Estrogen receptor-α in osteocytes is important for trabecular bone formation in male mice

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The bone-sparing effect of estrogen in both males and females is primarily mediated via estrogen receptor-α (ERα), encoded by the *Esr1* gene. ERα in osteoclasts is crucial for the trabecular bone-sparing effect of estrogen in females, but it is dispensable for trabecular bone in male mice and for cortical bone in both genders. We hypothesized that ERα in osteocytes is important for trabecular bone in male mice and for cortical bone in both males and females. *Dmp1-Cre* mice were crossed with $ERα^{flox/flox}$ mice to generate mice lacking ERα protein expression specifically in osteocytes ($Dmp1\text{-}ERα^{-/-}$). Male $Dmp1\text{-}ERα^{-/-}$ mice displayed a substantial reduction in trabecular bone volume (−20%, $P < 0.01$) compared with controls. Dynamic histomorphometry revealed reduced bone formation rate (−45%, $P < 0.01$) but the number of osteoclasts per bone surface was unaffected in the male $Dmp1\text{-}ERα^{-/-}$ mice. The male $Dmp1\text{-}ERα^{-/-}$ mice had reduced expression of several osteoblast/osteocyte markers in bone, including *Runx2*, *Sp7*, and *Dmp1* ($P < 0.05$). Gonadal intact $Dmp1\text{-}ERα^{-/-}$ female mice had no significant reduction in trabecular bone volume but ovariectomized $Dmp1\text{-}ERα^{-/-}$ female mice displayed an attenuated trabecular bone response to supraphysiological E2 treatment. $Dmp1\text{-}ERα^{-/-}$ mice of both genders had unaffected cortical bone. In conclusion, ERα in osteocytes regulates trabecular bone formation and thereby trabecular bone volume in male mice but it is dispensable for the trabecular bone in female mice and the cortical bone in both genders. We propose that the physiological trabecular bone-sparing effect of estrogen is mediated via ERα in osteocytes in males, but via ERα in osteoclasts in females.

Bone mass is maintained by highly regulated processes involving osteoblastic bone formation and osteoclastic bone resorption. Estrogen is the major sex hormone involved in the regulation of bone mass in females and several studies demonstrate that estrogen is also of importance for the male skeleton (1–6). The biological effects of estradiol (E2) are mainly mediated by the nuclear estrogen receptors (ERs), ERα encoded by the *Esr1* gene, and ERβ encoded by the *Esr2* gene. The bone-sparing effect of estrogen in both males and females is primarily mediated via ERα (6–8), although the effect of ERα activation in bone might be slightly modulated by ERβ in female mice (9–12).

Two studies using different strategies for the inactivation of *ERα* in osteoclasts have determined the role of ERα in osteoclasts for the bone-sparing effect of estrogen (13, 14). Nakamura et al. used *Ctsk* (Cathepsin K)-*Cre* mice to inactivate *ERα* in mature osteoclasts, resulting in trabecular bone loss caused by increased bone resorption in female but not male mice (14). In a separate study Martin-Millan et al. inactivated *ERα* in monocytes/osteoclast precursors using *LysM-Cre* mice and found that estrogen attenuates osteoclast generation and life span via cell-autonomous effects and that ERα in osteoclasts mediates the protective effect of estrogens on trabecular but not cortical bone in female mice (13). Collectively, these two studies clearly demonstrated that ERα in osteoclasts is crucial for trabecular bone in females, but it is dispensable for trabecular bone in male mice and for cortical bone in both males and females. However, not only osteoclasts but also osteoblasts/osteocytes express ERs (15–17). Several in vitro studies have suggested that ERα in osteoblasts/osteocytes is of importance for the regulation of bone metabolism, but this has not yet been demonstrated in vivo (2, 16, 18).

It is well established that mechanical loading is a major regulator of cortical bone dimensions (19, 20). Previous studies have demonstrated that female but not male mice with *ERα* inactivation display reduced cortical osteogenic bone response to mechanical loading (19, 21–24). In addition, we recently showed that ERα is required for the cortical osteogenic response to mechanical loading in a ligand-independent manner, involving activation function-1 but not activation function-2 in ERα in female mice (20). The primary ERα target cell for this role of ERα in the osteogenic bone response to mechanical loading is not yet characterized, but a plausible candidate is the osteocytes, which are the mechanosensors in bone.

Because ERα in osteoclasts is required for the bone-sparing effect of estrogen specifically in trabecular bone in female mice, we hypothesized that ERα in osteocytes might be crucial for trabecular bone in male mice, for cortical bone in both genders, and for the cortical osteogenic response to mechanical loading in female mice (13, 14). Therefore, dentin matrix protein (*Dmp*)*1-Cre* [*Tg(Dmp1-cre)1Jqfe*] mice were crossed with $ERα^{flox/flox}$ ($Esr1^{tm1.1Gust}$) mice to generate mice lacking ERα protein expression specifically in osteocytes ($Dmp1\text{-}ERα^{-/-}$). The main finding from this in vivo study shows that ERα in osteocytes is important for trabecular bone formation in male mice.

