Sex Steroid Actions in Male Bone

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Sex steroids are chief regulators of gender differences in the skeleton, and male gender is one of the strongest protective factors against osteoporotic fractures. This advantage in bone strength relies mainly on greater cortical bone expansion during pubertal peak bone mass acquisition and superior skeletal maintenance during aging. During both these phases, estrogens acting via estrogen receptor-α in osteoblast lineage cells are crucial for male cortical and trabecular bone, as evident from conditional genetic mouse models, epidemiological studies, rare genetic conditions, genome-wide meta-analyses, and recent interventional trials. Genetic mouse models have also demonstrated a direct role for androgens independent of aromatization on trabecular bone via the androgen receptor in osteoblasts and osteocytes, although the target cell for their key effects on periosteal bone formation remains elusive. Low serum estradiol predicts incident fractures, but the highest risk occurs in men with additionally low T and high SHBG. Still, the possible clinical utility of serum sex steroids for fracture prediction is unknown. It is likely that sex steroid actions on male bone metabolism rely also on extraskeletal mechanisms and cross talk with other signaling pathways. We propose that estrogens influence fracture risk in aging men via direct effects on bone, whereas androgens exert an additional antifracture effect mainly via extraskeletal parameters such as muscle mass and propensity to fall. Given the demographic trends of increased longevity and consequent rise of osteoporosis, an increased understanding of how sex steroids influence male bone health remains a high research priority. **(*Endocrine Reviews* 35: 906–960, 2014)**

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Abbreviations: AF-1, activation function 1; AF-2, activation function 2; AIS, androgen insensitivity syndrome; AP-1, activating protein-1; AR, androgen receptor; ARE, androgen response element; ARKO, AR knockout; BMD, bone mineral density; BMP, bone morphogenetic protein; ChIP, chromatin immunoprecipitation; COMT, catechol-O-methyltransferase; Ctsk, cathepsin K; DBD, DNA-binding domain; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; Dmp1, dentin matrix protein 1; DXA, dual-energy x-ray absorptiometry; E2, 17β-estradiol; EDC, E2 dendrimer conjugate; ER, estrogen receptor; ERE, estrogen response element; ERKO, estrogen-receptor knockout; FasL, Fas ligand; GPR30, G protein-coupled receptor 30; GWAS, genome-wide association studies; *hpg*, hypogonadal (mice); HRpQCT, high-resolution pQCT; IGFBP, IGF binding protein; LBD, ligand-binding domain; LRP, low-density lipoprotein receptor-related protein; LysM, lysozyme M; NERKI, nonclassical ER knock-in; NTD, N-terminal domain; OPG, osteoprotegerin; pQCT, peripheral QCT; QCT, quantitative computed tomography; RANK, receptor activator of nuclear factor κB; RANKL, RANK ligand; ROS, reactive oxygen species; RUNX2, Runt-related transcription factor 2; SARM, selective AR modulator; SERM, selective ER modulator; tau, transcriptional activation unit; Tfm, testicular feminization; vBMD, volumetric BMD.

I. Introduction

Osteoporosis in men, although less common than in women, still constitutes a major burden for public health (1, 2). Despite the higher competing risk of mortality from other causes in older men, the remaining lifetime risk of osteoporotic fractures after age 50 may be as high as 20–25% (vs 45–55% in women) in high-risk Caucasian populations (3–5). For hip fractures, a recent systematic review found that the male-female incidence ratio was also about 1:2 and was remarkably constant globally, despite greater than 10-fold variation between geographic regions (Figure 1) (6). Accordingly, men account for around one-third of fractures and associated expenditures (7, 8). Of the estimated 9 million incident osteoporotic fractures worldwide in the year 2000, men accounted for 30% of fractures at the hip, 39% at the spine, 25% at the humerus, 20% at the forearm, and 54% at other sites (7).

Fracture incidence increases exponentially around the age of menopause in women. In older men, the fracture rate rises in parallel, but with a delay of about 15 years (9, 10). Excess mortality endures for up to 10 years after hip fracture but more so in men, due to underlying frailty and comorbidities that appear characteristic of those men who do develop osteoporosis (11, 12). This is in line with the observation that the absolute risk of a second fracture is equal in both genders (ie, men have a lower risk of developing osteoporosis, but when they do so, they have a higher relative refracture risk) (13). Long-term care and institutionalization rates after fracture also seem higher in elderly men, and this greatly adds to the economic and societal burden of osteoporosis (14). Still, the general public, male patients, and many physicians perceive osteoporosis as a female disease. This contributes to underdiagnosis and undertreatment, which is considerable in both genders, but even more so in men (15–18). Recently, Boonen et al (19) provided formal evidence in a randomized controlled trial that this therapeutic inertia is unjustified because fracture risk in male osteoporosis can be reduced with similar effectiveness as that shown in women.

Thus, apart from younger age, male gender by itself is one of the strongest protective factors that approximately halves the risk of osteoporosis. As early as the 1940s, gender differences in fracture risk were attributed to the effects of estrogens in women and androgens in men. By the end of the 1990s, sex steroids, and especially estrogens, were deemed central mediators involved not only in postmenopausal osteoporosis but also in the slow, continued bone loss in older age (so-called involutional or type II osteoporosis), in male osteoporosis, and even in age-related secondary hyperparathyroidism (20, 21). However, this unitary model centered on estrogen deficiency has been modified with the recognition of generic aging mechanisms and other master regulator pathways involved in bone turnover (eg, Wnt/sclerostin, receptor activator of nuclear factor kappa B [RANK]/RANK ligand [RANKL]/osteoprotegerin [OPG], Runt-related transcription factor 2 [RUNX2], IGF-1, etc) (22–24). These pathways probably control bone metabolism in both genders (25, 26), and although many of them directly interact with sex steroid signaling, they have not been demonstrated as intrinsically sexually dimorphic (see also *Section VII*). Androgens and estrogens are therefore still considered the chief regulators of gender differences in bone metabolism.

The aim of this review is to critically summarize advances in our understanding of sex steroid actions on the male skeleton, and whether and how this contrasts to the situation in women. We will emphasize progress made since our previous review a decade ago (27). In *Sections II–VI*, we will discuss sex steroid physiology in relation to bone and how this regulates male bone strength and fracture risk at the organ, cellular, and genetic level during peak bone mass acquisition and subsequent maintenance. The clinical approach to the diagnosis and treatment of osteoporosis in men has been reviewed elsewhere (1, 28, 29) and will not be covered here.

In *Section VIII*, it will become clear that attention in this field has extended beyond the skeleton: sex steroids affect all aspects of body composition, which is overall more robust in men. Not only are men almost 10% taller than women, with 25% greater peak bone mineral content on average (1, 21), but also muscle mass and power are almost 50% higher and body fat percentage nearly 50% lower in young adult men compared to women (30, 31). In turn, interactions between bone, muscle, and fat are in-

Figure 1.

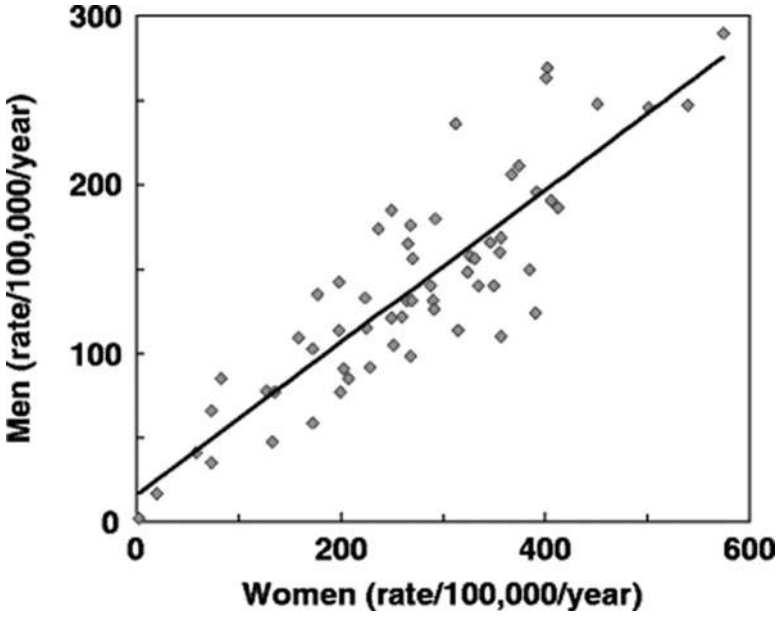

Figure 1. In a recent systematic review, age-standardized hip fracture rates (/100 000/y) in men were approximately half those in women (correlation: r = 0.82; $P < .001$). Thus, in countries where higher rates were observed in women, higher rates were also found in men and vice versa. [Reproduced from J. A. Kanis et al: A systematic review of hip fracture incidence and probability of fracture worldwide. *Osteoporos Int.* 2012;23:2239–2256 (6), with permission. © International Osteoporosis Foundation and National Osteoporosis Foundation.]

creasingly studied. In old age, decreasing bioavailable androgen and estrogen levels contribute to detrimental changes in these body composition domains (32–34). In that sense, sex steroid signaling may be considered an attractive drug target in the quest for shifting mesenchymal precursor cells toward osteogenic and myogenic differentiation and away from adipogenesis (see also *Section V* and Figure 5) (35). Unraveling these pathways may inform the design of better, preferentially gender-neutral drugs that not only inhibit bone resorption or stimulate bone formation, but also reinforce the musculoskeletal system as a whole, prevent falls, and decrease adiposity and its consequences on glucose and lipid metabolism. Given the demographic trends of increased longevity and consequent rise of osteoporosis, sarcopenia, and obesity in many societies worldwide, this is a high research priority.

II. Sex Steroid Physiology in Relation to Bone

A. Circulating sex steroids and SHBG

Androgens and estrogens are respectively C19 and C18 metabolites of cholesterol. The predominant gonadal androgen in men is T, 95% of which is secreted by the testes. The remaining 5% is produced by the adrenals after conversion of the precursor dehydroepiandrosterone (DHEA). In peripheral tissues, T can be converted by 5α-reductase enzymes into the more potent androgen dihydrotestosterone (DHT). T can also be converted into 17β-estradiol (E2) by the aromatase (CYP19A1) enzyme. Approximately 20% of circulating E2 in men is made by the testes; the remaining 80% is derived from DHEA in peripheral tissues (36, 37). Therefore, the actions of T can be mediated via T directly or DHT via the androgen receptor (AR), or T can be converted into E2 and act via the estrogen receptor (ER) α or β.

SHBG binds 50–60% of circulating T and E2 with high affinity, whereas 40–50% is bound nonspecifically to albumin and other proteins. Only 1–3% of the active sex steroids are unbound, and this is termed the free fraction (36). The free and non-SHBG-bound fractions together are referred to as the bioavailable sex steroid fraction. These fractions are often calculated using law-of-mass-action equations, based on the concentrations of total T or E2, SHBG, albumin, and fixed association constants. Several studies have demonstrated the limitations of these formulas, which tend to overestimate the free and/or bioavailable levels relative to the laboratory measurements, especially in the case of T (38–41). However, because direct measurements of free and bioavailable sex steroid concentrations are laborious and require considerable expertise, they are reserved for research purposes but not considered accurate enough in the clinical setting (42).

The circulating levels of total T and E2 decrease only marginally with age in men. However, the age-related increase in SHBG is more pronounced, resulting in a greater decrease of bioavailable or free sex steroid levels (43, 44). Importantly, older men generally have higher circulating E2 levels than postmenopausal women (45), making it plausible that E2 contributes to the conservation of the male skeleton and/or other tissues during aging.

Serum sex steroid levels are routinely measured by immunoassays, but due to limited specificity, especially at low E2 concentrations, mass spectrometry has been recommended (46–48). This is an important consideration when interpreting the results of both human and rodent studies using immunoassays for measurement of sex ste-

roid levels (see *Section II.E* for discussion of circulating sex steroids in rodents). Nevertheless, serum E2 levels measured either by a sensitive immunoassay or by mass spectrometry provide almost identical correlations with bone mineral density (BMD) in men (48, 49).

B. Local sex steroid metabolism in bone tissue

Not only sex steroid receptors, but also all the necessary enzymes to convert the adrenal sex steroid precursors DHEA and androstenedione into active androgens and estrogens, are expressed in bone tissue and osteoblast cell lines (27), supporting the notion of local skeletal paracrine and/or intracrine synthesis and action of sex steroids. In comparison, little is known about sex steroid-metabolizing enzymes in osteocytes and osteoclasts.

Transgenic male mice overexpressing human aromatase have an increased bone mass, but other, adverse systemic effects were also observed due to elevated circulating E2 levels (50). In contrast, aromatase overexpression specifically in osteoblasts increases bone mass without elevating serum E2 levels or systemic effects (51). Male mice genetically ablated of 5α-reductase type 1 have slightly reduced bone mass (52), although the relevance of this finding to human physiology remains to be established (see *Section IX.B*).

Androgens and estrogens are also inactivated locally, and the resulting metabolites can be measured in serum and urine. More specifically, androgens are glucuronidated by uridine glucuronosyl transferases, and this process can regulate the intracellular levels of active androgens and their local biological activity. Circulating levels of these specific glucuronidated androgen metabolites showed closer associations with BMD at several sites in older men than the serum levels of the active androgens (53). Polymorphism in the gene for catechol-O-methyltransferase (COMT; an estrogen-degrading enzyme) have also been associated with peak bone mass development (54, 55) as well as prevalent fractures in elderly men (56, 57).

In conclusion, the concept of intraskeletal sex steroid metabolism limits the interpretation of circulating levels of androgens and estrogens because they might not always reflect androgen and estrogen bioactivity within bone. Future studies should assess sex steroid levels not only in serum but also in the tissue of interest, although better methods to measure intraskeletal sex steroids remain to be established (58).

C. Classical androgen and estrogen receptor signaling

Androgens and estrogens can be defined as high-affinity ligands for AR or ERα/ERβ, which are ligand-inducible transcription factors of the nuclear receptor superfamily (NR3C4, NR3A1, and NR3A2, respectively). These receptors are comprised of a modular structure involving an intrinsically disordered N-terminal domain (NTD), a DNA-binding domain (DBD) consisting of two zinc fingers, the flexible hinge region, and the C-terminal ligand-binding domain (LBD) (Figure 2) (59, 60). Their classical action is initiated in the cytoplasm when ligands bind in a hydrophobic pocket of the LBD. This induces conformational changes revealing a nuclear translocation signal in the hinge region. The LBD also harbors activation function 2 (AF-2), a ligand-dependent interaction surface for coregulatory proteins or intra- and intermolecular interactions with the NTD (N-terminal/C-terminal interactions). Ligand-independent receptor activation can also occur through protein interactions and/or post-translational modifications of activation function 1 (AF-1), situated in the NTD. In both murine and human osteoblasts, a 46-kDa alternative splice variant of ERα lacking AF-1 has been demonstrated.

In the nucleus, these nuclear receptors bind as dimers to specific DNA sequences termed androgen or estrogen response elements (AREs, EREs), interact with other transcription factors, and recruit coregulatory proteins, chromatin

Figure 2.

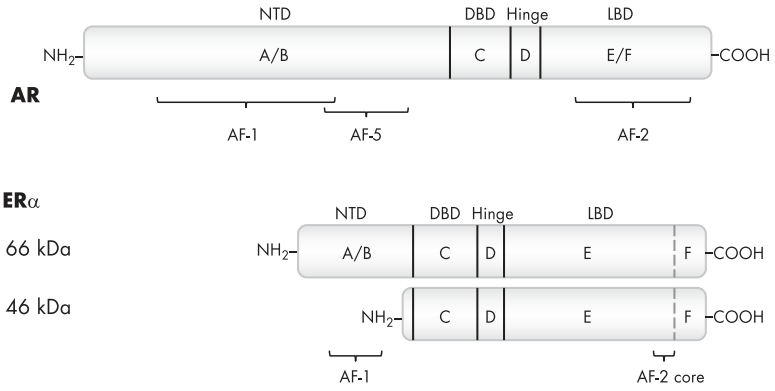

Figure 2. Diagram of the structure of AR and ERα with their different domains: the NTD (A/B domain), the C-domain containing the DBD, the D-domain containing the hinge region, and the C-terminal E- and F-domains containing the LBD. The AR A/B-domain contains two surfaces for interactions with coactivators AF-1 and AF-5 (also called transcriptional activation unit, tau-1 and tau-5) (60). The LBD contains AF-2, which is involved in N-terminal/C-terminal interactions and binding of coregulatory proteins, but it has weak activating capacity in AR. ERα also contains an AF-1 and AF-2 and has two isoforms: the full-length 66-kDa protein and a less abundant 46-kDa isoform lacking AF-1.

remodeling complexes, and components of the transcription machinery. Two types of AREs exist: 1) canonical AREs that form a clear palindromic repeat of the 5′-AGAACA-3′ consensus sequence; and 2) selective AREs with a more relaxed resemblance to direct or palindromic repeats of the same hexamer (61). Selective AREs play a role in the male reproductive system but not in the musculoskeletal system, as shown in AR knock-in mice with abrogated binding to selective AREs (62).

Nuclear receptors can also bind chromatin indirectly via tethering to other proteins: ER, for example, may associate with the activating protein-1 (AP-1; a complex containing Fos/Jun) or specificity proteins (eg, SP-1), which can bind to specific DNA motifs different from EREs. Both AR and ERα are able to form heterodimers with other transcription factors to modulate their activity in osteoblasts as shown for, eg, RUNX2 (see *Section VII.C*). Finally, coactivators or corepressors can modulate tissue-specific activity of AR/ER on target genes. The in vivo importance of coactivators in bone was illustrated by a study in ovariectomized steroid receptor coactivator-1 knockout mice; physiological doses of E2 restored cortical bone, but supraphysiological doses were required to restore trabecular bone (63). Further research on AR/ER coactivators as tissue-specific modulators of sex steroid responses in bone is warranted.

D. Ligand-independent and nongenomic actions

Nonclassical AR/ER actions fall into two categories. In nongenomic (synonym, nongenotropic) signaling, the liganded receptor activates second messengers like kinases, phosphatases, cytoplasmatic calcium release through ion channels, or nitric oxide synthesis. The second category of ligand-independent activation occurs without occupancy of the ligand-binding pocket; this mode is triggered mostly via phosphorylation of AF-1.

A hallmark of the former nongenomic effects is that these can occur within seconds or minutes. AR has been found to exert nongenomic effects via components of the SRC, ERK, PI3K, and AKT pathways. A particular form of nongenomic actions may occur through transmembrane receptors. For example, G protein-coupled receptor 30 (GPR30; see *Section IV.E*) has been proposed as a high-affinity membrane G protein-coupled ER (renamed GPER1), although this hypothesis is not unequivocally supported (64). ERα isoforms may signal at the plasma membrane as well (65). A transmembrane AR has been proposed but remains controversial, and a role in skeletal physiology has not been demonstrated.

The latter, ligand-independent activation mode has clearly been demonstrated for ERα but is more controversial for AR. Kinases responsible for AR/ER phosphorylation (eg, MAPK, AKT, MEK1/2, and ERK1/2) may be activated by several growth factors (eg, IGF-1, keratinocyte growth factor, epidermal growth factor) or by feedback from nongenomic sex steroid signaling. This may be sufficient to mediate ERα nuclear translocation, whereas for AR it may enhance androgen-induced translocation. In vitro ERα transfection in osteoblastic cells has been noted to activate transcription in the apparent absence of estrogens (66), whereas this is not the case for AR and androgens. In vivo, ERE reporter mice showed ER-dependent bone luciferase activity even when they were immature, gonadectomized, or treated with aromatase inhibitors (67), thus supporting ligand-independent ERα actions in bone.

To what extent these nonclassical mechanisms contribute to the skeletal effects of androgens, estrogens, or their respective receptors will be discussed in *Section IV.E*.

E. Rodent models in the study of sex steroids and bone: a critical appraisal

Animal models have proven invaluable to investigate the cellular and molecular mechanisms of sex steroid actions in bone. But before discussing the results of these studies, some caveats will be reviewed.

1. Differences in circulating sex steroid physiology

Circulating sex steroid physiology differs between humans, mice, and rats, mainly because the latter two lack circulating SHBG. This is thought to contribute to the observation that rodents have very low, highly fluctuating serum T levels. Indeed, SHBG transgenic mice have very high T levels in parallel with their SHBG overexpression (68).

Although it is clear that estrogens are important for the male rodent skeleton, circulating E2 levels measured with the "gold standard" of mass spectrometry are below the limit of detection of sensitive assays (69, 70), suggesting that local aromatization and intraskeletal effects are important. Even pharmacological doses of continuous T do not increase serum E2 in male mice, presumably because aromatase expression in rodents is mainly limited to the gonads and certain areas of the brain (71). Given the increased recognition in human studies that immunoassays may be unreliable and confounding to much of the literature, mass spectrometry will also be needed to reexamine serum T and E2 concentrations in different rodent models (72). Administration of E2 to (orchidectomized) male mice does produce measurable E2 serum levels (70), which are thus by definition unphysiological. This results in a marked increase in trabecular bone volume and formation (73, 74), which is unparalleled in humans (for example, unseen in male-to-female transsexuals given pharmacological E2 doses) (75). Together with the aforementioned findings on skeletal bioluminescence in ERE reporter mice

(67), these data argue against a purely endocrine role of circulating E2 in mediating genomic ER actions in rodents and point to the importance of intracrine effects of estrogens via local aromatization and/or ligand-independent ER activation in bone (see *Sections II.B*, *II.D*, and *IV.E*).

Gonadectomy is widely used as a preclinical model of osteoporosis, but because other gonadal endocrine bone-stimulating factors are lost as well, eg, inhibin A (76), control experiments with hormone replacement are necessary (eg, with T or nonaromatizable DHT). The role of the adrenal glands in rodents as a source for androgens and estrogens has been suggested as negligible, although several studies have suggested that adrenal androstenedione may represent an additional source of (aromatizable) androgens in mice and rats (27, 77, 78). To overcome limitations of assessing androgenic/estrogenic status based on serum sex steroid concentrations, the weight of sex steroid-sensitive organs is commonly used, as an in vivo bioassay (seminal vesicles and levator ani muscle in males and uterus weights in females). Unfortunately, there is no good tissue indicator of estrogen bioactivity in males.

2. Genetic rodent models and their limitations

Genetic models preventing androgen or estrogen actions at the level of the receptor preclude confounding by metabolites, eg, from aromatization or adrenal origin. Ubiquitous androgen- or estrogen-receptor knockout (ARKO, ERKO) mice have greatly contributed to our understanding of sex steroid actions in bone. Unfortunately, ubiquitous models suffer from disrupted negative feedback on the hypothalamic-pituitary axis as well as altered serum IGF-1 levels in some models (Table 1). As mentioned above, the low serum sex steroid concentrations in these animals (especially E2) were probably not reliably measured with previous immunoassays; serum E2 is undetectable with mass spectrometry in both wild-type and ARKO males (<0.6–1.3 pg/mL; our unpublished observations). Furthermore, serum levels may not accurately reflect intraskeletal metabolism or receptor activation. For example, IGF-1 expression and IGF-1 receptor levels have been found down-regulated in other tissues in ARKO mice (79).

