based on an appropriately conducted pre-study or historical data. If a toxicity study of a test article formulated in a vehicle reveals no toxicity, it follows that the vehicle is non-toxic at the concentration tested; thus, there is no need for a vehicle control.

EXPOSURE CONDITIONS

Administration of Concentrations

17. Animals are exposed to the test article for 6 hours per day on a 5 day per week basis for a period of at least 90 days (13 weeks). Animals may also be exposed 7 days per week (e.g. when testing inhaled pharmaceuticals). Justification should be provided if a species other than rat is tested or if it is necessary to conduct a long duration (e.g. 22 hours/day) whole-body exposure study (refer to GD 39). (2) Although males and females are always tested, they may be exposed at different concentration levels to yield optimal data and to identify gender differences in sensitivity. Feed should be withheld during the exposure period unless exposure exceeds 6 hours (4 hours for mice). Water may be provided throughout a whole-body exposure.

18. Animals are exposed to the test article as a gas, vapour, aerosol, or a mixture thereof. The physical state to be tested depends on the physico-chemical properties of the test article, the selected concentration, and/or the physical form most likely present during the handling and use of the test article. Hygroscopic and chemically reactive test articles should be tested under dry air conditions. Care should be taken to avoid generating explosive concentrations.

Particle-Size Distribution

19. Particle sizing should be performed for all aerosols and for vapours that may condense to form aerosols. To allow for exposure of all relevant regions of the respiratory tract, aerosols with mass median aerodynamic diameters (MMAD) ranging from 1 to 3 μm with a geometric standard deviation (σ g) in the range of 1.5 to 3.0 are recommended (4). Although a reasonable effort should be made to meet this standard, expert judgement should be provided if it cannot be achieved. For example, metal fume particles may be smaller than this standard, and charged particles, fibers, and hygroscopic materials (which increase in size in the moist environment of the respiratory tract) may exceed this standard.

Test Article Preparation in a Vehicle

20. Ideally, the test article should be tested without a vehicle. If it is necessary to use a vehicle to generate an appropriate test article concentration and particle size, water should be given preference. Whenever a test article is dissolved in a vehicle, stability and homogeneity of the tested solution must be demonstrated.

MONITORING OF EXPOSURE CONDITIONS

Chamber Airflow

21. The flow of air through the exposure chamber should be carefully controlled, continuously monitored, and recorded at least hourly during each exposure. Oxygen concentration should be at least 19% and carbon dioxide concentration should not exceed 1%.

Chamber Temperature and Relative Humidity

22. Chamber temperature should be maintained at 22 ±3° C. Relative humidity in the animals' breathing zone, for both nose-only and whole-body exposures, should be monitored continuously and recorded hourly during each exposure where possible. The relative humidity should ideally be maintained in the range of 30 to 70%, but this may either be unattainable (e.g., when testing water based formulations) or not measurable due to test article interference with the test method.

Test Article: Nominal Concentration

23. Whenever feasible, the nominal exposure chamber concentration should be calculated and recorded. The nominal concentration is the mass of generated test article divided by the total volume of air passed through the chamber. The nominal concentration is not used to characterize the animals' exposure, although it can be used to characterize gas exposure if an analytical method is not available (assuming 100% exposure efficiency with pressurized gases; not applicable to generated gases). The determination of nominal concentrations for solid test articles may require the dust generation system to be dismantled. If this results in day-to-day variability in actual test concentrations, nominal concentrations need not be measured.

Test Article: Actual Concentration

24. The actual concentration is the test article concentration at the animals' breathing zone in an inhalation chamber. Actual concentrations can be obtained either by specific methods (e.g., direct sampling, adsorptive or chemical reactive methods, and subsequent analytical characterisation) or by non-specific methods such as gravimetric filter analysis. When using non-specific methods, stability of the test compound over the duration of the study must be known. If this information is not available, a reanalysis of the test material at regular intervals during the course of the study may be necessary. For aerosolised agents that may evaporate/sublimate, it must be shown that all phases were collected by the method chosen.

25. One lot of the test article should be used, if possible, throughout the duration of the study, and the research sample should be stored under conditions that maintain its purity and stability. Preferably prior to the start of the study, there should be a characterization of the test article including its purity and, if technically feasible, the name and quantities of unknown contaminants and impurities.

26. The exposure atmosphere should be held as constant as practicable. A monitoring device, such as an aerosol photometer for aerosols or a total hydrocarbon analyser for vapours may be used to demonstrate the stability of the exposure conditions. Actual chamber concentration should be measured at least weekly for each exposure level. Individual chamber concentration samples should deviate from the mean chamber concentration by no more than ±10% for gases and vapours, and by no more than ±20% for liquid or solid aerosols. Time to chamber equilibration and decay (t_{95}) should be recorded for whole-body exposures, but is unnecessary for nose-only exposures due to low chamber volume.

