The role of estrogens for male bone health
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Abstract
Sex steroids are important for the growth and maintenance of both the female and the male skeleton. However, the relative contribution of androgens versus estrogens in the regulation of the male skeleton is unclear. Experiments using mice with inactivated sex steroid receptors demonstrated that both activation of the estrogen receptor (ER)\textsubscript{a} and activation of the androgen receptor result in a stimulatory effect on both the cortical and trabecular bone mass in males. ER\textsubscript{b} is of no importance for the skeleton in male mice while it modulates the ER\textsubscript{a}-action on bone in female mice. Previous \textit{in vitro} studies suggest that the membrane G protein-coupled receptor GPR30 also might be a functional ER. Our \textit{in vivo} analyses of GPR30-inactivated mice revealed no function of GPR30 for estrogen-mediated effects on bone mass but it is required for normal regulation of the growth plate and estrogen-mediated insulin-secretion. Recent clinical evidence suggests that a threshold exists for estrogen effects on bone in men: rates of bone loss and fracture risk seem to be the highest in men with estradiol levels below this threshold. Taken together, even though these findings do not exclude an important role for testosterone in male skeletal homeostasis, it is now well-established that estrogens are important regulators of bone health in men.

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Introduction
Peak bone mass is higher in men than in women. After peak bone mass is reached, both men and women lose bone but the bone loss is accelerated after menopause in women. Compared with women, men have greater bone strength, resulting in fewer fractures. This is mainly due to a larger cortical bone size in men compared with women, resulting from more bone deposition at the periosteal bone surface in men during sexual maturation. Sex steroids are important for the skeletal growth and maintenance of both the female and the male skeleton (1). The effects of testosterone (T) can be exerted either directly through the androgen receptor (AR) or indirectly via aromatization to estradiol (E\textsubscript{2}) and activation of estrogen receptor (ER)\textsubscript{a} and/or \textsubscript{b} (listed as ESR1 and ESR2 in the HUGO Database). All three of these sex steroid receptors are expressed in bone (2–4). In addition, it has recently been suggested from \textit{in vitro} experiments that the membrane G protein-coupled receptor GPR30 (listed as GPER in the HUGO Database) is also a functional ER (Fig. 1) (5). In this review, some of our experimental animal and clinical studies exploring the role of estrogens for the skeleton in males will be discussed.

Estrogen receptors and the male skeleton: lessons from mouse studies

Cortical bone
The larger cortical bone size in males compared with females is, at least partly, due to differences in sex steroid exposure during sexual maturation. Experiments by Venken \textit{et al.} using male AR\textsuperscript{−/−} mice have clearly demonstrated that AR activation results in cortical radial bone expansion (6). In addition, we found that male ER\textsubscript{a}−/− but not ER\textsubscript{b}−/− mice displayed reduced cortical radial bone growth during sexual maturation, demonstrating that ER\textsubscript{a} but not ER\textsubscript{b} activation is also required for a normal cortical radial bone expansion in males during growth (Fig. 2) (7). As the cortical radial bone growth was affected during sexual maturation in the male ER\textsubscript{a}−/− mice, one might speculate that the GH/insulin-like growth factor I (IGF-I) axis is involved in this effect. In this context, we and others have shown that a major part of serum IGF-I is liver-derived and that male ER\textsubscript{a}−/− mice had a similar cortical bone phenotype as mice with liver-specific IGF-I inactivation (7–11). Importantly, serum IGF-I levels were reduced in the ER\textsubscript{a}−/− mice, suggesting that these decreased serum IGF-I levels might mediate the reduced cortical radial bone growth seen in male ER\textsubscript{a}−/− mice during...
Regulation of bone mass in males

Androgens

Aromatization

Estrogens

AR

ERα

ERβ

GPR30

Figure 1 Summary of possible pathways for androgens to regulate bone mass in males. Experimental animal studies using mouse models with inactivation of the androgen receptor (AR), estrogen receptor α (ERα), ERβ or the recently suggested ER GPR30 have demonstrated that the AR and ERα, but not ERβ or GPR30, are involved in the regulation of cortical and trabecular bone in males.

