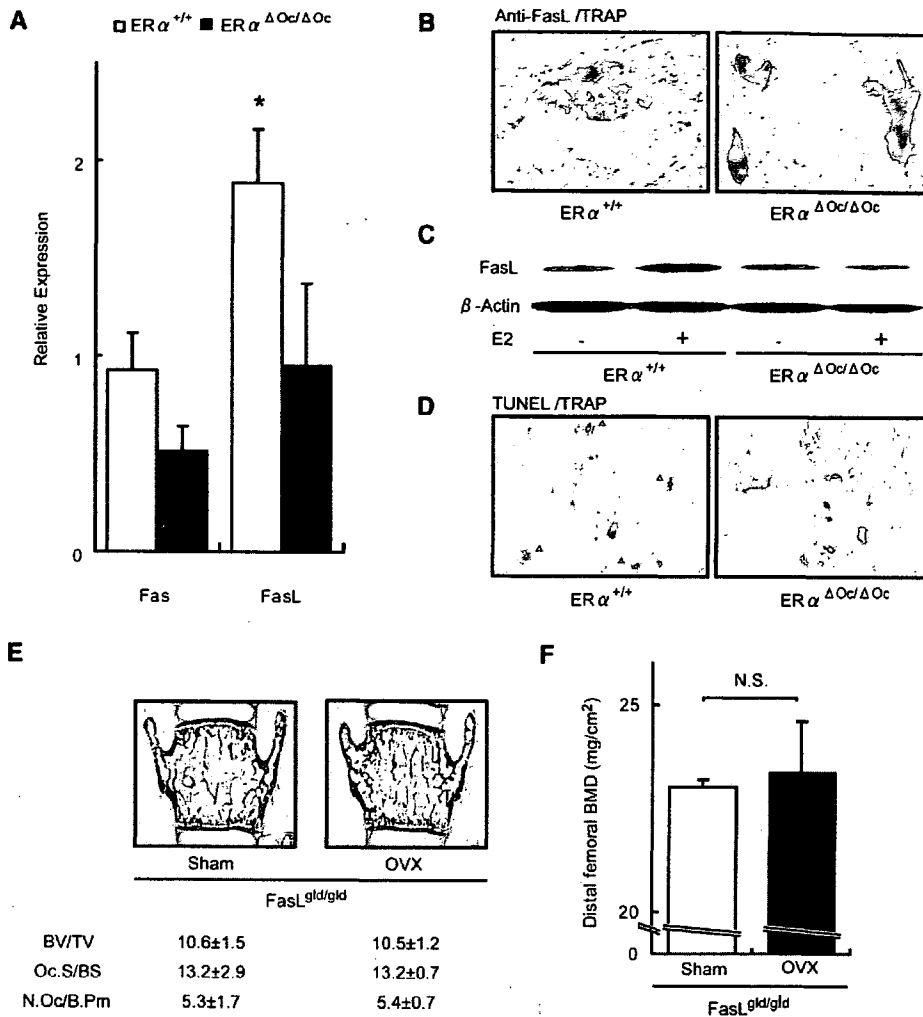


Figure 6. Activated ER α Induced Fas Ligand Expression and Apoptosis in Differentiated Osteoclasts of Intact Bone
 (A) Real-time RT-PCR analysis of *Fas* and *FasL*. Expression levels in bones from E2-treated ovariectomized ER $\alpha^{+/+}$ (open column) and ER $\alpha^{\Delta Oc/\Delta Oc}$ (filled 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

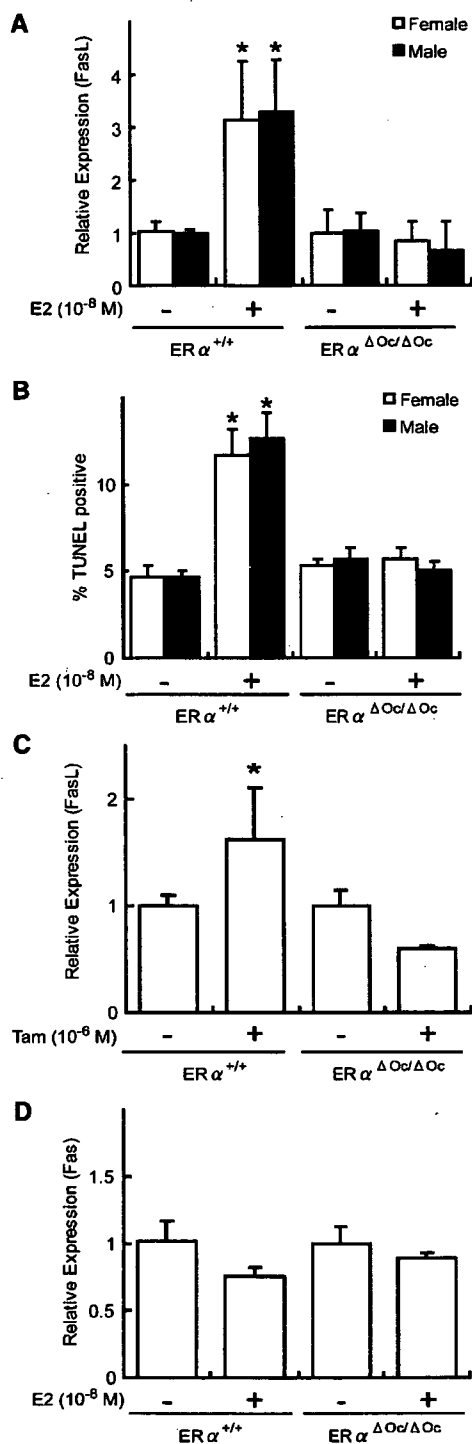


Figure 7. Estrogen-Induced *FasL* Expression and Apoptosis Required ER α in Cultured Osteoclasts

(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 \pm SEM.

markedly elevated levels of testosterone in ER α 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 ER α 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 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 ER α . 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 ER α is selectively ablated in osteoblasts are needed to define the role of ER α in these cells.

In osteoclastic cells, expression of the *FasL* gene, which leads to apoptosis, appears to be positive controlled by activated ER α . Not surprisingly, a direct binding site for ER α 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 ER α recruitment to specific promoter sites of given ER α target genes was cell-type specific (Carroll et al., 2005). Thus, there is significant impetus to identify the osteoclastic factor that associates with ER α 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 \pm SEM.

(C) *FasL* expression in each genotypic female osteoclastic cells treated with or without Tam (10⁻⁸ M) (**p* < 0.05 compared to the group treated without Tam). Data are represented as mean \pm SEM.

(D) Expression of *Fas* was measured as described in the legend of Figure 7A. Data are represented as mean \pm 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-Kar/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-Kar/Neo^r-FRT* cassette in the pSK2/3-*FRT-Neo* plasmid. The *nlsCre-FRT-Kar/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-Fipe* (Jackson Laboratory), to remove the *Kar/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. *Fast^{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^{-8} 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 α ^{flax/flax}*) (Dupont et al., 2000) and null alleles with a C57BL/6J background have been previously described. *ER α ^{flax/flax}* mice were crossed with *Ctsk^{Cre/+}* mice to generate *Ctsk^{Cre/+}; ER α ^{flax/+}* mice. *Ctsk^{Cre/+}; ER α ^{+/+}* (*ER α ^{+/+}*) and *Ctsk^{Cre/+}; ER α ^{flax/flax}* (*ER α ^{ΔOc/ΔOc}*) mice were obtained by crossing *Ctsk^{Cre/+}; ER α ^{flax/+}* with *ER α ^{flax/+}* mouse lines.

