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REVIEW

The role of estrogen receptor α in the regulation of bone and growth plate cartilage

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Abstract Estrogens are important endocrine regulators of skeletal growth and maintenance in both females and males. Studies have demonstrated that the estrogen receptor (ER)-α is the main mediator of these estrogenic effects in bone. Therefore, estrogen signaling via ERα is a target both for affecting longitudinal bone growth and bone remodeling. However, treatment with estradiol (E2) leads to an increased risk of side effects such as venous thromboembolism and breast cancer. Thus, an improved understanding of the signaling pathways of ERα will be essential in order to find better bone specific treatments with minimal adverse effects for different estrogen-related bone disorders. This review summarizes the recent data regarding the intracellular signaling mechanisms, in vivo, mediated by the ERα activation functions (AFs), AF-1 and AF-2, and the effect on bone, growth plate and other estrogen responsive tissues. In addition, we review the recent cell-specific ERα-deleted mouse models lacking ERα specifically in neuronal cells or growth plate cartilage. The newly characterized signaling pathways of estrogen, described in this review, provide a better understanding of the ERα signaling pathways, which may facilitate the design of new, bone-specific treatment strategies with minimal adverse effects.

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General introduction

Estrogens are important endocrine regulators of skeletal growth and maintenance in both females and males. Experimental and human studies have demonstrated that the estrogen receptor (ER)-α is the main mediator of these estrogenic effects in bone [1–8]. Therefore, estrogen signaling via ERα is a target both for affecting longitudinal bone growth and for bone remodeling. 17β-estradiol (E2) is the most potent estrogen and it is important for preventing bone loss. E2 also affects longitudinal bone growth and low levels of E2 during sexual maturation is essential for the longitudinal growth spurt, whereas high E2 levels in late puberty result in growth plate closure and thereby cessation of longitudinal bone growth in humans [9]. Since E2 is important for the different stages of longitudinal bone growth, it is possible to affect the longitudinal bone growth by manipulating the estrogen signaling in young patients with idiopathic short stature or constitutionally tall stature. Estrogen deficiency after menopause reduces bone mineral density (BMD), which leads to an increased risk of fractures. Therefore, it is possible to prevent bone loss in postmenopausal women by E2 administration [10]. However, there are several estrogen responsive tissues expressing ERs, e.g., the reproductive system, central nervous system (CNS), and blood vessels [11–13]. Therefore, estrogen treatment would not only have positive effects on the bone but could also lead to an increased risk of adverse effects, such as venous thromboembolism and breast cancer [14, 15]. Thus, it would be beneficial to develop bone-specific estrogen treatments. To achieve this, it is important to further characterize the estrogenic signaling pathways in bone versus other tissues in vivo.

Growth plate physiology

The growth plates consist of three principal zones of chondrocytes: the resting zone, the proliferative zone, and the hypertrophic zone, where the hypertrophic chondrocytes are the most differentiated [16, 17]. Longitudinal bone growth of the long bones occurs at the growth plates through a process called endochondral ossification. In this process, cartilage is formed and then replaced by bone tissue at the metaphyseal border of the growth plate [18].

Low E2 levels enhance skeletal growth during early sexual maturation (i.e. the pubertal growth spurt), whereas high E2 levels during late puberty result in growth plate fusion and thereby cessation of longitudinal bone growth in humans [9]. The mechanisms of action for these two seemingly opposite effects of estrogens on longitudinal bone growth are not fully understood but clearly depend on maturational stage and serum levels of E2 [9, 19]. Estrogens are crucial regulators of the growth hormone/insulin-like growth factor 1 (GH/IGF-I) axis, and, therefore, some of the effects of estrogens on skeletal growth may be indirect via modulation of the GH/IGF-I axis, while other effects may be direct [9, 20–22]. Low-dose E2 treatment has been shown to increase serum GH and IGF-I, which may contribute to the pubertal growth spurt. An effect via the GH/IGF-I axis is supported by the fact that ER blockade down-regulates the GH/IGF-I axis [3, 9]. A key role for E2 in the regulation of longitudinal bone growth and growth plate closure in humans was demonstrated by the findings that both males and females with estrogen deficiency, caused by a mutation in the aromatase gene, do not show a pubertal growth spurt and continue to grow after sexual maturation due to unfused growth plates [3, 23–26]. ERα was demonstrated to be the main mediator of this effect when a man with a similar phenotype was shown to have a non-functional ERα due to a point mutation in exon 2 (estrogen-resistant man). This man had elevated serum E2 levels, to which he was non-responsive [6, 27]. In contrast, the growth phenotype of the aromatase deficient patients could be rescued by E2 treatment [3, 23–25]. The mechanism for the estrogenic effects on growth plate fusion is not well understood. However, it has been demonstrated that estrogen accelerates the fusion of the growth plate in rabbits by advancing the senescence of the growth plate via proliferative exhaustion of the chondrocytes [28].

Estrogen and bone remodeling

The process in which osteoclasts resorb bone and osteoblasts form new bone is called bone remodeling. This process is important for enabling the bone to respond and adapt to load induced strain, replace old or damaged bone tissue, and to maintain calcium homeostasis [29]. Estrogen is important for maintaining the balance between bone resorption and bone formation. At menopause, when the E2 levels drop, there is an imbalance in bone resorption and bone formation leading to an accelerated bone loss [1]. It has been suggested that, although the trabecular bone loss is accelerated at menopause, the trabecular bone is not as sensitive as the cortical bone to the estrogen deprivation at menopause, since the trabecular bone loss starts already in young adult life when the levels of estrogens are normal [1].

Estrogen has fundamental effects on bone metabolism by several different mechanisms, acting both directly and indirectly on the skeleton. Estrogen affects the expression of certain factors in osteoblastic cells, e.g., it increases osteoprotegerin (OPG) and decreases receptor activator of nuclear κB ligand (RANKL) and tumor necrosis factor (TNF)-α, resulting in suppressed bone resorption [30–32]. It has been suggested by Khosla et al. [1]that the most consistent effect of estrogen on bone is to (1) induce commitment of precursor cells to the osteoblast lineage at the expense of the adipocyte lineage and (2) prevent apoptosis of osteoblastic cells [1, 33–37]. In addition, estrogen also reduces bone resorption by inhibiting differentiation and promoting apoptosis of osteoclasts [36, 38–42].