"E2 or vehicle. Both ERα and AR but not ERβ activation preserved the amount of trabecular bone. ERα activation resulted both in preserving the thickness and number of trabeculae. By contrast, AR activation exclusively preserved the number of trabeculae (13). Furthermore, the effects of E2 could not be mediated by the AR, and the effects of DHT were not reduced in ER-inactivated mice (Fig. 3). Thus, the in vivo bone-sparing effect of ERα activation is distinct from the bone-sparing effect of AR activation in adult male mice. Because these two pathways are clearly distinct from each other, we proposed that a combined treatment of selective ER and AR modulators might be beneficial in the treatment of male osteoporosis (13). In contrast to the IGF-I -mediated effects of E2 on cortical bone during sexual maturation, the effects of E2 on trabecular maintenance were not associated with the GH/IGF-I axis and seem to result from direct action of E2 on bone.

Trabecular bone

Similar to that seen in cortical bone, both AR and ERα but not ERβ activation regulate trabecular bone mass in male mice (Fig. 1) (12–14). To directly compare the effect of ER activation on trabecular bone in vivo with the effect of AR activation, orchidectomized wild type (WT) and ER-inactivated mice were treated with the non-aromatizable androgen dihydrotestosterone (DHT), E2 or vehicle. Both ERα and AR but not ERβ activation preserved the amount of trabecular bone. ERα activation resulted both in preserving the thickness and number of trabeculae. By contrast, AR activation exclusively preserved the number of trabeculae (13). Furthermore, the effects of E2 could not be mediated by the AR, and the effects of DHT were not reduced in ER-inactivated mice (Fig. 3). Thus, the in vivo bone-sparing effect of ERα activation is distinct from the bone-sparing effect of AR activation in adult male mice. Because these two pathways are clearly distinct from each other, we proposed that a combined treatment of selective ER and AR modulators might be beneficial in the treatment of male osteoporosis (13). In contrast to the IGF-I -mediated effects of E2 on cortical bone during sexual maturation, the effects of E2 on trabecular maintenance were not associated with the GH/IGF-I axis and seem to result from direct action of E2 on bone.

Estrogen receptor β modulates ERα activity in female but not male mice

Similar to that seen in males, ERα is the principal ER for the regulation of both trabecular and cortical bone in female mice (15–18). However, female ERβ-inactivated mice have an increased cortical bone area, resulting in a loss of feminization of the cortical bone size (Fig. 4A) (19). Furthermore, female ERβ−/− mice are partly protected against age-related trabecular bone loss (Fig. 4B) (20), suggesting that an ERβ antagonist might be useful in the treatment of post-menopausal osteoporosis. Thus, although ERα is the principal ER for the female skeleton, its activity can be modulated by ERβ. Interestingly, when we evaluated the effect of E2 in female bone using global gene expression analyses, we found that the magnitude of the stimulatory effect of E2 on estrogen-regulated transcripts was in general more pronounced in the ERβ−/− mice than in the WT mice (21). These data were the first to demonstrate in vivo that ERβ has the capacity to reduce ERα regulated gene transcription in a global manner. This inhibitory role of ERβ on ERα activity is now generally believed to be a major function of ERβ in several tissues, including for the regulation of tumorigenesis. These data support a ‘yin yang’ relationship between ERα and ERβ in the female bone and possibly in other tissues. It remains, however, to be determined why ERβ modulates the effect of ERα on the skeleton in female but not male mice.

Evaluation of GPR30 as a potential ER in bone

In vitro studies suggest that, besides the two known nuclear ERs α and β, the membrane GPR30 is also a functional ER (5, 22). GPR30 was shown to bind E2 with high affinity in vitro (5, 22) and to mediate estrogen-promoted proliferative signaling in an estrogen-sensitive but ER-negative breast cancer cell line.
Figure 3 The in vivo trabecular bone-sparing effect of ER activation is distinct from the trabecular bone-sparing effect of AR activation in males. To directly compare the effect of ER activation on trabecular bone in vivo with the effect of AR activation, 9-month-old orchidectomized (orx) wild type (WT) and ER-inactivated mice (ERα−/− β−/−) were treated with the non-aromatizable androgen dihydrotestosterone (DHT), E2 or vehicle (V). Trabecular volumetric BMD of the femur is shown, demonstrating that the effects of E2 could not be mediated by the AR, and the effects of DHT were not reduced in ER-inactivated mice. Values are given as means ± S.E.M. **P<0.01 versus orx + V; ††, P<0.01; †, P<0.05 versus sham. (Reproduced from Movérase et al. (13) with permission from the National Academy of Sciences, USA).