27. For very complex mixtures consisting of gases/vapours and aerosols (e.g. combustion atmospheres and test articles propelled from purpose-driven end-use products/devices), both phases may behave differently in an inhalation chamber. Therefore, at least one indicator substance (analyte) of each phase (gas/vapour and aerosol) must be selected. When the test article is a mixture (e.g. a formulation), the analytical concentration should be reported for the total formulation and not just for the active ingredient or the component (analyte). Additional information regarding actual concentrations can be found in GD 39. (2)

Test Article: Particle Size Distribution

28. The particle size distribution of aerosols should be determined at least weekly for each concentration level by using a cascade impactor or aerodynamic particle sizer (APS). A second device, such as a gravimetric filter or an impinger, should be used to confirm the accuracy of the primary instrument. The mass concentration obtained by particle size analysis should be within reasonable limits of the mass concentration obtained by filter analysis (see GD 39). (2) If equivalence of APS and impactor methods can be shown, then APS methods may be used throughout the study. Particle sizing should be performed for vapours if there is any possibility that vapour condensation will result in the formation of an aerosol.

OBSERVATIONS

29. The animals should be observed frequently during the exposure period. Careful clinical observations should be made during and following exposure, or more frequently when indicated by the response of the animals to treatment. When animal observation is hindered by the use of animal tubes or poorly lit whole body chambers, animals should be carefully observed after exposure and before the next exposure day in order to assess reversibility or exacerbation of toxic effects.

30. All observations are systematically recorded with individual records being maintained for each animal. Unless there are compelling reasons to do otherwise, animals found in a moribund condition and animals showing severe pain and/or enduring signs of severe distress should be humanely killed without delay for animal welfare reasons. When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible. It is important to note that poor appearance immediately following exposure is generally not a treatment-related clinical sign.

31. Cage-side observations should include changes in the skin and fur, eyes, and mucous membranes; changes in the respiratory and circulatory systems, changes in the autonomic and central nervous systems; and changes in somatomotor activity and behaviour patterns. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep, and coma. The measurement of rectal temperatures may provide supportive evidence of reflex bradypnea or hypo/hyperthermia related to treatment or confinement. Additional assessments may be included in the study protocol such as kinetics, biomonitoring, lung lavage, lung function, retention of poorly soluble materials that accumulate in lung tissue, and behavioural changes.

BODY WEIGHTS

32. Individual animal weights should be recorded shortly before the first exposure (day 0), twice weekly thereafter (preferably on Friday and Monday to demonstrate recovery over an exposure-free weekend), at the time of death or euthanasia, and at study termination. Satellite animals (if used) should continue to be weighed twice weekly throughout the recovery period and at study termination.

FOOD AND WATER CONSUMPTION

33. Food consumption should be measured weekly. Water consumption may also be measured.

CLINICAL PATHOLOGY

34. Clinical pathology assessments should be made for all animals, including controls and satellite animals, when they are sacrificed. Periodic testing throughout the study is recommended for large animals and interim sacrifice animals. The time interval between exposure and blood collection should be defined.

Rapid sampling after exposure is indicated for parameters with a short plasma half-time (e.g., COHb, CHE, and MetHb).

35. Table 1 lists the clinical pathology parameters that are generally required for all toxicology studies. Urinalysis is not required on a routine basis, but may be performed when deemed useful based on expected or observed toxicity. The study director may choose to assess additional parameters in order to better characterize a test article's toxicity (e.g., cholinesterase, lipids, hormones, acid/base balance, methaemoglobin, creatine kinase, myeloid/erythroid ratio, and blood gases).

Table 1. Standard Clinical Pathology Parameters

Haematology	
Erythrocyte count (RBC)	Heinz bodies
Haematocrit (Hct)	Total leukocyte count (WBC)
Haemoglobin concentration (Hb)	Differential leukocyte count (Diffs)
Mean corpuscular haemoglobin (MCH)	Platelet count (Plate)
Mean corpuscular volume (MCV)	Clotting potential (select one):
Mean corpuscular haemoglobin concentration (MCHC)	Prothrombin time (PT)
	Clotting time (CT)
Reticulocytes (Retics)	Partial thromboplastin time (PTT)

Clinical Chemistry	
Fasting glucose (GLU)	Alanine aminotransferase (ALT)
Total cholesterol (Chol)	Aspartate aminotransferase (AST)
Triglycerides (TRIG)	Alkaline phosphatase (ALP) or
Blood urea nitrogen (BUN)	Sorbitol dehydrogenase (SDH)
Total bilirubin (T. Bili)	Potassium (K)
Creatinine (Creat)	Sodium (Na)
Lactate dehydrogenase (LDH)	Calcium (Ca)
Total protein (T. Prot)	Phosphorus (P)
Albumin (Alb)	Chloride (Cl)
Globulin (Glob)	

Urinalysis (optional)	
Appearance (colour and turbidity)	PH
Volume	Total protein
Specific gravity or osmolality	Glucose