Structure of estrogen receptors

The first estrogen receptor discovered, ERα, was cloned in 1986, although its existence had already been suggested in 1962 [43–45]. Ten years later, a second estrogen receptor, ERβ, was cloned [46]. In 2005, there were reports suggesting that a seven-transmembrane G protein-coupled receptor (GPCR) named G protein-coupled ER-1 (GPER-1 or GPR30) was a membrane-associated ER [47–49]. However, there are contradictory studies suggesting that GPER-1 is not an estrogen receptor [49–53].

Both ERα and ERβ belong to the nuclear receptor superfamily of ligand-activated transcription factors, and they can dimerize to form both homo- and heterodimers. The primary structure of these receptors is divided into six functional domains, A–F, which display, overall, high sequence homology (Fig. 1) [46, 54, 55]. The A/B domains in the N-terminus contain the ligand-independent activation function-1 (AF-1) [56]. The C domain comprises the DNA binding domain (DBD) and the area important for dimerization. The D domain contains the nuclear localization signal and is also a hinge, which increases the flexibility between the C- and E/F domains. The ligand binding domain is found in the E/F domains in the C-terminus together with the ligand-dependent AF-2 [46, 55–57]. The most abundant ERα isoform is the 66-kDa protein, but there is also a truncated, less expressed 46-kDa ERα isoform that lacks most of the A/B domains (Fig. 1) [58].

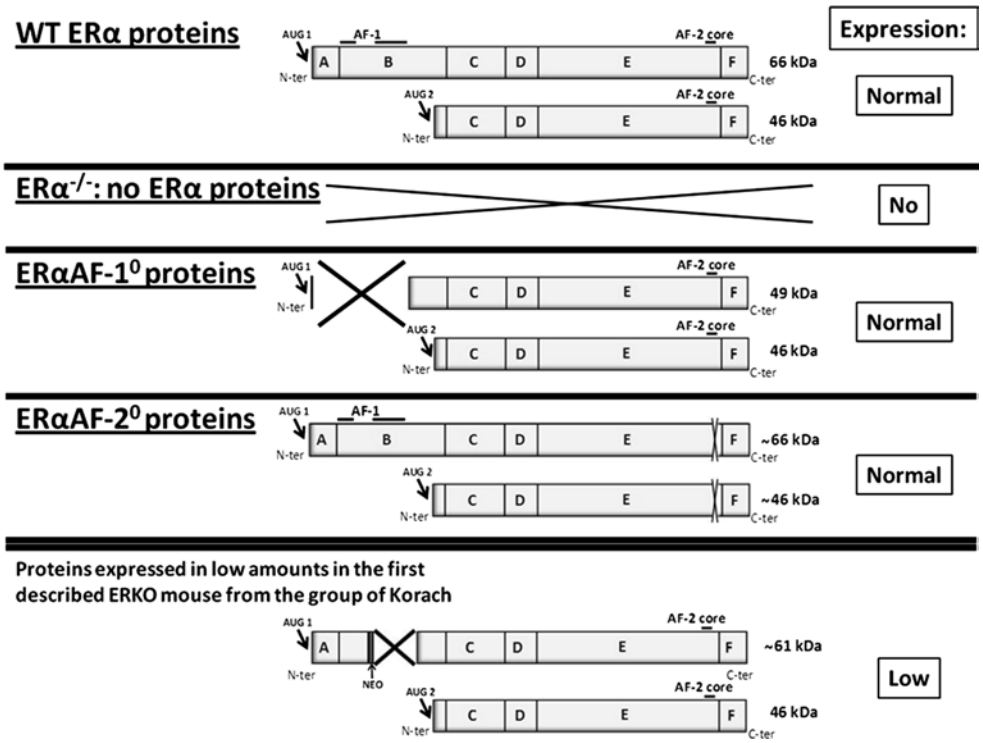

Fig. 1 Schematic presentation of the ERα proteins expressed in the different mouse models. There are two wild-type (WT) ERα isoforms; the full length 66-kDa protein and the less abundant, 46-kDa protein. The ERα$^{-/-}$ mice do not express any ERα protein. The ERαAF-1^0 mice have a normal expression of a 49-kDa protein, lacking amino acids 2–148 and also the 46-kDa isoform. The ERαAF-2^0 mice have a normal expression of two ERα proteins, which are slightly smaller than the WT proteins due to the deletion of amino acids 543–549. The first described ERKO mice from the group of Korach have a low expression of a 61-kDa isoform, lacking amino acids 92–155 which are replaced by 7 amino acids from the Neomycin sequence (NEO), and also an expression of the normal 46-kDa isoform. Adapted and reproduced with permission from *Am J Physiol Endocrinol Metab* (2012) 302:E1381–1389

Both AFs are important for recruiting cofactors essential for gene regulation. The cofactors that bind to the AFs are suggested to be dependent on the ER, the cell type and promoter context [59, 60]. When a ligand binds to an ER, the ER undergoes a conformational change so that helix 12 in the ligand binding domain folds in the agonistic orientation [61]. Helix 12 has a key role in forming the ERα AF-2, together with helices 3, 4, and 5, which can attract cofactors important for gene regulation, hence the AF-2 is ligand-dependent [61–63]. Although the ERβ AF-1 is suggested to be weaker than the ERα AF-1 [64], they both interact with cofactors independently of ligand being bound to the receptor or not, and therefore the AF-1 is ligand-independent [56, 65]. In vitro studies of ERα have shown that the E2-induced transactivation is dependent on either one of the AFs but that most promoter contexts require synergism between them for full transcriptional activity [56, 59, 66–68].

