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The role of estrogen receptor-α and its activation function-1 for growth plate closure in female mice

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Börjesson AE, Windahl SH, Karimian E, Eriksson EE, Lagerquist MK, Engdahl C, Antal MC, Krust A, Chambon P, Sävendahl L, Ohlsson C. The role of estrogen receptor-α and its activation function-1 for growth plate closure in female mice. *Am J Physiol Endocrinol Metab* 302: E1381–E1389, 2012. First published March 13, 2012; doi:10.1152/ajpendo.00646.2011.—High estradiol levels in late puberty induce growth plate closure and thereby cessation of growth in humans. In mice, the growth plates do not fuse after sexual maturation, but old mice display reduced longitudinal bone growth and high-dose estradiol treatment induces growth plate closure. Estrogen receptor (ER)-α stimulates gene transcription via two activation functions (AFs), AF-1 and AF-2. To evaluate the role of ERα and its AF-1 for age-dependent reduction in longitudinal bone growth and growth plate closure, female mice with inactivation of ERα ($ER\alpha^{-/-}$) or ERαAF-1 ($ER\alpha AF\text{-}1^{0}$) were evaluated. Old (16- to 19-mo-old) female $ER\alpha^{-/-}$ mice showed continued substantial longitudinal bone growth, resulting in longer bones (tibia: +8.3%, $P < 0.01$) associated with increased growth plate height (+18%, $P < 0.05$) compared with wild-type (WT) mice. In contrast, the longitudinal bone growth ceased in old $ER\alpha AF\text{-}1^{0}$ mice (tibia: −4.9%, $P < 0.01$). Importantly, the proximal tibial growth plates were closed in all old $ER\alpha AF\text{-}1^{0}$ mice while they were open in all WT mice. Growth plate closure was associated with a significantly altered balance between chondrocyte proliferation and apoptosis in the growth plate. In conclusion, old female $ER\alpha^{-/-}$ mice display a prolonged and enhanced longitudinal bone growth associated with increased growth plate height, resembling the growth phenotype of patients with inactivating mutations in ERα or aromatase. In contrast, ERαAF-1 deletion results in a hyperactive ERα, altering the chondrocyte proliferation/apoptosis balance, leading to growth plate closure. This suggests that growth plate closure is induced by functions of ERα that do not require AF-1 and that ERαAF-1 opposes growth plate closure.

estrogen receptor-α; growth plate; estrogen; chondrocytes

LONGITUDINAL BONE GROWTH OCCURS at the growth plate, which consists of three principal zones: the resting zone, the proliferative zone, and the hypertrophic zone (5, 17). This growth process is called endochondral ossification, in which cartilage is formed and then replaced by bone tissue (2). High estradiol (E_2) levels in late puberty result in growth plate closure and thereby cessation of longitudinal bone growth in humans (14). The key role for estrogen in the regulation of longitudinal bone growth and growth plate closure in humans is demonstrated by the findings that both males and females with estrogen deficiency, caused by a mutation in the aromatase gene, continue to grow after sexual maturation because of unfused growth plates (14, 29, 32). A similar phenotype was seen in a patient with a point mutation in exon 2 of the estrogen receptor (ER)α (estrogen-resistant man). This man experienced a continued growth long into adulthood, but at the age of 33.5 yr his growth plates were nearly fused. This indicates that ERα is the main mediator of the estrogenic effects on growth plate closure in humans (30, 31). It should be emphasized that the growth plate physiology differs between humans and rodents, since the growth plates do not fuse directly after sexual maturation in rodents and, therefore, one should be cautious when extrapolating data on humans from mouse models. However, old rodents have a reduced growth plate height, and long-term high-dose E_2 treatment results in growth plate closure in rodents (34). The mechanism for the estrogenic effect on growth plate fusion is not well understood, but, in a previous 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 (36).

ERα, ERβ, and the proposed ER G protein-coupled receptor GPR30 are expressed in the growth plate cartilage (8, 18, 22, 24, 25, 33, 37). ERα is the main functional ER in the growth plate, even though both ERβ and GPR30 have been suggested to have minor effects (22, 35, 37, 38). We recently showed that local expression of ERα in the growth plate cartilage is required for a high-dose E_2 treatment to reduce the growth plate height in adult mice and for age-dependent reduction of longitudinal bone growth in old mice (2). These results emphasize the importance of local ERα expression in the growth plate.

