0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(5):1994–1999

doi: 10.1210/en.2002-0074

Estrogen Receptor-α Dependency of Estrogen's Stimulatory Action on Cancellous Bone Formation in Male Mice

KATHLEEN E. McDOUGALL, MARK J. PERRY, RACHEL L. GIBSON, SHANE M. COLLEY, KENNETH S. KORACH, AND JONATHAN H. TOBIAS

Academic Rheumatology (K.E.M., M.J.P., R.L.G., S.M.C., J.H.T.), University of Bristol, Bristol BS2 8HW, United Kingdom; and National Institute of Environmental Health Sciences (K.S.K.), National Institutes of Health, Research Triangle Park, North Carolina 27709

We examined whether estrogen receptor (ER)α is required for estrogen to stimulate cancellous bone formation in long bones of male mice. 17β-Estradiol (E_2) was administered to ER$\alpha^{-/-}$ male mice or wild-type (WT) littermate controls at 40, 400, or 4000 μg/kg by daily sc injection for 28 d and histomorphometric analysis performed at the distal femoral metaphysis. In WT mice, treatment with E_2 (40 μg/kg·d) increased the proportion of cancellous bone surfaces undergoing mineralization and stimulated mineral apposition rate. In addition, higher doses of E_2 induced the formation of new cancellous bone formation surfaces in WT mice. In contrast, E_2 had little effect on any of these parameters in ER$\alpha^{-/-}$ mice. Immunohistochemistry was subsequently performed using an ERα-specific C-terminal polyclonal antibody. In WT mice, ERα was expressed both by cancellous osteoblasts and a significant proportion of mononuclear bone marrow cells. Immunoreactivity was also observed in cancellous osteoblasts of ER$\alpha^{-/-}$ mice, resulting from expression of the activation function-1-deficient 46-kDa ERα isoform previously reported to be expressed in normal osteoblasts and bones of ER$\alpha^{-/-}$ mice. Taken together, our results suggest that estrogen stimulates bone formation in mouse long bones via a mechanism that requires the presence of full-length ERα possessing activation function-1. (*Endocrinology* 144: 1994–1999, 2003)

ESTROGEN EXERTS A significant protective effect on the skeleton, the loss of which predisposes to the development of postmenopausal osteoporosis (1). This action has long been recognized to involve inhibition of osteoclastic bone resorption whereby estrogen acts to suppress bone turnover and prevent loss of cancellous bone (2, 3). In addition, recent observations suggest that estrogen, when administered at relatively high doses as estradiol implants, acts to stimulate osteoblast function at cancellous bone surfaces (4, 5). The latter effect may represent both direct actions of estrogen on osteoblasts involving the suppression of osteoblast apoptosis (6) and indirect effects mediated by locally produced growth factors in bone (7, 8).

The biological effects of estrogen are mediated by the estrogen receptor (ER), which exists in at least two distinct isoforms, ERα and ERβ, both of which are expressed in bone cells at significant levels as assessed under *in vitro* and *in vivo* conditions (9–17). Recent *in vitro* studies suggest that ERα predominantly acts to mediate ligand-induced transcription, whereas ERβ serves to modulate this response (18, 19). Consistent with this view, the finding of reduced bone mass in a man with ERα deficiency (20) suggests that ERα plays a central role in mediating the stimulatory action of estrogen on osteoblast function.

To date, analysis of the skeletal phenotype of ER$\alpha^{-/-}$ mice has made a limited contribution to our understanding of the role of ERα in estrogen's stimulatory action on osteoblast activity. For example, previous studies have found that rather than bone loss, ER$\alpha^{-/-}$ mice demonstrate preserved or even increased cancellous bone mass (21–23). Although Sims *et al.* (21) found that indices of osteoblast function were reduced in male ER$\alpha^{-/-}$ mice, this was associated with an increase in cancellous bone volume and a reduction in osteoclast surface and was thought to reflect reduced bone turnover rather than deficient ERα-dependent stimulation of osteoblast function.

We have used the mouse as an animal model to explore the stimulatory action of high-dose estrogen on cancellous bone formation, by analyzing changes in fluorochrome-based indices of bone formation in long bone sections following estrogen administration in intact female mice (24). In pharmacological studies, we confirmed that estrogen-induced cancellous bone formation in mouse long bones is ER dependent (25), but analysis of ER$\beta^{-/-}$ mice demonstrated that ERβ is not required for this response (26). In the present study, we aimed to use the same approach to determine whether ERα is necessary for estrogen-induced bone formation, by analyzing this response in ER$\alpha^{-/-}$ mice. Estradiol levels are grossly elevated in female ER$\alpha^{-/-}$ mice (21), and, although this can be prevented by ovariectomy, the latter might engender further skeletal effects. Therefore, the present investigation used male ER$\alpha^{-/-}$ mice, in view of previous findings that these show normal estradiol levels (21) and our unpublished observations that male mice show an equivalent cancellous bone response to estrogen to that in

Abbreviations: AF, Activation function; ALP, alkaline phosphatase; BV/TV, cancellous bone area expressed as a percentage of total tissue area; dlS/BS, percentage of the total length of cancellous bone perimeter; dlS/TV, tissue area referent; E_2, 17β-estradiol; ER, estrogen receptor; MAR, mineral apposition rate; WT, wild-type.

females as assessed by direct comparison of dose-response profiles.