ERα is the main ER in bone

The bone-sparing effects of estrogen are primarily mediated via ERα. This has been demonstrated using different mouse models lacking ERα, ERβ, both ERα and ERβ, or GPER-1. ERα has been shown to be crucial for mediating the estrogenic effects in both trabecular and cortical bone, while ERβ plays a less important role and GPER-1 has been demonstrated to be dispensable for the estrogenic preservation of bone mass [2, 4, 5, 49, 69, 70]. Female and male mice that are estrogen deficient due to ovariectomy (ovx) or orchidectomy (orx) lose bone. However, both trabecular and cortical bone mass can be restored by E2 treatment in both genders. In contrast, the bone mass cannot be restored in E2-treated ovx or orx mice lacking ERα (ERα$^{-/-}$ mice), demonstrating a crucial role for ERα in both the female and male skeleton [2, 4, 5, 69]. ERβ has only been shown to slightly modulate the effects of ERα in female mice but not in male mice [2, 8, 71–73]. This modulating effect of ERβ was shown by studying female adolescent ERβ$^{-/-}$ mice. These mice displayed an increased cortical bone mineral content but no trabecular phenotype [73]. One-year-old female ERβ$^{-/-}$ mice were also studied and shown to maintain the cortical phenotype seen in the adolescent mice, but also to have a higher trabecular bone mass compared to age-matched controls [72]. This suggests that ERβ normally has a slightly inhibiting effect

on ERα in the female bone [72, 73]. In addition, estrogen-regulated transcriptional activity in bone was evaluated in female ERα$^{-/-}$, ERβ$^{-/-}$, and ERαβ$^{-/-}$ mice, demonstrating that ERβ reduces ERα-regulated gene transcription in bone in the presence of ERα but partially replaces ERα in the absence of ERα [74]. Although both ERα and ERβ have an AF-1, this region is not well conserved. The ERβ AF-1 is weaker than the ERα AF-1, and it has been shown that the estrogenic effect mediated by ERα is slightly repressed when an ERα/ERβ heterodimer is formed [64].

Signaling pathways of estrogen

Primary target cells involved in bone regulation

Both ERs have been shown to be expressed throughout the skeleton in osteoblasts, osteoclasts, osteocytes, and chondrocytes [75–79]. The expression of ERs in these different bone cells demonstrates that the estrogenic effects can be exerted locally in the skeleton. The relative importance of the different estrogenic target cells for bone regulation is not yet completely understood. It would be valuable to fully characterize the primary target cells for estrogen, and how these cells exert effects on bone regulation when stimulated with E2, in order to develop cell-specific treatments. To investigate the importance of ERs in the different bone cells for the estrogenic skeletal effects, mouse models have been developed that specifically lack ERα in one cell type. Specific deletion of ERα in osteoclasts in female, but not male, mice led to trabecular bone loss, and this effect was due to a decreased apoptosis of the osteoclasts [38, 39]. Further experiments are required in order to find the target cells for the estrogenic effects in trabecular bone in male mice and in cortical bone in both male and female mice.

Bone is traditionally considered to be regulated by peripheral signaling, which is controlled by hormones (e.g., estrogen), autocrine/paracrine signals, and mechanical loading. However, bone is an innervated tissue and, lately, central signaling, via the CNS, has also been recognized to have a major role in bone regulation [80]. The first clear evidence showing this was when mice deficient of leptin were studied. These mice had, despite being hypogonadal, a high bone mass phenotype, which was reduced by intracerebroventricular injections of leptin [81]. In addition, mice lacking the β2-adrenergic receptor, which binds the main sympathetic neurotransmitter noradrenaline, were resistant to the central bone-reducing effects of leptin, demonstrating that central leptin signaling may exert its negative effects on bone via the sympathetic nervous system [82, 83].

Intracellular signaling

At the cellular level, the ERs are generally considered to have four signaling pathways, three of which are classified as ligand-dependent and one which is classified as ligand-independent [84]. The ERs are transcription factors that either directly or indirectly bind to the DNA. The AF-1 and AF-2 in the ERs are important for recruiting certain coactivators, which in turn can interact with the transcriptional preinitiation complex and thereby initiate transcription of specific estrogen-regulated genes [85, 86]. There are two genomic ligand-dependent signaling pathways involving dimerization and translocation of the ERs from the cytosol to the nucleus when ligand binds; (1) in the classical (direct) genomic pathway the ER-ligand complex binds directly to estrogen response elements (ERE) on the DNA and regulates gene transcription, and (2) in the non-classical (indirect) genomic pathway, other transcription factors are bound to the DNA and the ER-ligand complex binds to these transcription factors and thereby regulates gene transcription [54, 84, 87]. Fos/Jun and SP-1 are examples of transcription factors involved in the non-classical genomic pathway and these transcription factors can interact with both the ERs and specific sites on the DNA, not harboring EREs. Fos/Jun proteins form the transcriptional complex AP-1 (activator protein 1), which interacts with AP-1 responsive elements in the DNA [88, 89]. The SP-1 (specificity protein 1) complex interacts with GC-rich SP-1 motifs in the DNA [90]. Interaction between the ER-ligand complex and the described transcription factors results in activation of gene transcription. The ligand-dependent non-genomic pathway is not very well understood. The rapid responses of this pathway imply that no gene regulation occurs. Instead, it involves signaling cascades, which are activated when ligand binds to an ER that could be located either in the membrane or in the cytoplasm [84, 91]. Some of these rapid responses to E2 are mediated by second messenger systems, e.g., cAMP and protein kinase A (PKA), while other responses are mediated by membrane-based ion fluxes, involving, e.g., Ca^{2+} and Ca^{2+}-dependent K^+ channels, which are capable of responding to estrogens [91, 92]. GPER-1 has been suggested to be involved in this non-genomic pathway and there is also some evidence for a membrane-localized ERα [47, 49, 93]. The ligand-independent pathway does not involve any ligand binding to the ER. Instead, other factors are involved (e.g., growth factors (GFs) like epidermal growth factor (EGF) and IGF) that can activate extracellular-signal-regulated kinases (ERKs) which in turn can activate the ER by phosphorylation [84, 94–97]. It has also been shown that phosphorylation of the ER increases after E2 binding [98]. The phosphorylated ER translocates to the nucleus where it will recruit coactivators and mediate gene transcription [84, 98].

ERα has several sites for phosphorylation and most of them are found in the AF-1 [99].

Estrogen and modulation of longitudinal bone growth

Estrogens are important both for the longitudinal growth spurt and for cessation of growth by inducing growth plate closure [9]. It is possible to manipulate the longitudinal bone growth by affecting the estrogenic signaling pathways. High-dose E2 treatment will result in growth plate closure and thereby cessation of growth, leading to a shorter adult stature than expected. In contrast, treatment with aromatase inhibitors (attenuating the conversion of androgens into estrogens) will delay the closure of the growth plates, thereby increasing the final height [100–102]. The long-term effects of high-dose estrogen treatment or treatment with aromatase inhibitors are only recently being evaluated. It has been suggested that high-dose estrogen treatment in adolescent girls may lead to reduced fertility later in life [103–105]. There are also concerns for increased risks of breast or gynecological cancers [101, 106]. In addition, boys treated with aromatase inhibitors were shown to have a high rate of vertebral body deformities after treatment [107]. Further characterization of the estrogenic signaling pathways in bone and growth plate will therefore be important in order to find better treatments for manipulating longitudinal bone growth, without increasing the risk of adverse effects later in life.