Radiological Analysis

Bone radiographs of the femurs of 12-week-old *Ctsk^{Cre/+}; ER α ^{flax/flax}* (*ER α ^{ΔOc/ΔOc}*) and *Ctsk^{Cre/+}; ER α ^{+/+}* (*ER α ^{+/+}*) 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 α ^{flax/flax}* (*ER α ^{ΔOc/ΔOc}*) and *Ctsk^{Cre/+}; ER α ^{+/+}* (*ER α ^{+/+}*) 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 α ^{flax/flax}* (*ER α ^{ΔOc/ΔOc}*) and *Ctsk^{Cre/+}; ER α ^{+/+}* (*ER α ^{+/+}*) 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 α ^{flax/flax}* (*ER α ^{ΔOc/ΔOc}*) and *Ctsk^{Cre/+}; ER α ^{+/+}* (*ER α ^{+/+}*) 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 α ^{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.

Evaluation of Action Mechanisms of Toxic Chemicals Using JFCR39, a Panel of Human Cancer Cell Lines[Ⓢ]

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ABSTRACT

We previously established a panel of human cancer cell lines, JFCR39, coupled to an anticancer drug activity database; this panel is comparable with the NCI60 panel developed by the National Cancer Institute. The JFCR39 system can be used to predict the molecular targets or evaluate the action mechanisms of the test compounds by comparing their cell growth inhibition profiles (i.e., fingerprints) with those of the standard anticancer drugs using the COMPARE program. In this study, we used this drug activity database-coupled JFCR39 system to evaluate the action mechanisms of various chemical compounds, including toxic chemicals, agricultural chemicals, drugs, and synthetic intermediates. Fingerprints of 130 chemicals were determined and stored in the database. Sixty-nine of

130 chemicals (~60%) satisfied our criteria for the further analysis and were classified by cluster analysis of the fingerprints of these chemicals and several standard anticancer drugs into the following three clusters: 1) anticancer drugs, 2) chemicals that shared similar action mechanisms (for example, ouabain and digoxin), and 3) chemicals whose action mechanisms were unknown. These results suggested that chemicals belonging to a cluster (i.e., a cluster of toxic chemicals, a cluster of anticancer drugs, etc.) shared similar action mechanism. In summary, the JFCR39 system can classify chemicals based on their fingerprints, even when their action mechanisms are unknown, and it is highly probable that the chemicals within a cluster share common action mechanisms.

Determining the action mechanism or identifying the molecular target of a chemical with pharmacological activity or adverse side effects is highly desirable. Although various test methods are currently available for determining the action mechanisms of chemicals, such as methods based on animal models, methods based on cellular models, bacterial mutagenicity test, the uterotrophic assay (Kanno et al., 2002), Hershberger test (Hershberger et al., 1953), and the reporter assay for the nuclear receptor agonists, determination of the action

mechanisms of pharmacologically active chemicals, including the toxic chemicals, is still a difficult and challenging task. Therefore, it is highly desirable to develop efficient test methods for evaluating toxicity of chemicals.

A number of screening methods are currently available for discovering new anticancer drugs. One very powerful and unique approach using multiple cancer cell lines was developed at NCI (Paull et al., 1989; Weinstein et al., 1992, 1997) and in our laboratory (Yamori et al., 1999; Dan et al., 2002, 2003; Yamori, 2003; Nakatsu et al., 2005; Akashi and Yamori, 2007; Akashi et al., 2007; Nakamura et al., 2007). This bioinformatics-based approach enables mechanism-oriented evaluation of anticancer drugs. For example, we can evaluate the cell toxicity *in vitro* by determining the 50% growth inhibition (GI50), total growth inhibition, and 50% lethal concentration across a panel of 39 human cancer cell lines (JFCR39). We can also predict the molecular targets or evaluate the action mechanisms of the test compounds by comparing the cell growth inhibition profiles (termed "fingerprints") across the panel for these compounds with those of

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ABBREVIATIONS: GI50, 50% growth inhibition concentration; GI50, 50% growth inhibition; SN-38, 7-ethyl-10-hydroxycamptothecin; SV-NN, snake venom from *N. nigracollis*; SV-NNK; snake venom from *N. naja kaouthia*.

TABLE 1

List of chemicals tested. Chemical names, abbreviations, and applications/targets/mechanisms of the test compounds are summarized.

JCI No	Name	Abbreviation	Application/Target/Mechanism
-691	Trioctyltin	TOT	Organotin
-690	Triphenyltin	TPT	Organotin
-689	Dibutyltin		Organotin
-688	AM-580		RAR α
-687	TTNPB		RAR
-686	13-cis Retinoic acid	13-cis	RAR
-607	Methoprene		Agricultural chemical
-606	Methoprene acid		RXR
-605	5-aza-2'-deoxycytidine	5-AzaC	Methylation
-604	Carbaryl		Agricultural chemical
-603	Acephate		Agricultural chemical
-602	Sodium arsenite		Agricultural chemical
-601	Testosterone propionate	TP	Testosterone
-600	Ethinyl estradiol	EE	Estrogenic
-599	Thiram		Agricultural chemical
-598	Dimethylformamide	DMF	Solvent
-568	α -Bungarotoxin	α BuTX	Neurotoxin
-567	Snake venom from <i>Trimeresurus flavoviridis</i>	SV-TF	Snake venom
-566	Snake venom from <i>Crotalus atrox</i>	SV-CA	Snake venom
-565	Snake venom from <i>Aghkistrodon halys blomhoffii</i>	SV-AHB	Snake venom
-564	Dexamethasone	DEX	Steroid
-563	3-Methylcholanthrene	3-MC	Teratogenicity/carcinogenicity
-562	<i>N</i> -Ethyl- <i>N</i> -nitrosourea	ENU	Teratogenicity/carcinogenicity
-561	Diethylnitrosamine	DEN	Teratogenicity/carcinogenicity
-560	All <i>trans</i> -retinoic acid	ATRA	RAR + RXR
-559	9-cis Retinoic acid	9-cis	RAR
-558	Levothyroxine	T4	Thyroid hormone
-557	3-Amino-1 <i>H</i> -1,2,4-triazole	3AST	Agricultural chemical
-555	2-Vinylpyridine	2VP	Synthetic intermediate
-553	Phenobarbital	PB	Antiepileptic
-552	Acetaminophen	APAP	Analgetic
-551	Isoniazid		Phthisic
-549	4-Ethylnitrobenzene	4ENB	Synthetic intermediate
-548	1,2-Dichloro-3-nitrobenzene	1,2DC3NB	Pigment/synthetic intermediate
-546	<i>N</i> -Methylaniline	NMA	Synthetic intermediate
-545	2-Aminomethylpyridine	2AMP	Synthetic intermediate
-544	1 <i>H</i> -1,2,4-Triazole		Synthetic intermediate
-543	1 <i>H</i> -1,2,3-Triazole		Synthetic intermediate
-542	4-Amino-2,6-dichlorophenol	4A2,6DCP	Synthetic intermediate
-541	2,4-Dinitrophenol	2,4 DNP	Agricultural chemical
-513	Capsaicin		Food constituent
-485	2-Methoxyestradiol		Estrogenic
-466	Colcemid		Spindle inhibitor
-465	2,4-Dinitrochlorobenzene	2,4DCB	Pigment/mutagenesis
-464	Troglitazone		Diabetic
-463	Clofibrate		Antilipemic
-459	Bis(2-ethylhexyl)phthalate	DEHP	Plasticizer
-458	Thiourea		Agricultural chemical
-447	Cacodylic acid		Agricultural chemical
-446	Amitrole		Agricultural chemical
-445	4-Octylphenol	OP	Reproductive effector
-444	2,6-Dimethylaniline	2,6-Xylidene	Natural product
-443	1,2-Dibromo-3-chloropropane	DBCP	Agricultural chemical
-442	1,1-Dimethylhydrazine	1,1DMH	Reproductive effector
-441	Sulfanylamide		Agricultural chemical
-440	Streptozotocin		Agricultural chemical
-439	Spirolactone		Aldosterone antagonist
-438	<i>para</i> -Aminoazobenzene	pAAB	Pigment/mutagenicity/carcinogenicity
-437	<i>para</i> -Cresidine		Pigment/carcinogenicity
-436	Neostigmine bromide		Parasympathomimetics
-435	<i>para</i> -Dichlorobenzene	pDCB	Pigment/Agricultural chemical
-434	Phenytoin		Antiepileptic
-433	<i>ortho</i> -Toluidine	<i>o</i> Toluidine	Pigment
-432	Imipramine		Antidepressant
-431	Cobalt chloride		Teratogenicity/mutagenicity
-428	Atrazine		Agricultural chemical
-427	Propylthiouracil		Teratogenicity/carcinogenicity
-426	Thalidomide (L + D)		Teratogenicity
-425	Carbon tetrachloride	CCl ₄	Teratogenicity/carcinogenicity
-424	Hydroquinone		Oxidative stress
-423	Monocrotaline		Mutagenicity/carcinogenicity
-422	Vinyl chloride		Carcinogenicity
-421	Tributyltin chloride	TBT	Ship bottom paint/organotin
-420	Valproic acid		Antiepileptic
-419	Benzene		Carcinogenicity