Results

Osteocyte-Specific Inactivation of *ERα* in $Dmp1\text{-}ERα^{-/-}$ Mice. Previous studies have shown that the *Dmp1-Cre* mouse strain has the capacity to specifically inactivate *loxP* flanked genes in osteocytes expressing CRE-recombinase selectively in osteocytes under the control of a 10-kb promoter fragment of the osteocytic *Dmp1* marker gene (25, 26). To validate that the *Dmp1-Cre* mouse strain has the capacity to specifically recombine DNA in osteocytes postnatally, we mated *Dmp1-Cre* mice with *ROSA26-Cre*

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reporter mice. Femurs (Fig. 1*A*) of 3-wk-old offspring were stained with X-Gal to detect β-galactosidase activity. Specific β-galactosidase activity, as a result of CRE-recombinase action, was found in osteocytes of bone, whereas no specific β-galactosidase activity was seen in the bone marrow or liver (Fig. 1*A*).

Homozygous osteocyte-specific *ERα* mutant mice (*Dmp1-ERα*$^{-/-}$ mice) had no obvious morphological abnormalities or detectable differences in bone lengths, body weight, or organ weights (uterus, liver, fat, thymus) relative to control littermates (Table S1). *Dmp1-ERα*$^{-/-}$ mice had a significant reduction of *ERα* mRNA levels in cortical bone and calvaria but not in uterus, spleen, or kidney (Fig. 1*B*). Serum E2 and luteinizing hormone (LH) in females as well as serum testosterone and LH in males were normal in *Dmp1-ERα*$^{-/-}$ mice, demonstrating that neither serum sex steroids nor the negative-feedback regulation of serum sex steroids were affected in the *Dmp1-ERα*$^{-/-}$ mice (Table 1).

Reduced Trabecular Bone Mass in Male *Dmp1-ERα*$^{-/-}$ Mice. Peripheral quantitative computer tomography (pQCT) analyses of excised femurs and tibiae revealed a substantial reduction in trabecular volumetric bone mineral density (BMD) (Femur –42%, Tibia –25%, $P < 0.01$) but unaffected cortical bone parameters in male *Dmp1-ERα*$^{-/-}$ mice compared with control mice (Fig. 1*C* and Table S2). Neither the trabecular nor the cortical bone parameters were significantly affected in female *Dmp1-ERα*$^{-/-}$ mice (Fig. 1*C* and Table S2). MicroCT analyses of trabecular bone of L_5 vertebrae revealed a decreased trabecular bone volume/total volume (BV/TV, −20%, $P < 0.01$) as a result of both reduced thickness (−10%, $P < 0.01$) and number (−10%, $P < 0.05$) of trabeculae in male *Dmp1-ERα*$^{-/-}$ mice compared with WT mice (Fig. 1 *D*–*G*). To evaluate the biomechanical properties of the bone, three-point bending of tibiae, mainly corresponding to mechanical bone strength of cortical bone, was performed. Stiffness and maximal load at failure were unaffected in both male and female *Dmp1-ERα*$^{-/-}$ mice compared with WT mice (Table S3). These findings demonstrate that there is a trabecular but not a cortical bone phenotype in male *Dmp1-ERα*$^{-/-}$ mice.

Reduced Bone Formation but Normal Bone Resorption in Male *Dmp1-ERα*$^{-/-}$ Mice. The trabecular bone phenotype in the male *Dmp1-ERα*$^{-/-}$ mice was further characterized by static as well as dynamic histomorphometry. Histomorphometric analyses of the trabecular bone in the distal metaphyseal region of femur confirmed reduced trabecular BV/TV and trabecular thickness in male *Dmp1-ERα*$^{-/-}$ mice compared with WT mice ($P < 0.05$) (Table S4). Dynamic histomorphometry revealed a reduced trabecular bone formation