In vitro studies have shown that the E_2-induced transactivation of ERα is mediated by its transcription activation function-1 (AF-1), AF-2, or both (Fig. 1), and that this is dependent on the cell type and promoter context (33, 35, 37). There are two isoforms of ERα: the full-length 66-kDa ERα and the less abundant truncated, 46-kDa ERα lacking the NH_2-terminal A/B domains (Fig. 1).

Previous studies evaluating the role of ERα on longitudinal bone growth and growth plate closure have used an ERα knockout (KO) mouse model developed in the Korach and Smithies laboratories (ERKO mouse from the group of Korach). However, this mouse was later shown to have low

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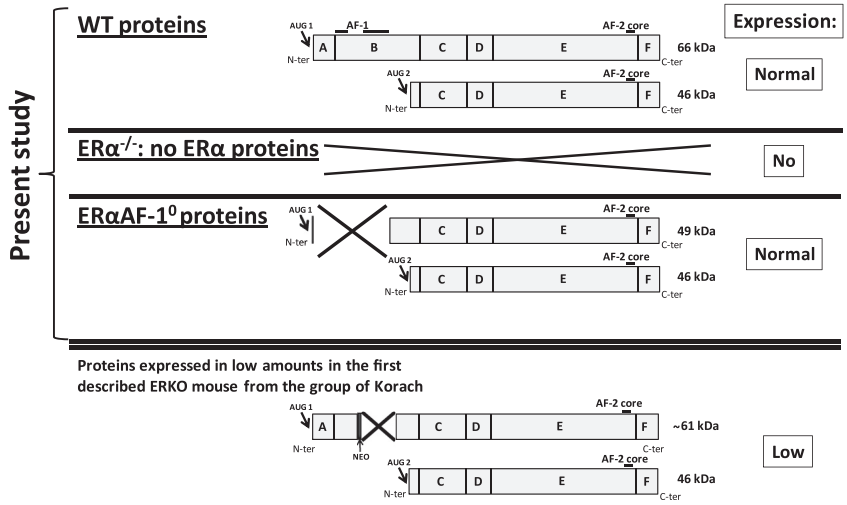

Fig. 1. Schematic presentation of the estrogen receptor (ER)α proteins expressed in the different mouse models. There are two wild-type (WT) ERα isoforms: the full-length 66-kDa protein with start codon AUG1 and the truncated, less-abundant, 46-kDa protein with start codon AUG2. Mice with inactivation of ERα ($ERα^{-/-}$) do not express any ERα protein. Mice with a specific inactivation of the activation function (AF)-1 in ERα ($ERαAF\text{-}1^{0}$) have normal expression of a 49-kDa protein, lacking amino acids 2–148, with start codon AUG1 and also the normal 46-kDa isoform. The first-described ERKO mice from the group of Korach have 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 (9, 11, 16).

expression of truncated ERα isoforms (9, 11, 16) (Fig. 1). The incomplete ERKO mouse from the group of Korach showed a decrease in bone length and had closed growth plates in old female mice (7, 26). These results are inconsistent with the estrogen-resistant man and aromatase-deficient patients who showed an increased bone length and unfused growth plates (29–31). We hypothesized that the conflicting growth plate phenotypes between mice and humans could be due to the remaining expression of truncated ERα isoforms in the ERKO mouse from the group of Korach.

To clarify the role of ERα for longitudinal bone growth and growth plate closure, we, therefore, studied a mouse model with a complete inactivation of ERα ($ERα^{-/-}$) (12) until old age (16–19 mo old). We hypothesized that this mouse model might have a similar longitudinal bone growth and growth plate phenotype as the estrogen-resistant man. In addition, to clarify the role of the AF-1 in ERα for growth plate regulation, a mouse model with a specific inactivation of the AF-1 in ERα ($ERαAF\text{-}1^{0}$; Fig. 1) was evaluated.