Figure 4 A role of ERβ for the skeleton in female mice. (A) Female E2-stimulated insulin release and is required for a normal estrogenic response in the growth plate (28, 29).

Serum E2 and bone growth in men

The traditional view was that estrogens and androgens were the main sex steroids influencing bone maturation and maintenance in women and men respectively. This concept has, however, been challenged in the 1990’s by the description of several ‘experiments of nature’. In 1994, Smith et al. described a 28-year-old man with a naturally occurring mutation in the estrogen receptor gene, making him resistant to estrogen (30). This patient had undergone normal early growth and developed normal male secondary sexual characteristics, but had unfused epiphyses and a markedly delayed bone age of 15 years. He was tall and had experienced no pubertal growth spurt, suggesting continued linear growth into adulthood. Moreover, despite normal serum T levels and elevated E2 levels, he had severe osteopenia associated with elevated markers of bone remodeling. Henceforth, two men with estrogen deficiency, due to mutations in the aromatase (CYP19) gene, were described (31, 32). These individuals had undetectable E2 levels and almost identical skeletal phenotypes as the estrogen-resistant man, regardless of normal or elevated T levels. But, contrary to the estrogen-resistant male who had a significantly increased bone mass, suppression of bone resorption and growth plate closure (32, 33).

(23, 24) and human endometrial cells (25). Furthermore, it was shown in vitro to mediate estrogen-dependent kinase activation as well as transcriptional responses (26, 27). However, it remains to be determined if GPR30 is an ER in vivo for bone metabolism or other phenotypes. We therefore, recently investigated the possible role of GPR30 as a functional ER for the regulation of skeletal parameters using GPR30 inactivated mice (28). The estrogenic responses on most of the investigated parameters, including increase in bone mass (total body bone mineral density (BMD), spine BMD, trabecular BMD, cortical thickness), increase in uterine weight, fat mass reduction, and increase in bone marrow cellularity, were similar for all of the investigated E2 doses in WT and GPR30 inactivated mice, demonstrating that GPR30 is not required for normal estrogenic responses on several major well-known estrogen-regulated parameters. On the other hand, high dose E2 treatment reduced longitudinal bone growth, reflected by decreased femur length and distal femur growth plate height, in the WT mice but not in the GPR30 inactivated mice, supporting a role of GPR30 for a normal estrogenic response in the growth plate. In addition, we found that GPR30 mediates E2-stimulated insulin release in vivo (29).
Since then, several new cases of aromatase deficiency have been reported, all with similar baseline skeletal phenotypes as the landmark case report by Smith et al. (30). Estrogen replacement therapy, using different doses of E2, in patients with aromatase deficiency has indicated that serum E2 levels above a threshold of ~20 pg/ml are required to complete bone maturation and mineralization (32–35).

**Serum E2 and bone maintenance in men**

In general, observational studies reported that serum E2 correlated better with BMD at various sites than T (36–45). Moreover, prospective studies have shown that serum E2 was the best predictor of both the increase in bone mass in young men (46) and the decrease of bone density in elderly men (46–48). In addition, treatment of elderly men with an aromatase inhibitor resulted in significant increases in bone resorption, together with decreases in bone formation markers (49). This pivotal role of estrogen with respect to male skeletal metabolism has since been confirmed in several other studies. Importantly, Khosla et al. (46) suggested that, in elderly men, a threshold exists for bioavailable E2 of 11 pg/ml (40 pmol/l), corresponding to a total E2 level of 31 pg/ml (114 pmol/l), below which the rate of bone loss at the radius and the ulna was clearly associated with bioavailable E2 levels. Above this level, no apparent association between the rate of bone loss and bioavailable E2 levels was found. Similar thresholds for serum E2 were reported in elderly men by Gennari et al. (48) for bone loss at the femoral neck and lumbar spine and by Szulc et al. (43) for changes in biochemical markers of bone turnover.