Selective ER modulators (SERMs)

SERMs have the ability to bind to an ER and act as an ER agonist or antagonist in a tissue-specific manner. Tamoxifene was the first developed SERM and is used for breast cancer treatment since it has an antagonizing effect in breast tissue. In addition, Tamoxifene was shown to be an agonist in bone but unfortunately also an agonist in uterus, increasing the risk of endometrial cancer [108, 109]. Another SERM, Raloxifene, shown to be an ER agonist in bone and an ER antagonist in breast, was the first SERM approved for the prevention and treatment of postmenopausal osteoporosis [110]. More SERMs have been developed, but all available SERM treatments for osteoporosis today, including Raloxifene, will not only lead to a reduced fracture risk but also to adverse effects such as increased risk of thromboembolism [110–112]. Therefore, further studies of the signaling pathways of estrogen are required in order to find more specific SERMs.

In vitro studies have shown that the SERMs have a bulky side chain, which upon binding to ERα protrudes from the ligand binding pocket. This hinders the optimal conformational change of the ligand binding domain of the ERα by preventing the folding of helix 12 in the agonistic orientation. Instead, helix 12 is able to bind to the static region of ERα AF-2; formed by residues from helices 3, 4, and 5. Helix 12 then prevents/limits the interaction between the static region of ERα AF-2 and certain coactivators and corepressors. Because of the lack of cofactors binding to the ERα AF-2 after SERM binding, ERα AF-1 is suggested to be the main mediator of the SERM effects [59, 61–63, 113–115]. Variation in the expression of cofactors and the recruitment of cofactors to the ER in different cell types appear to have an important role for the tissue-specific effects of the SERMs [61, 114]. Regarding treatment strategies for osteoporosis, development of new SERMs that have the positive effects of estrogen in bone but lack the negative effects in other tissues would be optimal. In addition, a SERM treatment directed specifically towards the growth plate in constitutionally tall adolescents could reduce their final height without leading to systemic adverse effects. To achieve this, the characterization of the ER signaling pathways in bone versus other tissues is of importance. Since the phenotypes of the different global ER-deleted mouse models have been described in detail in previous reviews [116–118], we do not focus on these mouse models in this review.

Roles of transactivating functions 1 and 2 of ERα in bone

The two transactivation functions; ERα AF-1 and AF-2, have been shown, in vitro, to interact with cofactors (coactivators and corepressors) and are therefore important for mediating the gene-regulating effects of ERα. Further characterization of these AFs in vivo is therefore of importance in understanding whether they can act independently of each other to mediate gene regulation and whether this is dependent on the tissue. Evaluation of these AFs in vivo was possible after the development of two new mouse models lacking either the ERα AF-1 (ERαAF-1^0; [119]) or the ERα AF-2 (ERαAF-2^0; [69]). The ERαAF-1^0 mice have a deletion of 441 bp of exon 1, corresponding to amino acid (aa) 2–148, with a preserved translational initiation codon in exon 1 (ATG1) (Fig. 1). The ERαAF-1^0 mice do not express any full-length 66-kDa protein [69, 119]. Instead, they express a truncated 49-kDa ERα protein that lacks AF-1 and also the physiologically occurring but less abundantly expressed 46-kDa ERα isoform, initiated by a second translational initiation codon in exon 2 (ATG2). ERαAF-2^0 mice have a deletion of the AF-2 core, which resides within exon 9 and corresponds to aa 543–549 (Fig. 1) [69]. The sizes of the ERαAF-2^0 proteins are slightly smaller than the WT ERα proteins of 66 and 46-kDa, respectively (corresponding to

the 7 aa truncation located in the AF-2 region; [69]). In order to evaluate the involvement of the AFs for the bone protective effects of estrogen the ERαAF-1^0 and ERαAF-2^0 mice were studied together with mice completely devoid of ERα protein (ERα$^{-/-}$; [120]) and WT control mice.

The ERαAF-1^0, ERαAF-2^0, and ERα$^{-/-}$ mice were shown to have elevated serum levels of E2, luteinizing hormone (LH) and testosterone compared to WT controls, demonstrating that these mouse models all have a disturbed negative feedback regulation of serum sex steroid levels [69]. Thus, both intact ERα AF-1 and AF-2 are important for a normal negative feedback regulation of serum sex steroids. Because of the elevated sex steroid levels in these mouse models, gonadal-intact mice were not ideal for studying the estrogenic response. Therefore, only ovx mice were studied when evaluating the roles of ERα AF-1 and AF-2 for the estrogenic effects in bone and other estrogen responsive tissues. The ovx ERαAF-1^0, ERαAF-2^0, ERα$^{-/-}$, and WT control mice were treated with either E2 or placebo, and thereafter the estrogenic effects on the skeleton and other estrogenic target tissues were evaluated. E2 treatment increased the amount of trabecular and cortical bone in ovx WT mice. Neither the trabecular nor the cortical bone responded to E2 treatment in ovx ERα$^{-/-}$ or ovx ERαAF-2^0 mice. Interestingly, ovx ERαAF-1^0 mice displayed a normal E2 response in cortical bone but no E2 response in trabecular bone (Fig. 2) [69]. This was shown by performing both pQCT and μCT analyses on femurs and μCT analyses on the L_5 vertebrae, demonstrating that the ERαAF-1^0 mice had a normal E2 response on cortical thickness and vBMD, while there was no response on trabecular bone volume/tissue volume (BV/TV) or trabecular number. Also, immune parameters and the major estrogen responsive tissues, uterus and liver, were evaluated. It was demonstrated that none of the tissues evaluated showed any estrogenic response in the ovx ERα$^{-/-}$ or ovx ERαAF-2^0 mice. However, the estrogenic response in ERαAF-1^0 mice was tissue-dependent with no significant estrogenic response on thymus, a clearly reduced estrogenic response on uterus weight and bone marrow cellularity, an intermediate response on the frequency of B-lymphocytes in the bone marrow, and a normal response on liver weight (Fig. 2) [69]. Together, these results suggest that ERα AF-2 is required for all the estrogenic effects in the evaluated tissues while the role of ERα AF-1 is tissue-dependent. This implies that, while some tissues are completely dependent or independent of ERα AF-1, other tissues require both ERα AF-1 and AF-2 for a full estrogenic effect.