MATERIALS AND METHODS

Generation of Mice

The generation of $ERα^{-/-}$ mice has previously been described (12). These mice have a deletion in exon 3 of the ERα gene, and they do not express any of the isoforms of the ERα protein (12) (Fig. 1). The $ERα^{-/-}$ mice and wild-type (WT) control ($ERα^{+/+}$) littermates were inbred C57BL/6 mice and generated by breeding male $ERα^{+/-}$ with female $ERα^{+/-}$ mice. The generation of ERα AF-1-deficient ($ERαAF\text{-}1^{0}$) mice has previously been described (1). These mice have a deletion of 441 bp of exon 1, corresponding to amino acids 2–148, with a preserved translational initiation codon (Fig. 1). The $ERαAF\text{-}1^{0}$ mice do not express any full-length 66-kDa protein. Instead, they have a normal expression of 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 (1) (Fig. 1). The $ERαAF\text{-}1^{0}$ mice and WT control ($ERαAF\text{-}1^{+/+}$) littermates were inbred C57BL/6 mice and were generated by breeding male $ERαAF\text{-}1^{+/-}$ with female $ERαAF\text{-}1^{+/-}$ mice. In contrast to the $ERα^{-/-}$ mice used in the present study, the first-described ERKO mouse from the group of Korach is not completely devoid of an ERα protein (9, 11, 16). Analyses of this mouse have shown a low expression of some truncated ERα mRNAs. The two resulting proteins are the physiologically occurring 46-kDa ERα isoform and also low levels of a truncated 61-kDa isoform with a deletion of amino acids 92–155, which have been replaced by a 7-amino acid-long fragment from the Neomycin sequence (9, 11, 16) (Fig. 1). Animal care was in accordance with institutional guidelines, and the study was approved by the local ethical committee.

The following primer pairs were used for genotyping the mice: for genotyping the $ERαAF\text{-}1^{0}$ mice primer pair 1 was used: P1:1 (5′-TGAAAGAACATTGAACCCGACACAAT-3′) and P1:2 (5′-GCCTTCTACAGGTACCCGCGCCACAT-3′). Primer pairs 2 and 3 were used to genotype the $ERα^{-/-}$ mice: P2:1 (5′-TTGCCCGATAACAATAACAT-3′) and P2:2 (5′-ATTGTCTCTTTCTGACAC-3′); as well as P3:1 (5′-GGCATTACCATTCTCCTGGGAGTCT-3′) and P3:2 (5′-TCGCTTTCCTGAAGACCTTTCATAT-3′) (12).

The mice were studied at different time points, including prepubertal mice (1 mo old), young adult mice (4 mo old), adult mice (8 mo old; only $ERαAF\text{-}1^{0}$), and old mice (16–19 mo old) (n = 7–15).

It should be emphasized that the growth plate physiology differs between humans and rodents, since the growth plates do not fuse directly after sexual maturation in rodents and, therefore, one should be cautious when extrapolating data on humans from mouse models.

Measurements of Serum Hormone Levels

Commercially available radioimmunoassay (RIA) kits were used to assess serum concentrations of insulin-like growth factor (IGF)-I (double-antibody IGF-binding protein-blocked RIA; Mediagnost, Tubingen, Germany) and E_2 (Siemens Medical Solutions Diagnostics, Los Angeles, CA).

Measurement of Bone Lengths

Dual-energy X-ray absorptiometry measurements of femur length were performed in 1-mo-old female mice using the Lunar PIXImus mouse densitometer (Wipro GE Healthcare, Madison, WI), Norland Medical Systems pDEXA Sabre (Norland Medical Systems, Fort Atkinson, WI), and the Sabre Research software (version 3.6; Norland Medical Systems). In the 4- and 16- to 19-mo-old mice, the femur and tibia were excised, and the bone lengths were measured with a micrometer.

Quantitative Histology of Growth Plates

Proximal tibiae and vertebrae were fixed in 4% paraformaldehyde, decalcified in 10% EDTA, and embedded in paraffin. Sections (5 μm thick) were stained with Alcian blue/van Gieson. Images were captured using a Nikon Eclipse E800 light microscope connected to a

Hamamatsu digital camera C4742–95 and a computer. All histological measurements were performed in the central three-fourths of the growth plate sections using Olympus MicroImage software (version 4.0; Olympus Optical, Hamburg, Germany). The heights of the growth plate, the proliferative zone, and the hypertrophic zone were calculated as an average of 10–20 measurements/growth plate. The height of the terminal hypertrophic chondrocyte, the cell in the last intact lacuna, was measured in 10–20 different columns per growth plate and averaged.