The majority of estrogens in elderly men do not originate from the testes but from peripheral conversion androgen action(55). Therefore, the extent of peripheral aromatase activity may also influence serum E2 levels. Elderly men with a high number of repeats in the aromatase gene have been reported to have higher E2 levels and decreased rates of bone loss compared with men with a low number of repeats (51). In addition, this CYP19 repeat was significantly associated with BMD change in elderly community-dwelling men (47). These findings suggest that a genetic variation in the aromatase gene may predispose men to increased age-related bone loss, by modulating bone metabolism either directly or indirectly via altered serum E2 levels. This notion is supported by our earlier report that CYP19 polymorphisms were associated with BMD and cortical bone size in young adult Swedish men (52). Recently, our extensive evaluation of 604 single nucleotide polymorphisms (SNPs) in 50 sex steroid-related candidate genes identified an SNP (rs2470152) in the 1.4 promoter region of the aromatase gene to be clearly associated with serum E2 levels in both young adults and elderly men ($P=10^{-14}$) (53).

Importantly, subjects with the GG genotype of this CYP19 polymorphism did not only have markedly elevated E2 levels but also higher lumbar spine BMD and fewer prevalent fractures than subjects with the GA or AA genotypes. Interestingly, the G to A transition of rs2470152 is predicted to alter a potential binding site for the transcription factor cyclic AMP response element binding protein, which is thought to be important in regulating aromatase expression (54). Further studies are required to test the functional significance of this CYP19 promoter polymorphism on the expression of the CYP19 gene in bone and other relevant tissues, and to evaluate the impact of this polymorphism on sex steroid-related disorders (53). In addition, a recent paper from the European Male Aging Study reported that the CAG repeat length in the AR correlated to calcaneus ultrasound parameters and this was associated with increased estrogen rather than decreased androgen action (55).

**Serum E2 and fractures in men**

Even though the above-mentioned studies provide strong evidence for an important role of estrogens for bone metabolism in men, little is known about the relative role of androgens and estrogens on fracture risk in men. In cross-sectional studies, inverse associations between both serum E2 (44, 56) and T (44) and prevalent fractures have been shown. Still, the roles of serum E2 and T as predictors of fracture risk in men analyzed in large prospective studies remain contradictory (57–60). Previous conflicting results might be due to the fact that these earlier prospective studies have been underpowered, including few incident fractures, and most of them (57–59) have analyzed the baseline sex steroid levels using immunoassay-based techniques, which have been shown to have a questionable

![Figure 5 A threshold for the inverse relation between serum E2 and fracture risk. Yearly incidence of fractures in relation to total E2. Poisson regression models were used to determine the relation between serum hormone levels and fracture risk (based on all validated fractures) in elderly subjects from the MDR Sweden cohort. (Adapted from Mellström et al. (61) with permission from the American Society for Bone and Mineral Research).](image-url)
specificity, especially at lower concentrations. Most recently, we analyzed the predictive role of serum E2 and T levels for incident fracture risk in the MrOS Sweden study, the largest population-based cohort so far with sex steroid levels measured at baseline with the mass spectrometry technique (61). We found that both serum E2 levels and T levels were inversely associated with fracture risk when analyzed separately. However, in multivariate analyses, serum free E2 was independently of free T, a predictor of all fractures in these elderly men. Moreover, when analyzing the effect of having low E2 and/or low T levels, subjects with low serum E2 levels had an increased risk of fractures, independent of T status. By contrast, subjects with low T levels but normal E2 levels were not at higher risk for fracture. In addition, the inverse relationship between serum E2 levels and fracture risk was nonlinear, with a strong relationship at total E2 levels below 16 pg/ml (59 pmol/l; Fig. 5). This observation further confirms the concept of a threshold E2 level for skeletal health in men (62). The threshold E2 level for fracture risk described in the MrOS Sweden Study (61) is slightly lower than those previously described for bone maturation, BMD and markers of bone resorption (35, 43, 46, 48). This difference could be due to the fact that in the latter studies, serum E2 was analyzed using immunoassay-based techniques, while it was analyzed by mass spectrometry in the Swedish cohort.

Conclusions
Experimental animal studies have demonstrated that both activation of ERz and AR result in a stimulatory effect on bone mass in males. Recent human studies have gathered substantial evidence for the existence of a threshold E2 level in men for bone maturation, bone loss as well as fracture risk. Even though these findings do not exclude an important role for T in male skeletal homeostasis, they do provide proof of an important role for estrogen in bone health in men.

Declaration of interest
The authors have no conflicts of interest.

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