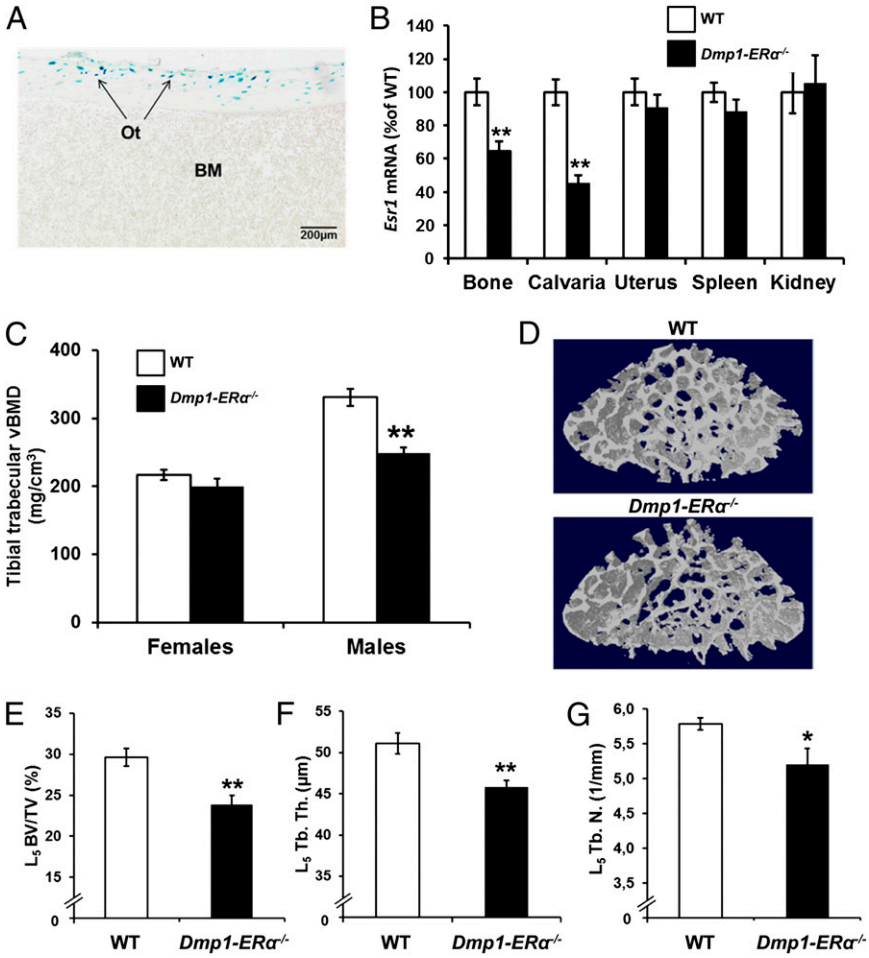

Fig. 1. Osteocyte-specific inactivation results in reduced trabecular bone mass in male mice. (*A*) Femur diaphyseal region stained with X-Gal to detect β-galactosidase activity of 3-wk-old *Dmp1-Cre* mice mated with *ROSA26-Cre* reporter mice demonstrating specific β-galactosidase activity in osteocytes (Ot) in bone but not in the bone marrow (BM). (*B*) *ERα* mRNA levels in cortical bone (Bone), calvaria, uterus, spleen, and kidney of *Dmp1-ERα*$^{-/-}$ mice expressed as percent of control mice. (*C*) Reduced tibia trabecular volumetric BMD (vBMD) in 11-wk-old male but not female *Dmp1-ERα*$^{-/-}$ mice. (*D*–*G*) MicroCT analyses of trabecular bone in L_5 vertebrae demonstrated a decreased trabecular BV/TV (*E*) as a result of both reduced thickness (*F*) (Tb. Th.) and number (*G*) (Tb. N.) of trabeculae in 11-wk-old male *Dmp1-ERα*$^{-/-}$ mice compared with WT mice. Values are given as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. WT (n = 7–12).

Table 1. Serum sex steroid levels

	Female		Male	
Steroid	WT	*Dmp1-ERα*$^{-/-}$	WT	*Dmp1-ERα*$^{-/-}$
17β-Estradiol (pg/mL)	17 ± 2	15 ± 1	ND	ND
Testosterone (pg/mL)	ND	ND	976 ± 749	720 ± 422
LH (ng/mL)	0.21 ± 0.06	0.26 ± 0.06	0.53 ± 0.09	0.55 ± 0.11

Values are given as mean ± SEM, (n = 7–12). ND, not determined.

rate (BFR) in male *Dmp1-ERα*$^{-/-}$ mice compared with WT mice (−45%, $P < 0.01$) (Fig. 2 *C* and *D*). The reduced BFR was reflected by a combination of decreased mineralizing surface (MS/BS) (Fig. 2*A*) and mineral apposition rate (MAR) (Fig. 2*B*). There was a nonsignificant trend of reduced serum bone γ-carboxyglutamate (Gla) protein (BGLAP; Osteocalcin) levels in the male *Dmp1-ERα*$^{-/-}$ mice compared with WT mice (−22%, $P = 0.12$). Neither the number of osteoclasts per bone surface nor serum C-terminal collagen cross-links (CTX-1), which is a degradation product from C-terminal telopeptides of type I collagen, were affected in the male *Dmp1-ERα*$^{-/-}$ mice (Fig. 2*E* and Table S5). These data provide evidence that trabecular bone formation is reduced but bone resorption is mainly unaffected, resulting in a net trabecular bone loss in the male *Dmp1-ERα*$^{-/-}$ mice. Osteocyte lacunae size was not affected in the cortical or trabecular bone in the *Dmp1-ERα*$^{-/-}$ mice (Table S6). There was a nonsignificant tendency of increased osteocyte lacunae density (13.3%, $P = 0.08$) in the trabecular bone but not in the cortical bone in male *Dmp1-ERα*$^{-/-}$ mice compared with WT mice (Table S6).