The first genetic models used to study androgen actions in bone were male testicular feminization (Tfm) rats that spontaneously developed a single base mutation in the AR LBD. Their bone phenotype is intermediate between females and males, which is, however, confounded by decreased IGF-1 and high estrogens (Table 1). Tfm mice, on the other hand, harbor a single base deletion and frameshift in the AR NTD but still express a truncated C-terminal AR peptide initiated upstream of the DBD with residual DNA- and hormone-binding activity (27).

Five different ubiquitous ARKO mouse models have been developed (97) that differ in their deletion of either exon 1, 2, or 3, resulting in abrogation of the NTD (98), the first zinc finger (80, 99), or the second zinc finger (100) of the DBD, respectively. The concern that AR is still expressed and may retain some of its actions in Tfm or resistant ARKO models is alleviated in the ARKO models that totally lack AR expression (80, 98, 99). Mice with abrogation of the AR second zinc finger ($AR^{\Delta ZF2}$ mice) purposely retain normal ligand binding and nongenomic effects on second messengers like ERK phosphorylation (100, 101) (see also *Section IV.E*). However, the possibility that the absence of an entire domain in this model results in protein misfolding and off-target effects cannot be completely excluded. Moreover, the floxed allele in $AR^{\Delta ZF2(flox/Y)}$ male mice results in increased baseline AR sensitivity (102).

The first reported ERαKO mouse model from the group of Korach and Smithies lacks the normal 66-kDa protein but still expresses the less abundant 46-kDa normal isoform as well as an unexpected 61-kDa splice vari-

Table 1. Summary of Endocrine Changes in Male Global Genetic Rodent Models Used in Studies on Sex Steroids and Male Bone Metabolism

	FSH/LH	Androgens	Estrogens[a]	IGF-1	Refs.
Tfm rats	↑	↑	↑	↓	27
Tfm mice	↑	↓	↓	NR	71
ARKO	↑	↓	<LOD	= *	80–84
Aromatase KO	↑	↑	↓	↓ (↑ F)	85–87
ERαKO	↑	↑	↑	↓	84, 88, 89
ERβKO	=	=	=	= (↑ F)	89–93
NERKI	NR	NS (↑ F)	=	↓	94–96

Abbreviations: LOD, limit of detection; ↑, increased; ↓, decreased; =, no change; NR, not reported; ↑ F, increase in female mice reported in some studies; = *, serum levels were reported as not decreased, but intraskeletal signaling may still be disturbed; NS, serum T was nonsignificantly increased due to high variability, and weights of androgen-responsive tissues were not reported.

[a] Low circulating E2 was measured with immunoassays in most studies, and changes paralleled aromatase activity; with mass spectrometry, however, serum E2 is below the limit of detection (<0.6–1.3 pg/mL; our unpublished results) in male wild-type and ARKO mice.

ant, which both have possible activity of AF-2 but not AF-1 (27). Subsequent models represent truly null mutants based on deletion of the first zinc finger of the ERα DBD. Whether a putative splice variant lacking the DBD in the original ERβKO mice could still harbor residual activity has not been clearly demonstrated, but the possibility has been used to explain differences to other ERβKO models (93, 103, 104). Due to a lack of negative feedback on the hypothalamic-pituitary axis, ERαKO models have increased levels of gonadotropins, androgens, and estrogens (84, 88)—whereas circulating T was normal in the ERαKO man (105). Male ERβKO mice do not have disturbed hypothalamic-pituitary feedback (90–93), although IGF-1 was increased in young ERβKO females in one study (90). Male mice with a knock-in ERα (NERKI mice) with abrogated DNA binding but capability to mediate nonclassical signaling had nonsignificantly increased T and were suggested to have elevated E2 serum levels on a mixed genetic background (94, 95).

The main revolution in our understanding of the mechanisms of action of androgens and estrogens comes from the use of conditional genetic mouse models. Bone cell-specific ARKO/ERKO models have the potential to avoid disrupted feedback on other pathways (eg, systemic IGF-1) or indirect effects on bone via ER and AR in other tissues (eg, decreased voluntary physical activity in ARKO mice) (106, 107). However, Cre-LoxP technology has its own pitfalls, including independent effects of inserting a Cre somewhere in the genome or LoxP sites in a gene, incomplete deletion (which is typically still only around 80% in efficient conditional models), off-target Cre activity in cells and tissues other than those targeted, "leaky" Cre activity in inducible models or confounding effects of hormone therapy used to induce the recombinase (which probably explains why no studies on sex steroids and bone have yet used tamoxifen- or progesterone-inducible Cre systems). Moreover, the phenotype of noninducible models could result from embryonic or perinatal imprinting effects, even when this phenotype becomes evident at later ages.

In summary, sex steroid physiology differs between male mice, rats, and humans, mainly because of the absence of SHBG and differences in peripheral, extraskeletal aromatization. Genetic models exclude AR or ER activation altogether but harbor many caveats including the presence of residual receptor isoforms, nonclassical activation, baseline confounding phenotypes in Cre or LoxP controls, intrinsic perturbations of the hypothalamic-pituitary axis in global, or eg, neuron-specific models (108), and indirect effects on bone via other tissues. As such, use of selective ligands in gonadectomized, completely null genetic models with careful attention to controls should probably be regarded as the highest level of evidence in the face of conflicting results. Future studies using inducible Cre systems are needed to avoid developmental effects and dissect the timeframe in which AR/ER actions on bone are determined.

III. Structural Basis of Male Bone Strength

Fracture risk is determined by bone strength on the one hand, and fall risk and defense mechanisms (eg, soft tissue absorption of kinetic energy) on the other hand. Bone strength in turn is determined not only by BMD but also by bone dimensions, microstructure, and material properties (2). BMD is usually measured by dual-energy x-ray absorptiometry (DXA), but because it is a projectional (two-dimensional) technique, it cannot truly differentiate between cortical and trabecular bone. Quantitative computed tomography (QCT), on the other hand, can separately measure the volumetric BMD (vBMD) of both bone compartments and provides bone geometrical parameters together with indices of bone strength. Still, this technology is limited by its resolution (about 400 μm) to accurately measure bone microstructural properties. This has been overcome by the advent of high-resolution peripheral QCT (HRpQCT), which permits the noninvasive measurement of the microstructural properties of the trabecular and cortical bone compartments at the distal radius and distal tibia with a resolution of 82 μm or, more recently, even less. This is sufficient to quantify the larger pores in bone (cortical porosity). HRpQCT also provides the highest quality data to generate finite element analysis models for estimation of bone strength. Emerging new technologies like microindentation may be able to determine bone material properties and sexual dimorphism herein, but further validation is needed.

In the following paragraphs, we will discuss the structural basis of male bone strength, which determines the decreased risk of osteoporotic fractures in men. Understanding how gender dimorphism in bone geometry and microstructure is established during adolescence and young adulthood may offer insights in fracture susceptibility and its prevention later in life. Sex steroids probably determine sexual dimorphism in these (HR)pQCT parameters, but because current evidence is limited by its mostly cross-sectional and observational nature, sex steroid regulation of cortical and trabecular peak bone mass acquisition is better understood from murine and sporadic human genetic models discussed in *Section IV*.

A. Favorable cortical bone size and trabecular microstructure in young adult men

Men are on average 10% taller than women because of their greater bone length. Even after correcting for bone length, men have greater bone width than women, with cortical bone placed further away from the neutral axis of the bone. This results in an exponential increase in strength because the rigidity of a tubular bone scales as the fourth power of its diameter (2). Using QCT, Riggs et al (109) investigated sexual dimorphism in bone geometry in an age- and sex-stratified, population-based, cross-sectional sample. At both central (lumbar spine, femoral neck) and peripheral (distal radius, distal tibia) sites, young adult men had a 25–33% larger cross-sectional bone area than young adult women, and these relative differences were maintained throughout life. Trabecular vBMD was greater in men at all sites, whereas cortical vBMD was mostly similar between sexes (109). The larger bone width in men results from greater periosteal expansion during puberty and early adulthood in men, whereas girls predominantly increase their cortical thickness by limiting endocortical expansion.

Serum levels of calculated free T were positively associated with the cortical cross-sectional area and periosteal circumference at both the tibia and the radius of young adult Swedish men (110). Also in young men, higher serum SHBG levels were associated with larger cortical bone area and periosteal and endocortical circumference, independently from free sex steroid levels, suggesting an independent role of SHBG in determining cortical bone size at the age of peak bone mass (110, 111). The reduced bone size in women may predispose elderly women to a greater fracture risk than elderly men after similar age-related bone losses have occurred (see *Section IX*).

Structural differences between male and female bone have more recently also been assessed by HRpQCT. Khosla et al (112) found that young adult men had a 26% greater trabecular bone volume with more plate-like, thicker trabeculae but similar values for trabecular number and separation compared to women (Figure 3, A–C). Bone area and medullary area were also larger in young men compared with young women. Cortical thickness at the age of peak bone mass was similar in both sexes (Figure 3D), and cortical vBMD was slightly lower (5%) in young men (112). These gender differences have been confirmed by several other cross-sectional HRpQCT studies (113–119).

Correlation analyses between serum levels of sex steroids and the differences in bone structure during pubertal growth showed that the increases in trabecular bone volume and trabecular thickness in boys were correlated to

Figure 3.

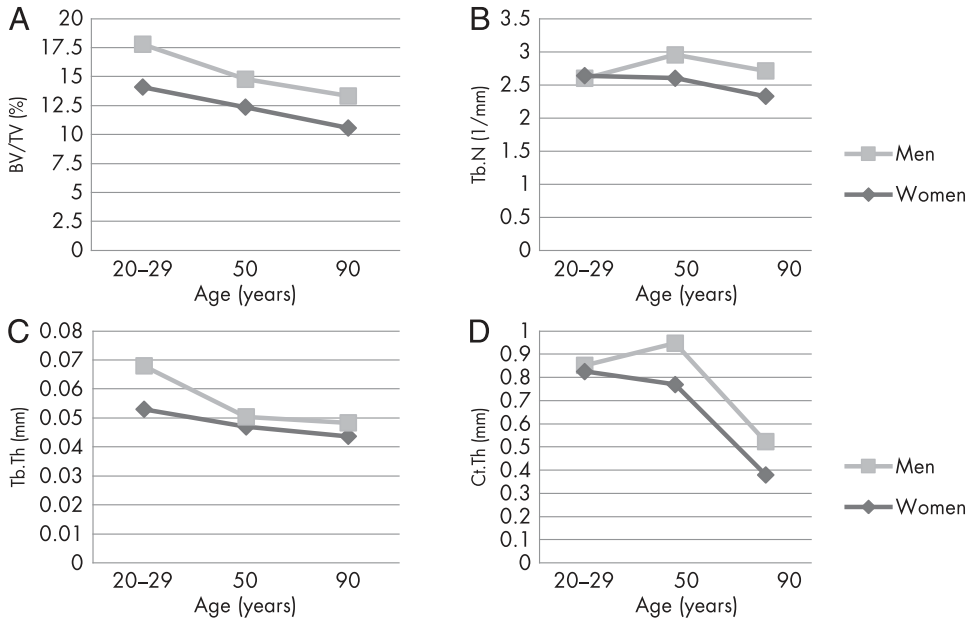

Figure 3. Age-related changes in structural determinants of bone strength in men and women based on a cross-sectional study using high-resolution peripheral CT at the ultradistal radius. A, BV/TV, trabecular bone volume/tissue volume; B, Tb.N, trabecular number; C, Tb.Th, trabecular thickness; D, Ct.Th, cortical thickness. [Adapted and reproduced from S. Khosla et al: Effects of sex and age on bone microstructure at the ultradistal radius: a population-based noninvasive in vivo assessment. *J Bone Miner Res.* 2006;21:124–131 (112), with permission. © American Society for Bone and Mineral Research.]

higher levels of both serum T and IGF-1 (116). The larger bone width in boys, evidenced by the larger periosteal circumference, was associated mainly with serum T. Although serum E2 was not an independent predictor of skeletal dimorphism in this multivariate model (116), part of the effects of T could still be mediated by aromatization into E2 locally in bone, which may not always be reflected by serum E2 levels (see *Section II.B*). Indeed, human cases of ERα and aromatase deficiency have clearly illustrated that estrogen actions are crucial in peak bone mass acquisition in both genders (see *Section IV*). Other studies, however, found no association between serum levels of sex steroids and trabecular or cortical microstructure in young adult men (112, 119).

Intracortical porosity (calculated as the pore volume normalized to the cortical bone volume) appears significantly greater in men in their 20s and 30s than in women (113, 115, 117–119). Although a more porous cortex would be a disadvantage in biomechanical terms, this was counterbalanced by a larger cross-sectional bone area. Size-dependent indices of bone strength such as stiffness and estimated failure load were higher in young adult men compared to young adult women, whereas apparent modulus, which is size-independent, was similar in both sexes (113, 115, 117–119). These findings indicate the importance of geometry with respect to bone strength. Cortical thickness, cross-sectional area, and cortical vBMD of the tibia and/or radius were all greater in young adult men than in late adolescent men, showing that early adulthood is a period of ongoing cortical modeling with further increases in cortical thickness and estimated bone strength (119, 120).

In summary, men have a structural advantage over women from early adult life characterized by wider bones due to greater cortical bone expansion and greater trabecular bone volume due to thicker trabeculae translating into superior indices of bone strength (Figure 3), which may protect against fractures later in life. The larger periosteal expansion and trabecular bone volume in men have been independently associated with serum T, SHBG, and IGF-1 in some (but not all) studies, although genetic disruption of ERα actions (discussed in *Section IV.A*) is certainly also detrimental for male peak bone mass acquisition.

B. Better maintenance of trabecular and cortical bone structure in men across the lifespan

Studies using central and peripheral QCT and HRpQCT have shown that, in general, age-related deterioration in bone geometry, vBMD, and bone microstructure affects men and women equally, but because older men maintain their sex steroid levels better, they do not experience an equivalent to the accelerated phase of bone loss associated with menopause in women (ie, no "andropause" equivalent).

1. Cortical geometry

Riggs et al (109) nicely demonstrated, using QCT in both a cross-sectional and a 3-year longitudinal study (121) in an age- and sex-stratified population sample of men and women, that the total bone cross-sectional area continues to increase over a lifetime by about 15% in both sexes. This is, however, outpaced by endocortical resorption, estimated from the 25–40% increase in total medullary area at the femoral neck and distal radius, resulting in an age-related decrease in cortical thickness in both sexes (Figure 3D). Longitudinal pQCT data from the InCHIANTI study confirm that older women show a dramatically greater medullar expansion and resulting greater cortical thinning compared with older men, although periosteal expansion (estimated from the increase in total cross-sectional area) is slightly greater in older women than in older men (122). These changes in cortical geometry were also confirmed by cross-sectional age-stratified HRpQCT studies (112–115, 117).

2. Cortical and trabecular vBMD

Loss of trabecular and cortical vBMD with age is more pronounced in women than in men, although the rates of trabecular bone loss vary according to site. Remarkably, loss of trabecular vBMD starts already in young adult life in both women and men and continues throughout life with acceleration in women at perimenopause. In men, the rate of trabecular bone loss appears to attenuate in older men at the distal radius and tibia but not at the spine. In contrast, cortical vBMD remains relatively stable until midlife in both sexes with substantial loss of cortical vBMD after menopause in women, but only from about age 75 in men (109, 112, 117, 121, 122). In the Osteoporotic Fractures in Men (MrOS) study, men over 85 years of age had 22% lower trabecular vBMD but similar cortical vBMD of the femoral neck compared with men aged 65–69 years (123). At the femoral shaft, bone cross-sectional area increased by 9% in the oldest men, consistent with ongoing periosteal apposition with age, but because the endocortical area increased by 22%, the cortical area decreased significantly. It is plausible that the decreases in cortical thickness and cortical and trabecular vBMD after the age of 50 in women and in old age in men are driven by sex steroid deficiency both in women (by menopause) and at least partly in men (by the age-related decline in bioavailable sex steroids). However, the continuous decrease in trabecular bone even in young adult men and women, accounting for between one-third and one-half of

the total trabecular bone loss over life (121), occurs during sex steroid sufficiency, and the mechanism responsible for this remains unexplained.

3. Trabecular bone microstructure

Age-related microstructural changes at the trabecular compartment in the radius include similar decreases in trabecular bone volume in men (−26%) and women (−23 to −27%) (Figure 3A), although the microstructural changes underpinning this trabecular bone loss differ between the sexes (112, 117). Women undergo loss of trabeculae, resulting in an increase in trabecular spacing, whereas in men between the ages of 20 and 49, the number of trabeculae increased while at the same time trabecular thickness decreased dramatically (Figure 3, B and C). These findings are consistent with the idea that in men, bone loss is mainly due to thinning of the trabeculae because of decreased rates of bone formation, whereas in women, the increased rates of bone resorption associated with menopause contribute to the loss of trabeculae (124). Importantly, this pattern of trabecular bone loss appears to vary with skeletal site. In contrast to the findings at the radius, Macdonald et al (117) and Hansen et al (115) recently demonstrated in population-based samples of men and women that in the distal tibia, both trabecular thinning and loss of trabeculae contributed to the loss of trabecular bone volume in both men and women. These studies also suggested site-specific differences in the onset of trabecular bone loss. There is clearly a need for prospective HRpQCT studies to confirm the age of onset, exact nature, and underlying mechanism of microstructural deterioration in men and women.

4. Cortical porosity

Age-related microstructural changes at the cortical compartment are dominated by significant increases in cortical porosity in both men and women (113, 115, 117, 125), coinciding with (and possibly explaining to a large extent) the decline in cortical vBMD. The increase in cortical porosity in men is less pronounced than in women and does not occur until old age in men, in accordance with the findings for loss of cortical vBMD. Recent data demonstrate that serum E2 levels associate inversely with cortical porosity in elderly Swedish men, indicating that E2 might regulate cortical bone microstructure (126).

In summary, men have favorable bone structure at peak bone mass, and age-related decreases in bone structure and strength at both the trabecular and cortical compartment are delayed and less pronounced in aging men compared to women. The patterns of age-related structural changes are gender-specific and also differ between weight-bearing (tibia) and non-weight-bearing (radius) sites. The larger number of thicker trabeculae and better maintenance of cortical thickness together with their larger bone width all contribute to the observed lower fracture incidence in men. This occurs most specifically at the radius, where the risk in childhood is greater in boys (at least in part due to a transient deficit during cortical bone expansion) (116), whereas there is a marked increase in Colles' fracture early after menopause in women compared with an almost complete absence in men (127). Longitudinal HRpQCT studies are needed to more accurately determine age-related changes in trabecular and cortical bone microstructure in men and women because the available cross-sectional studies cannot determine true age-related changes because of possible confounding by secular trends, as nicely illustrated by the InCHIANTI study (122).

The relative contributions of androgens, estrogens, and SHBG in determining bone loss and fracture risk in older men will be the focus of *Section IX*. More studies are needed to determine the role of sex steroids in age-related microstructural bone decay.

IV. Regulation of Skeletal Sexual Dimorphism

In this section, we will examine the relative contributions of androgens and estrogens for cortical and trabecular bone development. Puberty is a critical period for peak bone mass acquisition and establishment of sexual dimorphism in bone mass and geometry. This period may be even more important than age-related bone loss in determining the risk of osteoporotic fractures in later life (128). Cell-specific ERαKO and ARKO mice have recently advanced our understanding of sex steroid regulation in cortical and trabecular bone. These studies will be discussed in detail in *Section V*. The principal conclusions on the effects of sex steroids and their receptors in male and female peak bone mass acquisition are summarized schematically in Figure 4.

A. Estrogen receptor α: crucial for peak bone mass

1. Role in cortical and trabecular bone development in females

Estrogens have traditionally been viewed as promoting trabecular and cortical bone development in females. Compared to males, however, periosteal bone expansion, endocortical widening, and trabecular thickness are lower in females (see *Section III*). The importance of estrogens is confirmed in aromatase knockout mice: females have poor trabecular and cortical bone development despite increased T and IGF-1 (85, 87). Female ERαKO mice have been reported as having paradoxically increased trabecu-

Figure 4.

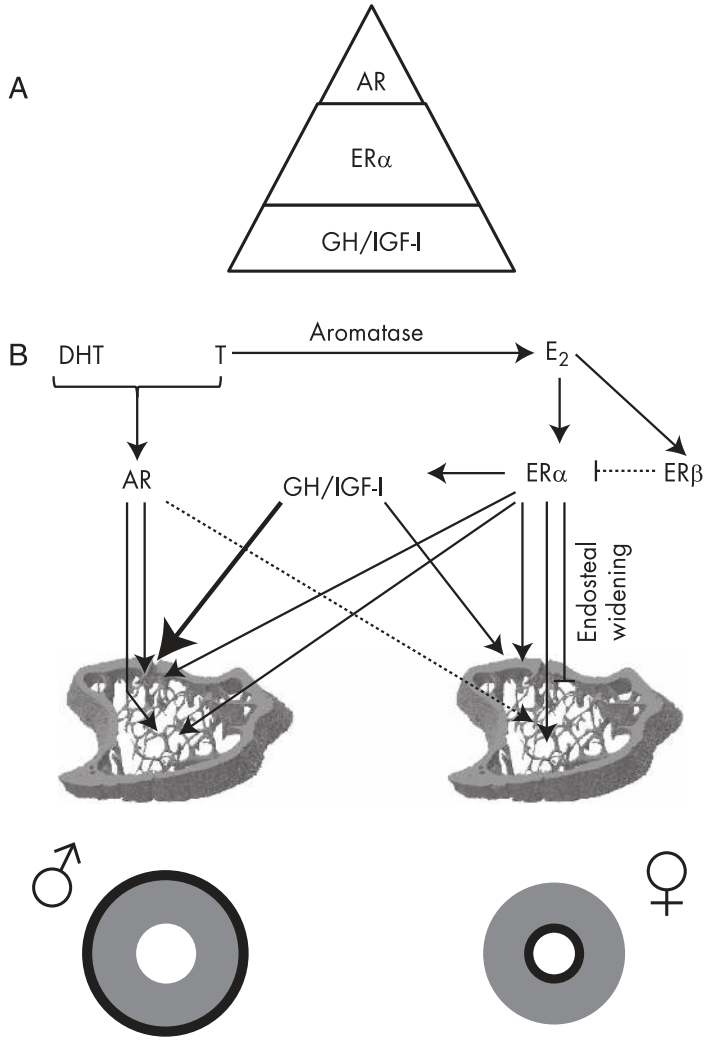

Figure 4. Schematic representation of the effects of sex steroids and their receptors in the development of male and female pubertal bone development. A, Pyramid hierarchy of sexual dimorphism in bone. Given the severely disordered bone development in case reports of ERα mutations or aromatase deficiency, compared to the normal female-like bone structure in XY females with AIS, the effects of ERα should be considered critical, whereas AR provides further benefits that, however, are unable to compensate for severely decreased ERα activation. Even more severe is the skeletal phenotype of developmental disruption of the GH/IGF-1 axis in both genders, and skeletal sexual dimorphism is completely lacking in GH-receptor knockout mice (with very low circulating IGF-1 levels) (78). B, Androgens like T can be converted via aromatization to estrogens and can thus activate both AR and ERα. In males, both AR and ERα stimulate cortical and trabecular bone development. During puberty, males have greater periosteal expansion than females (bottom left, black outer circle), which depends both on AR (in late puberty) and ERα (in early puberty), but especially on IGF-1 (probably via central aromatization of androgens) (78, 82). Trabecular bone formation is increased by ERα in males (220), whereas both ERα and AR can inhibit trabecular bone resorption. Additionally, AR inactivation has been shown to slow mineralization in early growth, when bone turnover is high (170). In females, ERα is responsible for both trabecular and cortical bone mass accretion, but their cortical thickness relies more on limiting endocortical expansion (bottom right, black inner circle). ERα inhibits endosteal bone resorption and stimulates periosteal bone formation (179), although the latter is lower compared to males (82). Females have higher endosteal bone formation (78) due to actions of ERα (95). Estrogens also stimulate trabecular bone formation in female mice (219, 221, 222). Both androgens and estrogens can decrease trabecular bone resorption in female mice (130, 212), although the physiological importance of androgens in female mice is probably limited. ERβ is thought to repress ERα's stimulatory actions on cortical and trabecular bone in female mice and to play no role in male mice (91, 150, 151), but definitive evidence for a role in humans is lacking.

lar bone in the peripheral skeleton due to very high levels of T stimulating AR (90, 93, 129). This confirms other studies suggesting that compensatory actions via AR can maintain trabecular but not cortical bone mass in female rodents (130, 131), although this probably has minor physiological importance. Complete ERαβKO female mice again have a clear cortical and trabecular bone deficit, and ERα is required for the cortical and trabecular bone response to estrogens in ovariectomized mice (93, 129).