36. If bronchoalveolar lavage (BAL) is performed, it should be carried out in the same animals used for histopathological examination of the lungs in order to minimize animal usage. This is accomplished by using the left lung for lavage and the right unlavaged lung for microscopic examination. The BAL fluid is analysed for total and differential cell counts, total protein, and lactate dehydrogenase. Other parameters that may be considered are those indicative of lysosomal injury, phospholipidosis, fibrosis, and irritant or allergic inflammation which may include the determination of pro-inflammatory cytokines/chemokines. The targeted lavage volume (per cycle) should be 20 mL/kg-rat. If the focus of the study is structural lung changes such as emphysema and fibrosis, then lavaged lungs may be suitable for histopathology.

OPHTHALMOLOGICAL EXAMINATION

37. Using an ophthalmoscope or an equivalent device, ophthalmological examinations of the fundus, refractive media, iris, and conjunctivae should be performed for all animals prior to the administration of the test article, and for all high concentration and control groups at termination. If changes in the eyes are detected, all animals in the other groups should be examined.

GROSS PATHOLOGY AND ORGAN WEIGHTS

38. All test animals, including those which die during the test or are removed from the study for animal welfare reasons, should be subjected to complete exsanguination and gross necropsy. The time between the end of each animal's last exposure and their sacrifice should be recorded. If a necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated (not frozen) at a temperature low enough to minimize autolysis. Necropsies should be performed as soon as possible, normally within a day or two. All gross pathological changes should be recorded for each animal with particular attention to any changes in the respiratory tract.

39. Table 2 lists the organs and tissues that should be preserved in a suitable medium during gross necropsy for histopathological examination. The preservation of the bracketed organs and tissues and any other organs and tissues is at the discretion of the study director. The **bolded** organs should be trimmed and weighed wet as soon as possible after dissection to avoid drying. The thyroid and epididymides should only be weighed if needed because trimming artefacts may hinder histopathological evaluation. Tissues and organs should be fixed in 10% buffered formalin or another suitable fixative as soon as necropsy is performed, and no less than 48 hours prior to trimming.

Table 2. Organs and Tissues Preserved During Gross Necropsy

Trachea (at least 2 levels including 1 longitudinal section through the carina and 1 transverse section)
Lung (all lobes at one level, including main bronchi)
Nasopharyngeal tissues (at least 4 levels; 1 level to include the nasopharyngeal duct)
Nasal associated lymphoid tissue (NALT)
Larynx (3 levels including the base of the epiglottis)
Nasal turbinates (at least 4 levels)
Upper (cervical/submandibular) and lower (mediastinal/tracheobronchial/hilar) respiratory tract draining lymph nodes
[Tongue]
Teeth
Salivary glands
Oesophagus
Stomach
Duodenum
Jejunum
Ileum
Caecum
Colon
Rectum
Liver
Pancreas
Gallbladder (where present)
Brain (including sections of cerebrum, cerebellum, and medulla/pons)
Pituitary
Olfactory bulb
[Eyes (retina, optic nerve) and eyelids]
Peripheral nerve (sciatic or tibial, preferably close to muscle)
Spinal cord (cervical, mid-thoracic, and lumbar)
Adrenals
Thyroids
Parathyroids
Heart
Aorta
Bone marrow (and/or fresh aspirate)
Spleen
Thymus
Lymph nodes (preferably one node covering the portal-of-entry, and one distal from the portal-of-entry)
Kidneys
Urinary bladder
[Uteter]
[Urethra]
Prostate
Testes
[Epididymides]
Seminal vesicles
Uterus
Ovaries
Skin
Mammary gland (female)
Muscle (thigh)
[Harderian gland]
[Lacrimal gland (extraorbital)]
Femur and joint
Sternum
Target organs
All gross lesions and masses

40. The lungs should be removed intact, weighed, and instilled with a suitable fixative at a pressure of 20-30 cm of water to approximately 80-90% of total lung capacity to ensure that lung structure is maintained (5). Sections should be collected for all lobes at one level, including main bronchi, but if lung lavage is performed, the unlavaged lobe should be sectioned at three levels (not serial sections). Additional guidance is provided in GDXX.

41. At least 4 levels of the nasopharyngeal tissues should be examined, one of which should include the nasopharyngeal duct, (5, 6, 7, 8, 9) to allow adequate examination of the squamous, transitional (non-ciliated respiratory), respiratory (ciliated respiratory) and olfactory epithelium, and the draining lymphatic tissue (10, 11). Three levels of the larynx should be examined, and one of these levels should include the base of the epiglottis (12). At least two levels of the trachea should be examined including one longitudinal section through the carina and 1 transverse section. Additional guidance is provided in GD XX.