To identify the molecular mechanism behind the reduced trabecular bone formation rate in male *Dmp1-ERα*$^{-/-}$ mice, we analyzed the expression of osteoblast and osteocyte specific transcripts in bone derived from the vertebral body, a bone with relatively high trabecular bone content. Male *Dmp1-ERα*$^{-/-}$ mice had reduced levels of the early osteoblast markers *Runx2* and *Sp7* (Osterix), the late osteoblast marker *Ibsp* (Bone-sialoprotein), and the osteocyte marker *Dmp1* (Fig. 2 *F* and *G* and Table S7). In contrast, the mRNA levels of the two osteoclast markers *Acp5* (Trap) and *Tnfrsf11a* (Rank) were not significantly altered in the *Dmp1-ERα*$^{-/-}$ mice (Table S7).

Female *Dmp1-ERα*$^{-/-}$ Mice Displayed an Attenuated Trabecular Bone Response to Supraphysiological E2 Treatment. As described above, gonadal-intact female (Sham) *Dmp1-ERα*$^{-/-}$ mice did not have a trabecular bone phenotype (Figs. 1*C* and 3*D*). Thus, ERα in osteocytes was not required for the physiological regulation of trabecular bone in female mice. To determine if the estrogenic response to supraphysiological E2-treatment was affected in trabecular bone in female *Dmp1-ERα*$^{-/-}$ mice, ovariectomized (ovx) WT and *Dmp1-ERα*$^{-/-}$ mice were treated with E2 (0.17 μg/d) for 4 wk. As expected, E2 treatment increased uterine weight,