Materials and Methods

Experimental design

ERα$^{-/-}$ mice were generated at the National Institute of Environmental Health Sciences, back-crossed onto a C57Bl/6 genetic background, transferred to the University of Bristol animal facility, and crossed with wild-type C57Bl/6 mice from the local breeding stock (27). PCR-based genotyping was performed on DNA extracted from tail tips at 4–6 wk of age, based on previously published primer sets. Intact 14-wk-old male ERα$^{-/-}$ mice and age-matched littermate controls were subsequently administered vehicle [0.1 ml corn oil (Sigma, Poole, Dorset, UK)], or 17β-estradiol (E_2; Sigma) 40, 400, or 4000 μg/kg by daily sc injection (4–7 animals/group). This protocol was employed on the basis of our previous study in which we defined the dose responsiveness of estrogen-induced osteogenesis in intact female mice (25).

Throughout the study animals received a standard diet (Rat and Mouse Standard Diet, B&K Ltd., Humberside, UK) and water *ad libitum* and were kept with a cycle of 12-h light and 12-h darkness. The experimental duration was 28 d, with tetracycline hydrochloride (25 mg/kg; Sigma) and calcein (30 mg/kg; Sigma) being injected ip at 5 and 1 d, respectively, before the animals were killed. At termination of the study, animals were killed by cervical dislocation and long bones removed for histomorphometric analysis. All experimental procedures were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and performed under appropriate licenses from the United Kingdom Home Office.

Histomorphometry

Femurs were cleared of soft tissue, separated into proximal and distal halves, fixed in 70% ethanol for 48 h, and then dehydrated through a graded series of alcohols: 80% ethanol, 90% ethanol, and three changes of 100% ethanol for 24 h each. Femurs were then cleared in chloroform for 24 h, placed for another 24 h in 100% ethanol and embedded without decalcification in LR White Hard Grade (London Resin Co., Reading, UK). Longitudinal sections of the distal portion of the femur were then prepared for histomorphometric analysis of the proximal tibial metaphysis, using a Reichert-Jung 2050 microtome (Cambridge Instruments GmbH, Heidelberg, Germany) with a "d" profile tungsten carbide knife; 7-μm sections were stained with 1% toluidine blue in 0.01 M citrate phosphate buffer for bone area measurement; 10-μm sections were mounted unstained in fluoromount (BDH Laboratory Supplies, Poole, UK) for assessment by fluorescent microscopy.

Histomorphometric analysis was performed using transmitted and epifluorescent microscopy linked to a computer-assisted image analyzer (Osteomeasure, Osteometrics, Atlanta, GA). Two nonconsecutive sections per animal were analyzed for each parameter in a blinded manner. A standard area of 0.36 mm^2 was used, the distal border of which was situated 0.25 mm above the growth plate to exclude the primary spongiosa. Cancellous bone area was expressed as a percentage of total tissue area (BV/TV).

The length of trabecular bone perimeter covered by double label was expressed with reference to the total tissue area (tissue area referent; dlS/TV) and as a percentage of the total length of cancellous bone perimeter (cancellous perimeter referent; dlS/BS). The former parameter (*i.e.* dlS/TV) was analyzed because this gives a better reflection of estrogen's tendency to induce the appearance of new sites of cancellous bone formation than dlS/BS (24). Mineral apposition rate (MAR) was determined by dividing the mean distance between the tetracycline and calcein labels by the time interval between the administration of the two labels (values were not corrected for the obliquity of the plane of section).

ERα immunostaining

Fourteen-week-old male wild-type (WT) and ERα$^{-/-}$ male mice were administered vehicle or E_2, 4000 μg/kg, by weekly sc injection for 8 d. After the end of the experiment, tibiae were removed, freed from soft tissue, fixed in formol saline, and decalcified in EDTA. The metaphysis and diaphysis were separated, dehydrated, and paraffin embedded. Longitudinal sections (5 μm thick) were obtained at the proximal tibial metaphysis. Immunoreactivity for ERα was subsequently detected by incubating sections with a polyclonal rabbit antibody directed against a peptide mapping to the C terminus of murine ERα (MC-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Previous studies demonstrate that this antibody is not cross-reactive with ERβ (28).