Immunostaining for Proliferating Cell Nuclear Antigen

Immunohistochemistry was performed as previously described (25) with the following modifications. Antigen retrieval was carried out in citrate buffer (0.1 M) at +80°C in water bath for 1 h and then remained in the citrate buffer overnight cooling down to reach room temperature (RT), to prevent detachment of sections. Nonspecific antibody binding was blocked with 3% serum in PBS at RT for 45 min, and then the primary antibody for proliferating cell nuclear antigen (PCNA) (mouse monoclonal anti-PCNA, ab29; Abcam, Cambridge, UK) was added, and the sections were incubated for 1 h at RT. The sections were incubated with secondary antibody (biotinylated polyclonal rabbit anti-mouse, E0464; DakoCytomation) for 1 h at RT, and the signal was then enhanced with the avidin-biotin complex (ABC kit, PK-4001; Vectastain) and visualized by diaminobenzidine staining.

Apoptosis Assay (Terminal Deoxynucleotidyl Transferase-Mediated Deoxy-UTP Nick End Labeling)

Apoptotic cells in the growth plate sections were identified employing the terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end-labeling (TUNEL) technique (TdT-FragELTM DNA fragmentation kit; Calbiochem, Damstadt, Germany), as described previously (6).

RESULTS

Old Female ERα$^{-/-}$ Mice Have Increased Bone Lengths While Old Female ERαAF-1^0 Mice Have Decreased Bone Lengths

The growth of both the appendicular (femur and tibia) and axial (vertebra L_6) skeleton was followed in female ERα$^{-/-}$ and ERαAF-1^0 mice and compared with their respective WT controls. At the prepubertal age, both the female ERα$^{-/-}$ and ERαAF-1^0 mice had a normal size of the appendicular and axial skeleton (Fig. 2 and data not shown). The female ERα$^{-/-}$ mice had normal longitudinal bone growth as young adults (Figs. 2 and 3*A*). However, the old ERα$^{-/-}$ mice showed continued substantial longitudinal bone growth, resulting in clearly longer bones compared with WT controls (16–19 mo of age; tibia length: +8.3%, $P < 0.01$; femur length: +6.0%, $P < 0.01$; Figs. 2 and 3*B*). The axial skeletal growth (vertebral height of L_6; Fig. 4*A*) was normal in old female ERα$^{-/-}$ mice.

After sexual maturation, the young adult ERαAF-1^0 mice showed a slight decrease in both femoral and tibial lengths compared with their WT controls ($P < 0.05$; Figs. 2 and 3*A*). The length difference between the ERαAF-1^0 and WT mice increased with age and was substantial in the old mice (16–19 mo of age, femur length: −4.0%, tibia length: −4.9%, $P < 0.01$; Figs. 2 and 3*B*). The height of the L_6 vertebra was also reduced in the old ERαAF-1^0 mice (L_6: −6.1%, $P < 0.01$; Fig. 4*A*). The length differences seen in the old female ERα$^{-/-}$ and ERαAF-1^0 mice were not associated with any changes in serum IGF-I levels (Table 1). However, the serum E_2 levels

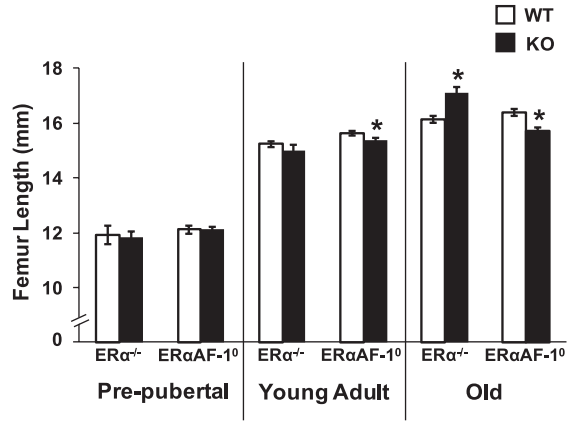

Fig. 2. Increased femur lengths in old ERα$^{-/-}$ mice while decreased femur lengths in old ERαAF-1^0. Femur lengths of prepubertal (1-mo-old), young adult (4-mo-old), and old (16- to 19-mo-old) female ERα$^{-/-}$ and ERαAF-1^0 mice. *$P < 0.05$, Student's *t*-test vs. WT. Values are means ± SE (n = 7–15). KO, knockout.

were elevated in both the old female ERα$^{-/-}$ (+724 ± 224%, $P < 0.05$) and ERαAF-1^0 (+119 ± 28%, $P < 0.05$) mice compared with WT controls, due to disturbed negative feedback regulation.