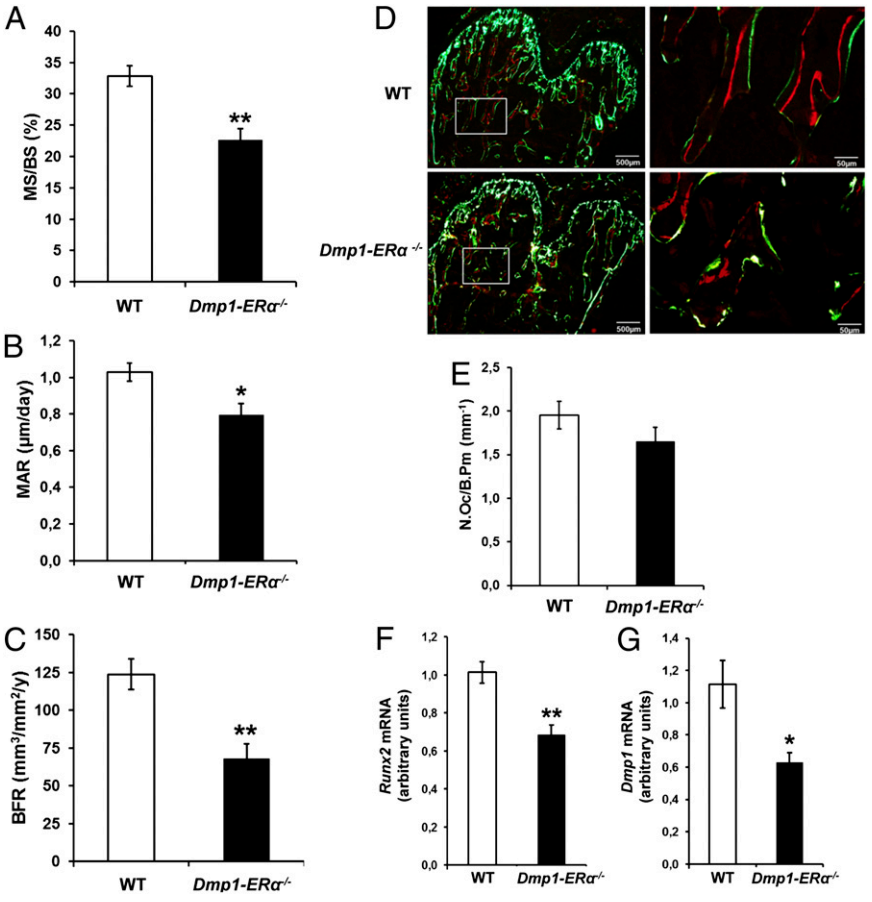

Fig. 2. Reduced trabecular bone formation in male *Dmp1-ERα*$^{-/-}$ mice. (*A–E*) Histomorphometric analyses of the distal metaphyseal region of femur in 11-wk-old male *Dmp1-ERα*$^{-/-}$ mice compared with WT mice. (*A–D*) Dynamic histomorphometry demonstrating reduced (*A*) mineralizing surface (MS/BS), (*B*) MAR, and (*C*) BFR in *Dmp1-ERα*$^{-/-}$ mice compared with WT mice. (*D*) Representative sections demonstrating less trabecular bone formation in the *Dmp1-ERα*$^{-/-}$ mice compared with WT mice (calcein/green and alizarin/red). (*Left*) Distal femur metaphyseal region; (*Right*) close up of the indicated area in the metaphysis. (*E*) Number of osteoclasts per bone perimeter (N.Oc/B.Pm). (*F* and *G*) Reduced expression of transcripts related to bone formation in male *Dmp1-ERα*$^{-/-}$ mice. (*F*) *Runx2* and (*G*) *Dmp1* mRNA levels in bone from the vertebral body analyzed by RT-PCR. Values are given as mean ± SEM, *$P < 0.05$, **$P < 0.01$ vs. WT (n = 7–12).

trabecular BV/TV in L_5 vertebrae, and cortical thickness and decreased thymus weight and gonadal fat weight in ovx WT mice (Fig. 3 and Table S8). The E2 responses on uterine weight, gonadal fat weight, thymus weight, and cortical thickness were unaffected in ovx *Dmp1-ERα*$^{-/-}$ mice compared with ovx WT mice (Fig. 3 *A* and *B* and Table S8). In contrast, the E2 response on trabecular bone parameters (BV/TV, thickness, and number) was significantly reduced in ovx *Dmp1-ERα*$^{-/-}$ mice compared with the E2 response in ovx WT mice (Fig. 3 *C*–*F*).

Normal Osteogenic Response to Mechanical Loading in *Dmp1-ERα*$^{-/-}$ Mice. Earlier studies have demonstrated that female but not male mice with *ERα* inactivation display reduced cortical osteogenic bone response to mechanical loading (19, 21–24). To determine if the reduced osteogenic response to mechanical loading in female *ERα*$^{-/-}$ mice is caused by a lack of ERα in osteocytes, we loaded tibiae of female *Dmp1-ERα*$^{-/-}$ mice and WT mice using a standardized axial loading procedure. The cortical osteogenic bone response was similar in *Dmp1-ERα*$^{-/-}$ mice (tibia cortical bone area 28.2 ± 1.2% over unloaded control tibia, $P < 0.001$) and WT mice (tibia cortical bone area 29.2 ± 2.6% over unloaded control tibia, $P < 0.001$) (Fig. S1), demonstrating that ERα in osteocytes is not crucial for the osteogenic response to mechanical loading in female mice.

Discussion

Bone tissue consists of bone-forming osteoblasts and bone-resorbing osteoclasts, which are located on the surface of the bone, and osteocytes located in the extracellular bone matrix (27). Osteoblasts/osteocytes are derived from mesenchymal stem cells but osteoclasts are macrophage lineage multinuclear giant cells that originate from hematopoietic stem cells (28). It is well established that ERα is crucial for the bone-sparing effect of estrogen and that ERα in osteoclasts is indispensable for the bone-sparing effect of E2 in trabecular bone in female mice, but the importance of ERα in osteoblasts/osteocytes is not yet