HISTOPATHOLOGY

42. A histopathological evaluation of all the organs and tissues listed in Table 2 should be performed for the control and high concentration groups, and for all animals which die or are sacrificed during the study. Particular attention should be paid to the respiratory tract, target organs, and gross lesions. When a satellite group is used, histopathological evaluation should be performed for all tissues and organs identified as showing effects in the treated groups. If there are excessive early deaths or other problems in the high exposure group that compromise the significance of the data, the next lower concentration should be examined histopathologically. An attempt should be made to correlate gross observations with microscopic findings.

DATA AND REPORTING

Data

43. Individual animal data on body weights, food consumption, clinical pathology, gross pathology, organ weights, and histopathology should be provided. Clinical observation data should be summarized in tabular form showing for each test group the number of animals used, the number of animals displaying specific signs of toxicity, the number of animals found dead during the test or killed for humane reasons, time of death of individual animals, a description and time course of toxic effects and reversibility, and necropsy findings. All results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used and the statistical methods should be selected during the design of the study.

Test Report

44. The test report should include the following information, as appropriate:

Test animals and husbandry

- Description of caging conditions, including: number (or change in number) of animals per cage, bedding material, ambient temperature and relative humidity, photoperiod, and identification of diet.
- Species/strain used, including source and historical data, and justification for using a species other than the rat.
- Number, age, and sex of animals.
- Method of randomization.
- Description of any pre-test conditioning including diet, quarantine, and treatment for disease.

Test article

- Physical nature, purity, and, where relevant, physico-chemical properties (including isomerization).
- Identification data and Chemical Abstract Services (CAS) Registry Number, if known.

Vehicle

- Justification for use of vehicle and justification for choice of vehicle (if other than water).
- Historical or concurrent data demonstrating that the vehicle does not interfere with the outcome of the study.

Inhalation chamber

- Description of the inhalation chamber including dimensions and volume.
- Source and description of equipment used for the exposure of animals as well as generation of atmosphere.
- Equipment for measuring temperature, humidity, particle-size, and actual concentration.
- Source of air and system used for conditioning.
- Methods used for calibration of equipment to ensure a homogeneous test atmosphere.
- Pressure difference (positive or negative).
- Exposure ports per chamber (nose-only); location of animals in the chamber (whole-body).
- Temporal homogeneity/stability of test atmosphere.
- Location of temperature and humidity sensors and sampling of test atmosphere in the chamber.
- Treatment of air supplied/extracted.
- Air flow rates, air flow rate/exposure port (nose-only), or animal load/chamber (whole-body).
- Time to inhalation chamber equilibrium and decay (t_{95}) for whole-body exposures.
- Number of volume changes per hour.
- Metering devices (if applicable).

Exposure data

- Rationale for target concentration selection in the main study.
- Nominal concentrations (total mass of test article generated into the inhalation chamber divided by the volume of air passed through the chamber).
- Actual test article concentrations collected from the animals' breathing zone; for test mixtures that produce heterogeneous physical forms (gases, vapours, aerosols), each may be analysed separately.
- All air concentrations should be reported in units of mass (mg/L, mg/m^3, etc.) rather than in units of volume (ppm, ppb, etc.).
- Particle size distribution, mass median aerodynamic diameter (MMAD), and geometric standard deviation (σ g), including their methods of calculation. Individual particle size analyses must be reported.

Test conditions

- Details of test article preparation, including details of any procedures used to reduce the particle size of solid materials or to prepare solutions of the test article.
- A description (preferably including a diagram) of the equipment used to generate the test atmosphere and to expose the animals to the test atmosphere.
- Details of the equipment used to monitor chamber temperature, humidity, and chamber airflow (i.e. development of a calibration curve).
- Details of the equipment used to collect samples for determination of chamber concentration and particle size distribution.
- Details of the chemical analytical method used and method validation (including efficiency of recovery of test article from the sampling medium).

- Method of randomization in assigning animals to test and control groups.
- Details of food and water quality (including diet type/source, water source).
- The rationale for the selection of test concentrations.

Results

- Tabulation of chamber temperature, humidity, and airflow.
- Tabulation of chamber nominal and actual concentration data.
- Tabulation of particle size data including analytical sample collection data, particle size distribution, and calculations of the MMAD and σ g.
- Tabulation of response data and concentration level for each animal (i.e., animals showing signs of toxicity including mortality, nature, severity, time of onset, and duration of effects).
- Tabulation of individual animal weights.
- Tabulation of food consumption
- Tabulation of clinical pathology data
- Necropsy findings and histopathological findings for each animal, if available.

Discussion and interpretation of results

- Particular emphasis should be made to the description of methods used to meet the criteria of this test guideline, e.g., the limit concentration or the particle size.
- The respirability of particles in light of the overall findings must be addressed, especially if the particle-size criteria could not be met.
- The consistency of methods used to determine nominal and actual concentrations, and the relation of actual concentration to nominal concentration must be included in the overall assessment of the study.
- The likely cause of death and predominant mode of action (systemic versus local) should be addressed.
- The target organ(s) should be identified.
- The NOAEL and LOAEL should be determined.