TABLE 1—(Continued)

JCI No	Name	Abbreviation	Application/Target/Mechanism
-418	Acrylamide		Neurotoxin/carcinogenicity
-417	Hexachlorobenzene	BHC	Agricultural chemical/carcinogenicity
-346	2-Deoxyglucose	2-DG	Glycolytic pathway/glycosylation inhibitor
-325	Pentachlorophenol	PCP	Agricultural chemical/teratogenicity/carcinogenicity
-324	Aniline		Oxidative stress/methemoglobinemia/carcinogenicity
-323	Triazine		Agricultural chemical
-322	Edifenphos	EDDP	Agricultural chemical/antibiotics/choline esterase
-321	γ -1,2,3,4,5,6-Hexachlorocyclohexane	γ -BHC	Agricultural chemical/carcinogenicity
-320	Dichlorvos	DDVP	Agricultural chemical/teratogenicity/carcinogenicity
-319	O-Ethyl O-4-nitrophenyl phenylphosphonothioate	EPN	Agricultural chemical
-318	Cadmium chloride	CdCl ₂	Teratogenicity/carcinogenicity
-317	Phenylmercury acetate	PMA	Fungicides/mutagenicity
-316	Mercaptoacetic acid		Synthetic intermediate
-315	1,3-Diphenylguanidine	DPG	Vulcanizing agent
-314	3,4,4'-Trichlorocarbanilide	TCC	Cosmetics/antibacterial agent
-313	3-Iodo-2-propynyl butylcarbamate	IPBC	Antibacterial agent
-311	2,3,3,3'-2',3',3'-Octachlorodipropylether	S-421	Agricultural chemical/antibacterial agent
-310	1,2-Benzisothiazolin-3-one	BIT	Antibacterial agent
-309	Isobornylthiocyanacetate	IBTA	Antibacterial agent
-308	p-Chlorophenyl-3-iodopropargylformal	CPIP	Antibacterial agent
-307	Zinc butylxanthate	ZBX	Vulcanizing agent
-306	Polypropylene glycol	PG	Synthetic intermediate
-305	10,10'-Oxy-bis(phenoxyarsine)	OBPA	Antibacterial agent
-296	Snake venom from <i>Naja naja kaouthia</i>	SV-NNK	Snake venom
-295	Snake venom from <i>Naja nigricollis</i>	SV-NN	Snake venom
-294	2,5-di(tert-butyl)-1,4-Hydroquinone	DTBHQ	Oxidative stress
-293	Ibotenic Acid		Mushroom toxin/neurotoxin
-292	N-Methy-4-phenyl-1,2,3,6-tetrahydropyridine	MPTP	Neurotoxin
-289	Tetrodotoxin		Natural product/Na ⁺ channel inhibitor
-288	ICI 182,780		Estrogen antagonist
-275	Benzophenone		Agricultural chemical
-274	1,2-Dibromo-3-chloropropane	DBCP	Antibacterial agent/insecticide/carcinogenicity
-273	Zineb		Agricultural chemical
-272	Dieldrin		Insecticide
-271	Hexachlorobenzene	HCB	Antibacterial agent/carcinogenicity
-270	Ziram		Antibacterial agent/vulcanizing agent
-269	Chlordane		Insecticide/carcinogenicity
-268	4,4'-Dichlorodiphenyltrichloroethane	p,p'-DDT	Insecticide/carcinogenicity/teratogenicity
-267	Bisphenol A	BPA	Estrogenic
-266	17- β -Estradiol	E2	Estrogenic
-265	Diethylstilbestrol	DES	Estrogenic
-261	Paraquat		Agricultural chemical/oxidative stress
-247	Ouabain		Cardiac glycosides
-245	Okadaic acid		Natural product/PP1, PP2A inhibitor
-242	Antimycin A1		Agricultural chemical
-232	Digoxin		Cardiac glycosides
-201	OH-Flutamide		Flutamide derivative/androgen antagonist
-200	Flutamide		Anticancer drugs/androgen antagonist
-185	30% H ₂ O ₂		Oxidative stress
-182	N-Acetyl-L-cysteine	NAC	Super oxyide scavenger
-181	L-Ascorbic acid		Food constituent
-179	Dopamine		Neurotransmitter
-177	Caffeine		Food constituent
-168	Cycloheximide		Protein synthesis inhibitor
-144	4-Hydroxyphenylretinamide	4-HPR	RAR
-137	Indomethacin		COX inhibitor
-99	SN-38		Irinotecan derivative/Topo I
-96	Toremifene		Anticancer drugs/estrogen antagonist
-95	Tamoxifen		Anticancer drugs/estrogen antagonist
-63	Cyclosporin A		Anticancer drugs/helper T cell
-46	HCFU		Anticancer drugs/antimetabolite(pyrimidine)
-36	Docetaxel		Anticancer drugs/tubulin
-35	Paclitaxel		Anticancer drugs/tubulin
-34	Colchicine		Antipodagric/tubulin
-33	Cisplatin		Anticancer drugs/DNA cross linker
-32	Carboplatin		Anticancer drugs/DNA cross linker
-31	Irinotecan		Anticancer drugs/Topo I
-30	Camptothecin	CPT	Anticancer drugs/Topo I
-24	Methotrexate		Anticancer drugs/DHFR
-19	Vincristine		Anticancer drugs/tubulin
-18	Vinblastine		Anticancer drugs/tubulin
-16	Mitomycin-C	MMC	Anticancer drugs/DNA alkylator
-9	Tegafur		Anticancer drugs/antimetabolite(pyrimidine)
-8	5-Fluorouracil	5-FU	Anticancer drugs/antimetabolite(pyrimidine)
-5	Cytarabine		Anticancer drugs/antimetabolite(pyrimidine)
-4	Nitrogen mustard		Anticancer drugs/DNA alkylator

RAR, retinoic acid receptor; RXR; retinoid X receptor.