Osteoclast-, osteoblast-, and osteocyte-specific ERαKO models have now been used to avoid systemic endocrine alterations. These studies have shown that ERα actions are important to stimulate periosteal and trabecular bone formation and inhibit endocortical and trabecular bone resorption via different bone cells (see *Section VI*). Female mice also have increased endosteal bone formation compared to males (78), which may rely mainly on nonclassical actions (see *Section IV.E*) of ERα (95). In summary, ERα activation is crucial for cortical and trabecular bone development in female mice.

2. Role in cortical and trabecular bone development in males

The historic dichotomous view that estrogens were important for female bone health and androgens for male bone health were challenged by a unique report of a man with an ERα null mutation (105). He presented with tall stature, incomplete epiphyseal closure in adulthood, markedly decreased BMD, normal T, and increased E2, gonadotropin, and IGF-1 levels, demonstrating the importance of ERα for peak bone mass and growth plate closure in men. Further pQCT and histomorphometric evaluation in this man showed significant impairment of cortical thickness (due to increased endosteal expansion, whereas radial periosteal circumference was similar), as well as deficient cortical and trabecular vBMD with thinner trabeculae and decreased

bone formation (132). Similarly, men with aromatase deficiency have high T but suboptimal bone mass, which cannot improve without estrogen replacement (133). A pQCT study in an adolescent male with congenital aromatase deficiency revealed that increases in BMD during estrogen therapy were not the result of trabecular or cortical vBMD gains but rather increases in cortical thickness and cross-sectional bone area, suggesting that estrogens are required for optimal periosteal pubertal bone expansion typical of the male bone phenotype (134).

In mice, ERα and ERαβKO males also have cortical bone deficits despite increased serum T, in line with observations in male rats that pure androgens cannot compensate for a complete loss of estrogen signaling in cortical bone development and maintenance (89, 93, 135). Trabecular bone development, on the other hand, has been reported as normal (89, 136) or even increased in male ERαKO mice due to increased serum T acting via the AR (84, 93, 129), suggesting that AR is sufficient for trabecular bone development in male mice. On the other hand, E2 is a strong anabolic stimulus for trabecular bone in male mice, and this effect is ERα-dependent (129, 137), although it is currently impossible to define physiological E2 levels in male mice (see *Section II.E*). Male aromatase knockout mice have clearly reduced trabecular bone volume and increased endocortical bone resorption (77). In more recent conditional knockout models, some studies also suggest that ERα is involved in male bone development (see *Section V*). The main conclusion from both human and mouse genetic models is that male cortical and trabecular peak bone mass acquisition is dependent on both AR and ERα actions.

B. Estrogen receptor β

Estrogens act via at least two receptors (ERα and ERβ), so the question is which of these is chiefly responsible for the effects of estrogens. ERβ seems to play no role in bone development in male mice because male ERβKO mice have normal bones and ERαβKO mice show no difference compared to ERαKO males (89, 91, 129, 137, 138). Female ERβKO mice also show normal trabecular bone response to E2 after ovariectomy (129, 139), and selective ERβ agonists are unable to protect rats from ovariectomy-induced bone loss (140, 141). Thus, ERα is definitely the dominant mediator of the skeletal actions of estrogens. Still, three independent groups have shown in female ERβKO mice that longitudinal and transversal bone growth are enhanced and age-related trabecular and endocortical bone resorption are decreased, the latter of which was associated with increased ERα mRNA levels (91, 92, 139, 142, 143). In a fourth ERβKO model, however, a transiently increased trabecular bone volume but no effect on longitudinal or radial bone growth was observed in female mice (93). Some studies also suggest that ERβ is present in cancellous bone whereas cortical bone has little or no ERβ (63, 144, 145). Less rapid trabecular bone loss and a greater increase in cortical area have in turn been observed in long-term follow-up of ovariectomized adult ERβKO mice (146). Female ERβKO also display enhanced fracture healing and increased mandibular condylar growth (147, 148). Overall, ERβ appears to have a minor inhibiting effect on the positive actions of ERα in female mice.

Conflicting results have been presented on whether or not ERβ can also partly compensate for bone loss due to the absence of ERα when ovariectomized complete ERαKO female mice were treated with E2 (129, 149). These data led Lindberg et al (150) to propose a "yin-yang" relationship in female mice, with a moderate repressive function of ERβ on normal ERα signaling, whereas ERβ might partially compensate when ERα is lost (93, 150). Interestingly, ERβ expression also appeared higher in stromal cells isolated from the ERαKO man (132).

Recently, to avoid putative activity from ERβ transcript variants (104) and systemic influences, Khosla's group (151) selectively deleted ERβ in osteoblasts (*Col1a1* 2.3 kb-Cre) and osteoprogenitor cells (*Prx1*-Cre). The latter increased trabecular bone volume by 93% in female mice (151).

We conclude that ERα is the dominant mediator of estrogen actions in bone. Studies in ERβKO mice support a repressive function of ERβ in bone of females. The reason behind the gender difference in the role of ERβ is unknown. Evidence for a role of ERβ in male mice is lacking. In humans, several observational studies suggest that ERβ dinucleotide repeat polymorphisms are associated with BMD (although with opposite effects in different studies) (152–158) and a lower risk of femoral fracture (159), but this has not been confirmed in meta-analyses of genome-wide association studies (GWAS) (25, 26, 160–162). Thus, a definite role for ERβ in humans remains hitherto unconfirmed.

C. ERα AF-1 and AF-2

Recently, the effects of ERα in bone and other tissues have been further analyzed in terms of whether they require ERα AF-1 or AF-2 (see *Section II.C*).

Both gonadectomized male and female mice lacking AF-2 showed no response to E2, demonstrating the importance of the ligand-dependent AF-2 for the effects of E2 on cortical and trabecular bone. Importantly, female mice lacking AF-1 of ERα still displayed normal response in cortical bone, but not in trabecular bone, and showed only

weak effects on the uterus (149). Similarly, ERαAF-2 was required for skeletal E2 responsiveness in male mice, whereas the role of ERαAF-1 was again tissue-specific and was seen for cortical but not trabecular bone (136). Thus, selective ERα agonists with minimal effects on ERαAF-1 could retain efficacy on cortical bone (which constitutes most of the skeleton) with minimal stimulation of reproductive tissues. Unfortunately, none of the existing selective ER modulators (SERMs) tested in a recent study were able to exert these actions in ERαAF-1 null males (136). Surprisingly, the ERα antagonist ICI 182,780 (fulvestrant, Faslodex) behaves as a partial agonist on trabecular bone and uterine weights (but not cortical bone or other estrogen-sensitive organs) in ovariectomized ERαAF-2 null mice (163).

The role of AF-1 and AF-2 in mediating ERα's modulation of the response to mechanical loading is discussed in *Section VIII.D*. Regarding growth plate closure, ERαAF-1 seems not to be required—in fact, ERαAF-1 null females show accelerated growth plate closure, suggesting that ERαAF-1 opposes normal ERα-mediated growth plate closure (164). In ovariectomized ERαAF-2 null mice, on the other hand, ICI 182,780 behaved as an inverse agonist, increasing growth plate height in the absence of estrogens (163).

Taken together, these data suggest that estrogen actions do not require full-length ERα, which opens perspectives for tissue-selective ERαAF-1 or -AF-2 modulators.

D. Role of the androgen receptor

1. Providing an edge to cortical and cancellous bone

Following the realization that estrogens were crucial for male peak bone mass as evidenced by the severe consequences of ERα mutation in a man (105), confirmed by studies in male ERαKO and aromatase knockout mice discussed above, doubts arose as to whether aromatization alone would be sufficient to explain greater peak bone mass development in men.

It is important to emphasize here that estrogen deficiency has severe consequences in both genders, whereas androgens may merely serve to enhance bone (and muscle) mass in men compared to women. In other words, the worst consequence of impaired AR actions will probably still only be a lack of sexual dimorphism and consequent feminization of bone development, ie, lack of male-type musculoskeletal enhancement. This prediction was confirmed in a study in pubertal male ARKO and wild-type female mice, which had identical cortical bone parameters (165). Trabecular bone development, on the other hand, was worse in ARKO males compared to females. Male aromatase knockout mice also show additional trabecular and endocortical bone resorption after orchidectomy (77). Further attesting to the pubertal skeletal actions of androgens independent of aromatization is a unique pQCT study in a man with concomitant aromatase deficiency and mild hypogonadism, in whom adding T to E2 replacement increased BMD, vBMD, cortical thickness, periosteal expansion, and normalized bone turnover (166). In the ERαKO man, all pQCT parameters were markedly lower compared to a control population except periosteal circumference, which was not significantly decreased (132).

Another question is whether androgens are directly responsible for the male skeletal phenotype or whether this is caused by other genetic determinants present on the Y chromosome. This can be examined, for example, in transsexual men and women, in whom gonadectomy and prolonged cross-gender hormone therapy reproduce the effects of karyotype on cortical bone size, trabecular vBMD, and muscle mass and strength (75, 167). Similarly, bone size has been reported intermediate between male and female as judged by pQCT in an XY woman with androgen insensitivity syndrome (AIS) (168). In contrast, BMD is much lower in both XX and XY women if gonadal dysgenesis is present, implying that gonadal factors (presumably sex steroids) are dominant over other chromosomal determinants (169).

2. Evidence from Tfm and ARKO rodent models

Following earlier studies in Tfm rats (reviewed in Ref. 27), it was shown in orchidectomized Tfm mice and also in orchidectomized growing ARKO mice that T could no longer prevent bone turnover and trabecular bone loss or stimulate periosteal bone formation, pointing to a direct role for AR in these processes (71, 82). A similar conclusion was reached in double AR-ERαKO and ERαβKO mice in which AR activation was sufficient for normal trabecular bone development and maintenance, whereas both AR and ERα activation was required for optimal cortical bone development (84). The observation that E2 is a more potent stimulus for trabecular bone than DHT (135) is in apparent contradiction with these findings, but the physiological relevance of pharmacological E2 doses in male rodents with undetectable circulating E2 levels remains questionable (see also *Section II.E*) (74, 135). Thus, AR actions independent of aromatization contribute to greater periosteal bone formation and enhanced trabecular bone development during puberty in male mice. This fits with the observed gender dimorphism in adults at the age of peak bone mass (see *Section III*).

The effects in knockout mice should be considered developmental; studies on the role of perinatal androgen secretion are discussed in the next paragraph, and studies on cortical and trabecular bone maintenance using selec-

tive ER or AR agonists in adult male ERαKO or ARKO mice are discussed in *Section IX*. Importantly, the effect of androgens on skeletal sexual dimorphism, in particular periosteal expansion, may partially depend on ERα (via aromatization) and stimulation of the GH/IGF-1 axis (see *Section VIII.A*).

Collectively, the murine and human data continue to support the dual mode of action of T on the male skeleton via both AR and ERα after aromatization (27). Nevertheless, key actions of AR (especially on periosteal bone formation) remain unexplained by cell-specific ARKO models (170–172) (see *Section V*), implying that AR may mediate some of its effects on bone via yet unidentified target cells (see *Section VIII*).

3. Importance of the perinatal T surge

Although skeletal sexual dimorphism is not so evident before puberty, sex steroids influence adult development even in the perinatal period. Especially in the first 2 weeks of life, there is a peak in serum T to low-adult male levels in boys due to high FSH and LH secretion (173). As a result, male mammals undergo neonatal imprinting, which determines male-pattern GH secretion, body weight, longitudinal bone growth, and hepatic steroid metabolism during puberty (174). An important question is whether the perinatal T surge and imprinting of the GH/IGF-1 axis also regulates radial bone expansion during puberty. Sims et al (175) addressed this in an elegant study comparing hypogonadal mice (*hpg*, which have a large spontaneous deletion in the gene for GnRH) with intact, orchidectomized, and T-treated mice. At 3 weeks of age (before the start of puberty), *hpg* mice had lower osteoblast surface and higher osteoid volume and osteoclast surface. At 9 weeks, high-turnover osteopenia was similarly present in both *hpg* and orchidectomized mice, and T or DHT treatment from 3 weeks inhibited trabecular bone resorption and stimulated periosteal bone formation equally in both groups. However, a short bone phenotype remained as in previous studies (174). Thus, the perinatal T surge and consequent imprinting of the GH/IGF-1 axis has important effects at the growth plate but appears redundant for cortical or trabecular bone (175).

E. Nongenomic signaling

As mentioned in *Section II.D*, sex steroid receptor signaling can deviate from its classical mechanism in two ways: it can be activated in the absence of ligand, or it can have rapid nongenomic effects via cytoplasmatic second messengers.

As mentioned above, ERα transfection in osteoblasts can activate transcription in the apparent absence of estrogens (66), although this is not evident for AR and androgens. Estrogen-independent ER activation in the skeleton was suggested to occur in immature, gonadectomized, or aromatase inhibitor-treated ERE-luciferase reporter mice (67), but intracrine signaling could be an alternative explanation. Several studies discussed in *Section VIII* show that ligand-independent actions of ERα mediate the osteogenic response to mechanical loading (176–178). Also, in the study of Almeida et al (179), the unliganded ERα was found responsible for stimulation of osteoblastogenesis and potentiation of Wnt/β-catenin signaling. However, the skeletal phenotype of gonadectomized mice does not differ significantly from ERαKO, ERαβKO, or ARKO animals, showing that in vivo, remaining ligand-independent ERα or AR actions generally do not compensate for sex steroid deficiency. In the following section, we will discuss studies on the latter, nongenomic mechanisms of sex steroid receptor actions in the skeleton.

1. Knock-in models with impaired DNA binding show that classical pathways have a dominant phenotypic role in bone

Mice expressing a mutant AR without the second zinc finger ($AR^{\Delta ZF2}$) have a similar overall phenotype as other ARKO models but, notwithstanding the limitations of this model (see *Section II.E*), allow the study of nongenomic AR effects. DHT treatment in male $AR^{\Delta ZF2}$KO mice results in AR-dependent ERK-1/2 phosphorylation and a small further decrease in cortical bone growth (101), whereas DHT has no effect in complete ARKO models (82).

Knock-in mice with an ERα incapable of DNA binding (nonclassical ER knock-in [NERKI]) present an opportunity to study the contribution of ERE-dependent classical signaling. Female NERKI mice had deficits in cortical but not trabecular bone (although the latter may be confounded by elevated T) (94), whereas male NERKI mice had decreases in both trabecular and cortical bone development (95, 96). NERKI mice have decreased osteoblast activation markers and Wnt signaling (consistent with a direct role for estrogens in osteoblastogenesis) (66, 180). They also lost trabecular bone as expected after ovariectomy (94, 181), confirming that classical ERE-dependent mechanisms are responsible for the protean manifestations of estrogen deficiency. Remarkably, ovariectomized NERKI mice gained cortical vBMD compared to wild-type controls (who lost cortical bone as expected), and estrogens suppressed this (94). This again suggests a generally repressive function of non-ERE-mediated signaling on classical ERα signaling, with a paradoxical partial compensation in the complete absence of classical ERE-dependent signaling. The inhibitory effects of estrogens on

osteoblastogenesis, oxidative stress (see *Section VIII.D*), and osteoblast apoptosis were unaffected in NERKI mice and thus appear mediated by nongenomic ERα actions via ERKs (181). Interestingly, the majority (72%) of estrogen-regulated genes were unaltered in NERKI mice, suggesting that classical pathways control only a minority of the transcriptional response to estrogens, but that these are the genes critical for the ultimate phenotype (182).

In summary, both AR and ERα can induce non-ARE/ERE dependent signaling, but in both cases, the skeletal effects are small and in opposite direction to the dominant, classical effects.

2. Nongenomic sex steroid receptor agonists

In a seminal series of papers, Kousteni et al (183–185) showed that ERα, ERβ, and AR through ligand-dependent, nongenomic signaling can induce bone-sparing and antiapoptotic effects in osteoblasts and osteocytes, regardless of whether the ligands were androgens or estrogens. These effects were accompanied by rapid and transient phosphorylation of common transcription factors (such as ERKs, c-Fos/c-Jun, CCAAT enhancer-binding protein β). Furthermore, a novel class of synthetic ligands called estrens could reproduce these nongenomic bone-sparing effects without stimulating reproductive organs. Substances acting via this new mechanism of action were termed ANGELS (activators of nongenotropic estrogen-like signaling). The same authors later showed that these ER-agonists, similarly to E2 treatment in NERKI mice, preferentially activate kinase signaling and stimulate osteoblasts via Wnt and bone morphogenetic protein (BMP) signaling (186). However, independent groups suggested that estrens do induce sex organ hypertrophy and activate classical signaling through both the AR and ERα (187–190).

E2 dendrimer conjugate (EDC) is a macromolecule in which E2 is attached to a large, positively charged, nondegradable poly(amido)amine dendrimer. This affords unhindered ligand access to ERs and initiation of nonnuclear signaling, without triggering classical nuclear transcription. Recently, the group of Manolagas (191) showed that EDC protects ovariectomized mice from cortical bone loss without affecting trabecular bone or the uterus. EDC achieved this by preventing ovariectomy-induced increased bone turnover and oxidative stress via the aforementioned non-nuclear signaling pathways. The authors suggested that the lack of effect on trabecular bone argues against a direct action of EDC on osteoclast apoptosis in vivo, contrary to their earlier in vitro findings (192). In summary, these promising results with EDC and the cortical bone phenotype of NERKI mice provide further impetus to exploit the benefits of sex steroid signaling as a gender-neutral, tissue-specific, pharmacotherapeutic strategy.

3. GPR30: a membrane ER present in bone cells

As mentioned above, it is still debated whether or not GPR30 is an ER (see *Section II.D*). GPR30 is expressed in osteoclasts, osteoblasts, and osteocytes but appears to be redundant for the effects of estrogens on most tissues, including fat, reproductive organs, and cortical and trabecular bone in female mice (193, 194), although it may have an effect in the growth plate. Female *Gpr30*-KO mice are shorter and have less body weight and decreased IGF-1 serum levels (195). Ovariectomized *Gpr30*-KO mice have diminished estrogen-mediated growth plate closure (194). In another *Gpr30*-KO mouse model, males also showed delayed growth plate closure and greater body length, but in contrast to previous studies, lean, fat, and bone mass were increased although serum IGF-1 was normal (196). Interestingly, GPR30 expression in the growth plate in humans seems to decline as puberty progresses in both genders (197). Thus, GRP30 may be important to mediate estrogenic inhibition of growth plate chondrocytes, but further studies are needed to delineate the contribution of GPR30 to sex steroid regulation of male bone metabolism.

F. Independent effects of gonadotropins

Earlier observations that accelerated bone turnover in women precedes menopause by several years, when serum E2 is still normal, led to the hypothesis that other endocrine alterations during the early menopausal transition may be involved, such as FSH, progesterone, androgens, or inhibins (198). This topic received substantial attention when the group of Zaidi (199, 200) observed that neither FSHβ nor FSH-receptor null female mice exhibit bone loss despite severe hypogonadism, whereas osteoclastic bone resorption is increased in haploinsufficient FSH$\beta^{+/-}$ mice via osteoclastic and immune cell FSH receptors that enhance TNF-α production and osteoclast formation and function. More recently, they and others showed that an exogenous FSHβ-subunit neutralizing antibody or an endogenous FSHβ immunization strategy prevents ovariectomy-induced bone loss (201, 202). These findings have, however, been challenged by others. Previous reports had suggested that the effects of LH, FSH, and chorionic gonadotropin on bone in mice were indirect via their stimulatory actions on sex steroid synthesis (203). This led to concerns that increased serum LH and T concentrations in the aforementioned models may explain the observed bone preservation (204), which was confirmed by another group (205). Another way of examining this issue is to examine *hpg* mice, which have a bone deficit entirely correctable by sex steroid replacement (175). Another study

in ovariectomized rats showed that GnRH analog (decapeptyl) treatment did not rescue bone loss, except for a partial recovery of trabecular bone volume (206). Furthermore, female mice overexpressing human FSH on the *hpg* background showed a striking accrual in bone mass via a nonestrogen, ovary-dependent pathway, which probably includes T, inhibin A, and potentially other factors (207). Importantly, the presence of FSH receptors in bone or cultured osteoblasts or osteoclasts could not be confirmed (207). A direct interventional study using aromatase inhibition and the GnRH agonist leuprolide in postmenopausal women found that FSH suppression did not rescue the effects of estrogen deficiency (198).

In summary, the suggested independent effects of gonadotropins remain highly controversial. In any case, it should be noted that all previous reports have been limited to females. In the eagerly awaited bone substudy of the recent investigation by Finkelstein et al (208), an independent role of FSH suppression in men could not be confirmed (209). Thus, for male bone metabolism, any role of gonadotropins independent of sex steroids remains to be proven.

V. Target Cells of Sex Steroids in Bone

Although ERα and AR have been established to be important mediators of the estrogenic and androgenic effects in bone, the target cells for these effects are not completely known. The same goes for the target cell/s for the ERα-modulating effect of ERβ observed in female mice. ERα, ERβ, and AR expression have been described in all resident bone cells including osteoblasts, osteocytes, osteoclasts, and bone marrow stromal cells (although AR expression in osteoclasts remains controversial; see *Section V.A.2*) (27). During recent years, the Cre-LoxP system has allowed conditional deletion of ERα, ERβ, and AR in specific bone cells, which has made it possible to clarify the roles of different cells for the effects of sex steroids in bone. This approach is also favorable because the negative feedback regulation of serum sex steroid levels in these bone cell-specific mouse models is not disrupted, leading to normal sex steroid levels. These cell-specific ERαKO, ERβKO, and ARKO mouse models will be discussed in this Section. Although these mouse models have considerably advanced our understanding of direct sex steroid actions in bone, the Cre-LoxP technique comes with its own limitations (see *Section II.E*). The promoter driving the recombinase determines at what stage of cell differentiation Cre expression becomes activated (Figure 5). It is important to realize that the phenotype of the mouse models where ER or AR is deleted in early osteoprogenitor cells may also rely on downstream effects in osteoblasts or even osteocytes. However, not all phenotypes of osteoblast- and osteocyte-specific knockout models are reproduced by knockout in earlier stages. Thus, it remains unclear to what extent alternative cell maturation pathways exist, whether opposing effects at different maturation stages cancel each other out, or whether the recombined cells have an altered survival rate compared to the cells where no recombination has occurred.