Sections were dewaxed, rehydrated, and rinsed in PBS with 0.2% Triton X-100, incubated with 10% normal goat serum (Sigma) in PBS with 0.2% Triton X-100 for 2 h to block any nonspecific binding of the secondary antibody, and then incubated with primary ERα antibody diluted 1:50 in normal goat serum overnight at 4 C. Sections were rinsed and incubated in alkaline phosphatase (ALP)-conjugated goat antirabbit IgG secondary antibody (Sigma) diluted 1:100 in PBS for 2 h at room temperature. The ALP conjugate was visualized using Fast Fast Red TR/Naphthol AS-MX tablet set (Sigma) containing 0.15 mg/ml levamisole to block endogenous ALP activity. Sections were rinsed, counterstained with hematoxylin, and mounted in Faramount aqueous mounting medium (DAKO Corp., Carpinteria, CA). To confirm specificity of the antibody, control sections were analyzed after preincubation with blocking peptide (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature.

Statistical analysis

Results are expressed as mean ± SEM. Two-way ANOVA was used to examine whether E_2 treatment or genotype exerted statistically significant effects. The cut-off for statistical significance was taken as $P < 0.05$.

Results

Histomorphometry

As previously found, treatment with high-dose E_2 led to the appearance of new cancellous bone throughout the distal femoral metaphysis in WT male mice (Fig. 1). A similar response was not observed in ERα$^{-/-}$ animals. These findings were confirmed by histomorphometric analysis, which revealed an increase in cancellous bone volume following treatment with E_2, 400 and 4000 μg/kg·d, within the distal femoral metaphysis of WT but not ERα$^{-/-}$ mice (Fig. 2A).

Dynamic histomorphometry was employed to analyze the osteogenic response of cancellous bone to E_2 in more detail. Following treatment with E_2, an increase in the extent of cancellous mineralizing surfaces was observed in WT but not ERα$^{-/-}$ male mice (Fig. 3). Histomorphometric analysis demonstrated that in WT mice, treatment with E_2, 40 μg/kg, acted to increase the proportion of cancellous bone surfaces undergoing mineralization (*i.e.* dlS/BS; Fig. 2B). In contrast, treatment with higher doses of E_2 led to a significant increase in the absolute extent of mineralizing surfaces (*i.e.* dlS/TV; Fig. 2C), indicating the formation of new cancellous bone surfaces as previously observed following high-dose estrogen (24). In ERα$^{-/-}$ mice, relatively little change was observed in either dlS/BS or dlS/TV following administration of E_2. An increase in MAR also contributed to the stimulatory action of E_2 on bone formation in WT but not ERα$^{-/-}$ mice, following treatment with both low- and high-dose E_2 (Fig. 2D).

ERα immunostaining

We explored the role of ERα in regulating osteoblast function in cancellous bone by analyzing its expression in the proximal tibial metaphysis by immunohistochemistry. In WT vehicle-treated mice, ERα was found to be expressed by the majority of osteoblasts on cancellous bone surfaces (Fig.

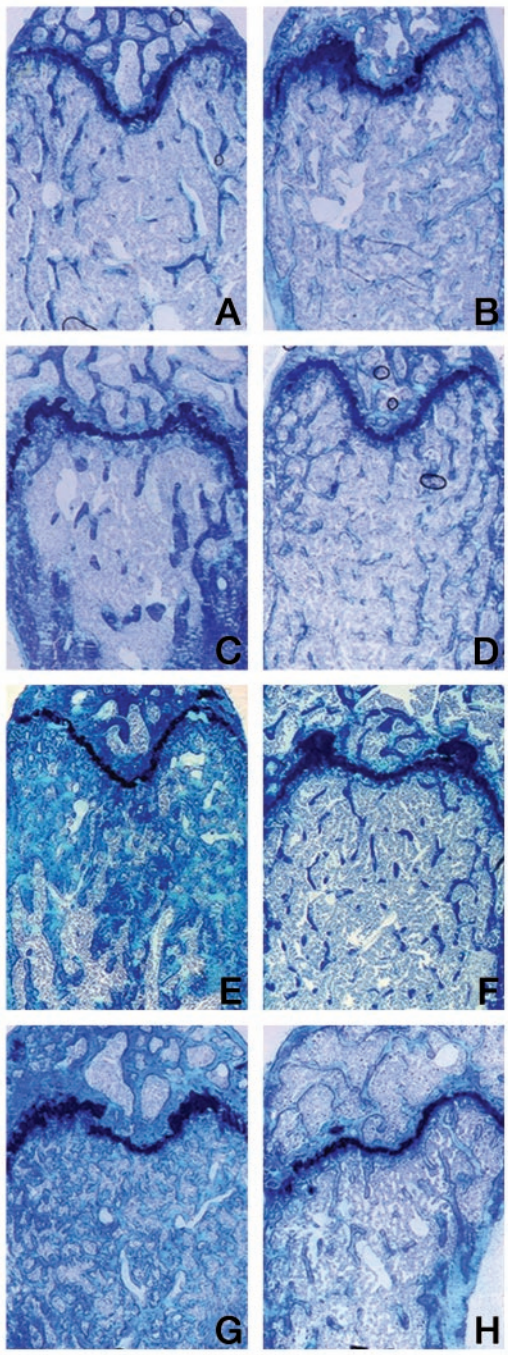

FIG. 1. Histological appearance of longitudinal sections of distal femoral metaphyses viewed by light microscopy. WT (A, C, E, and G) and ERα$^{-/-}$ (B, D, F, and H) mice were treated with vehicle (A and B) or E_2 at 40 (C and D), 400 (E and F), or 4000 (G and H) μg/kg·d for 4 wk. Note the increase in cancellous bone formation following treatment with E_2 in WT but not ERα$^{-/-}$ mice (×15).