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研究成果の刊行に関する一覧表

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Cell

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;

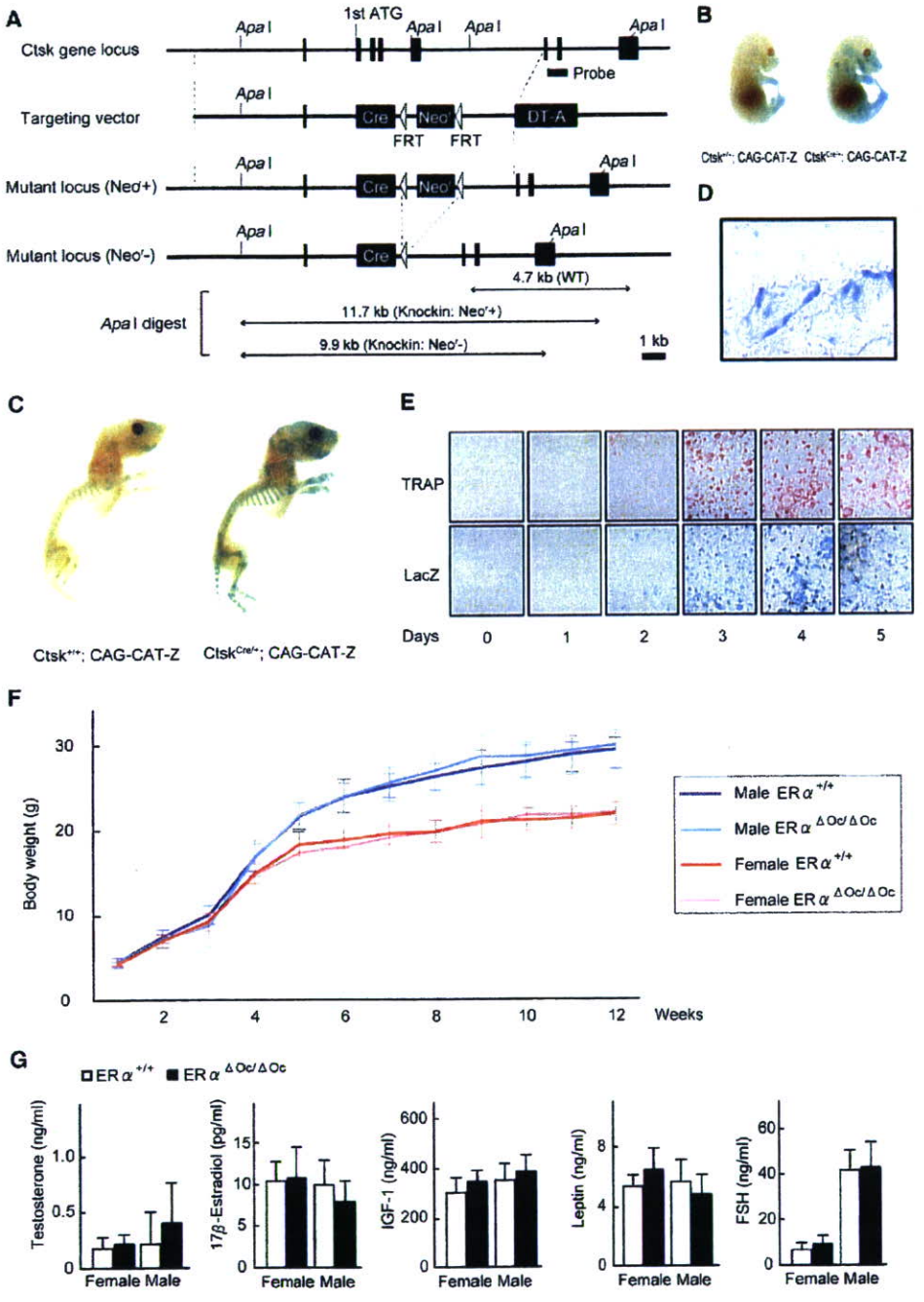

Figure 1. Generation of Knockin Mice Selectively Expressing Cre in Mature Osteoclasts