A. Osteoclasts

1. Estrogen receptor-α

One of the pathways through which estrogens have been shown to reduce bone resorption is by promoting apoptosis and inhibiting differentiation of osteoclasts (192, 210, 211). ERα has been deleted in osteoclasts using the cathepsin K (*Ctsk*) promoter, which deletes ERα in mature osteoclasts (ER$\alpha^{Oc;Ctsk}$) (212), and the lysozyme M (*LysM*) promoter, which deletes ERα in early osteoclast precursors (ER$\alpha^{Oc;LysM}_{Martin\text{-}Millan}$ and ER$\alpha^{Oc;LysM}_{Seitz}$) (192, 213) (Figure 5).

The female ER$\alpha^{Oc;Ctsk}$ and ER$\alpha^{Oc;LysM}_{Martin\text{-}Millan}$ mice had decreased trabecular bone mass, whereas the cortical bone mass was normal (Table 2) (192, 212, 213). Male mice lacking ERα in osteoclasts have both normal cortical and trabecular bone (192, 212).

The osteoclast number was increased, whereas there was no change in osteoblast number in the female ER$\alpha^{Oc;Ctsk}$ and ER$\alpha^{Oc;LysM}_{Martin\text{-}Millan}$ mice (192, 212). The female ER$\alpha^{Oc;Ctsk}$ had increased bone formation and resorption rate, indicating a high bone turnover (212) (Table 2). The molecular mechanism explaining the increased osteoclast number in ER$\alpha^{Oc;Ctsk}$ females was suggested to be disrupted osteoclast apoptosis via Fas ligand (FasL) (212). However, this could not be shown for the mice lacking ERα in the monocyte/macrophage lineage (192). Furthermore, it should not be assumed that FasL is transcriptionally regulated by ERα in osteoclasts, because Krum et al (214) found evidence for a paracrine mechanism by which ERα regulates FasL in osteoblasts, not osteoclasts (see *Section VII*).

ERα in osteoclasts is hence shown to be of importance for trabecular but not cortical bone in female mice, whereas it has no effect in male mice.

2. Androgen receptor

Thus far, AR expression has not been demonstrated in human osteoclasts, although it has been reported in avian and rodent osteoclasts (27). Studies on androgen modulation of osteoclast generation or activity remain conflicting (27, 215, 216). Because there is no published report of a mouse model lacking the AR in osteoclasts, this is a high research priority.

Figure 5.

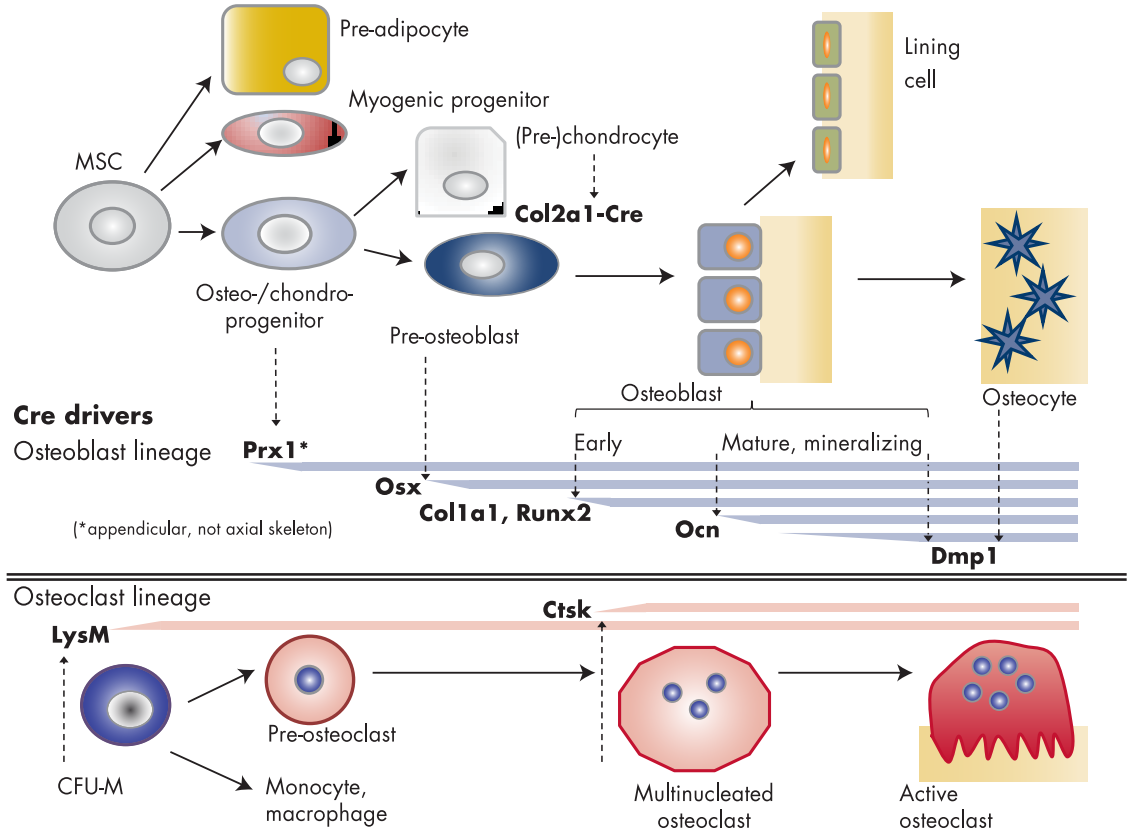

Figure 5. Overview of Cre promoter mouse strains (in bold) that have been crossed with floxed ERα, ERβ, or AR transgenic mice to generate conditional knockout models discussed in *Section V*. Osteoblast and osteoclast differentiation are schematically represented above and below, respectively, and the maturation stage at which the respective promoters become active is indicated by the blue and red horizontal bars, respectively. Note that Cre-mediated target gene excision occurring at a specified maturation stage also affects all downstream differentiation stages. *Col2a1*, collagen, type II, α1; *Prx1*, paired related homeobox 1; *Osx*, osterix (transcription factor Sp7); *Col1a1*, collagen, type I, α1; MSC, mesenchymal stem cell; CFU-M, colony-forming unit-monocyte.

B. Osteoblast lineage

1. Osteoblasts—ERα

To clarify the importance of ERα in osteoblast lineage cells, six different promoters have been used to delete ERα at different stages in osteoblast/osteocyte differentiation. These promoters have deleted ERα in osteoblast progenitors and all downstream cells (*Prx1* and osterix promoter), from the mature osteoblast stage onward (*Col1a1* [2.3-kb fragment], *Runx2*, and osteocalcin promoter) or only in late osteoblasts/osteocytes (dentin matrix protein 1 [*Dmp1*] promoter) (217) (Figure 5).

In males, cortical bone was transiently reduced when ERα was deleted in osteoblast precursors and downstream cells ($ERα^{Ob;Prx1}$ mice) (179, 218), whereas no cortical phenotype was evident when ERα was deleted in mature osteoblasts ($ERα^{Ob;Osteocalcin}_{Määttä}$ and $ERα^{Ob;Col1a1}$) (Table 2) (179, 219). These studies demonstrate that ERα in osteoblast precursors regulates cortical bone in young male mice.

Extensive studies by Almeida et al (179) revealed that, in male mice, trabecular bone was not affected when ERα was deleted in osteoblast precursors ($ERα^{Ob;Prx1}$ and $ERα^{Ob;Osx1}$) or using *Col1a1*-Cre. In contrast, a study by Määttä et al (219) demonstrated that trabecular bone volume was reduced in male mice when ERα was deleted in mature osteoblasts ($ERα^{Ob;Osteocalcin}_{Määttä}$). Although the latter study stated that the osteocalcin-Cre mouse strain used did not have a bone phenotype in itself, this was, as emphasized in a recent review by Manolagas et al (218), not excluded quantitatively. However, a role of ERα in late osteoblasts/osteocytes for trabecular bone in males is supported by the finding that male $ERα^{Ocy;Dmp1}_{Windahl}$ mice have reduced trabecular bone (see *Section V.B.2*) (220). Based on these studies, we conclude that there is moderate evidence for a role of ERα in mature osteoblasts/osteocytes in the regulation of trabecular bone in male mice.

Cortical bone in female mice is decreased when deleting ERα from the early osteoblast progenitor stage

Table 2. Skeletal Phenotypes of Osteoclast-, Osteoblast- or Osteocyte-Specific Conditional Sex Steroid Receptor Ablation Models

			Male			Female		
Cell Type	**Mouse Strain**	**Ref.**	**Trabecular**	**Cortical**	**F/R**	**Trabecular**	**Cortical**	**F/R**
ER-α								
Osteoclast	$ER\alpha^{Oc;Ctsk}$	212	→	→	ND	↓	→	Tr: F + R ↑ Ocl ↑, Obl →
	$ER\alpha^{Oc;LysM}_{Martin\text{-}Millan}$	192	ND	ND	ND	↓	→	Tr: Ocl ↑, Obl →
	$ER\alpha^{Oc;LysM}_{Seitz}$	213	ND	ND	ND	→	→	ND
Osteoblast	$ER\alpha^{Ob;Prx1}$	179	→	(↓)	ND	→	↓	Co: Endosteal R ↑, periosteal F ↓
	$ER\alpha^{Ob;Osx1}$	179, 218	→	→	ND	→	↓	ND
	$ER\alpha^{Ob;Col1a1}$	179	→	→	ND	→	→	ND
	$ER\alpha^{Ob;Runx2}$	213	ND	ND	ND	↓	ND	ND
	$ER\alpha^{Ob;Osteocalcin}_{Määttä}$	219	↓	→	ND	↓	↓	Tr: Obl ↓, Ocl ↓
	$ER\alpha^{Ob;Osteocalcin}_{Melville}$	221	ND	ND	ND	↓	↓	Tr: Obl ↓, Ocl →
Osteocyte	$ER\alpha^{Ocy;Dmp1}_{Windahl}$	220	↓	→	Tr: F↓, R→	(↓)	→	ND
	$ER\alpha^{Ocy;Dmp1}_{Kondoh}$	222	ND*	ND*	ND	↓	→	Tr: Obl ↓, Ocl →
ER-β								
Osteoblast	$ER\beta^{Ob;Prx1}$	151	ND	ND	ND	↑	→	Tr: Obl, Ocl →
AR								
Osteoblast	$AR^{Ob;Prx1}$	223	↓	→	ND	→	→	ND
	$AR^{Ob;Osteocalcin}_{Määttä}$	130	↓	→	Tr: F→, R ↑	↓	→	Tr: Obl →, Ocl →
	$AR^{Ob;Osteocalcin}_{Chiang}$	170	↓	(↓)	Tr: F→, R ↑	ND	ND	ND
	$AR^{Ob;Col1a1}$	171	↓	→	Tr: R ↑	ND	ND	ND
Osteocyte	$AR^{Ocy;Dmp1}$	172	↓	→	→	ND	ND	ND

Abbreviations: Tr, trabecular; Co, cortical; F, bone formation; R, bone resorption; Obl, osteoblast; Ocl, osteoclast; ↑, increased; ↓, decreased; →, unchanged; ND, not determined; ND*, only BMD determined by DXA; (↓), reduced E2 response in ovariectomized mice but no phenotype in gonadal intact mice; ((↓)), transient reduction.

($ER\alpha^{Ob;Prx1}$ and $ER\alpha^{Ob;Osx1}$) (179) as well as from the mature osteoblast stage when using the osteocalcin promoter ($ER\alpha^{Ob;Osteocalcin}_{Määttä}$, $ER\alpha^{Ob;Osteocalcin}_{Melville}$) (219, 221) but not with the *Col1a1* promoter ($ER\alpha^{Ob;Col1a1}$) (179) (Table 2). Collectively, these studies clearly demonstrate that ERα in the osteoblast lineage is crucial for cortical bone in female mice. Dynamic histomorphometry in female and male $ER\alpha^{Ob;Prx1}$ and female $ER\alpha^{Ob;Osx1}$ mice revealed that there was a reduction in cortical thickness as a result of a decreased mineral apposition rate at the periosteal but not the endosteal bone surface (179). The importance of ERα in osteoblast progenitors at the periosteal surface was concluded to be ligand-independent, and the effects of ERα were suggested to be induced by mechanical strain (179).

Studies by Almeida et al (179) revealed that trabecular bone was not affected in female mice when ERα was deleted in osteoblast precursors and downstream cells ($ER\alpha^{Ob;Prx1}$ and $ER\alpha^{Ob;Osx1}$) or in mature osteoblasts using *Col1a1*-Cre. In contrast, in three independent models ($ER\alpha^{Ob;Osteocalcin}_{Määttä}$, $ER\alpha^{Ob;Osteocalcin}_{Melville}$, and $ER\alpha^{Ob;Runx2}$) trabecular bone was reduced in female mice when ERα was deleted from the mature osteoblast stage (213, 219, 221). Although it was not excluded quantitatively in any of these three studies that the osteocalcin-Cre or *Runx2*-Cre mouse models used have a bone phenotype themselves, collectively we propose that there is at least moderate evidence that ERα in mature osteoblasts contributes to the regulation of trabecular bone in female mice.

As described above, the Cre-LoxP technique has some limitations, and the discordant findings described above regarding the role of ERα in osteoblasts (especially for trabecular bone) could therefore possibly be due to residual ERα expression in the osteoblasts of some mouse models. Alternatively, the cells that are recombined may have a survival disadvantage compared to the fraction of cells that are not recombined. This could lead to a decreased amount of downstream cells with deletion of ERα. Another explanation is that the bone phenotype seen is due to unspecific deletion of ERα also in other cell types. Finally, it is possible that the Cre mouse strains used have a phenotype in themselves, confounding the results. In any case, genetic manipulation of mice, powerful as it is, requires controls that are not always obvious; without such controls, one can come to erroneous conclusions (218).

2. Osteocytes—ERα

Osteocytes are differentiated osteoblasts that are found within mature bone and represent a possible target cell for estrogens. There are two publications (220, 222) where ERα was deleted in osteocytes and late osteoblasts by using the *Dmp1* promoter ($ER\alpha^{Ocy;Dmp1}_{Windahl}$ and $ER\alpha^{Ocy;Dmp1}_{Kondoh}$ mice). The young adult male $ER\alpha^{Ocy;Dmp1}_{Windahl}$ mice had a reduced trabecular bone volume but a normal cortical

bone, which is in line with the results from the male $ER\alpha^{Ob;Osteocalcin}_{Määttä}$ mice (219). The reduced trabecular bone volume in the male $ER\alpha^{Ocy;Dmp1}_{Windahl}$ mice was due to reduced bone formation (220). The bone phenotype in the male $ER\alpha^{Ocy;Dmp1}_{Kondoh}$ mice was, however, only analyzed with DXA, which showed normal BMD (222).

The female $ER\alpha^{Ocy;Dmp1}_{Kondoh}$ mice had reduced trabecular bone volume but normal cortical bone (222), whereas the female $ER\alpha^{Ocy;Dmp1}_{Windahl}$ mice had both normal trabecular and cortical bone (220). However, when female $ER\alpha^{Ocy;Dmp1}_{Windahl}$ mice were treated with slightly pharmacological doses of E2, they had a substantially attenuated estrogenic response in trabecular bone, suggesting that the deletion of ERα in the osteocytes in these female mice also affects trabecular bone (220). These results are in agreement with the decreased trabecular bone volume observed in the female mouse models lacking ERα in mature osteoblasts (179, 213, 219, 221) (Table 2).

In summary, ERα in osteocytes is not required for cortical bone but seems to regulate trabecular bone in both genders.

3. Osteoblasts—ERβ

Thus far, *Prx1* and *Col1a1* (2.3-kb fragment) have been used to delete ERβ in specific bone cells ($ER\beta^{Ob;Prx1}$ and $ER\beta^{Ob;Col1a1}$) in only one study, and only the phenotype of female mice has been reported (151). The $ER\beta^{Ob;Prx1}$ mice showed an increase in trabecular bone volume, whereas no difference was observed in the cortical bone. This increase in trabecular bone was accompanied by an increase in osteoblast numbers without any difference in osteoclast numbers, suggesting an increased bone formation rate (Table 2). The female $ER\beta^{Ob;Col1a1}$ had increased BMD in the spine and femur (151). The increase in trabecular bone confirms the inhibitory actions of ERβ in bone of female mice (150) (see *Section IV.B*).

4. Osteoblasts—AR

The AR has been deleted both in osteoblast progenitors (223) and in mature osteoblasts (130, 170, 171). AR deletion in osteoblast progenitors using the *Prx1* promoter ($AR^{Ob;Prx1}$) in male mice reduces trabecular bone volume and number but not cortical thickness (223). Deletion of the AR in mature osteoblasts using the *Col1a1* promoter 2.3-kb fragment ($AR^{Ob;Col1a1}$) (171) or the osteocalcin promoter ($AR^{Ob;Osteocalcin}_{Chiang}$ and $AR^{Ob;Osteocalcin}_{Määttä}$) (130, 170) also reduces trabecular bone volume and number in male mice, whereas cortical bone was again unaffected in the $AR^{Ob;Col1a1}$ and the $AR^{Ob;Osteocalcin}_{Määttä}$ mice (130, 171) (Table 2). However, a slight and transient reduction in cortical bone was seen in the male $AR^{Ob;Osteocalcin}_{Chiang}$ mice when bone turnover peaked at the age of 6 weeks, but no difference was observed at later ages (170). The difference between $AR^{Ob;Osteocalcin}$ mice could be explained by the fact that Chiang et al (170) deleted exon 3, whereas Määttä et al (130) deleted exon 2 of the AR gene. Although exon 2 deletion leads to a total ablation of AR activity (99, 130), the deletion of exon 3 results in an AR transcript that has preserved DNA-binding capacity and could possibly still mediate AR signaling (see *Section II.E*). The male $AR^{Ob;Osteocalcin}_{Määttä}$ mice had an increase in osteoclast but not osteoblast number in trabecular bone, suggesting an increased resorption (130) (Table 2).

In female $AR^{Ob;Prx1}$ mice, the bone phenotype was normal (223), whereas the female $AR^{Ob;Osteocalcin}_{Määttä}$ mice had a decreased trabecular bone volume due to a reduced trabecular number (130). Cortical bone was normal in female $AR^{Ob;Prx1}$ and $AR^{Ob;Osteocalcin}_{Määttä}$ mice (130, 223).

In conclusion, AR in mature osteoblasts appears to have an important role in the maintenance of trabecular bone in adult male mice, whereas AR in osteoblasts seems to have no significant effect on cortical bone. The impact on trabecular bone in male ARKO mice is probably mainly mediated via AR in osteoblasts. In contrast, the AR target cell responsible for the reduced cortical bone dimensions in male ARKO mice remains currently unknown (Figure 6).

5. Osteocytes—AR

AR expression increases toward osteocyte differentiation, and thus osteocytes are likely target cells for the skeletal effects of androgens (172). AR deletion in late osteoblasts and osteocytes using the *Dmp1* promoter ($AR^{Ocy;Dmp1}$) decreases trabecular bone volume in the appendicular skeleton in 32-week-old male $AR^{Ocy;Dmp1}$ mice, due to a reduced trabecular number. Trabecular number was also reduced in the vertebra of male $AR^{Ocy;Dmp1}$ mice, although trabecular bone volume was normal. Again, cortical bone was unaffected (172). No differences in bone formation, resorption, or the response to mechanical loading could be identified. The decreased trabecular bone volume and normal cortical bone observed in these male mice are in accordance with the results from the mice lacking AR in osteoprogenitor cells and osteoblasts (130, 170, 171, 223). In conclusion, the AR in osteocytes is important for trabecular bone in older mice but not for cortical bone development.

6. Osteoblast overexpression of AR

The bone phenotype resulting from overexpression of the AR in osteoblasts has been evaluated by using two different fragments of the *Col1a1* promoter: 3.6-kb and 2.3-kb fragments ($AR\text{-transgene}^{Ob;3.6\text{-}Col1a1}$ and $AR\text{-transgene}^{Ob;2.3\text{-}Col1a1}$). The 3.6-kb promoter is expressed throughout the osteoblast lineage (and also in stromal precursors and periosteal fibroblasts), whereas the 2.3-kb promoter is expressed in

Figure 6.

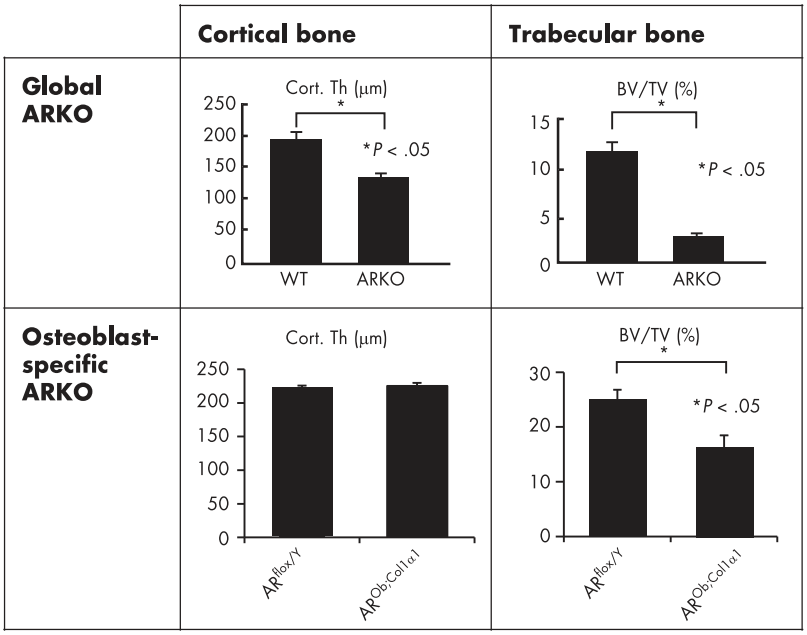

Figure 6. Cortical thickness (Cort.Th) and trabecular bone volume fraction (BV/TV) in global ARKO mice (81) compared to osteoblast-specific ARKO ($AR^{Ob;Col1a1}$) mice (171). Cortical bone deficits in global ARKO mice are not reproduced in osteoblast-specific conditional models, although clearly and selectively related to androgens (see *Section IV.D*). Thus, the target cell for androgen actions on cortical bone remains to be identified. Trabecular bone is decreased in both global and conditional ARKO models, although less pronounced in the conditional models, probably due to inherent incomplete deletion. [Adapted and reproduced from H. Kawano et al: Suppressive function of androgen receptor in bone resorption. *Proc Natl Acad Sci USA*. 2003;100:9416–9421 (81), with permission. © National Academy of Sciences. And from A. J. Notini et al: Osteoblast deletion of exon 3 of the androgen receptor gene results in trabecular bone loss in adult male mice. *J Bone Miner Res*. 2007;22:347–356 (171), with permission. © American Society for Bone and Mineral Research.]

mature osteoblasts (224, 225). The male AR-transgene$^{Ob;Col1a1}$ mice in both models had an increased trabecular bone volume due to an increased trabecular number (224, 225). Bone formation rate and mineral apposition rate in the trabecular bone were reduced, indirectly indicating that the increased trabecular bone volume results from decreased bone resorption (224). In the calvaria, AR overexpression resulted in cortical thickening that was most pronounced at the periosteal surface (224). Transgenic males demonstrated a dramatic lack of labeling at the endosteal surface and an increase on the anterior periosteal surface of the femur (224). Because the effect of decreased endosteal bone formation was dominant, cortical bone area and overall bone strength were reduced in both of the AR-transgene$^{Ob;Col1a1}$ mice (224, 225). The female AR-transgene$^{Ob;2.3\text{-}Col1a1}$ mice did not show any bone phenotype, whereas the female AR-transgene$^{Ob;3.6\text{-}Col1a1}$ only had a very mild phenotype (224, 225).