4A). A significant number of bone marrow cells were also noted to express ERα, which tended to occur within groupings of positive cells randomly situated within the marrow cavity (Fig. 4B). In addition, megakaryocytes were consistently ERα positive. Little immunoreactivity was observed in the presence of blocking peptide (Fig. 4C).

Following E_2 treatment, a substantial increase in number of ERα-positive cancellous osteoblasts was seen in WT mice, whereas the degree and pattern of bone marrow expression was unchanged (Fig. 4D). Interestingly, the majority of cancellous osteoblasts in ERα$^{-/-}$ mice also demonstrated immunoreactivity to ERα, both in vehicle- and E_2-treated animals (Fig. 4, E and F). Presumably the latter observations reflect the fact that an N-terminal-truncated ERα isoform also exists, which is known to be expressed in ERα$^{-/-}$ mice (29), and would have been detectable by the MC-20 antibody, which is directed against the ERα C terminus. Cells that expressed ERα generally demonstrated a combination of nuclear and cytoplasmic staining, with no differences in localization observed between WT and ERα$^{-/-}$ mice.

Discussion

We found that different doses of E_2 exerted distinct actions on osteoblast function in cancellous bone of male mice, all of which were abolished in ERα$^{-/-}$ animals. E_2 as administered at the dose of 40 μg/kg·d increased MAR and the proportion of cancellous bone surfaces undergoing mineralization. These findings are consistent with the effects of E_2 treatment on cancellous bone formation as previously documented in female rats (30) and reports that estradiol implants increase osteoblast lifespan and/or work rate in postmenopausal women as assessed by measurement of mean wall thickness (4, 5) and may reflect a tendency of E_2 to suppress osteoblast apoptosis (6). Because the stimulatory effect of E_2, 40 μg/kg·d, on osteoblast function was found to be absent in ERα$^{-/-}$ mice, we concluded that this action is ERα dependent. As well as stimulating mineral apposition rate, E_2 at 400 and 4000 μg/kg·d induced the formation of new cancellous bone formation surfaces as reflected by an increase in the absolute extent of cancellous mineralization surfaces and a substantial gain in cancellous bone volume, as previously reported in female mice (24). This response to high-dose E_2, which our previous observations suggest involves the generation of osteoblast precursors from osteoprogenitors within bone marrow (31, 32), was also abrogated in ERα$^{-/-}$ mice.

To our knowledge, the present findings are unique in that no previous study has directly examined whether estrogen's stimulatory action on osteoblast function in cancellous bone is impaired following targeted gene deletion of ERα. Previous reports indicated that, whereas E_2 as administered at levels similar to the 40-μg/kg dose in the present study increases cancellous bone volume in orchidectomized male mice, no response occurs in ERα$^{-/-}$ animals (22, 33). However, prevention of bone loss by estrogen following orchidectomy may partly reflect this hormone's antiresorptive action, and because the latter studies did not analyze fluorochrome-based indices of cancellous bone formation, it is not possible to determine whether lack of estrogen's stimulatory action on osteoblast function underlies these previous observations.

To explore the role of ERα in mediating the osteogenic response to estrogen, immunohistochemical studies were performed, which revealed that ERα is expressed at significant levels within cancellous bone by osteoblasts. These results are in keeping with previous findings that ERα is