(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

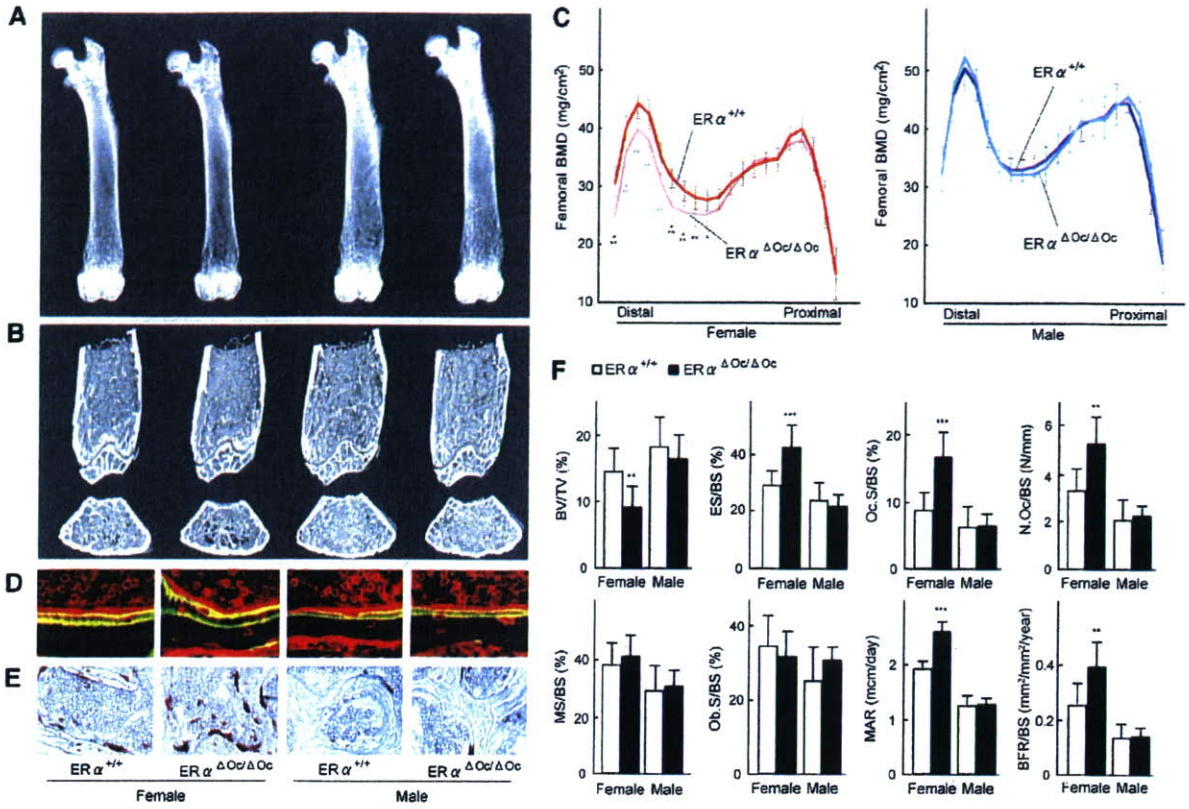

Figure 2. High Bone Turnover Osteopenia Was Observed in $ER\alpha^{\Delta Oc/\Delta Oc}$ Females But Not Males

(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).