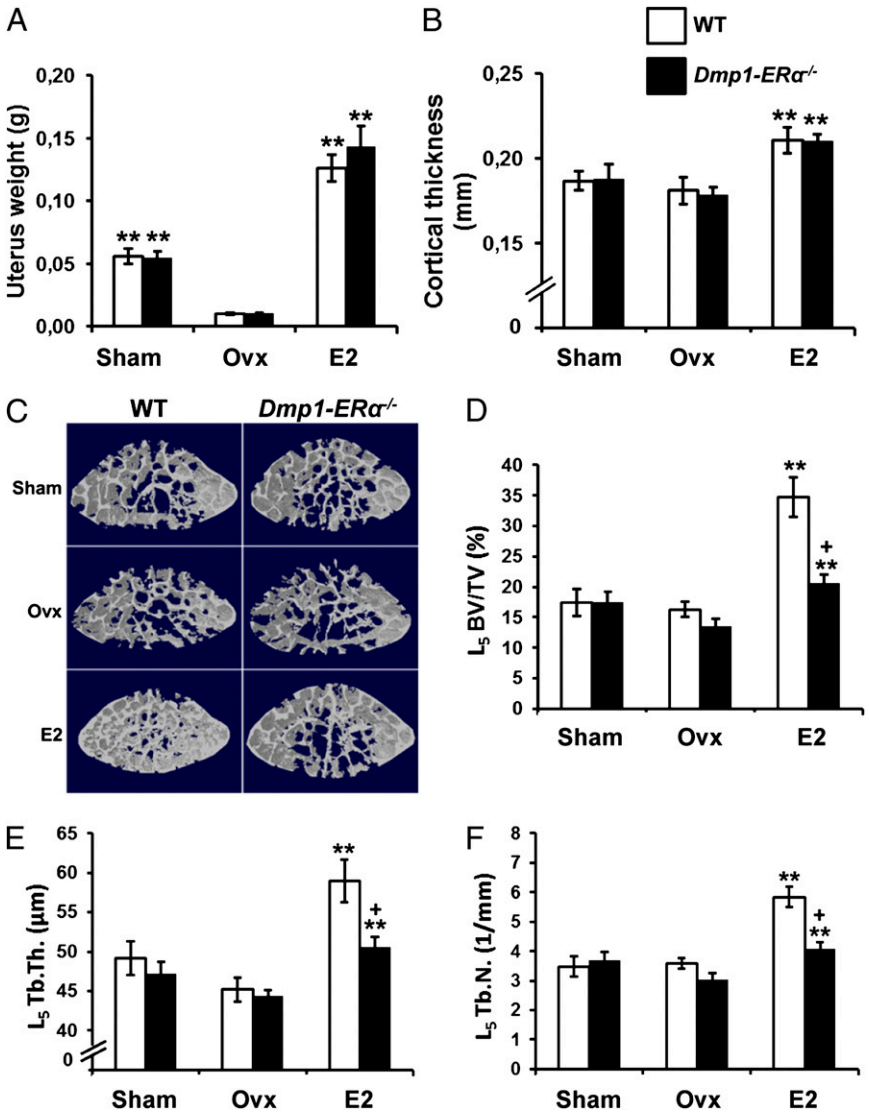

Fig. 3. Female *Dmp1-ERα*$^{-/-}$ mice displayed an attenuated trabecular bone response to supraphysiological E2 treatment. Three-month-old ovx *Dmp1-ERα*$^{-/-}$ and littermate control mice (WT) were treated with placebo or E2 for 4 wk using slow release pellets (Innovative Research of America), delivering placebo or a slightly supraphysiological dose of E2 (0.17 μg per mouse per day). (*A*) Uterine weight. (*B*) Cortical bone thickness. (*C*–*F*) MicroCT analyses of trabecular bone in L_5 vertebrae, (*D*) BV/TV, (*E*) trabecular thickness (Tb. Th.) and (*F*) trabecular number (Tb. N.). Sham, sham operated gonadal-intact mice. Values are given as mean ± SEM, (n = 8–10). $^{**}P < 0.01$ vs. OVX, $^{+}P < 0.05$ E2 effect in *Dmp1-ERα*$^{-/-}$ mice vs. E2 effect in WT mice.

determined in vivo (13, 14). To evaluate the role of ERα in osteocytes, *ERα* was specifically inactivated in *Dmp1*-expressing cells using the *Cre/loxP* system. The main finding in the present study is that ERα in osteocytes is important for trabecular bone formation in male mice.

In this study we used the *Dmp1-Cre* mouse model, which is a widely used and well-described mouse model for deleting gene expression in osteocytes (25, 26). We confirmed the specificity of the *Dmp1-Cre* mouse model using *ROSA26-Cre* reporter mice, demonstrating specific recombination in osteocytes. Furthermore, *Dmp1-ERα*$^{-/-}$ mice had clearly reduced *ERα* mRNA levels in bone but the *ERα* mRNA levels in several other tissues were unchanged. Previous studies have shown that total deletion of ERα results in a disturbed negative feedback regulation of sex steroids (10, 16). The *Dmp1-ERα*$^{-/-}$ mice, with osteocyte specific *ERα* inactivation, had normal serum sex steroid levels. Thus, the bone phenotype in *Dmp1-ERα*$^{-/-}$ mice is not confounded by elevated serum testosterone levels, having the capacity to preserve bone via activation of the androgen receptor in *ERα*$^{-/-}$ mice. *Dmp1-ERα*$^{-/-}$ mice had no obvious morphological abnormalities or detectable differences in bone lengths, body weight, or organ weights, demonstrating that the reduced trabecular bone volume in the male mice was not secondary to a general poor health status.