Increased Growth Plate Height in Old ERα$^{-/-}$ Mice but Growth Plate Closure in Old ERαAF-1^0 Mice

There was no change in the proximal tibial growth plate height in the young adult female ERα$^{-/-}$ mice, whereas the old ERα$^{-/-}$ mice had an increased growth plate height (+18%, $P < 0.05$) compared with the WT controls (Fig. 3, *C-E*). In contrast, the growth plate height was slightly reduced in the young adult ERαAF-1^0 mice, and in the old (16–19 mo of age) ERαAF-1^0 mice the proximal tibial growth plates were closed (Fig. 3, *C–E*). Further evaluation of the timing of the growth plate closure showed an initiation of growth plate closure in adult (8-mo-old) female ERαAF-1^0 mice (1/9 was completely closed, 3/9 were half-closed, and 5/9 were open). In contrast, all adult and old female WT controls had open growth plates in the long bones. Thus, the growth plate heights in the proximal tibia corresponded well with the longitudinal bone growth phenotypes observed in the old female ERα$^{-/-}$ and ERαAF-1^0 mice. Both the female ERα$^{-/-}$ and ERαAF-1^0 mice had normal vertebral growth plate heights at an old age (Fig. 4*B*).

The Growth Plate Closure in Female ERαAF-1^0 Mice is Associated With an Altered Balance Between Proliferation and Apoptosis

The growth plate morphology was further analyzed to characterize the mechanisms behind the enhanced longitudinal bone growth in old female ERα$^{-/-}$ mice and the growth plate closure observed in the old ERαAF-1^0 mice. In the old ERα$^{-/-}$ mice, which have an increased growth plate height (Fig. 3, *D* and *E*), there was a nonsignificant tendency of increases in height of both the proliferative and hypertrophic zones compared with WT controls (Fig. 5, *A* and *B*). The proliferative zone was slightly decreased in the young adult ERαAF-1^0 mice compared with WT controls. This decrease became more pronounced in the adult mice, and, finally, the