It is evident from these overexpression studies that the AR in osteoblasts has a direct role in modulating trabecular bone resorption and cortical bone formation in male mice. The decreased trabecular bone resorption is consistent with global and conditional ARKO models. The tendency to increase periosteal bone formation and limit endosteal contraction also confirms a direct role for AR in these processes, although this is not mirrored in conditional ARKO models. The rather complex bone phenotype in the AR-transgene$^{Ob;Col1a1}$ mice suggests that excessive AR signaling in osteoblasts does not have a positive effect on cortical bone development. Taken together with the results from the global and osteoblast-specific ARKO mice, it is reasonable to believe that neither too high nor too low AR activity is favorable for bone development.

C. Chondrocytes

1. Estrogen receptor-α

Estrogens have long been known to affect both the longitudinal growth spurt in early puberty and the epiphyseal closure, and thereby cessation of growth, in late puberty. These seemingly opposite effects of estrogens on longitudinal growth depend on the maturational stage of the skeleton and the E2 serum levels; low levels of E2 induce the pubertal growth spurt, whereas higher E2 levels later in puberty induce growth plate closure (226). ERα is chiefly responsible for the effects of estrogens on growth, as discussed in *Section IV.B*.

Growth plate physiology is somewhat different between mice and humans because fusion occurs shortly after puberty in humans but not in mice. Still, it is possible to fuse the growth plates in mice by long-term E2 treatment (227), and their growth plate height decreases with aging. It was previously believed that there was a discrepancy between the man lacking ERα (105, 132) and the mouse model lacking ERα (228) because the human had unfused growth plates and continued to grow, whereas the mouse had fused growth plates. This discrepancy was shown to be due to the first ERα-deleted mouse still expressing low levels of truncated forms of ERα, lacking the ERαAF-1 (see *Sections II.C* and *II.E*). The E2 levels in this mouse model were elevated, and the truncated ERα was able to induce growth plate closure (164, 228). A study of mice completely devoid of ERα isoforms showed that

these mice indeed resembled the ERαKO man in that they had wider growth plates, prolonged growth, and greater ultimate length (164).

Estrogen is a potent regulator of the GH/IGF-1 axis (see *Section VII.A*), and the effects of estrogens on longitudinal growth and epiphyseal fusion are generally believed to be mediated either indirectly via central regulation of the GH/IGF-1 axis or directly via ERα expressed in the growth plate. To investigate what mechanisms control longitudinal growth, a mouse model lacking ERα specifically in chondrocytes ($ER\alpha^{Chond;Col2a1}$) has been developed (229). Both female and male $ER\alpha^{Chond;Col2a1}$ mice showed a normal growth pattern during sexual maturation, when estrogen activity is low, suggesting that local ERα in the growth plate is not crucial for pubertal growth (229). To investigate whether local ERα is of importance for growth plate closure during late sexual maturation, when estrogen activity is high, the $ER\alpha^{Chond;Col2a1}$ mice were gonadectomized and treated with a high E2 dose. In control mice, this treatment reduced growth plate height, but this effect was not observed in either female or male $ER\alpha^{Chond;Col2a1}$ mice (229). This shows that local ERα in the growth plate is required for the ability of estrogens to reduce growth plate height in both female and male mice (Figure 7). Osteocalcin is also expressed in hypertrophic chondrocytes, which leads to a deletion of ERα in these cells in the $ER\alpha^{Ob;Osteocalcin}_{Määttä}$ mice. Analyses of the growth plates in the female $ER\alpha^{Ob;Osteocalcin}_{Määttä}$ mice showed that the growth plate height was increased compared to controls (219), which is in agreement with the lack of E2 effect in the $ER\alpha^{Chond;Col2a1}$ mice. Moreover, female $ER\alpha^{Chond;Col2a1}$ mice continued to grow after 4 months of age, whereas essentially no growth was observed in the control mice, resulting in a significant difference in bone lengths at 12 months of age (229).

Male complete ERαKO mice have reduced bone lengths at 17 weeks of age, which was not seen in the $ER\alpha^{Chond;Col2a1}$ mice (229). This reduction in length was associated with a disturbed GH secretory pattern and low serum IGF-1 levels. Therefore, indirect effects of ERα, probably via the GH/IGF-1 axis, are crucial for a normal growth during sexual maturation. In contrast, local ERα in the growth plate cartilage is crucial for high-dose E2-mediated growth plate closure in adult mice and for reducing longitudinal bone growth in elderly female mice (Figure 7).

Figure 7.

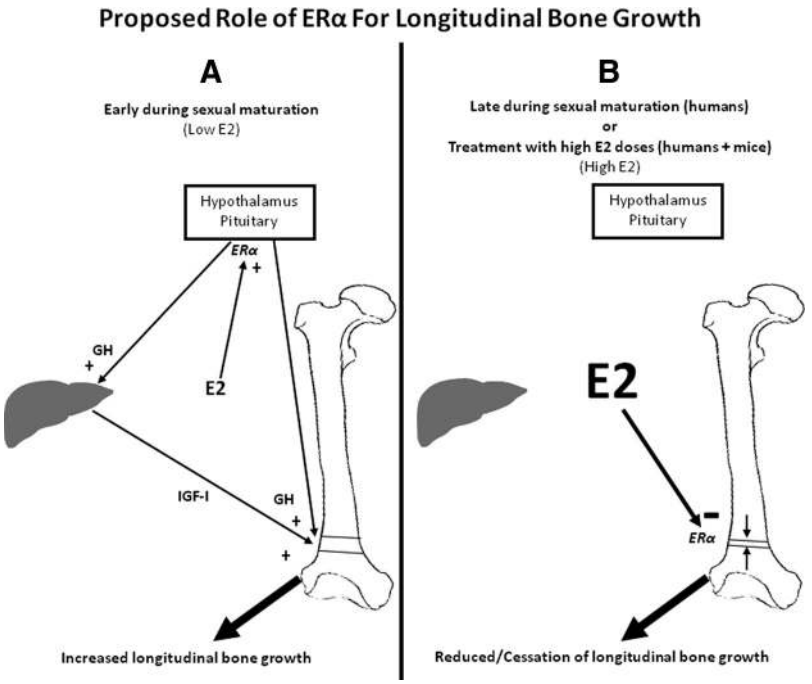

Figure 7. A, Longitudinal growth during early sexual maturation is mediated by nonbone/noncartilage ERα, probably by indirect effects of ERα via the GH/IGF-1 axis. B, In contrast, local ERα in the growth plate cartilage is crucial for high dose E2-mediated growth plate closure in adult mice and for reducing longitudinal bone growth in elderly female mice. [Reproduced from A. E. Börjesson et al: The role of estrogen receptor α in growth plate cartilage for longitudinal bone growth. *J Bone Miner Res*. 2010;25:2690–2700 (229), with permission. © American Society for Bone and Mineral Research.]

2. Androgen receptor

The AR is expressed in growth plate chondrocytes, but there is no clear evidence that it should be of importance for growth and growth plate closure. So far, there are no reports of a mouse depleted of AR in chondrocytes. Most studies of ARKO mice show normal bone lengths, although one study reported male ARKO mice as having a slightly increased femur length (84), and osteoblast-specific AR-overexpressing mice showed reduced femur length (224). Therefore, although most studies in humans and mice indicate that the effect of T on the growth plate is due to its aromatization and stimulation of ERα, a chondrocyte-specific ARKO model could still be of interest.

D. Conclusions from conditional mouse models

1. Target cells of sex steroid actions in males

The studies from total ERαKO, ARKO, and aromatase knockout

mice, together with the different conditional knockout and overexpression models, show that both ERα and AR are required for normal trabecular and cortical bone development (Figure 8A). However, the trabecular and cortical bone compartments are not always regulated by the same bone cells.

ERα in early osteoblasts has been found to mediate a transient positive effect on cortical bone during growth, although there is no evidence that this effect requires estrogens (179). ERα in mature osteoblasts, osteocytes, and osteoclasts appears not to be important for cortical bone in male mice (Figure 8A). Importantly, although osteoblast-lineage cells may be involved (170, 224), the AR-expressing cell type mediating the regulation of male cortical bone remains largely unknown. It is possible that the AR in osteoclasts or in periosteal cell populations is important, but it is also possible that at least some of the AR bone effects are mediated via target cells outside bone.

For trabecular bone, we suggest that the effect in males comes from T acting via the AR in osteoblast precursors, mature osteoblasts, and (at older ages) osteocytes, which decreases trabecular bone resorption by indirectly affecting osteoclasts (Figure 8A). Estrogens may also have an effect on trabecular bone in male mice by acting via ERα in mature osteoblasts/osteocytes, by increasing bone formation. ERα exerts no actions in osteoclasts in male mice; AR actions in osteoclasts require further investigation.

The regulation of longitudinal growth in male mice is clearly regulated by ERα, where the growth plate closure is regulated by ERα in chondrocytes, whereas the longitudinal growth during early sexual maturation is mediated by nonbone/noncartilage ERα, probably involving the GH/IGF-1 axis via a central mechanism (Figure 7).

2. Target cells of sex steroid actions in females

ERα is the most important sex steroid receptor in female bone (Figure 8B and Table 2). We propose that estrogen regulation of trabecular bone is mediated via the ERα in osteoclasts, which directly leads to a reduction of bone resorption. ERα in mature osteoblasts/osteocytes increases trabecular bone formation, which can be inhibited by ERβ.

For cortical bone, ERα actions are evident in early and mature osteoblasts, whereas ERα in osteoclasts and osteocytes is not required. The estrogenic effect mediated by ERα in the early osteoblasts affects endocortical osteoclasts, thereby decreasing endocortical resorption. Ligand-independent activation of the ERα in early osteoblasts increases periosteal bone formation and is also required for the periosteal response to mechanical loading (see also *Section VII.E*).

ERα in chondrocytes in female mice is clearly required for growth plate closure and cessation of longitudinal growth, whereas the longitudinal growth during early sex-

Figure 8.

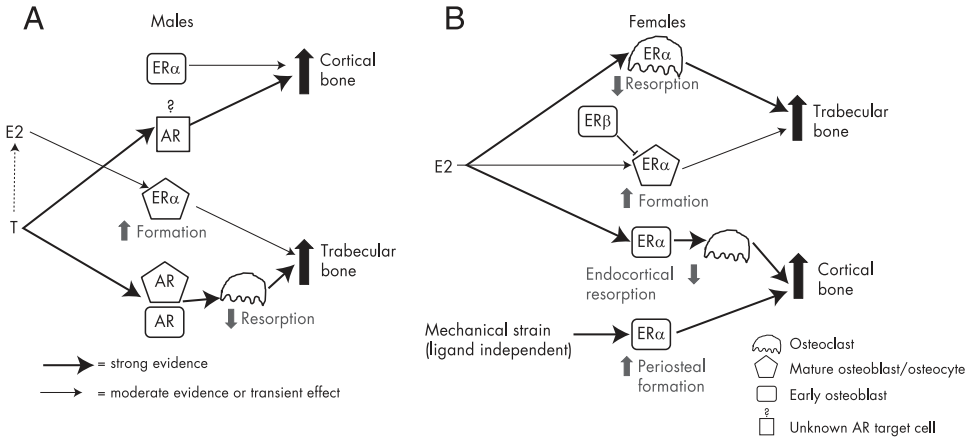

Figure 8. Proposed pathways for ER and AR actions on bone cells. A, In males, ERα in early osteoblasts has a transient effect on cortical bone mass. Some but not all studies indicate that estrogens, via the ERα in mature osteoblasts/osteocytes, lead to an increased bone formation rate that in turn leads to an increase in trabecular bone mass. Acting via the AR in osteoblast lineage cells, T affects the osteoclasts to decrease their resorption rate, which maintains trabecular bone mass. It is known that androgens via the AR stimulate cortical bone expansion, but the target cell for this is still unclear. B, In females, E2 acts via the ERα in osteoclasts to decrease bone resorption, which maintains trabecular bone mass. In addition, some but not all studies indicate that trabecular bone mass is increased by E2 acting via ERα in mature osteoblasts/osteocytes, which induces an increased bone formation rate. ERβ in osteoblasts can inhibit this positive effect of ERα in osteoblasts. E2 acting via the ERα in early osteoblasts can affect endocortical osteoclasts to decrease their resorption rate, which leads to an increased cortical bone mass. Cortical bone mass can also be increased via mechanical strain activating ERα in early osteoblasts, in a ligand-independent manner, to increase periosteal bone formation. AR actions have also been reported to contribute to female bone mass, but the physiological significance of these findings is difficult to establish.

ual maturation seems to be mediated by ERα in cells of nonbone/noncartilage origin, probably involving the GH/IGF-1 axis via a central mechanism (Figure 7).

VI. Direct Skeletal Target Genes of the Androgen and Estrogen Receptor

From the previous section, it is clear that ERα exerts direct skeletal effects in osteoblasts, osteocytes, osteoclasts, and chondrocytes, whereas direct AR actions have been demonstrated in osteoblasts and osteocytes. The logical next question regards the downstream mechanism by which these receptors act. As discussed in *Section IV.E*, important aspects of their effects likely rely on non-ARE/ERE-dependent mechanisms at the cell membrane, in the cytoplasm, or in the nucleus (like ERα and AR modulation of RUNX2 activity) (see *Section VII.C*). This section will, however, focus on the traditional direct transcriptional targets of AR and ER in bone cells. Consistent with their role in regulating bone turnover, androgen or estrogen deficiency has been associated with up-regulation of osteoblast and osteoclast markers. For example, a recent microarray study using whole bones isolated from osteocalcin-driven ARKO mice confirmed androgen-responsive expression of *Col1a1, Bglap, Ctsk*, and *Tnfsf11* (RANKL) (230). An elegant example also comes from Wiren et al (231), who examined RNA expression from cortical bone of osteoblast-specific, AR-overexpressing male mice and calvarial osteoblasts continuously treated with DHT. Up-regulation was found for genes like *Tnfrs11b* (osteoprotegerin), *Runx2*, *Tgfb2* (TGFβ2), *Fos*, and *Jun*, and down-regulation of *Il6*, *Il1a*, *Tnfrsf11a* (encoding RANK), and several other nuclear factor-κB, TNF, Wnt, and BMP family members (231). More recently, Khosla's group (232) studied associations between gene expression in human bone samples and estrogen status. But to ascertain whether these transcriptional differences are a direct or indirect consequence of AR/ER signaling and to identify the associated target cells, other techniques are needed. In an excellent recent review on this topic, Krum (233) defined a direct transcriptional target as a gene of which the expression is regulated by AR or ER binding at a promoter or enhancer of that gene. However, evidence from techniques such as chromatin immunoprecipitation (ChIP) remains a major hiatus in the bone field, with a few notable exceptions for ERα target genes in osteoblasts (Table 3). These have advanced our knowledge from previous studies examining early changes in gene expression (after 24 h or less) in cell lines or ex vivo cell cultures (27, 233). To our knowledge, no studies have examined early changes in differential gene expression induced by sex steroids after in vivo stimulation (234).

A. Estrogen receptor target genes

1. ER target genes in osteoclasts

Several E2 targets in osteoclasts have been known from studies in the 1990s, yet surprisingly, systematic studies using microarray approaches or ChIP studies are lacking. In vitro studies using mammalian osteoclasts found E2-induced up-regulation of c-Fos and c-Jun within minutes and down-regulation of *Ctsk*, lysosomal enzyme secretion (cathepsins and tartrate-resistant alkaline phosphatase), and IL-1R within a few hours (27, 233). C-Fos and c-Jun are essential transcription factors for osteoclast development and also belong to the AP-1 family through which ERα signals indirectly (see *Section II.C*); hence, this has been suggested to represent an amplification mechanism for estrogen signaling (233). Regulation of *Ctsk* in rabbit osteoclasts has been observed after only 3 hours and is affected by transcription blockers but not the translation inhibitor cycloheximide (233). However, another study using murine osteoclasts could not confirm altered *Ctsk* expression within 3 hours of E2 treatment (214). Estrogen regulation of FasL was suggested as a mechanism from osteoclast-specific ERαKO (212), but this study relied again on whole bones. However, Krum et al (214, 235) found evidence for a paracrine mechanism by which estrogens regulate osteoblast FasL to induce osteoclast apoptosis (see *Section VI.A.2*). Another mechanism of estrogen signaling in osteoclasts comes from the group of Manolagas (192), who showed that E2 can stimulate os-

Table 3. Direct Transcriptional Target Genes of ERα in Osteoblasts, Confirmed by ChIP of Target Gene Promoter DNA

Target Gene	Regulation	Stimulation	Cell Origin	Refs.
Fasl	↑	3 h	Mouse calvaria	214
ALPL	↑	3 h	U2OS-ERα (249), mouse calvaria	236
Svep1	↑	24 h	Mouse preosteoblastic MBA-15 cell line	245
IGFBP4	↑	24 h	Primary human Ob	246
ESR1	↑	15–90 min to 24 h	SaOS2 cell line	247

Abbreviations: ↑, increased; *ALPL*, alkaline phosphatase, liver/bone/kidney; *Svep1*, sushi, von Willebrand factor type A, EGF, and pentraxin domain containing protein 1 (a multidomain cell adhesion molecule); *ESR1*, ER α.

teoclast apoptosis via DNA-binding independent actions of ERα.

2. ERα target genes in osteoblasts

Krum et al (214, 236) revealed, using ChIP-on-chip, that ERα controls expression of many key osteoblast genes (including alkaline phosphatase (*Alpl*), *Nfatc1* and *Fasl*), nuclear receptors (including *Gper1*/*Gpr30* and *Nr5a2*), and other transcription factors (including *Pax7*, *Sox5*, *Foxo1*, and *Foxo4*). Interestingly, whereas FoxA1 serves as a critical pioneering factor for ERα in breast cancer cells, GATA4 may serve this role in osteoblasts (237, 238). More recently, the same authors demonstrated that ERα regulation of matrix metalloproteinase 3 was responsible for osteoblast membrane-bound FasL cleavage and release to mediate osteoclast apoptosis (235). Spelsberg's group (239–244) has identified retinoblastoma-binding protein 1 and TGF-β early inducible gene 1 as highly regulated by ERα and ERβ, respectively, and these factors directly modulate RUNX2 and OPG in osteoblasts as well as important osteoclast pathways. However, confirmation by ChIP is lacking (233). Others found that *Svep1* is directly ERα-regulated in a preosteoblast cell line, in collaboration with other transcription factors recruited to its promoter by E2 stimulation, including transcription factor IIB, nuclear factor-κB, and specificity protein 1 (245). The identification of the IGF binding protein 4 (*IGFBP4*) gene as a direct ERα target (246) underlines the importance of the interplay between sex steroid and IGF signaling. Finally, ERα and AP-1 regulation of ERα itself has been demonstrated in human osteoblasts (247), confirming earlier studies identifying a positive feedback regulation (248).

3. ERβ target genes

In a classical study from the group of Spelsberg (249) using inducible ERα or ERβ U2OS osteosarcoma cell lines, 24-hour treatment with E2 detected 63 and 59 genes regulated solely by ERα and ERβ, respectively, and only 17 by both ERs. Some of the identified ERα target genes were similar to those identified by ChIP (like *Alpl*), but for many of the historically described target genes of estrogens (eg, c-Fos/Jun, cathepsins, IL-1 receptor), we do not know for certain whether they are selective for ERα or ERβ.

B. Androgen receptor target genes

As discussed in *Section V*, there are doubts about the in vivo role of AR in osteoclasts. In an early in vitro study, 24-hour treatment of avian osteoclasts up-regulated genes for TGF-β and down-regulated genes for cathepsin B and tartrate-resistant acid phosphatase (250), but further evidence of AR regulation of osteoclast genes is lacking.

No direct AR target genes have been identified by ChIP, although ChIP has been used to demonstrate that AR binding to RUNX2 abrogates its recruitment to Runx response elements (251). In murine calvarial osteoblasts, in vitro stimulation with androgens for 10 hours also up-regulates TGF-β (27, 233). In a more recent microarray study, 12 genes were specifically reported as induced ≥ 2-fold after 12-hour DHT stimulation; of five genes tested, three (*MYBL2*, *HOXD11*, and *ADCYAP1R1*) were confirmed by quantitative RT-PCR, and an AR antagonist only abrogated up-regulation of the latter two (252). There is evidence for androgen regulation of IL-6 and OPG (although both stimulation and repression of the latter have been reported), but there is no definitive evidence that these are direct transcriptional targets (233).

In summary, studies using ChIP have begun to elucidate direct transcriptional targets of ERα in osteoblasts. The direct transcriptional targets of AR in bone cells and of ERα in osteoclasts and osteocytes remain, however, poorly investigated. Although most evidence points to a convergence of sex steroid signaling on master regulators like RUNX2, identification of highly regulated downstream targets such as TGF-β early inducible gene 1 or retinoblastoma-binding protein 1 is useful as a proxy for AR/ER activity.

VII. Interaction of Sex Steroids with Other Signaling Pathways

Sex steroids probably control musculoskeletal sexual dimorphism largely by converging onto more fundamental signaling pathways, which by themselves would seem unlikely to have gender-specific set points. This view is supported by the fact that the only gender-specific locus (at Xp22.31) associated with BMD in GWAS meta-analyses is also associated with male serum T levels, whereas no autosomal gene-by-sex interactions have yet been confirmed (25, 26, 40). Furthermore, interfering with master regulatory pathways in bone can abolish skeletal sexual dimorphism, as shown for GH receptor knockout mice, for example (78). Numerous animal and human studies have suggested gender-specific effects in other pathways (eg, Wnt signaling, discussed in *Section VII.D*), but whether these observations represent downstream consequences of cross talk with sex steroids rather than independent causes of sexually dimorphic bone metabolism requires further investigation.