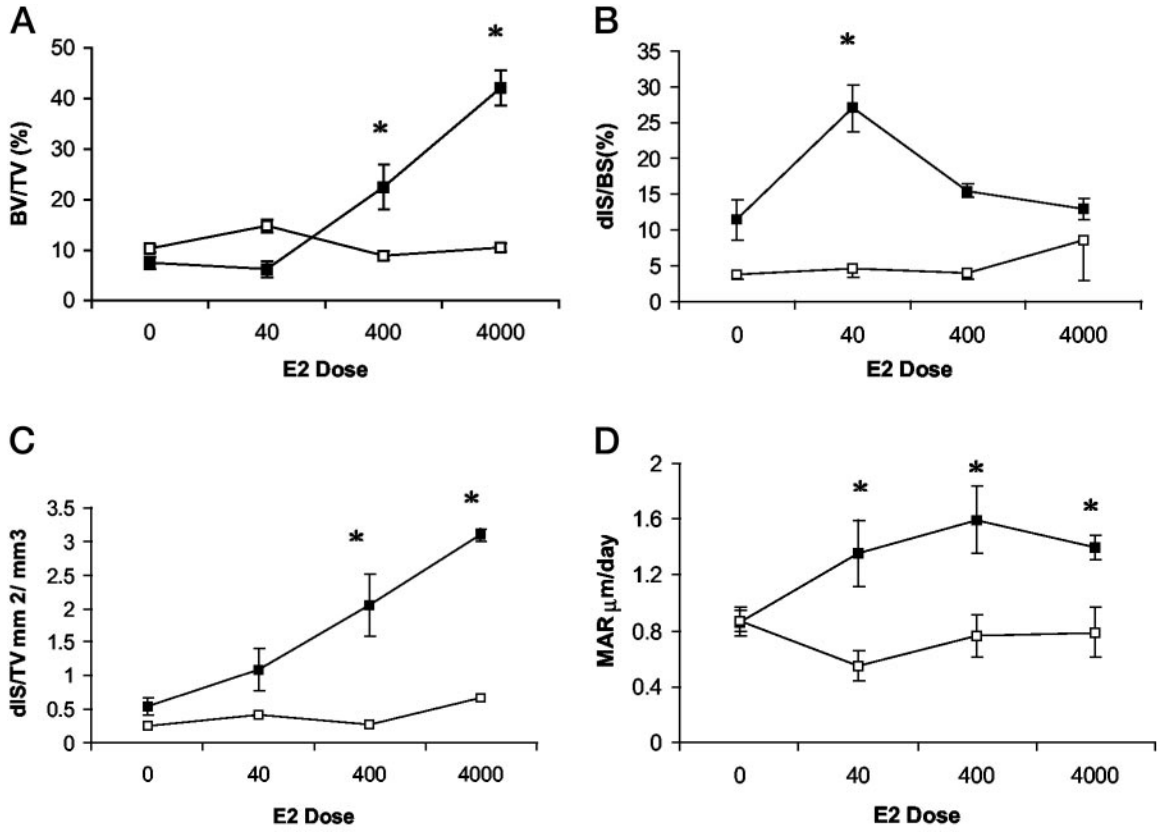

FIG. 2. Cancellous bone histomorphometric indices as measured at the distal femoral metaphysis of WT (*black squares*) and ER$^{-/-}$ (*white squares*) mice, following treatment with vehicle or E_2 at 40, 400, or 4000 μg/kg·d for 4 wk. Results show the following indices (mean ± SEM): cancellous bone volume (BV/TV; A); mineralizing surfaces (dlS/BS; B); mineralizing surfaces [tissue volume referent (dlS/BV; C]; and MAR (D). Two-way ANOVA revealed significant effects of genotype ($P < 0.0001$ for all indices), dose ($P < 0.005$ for BV/TV, dlS/BS, and dlS/BV), and genotype-dose interaction ($P < 0.001$ for BV/TV and dlS/BV, $P < 0.05$ for dlS/BS and MAR). *, $P < 0.05$ *vs.* vehicle (one-way ANOVA performed on data from WT animals).

expressed by isolated osteoblasts as assessed *in vitro* (9–12) and osteoblasts and bone marrow at cancellous-rich sites within neonatal human ribs (13). These findings may reflect the fact that direct ERα-dependent activation of osteoblasts contributes to estrogen-induced bone formation, which is consistent with a recent report that estrogen acts directly on osteoblasts to suppress their apoptosis (6). The observation that ERα showed cytoplasmic as well as nuclear staining in bone tissue sections is consistent with this mode of action, in view of the suggestion that this involves a non-genotropic pathway (6). Significant ERα expression was also found in megakaryocytes, as previously reported in human bone (34) and a subset of mononuclear bone marrow cells. The latter may have included stromal cells, which may also contribute to estrogen-induced bone formation in mouse long bones by stimulating the formation of osteoblast precursors in bone marrow following the release of osteogenic growth factors (8).

Interestingly, significant ERα immunoreactivity was observed in cancellous bone of ERα$^{-/-}$ mice with a similar intracellular and tissue distribution to that of vehicle-treated WT animals. This finding is consistent with recent reports that osteoblasts also express significant levels of an N-terminal-deficient 46-kDa isoform of ERα and that the latter is expressed in bones from ERα$^{-/-}$ mice as used in the present study (29). Presumably, ERα immunoreactivity that we observed in ERα$^{-/-}$ mice was related to the presence of this 46-kDa ERα isoform. The MC-20 antibody used in the present study was raised against a peptide mapping to the C-terminal of mouse ERα and is therefore expected to detect the 46-kDa ERα isoform in which the C-terminal is intact, although confirmatory studies are required to test this assumption. In view of the lack of estrogen response in ERα$^{-/-}$ mice, our results imply that estrogen-induced bone formation requires full-length ERα. This finding may represent an important role of the activation function (AF)-1 of ERα, which is deficient in the N-terminal-truncated 46-kDa isoform of ERα (29).