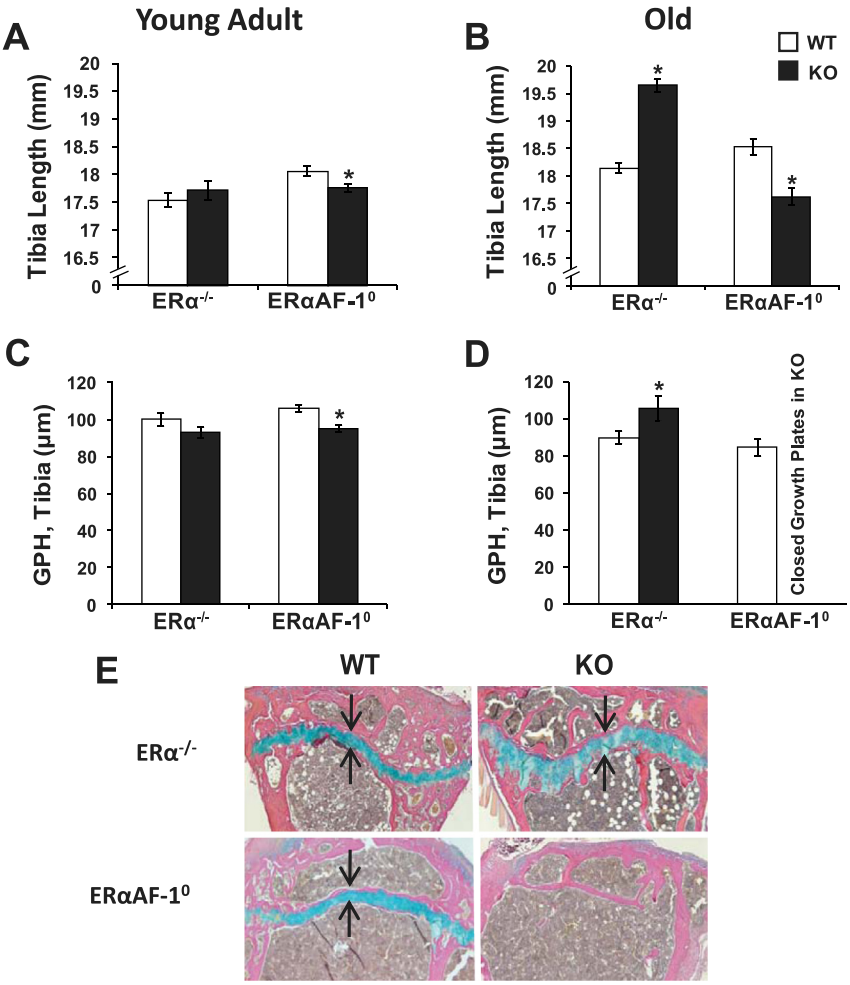

Fig. 3. Altered tibia lengths and growth plate heights in ERα$^{-/-}$ and ERαAF-1^0 mice. Tibia lengths of young adult (4-mo-old, *A*) and old (16- to 19-mo-old, *B*) female ERα$^{-/-}$ and ERαAF-1^0 mice. Growth plate heights (GPH) from proximal tibia of young adult (*C*) and old (*D*) female ERα$^{-/-}$ and ERαAF-1^0 mice. *E*: representative images of growth plates from proximal tibiae of old female ERα$^{-/-}$ and ERαAF-1^0 mice. *$P < 0.05$, Student's *t*-test vs. WT. Values are means ± SE (n = 7–15).

growth plates were closed in the old ERαAF-1^0 mice (Fig. 5*A*). The height of the terminal hypertrophic chondrocytes was not altered in the ERα$^{-/-}$ or ERαAF-1^0 mice (Fig. 5*C*).

Immunostaining for PCNA and TUNEL was performed to evaluate proliferation and apoptosis, respectively. Neither the numbers of PCNA-positive nor TUNEL-positive cells were significantly altered in the ERα$^{-/-}$ mice compared with WT controls (Fig. 6*A*). Interestingly, there was an altered balance between proliferation and apoptosis in the proximal tibial growth plate in young adult (4-mo-old) female ERαAF-1^0 mice ($P < 0.05$, Fig. 6*B*). The altered balance is the result of a combination of nonsignificant tendencies of reduced proliferation (−51%, P = 0.08) and increased apoptosis (+77%, P = 0.12) in the ERαAF-1^0 mice compared with WT controls (Fig. 6*B*).

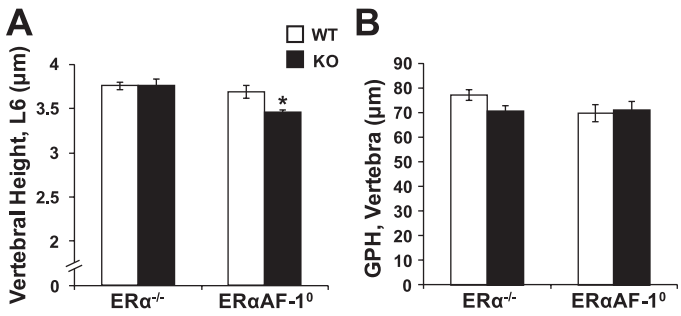

Fig. 4. Decreased vertebral height in old ERαAF-1^0 mice. The vertebral heights (*A*) and the growth plate heights (GPH, *B*) of the L_6 vertebrae in old (16- to 19-mo-old) female ERα$^{-/-}$ and ERαAF-1^0 mice. *$P < 0.05$, Student's *t*-test vs. WT. Values are means ± SE (n = 7–15).

DISCUSSION

In humans, estrogens induce growth plate fusion after sexual maturation as a result of the prolonged exposure to increased serum E_2 levels (21, 28). The timing of growth plate fusion can be manipulated by different pharmacological treatments, which all alter the timing of the peak of estrogenic activity (2, 13, 21, 27, 28). It should be emphasized that the growth plate physiology differs between humans and rodents, since the growth plates do not fuse directly after sexual maturation in rodents and, therefore, one should be cautious when extrapolating data on humans from mouse models, but old mice have a reduced growth plate height, and it is clear that growth plate closure can be reached by supraphysiological E_2 treatment (34). There has thus far been a substantial discrepancy between the longitudinal bone growth phenotype observed in the previously evaluated ERKO mouse from the group of Korach and the phenotype in the estrogen-resistant man and aromatase-deficient patients (7, 26), and we hypothesized that this could be due to the remaining expression of the truncated ERα isoforms in the