Lately, it has been suggested that the trabecular and cortical bone compartments are different and that they could have somewhat different estrogenic signaling mechanisms [1, 39, 121]. Data supporting that the cellular environment as well as the specific cell type decide the expression levels of different cofactors have also been reported [122–124]. The mechanism(s) behind the crucial role of ERα AF-1 in the trabecular but not the cortical bone is still unknown.

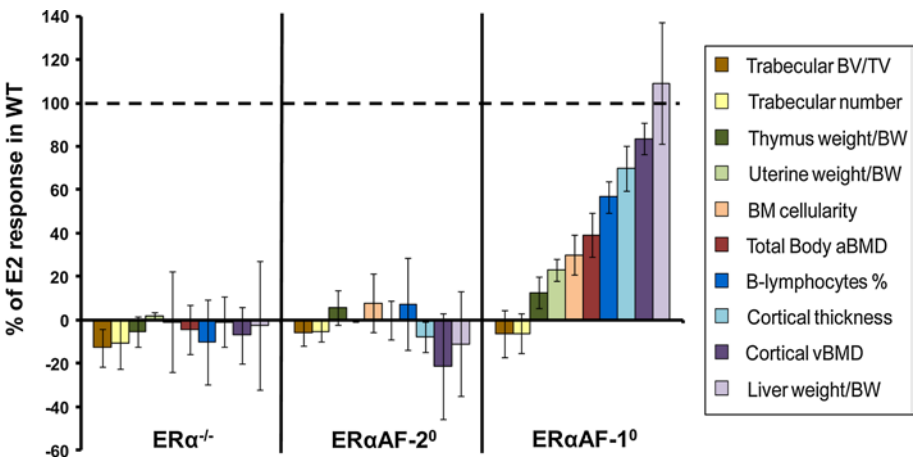

Fig. 2 The role of ERα AF-1 is tissue-dependent. Ovariectomized (ovx) ERα$^{-/-}$, ERαAF-2^0 and ERαAF-1^0 and their corresponding wild-type (WT) mice were treated with either vehicle or estradiol (E2) for 4 weeks. As expected, E2 treatment resulted in a significant effect on several estrogen responsive bone parameters (increased total body aBMD, cortical thickness, cortical volumetric BMD, trabecular BV/TV, and trabecular number), bone marrow parameters (reduced bone marrow cellularity and frequency of B-lymphocytes), and non-bone parameters (increased uterine weight and liver weight but reduced thymus weight) in ovx WT mice. To illustrate the role of ERα AF-1 and ERα AF-2 for the effect of E2 on these parameters, the estrogenic response in E2 treated ovx WT mice is for each parameter set to 100 %. The *bars* represent the estrogenic response in % for the E2-treated ovx ERα$^{-/-}$, ERαAF-2^0, and ERαAF-1^0 mice compared to the E2 response in their ovx WT mice, respectively. Thus, 0 % means no E2 response while 100 % is a normal WT E2 response. Values are mean ± SEM (n = 8–12). *BM* bone marrow, *BV/TV* bone volume/total volume, *BW* body weight, *vBMD* volumetric bone mineral density, *aBMD* areal bone mineral density. Reproduced with permission from *Proc Natl Acad Sci USA* (2011) 108:6288–6293

A possible explanation for this difference may be that the trabecular and cortical bone compartments have different cofactor expression patterns and this would then explain why the estrogen signaling pathways in these bone compartments differ. One possible pathway involves the steroid receptor coactivator (SRC)-1, which has been shown, in vitro, to be cooperatively recruited by AF-1 and AF-2 in ERα and thereby mediate synergism between the two AFs [125]. Another study has shown that different cell types express different amounts of SRC-1, which in turn lead to different responses to SERM treatment. Since SERMs are suggested to act mainly via ERα AF-1, these results suggestthat SRC-1 is important for mediating the effect of SERMs by interacting with ERα AF-1 [126, 127]. In addition, when mice with a specific inactivation of SRC-1 (SRC-1 KO) were ovx and treated with E2, the normal estrogenic response was absent in the trabecular bone, while it was normal in cortical bone [121]. Thus, the estrogenic response in the SRC-1 KO and ERαAF-1^0 mice showed a similar pattern. This suggests that SRC-1 is involved in the AF-1-dependent E2 effects in trabecular bone but not in the AF-1-independent E2 effects in cortical bone in female mice [69].

It has also been suggested that the estradiol-mediated phosphorylation of residues in ERα AF-1 is different, dependent on the cell type [98]. This suggests that phosphorylation of residues of ERα AF-1 could be involved in the AF-1-dependent estrogenic effects in trabecular bone but not in cortical bone. This may be due to different expression patterns of kinases and/or phosphatases in trabecular and cortical bone.

Finding the cell types responsible for the estrogenic effects in bone have been the focus of several research groups during recent years. So far, ERα has been specifically inactivated in osteoclasts [38, 39] using the Cre-loxP system in mice. These studies have shown that ERα in osteoclasts is of importance for the estrogenic effect in the trabecular but not the cortical bone compartment in female mice [38, 39]. These findings, together with the results from ERαAF-1^0 mice, suggest that ERα AF-1 is crucial for the estrogenic effect in trabecular but not cortical bone, and that estrogen preserves the trabecular bone via osteoclast ERα involving AF-1 [69].

The mice lacking ERα AF-1 have also been reported to have retained vasculoprotective effects of E2 treatment [119]. The results from the mice lacking ERα AF-1 or AF-2 could be useful for the development of new SERMs, and suggest that SERMs activating ERα AF-1 minimally could exert beneficial actions in cortical bone and the vascular system, while minimizing the AF-1-dependent effects on reproductive organs [69].