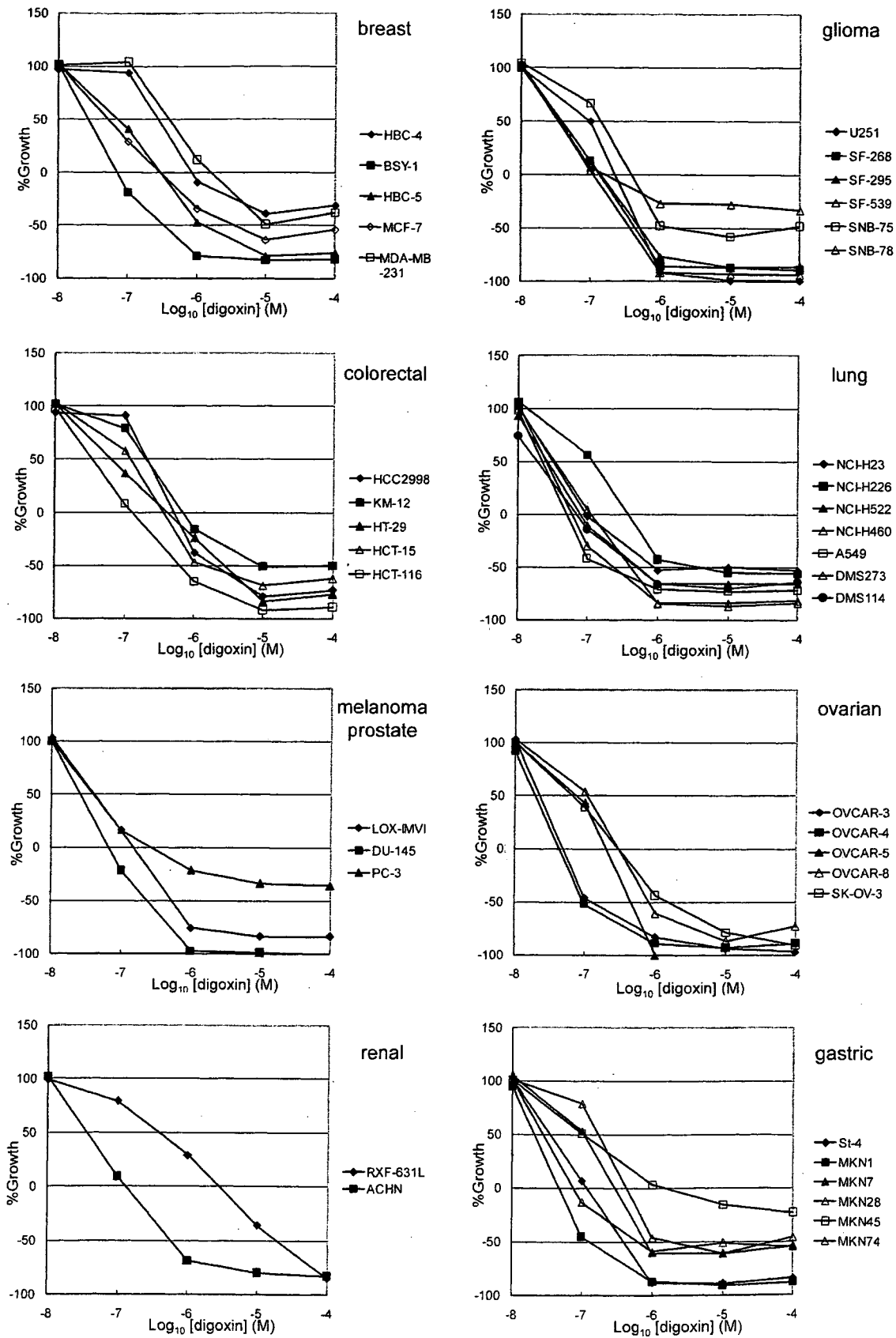


Fig. 1. Dose response curves of digoxin against growth of JFCR-39 cells. The x-axis represents concentration of digoxin and the y-axis represents percentage growth. The GI50 represents the concentration required to inhibit cell growth by 50% compared with untreated controls.

the standard anticancer drugs using the COMPARE algorithm (Yamori et al., 1999). We have used this system successfully and demonstrated that the molecular targets of the novel chemicals MS-274, FJ5002, and ZSTK474 were topoisomerases I and II (Yamori et al., 1999), telomerase (Naasani et al., 1999), and phosphatidylinositol 3-kinase (Yaguchi et al., 2006), respectively. Several other interesting studies, based on a panel of cancer cells, classified anticancer drugs according to their action mechanisms or molecular targets by cluster analysis of their GI50 values (Weinstein et al., 1992, 1997; Dan et al., 2002). Correlation analysis has also been used to explore the genes associated with the sensitivity of the cells in the panel to anticancer drugs (Scherf et al., 2000; Okutsu et al., 2002; Zembutsu et al., 2002; Nakatsu et al., 2005).

In this study, we have examined the potential of the JFCR39 system in classifying various chemicals, and predicted their action mechanisms. For this purpose, we have determined the fingerprints of 130 different types of chemicals including toxic chemicals, pesticides, drugs and synthetic intermediates, and then classified these chemicals according to the cluster analysis of their fingerprints.

Materials and Methods

Cell Lines and Cell Cultures. The panel of human cancer cell lines has been described previously (Yamori et al., 1999; Dan et al., 2002) and consists of the following 39 human cancer cell lines: lung cancer, NCI-H23, NCI-H226, NCI-H522, NCI-H460, A549, DMS273, and DMS114; colorectal cancer, HCC-2998, KM-12, HT-29, HCT-15, and HCT-116; gastric cancer, MKN-1, MKN-7, MKN-28, MKN-45, MKN-74, and St-4; ovarian cancer, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, and SK-OV-3; breast cancer, BSY-1, HBC-4, HBC-5, MDA-MB-231, and MCF-7; renal cancer, RXF-631L and ACHN; melanoma, LOX-IMVI; glioma, U251, SF-295, SF-539, SF-268, SNB-75, and SNB-78; and prostate cancer, DU-145 and PC-3. All cell lines were cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) with 5% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37°C under 5% CO₂.