Table 1. *Body weight and IGF-I serum levels in ERα$^{-/-}$ and ERαAF-1^0 mice*

	Prepubertal				Young Adult				Old			
	ERα$^{-/-}$		ERαAF-1^0		ERα$^{-/-}$		ERαAF-1^0		ERα$^{-/-}$		ERαAF-1^0	
	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO
Body wt, g	15.9 ± 0.5	16.2 ± 0.6	15.4 ± 0.3	15.8 ± 0.3	21.1 ± 0.5	22.3 ± 0.8	22.1 ± 0.5	24.7 ± 1.0*	33.4 ± 2.4	46.2 ± 4.8*	28.9 ± 1.0	31.5 ± 1.6
IGF-I, ng/ml	ND	ND	ND	ND	223 ± 11	188 ± 23	266 ± 10	272 ± 14	385 ± 41	388 ± 25	421 ± 24	420 ± 15

Values are given as means ± SE; n = 7–15 mice in each group. IGF-I, insulin-like growth factor-I; ERα$^{-/-}$, mice with inactivation of estrogen receptor-α (ERα); ERαAF-1^0, mice with a specific inactivation of the activation function-1 in ERα; prepubertal, 1-mo-old mice; young adult, 4-mo-old mice; old, 16- to 19-mo-old mice; WT, wild type; KO, knockout. *$P < 0.05$ vs. WT. ND, not determined.

ERKO mouse from the group of Korach (9, 11). We herein show that mice with complete inactivation of ERα (ERα$^{-/-}$) have a similar growth plate phenotype as the estrogen-resistant man, showing continued substantial longitudinal bone growth, associated with wide growth plates at an old age. In addition, we demonstrate that mice specifically devoid of AF-1 in ERα display growth plate closure and, therefore, the ERαAF-1^0 mouse model might be a useful animal model for studying mechanistic aspects of growth plate closure.

ERα is Required for a Normal Age-Dependent Reduction of Longitudinal Bone Growth

Low E_2 levels enhance skeletal growth during early sexual maturation (i.e., the pubertal growth spurt), whereas high E_2 levels during late puberty result in growth plate fusion and thereby cessation of longitudinal bone growth in humans and reduced longitudinal bone growth in rodents (4, 5, 10, 14). 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 E_2 (2, 4, 10, 14). E_2 treatment is used to induce growth plate fusion in aromatase-deficient patients, and it has been shown that growth plate fusion only occurs when the E_2 serum levels are above a certain E_2 "threshold" (15, 19). This finding indicates that growth plate fusion is initiated when a certain E_2 threshold is reached during the pubertal development. The estrogen-resistant man did not respond to E_2 treatment, but at the age of 33.5 yr his growth plates were nearly fused. This suggests that it is possible to attain growth plate closure even without a functional ERα in humans, but that this fusion is clearly delayed (31). There seems to be species differences regarding ERα-independent growth plate fusion, since elderly female ERα$^{-/-}$ mice still have open growth plates.

Effects of estrogen on the growth spurt during early sexual maturation. We have previously demonstrated that male mice with complete ERα inactivation, with no remaining truncated ERα transcripts, display reduced longitudinal bone growth during sexual maturation associated with reduced serum IGF-I levels (2). The low IGF-I levels in these mice are in accordance with the impaired growth hormone (GH) secretion and low IGF-I levels in male aromatase-deficient patients (19). In contrast, the female mice with complete ERα inactivation were, in the present study, shown to have a normal longitudinal bone growth during sexual maturation and normal serum IGF-I levels. These findings indicate that ERα via altered activity in the GH/IGF-I axis is of importance for the pubertal growth spurt in male but not female mice.