Our previous results, which suggest that ERβ does not mediate the osteogenic response to estrogen, are consistent with an important role of AF-1 in bone formation (26) because ERβ is also thought to be deficient in terms of its AF-1 function (35). This possibility is supported by findings with the estrogen antagonist, tamoxifen, which paradoxically acts as an estrogen agonist in bone (36), and stimulates bone formation in mouse long bones with a potency approaching that of E_2 (our unpublished results). Because tamoxifen is thought to regulate gene activity through AF-1 (37), the latter domain is also likely to play a role in mediating ERα-

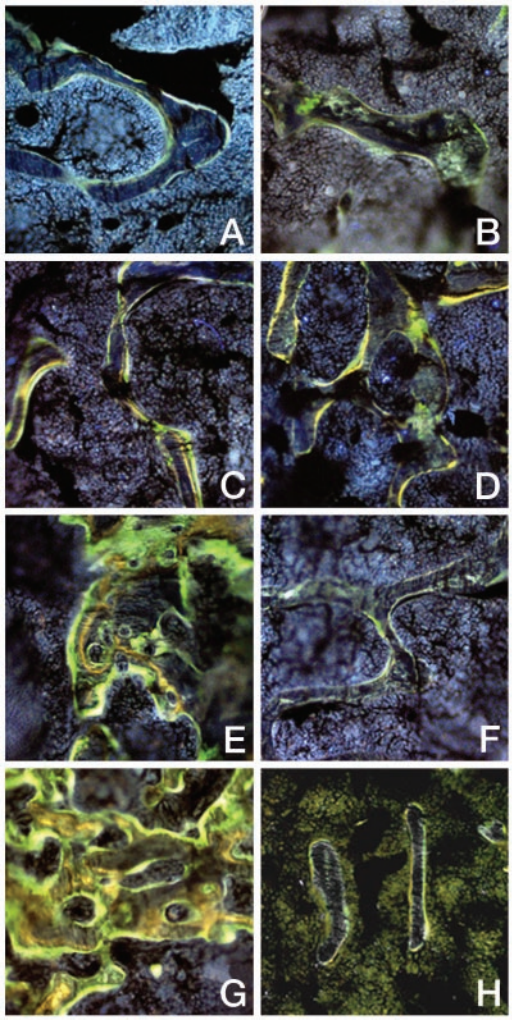

FIG. 3. Mineralizing surfaces in longitudinal sections of distal femoral metaphyses viewed under UV light. WT (A, C, E, and G) and ERα$^{-/-}$ (B, D, F, and H) mice were treated with vehicle (A and B) or E_2 at 40 (C and D), 400 (E and F), and 4000 μg/kg·d (G and H) for 4 wk. WT mice showed an increase in the extent of cancellous mineralizing surfaces following treatment with all doses of E_2. An equivalent response was not observed in ERα$^{-/-}$ mice (× 50).

dependent activation of bone formation in response to estrogen antagonists.

The absence of an osteogenic response of male ERα$^{-/-}$ mice to estrogen contrasts with a previous report that in female double-ER knockout mice generated by crossing ERα$^{-/-}$ animals as used in the present study with ERβ$^{-/-}$ mice, ovariectomy leads to bone loss, which can be prevented by high-dose E_2 (38). Prevention of ovariectomy-induced bone loss by estrogen is largely thought to reflect this hormone's antiresorptive action (3). Therefore, taken with results of the present study, these findings suggest that whereas AF-1 is required for estrogen's osteogenic action, estrogen's antiresorptive effect is mediated by a distinct, AF-1-independent pathway.

Whether estrogen's stimulatory action on osteoblast function in humans is also mediated by ERα is currently unclear. Estrogen's tendency to stimulate cancellous bone formation in mouse long bones is somewhat exaggerated, compared

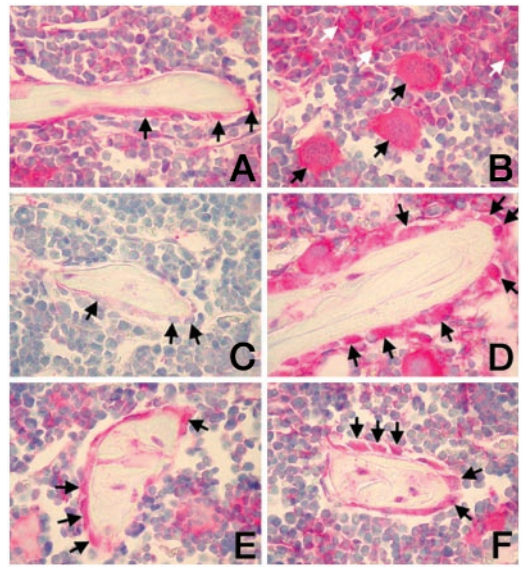

FIG. 4. ERα expression in longitudinal sections of the proximal tibial metaphysis as assessed by light microscopy. WT and ERα$^{-/-}$ mice were treated with vehicle or E_2, 4000 μg/kg·d, for 8 d. Sections from WT vehicle-treated mice showed ERα expression by the majority of osteoblasts on cancellous bone surfaces (A; *arrows*); ERα expression by megakaryocytes (B; *black arrows*) and mononuclear bone marrow cells (B; *white arrows*); and osteoblasts showed reduced immunostaining following preincubation with blocking peptide (C; *arrows*). In WT mice treated with E_2, a substantial increase in number of cancellous osteoblasts was observed (D), the majority of which expressed ERα (*arrows*). In ERα$^{-/-}$ mice treated with vehicle (E) or E_2 (F), comparable numbers of osteoblasts were observed with those in vehicle-treated WT animals, and the majority of osteoblasts expressed ERα (*arrows*; ×250).