A. Growth hormone and IGF-1

1. Physiology

Bone matrix is a storehouse replete with polypeptide growth factors, among which IGFs and TGF-β feature

most abundantly. Under control of pituitary GH, the liver produces systemic IGFs that exert endocrine actions on bone and cartilage, but osteoblasts and chondrocytes also secrete these factors locally in an autocrine/paracrine pathway (253). Osteoclast digestion of bone's extracellular matrix releases growth factors like IGFs, which act as coupling factors by stimulating osteoblast refilling of the resorption cavity (254). Human bone contains about 10-fold higher concentrations of IGF-2 than IGF-1, but the latter is much more potent. The activity and storage of IGFs is regulated by no less than six IGFBPs: IGFBP-3 and -5 can form a ternary complex with the acid labile subunit. Because IGFBPs are produced in molar excess, less than 1% of IGF-1 circulates as a free hormone (255). However, IGFBPs also prolong the half-life of IGF-1, thus enabling its activity, as evidenced by reduced bone length and width in humans and mice with defects in the acid labile subunit (256). The precise role of IGFBPs may be even more complex and remains incompletely understood; they may also enhance IGF activity by enhancing extracellular matrix storage or even have intracellular and/or IGF-independent effects. Studies in rodents dissecting the skeletal influence of different components of the GH/IGF-1 system have been reviewed elsewhere (253, 254). Briefly, genetic models of GH or IGF-1 deficiency display osteopenia with reduced periosteal bone expansion and longitudinal bone growth. Conditional overexpression of IGF-1 or deletion of the GH or IGF-1 receptor, either ubiquitously, in the liver or locally in osteoblasts and chondrocytes has shown that: 1) IGF-1 has both GH-dependent and -independent effects; 2) GH has direct effects on osteoblasts as well as on hepatic IGF-1 production; and 3) IGF-1 acts both in an endocrine and intracrine/paracrine manner. Furthermore, IGF-2 knockout mice have only modest reductions in cortical bone size compared to the previous models (257). Finally, paracrine overexpression of IGFBP-3, -4, and -5 has been shown to impair osteoblast function (255).

2. Sex steroid regulation of local IGF signaling in bone

IGF-1, IGF-2, their receptors, and several IGFBPs have been suggested to be regulated by androgens and estrogens in vitro in some studies, although the data remain as conflicting as a decade ago (27, 66, 258). For example, E2 treatment increased IGF-1 expression in osteoblastic U2OS cells stably transfected with ERα but not a NERKI vector, suggesting an ERE-dependent pathway (66). However, E2 had no direct effect on IGF-1 expression in rat osteoblasts in vitro; rather, ERα suppressed IGF-1 induction by other hormones indirectly via contact with other transcription factors (258). In a highly AR-expressing human fetal osteoblast cell line, DHT increased cell proliferation and expression of IGF-1 and IGFBP-2 and -3 levels, whereas IGFBP-4 decreased (259). Another group, however, showed that DHT increased TGF-β but not IGF-1 or IGF-2 in conditioned media of murine calvarial osteoblast cultures, although IGF-2 responsiveness was increased due to increased expression of its receptor (260, 261). IGFBP-4 has been confirmed by ChIP as a direct ERα target gene in osteoblasts (see *Section V.B*). Gender differences in the skeletal response to acid labile subunit deficiency have been described in mice, but regulation by sex steroids has not been examined (256). Estrogens may also enhance GH signaling in human osteoblasts, at least in vitro (262). No firm conclusions can be drawn from the conflicting evidence available.

3. Indirect skeletal effects of sex steroids via the somatotropic axis

The GH/IGF-1 axis is considered critical in determining sexual dimorphism in both longitudinal growth and periosteal bone expansion. The perinatal T surge is believed to cause imprinting of the hypothalamic GnRH pulse generator and consequent male-type GH secretion patterns during adulthood (see also *Section IV.D*) (175). At the start of puberty, the GH pulse amplitude rises, IGF-1 increases, and both contribute to an acceleration in longitudinal and transversal bone growth. This occurs later in boys but lasts longer, resulting in enhanced pubertal cross-sectional and longitudinal bone development. However, more recent studies show that gender differences in adult GH secretion are determined not only perinatally and during puberty but also during adulthood. Indeed, experimental alterations in the gonadal steroid environment in adulthood rapidly alter GH pulsatility patterns (263). Male-pattern GH secretion may also be more directly involved in periosteal bone expansion than previously realized, as evidenced by a striking feminization of bone geometry in male mice with conditional GH-receptor knockout in osteoblasts (264).

As mentioned in *Section II.E*, Tfm rats display decreased circulating IGF-1, but this is not the case in orchidectomized or ARKO mice (82, 84); thus, liver-derived IGF-1 cannot account for decreased periosteal bone formation in the latter two models (although disrupted local IGF-1 production has not been ruled out). Conversely, male ERαKO, aromatase knockout, ER$\alpha\beta$KO and aromatase inhibitor-treated mice all show decreased IGF-1 and radial bone expansion despite increased androgens (see *Section II.E* and Table 1); thus, androgens seem to regulate systemic IGF-1 in males via aromatization and activation of ERα. Female ERβKO mice had increased IGF-1 levels in some studies; the inhibitory effect of ERβ on female bone (see *Section IV.B*) could thus be related to restraints on IGF-1.

Callewaert et al (78) dissected the relative contributions of androgens, aromatization, and IGF-1 for skeletal sexual dimorphism in a careful longitudinal study. Skeletal sexual dimorphism was mainly established in early puberty (age 3–5 wk), when male mice have higher periosteal and lower endocortical bone formation, along with higher serum IGF-1 compared to females (78). From age 6 weeks, circulating IGF-1 was not significantly different between genders, and body weight and bone cross-sectional area increased in parallel. GH-receptor knockout mice, on the other hand, showed very low IGF-1 and no skeletal sexual dimorphism. Androgen deficiency did not decrease IGF-1 and only altered radial bone expansion during late puberty. Treatment with an aromatase inhibitor, however, significantly reduced IGF-1 and radial bone expansion from early puberty in male mice. Along the same lines, E2 was found to be a major positive regulator of hepatic IGF-1 synthesis in male GH-receptor knockout mice, which fully restored periosteal bone formation in these mice (265).Ovariectomy increased radial bone expansion during early puberty in females independent of IGF-1 levels, as confirmed by others (266). Thus, previous observations that (bioavailable) E2 levels correlate with the earlier onset of puberty in females by direct activation of their GH/IGF-1 axis and consequent bone development may be confounded by other factors, eg, leptin or signals from the central nervous system.

In conclusion, these mouse studies suggest that skeletal sexual dimorphism is mainly established in early puberty, when the stimulation of radial bone expansion by androgens in males appears to depend partially on both aromatization and IGF-1, whereas in females, estrogens stimulate endocortical bone formation and limit periosteal expansion independent of IGF-1 (Figure 3). In late puberty, androgens maintain periosteal bone formation in males independently of systemic IGF-1 (267). This fits with human observations that aromatizable androgens are more effective to stimulate bone growth in boys with delayed puberty, whereas only aromatase- or ERα-deficient men lack a pubertal growth spurt but not AIS individuals (254). Estrogens appear to exert a biphasic influence on periosteal bone formation and longitudinal bone growth (see *Section V.C*). In both genders, low levels during early puberty have stimulatory effects on the GH/IGF-1 axis, but this effect lasts longer in boys. In late puberty, growth plate chondrocytes and periosteal expansion are directly inhibited by high estrogen levels, which are reached more quickly in girls (268).

4. Involvement of GH/IGF-1 in male osteoporosis

From the previous section, it is clear that sex steroids interact with the GH/IGF-1 axis to determine sexual dimorphism during pubertal bone development. It is not clear, however, whether sex steroids also influence bone maintenance in older men via the somatotropic axis—there is even uncertainty whether the somatotropic axis plays a role altogether (33). Observational studies have shown that IGF-1 decreases with age in both genders, but both positive association and no association with bone turnover or BMD in men have been reported, as well as associations only in women or only in men in different studies (269–271). Recently, Ohlsson et al (272) found that older men with IGF-1 below the median had increased risk of incident hip and vertebral fractures. However, most of these studies showed no association between IGF-1 and sex steroid levels (270, 272). Although treatment with an aromatase inhibitor decreases IGF-1 in younger men and aged male rodent models, this has either not been confirmed (273) or has been left unreported in elderly men (274–276). This issue should be further examined in human interventional studies.

B. Vitamin D and PTH

PTH regulates formation of the active vitamin D metabolite 1,25-$(OH)_2$-vitamin D, and both maintain serum calcium by stimulating bone resorption, intestinal calcium absorption, and renal calcium reabsorption (277). Secondary hyperparathyroidism, low serum 25-OH-D and increased levels of 1,25-$(OH)_2$-vitamin D have shown independent and additive associations with bone loss in men (278, 279). At least in postmenopausal women, estrogens stimulate PTH-induced calcium absorption and reabsorption, although their overriding effect is a PTH increase due to decreased calcium mobilization from the skeleton (23). Whether the same holds true in men remains unconfirmed.

In MrOS USA, 25-OH-vitamin D levels were associated with hip BMD loss only when $<$20 ng/mL in men $>$75 years of age (280). In a case-control analysis, 25-OH-vitamin D levels were associated with incident hip fracture only, and not with other types of fractures (281). Isolated vitamin D deficiency without sex steroid deficiency was not related to any skeletal measure, and incident nonspine fractures were not related to low bioavailable T/E2 or high SHBG alone; only concurrent vitamin D deficiency and low bioavailable E2 were associated with these fractures (282). The authors concluded that the adverse effects of sex steroid deficiency were more pronounced in older men with low 25-OH-vitamin D levels. However, interventional studies are needed to confirm causality because in some other studies, androgen and vitamin D deficiency were associated (283, 284). In a small randomized trial, vitamin D supplementation increased (total and bioactive) T levels in healthy overweight men in a weight reduction program (285). Interestingly, DHT has been shown to

increase 1α-hydroxylation of 25-OH-vitamin D in osteoblasts from men, whereas E2 had no effect (286). In mice, it was shown that males have higher calcium excretion along with increased expression of renal calcium transporters, which are both decreased by orchidectomy and restored by T replacement; PTH and 1,25-$(OH)_2$-vitamin D were not affected (287). A cross-sectional study in MrOS USA and Sweden found that both T and E2 were independently associated with lower serum phosphorus levels in men (288).

In summary, sex steroid deficiency might amplify the association between vitamin D deficiency and fracture risk in older men. There might exist more direct interactions between the gonadal and calciotropic axis in men, which could have important repercussions for skeletal metabolism, but the existing data remain hypothesis-generating.

C. Biphasic modulation of RUNX2 activity

The interplay between RUNX2 and sex steroids has been excellently reviewed by Frenkel et al (289). Briefly, both ERα and AR have been shown to directly bind and modulate the transcriptional activity of RUNX2 (formerly CBFA1, a master transcription factor in osteoblast and chondrocyte differentiation) (Figure 9). In late-stage MC3T3-E1 osteoblast cultures (when RUNX2 activity is maximal; Figure 9A, right side), liganded ERα can bind with its DBD to RUNX2, hijacking its recruitment and activity on Runx DNA response elements (290). In early MC3T3-E1 osteoblasts, however, ERα binding has been found stimulatory to RUNX2 activity and repressive to ERE activation (291, 292) (Figure 9A, left side). These findings may seem contradictory but have been suggested as indicative of a stage-specific, biphasic regulation by which ERα keeps RUNX2 in check, preventing both insufficient and excessive RUNX2 activity. AR, also through its DBD, binds RUNX2 even more strongly than ERα, and a mutual repression of their subnuclear localization and transactivational capacity has been reported (251, 292) (Figure 9B). RUNX2 in turn regulates expression of several major osteoblast proteins such as osteocalcin, *Col1a1*, bone sialoprotein, osteopontin, osterix, and matrix metallopeptidases. Furthermore, the aromatase gene has recently been reported as a downstream target of RUNX2 in bone (293). Because AR, ERα, and RUNX2 are beneficial for osteoblast development and bone formation, a repressive action of AR and ERα on RUNX2 target genes may seem counterintuitive at first. However, attenuation of RUNX2 activity may prevent the deleterious effects of excessively high bone turnover as seen in transgenic mice overexpressing RUNX2 in osteoblasts, whereas transgenic mice expressing a dominant-negative RUNX2 resist ovariectomy-induced bone loss (294).

In summary, sex steroid nuclear receptors and RUNX2 can heterodimerize and are proposed to exert a mutual and biphasic modulation of each other's activity, which prevents both overstimulation and (as demonstrated for ERα) underactivity (289).

D. Wnt signaling and sclerostin

1. Physiology

Genetic investigations into the cause of several high bone mass disorders revealed an important role for the Wingless-type mouse mammary tumor virus integration site (Wnt) pathway in bone formation control (295). Briefly, canonical Wnt signaling involves several ligands

Figure 9.

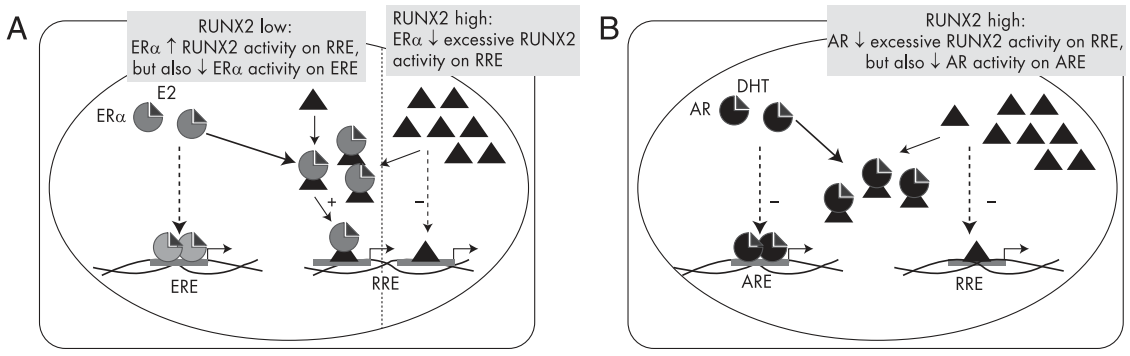

Figure 9. A, ERα and B, AR may interact with other nuclear transcription factors, thus binding and influencing chromatin indirectly. This is illustrated by the interactions between ERα and AR on the one hand and RUNX2 on the other hand. ERE, estrogen response element; ARE, androgen response element; RRE, Runx response element. [Adapted and reproduced from T. L. McCarthy et al: Runx2 integrates estrogen activity in osteoblasts. *J Biol Chem*. 2003;278:43121–43129 (291), with permission. © American Society for Biochemistry and Molecular Biology. And from B. Frenkel et al: Regulation of adult bone turnover by sex steroids. *J Cell Physiol*. 2010;224:305–310 (289), with permission. © Wiley Periodicals Inc.]

binding at the cell surface with Frizzled and low-density lipoprotein receptor-related proteins (LRP) 5 and LRP6 as coreceptors, activating downstream factors like GSK3β and LEF1 and ultimately resulting in intracellular β-catenin activation. The result is a striking increase in bone formation, which requires regulation by endogenous inhibitors like sclerostin (encoded by the *SOST* gene and almost exclusively expressed in osteocytes), Dickkopf-1, soluble frizzled-related proteins, and Wnt-inhibitory factors.

2. Sexual dimorphism in mouse models of the Wnt/β-catenin pathway

Several studies have shown gender differences resulting from genetic manipulation of the Wnt/β-catenin pathway, but few studies have yet examined whether this is directly related to sex steroid signaling or their interaction with bone mass, turnover, or response to mechanical loading (see *Section VII.E*). For example, trabecular bone volume showed greater increases in female compared to male *Sost* knockout mice (296). Conversely, progression of bone loss was generally faster and more severe in female compared to male *Dmp1*-Cre conditional β-catenin knockout mice (297), and in a haploinsufficient model, female mice showed markedly decreased trabecular bone volume, whereas males were unaffected (298). Along the same lines, *Gsk3β* and *Lef1* haploinsufficient females displayed enhanced and reduced trabecular bone mass, respectively, whereas males were unaffected (299). Interestingly, *Lef1* haploinsufficient males did show low bone mass on a *Tfm* background, although the AR may not be completely disrupted in this model (see *Section II.E*). On the other hand, secreted frizzled-related protein overexpression decreased trabecular bone to a greater extent in male mice (300). Osteocyte-specific deletion of the transcription factor myocyte enhancer factor 2c (involved in sclerostin expression) reduced sclerostin to a greater extent in male mice, resulting in more pronounced cortical and trabecular bone gains in males (301). Female mice with activating *Lrp5* mutations were slightly more load-responsive and less sensitive to disuse compared to males (302).

As mentioned above, several studies have suggested that sex steroid actions alter expression of Wnt family members, although direct regulation needs to be confirmed by ChIP. At least in vitro, Almeida et al (179) showed that ERα stimulation of periosteal cells occurs via potentiation of Wnt signaling. Androgens have also been shown to increase differentiation of MC3T3 preosteoblasts with an accompanying increase in Wnt signaling, and soluble frizzled-related protein can inhibit this (303). Several in vivo studies have suggested sex steroid regulation of sclerostin. Ovariectomy decreased skeletal sclerostin expression in one study (304), whereas another study came to the opposite conclusion: ovariectomy increased skeletal sclerostin expression, whereas E2 prevented this (305). The former study found some variation, depending on the type of bone and the time point examined.

In conclusion, male mice have displayed either enhanced or decreased sensitivity to manipulation of the Wnt/β-catenin pathway, depending on which component is genetically modified. ERα and AR have been shown to potentiate Wnt signaling in osteoblasts in vitro. Future in vivo studies are needed to examine whether sex steroid actions involve a direct, short-term regulation of Wnt/β-catenin signaling, and whether this contributes to skeletal gender differences. Clinical trials may in the future reveal whether or not sexual dimorphism in the skeletal response to Wnt/β-catenin manipulation also exist in humans, although this pathway is most likely of great importance in both genders.

3. Role of circulating sclerostin

Sclerostin serum levels have been examined in several studies, but the results differ markedly depending on the immunoassay used and should thus be interpreted cautiously (306). In animal models (304) and more recently in humans (307), discordance has been noted between circulating sclerostin and expression locally in bone. Several studies found higher (instead of lower) sclerostin serum levels in men compared to women, and a positive (instead of a negative) association with age, whole-body bone mineral content, and HR-pQCT parameters (306, 308–314). The gender difference in circulating sclerostin seems to be established during puberty, and serum sclerostin tends to decline in late puberty in both genders (314). In the MINOS study, older men with higher serum sclerostin had lower fracture risk, even independent of BMD and age (310). Although not unequivocally (312), most observational (310) and also interventional studies showed that E2, and not T, regulates serum sclerostin in elderly men and both circulating sclerostin and intraskeletal mRNA in postmenopausal women (232, 315). This correlated with bone resorption but not formation markers. Taken together, these results suggest that circulating sclerostin is more closely related to the amount of bone (and probably osteocytes) present and may also reflect increased bone turnover per se, but sclerostin serum levels do not necessarily reflect its known role as a local inhibitor of bone formation. Circulating sclerostin was related to estrogen status in both genders in most studies, but whether sex steroids also regulate local sclerostin signaling in male bone and whether this represents a direct AR/ER effect requires further study.

The interaction between sex steroids and the Wnt/sclerostin pathway in the osteogenic response to mechanical loading is discussed in the next section.

E. Physical activity and response to mechanical loading

In a seminal study, the group of Lanyon (316) showed that female ERαKO mice displayed decreased periosteal and endosteal bone formation after mechanical loading and that strain induced less proliferation in osteoblast-like cells derived from these animals. Load-induced cancellous bone formation on the other hand is normal in these female ERαKO mice (317). Strain-induced osteoblast proliferation is also prevented by an ERα antagonist (177). The antiapoptotic effect of mechanical stimulation on osteoblasts and osteocytes, however, seems to require ligand-independent actions of both ERα and ERβ (176). In vivo, the cortical osteogenic response in female mice was enhanced by tamoxifen (an ERα agonist in bone, but a pure ERβ antagonist), and this effect could be abrogated by adding fulvestrant (which inhibits both ERs and had no effect on its own). ERβKO had the opposite effect, ie, increased cortical loading response, in line with the known inhibition of normal ERα functions by ERβ (see *Section IV.B*) (317). Ovariectomy on its own does not reproduce this effect (318). Along the same lines, several studies in rats showed that ovariectomy increases and E2 decreases load- or exercise-induced bone formation (319–323), whereas another study showed no interaction (324). A recent study confirmed that these effects are indeed ligand-independent and further showed that ERαAF-1, but not AF-2, mediated the osteogenic response of cortical bone to mechanical loading (178). Furthermore, loading did not induce in vivo luciferase activity in ERE-Luc reporter mice (178). Thus, the osteoanabolic effects of mechanical loading are enhanced in female mice by ERα actions via its AF-1 and nonclassical signaling.

In male mice, however, the group of Lanyon (317) showed that ERαKO increased both the cortical and trabecular response to mechanical loading (contrary to the situation in female mice), whereas ERβKO increased the cortical response (similarly to what was observed in females). In an earlier study using the Korach's ERαKO model and ulna loading, however, there was no effect of ERα on the osteogenic response to loading in male mice (325). Apart from methodological issues, the latter difference could also imply that ERα-AF-1 might be involved in males, too, but this requires confirmation. In ARKO male mice, the cortical response to loading is increased (325). Whether this effect is ligand-dependent or not requires confirmation.

The exact mechanism upstream of ERα linking it to mechanical stimuli remains unidentified. The early Wnt/β-catenin response to mechanical loading seems to require ERα, at least in vitro (326). Almeida et al (179) confirmed this in periosteal cells from $ER\alpha^{Ob;Prx1}$ and $ER\alpha^{Ob;Osx1}$ mice, which showed blunted proliferative and osteoblastogenic responses to Wnt3 stimulation. E2 on the other hand had no effect on proliferation and even attenuated Wnt-induced osteoblastogenesis, showing that the receptor has independent or even opposite effects to those of its ligand (179). Suggested downstream effects explaining the increased loading response include sensitization of the IGF-1 receptor via ERα and down-regulation of *Sost*/sclerostin via ERβ (whereas ERα tends to antagonize this) in female mice (327). In male ARKO mice, a stronger inhibitory effect of loading on sclerostin expression and a higher nitric oxide production by osteocytes in vitro were observed (325).

There are several methodological caveats in these studies, including the reliability of strain-gauge techniques in one cortical site (but not in other sites or in trabecular bone), reporting relative differences, which obscures baseline absolute differences, and confounding, for example, by background physical activity (328). For example, ARKO mice have decreased physical activity and lack of motivation, whereas forced activity improves their musculoskeletal deficit (106). Interestingly, spontaneous locomotor activity was increased in gonadally intact but not orchidectomized central nervous system (Nestin-Cre) ARKO mice, suggesting a role for increased estrogen levels in this model (108). This is in line with evidence that female mice and rats have higher voluntary running activity than their male counterparts (329, 330).

The osteocyte would be an obvious candidate target cell given its mechanosensitivity and mechanotransduction capabilities. Strain has been shown to induce nuclear and membrane localization of ERα in vitro in osteocytes (331), and E2 enhances connexin 43-based gap junction intercellular communication in the osteocyte-like MLO-Y4 cell line (332). However, both the osteocyte-specific ERαKO and ARKO mice showed no altered loading responses (172, 220). Instead, ERα stimulation of mechanoresponsiveness in periosteal cells has been proposed to be responsible for the cortical bone deficits observed in osteoprogenitor- and early osteoblast-specific conditional knockout models (179).

In summary, the cortical osteogenic response to mechanical loading in female mice requires ERα via its AF-1 and ligand-independent, non-ERE-mediated signaling. More studies are needed in male mice, but there seems to be an opposite, inhibitory effect of AR. Many questions remain regarding the target cells, the molecular mechanism, as well as the importance of these findings (not the least of which is their generalizability to humans). Further

studies on the interaction between hormones, mechanical signals, and physical activity in bone regulation are eagerly awaited.