Determination of Cell Growth Inhibition Profiles. Growth inhibition experiments were performed to assess the sensitivity of the cells to various chemicals as described before (Yamori et al., 1999; Dan et al., 2002). Growth inhibition was measured by determining the changes in the amounts of total cellular protein after 48 h of chemical treatment using a sulforhodamine B assay. For each chemical, the growth assay was performed using a total of five different concentrations of the chemical (for example, 10⁻⁴, 10⁻⁵,

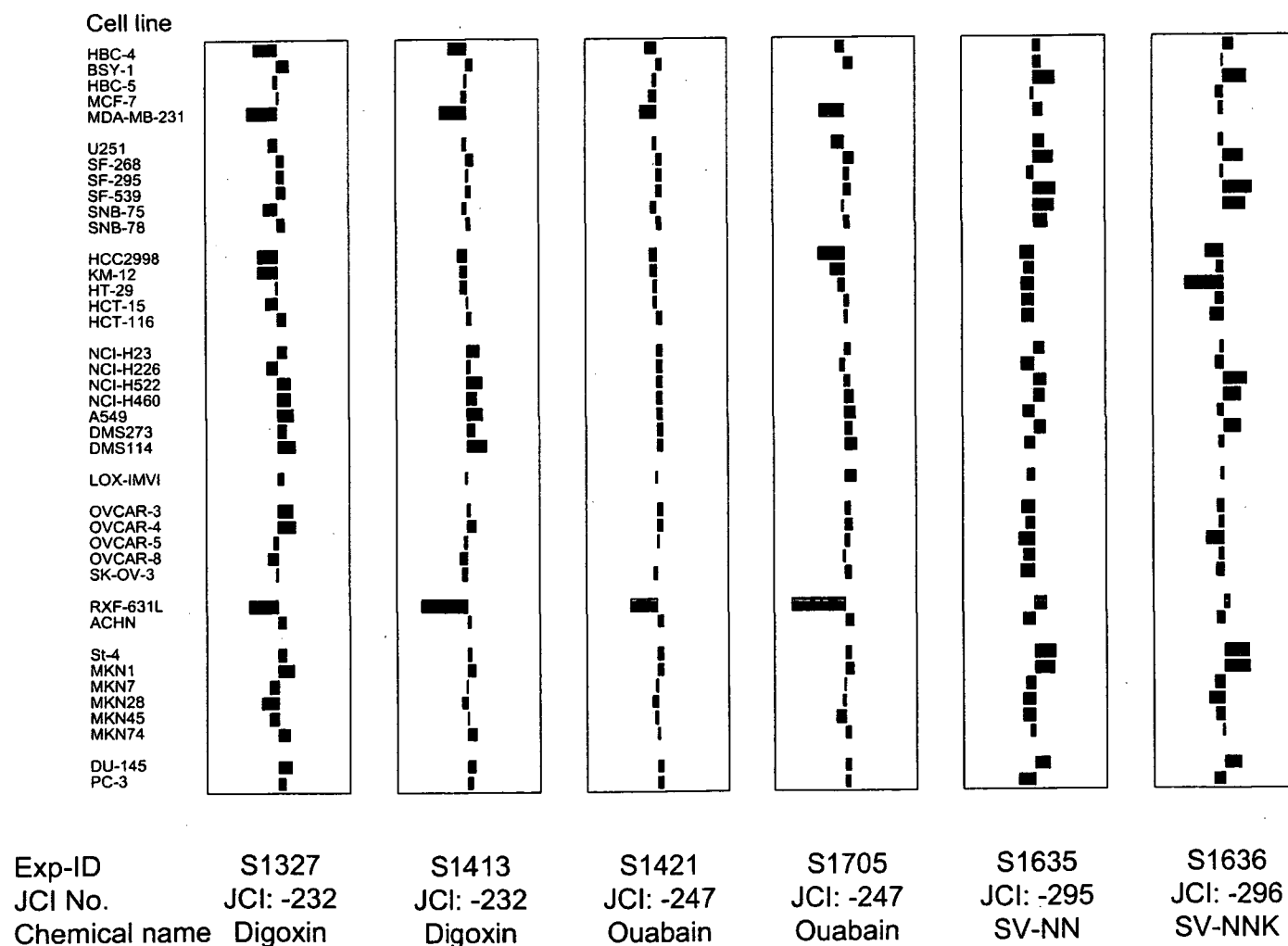


Fig. 2. Fingerprints of digoxin, ouabain, SV-NN, and SV-NNK. Fingerprint shows the differential growth inhibition pattern of the cells in the JFCR-39 panel against the test chemical. The X-axis represents relative value of GI50; $(-1) \times (\log \text{GI50} - \text{MG-MID})$; MG-MID is the mean value of the log GI50. Zero means the mean GI50 and one means the GI50 value is 10-fold more sensitive than the mean GI50. Exp-ID and JCI numbers are the ID for the experiment and ID for the chemical, respectively, in our database.

10^{-6} , 10^{-7} , and 10^{-8} M) and one negative control. All assays were performed in duplicate. This GI50 calculation method is well established and reliable through anticancer drug screen using NCI60 as well as JFCR39 (Paull et al., 1989; Yamori et al., 1999; Yamori, 2003). At each test concentration, the percentage growth was calculated using the following seven absorbance measurements: growth at time 0 (T0), growth of the control cells (C), and test growth in the presence of five different concentrations (T) of a drug. The percentage growth inhibition was calculated as: % growth = $100 \times [(T - T0)/(C - T0)]$ when $T \geq T0$, and % growth = $100 \times [(T - T0)/T]$ when $T < T0$. The GI50 values, which represent 50% growth inhibition concentration, were calculated as $100 \times [(T - T0)/(C - T0)] = 50$. When the GI50 of a chemical could not be calculated, the highest used concentration was assigned as its GI50 value. Absolute values of GI50 were then log transformed for further analysis. We certified the accuracy of measured GI50 data by using reference control chemicals, such as mitomycin-C, paclitaxel, and SN-38, in every experiment and by checking the dose response curves.

Chemicals. Spirolactone, *para*-aminoazobenzene, *para*-cresidine, neostigmine bromide, *para*-dichlorobenzene, phenytoin, *ortho*-toluidine, imipramine, cobalt chloride, atrazine, propylthiouracil, (D,L)-thalidomide, carbon tetrachloride, hydroquinone, monocrotaline, vinyl chloride, tributyl-tin chloride, valproic acid, benzene, acrylamide, pentachlorophenol, aniline, 1,3-diphenylguanidine, polypropylene glycol, 10,10'-oxy-bis(phenoxyarsine), testosterone propionate, carbaryl, acephate, bisphenol A, 17- β -estradiol, diethylstilbestrol, and α -bungarotoxin were purchased from Wako (Tokyo, Japan). Snake venoms from *Akistrodon halys blomhoffii*, *Trimeresurus flavoviridis*, *Crotalus atrox*, *Naja nigricollis*, and *Naja naja kaouthia* were purchased from Latoxan (Valence, France).