The role of ERα and its AF-1 for growth plate closure in female mice

Although the rodent growth plates do not fuse during their lifetime, elderly mice do have a reduced longitudinal bone growth, and growth plate closure can be induced in adult mice by high-dose E2 treatment [128]. The estrogen-resistant man, lacking a functional ERα, had a continued linear growth long into adulthood, due to unfused growth plates [6, 27]. A mouse model, believed to lack the entire ERα (K-ER$\alpha^{-/-}$; [129]) had an opposite growth plate phenotype compared to the estrogen-resistant man, in that these mice completely fused their growth plates [130, 131]. However, the K-ER$\alpha^{-/-}$ mouse model, developed in the Korach and Smithies laboratories, was later shown to have low expression of truncated ERα isoforms, lacking AF-1 (Fig. 1) [129, 132–134]. The question then arose whether these truncated ERα isoforms could explain the opposite effects in the

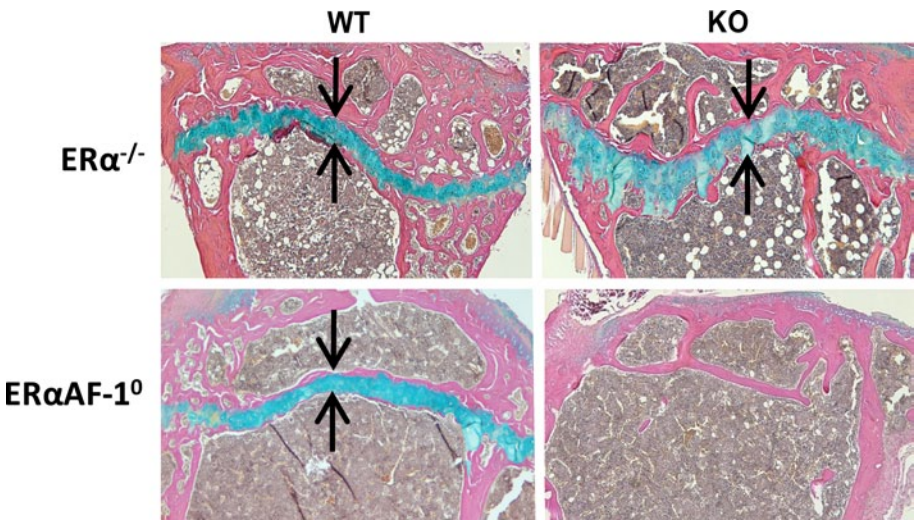

Fig. 3 Altered growth plate heights in old ER$\alpha^{-/-}$ and ERαAF-1^0 mice. Representative pictures of growth plates from proximal tibiae of old female ER$\alpha^{-/-}$ and ERαAF-1^0 mice and their wild-type (WT) controls. *KO* knock-out. Adapted and reproduced with permission from *Am J PhysiolEndocrinol Metab* (2012) 302:E1381–1389

growth plates in mouse and man, and also what role ERα AF-1 has in regulating growth plate fusion. To answer these questions, both longitudinal bone growth and growth plate closure were studied in female ERα$^{-/-}$ [120] and ERαAF-1^{0} mice [119, 132].

Young adult (4 months old) female ERα$^{-/-}$ mice had normal longitudinal bone growth. Interestingly, old (16–19 months) female ERα$^{-/-}$ mice showed continued longitudinal bone growth, resulting in longer bones, associated with increased growth plate height compared with WT mice (Fig. 3). The increased growth was not associated with any changes in serum IGF-I levels [132]. These results showed that the ERα$^{-/-}$ mice and the estrogen-resistant man, with a dysfunctional ERα, have both increased longitudinal bone growth and wide, unfused growth plates [6], confirming the ERα to be the main mediator of the estrogenic effects on growth plate reduction in mouse and growth plate closure in man [132].

In contrast, the longitudinal bone growth in ERαAF-1^{0} female mice had already ceased in the young adult mice, and this was even more pronounced in the old ERαAF-1^{0} female mice, due to growth plate closure (Fig. 3). The changes in longitudinal bone growth were not related to changes in the serum IGF-I levels [132]. These results are similar to what was earlier shown in the K-ERα$^{-/-}$ mice [130], but more specifically show that it is the lack of the ERα AF-1 that is responsible for this phenotype. In a study of growth plate fusion in rabbits, it was demonstrated that estrogen accelerates growth plate fusion by advancing the senescence of the growth plate via proliferative exhaustion of the chondrocytes [28]. Consistent with these findings, the ERαAF-1^{0} mice, prior to growth plate closure, displayed reduced height of the proliferative zone in the growth plate [132]. In addition, immunostaining showed that there was a tendency towards a reduction in chondrocyte proliferation and an increase in chondrocyte apoptosis. This led to a significantly altered balance between chondrocyte apoptosis and proliferation, which is suggested to be involved in the mechanism for growth plate closure in the ERαAF-1^{0} mice [132].

The signaling of estrogen via the ERα involves the interaction of cofactors with the ERα AFs. It is likely that there are cofactors specific for ERα AF-1 that have repressive effects on the ERα-mediated growth plate senescence pathway. This could explain the growth plate phenotype in the ERαAF-1^{0} mice, where the ERα AF-1 interaction with corepressors would not be present and, thus, the repressive effect of ERα AF-1 on the growth plate senescence would be lost. The premature growth plate fusion, and thereby cessation of longitudinal bone growth, in the ERα AF-1-deficient mice could, therefore, be the result of a hyperactive ERα that is activated by the ERα AF-2 with no interference from the ERα AF-1 [132]. However, ERαAF-1^{0} mice have been shown to have elevated serum levels of E2 which could lead to the threshold level for estrogen-induced growth plate closure being reached. This would mean that the AF-1-deleted ERα still has the capacity to mediate growth plate closure when exposed to supra-physiological E2 levels. Regardless of the process occurring in the growth plate, these results suggest that growth plate closure is induced by functions of the ERα that do not require AF-1 [132].

Although the growth plate physiology in mouse and human is different, and one should be careful to extrapolate data from mice to humans, the recent results from the ERα$^{-/-}$ and ERαAF-1^{0} mice indicate that mice are more similar to humans than was previously believed [132]. The prolonged substantial longitudinal bone growth observed in elderly ERα$^{-/-}$ mice resembles the continued post-pubertal growth seen in humans with ERα inactivation or aromatase deficiency. In contrast, the spontaneous growth plate closure in the ERαAF-1^{0} mice implies that it may be beneficial to use ERαAF-1^{0} mice as a model for studying E2-mediated signaling pathways in growth plate closure. Also, it would be interesting to study whether some patients with short stature have an inactivating mutation in the ERα AF-1 region [132].