There are several possible primary ERα target cells for the bone-sparing effect of estrogen in bone, including osteoclasts, osteoblasts, osteocytes, T lymphocytes, and dendritic cells (13, 14, 18). In addition, it was recently proposed that neuronal ERα modulates bone mass in female mice (29). The most compelling evidence for a role of cell-specific ERα for bone metabolism is the results from two separate in vivo studies inactivating *ERα* either in mature osteoclasts (14) or monocytes/osteoclast precursors (13). These two studies demonstrated that ERα in osteoclasts is indispensable for trabecular bone in females, but it is dispensable for trabecular bone in male mice and for cortical bone in both males and females. The present study is unique in reporting a clear bone phenotype in a mouse model with specific inactivation of *ERα* in a mesenchymal cell type. We hypothesized that ERα in osteocytes is important for trabecular bone in male mice and for cortical bone in both male and female mice.

Importantly, male *Dmp1-ERα*$^{-/-}$ mice had reduced trabecular bone volume observed both in the metaphyseal area of the long bones and in the L_5 vertebrae of the axial skeleton. MicroCT analyses of L_5 vertebrae suggested that the reduced trabecular bone volume was the result of a combination of reduced number and thickness of trabeculae, but histomorphometry of the distal metaphyseal region of femur indicated that it was mainly a result of reduced trabecular thickness. The previously reported reduced trabecular bone volume in the female mice with inactivation of *ERα* in osteoclasts (13, 14) was caused by increased osteoclastic bone resorption but neither the number of osteoclasts per bone surface nor CTX-1, a serum marker of bone resorption, was elevated in the male *Dmp1-ERα*$^{-/-}$ mice. This finding suggests that the reduced trabecular bone volume in the male *Dmp1-ERα*$^{-/-}$ mice is primarily caused by reduced bone formation. As expected, dynamic histomorphometry revealed a 45% reduction in trabecular bone formation rate in the male *Dmp1-ERα*$^{-/-}$ mice. Bone tissue from the vertebral body, with a relatively high trabecular bone content, of male *Dmp1-ERα*$^{-/-}$ mice had reduced mRNA levels of both early (*Runx2* and *Sp7*) and late (*Ibsp*) osteoblast markers, demonstrating that osteoblastic differentiation was affected. The osteocyte marker *Dmp1* was also reduced in the male *Dmp1-ERα*$^{-/-}$ mice. We propose that estrogen via activation of ERα in osteocytes results in paracrine signals to osteoblasts enhancing osteoblast activity.

The influence of sex steroids on growth and maintenance of the skeleton in both men and women has long been recognized. In men, however, the relative contribution of androgens versus estrogens in the regulation of bone metabolism remains uncertain. Animal studies demonstrate that both E2, via activation of ERα, and testosterone, via activation of the androgen receptor, regulate bone mass in male rodents (30). The present finding of a substantially reduced trabecular bone volume in male *Dmp1-ERα*$^{-/-}$ mice supports previous studies suggesting that estrogen action via ERα is indeed crucial for male bone health.

Gonadal-intact female *Dmp1-ERα*$^{-/-}$ mice had no significant reduction in trabecular bone volume but ovx *Dmp1-ERα*$^{-/-}$ mice displayed an attenuated trabecular bone response to supraphysiological E2 treatment. This finding indicates that ERα in osteocytes is dispensable for the physiological regulation of trabecular bone in gonadal-intact female mice, but that ERα in osteocytes contributes to a maximal E2 response to supraphysiological E2 treatment in ovx mice. Because the trabecular bone volume was reduced in gonadal-intact female mice with inactivation of *ERα* in osteoclasts but not in female mice with *ERα* inactivation in osteocytes, we propose that ERα in osteoclasts is the major target for the physiological trabecular bone-sparing effect of estrogen in females. However, the present study evaluating a mouse model with inactivation of *ERα* in osteocytes does not preclude a role of ERα in osteoblast progenitors or early osteoblasts.

Estrogen acting via ERα exerts important effects also in cortical bone (2, 6, 16). However, the cortical bone was not affected in the two mouse models with *ERα* inactivation in osteoclasts or in the present mouse model with osteocyte-specific *ERα* inactivation (13, 14). This finding suggests that ERα in other cells, such as osteoblasts or other nonosteocyte/nonosteoclast cells to be identified, mediate the cortical bone-sparing effect of estrogens.

As female mice with total *ERα* inactivation display reduced cortical osteogenic bone response to mechanical loading (19, 21–24), and osteocytes are thought to function as the main mechanosensors in bone, it was biologically plausible to hypothesize that ERα in osteocytes is important for the osteogenic response to mechanical loading. However, the cortical osteogenic bone response to mechanical loading was normal in *Dmp1-ERα*$^{-/-}$ mice, demonstrating that ERα in osteocytes is not crucial for the osteogenic response to mechanical loading and, therefore, further studies are required to identify the ERα target cell type contributing to the osteogenic response to mechanical loading. Further studies are also required to determine the role of ERα in osteocytes for age-dependent bone loss in aging mice. In addition, it would be valuable in future studies to evaluate the estrogenic response to several different doses of E2 in ovx, as well as in orchidectomized *Dmp1-ERα*$^{-/-}$ mice. In the present study, the ovx-induced trabecular bone loss in WT mice was relatively modest, not reaching statistical significance, and we speculate that dietary phytoestrogens might have attenuated the effect of ovx on trabecular bone.