VIII. Indirect Effects of Sex Steroids on Bone

Because the skeletal phenotype of bone cell-specific ARKO and ERKO models are generally mild compared to their ubiquitous counterparts, it follows that sex steroids might have important indirect effects on bone via other tissues. The primary suspects in this regard are muscle and the nervous system. In vivo evidence from muscle- and neuron-specific ARKO/ERKO models will be reviewed in the following paragraphs. Perhaps other tissues known to affect bone metabolism like fat, the gut, the kidney, etc, may also be involved in sex steroid control of bone metabolism, which needs to be investigated in future studies.

A. Muscle

Given the well-known trophic and anabolic effects of androgens on muscle in humans (208), as evidenced most strongly by androgen abuse in weight lifters (333), the possibility arose that some of the effects of androgens in rodent models might indirectly result from bone-muscle interactions (334). In ARKO mice, males (but not females) have impaired muscle development (83). Additional ERαKO further reduces muscle mass in ARKO males (84), in line with other evidence for an independent effect of estrogens on muscle in mice and rats (135, 335). In humans, however, T alone via the AR seems sufficient for maintaining muscle mass in older men because neither aromatase nor 5α-reductase inhibition diminishes the efficiency of T replacement (208, 336, 337). However, bone and muscle development are highly integrated and controlled by common signals including IGF-1 (338). Muscle-specific ARKO mice do not show altered bone metabolism, and even appendicular muscle mass is only slightly reduced, possibly because only perineal muscles display high AR content and androgen regulation in mice (339–341). In human studies, the effects of androgens on bone could be examined by correcting for muscle parameters. In male-to-female transsexuals, for example, bone mass seems to be maintained during pharmacological estrogen therapy, despite substantial muscle loss related to androgen deficiency (75). Such associations are interesting, but they offer no definitive proof that estrogens alone are sufficient for skeletal maintenance in older men.

Although the effects of androgens on bone in mice are unlikely to be mediated via the AR in muscle, it remains unclear whether sarcopenia in older men with late-onset hypogonadism contributes directly or is merely associated with bone loss and fractures (32, 33, 342). Future studies are necessary to examine whether maintaining or increasing muscle mass is useful to prevent falls, bone loss, and ultimately fractures. In any case, exercise seems an effective countermeasure for hypogonadal bone loss in preclinical models (106). Conversely, hypogonadism is commonly seen in clinical situations of disuse, and nandrolone or T have been found beneficial for bone and muscle decay in the absence of mechanical stimuli in some preclinical studies (343–345), but not others (346). Whether these effects are mediated by muscle or skeletal AR requires further investigation.

B. Nervous system

It has long been known that bone cells are connected to sympathetic, parasympathetic, and sensory nerve fibers and contain various receptors for neurotransmitters, although the notion that these nervous systems control bone metabolism is still controversial. Sex steroid receptors in turn are expressed in neurons throughout the nervous system, opening the possibility that sex steroids may affect the skeleton via the nervous system (108, 347). Interestingly, ovariectomy has been demonstrated to produce a dramatic decrease of innervation in the rat tibia (348). In some mouse models, bone loss due to estrogen deficiency was diminished when nervous signaling was disrupted, although other studies could not confirm this (349).

Farr et al (350) recently demonstrated, for the first time in humans, an increase in sympathetic activity in postmenopausal women, which was negatively associated with trabecular microstructure and bone strength.

A role of estrogen signaling in nervous tissue for bone metabolism was recently suggested by an investigation in female mice lacking ERα in neuronal cells (351). Pan-neuronal ERα inactivation using Nestin-Cre resulted in increased bone mass, indicating that estrogen signaling in neuronal cells may have a negative impact on bone mass in contrast to the positive effects of peripheral estrogen signaling. The exact mechanism underlying the increased bone mass observed in this study was not completely understood, but a subsequent study suggested that pro-opiomelanocortin neurons were involved (mainly found in the arcuate nucleus in the ventral hypothalamus, a region previously implicated in regulation of bone mass) (352).

In conclusion, apart from their well-known actions on the gonadotropic and somatotropic neuroendocrine axis, estrogens may influence bone indirectly via ERα signaling in other neuronal cells. Future mechanistic studies in mice as well as more studies in humans are needed to confirm whether and how both estrogens and androgens affect neuronal regulation of bone metabolism.

C. Adipose tissue

According to the traditional view, obesity, both through increased mechanical forces imposed on the skeleton and estrogen production via adipocyte aromatization, has beneficial effects on BMD, and thus fracture risk. This paradigm has, however, been challenged recently by reports demonstrating unaltered fracture susceptibility in obese men despite higher BMD (353). Moreover, the relationship between adiposity and bone mass may depend on gender, age, menopausal status as well as type of adipose depot (sc vs visceral) (354). Premenopausal women predominantly accumulate sc fat that is positively correlated with bone mass; men, however, accumulate visceral fat, which associates negatively with BMD (35). Marrow fat can also be assessed with novel imaging techniques and has, in most reports, been found inversely correlated with bone parameters (35). However, thus far no prospective study has related marrow fat to fracture.

T promotes a reduction in total body fat and visceral adiposity in elderly men (355–357). Finkelstein et al (208) have suggested that increased body fat in male late-onset hypogonadism is dependent on reduced E2. However, anabolic doses of nonaromatizable androgens have also demonstrated independent lipolytic actions of AR (358, 359), which may depend on shifting mesenchymal precursor cells away from adipogenesis and toward osteogenic and myogenic differentiation (360). Global inactivation of ERα and AR in mice of both sexes results in obesity (84, 107, 361, 362). In contrast, ERβKO mice of both sexes have normal adipose tissue under normal diet conditions (363). Sex steroid receptors are expressed in adipocytes, and recently, adipocyte-specific ARKO mice were shown to have impaired insulin secretion and increased susceptibility to visceral obesity (364). Plasma levels and adipocyte expression of adipokines like leptin, adiponectin, and resistin have been associated with circulating sex steroid levels in humans, and receptors for these adipokines are expressed in osteoblasts and osteoclasts and regulate bone mass in transgenic mouse models (353).

In conclusion, adipose tissue influences the skeleton, and this effect seems depot-specific. Sex steroids also exert depot-specific effects on adipocytes and may regulate adipokine secretion. Whether effects of sex steroids on bone also involve indirect effects via AR or ER in adipocytes remains to be established.

D. Oxidative stress

Reactive oxygen species (ROS) are natural products from the aerobic metabolism of cells, but they can also be generated from oxidation of fatty acids or as a response to external stimuli, eg, inflammatory cytokines, growth factors, or UV light. Although high levels of ROS are harmful, low levels are important for triggering essential signaling cascades. Oxidative stress is caused by excess ROS, which cumulatively leads to age-related damage in many tissues including bone. Generic aging mechanisms including oxidative stress and their link with sex steroids were recently the subject of an excellent review by Manolagas (22), to which we refer for a more extensive summary on this topic.

ROS influence the generation and apoptosis of osteoclasts, osteoblasts, and osteocytes, and it has been shown that oxidative stress is temporally linked to reduced bone mass and strength in aging mice (which remain sex steroid sufficient) (210). Estrogens and androgens may protect against bone loss by increasing defenses against excessive oxidative stress. Gonadectomy of both female and male mice increases ROS in the bone marrow. These changes can be reversed by E2 (females), DHT (males), or an antioxidant (210). Antioxidant treatment also prevents the gonadectomy-induced spinal BMD loss in both male and female mice (210). In vitro studies using osteoblastic cells confirm that E2 and DHT attenuate osteoblast apoptosis induced by oxidative stress (210). There is also evidence that E2 and DHT restrain osteoclastogenesis and stimulate osteoclast apoptosis by reducing oxidative stress via promotion of antioxidative pathways (210). The effect of estrogens on oxidative stress does not appear to involve binding of the ERα to DNA response elements because E2-treated ovariectomized NERKI and control mice show a similar reduction of ROS in bone marrow (181). Instead, the normal increase in ROS in bone marrow in ovariectomized mice was reversed by EDC treatment (an estrogenic ligand incapable of mediating nuclear ER signaling; see *Section IV.E*). This suggests that oxidative stress is reduced by extranuclear ERα actions via ERKs (191).

In conclusion, age-related bone loss may partly result from oxidative stress, which may be exacerbated by combined androgen and estrogen steroid deficiency in late-onset male hypogonadism. This hypothesis should be examined further in human interventional studies.

E. Immune system

Since the 1990s, it has been known from seminal studies by Pacifici (365) and other groups that estrogen deficiency increases systemic and bone marrow levels of proinflammatory cytokines like IL-1, TNF-α, interferon-γ, IL-6, and IL-17, which may all contribute to osteoporosis. T-lymphocytes are considered critical in the field of osteoimmunology because T-cell-deficient mice have been shown to be resistant to bone loss induced by ovariectomy in several (but not all) studies (365). Androgen deficiency also produces a proinflammatory response (366), but it is un-

known whether immunodeficient mice resist orchidectomy-induced bone loss. Recently, Onal et al (367) showed that RANKL expression in B-lymphocytes but not T-lymphocytes contributes to ovariectomy-induced cancellous bone loss.

The key question is whether ERα, ERβ, and AR in T-lymphocytes and/or B-lymphocytes mediate some of the skeletal effects of sex steroid deficiency. Conditional AR inactivation has recently been shown to expand B-cell repertoires (368), and ERα actions in T-lymphocytes account for the protective effects of estrogens in experimental autoimmune encephalitis (369). Future studies examining sex steroid actions in osteoimmunology using similar conditional models are eagerly awaited.

IX. Bone Loss and Fractures in Men

In the previous sections, we have reviewed evidence, mostly from genetic mouse models and rare human mutations, on how androgens and estrogens, via the AR and ERs, promote male skeletal development. Although further validation of these insights in human studies is needed, the conclusions that emerge from the bulk of available evidence are that the effects of T on male cortical and trabecular bone development rely on several mechanisms including: 1) direct actions via AR in osteoblasts and possibly other target cells; 2) ERα actions in osteoblast-lineage cells; 3) interaction with pubertal systemic GH/IGF-1 signaling; and 4) possibly via interaction with other signaling pathways and indirect effects (see Figures 4 and 8). These mechanisms can be linked to the greater cortical bone size and superior trabecular microstructure observed in young adult men compared to women. As mentioned in *Section III*, part of the advantage men have in terms of fracture risk later in life is probably determined by optimal pubertal peak bone mass acquisition.

In the last part of our review, we will now focus on age-related bone decay as well as fracture risk and the contribution of (acquired) sex steroid deficiency in late-onset male hypogonadism (see *Section II.A*).

A. Estrogen deficiency: the primary mediator of hypogonadal bone loss in older men

1. Observational studies

The importance of E2 for maintenance of bone mass in adult and elderly men has been established by numerous cross-sectional observational studies showing a strong association between serum levels of total and especially bioavailable E2, on the one hand, and BMD at various sites on the other (27, 370–374). Prospective studies also confirm that serum E2 levels associate with BMD loss in older men (373, 375–378). In a cohort of elderly men from Rochester, Minnesota, BMD at the radius and ulna declined by 0.49 to 0.66% per year, and these decreases were associated with both bioavailable E2 and bioavailable T levels, although the strongest correlations were found with bioavailable E2 (375). In this study, a threshold below which annual rates of bone loss started to decrease was found at a bioavailable E2 level of 40 pmol/L (11 pg/mL; corresponding to a total E2 level of 114 pmol/L [31 pg/mL]) (Figure 10). Other investigators independently reported similar threshold levels below which bone loss in older men accelerated at the lumbar spine or femoral neck (376, 378). Using QCT, further studies in men found that serum bioavailable E2 levels were con-

Figure 10.

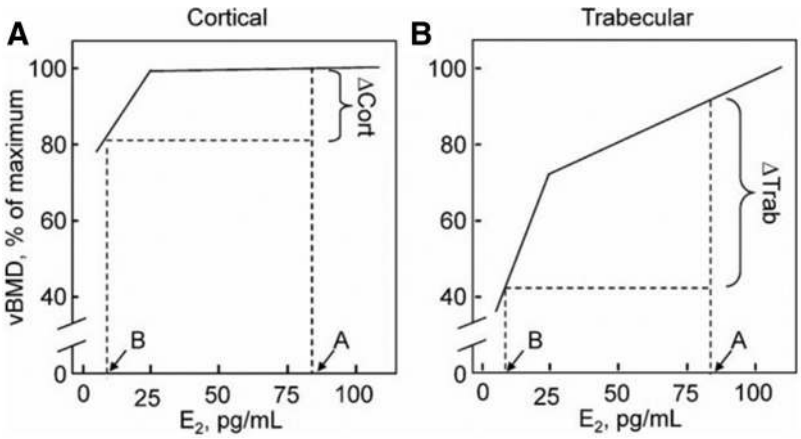

Figure 10. The possible threshold for skeletal E2 deficiency is more evident at cortical than trabecular sites in men. Schematic illustration is based on the data of Khosla et al (379) of the relationship between femoral neck cortical vBMD (A) and vertebral trabecular vBMD (B) and serum E2 levels. Note that whereas cortical vBMD is correlated with E2 levels at low E2 levels (below 25 pg/mL, corresponding to bioavailable E2 of 30 pmol/L), no relationship is evident at high E2 levels, consistent with a threshold below which cortical bone becomes estrogen-deficient. By contrast, trabecular vBMD remains associated with E2 levels at both low and high levels, suggesting either the absence of a threshold or a threshold considerably higher than that present for cortical bone. Also shown are the predicted relative changes in cortical (Δcort) and trabecular bone (Δtrab) as E2 levels fall from estrogen sufficiency (point A) to estrogen deficiency (point B). [Reproduced from S. Khosla et al: The unitary model for estrogen deficiency and the pathogenesis of osteoporosis: is a revision needed? *J Bone Miner Res*. 2011;26:441–451 (23), with permission. © American Society for Bone and Mineral Research.]

sistently associated with vBMD at trabecular and cortical sites (379, 380). Interestingly, there appear to be differences in the dose relationships between serum E2 levels and cortical vs trabecular bone. In an elderly population of men, significant associations were observed between E2 levels and cortical vBMD when bioavailable E2 was below the median of 30 pmol/L (corresponding to a total E2 level of about 25 pg/mL), but not for bioavailable E2 levels above this value (379) (Figure 10A). In contrast, the correlations between trabecular vBMD and bioavailable E2 were rather similar for bioavailable E2 levels below or above 30 pmol/L (Figure 10B). Thus, the proposed threshold effect mentioned above appears to be more pronounced at cortical sites than trabecular sites in elderly men (379), although this was not confirmed in middle-aged men (380).

Only one prospective study investigated the associations between sex steroids and vBMD loss at trabecular and cortical sites (121). In older men (>50 y), cortical bone loss correlated with bioavailable levels of both T and E2 at the distal radius, but not at the distal tibia. Serum bioavailable E2 levels associated with trabecular vBMD loss at the lumbar spine. Cross-sectional HRpQCT analyses in older men show moderate associations between serum bioavailable E2 and trabecular bone volume, trabecular number, and trabecular spacing at the distal radius (381), whereas serum total E2 levels associate inversely with cortical porosity at the distal tibia in the MrOS Sweden (126). In the STRAMBO cohort, a threshold was found of 25 pmol/L (quite similar to the one proposed above), below which cortical thickness and cortical vBMD were decreased when combined with a low apparent free T concentration (382). Lastly, data from the MINOS study (383) and the BACH study (384) showed that circulating E2 levels associated positively with estimated bone strength at the femur neck and distal radius in men. Longitudinal HRpQCT studies are required, however, to carefully elucidate the role of sex steroids in the age-related changes in trabecular and cortical microstructure and related bone strength indices in men.

Studies in older men with prostate cancer receiving androgen deprivation therapy (by chronic administration of GnRH agonists) further demonstrate the importance of E2 for skeletal maintenance in older men. These patients have reduced BMD and increased fracture risk (385) because of significantly suppressed levels of serum T and E2. In contrast, in patients treated with antiandrogen monotherapy, serum concentrations of T and E2 are increased, BMD is maintained, and bone turnover is unaffected (386, 387), indicating the importance of estrogen actions. Also in male-to-female transsexuals, androgen suppression and pharmacological estrogen therapy seems to be sufficient to maintain BMD (75, 388, 389), although this requires confirmation in prospective pQCT studies.

Several genetic studies also support the primary role of estrogens for bone health in men. Older men with more TTTA repeats in the aromatase (CYP19A1) gene have higher serum E2, less rapid BMD loss, and reduced fracture risk (377, 390, 391). In a sex steroid-related candidate gene study, a single nucleotide polymorphism in CYP19A1 conferred elevated E2 levels, higher lumber spine BMD, and reduced fracture incidence in older men (391). Bone quantitative ultrasound parameters in middle-aged and elderly men were also associated with several polymorphisms in CYP19A1 (392). The fact that peripheral aromatase activity may modulate circulating E2 levels is particularly interesting because estrogens in men are mostly derived from peripheral conversion of androgens (36, 37). A polymorphism in the gene for COMT (an estrogen-degrading enzyme) is also associated with prevalent fractures in elderly men (56, 57). Moreover, men with higher CAG repeat length in the AR gene have superior calcaneal ultrasound parameters because of estrogen activity after aromatization of their increased T levels (393). Finally, GWAS meta-analyses have identified the *ESR1* locus (encoding ERα) to be strongly associated with lumbar spine and femoral neck BMD (394, 395), as well as trabecular and cortical vBMD in middle-aged and older European men (396). Finally, Paternoster et al (162) recently provided the first genetic evidence using GWAS that a single nucleotide polymorphism in *ESR1* was associated with cortical but not trabecular vBMD in men.

Taken together, these observational studies suggest that estrogens play a vital role in maintaining bone mass in adult and elderly men (Table 4).

2. Intervention studies

Although highly indicative, the above-mentioned association studies do not provide evidence for a causal role of E2 for skeletal health in aging men. The relative contributions of androgens and estrogens to male bone metabolism have been elegantly demonstrated in direct intervention studies. Administration of an aromatase inhibitor in older men with low T levels increases T but decreases E2 levels and reduces BMD, suggesting that the increase in T cannot overcome the detrimental effect of selective E2 deficiency on the skeleton (274). Conversely, SERMs have proven efficient in treating bone loss associated with androgen deprivation therapy in prostate cancer patients (397). Raloxifene treatment modestly increases BMD in men on androgen deprivation therapy (398), whereas toremifene not only increased BMD and reduced bone turnover markers, but also reduced the incidence of new vertebral fractures after 2 years in a randomized, placebo-

Table 4. Different Lines of Evidence Regarding the Role of Estrogen or Androgen Actions in Male Hypogonadal Bone Loss

	E2/ERα	T/AR	Refs.
Population-based studies	Independent association with BMD, BMD losses, (micro)structure and fracture risk in multiple independent studies	Same associations less strong, not independent or weakened after correcting for E2 levels; or limited to cortical bone sites and BMD-independent fracture prevention	121, 126, 370–384, 417, 418[a] 271, 373, 380, 382, 413, 416, 419, 420, 422, 427[b]
Genetic evidence	Polymorphisms in *ESR1*, *CYP19A1*, or *COMT* associated with E2, BMD, and/or fracture risk	Long CAG repeats inhibit AR but increase T and E2; not negatively associated with BMD	56, 57, 162, 377, 390–392, 394–396[a] 393, 425[b]
Clinical studies	Androgen (and estrogen) deprivation therapy for prostate cancer increases bone loss and fracture risk; SERMs prevent bone loss and fractures in this setting	Antiandrogen monotherapy does not increase bone loss or fracture risk	385, 387, 398, 399[a]
		SARMs do not reduce BMD loss or bone turnover in human trials	359, 446[b]
	Pharmacological E2, even with androgen suppression, prevents bone loss in male-to-female transsexuals	In female-to-male transsexuals, effects of T on BMD are equivocal; cortical bone size may increase and cortical vBMD decrease, but may depend on aromatization	75, 388[a] 167, 389, 423, 424[b]
Intervention studies	Aromatase inhibition increases T but decreases E2 and BMD	DHT therapy decreases BMD, probably due to E2 suppression	274, 275[a] 438[b]
		5α-reductase inhibitors reduce muscle-anabolic effects of T but do not affect bone	336, 337, 447–450[b]
	GnRH suppression with T, E2, or T + aromatase inhibition shows dominant role for E2 in short-term BTM and HRpQCT changes	Androgens selectively linked to bone formation markers	276, 404, 405[a,b]

Abbreviation: BTM, bone turnover marker.

[a] These references pertain to estrogens/ER.

[b] These references pertain to androgens/AR.

39controlled trial (399). Treatment with the sex steroid precursor DHEA in older men with low levels of DHEA sulfate modestly increased BMD at the femoral neck in some (400, 401) but not all (402, 403) studies. Jankowski et al (400) showed that the changes in regional hip BMD during DHEA treatment were associated with the serum levels of E2 at the end of the intervention. Short-term pharmacological suppression of endogenous E2 and T production in elderly men (through treatment with a GnRH agonist in combination with an aromatase inhibitor), followed by complete or selective add-back of E2 and T, shows that E2 significantly restores bone resorption and formation markers, although T also independently maintains the bone formation marker osteocalcin (276). Leder et al (404) randomized adult men to chemical castration and T replacement, with or without an aromatase inhibitor, to show that especially estrogens but possibly also androgens independently regulate bone resorption, whereas bone formation markers showed more complex patterns and only bordered significance. Most recently, a 16-week intervention study in 20- to 50-year-old men with gonadotropin inhibition, aromatase inhibition, and different doses of T treatment was performed by the same investigators in order to investigate the effects of T and E2 on vBMD and bone microstructure as assessed by HRpQCT (208, 405). Changes in vBMD and bone microstructural parameters were similar across the different T dose groups, indicating a lack of effect of physiological androgen levels in the near-absence of E2. In contrast, this suppression of E2 levels reduced trabecular and cortical vBMD at the tibia and radius and altered bone microstructure in a pattern consistent with trabecularization of the cortex.

These results strongly indicate that estrogen deficiency is the primary mediator of bone loss in hypogonadal men.

3. Observational studies with fracture endpoints

Several large-scale, prospective cohort studies using fractures as the hard endpoint further support a dominant role for E2 in male bone health (406). However, it should be mentioned that the early fracture studies, both cross-sectional and prospective, showed conflicting results regarding the relationship between serum E2 and fracture risk (407–414) (Table 5). This was most probably due to

Table 5. Overview of Longitudinal Studies Investigating the Relationship Between Serum Levels of Sex Steroids and SHBG and Fractures

First Author, Year (Ref.), Study	No. of Subjects/ Age, y	Follow-Up Time, y	No. of Subjects With Fracture/ Fracture Type	Sex Steroid Assay	E2	T	SHBG
Nyquist, 1998 (453)	242/mean, 67	7	22/all	RIA	–	No	No
Barrett-Connor, 2000 (407), Rancho Bernardo study	352/median, 66	8	28/vertebral	RIA	Yes	No	–
Goderie-Plomp, 2004 (410), Rotterdam study	178/mean, 66	6.5	45/vertebral	RIA	No	No	No
Amin, 2006 (416), Framingham study	793/mean, 71	18	39/hip	RIA	Yes	No	–
Bjørnerem, 2007 (408), Tromsø study	1364/mean, 63	8.4	105/nonvertebral	IA	No	No	Yes
Meier, 2008 (427), Dubbo study	609/mean, 73	5.8	113/low-trauma	MS	No	Yes	Yes
Mellström, 2008 (417), MrOS Sweden	2639/mean, 75	3.3	209/all	MS	Yes	No	Yes
Roddam, 2009 (414), EPIC-Oxford study	464/mean, 51	5	155/all	RIA	Yes	No	No
LeBlanc, 2009 (418), MrOS USA	1978/mean, 73	4.7	342/nonvertebral	MS	Yes	No[a]	Yes
Woo, 2012 (373), MrOS Hong Kong	1489/mean, 73	4	108/all	MS	Yes	No	No

Abbreviations: –, not reported; IA, immunoassay; MS, mass spectrometry.