2-Aminomethylpyridine, 1H-1,2,4-triazole, 1H-1,2,3-triazole, 3,4,4'-trichlorocarbonyl, edifenphos, dichlorvos, *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate, 2,4-dinitrophenol, *N*-methylaniline, 1,2-dichloro-3-nitrobenzene, 4-ethylnitrobenzene, 2-vinylpyridine, 3-amino-1H-1,2,4-triazole, *N*-ethyl-*N*-nitrosourea, 5-aza-2'-deoxycytidine, ethynyl estradiol, 3-methylcholanthrene, phenobarbital, acetaminophen, isoniazid, capsaicin, *N*-deacetyl-*N*-methylcolchicine (Colceid), 2,4-dinitrochlorobenzene, and dexamethasone were from Sigma Chemicals (St. Louis, MO). Methoprene acid, methoprene, all-*trans* retinoic acid, and 9-*cis* retinoic acid were from BIOMOL International L.P. (Plymouth Meeting, PA). Levothyroxine was from MP Biomedicals (Irvine, CA). 3-Iodo-2-propynyl butylcarbamate was from Olin Japan Inc. (Tokyo, Japan), *p*-chlorophenyl-3-iodopropargylaldehyde was from Nagase ChemteX (Osaka, Japan), and 2,3,3,3'-2',3',3',3'-octachlorodipropylether was from Sankyo Chemical Industries, Ltd. (Tokyo, Japan). 1,2-Benzisothiazolin-3-one was from Riverson (Osaka, Japan), zinc butylxanthate was from Ouchishinko Chemical Industrial Co., Ltd. (Tokyo, Japan), and 4-amino-2,6-dichlorophenol was from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan).

Hierarchical Clustering. Hierarchical clustering analysis was carried out using the average linkage method and the "GeneSpring" software (Silicon Genetics, Inc., Redwood, CA). Pearson correlation coefficients were used to determine the degree of similarity.

Results

Sensitivity of JFCR39 to Chemicals. Sensitivity of the JFCR39 panel of cells to 130 chemicals was determined as described under *Materials and Methods*. Table 1 summarizes

TABLE 2

Log₁₀ GI50 values of chemicals for each cell line in the JFCR-39 panel

Hi-conc means the highest concentration of the test chemical used. When the growth inhibition was over 50% at the Hi-Conc, GI₅₀ was assigned the Hi-Conc value.

Exp-ID	S3416	S3415	S3413	S3245	S3117	S3414	S3118	S3246	S3125	S3124	S3123	S1636	S1635	S1634	S1718
JCI No	-687	-686	-559	-559	-559	-560	-560	-560	-567	-566	-565	-296	-295	-294	-294
Name or Abbr.	TTNPB	13- <i>cis</i>	9- <i>cis</i>			ATRA			SV-TF	SV-CA	SV-AHB	SV-NNK	SV-NN	DTBHQ	
Hi-Conc.	-4	-4	-4	-4	-4	-4	-4	-4	-4	-4	-4	-4	-4	-4	-4
HBC-4	-4.76	-4.00	-4.53	-4.40	-4.43	-4.42	-4.41	-4.41	-5.87	-5.80	-5.66	-7.25	-7.31	-4.72	-4.80
BSY-1	-4.78	-4.16	-4.60	-4.73	-4.73	-4.69	-4.70	-4.81	-6.31	-6.06	-5.76	-6.93	-7.34	-5.07	-4.93
HBC-5	-4.80	-4.41	-4.56	-4.57	-4.61	-4.61	-4.47	-4.51	-6.98	-6.45	-5.73	-7.64	-7.72	-4.89	-4.78
MCF-7	-4.73	-4.35	-4.40	-4.39	-4.48	-4.48	-4.54	-4.66	-5.87	-5.78	-5.68	-6.77	-7.08	-5.29	-5.25
MDA-MB-231	-4.75	-4.21	-4.70	-4.55	-4.69	-4.63	-4.53	-4.65	-5.90	-5.86	-5.84	-6.84	-7.39	-5.52	-5.30
U251	-4.77	-4.14	-4.61	-4.51	-4.61	-4.57	-4.45	-4.63	-6.45	-5.76	-5.70	-6.85	-7.44	-4.96	-5.11
SF-268	-4.75	-4.00	-4.24	-4.55	-4.40	-4.47	-4.48	-4.76	-5.90	-5.79	-5.70	-7.53	-7.67	-4.77	-4.81
SF-295	-4.80	-4.29	-4.54	-4.66	-4.60	-4.59	-4.48	-4.57	-6.19	-5.80	-5.74	-6.89	-6.97	-4.87	-4.97
SF-539	-4.95	-4.35	-4.75	-4.80	-4.79	-4.80	-4.71	-4.76	-6.39	-5.96	-5.81	-7.79	-7.75	-4.79	-4.86
SNB-75	-5.31	-5.28	-5.13	-5.19	-4.71	-4.69	-4.69	-4.87	-6.41	-6.33	-5.93	-7.60	-7.70	-4.67	-4.80
SNB-78	-4.77	-4.25	-4.69	-4.78	-4.86	-4.49	-4.70	-4.68	-6.19	-6.00	-5.95	-6.97	-7.53	-4.75	-4.75
HCC2998	-4.68	-4.00	-4.48	-4.61	-4.62	-4.55	-4.62	-4.76	-5.91	-5.75	-5.67	-6.47	-6.77	-4.82	-4.75
KM-12	-4.70	-4.00	-4.46	-4.51	-4.48	-4.51	-4.47	-4.58	-5.93	-5.80	-5.65	-6.77	-6.87	-4.74	-4.77
HT-29	-4.73	-4.00	-4.47	-4.53	-4.50	-4.60	-4.52	-4.56	-5.90	-5.80	-5.56	-5.89	-6.78	-4.80	-4.89
HCT-15	-4.72	-4.25	-4.45	-4.49	-4.48	-4.52	-4.52	-4.53	-5.88	-5.76	-5.57	-6.73	-6.82	-4.72	-4.77
HCT-116	-4.77	-4.07	-4.67	-4.59	-4.67	-4.71	-4.61	-4.64	-6.46	-6.10	-5.77	-6.58	-6.82	-4.98	-5.13
NCI-H23	-4.74	-4.00	-4.47	-4.60	-4.59	-4.61	-4.55	-4.63	-6.11	-5.75	-5.72	-6.86	-7.42	-4.76	-4.90
NCI-H226	-4.72	-4.00	-4.61	-4.68	-4.78	-4.80	-4.54	-5.48	-5.95	-5.81	-5.76	-6.73	-6.78	-4.89	-4.91
NCI-H522	-4.72	-4.45	-4.68	-4.82	-4.77	-4.71	-4.71	-4.68	-6.45	-5.99	-5.78	-7.62	-7.46	-5.37	-5.37
NCI-H460	-4.70	-4.00	-4.55	-4.63	-4.58	-4.68	-4.55	-4.49	-5.96	-5.82	-5.72	-7.44	-7.42	-4.84	-4.84
A549	-4.79	-4.00	-4.72	-4.77	-4.78	-4.70	-4.62	-4.53	-5.91	-5.79	-5.71	-6.80	-6.83	-4.83	-4.87
DMS273	-4.57	-4.21	-4.50	-4.62	-4.55	-4.57	-4.51	-4.49	-6.20	-5.81	-5.72	-7.43	-7.44	-4.91	-4.98
DMS114	-4.77	-4.16	-4.33	-4.62	-4.49	-4.51	-4.53	-4.61	-6.66	-6.33	-5.77	-6.83	-6.88	-5.12	-5.21
LOX-IMVI	-4.77	-4.69	-4.68	-4.66	-4.70	-4.77	-4.74	-4.74	-6.75	-6.59	-5.76	-6.86	-6.94	-5.05	-5.15
OVCAR-3	-4.77	-4.38	-4.56	-4.67	-4.72	-4.64	-4.62	-4.71	-6.61	-6.13	-5.89	-6.77	-6.79	-4.89	-4.86
OVCAR-4	-4.72	-4.05	-4.63	-4.64	-4.64	-4.58	-4.39	-4.54	-6.73	-6.23	-5.80	-6.82	-6.90	-5.13	-4.90
OVCAR-5	-4.75	-4.00	-4.33	-4.39	-4.42	-4.44	-4.34	-4.44	-5.92	-5.74	-5.67	-6.46	-6.71	-5.22	-5.26
OVCAR-8	-4.75	-4.23	-4.50	-4.53	-4.59	-4.66	-4.67	-4.70	-5.95	-5.77	-5.69	-6.82	-6.84	-4.64	-4.70
SK-OV-3	-4.79	-4.00	-4.49	-4.51	-4.81	-4.52	-4.54	-4.50	-5.76	-5.64	-4.91	-6.75	-6.76	-4.64	-4.74
RXF-631L	-4.77	-4.00	-4.54	-4.58	-4.60	-4.72	-4.63	-4.61	-5.91	-5.80	-5.59	-7.13	-7.46	-4.81	-4.84
ACHN	-4.73	-4.00	-4.56	-4.66	-4.56	-4.50	-4.40	-4.76	-5.90	-5.79	-5.73	-6.74	-6.80	-4.71	-4.83
St-4	-4.74	-4.00	-4.42	-4.54	-4.65	-4.53	-4.49	-4.57	-5.91	-5.81	-5.76	-7.65	-7.70	-4.68	-4.75
MKN1	-4.75	-4.33	-4.56	-4.63	-4.62	-4.56	-4.45	-4.48	-6.15	-5.81	-5.78	-7.67	-7.68	-4.59	-4.81
MKN7	-4.78	-4.40	-4.68	-4.59	-4.70	-4.73	-4.56	-4.65	-6.29	-5.85	-5.76	-6.70	-6.90	-4.79	-4.84
MKN28	-4.71	-4.28	-4.56	-4.59	-4.59	-4.65	-4.56	-4.60	-6.10	-5.93	-5.68	-6.51	-6.81	-4.72	-4.89
MKN45	-4.72	-4.00	-4.51	-4.41	-4.46	-4.73	-4.41	-4.43	-6.06	-5.90	-5.69	-6.71	-6.82	-4.71	-4.87
MKN74	-4.74	-4.40	-4.61	-4.63	-4.61	-4.73	-4.68	-4.67	-5.97	-5.92	-5.61	-6.92	-7.00	-5.10	-5.42
DU-145	-4.68	-4.00	-4.25	-4.78	-4.41	-4.42	-4.44	-4.54	-6.08	-5.82	-5.75	-7.43	-7.55	-4.59	-5.02
PC-3	-4.74	-4.00	-4.58	-4.65	-4.48	-4.74	-4.39	-4.51	-5.83	-5.77	-5.61	-6.67	-6.69	-4.89	-4.74