with the response observed in postmenopausal women treated with estradiol implants (4, 5), and it is possible that certain species differences exist between the molecular pathways involved. Nevertheless, the observation that ERα deficiency in an adult male is associated with impaired acquisition of peak bone mass (20) supports the possibility that ERα is also required for estrogen's stimulatory action on osteoblast function in humans. To the extent that our findings can be extrapolated to humans in this way, our results indicate that it may be possible to develop novel bone-forming therapies for postmenopausal osteoporosis, based on agents that target ERα/AF-1-dependent responses in osteoblasts.

Acknowledgments

Received November 25, 2002. Accepted January 9, 2003.

Address all correspondence and requests for reprints to: Dr. J. H. Tobias, Rheumatology Unit, Bristol Royal Infirmary, Bristol BS2 8HW, United Kingdom. E-mail: Jon.Tobias@bristol.ac.uk.

This work was supported by the Oliver Bird Fund, Nuffield Foundation.

References

1. **Richelson LS, Heinz HW, Melton III LJ, Riggs BL** 1984 Relative contributions of aging and estrogen deficiency to postmenopausal bone loss. N Engl J Med 311:1273–1275
2. **Christiansen C, Christiansen MS, Larsen N-E, Transbol I** 1982 Pathophysiological mechanism of estrogen effect on bone metabolism: dose-response relationship in early postmenopausal women. J Clin Endocrinol Metab 55: 1124–1130