The role of ERα in growth plate cartilage for longitudinal bone growth

The effects of estrogens on longitudinal bone growth and growth plate closure are generally believed to be mediated either indirectly via regulation of the GH/IGF-I axis or via direct effects on locally expressed ERα in the growth plate chondrocytes. Until the development of a mouse model lacking ERα specifically in cartilage (Col2α1-ERα$^{-/-}$; [135]), it was not possible to evaluate the relative roles of these two pathways for the effects of estrogens on longitudinal bone growth in vivo.

The Col2α1-ERα$^{-/-}$ mice were developed by crossing mice expressing Cre recombinase under the control of the collagen type II, α 1 (Col2α1) promoter [136] with mice in which exon 3 of the ERα gene was flanked by the loxP sequence (ERα$^{flox/flox}$; [120]). The offspring of this breeding were then bred in a specific scheme in order to generate littermate conditional mutant Col2α1-ERα$^{-/-}$ and control (ERα$^{flox/flox}$) mice [135].

The role of ERα for longitudinal bone growth during sexual maturation was investigated by studying ERα$^{-/-}$ and Col2α1-ERα$^{-/-}$ mice until 4 months of age. The male ERα$^{-/-}$ mice had a reduced longitudinal bone growth during sexual maturation, resulting in shorter bone length, while the male Col2α1-ERα$^{-/-}$ mice had a normal growth [135]. The reduced growth in male ERα$^{-/-}$ mice was associated with a significant reduction of serum IGF-I levels and disturbed GH secretion as indicated by reduced major urinary protein

(MUP) expression in the liver [21]. This demonstrates that in male mice, indirect, probably GH/IGF-I-mediatedeffects not requiring ERα in growth plate cartilage are responsible for the role of ERα to modulate skeletal growth during early sexual maturation. Furthermore, this indicates that ERα via altered activity in the GH/IGF-I axis is of importance for the pubertal growth spurt in male mice [135]. One-year-old female Col2α1-ERα$^{-/-}$ mice were also evaluated, and it was found that local ERα in the growth plate is important for the normal reduction in longitudinal bone growth seen in elderly mice [135]. This resembles the phenotype seen in old ERα$^{-/-}$ mice, which also displayed a continued growth after sexual maturation [132]. Thus, ERα in the growth plate is important for mediating normal age-related reduction in longitudinal bone growth [135].

High-dose E2 treatment induces growth plate closure in rodents [128]. To examine the importance of local ERα for this effect, sexually mature female and male Col2α1-ERα$^{-/-}$ and WT mice were gonadectomized (gnx) and treated with supraphysiological levels of E2 or placebo. The E2 treatment resulted in normal estrogenic responses on bone mass, uterus, and thymus in both WT and Col2α1-ERα$^{-/-}$ mice [135]. As expected, both female and male WT mice displayed reduced growth plate height after E2 treatment compared to placebo-treated mice. Interestingly, this effect was not seen in either female or male Col2α1-ERα$^{-/-}$ mice, demonstrating an essential role of cartilage-located ERα for the effect of high-dose E2 to reduce growth plate height in both males and females [135]. Quantitative histological measurements of the growth plates in female mice showed that the reduced growth plate height in the WT mice was due to a reduction of the proliferative zone, while the height of the hypertrophic zone was unchanged. The Col2α1-ERα$^{-/-}$ mice did not display any changes in the proliferative or hypertrophic zones [135]. This is consistent with previous experiments showing that E2 accelerates the proliferative exhaustion, and thereby senescence, of growth plate chondrocytes [28, 132]. A reduced height in the proliferative zone was also seen in the ERαAF-1^0 mice before growth plate closure occurred [132]. This suggests that the

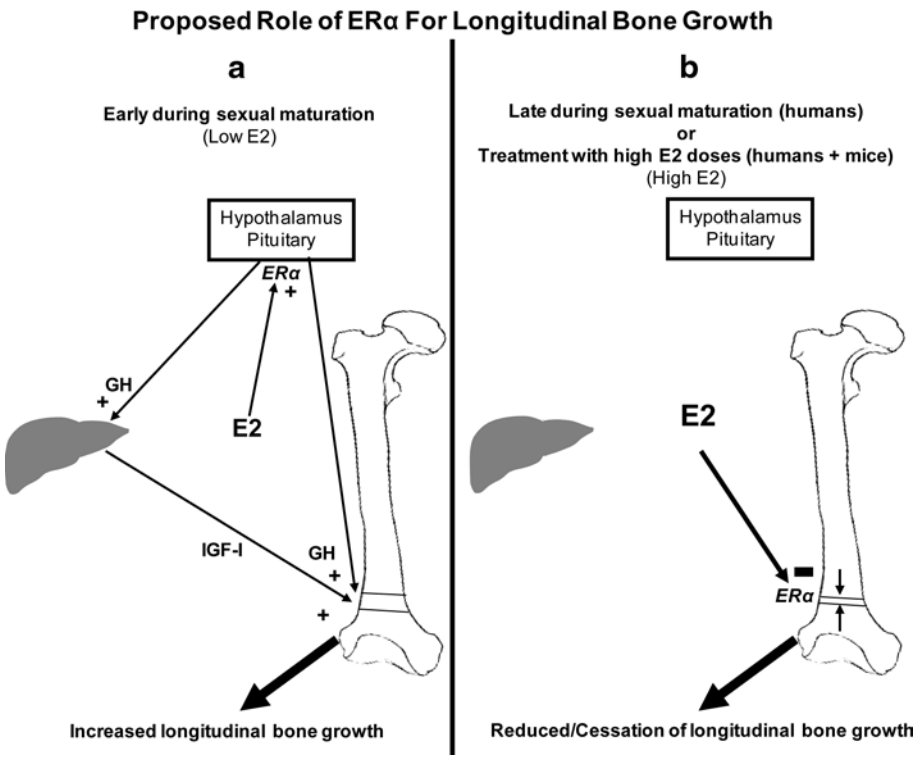

Fig. 4 Proposed role of ERα for longitudinal bone growth. Our findings demonstrate that **a** indirect, probably GH/IGF-I-mediated effects, not requiring ERα in growth plate cartilage, are responsible for the role of ERα to modulate skeletal growth during early sexual maturation associated with low serum E2 levels, while **b** direct effects of ERα in growth plate cartilage are required for the effect of a high E2 dose to reduce the growth plate height in adult mice and for reduction of longitudinal bone growth in elderly mice. We propose that low E2 levels in early puberty enhance longitudinal bone growth via actions on the GH/IGF-I axis, while the higher E2 levels during late puberty (humans) or after high-dose E2 treatment (humans and mice) reduce bone growth via a direct effect on ERα in the growth plate cartilage. + Stimulatory action, − inhibitory action. Reproduced from *J Bone Miner Res* (2010) 25:2690–2700 with permission of the American Society for Bone and Mineral Research