abbreviations, applications, targets, and known mechanisms of 130 chemicals and 21 anticancer drugs. Approximately 15% of the chemicals were assessed twice or more. Approximately 40% of the chemicals tested had little effect on the growth of cells in the JFCR39 panel. However, the rest of the chemicals significantly inhibited the cell growth across the JFCR39 panel. For example, Fig. 1 shows the dose response curves of the cells in the JFCR39 panel against digoxin. The concentration at which the cell growth is inhibited by 50% represents GI50. Figure 2 shows the fingerprints of four chemicals [digoxin, ouabain, snake venom from *N. nigricollis* (SV-NN), and snake venom from *N. naja kaouthia* (SV-NNK)], which differentially inhibited the growth of cells in the JFCR39 panel; these fingerprints were drawn based on a calculation using a set of GI50s and clearly represented the GI50 pattern. These results were highly reproducible in that the Pearson correlation coefficient of the duplicate experiments for digoxin was 0.839 ($p < 0.001$) and that for ouabain was 0.864 ($p < 0.001$). It is noteworthy that, digoxin and ouabain, both of which are cardiac glycosides and inhibit Na-K ATPase, showed similar fingerprints. The fingerprints of SV-NNK and SV-NN, which belong to the elapidae, known as cobras, were also similar, but were different from the fingerprints of digoxin and ouabain. Table 2 summarizes only a portion of the GI50 values from 160 experiments involving 130 chemicals and 42 experiments involving 21 anticancer drugs. GI50 values from all experiments are described in the

Supplemental Data (Table S1). All these data were stored in a chemosensitivity database and used for further analysis.

Classification of the Chemicals by Hierarchical Clustering. Sixty-nine chemicals were selected for further analysis based on the following criteria: 1) GI50 values for the test chemical can be determined for at least 10 cell lines in the JFCR39 panel, and 2) the range of log GI50 for the test chemical is more than 0.6, suggesting differential growth inhibition. We analyzed the GI50 values of these 69 chemicals and 20 anticancer drugs by hierarchical clustering analysis (Fig. 3). We found approximately 12 clusters (threshold: $r = 0$, Fig. 3, clusters A–L), which were further divided into 49 subclusters (threshold: $r = 0.408$, Fig. 3, clusters A1–L6).

Analysis of Clusters. Most anticancer drugs we have tested belonged either to cluster A or cluster H, depending on their modes of action (Dan et al., 2002). The targets of the anticancer drugs belonging to the cluster A were related to DNA (Topo I, antimetabolite of pyridine, DNA alkylator) and the target of the anticancer drugs belonging to the cluster H was tubulin. We presently found that cisplatin exceptionally belonged to cluster F2, not cluster A, although it is known to cross-link DNA strands (Jamieson and Lippard, 1999; Wong and Giandomenico, 1999). We were also able to precisely group the clusters into several subclusters having similar characteristics. For example, the cardiac glycosides digoxin and ouabain were grouped in one cluster (cluster F3). SV-