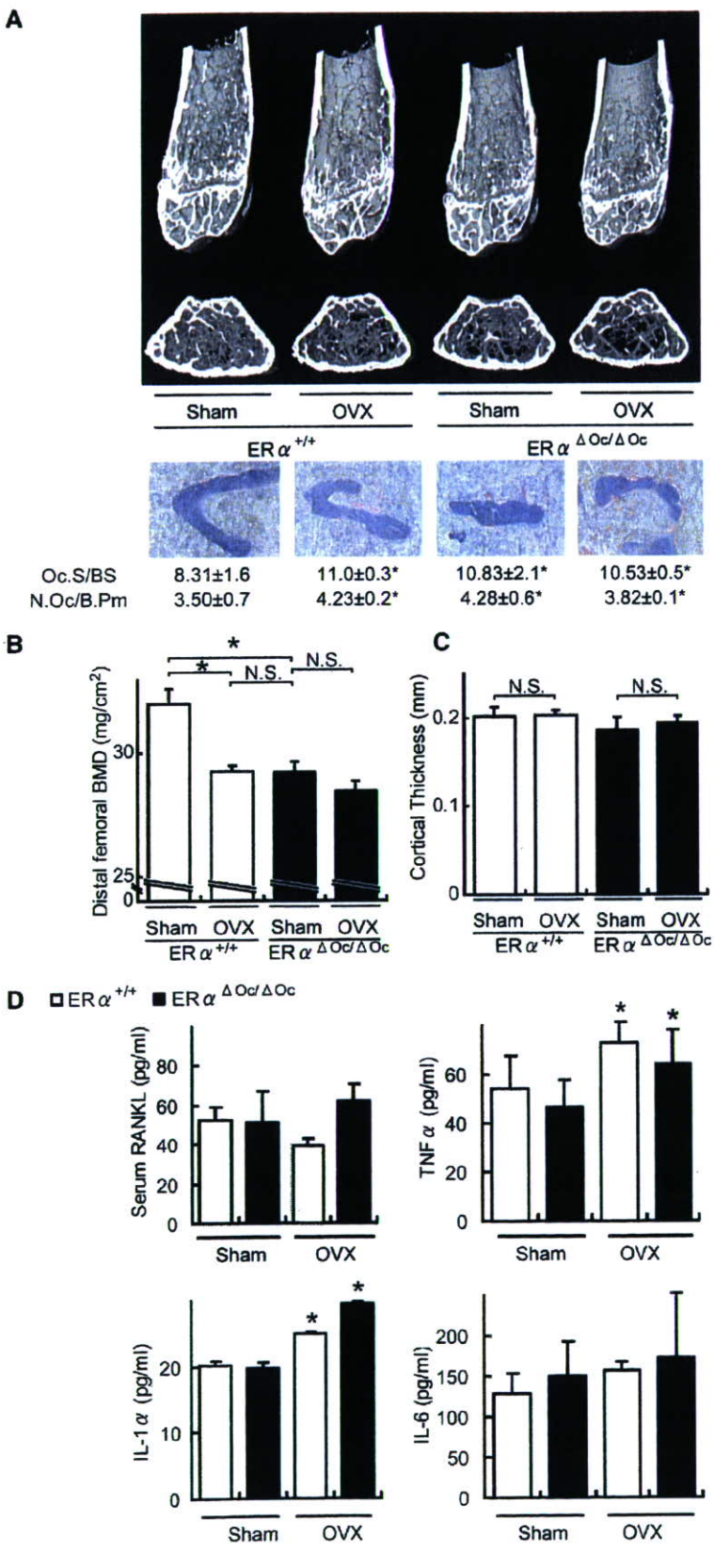

Figure 3. No Further Bone Loss of $ER\alpha^{\Delta Oc/\Delta Oc}$ Females by Ovariectomy

(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.

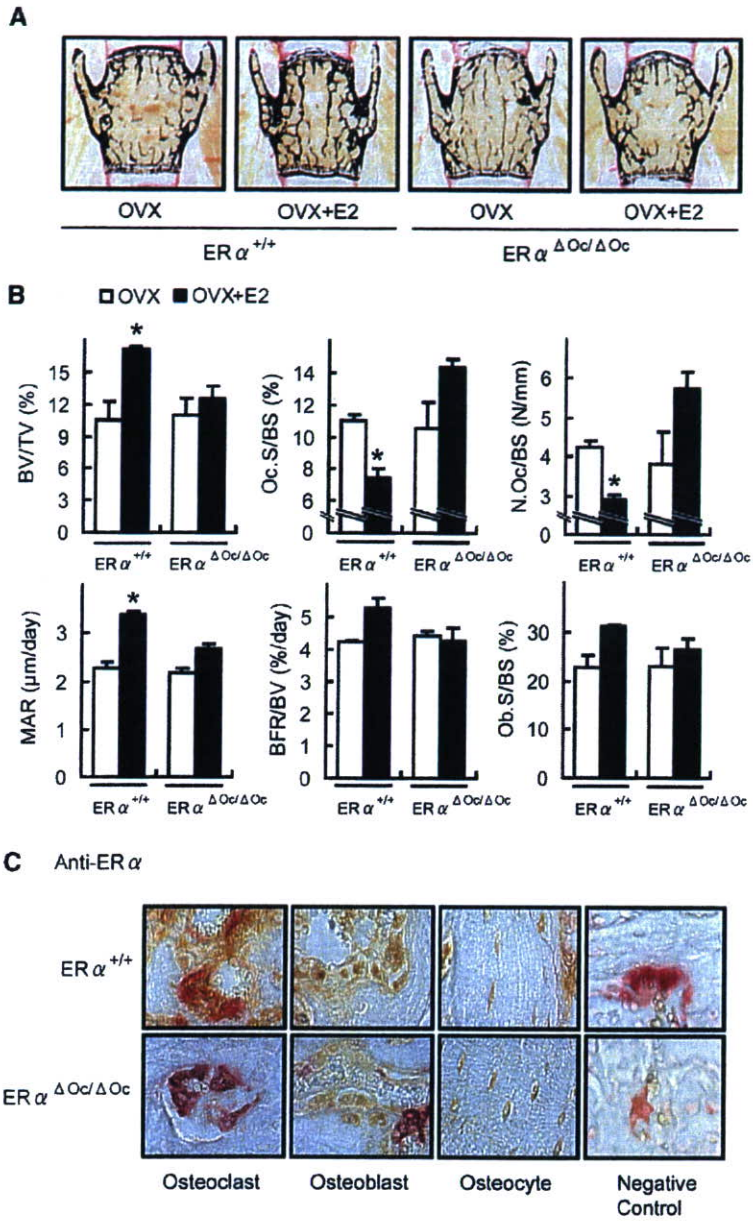

Figure 4. Estrogen treatment failed to reverse trabecular bone loss of ovariectomized $ER\alpha^{\Delta Oc/\Delta Oc}$ females