In conclusion, ERα in osteocytes regulates trabecular bone formation and thereby trabecular bone volume in male mice, but it is dispensable for the physiological regulation of trabecular bone in female mice and cortical bone in both males and females. This finding also supports previous studies demonstrating that estrogen action via ERα is crucial for male bone health (6). In addition, the reduced osteogenic response to mechanical loading in female mice with a total *ERα* inactivation is not mediated via an effect of ERα in osteocytes. We propose that the trabecular bone-protective effect of physiological E2 levels is, at least in part, mediated via ERα in osteocytes in males, but via ERα in osteoclasts in females. The cellular location of ERα with an impact on cortical bone and for the osteogenic response to mechanical loading remains to be determined.

Materials and Methods

Generation of Mice Depleted of ERα in Osteocytes. The mice were housed in a standard animal facility under controlled temperature (22 °C) and

photoperiod (12-h light, 12-h dark) and fed pellet diet ad libitum. All efforts were made to minimize suffering. All animal experiments had been approved by the local Ethical Committees for Animal Research (Göteborgs djurförsöksetiska nämnd, application no. 135-2011). Mice expressing CRE-recombinase driven by the 10-kb *Dmp1* promoter specifically in osteocytes [*Tg(Dmp1-cre)1Jqfe* mice] (31) were mated with $ER\alpha^{flox/flox}$ ($Esr1^{tm1.1Gust}$) (32) mice to generate mice specifically lacking ERα in osteocytes. Double-heterozygous $Cre^{+/-}ER\alpha^{flox/-}$ mice were mated with $ER\alpha^{flox/flox}$ mice to generate $Cre^{+/-}ER\alpha^{flox/flox}$ (*Dmp1-ERα*$^{-/-}$) mice, and $Cre^{-/-}ER\alpha^{flox/flox}$ (control; WT) mice. Further details are provided in *SI Materials and Methods*.

X-Ray Analyses. CT scans of the femur and tibia were performed by using pQCT XCT RESEARCH M (v4.5B; Norland) as described previously (12, 33). The μCT analyses were performed on the lumbar vertebra (L_5) using a model 1072 scanner (Skyscan) (29, 34, 35). Further details are provided in *SI Materials and Methods*.

In Vivo Loading of the Tibia. When under inhalation anesthesia with Isoflurane (Forene; Abbot Scandinavia), the right tibia of 3-mo-old female *Dmp1-ERα*$^{-/-}$ and WT mice were axially loaded on 3 alternate days per week for 2 wk for 40 cycles per day with a trapezoid waveform, with 10-s rest between cycles. Further details are provided in *SI Materials and Methods*.

Histomorphometry. Trabecular bone from femur was evaluated using static and dynamic histomorphometric analyses (36). Further details are provided in *SI Materials and Methods*.

Gene Expression Analyses. Total RNA from humerus, calvaria, vertebral body, uterus, spleen, and kidney was prepared for real-time PCR analysis. Further details are provided in *SI Materials and Methods*.

Measurement of Serum Hormone Levels. Commercially available RIA kits were used to assess serum concentrations of E2 (Siemens Medical Solutions), testosterone (RIA; ICN Biomedicals), LH (RIA AHR002, IDS), BGLAP (IRMA; Immutopics), and CTX-1 (RatLaps EIA, IDS Biocode-Hycel). Procollagen type 1 amino-terminal propeptide (P1NP) was measured in serum using a mouse P1NP EIA assay (Immunodiagnostic Systems) according to the manufacturer's instructions.

Three-Point Bending. The three-point bending test (span length 5.5 mm, loading speed 0.155 mm/s) at the mid tibia was made using the Instron universal testing machine (Instron 3366, Instron). Based on the recorded load deformation curves, the biomechanical parameters were acquired from raw-files produced by Bluehill 2 software v2.6 (Instron), with custom-made Excel macros.

Statistical Analyses. Values are given as mean ± SEM. The statistical differences between WT and *Dmp1-ERα*$^{-/-}$ mice and between ovx E2 and ovx placebo were calculated using Student´s *t* test. The statistical differences in E2 response between ovx WT and ovx *Dmp1-ERα*$^{-/-}$ mice were calculated by the interaction *P* value from a two-way-ANOVA including genotype (WT or *Dmp1-ERα*$^{-/-}$) and treatment (ovx or ovx+E2).

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