[a] Men with low bioavailable T and high SHBG have increased fracture risk.

the limited number of fractures in these studies, as well as reliance on immunoassays, which have inadequate specificity at lower sex steroid concentrations (46, 48, 415) (see *Section II.A*).

Amin et al (416) assessed the risk for hip fracture in aging men from the Framingham Study who were stratified in tertiles according to serum E2 levels. During 18 years of follow-up, 39 men sustained a hip fracture, and men in the low E2 group had three times greater risk of hip fracture compared to men in the highest E2 group. This was consistent with a threshold E2 level of around 66 pmol/L (18 pg/mL), below which fracture risk increased exponentially. The most conclusive data addressing this issue have been reported by investigators from the MrOS study. In the MrOS Sweden cohort, Mellström et al (417) analyzed baseline serum sex steroid levels by mass spectrometry in 2639 elderly men and evaluated fractures over a relatively short follow-up of 3.3 years. Serum free E2 levels were independently associated with fracture risk. Fracture incidence correlated negatively with serum E2 levels, but only below a level of 59 pmol/L (16 pg/mL), whereas no relationship between fracture incidence and E2 levels was seen above this level (Figure 11) (417). These findings were subsequently extended in men ≥ 65 years old in MrOS USA (n = 1978) by LeBlanc et al (418), who found that men in the lowest quartile of bioavailable E2 (<12 pg/mL) had the greatest risk of nonvertebral fractures. A third very similar study in the MrOS Hong Kong cohort observed comparable findings (373). Also in this study, nonlinear relationships were described between total E2 and bioavailable E2 and fracture risk, with thresholds at 16 and 12 pg/mL, respectively, similar to the ones described previously for fractures (416–418).

In all studies, the association between serum free/bioavailable E2 levels and fracture risk was attenuated after adjustment for BMD, suggesting that this is partly explained by effects on bone density and partly by additional effects. The threshold E2 level observed in the different fracture studies is also somewhat lower than the threshold described previously for BMD and BMD loss.

Figure 11.

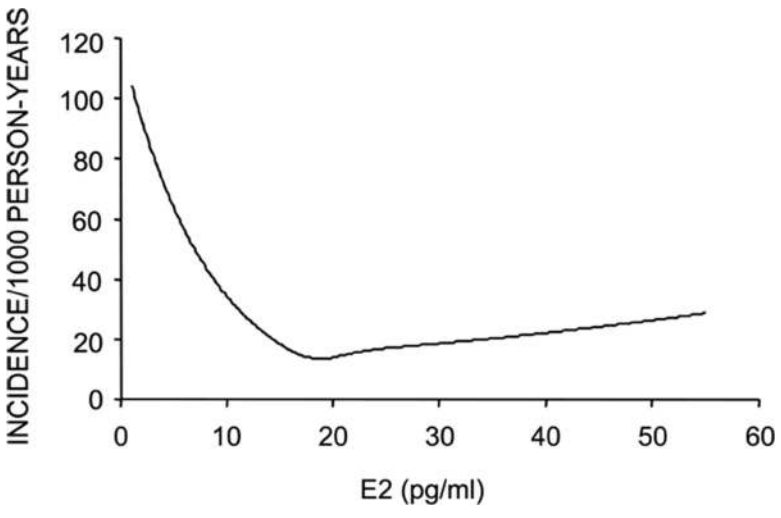

Figure 11. Annual incidence of fractures in relation to serum E2 levels in older men. Poisson regression models were used to determine the relationship between serum E2 and fracture risk. [Reproduced from D. Mellström et al: Older men with low serum estradiol and high serum SHBG have an increased risk of fractures. *J Bone Miner Res*. 2008;23:1552–1560 (417), with permission. © American Society for Bone and Mineral Research.]

This may indicate that the mechanism through which E2 affects fracture risk differs to some extent from that affecting bone maintenance in aging men. Alternatively, the different E2 assays used may also explain the difference in threshold values because in the association studies serum E2 was most often measured by immunoassay, whereas mass spectrometry was used in recent fracture studies.

In summary, these results provide compelling evidence for the essential effects of estrogens on skeletal health in aging men. Accumulating evidence shows a threshold below which E2 is associated with lower BMD, accelerated bone loss, and elevated fracture risk in older men.

B. High SHBG and low T: the second and third fiddle in male osteoporosis?

1. Independent actions of androgens in aging men

Although the above-mentioned data indicate that E2 is pivotal for maintaining bone health in aging men, this does not preclude independent contributions of androgens. Yet, observational studies have reported conflicting results on the relation between serum T levels and bone turnover markers, BMD, or BMD loss, with the associations being absent, less strong compared to E2, limited to cortical bone sites, or lost after adjusting for E2 or other confounders (271, 380, 382, 413, 419, 420). Most of these studies are limited by the small number of older men with frank hypogonadism, ie, ability only to show lack of association between BMD and T variations within the normal range for older men. A more strict definition of late-onset hypogonadism from the European Male Ageing Study (EMAS) study is based on the presence of at least three sexual symptoms, total T levels of less than 11 nmol/L, and free T level of less than 220 pmol/L (421). These criteria more accurately associated with ultrasound-estimated BMD than biochemically low T alone (422). Although T therapy in female-to-male transsexuals has no clear effect on BMD (167, 389, 423), it increased cortical bone size in a recent prospective QCT study (167). In another study, however, BMD loss was again seen when an aromatase inhibitor was added (424).

CAG repeats, which reduce AR sensitivity, have been shown to exert a positive effect on BMD in EMAS by increasing T and consequently E2 levels (393), whereas previous smaller studies were inconsistent (425). Genetic evidence recently implicated T in male bone health by identifying a locus on the X chromosome that was genome-wide significantly associated with both serum T levels (40) and lumbar spine BMD specifically in men (25), although this could be due to aromatization.

The influence of T on bone turnover markers is complex, with T treatment inducing a decrease in bone resorption, which is followed by a decrease in bone formation (426). Still, T was suggested to be independently related to indices of bone formation and resorption in the intervention studies described above (276, 404, 405).

The contribution of serum T to fracture risk in men is more complex than that of E2. In several studies, serum levels of total or bioavailable T were not independently related to fracture incidence in older men (373, 416–418) (Table 5). Nonetheless, Meier et al (427) found that T was independently and more strongly associated with low-trauma fractures than serum E2 in 609 older men during a 5.8-year follow-up, although spine and hip BMD in that study related to E2 but not T levels. Also in the Framingham Study, men with low T faced additional hip fracture risk in the presence of similarly low E2 levels (416), and in MrOS Hong Kong, older men within the lowest quartiles of both E2 and T faced approximately twice the risk of incident nonvertebral fractures (373).

Despite the general lack of association with BMD, androgens may still play a role in cortical density (382) and cortical appositional growth because older men with high T levels have been reported to have larger cortical bone areas (378, 380, 413). Whether or not this occurs through effects on peak bone mass acquisition (see *Sections III* and *IV*) or during aging is unknown. Furthermore, we agree with the view of Khosla (406), who suggested that androgen status probably affects fracture risk in elderly men to a large extent via nonskeletal effects, such as on muscle mass and propensity to fall. Indeed, in the MrOS USA study, it was found that serum T levels affect falling; men with T levels in the lowest quartile had a 40% higher fall risk than those in the highest quartile (428). Falls are independent predictors of fracture risk in older men (429). Serum T levels have been associated with lean mass, muscle strength, and frailty in cross-sectional (32) and prospective studies (34, 430). Also, intervention studies have shown that T increased lean body mass and muscle strength in men (33, 208). Moreover, (free) T levels have been associated with cognition in men (431–433), although randomized trials have not confirmed this. Clinical trials with T or selective AR modulators (SARMs) looking at BMD, sarcopenia, frailty, fractures, and falls as endpoints are needed to provide direct evidence for the clinical efficacy (and safety) of T for prevention and treatment of age-related musculoskeletal involution, falls, and fractures.

2. Randomized trials

The use of androgen replacement in elderly men to preserve musculoskeletal integrity, physical functioning, and quality of life remains controversial and poorly supported by evidence (406). Nevertheless, an increasing number of

older men are prescribed T for age-related symptoms or age-related decline in T levels (33). However, T replacement in aging men is only warranted in the case of late-onset hypogonadism, with consistent symptoms and signs and unequivocally low T levels (434). The effects of T therapy on bone health and physical function outcomes have been reviewed recently (33). Its clinical efficacy on BMD in older men with low or borderline serum T levels varies depending on the route of administration, with im but not transdermal T increasing lumbar spine BMD (435). Also, short follow-up and relatively normal baseline serum T levels of the participants may explain the lack of effect in some studies. Lumbar spine BMD increased moderately in most studies after at least 1 year of T therapy, whereas BMD increased at the hip in some studies but not others (355–357, 436, 437).

Whether T exerts its effects directly via the AR or indirectly on the ERs after aromatization cannot be determined from these studies. However, a 2-year study in older men showed that transdermal DHT (which cannot be aromatized) reduced spinal but not hip BMD, concomitant with decreases in serum E2 levels (438). These findings are at odds with studies in adult rodents and ARKO, ERαKO, and/or ERβKO mice treated with selective ligands, which showed that both ERα and AR can mediate trabecular bone maintenance during aging, whereas both together are required for cortical bone maintenance (73, 131, 135, 137). It is important to realize that all of the human intervention studies described above (274, 276, 404, 405, 438) have examined the role of androgens on the background of very low instead of normal E2 levels. Moreover, effects on periosteal apposition may occur too slowly to be detected in short-term experimental studies.

There is growing concern regarding the effects of T therapy on cardiovascular outcomes and mortality. A generally modest association has been observed between low endogenous T and incident cardiovascular endpoints in men (439). A randomized clinical trial of T therapy in men with impaired mobility and a high prevalence of cardiovascular diseases was, however, stopped prematurely due to adverse cardiovascular events (440), raising concerns about the safety of T therapy. In multivariable analysis, these cardiovascular events were related to the change in free T levels, and not E2 (441). The ongoing Testosterone Trial in Older Men (ClinicalTrials.gov identifier: NCT00799617), a large, randomized trial of T in older men with unequivocally low T levels and symptoms—sexual dysfunction, physical dysfunction, or low vitality—will determine whether T improves these symptoms and also address safety issues.

Preclinical animal studies have demonstrated increased bone and muscle mass and decreased fat mass without adverse effects on reproductive organs with SARMs (358, 442–445). Yet the few available human trials with SARMs haven't shown any beneficial effect on BMD or bone turnover markers, despite improvements in lean mass and physical performance (359, 446). Furthermore, the lack of aromatizability of most (nonsteroidal) SARMs remains a concern, given the possibility of (further) suppressing endogenous T and the importance of aromatization for the male skeleton.

3. Role of 5α-reductase

Part of the effects of T on the skeleton may be mediated by the more potent androgen DHT after conversion by 5α-reductase enzymes. 5α-Reductase type 2 is expressed predominantly in male reproductive tissues, whereas type 1 is moderately expressed in bone and also in other tissues (52). Finasteride, the most commonly used 5α-reductase inhibitor, is specific for type 2 and does not affect bone turnover, BMD, fracture risk (447–449), or the effects of androgens on muscle mass and the skeleton (337, 450). The skeletal effects of dutasteride (a dual type 1 and 2 inhibitor) are less well studied, but available evidence suggests that it also does not reduce BMD, increase bone turnover markers (448), or impair the muscle anabolic effects of T (336), suggesting that the conversion of T to DHT is not necessary for the effects of T on the musculoskeletal system in humans. 5α-Reductase type 1-inactivated male mice have slightly reduced bone mass (52) (see *Section II.B*), but the relevance of this finding to human bone physiology requires further investigation.

4. Role of SHBG

SHBG levels have also been associated with bone turnover markers, BMD, and BMD loss in older men (372, 378, 411, 412, 451). Polymorphisms in the promoter of the SHBG gene associated with serum levels of SHBG and hip BMD in elderly men (452). Several observational studies showed increased prevalent fractures with increased levels of SHBG (409, 411, 412). High SHBG levels were also independently associated with incident fracture risk in older men in most (408, 417, 418, 427), but not all studies (373, 410, 453) (Table 5). Interestingly, the MrOS USA study found evidence of an interaction between bioavailable T and SHBG: older men with low bioavailable T and high SHBG were at substantially increased risk of fracture, and the highest fracture risk occurred in men with low bioavailable E2, low bioavailable T, and high SHBG (418). Similar results were found when analyzing bone loss in the same cohort: men who had the lowest bioavailable E2, the lowest bioavailable T, and the highest SHBG experienced the fastest annualized rate of bone loss (378). A significant threshold was also found for SHBG at

Figure 12.

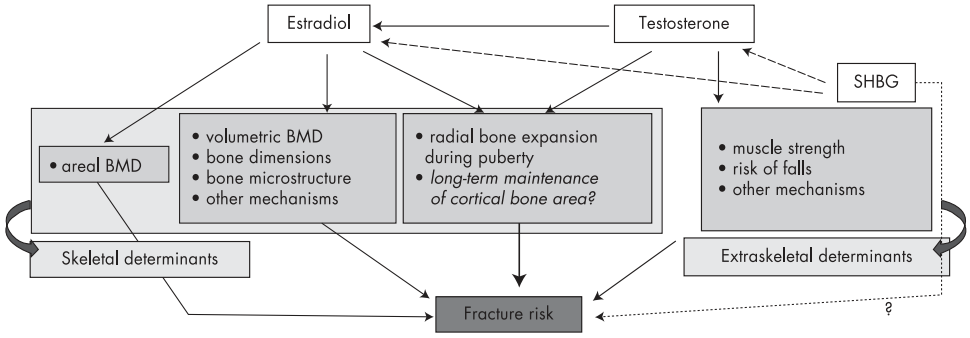

Figure 12. Proposed mechanism for the effects of E2, T, and SHBG on fracture risk in older men.

around 50 nmol/L; men with SHBG levels above this level experienced significantly faster rates of bone loss. This threshold is consistent with findings from the MrOS Sweden cohort in which men with SHBG levels in the highest quartile had an increased fracture risk (417). In the latter cohort, the greatest fracture risk was seen in men with low serum E2 and high SHBG levels. Serum SHBG levels are also related to nonskeletal fracture determinants like frailty (433, 454).

SHBG may affect fracture risk indirectly; by binding T and E2, SHBG reduces the free and bioavailable sex steroid fraction. However, there is discussion about whether SHBG may also enhance sex steroid sufficiency or deficiency, regulate the androgen/estrogen balance, or regulate mostly total sex steroid levels (because hypothalamic feedback mechanisms may correct free/bioavailable concentrations in vivo) (455, 456). Alternatively, it has been speculated that high levels of SHBG may directly potentiate sex steroid actions via putative membrane uptake of SHBG-sex steroid complexes or sequestration of sex steroids interstitially or intracellularly (41). Nonetheless, the results summarized here should be interpreted with caution because of strong correlations between each of the sex steroids and SHBG, particularly for the calculated free and bioavailable levels because these are derived from equations that already include SHBG (see also *Section II.A*). Furthermore, putative nonclassical actions of SHBG remain highly controversial, and it is not known whether these mechanisms bear any relevance to bone tissue. Finally, the observed associations could be due to residual confounding, eg, by IGF-1 levels, which may be associated with SHBG levels and predict fracture risk in older men (272).

In summary, strong evidence suggests that E2 plays a pivotal role in bone maintenance and fracture risk in aging men. These findings also suggest that each hormone—E2, T, and SHBG—or combinations thereof play a role in male bone health. The proposed mechanisms for the effects of serum E2, T, and SHBG on bone loss and fracture risk in men are depicted in Figure 12. Circulating E2 is consistently associated with BMD and bone loss and inversely predicts fracture risk. Because the association between serum E2 and fractures is only partially attenuated after adjustment for BMD, other mechanisms besides a BMD-dependent effect can also contribute to E2's effect on fracture risk. These can include effects on bone dimensions, vBMD, and bone microstructure, which cannot be captured by DXA. T has a moderate effect on fracture risk that appears independent of BMD but may be related to maintenance of bone formation markers, cortical bone area, and especially to extraskeletal factors such as muscle mass, strength, and propensity to fall. Nevertheless, because T also functions as the substrate for aromatization into E2, it can affect BMD and thus fracture risk indirectly via E2. Finally, SHBG may indirectly influence fracture risk by regulating the bioavailable concentrations of E2 and T or, alternatively, affect fracture risk directly.

Regardless of their importance in pathophysiology, recent guidelines on male osteoporosis (28) do not advise routine measurement of E2, T, or SHBG in the absence of symptomatic hypogonadism because: 1) mass spectrometry is required to accurately determine low E2 levels, but this is not routinely available in most clinical settings; 2) there are no validated algorithms available to incorporate the sex steroid or SHBG in clinical decision making, eg, demonstrating that their measurement improves fracture prediction beyond DXA results and clinical risk factors (457); and 3) there are no randomized trials of T showing fracture prevention, whereas traditional antiresorptive drugs with established antifracture efficacy in women have demonstrated similar efficacy in men, regardless of normal or low T levels (19, 458). However, T replacement is an option in older men with morning serum T repeatedly < 6.9 nmol/L (200 ng/dL) who are not candidates for

other osteoporosis treatments (28), in order to prevent the deleterious effects on bone that are mainly related to the concomitant estrogen deficiency.

X. Summary and General Conclusions

Although male osteoporosis constitutes a major burden for public health and can be effectively treated, underrecognition and undertreatment of this condition remains. Yet male gender is one of the strongest protective factors that approximately halves the risk of hip and other osteoporotic fractures (Figure 1). During the last decade, studies using (p)QCT and more recently HR-pQCT have refined our insights into the structural basis of sex differences in bone strength across the human lifespan (Figure 3).

The effects of androgens and estrogens on male bone health can be divided into two phases: 1) peak bone mass acquisition; and 2) subsequent maintenance. The male advantage in bone strength is mainly established during the first phase by placing cortical bone further from its central axis due to greater periosteal bone formation. Young adult women achieve similar cortical thickness by limiting endosteal expansion, but this doesn't provide the same biomechanical advantages. Men also have greater peak trabecular bone volume due to thicker, more plate-like trabeculae. However, these gender differences are probably site-specific and require further confirmation in more prospective studies.

How then do androgen and estrogen bioactivity regulate the establishment of this skeletal sexual dimorphism in cortical and trabecular bone? Human case reports of rare genetic conditions such as inactivating ERα mutations, aromatase deficiency, or AIS suggest that the lack of ERα activation is devastating for male cortical and trabecular bone acquisition, whereas androgen signaling via AR merely offers enhancement in these compartments in males. Genetic mouse models have allowed more systematic mechanistic studies, which confirm the dual mode of action of T on cortical and trabecular bone via both the AR and ERα. At the periosteal surface specifically, the stimulation by T in these models occurs mainly via the AR but also requires ERα as well as systemic GH/IGF-1 actions, which are regulated by ERα after aromatization (Figure 4).

Which target cells are then responsible for the skeletal effects of AR and ERα? Throughout the osteoblast lineage, AR decreases trabecular bone resorption in male mice, whereas there is moderate evidence that ERα in mature osteoblasts and osteocytes regulates trabecular bone in male mice. ERα in early osteoblasts is crucial for the cortical bone in female mice, whereas it exerts a transient effect on cortical bone mass in male mice (Table 2 and Figure 8). Direct effects of ERα in osteoclasts have been shown in female, but not male mice. In addition, ERβ seems to exert some repressive actions in the female murine skeleton, but a role in male mice or in humans remains to be established. Longitudinal growth has been known to be regulated by ERα in males, but only recently it was confirmed in mice that ERα in growth plate chondrocytes mediates growth plate closure, whereas ERα in non-bone/non-cartilage cells (probably via hypothalamic-pituitary regulation of the GH/IGF-1 axis) mediates longitudinal growth during early sexual maturation (Figure 7). Importantly, the target cell(s) for the AR effects on periosteal bone formation remains elusive, suggesting that sex steroid actions on the male skeleton may also rely on other organs. This is most evident for the effects of androgens on muscle in humans. Additional examples from mice include effects of ERα and AR on oxidative stress and possibly other organ systems, which requires further confirmation and investigation in humans.

How do AR and ERs regulate bone cells at the molecular level? With a few exceptions like FasL and alkaline phosphatase regulation by ERα in osteoblasts, the direct transcriptional targets of sex steroid actions in bone cells remain poorly understood. Nongenomic signaling pathways and specific actions of different ERα domains have shown promise as potential skeletal modulators in vivo. AR and ERα also cross talk with other signaling pathways and transcription factors in bone cells, as has been shown for GH/IGF and RUNX2. These molecular mechanisms require confirmation in humans.

Apart from their favorable influence on peak bone mass, how do sex steroid actions influence the second phase of male skeletal maintenance? Age-related bone loss is generally similar between men and women, only men lack an accelerated phase of bone loss compared to perimenopausal women, probably because men better maintain their bioactive sex steroids and especially estrogen levels. In rodent models, both ERα- and AR-mediated effects are required for male cortical and trabecular bone maintenance. In humans, however, recent epidemiological and interventional studies show that (bioactive) E2 levels in aging men are pivotal determinants of bone loss, microarchitectural decay, and increased fracture incidence. Yet the highest fracture risk is observed in men with a combination of low E2, low T, and high SHBG. Contrary to the situation for E2, the associations of T and SHBG with fractures appear largely independent of BMD, thereby suggesting that extraskeletal parameters like sarcopenia, frailty, and falls determine such associations (Figure 12). Interventional studies show that androgens alone cannot overcome bone loss due to (severe) estrogen defi-

ciency in aging men. On the other hand, none of the existing studies have examined the possibility of an independent skeletal effect of androgen deficiency in the face of (relatively) normal estrogen levels. Androgens could still have an independent effect on bone formation markers and at the periosteal surface, but the latter may not become evident in short-term interventional studies.

Regardless of their importance in pathophysiology, recent guidelines in male osteoporosis rightly point out that the role of T replacement in men with late-onset hypogonadism remains controversial. Evidence supporting the clinical utility of serum sex steroid measurements for fracture prediction beyond clinical risk factors and BMD also remains lacking. Further studies on sex steroid signaling in the musculoskeletal system are a high research priority because they may help in the design of additional, preferentially gender-neutral, therapeutic strategies to reach the ultimate goal of not merely preventing bone loss but also reinforcing the musculoskeletal system as a whole to prevent osteoporotic fractures in both genders.

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