(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

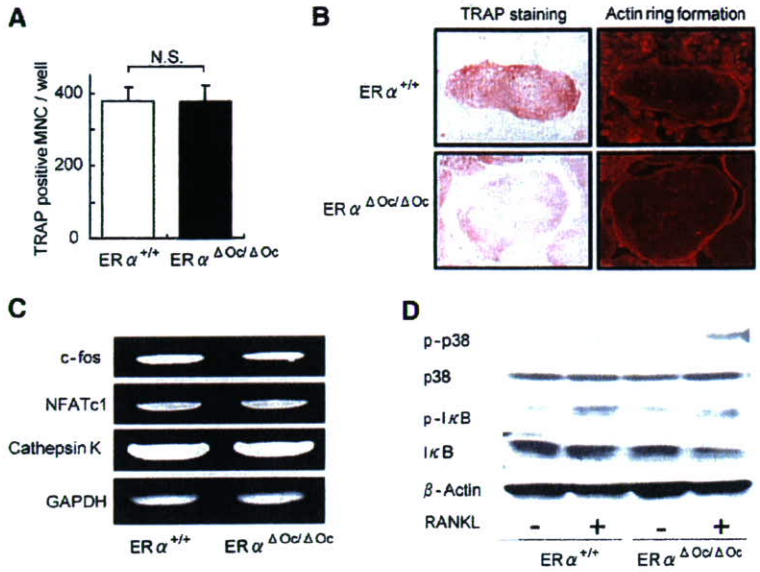

Figure 5. ERα Deficiency Did Not Affect Osteoclastogenesis

(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 $ER\alpha^{+/+}$ 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 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 $ER\alpha^{+/+}$ 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 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 $ER\alpha^{+/+}$ 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). ERα 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 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.

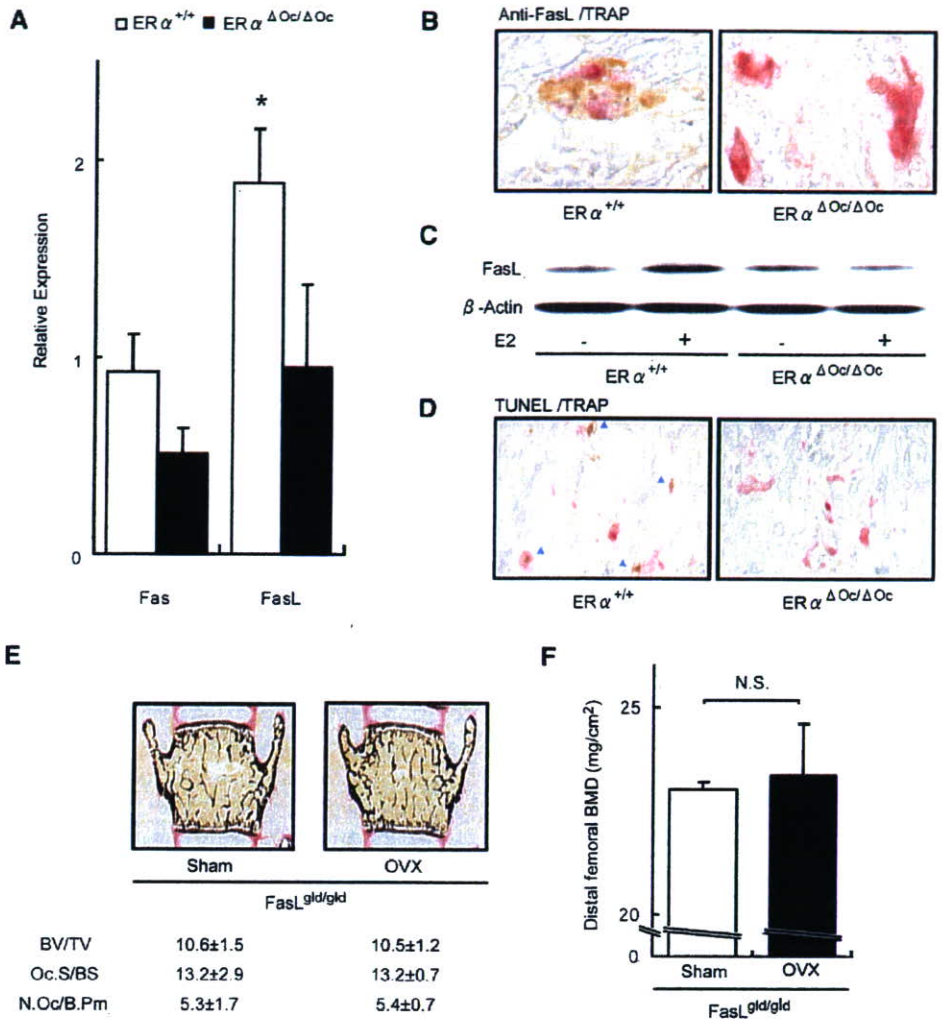

Figure 6. Activated ERα Induced Fas Ligand Expression and Apoptosis in Differentiated Osteoclasts of Intact Bone

(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