process of growth plate senescence and closure is dependent on growth plate-located ERα via a signaling pathway that does not require ERα AF-1.

Although there are clear species differences between man and mouse, the findings of the importance of ERα in growth plate cartilage, both for high-dose E2-induced reduction of the growth plate height in adult mice and for age-dependent reduction of longitudinal bone growth in mice, suggest that growth plate-located ERα might also be essential for growth plate fusion and cessation of longitudinal bone growth in humans [135].

The results from the Col2α1-ERα$^{-/-}$ mice suggest that the high E2 levels during late puberty (humans) or after E2 treatment (humans and mice) reduce bone growth via a direct effect on ERα in growth plate cartilage (Fig. 4) [135]. Also, growth plate-located ERα is of importance for the reduced longitudinal bone growth in elderly mice. Therefore, studies in both mice [132] and humans [6] strongly suggest that ERα is the dominant ER for the normal age-dependent reduction and cessation of longitudinal bone growth observed in mouse and man, respectively, and that this effect is mediated via growth plate-located ERα (Fig. 4) [135].

ERα expression in neuronal cells affects bone mass

Peripheral estrogen signaling via ERα is important for the regulation of bone, which has been shown by evaluating mouse models that lack ERα in either chondrocytes [135] or osteoclasts [38, 39]. The CNS is a target for estrogen, and ERs are expressed in many different parts of the brain. In order to evaluate the importance of central ERα, a mouse model with a specific inactivation of ERα in nervous tissue (nestin-ERα$^{-/-}$) was developed [137].

The nestin-ERα$^{-/-}$ mice were generated by crossing mice which express the Cre recombinase specifically in neuronal and glial precursor cells (nestin-Cre; [138]) with ERα$^{flox/flox}$ mice [120]. The offspring were then bred according to a specific scheme in order to generate littermate conditional mutant nestin-ERα$^{-/-}$ and control (ERα$^{flox/flox}$) mice [137].

Female nestin-ERα$^{-/-}$ mice had an increased trabecular and cortical vBMD together with increased femoral bone strength. The high bone mass seen in nestin-ERα$^{-/-}$ mice was mainly caused by increased bone formation. Thus, prevention of ERα signaling in the nervous system leads to increased bone density and increased bone strength, which suggests that central estrogen signaling has a negative impact on bone [137]. The female nestin-ERα$^{-/-}$ mice did not have a disturbed negative feedback regulation of sex steroids, indicating that increased levels of sex steroids are not the primary cause of the increased bone mass. The main mechanism for the increased bone mass in nestin-ERα$^{-/-}$ mice was suggested to be mediated via decreased central leptin sensitivity in the hypothalamus, and thereby increased secretion of leptin from white adipose tissue, which in turn results in increased peripheral leptin-induced bone formation [137]. It was also shown that the increased bone mass seen in nestin-ERα$^{-/-}$ mice was not due to a higher bone accrual during early development, but is a dynamic event. In addition, there was no evidence that altered serotonin regulation or sympathetic tone was responsible for the bone phenotype seen in nestin-ERα$^{-/-}$ mice. These data are in accordance with previous results, suggesting a more prominent role for ERβ in the regulation of the central serotonergic system [137, 139].

In conclusion, the balance between peripheral and central ERα actions is important for the regulation of bone mass, since central ERα signaling was shown to have inhibitory effects, and peripheral ERα signaling is known to have stimulatory effects, on bone mass [137]. This suggests that it would, probably, be favorable to develop an ERα-specific agonist with low penetrance to the CNS for having positive effects on bone. Such an agonist would lead to stimulatory peripheral bone effects without having inhibitory central bone effects. However, further studies are required to characterize the relative importance of centralversus peripheral ERα-signaling for other nonbone-related estrogen effects [137].

Concluding remarks

The ongoing research about the relative importance of local versus central effects of ERα and the understanding of how estrogen signals in different tissues and cell types, together with the characterization of the roles of the different domains in ERα, is important in order to map the signaling pathways of estrogen. A better knowledge of the estrogenic signaling pathways may in turn facilitate the design of new, improved SERMs with minimal adverse effects.

The results described in this review recognize that an ERα-specific SERM that would not activate the ERα AF-1 could have beneficial effects on cortical bone, while the effects on reproductive tissues would be minimal. Such a SERM could perhaps also be considered for inducing growth plate closure in constitutionally tall adolescents with reduced risks of adverse effects. The importance of local ERα for reducing growth plate height suggests that, if it was possible to modulate the ERα signaling pathways locally in the growth plate chondrocytes, this could potentially reduce or increase the final height of the patient without the risk of systemic adverse effects. In addition, the results from the mice lacking ERα in neuronal tissue suggest that a SERM with low penetrance to the CNS or with antagonistic effects in the CNS would have a more pronounced effect on bone mass.

It would be optimal to attain local estrogenic effects without having systemic effects. One appealing approach for this emerged when Finan et al. [140] recently reported a method for targeted estrogen delivery. They showed that estrogen conjugated to a glucagon-like peptide-1 (GLP-1) only affected tissues expressing the GLP-1 receptor. This GLP-1-estrogen conjugate did not lead to adverse estrogenic effects in the reproductive system or oncogenicity [140]. If estrogen could be conjugated to a peptide which specifically targets skeletal tissue or the growth plate, this could potentially lead to beneficial estrogenic effects locally, without resulting in systemic adverse effects.

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