3. **Wronski TJ, Cintron M, Doherty AL, Dann LM** 1988 Estrogen treatment prevents osteopenia and depresses bone turnover in ovariectomized rats. Endocrinology 123:681–686
4. **Khastgir G, Studd J, Holland N, Alaghband-Zadeh J, Fox S, Chow J** 2001 Anabolic effect of estrogen replacement on bone in postmenopausal women with osteoporosis: histomorphometric evidence in a longitudinal study. J Clin Endocrinol Metab 86:289–295
5. **Vedi S, Purdie DW, Ballard P, Bord S, Cooper AC, Compston JE** 1999 Bone remodeling and structure in postmenopausal women treated with long-term, high-dose estrogen therapy. Osteoporos Int 28:52–58
6. **Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC** 2001 Nongenotropic, sex-nonspecific signaling through estrogen or androgen receptors: dissociation from transcriptional activity. Cell 104:719–730
7. **Bord S, Beavan S, Ireland D, Horner A, Compston JE** 2001 Mechanisms by which high-dose estrogen therapy produces anabolic skeletal effects in postmenopausal women: role of locally produced growth factors. Bone 29:216–222
8. **Plant A, Tobias JH** 2002 Increased bone morphogenetic protein 6 expression in mouse long bones following estrogen administration. J Bone Miner Res 17:782–790
9. **Bodine PVN, Henderson RA, Green J, Aronow M, Owen T, Stein GS, Lian JB, Komm BS** 1998 Estrogen receptor α is developmentally regulated during osteoblast differentiation and contributes to selective responsiveness of gene expression. Endocrinology 139:2048–2057
10. **Komm BS, Terpening CM, Benz DJ, Graeme KA, Gallegos A, Korc M, Greene GL, O'Malley BW, Haussler MR** 1988 Estrogenic binding, receptor mRNA and biologic response in osteoblast-like osteosarcoma cells. Science 241:81–83
11. **Eriksen EF, Colvard DS, Berg NJ, Graham ML, Mann KG, Spelsberg TC, Riggs BL** 1988 Evidence of estrogen receptors in normal human osteoblast-like cells. Science 241:84–86
12. **Arts J, Kuiper GGJM, Janssen JMMF, Gustafsson J-A, Lowik CWGM, Pols HAP, Van Leeuwen JPTM** 1997 Differential expression of estrogen receptors α and β mRNA during differentiation of human osteoblast SV-HFO cells. Endocrinology 138:5067–5070
13. **Bord S, Horner A, Beavan S, Compston J** 2001 Estrogen receptor α and β are differentially expressed in developing human bone. J Clin Endocrinol Metab 86:2309–2314
14. **Braidman IP, Hainey L, Batra G, Selby PL, Saunders PTK, Hoyland JA** 2001 Localisation of estrogen receptor β protein expression in adult human bone. J Bone Miner Res 16:214–220
15. **Onoe Y, Miyaura C, Ohta H, Nozawa S, Suda T** 1997 Expression of estrogen receptor β in rat bone. Endocrinology 138:4509–4512
16. **Vidal O, Kindblom L-G, Ohlsson C** 1999 Expression and localisation of estrogen receptor β in murine and human bone. J Bone Miner Res 14:923–929
17. **Windahl SH, Norgard M, Kuiper GGJM, Gustafsson J-A, Andersson G** 2000 Cellular distribution of estrogen receptor β in neonatal rat bone. Bone 26:117–121
18. **Hall JM, McDonnell DP** 1999 The estrogen receptor β isoform (ERβ) of the human estrogen receptor modulates ERα transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 140:5566–5578
19. **Pettersson K, Delaunay F, Gustafsson J-A** 2000 Estrogen receptor β acts as a dominant regulator of estrogen signalling. Oncogene 19:4970–4978
20. **Smith EC, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS** 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 331:1056–1061
21. **Sims NA, Dupont S, Krust A, Clement-Lacroix P, Minet D, Resche-Rigon M, Gaillard-Kelly M, Baron R** 2002 Deletion of estrogen receptors reveals a regulatory role for estrogen receptor-β in bone remodeling in females but not males. Bone 30:18–25
22. **Lindberg MK, Moverare S, Skrtic S, Alatalo S, Halleen J, Mohan S, Gustafsson J-A, Ohlsson C** 2002 Two different pathways for the maintenance of trabecular bone in adult male mice. J Bone Miner Res 17:55–562
23. **Vidal O, Lindberg MK, Hollberg K, Baylink DJ, Andersson G, Lubahn DB, Mohan S, Gustafsson J-A, Ohlsson C** 2000 Estrogen receptor specificity in the regulation of skeletal growth and maturation in male mice. Proc Natl Acad Sci USA 97:5474–5479
24. **Samuels A, Perry MJ, Tobias JH** 1999 High-dose estrogen induces *de novo* medullary bone formation in female mice. J Bone Miner Res 14:178–186
25. **Samuels A, Perry MJ, Goodship AE, Fraser WD, Tobias JH** 2000 Is high-dose estrogen-induced osteogenesis in the mouse mediated by an estrogen receptor? Bone 27:41–46
26. **McDougall KE, Perry MJ, Gibson R, Bright J, Colley S, Hodgin JB, Smithies O, Tobias JH** 2002 Estrogen-induced osteogenesis in intact female mice lacking ERβ. Am J Physiol 283:E817–E823
27. **Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O** 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci USA 90:11162–11166
28. **Saji S, Jensen EV, Nilsson S, Rylander T, Warner M, Gustafsson J-A** 2000 Estrogen receptors α and β in the rodent mammary gland. Proc Natl Acad Sci USA 97:337–342
29. **Denger S, Reid G, Kos M, Flouriot G, Parsch D, Brand H, Korach KS, Sonntag-Buck V, Gannon F** 2001 ERα gene expression in human primary osteoblasts: evidence for the expression of two receptor proteins. Mol Endocrinol 15:2064–2077
30. **Chow J, Tobias JH, Colston KW, Chambers TJ** 1992 Estrogen maintains trabecular bone volume in rats not only by suppression of bone resorption but also by stimulation of bone formation. J Clin Invest 89:74–78
31. **Perry MJ, Samuels A, Bird D, Tobias JH** 2000 The effects of high-dose estrogen on murine hematopoietic marrow precede those on osteogenesis. Am J Physiol 279:E1159–E1165
32. **Plant A, Samuels A, Perry MJ, Colley S, Gibson R, Tobias JH** 2001 Estrogen-induced osteogenesis in mice is associated with the appearance of Cbfa1-expressing bone marrow cells. J Cell Biochem 84:285–294
33. **Vandenput L, Ederveen AG, Erben RG, Stahr K, Swinnen JV, Van Herck E, Verstuyf A, Boonen S, Bouillon R, Vanderschueren D** 2001 Testosterone prevents orchidectomy-induced bone loss in estrogen receptor-α knockout mice. Biochem Biophys Res Commun 285:70–76
34. **Bord S, Vedi S, Beavan SR, Horner A, Compston JE** 2000 Megakaryocyte population in human bone marrow increases with estrogen treatment: a role in bone remodeling? Bone 27:397–401
35. **Aranda A, Pascual A** 2001 Nuclear hormone receptors and gene expression. Physiol Rev 81:1269–1304
36. **Turner RT, Wakeley GK, Hannon KS, Bell NH** 1987 Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. J Bone Miner Res 2:449–456
37. **Berry M, Metzger D, Chambon P** 1990 Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. EMBO J 9:2811–2818
38. **Gentile MA, Zhang H, Harada S, Rodan GA** 2001 Bone response to estrogen replacement in ovx double estrogen receptor knockout mice. J Bone Miner Res 16(Suppl 1):S147