S3243	S3244	S1534	S3237	S3238	S1525	S3236	S1928	S1421	S1705	S1327	S1413	S1413	S3408	S3409	S2421
-599	-599	-270	-270	-270	-261	-261	-261	-247	-247	-232	-232	-232	-421	-421	-421
Thiram		Ziram			Paraquat			Ouabain		Digoxin			TBT		
-4	-4	-4	-4	-4	-4	-3	-4	-4	-6	-4	-4	-4	-4	-4	-4
-4.71	-4.79	-5.80	-5.73	-5.70	-4.00	-3.61	-4.00	-7.54	-7.28	-6.57	-6.96	-6.96	-6.79	-6.77	-6.72
-6.97	-7.12	-6.85	-6.76	-6.60	-4.00	-4.45	-4.51	-8.00	-7.76	-7.58	-7.68	-7.68	-7.03	-7.01	-6.83
-7.41	-7.66	-7.18	-7.47	-7.47	-4.68	-4.70		-7.76	-7.51	-7.15	-7.44	-7.44	-6.76	-6.88	-6.83
-4.77	-4.80	-6.00	-5.84	-5.83	-4.06	-3.72	-4.00	-7.64	-7.51	-7.29	-7.39	-7.39	-6.86	-6.84	-6.79
-4.66	-4.68	-5.64	-5.75	-5.63	-4.00	-3.57	-4.00	-7.40	-6.81	-6.41	-6.72	-6.72	-6.83	-6.81	-6.70
-4.75	-4.78	-5.71	-5.79	-5.82	-4.00	-3.69	-4.00	-7.75	-7.16	-7.01	-7.40	-7.40	-6.79	-6.77	-6.72
-4.86	-4.96	-5.74	-5.83	-7.01	-4.00	-4.08	-4.00	-8.00	-7.77	-7.42	-7.70	-7.70	-6.84	-6.85	-6.71
-4.77	-4.89	-5.71	-5.70	-5.79	-4.47	-4.37	-4.20	-8.00	-7.64	-7.42	-7.55	-7.55	-6.75	-6.73	-6.76
-4.75	-4.88	-5.73	-5.75	-5.77	-4.00	-4.03	-4.00	-8.00	-7.70	-7.46	-7.63	-7.63	-6.77	-6.72	-6.67
-4.71	-4.96	-5.79	-5.92	-5.80	-4.00	-3.94	-4.00	-7.70	-7.45	-6.86	-7.40	-7.40	-6.99	-6.95	-7.05
-4.70	-4.78	-5.69	-5.64	-5.69	-4.00	-3.78	-4.00	-7.98	-7.64	-7.45	-7.60	-7.60	-6.72	-6.79	-6.70
-4.82	-4.69	-5.76	-5.79	-5.81	-4.00	-3.70	-4.00	-7.64	-6.77	-6.68	-7.25	-7.25	-6.77	-6.79	-6.72
-4.80	-4.80	-5.43	-5.74	-5.73	-4.00	-3.58	-4.00	-7.67	-7.12	-6.69	-7.34	-7.34	-7.00	-6.98	-6.74
-4.68	-4.85	-5.75	-5.77	-5.76	-4.10	-4.03	-4.07	-7.75	-7.31	-7.20	-7.34	-7.34	-6.89	-6.84	-6.66
-4.68	-4.75	-5.70	-5.72	-5.83	-4.00	-3.64	-4.00	-7.74	-7.63	-6.92	-7.54	-7.54	-6.88	-6.84	-6.70
-4.72	-4.72	-5.74	-5.68	-5.77	-4.00	-3.60	-4.00	-8.00	-7.57	-7.47	-7.62	-7.62	-6.90	-6.85	-6.74
-4.69	-4.78	-5.96	-5.85	-5.84	-4.19	-4.18	-4.00	-8.00	-7.67	-7.50	-7.84	-7.84	-6.90	-6.85	-6.76
-6.33	-6.74	-5.63	-5.96	-6.12	-4.41	-4.41	-4.00	-8.00	-7.37	-6.93	-7.61	-7.61	-6.99	-6.91	-6.74
-7.49	-7.50	-7.44	-7.66	-8.00	-4.49	-4.71	-4.59	-8.00	-7.64	-7.59	-7.91	-7.91	-6.83	-6.80	-6.25
-6.14	-6.16	-6.30	-6.10	-6.15	-4.30	-4.45	-4.37	-8.00	-7.74	-7.60	-7.77	-7.77	-6.98	-6.98	-6.56
-4.84	-4.82	-5.97	-5.91	-5.91	-4.49	-4.49	-4.41	-8.00	-7.80	-7.66	-7.91	-7.91	-6.82	-6.87	-6.73
-6.64	-6.58	-6.43	-6.84	-6.82	-4.25	-4.43	-4.30	-8.00	-7.71	-7.48	-7.72	-7.72	-6.74	-6.75	-6.70
-7.18	-7.39	-7.37	-7.38	-7.43	-4.50	-4.63	-4.27	-8.00	-7.84	-7.73	-8.00	-8.00	-7.11	-7.12	-7.02
-4.68	-4.71	-5.66	-5.71	-5.70	-4.00	-3.51	-4.00	-7.80	-7.80	-7.39	-7.46	-7.46	-6.93	-6.94	-6.76
-4.86	-6.25	-6.07	-6.35	-6.32	-4.00	-4.46	-4.28	-8.00	-7.66	-7.64	-7.59	-7.59	-6.80	-6.82	-6.74
-4.77	-6.67	-5.90	-5.91	-5.87	-4.00	-4.21	-4.48	-8.00	-7.71	-7.71	-7.72	-7.72	-6.91	-7.20	-6.80
-4.90	-6.00	-6.11	-6.91	-6.74	-4.00	-3.98	-4.00	-7.89	-7.62	-7.12	-7.42	-7.42	-6.90	-6.93	-6.75
-4.62	-4.74	-5.59	-5.68	-5.67	-4.00	-3.84	-4.00	-7.85	-7.44	-6.97	-7.29	-7.29	-6.73	-6.67	-6.57
-4.39	-4.38	-4.92	-5.49	-5.53	-4.00	-3.39	-4.00	-7.74	-7.67	-7.18	-7.38	-7.38	-6.80	-6.77	-6.68
-4.75	-4.65	-5.57	-5.63	-5.60	-4.00	-3.60	-4.00	-7.10	-6.00	-6.42	-6.20	-6.20	-6.78	-6.76	-6.68
-4.52	-4.60	-5.64	-5.68	-5.69	-4.00	-3.51	-4.00	-8.00	-7.72	-7.44	-7.59	-7.59	-6.78	-6.77	-6.76
-4.59	-4.72	-5.99	-5.73	-5.81	-4.00	-3.58	-4.00	-8.00	-7.65	-7.45	-7.60	-7.60	-6.80	-6.80	-6.72
-4.80	-4.85	-6.82	-5.84	-5.92	-4.41	-4.61	-4.48	-8.00	-7.72	-7.68	-7.72	-7.72	-7.25	-7.15	-6.87
-4.79	-4.82	-6.56	-5.84	-5.82	-4.08	-4.29	-4.32	-7.80	-7.48	-6.98	-7.47	-7.47	-7.34	-7.07	-6.86
-7.18	-7.21	-5.82	-7.09	-7.13	-4.00	-4.40	-4.18	-7.70	-7.42	-6.77	-7.37	-7.37	-6.84	-6.82	-6.87
-6.65	-6.71	-6.05	-7.18	-6.86	-4.00	-4.29	-4.35	-7.77	-7.25	-6.99	-7.52	-7.52	-6.96	-6.97	-6.87
-7.05	-7.08	-6.35	-5.86	-7.05	-4.00	-4.47	-4.06	-7.91	-7.65	-7.55	-7.74	-7.74	-6.97	-7.37	-7.05
-4.47	-4.70	-5.68	-5.68	-5.65	-4.00	-3.57	-4.00	-8.00	-7.64	-7.59	-7.71	-7.71	-6.90	-6.89	-6.70
-4.42	-4.77	-5.61	-5.53	-5.56	-4.00	-3.64	-4.37	-8.00	-7.62	-7.41	-7.62	-7.62	-6.77	-6.78	-6.73