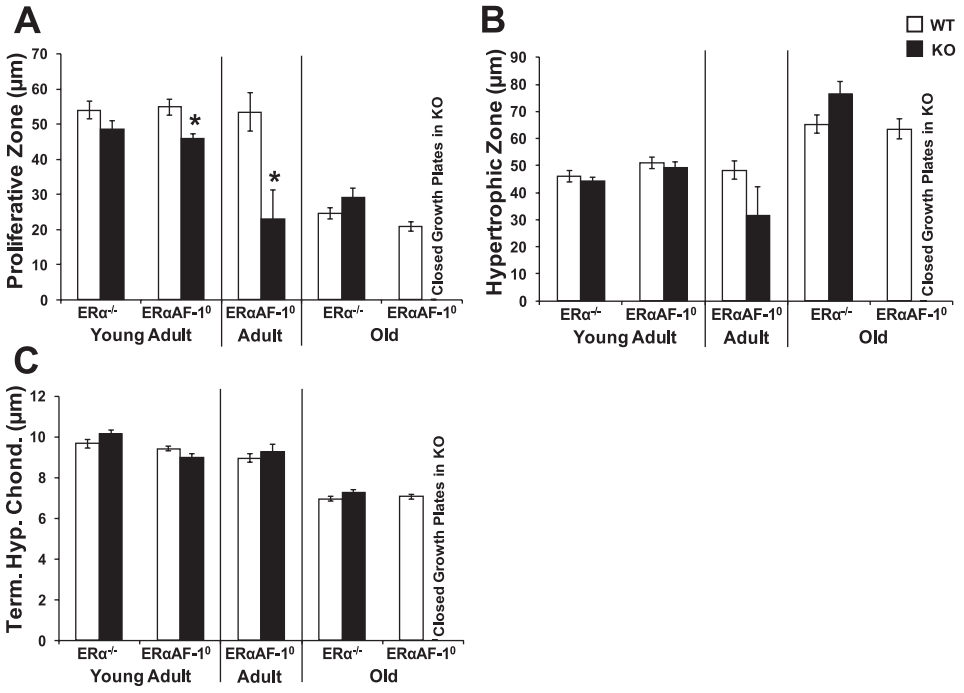

Fig. 5. Decreased proliferative zone in the ERαAF-1^0 mice. Heights of the proliferative zone (*A*), hypertrophic zone (*B*), and terminal hypertrophic chondrocyte (*C*) of the proximal tibial growth plates in young adult (4-mo-old), adult (8-mo-old), and old (16- to 19-mo-old) female ERα$^{-/-}$ and ERαAF-1^0 mice. *$P < 0.05$, Student's t-test vs. WT. Values are means ± SE (n = 7–15).

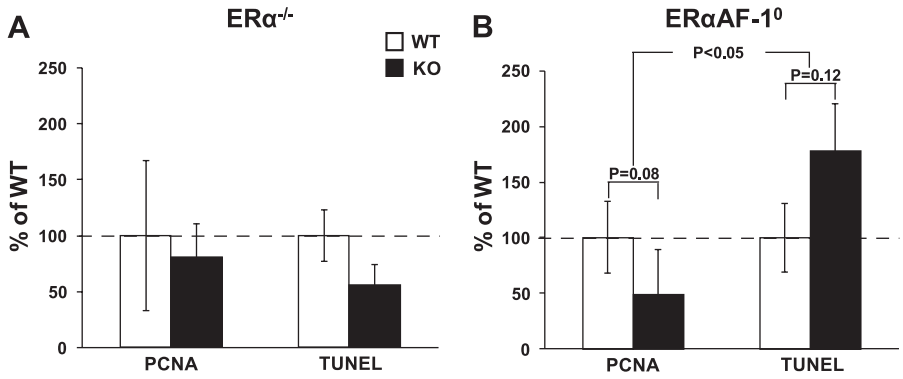

Fig. 6. Altered balance between apoptosis and proliferation in ERαAF-1^0 mice. Immunostaining for proliferation [proliferating cell nuclear antigen (PCNA)] and apoptosis [terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end-labeling (TUNEL)] in the proximal tibial growth plates of young adult (4-mo-old) ERα$^{-/-}$ (*A*) and ERαAF-1^0 (*B*) mice. The values are expressed as percent of WT mice, and the value of the WT mice is set to 100%. Immunostaining of the growth plates shows that the growth plate closure in the ERαAF-1^0 mice is associated with an altered balance between apoptosis and proliferation ($P < 0.05$). Values are means ± SE (n = 7–15).

Effects of high estrogen levels to reduce growth after sexual maturation. When studying old female ERα$^{-/-}$ mice, we could, for the first time, show that the bone length and growth plate phenotypes in these mice correspond well with those found in postpubertal patients with aromatase deficiency or a nonfunctional ERα (29–31). Accordingly, the old female ERα$^{-/-}$ mice show increased bone lengths as a result of substantial continued longitudinal bone growth associated with wider growth plates. The ERα$^{-/-}$ mice have increased E_2 levels due to disturbed negative feedback regulation. These high E_2 levels could have led to reduced growth plate height in normal WT mice, but in the ERα$^{-/-}$ mice these high E_2 levels are probably not enough to alone mediate a reduction of growth plate height, since the ERα$^{-/-}$ mice have wider growth plates. Therefore, the timing of growth plate closure seems to be dependent on a functional ERα, although the mechanisms within the growth plate for these ERα-mediated estrogenic effects are unknown. We have previously shown that old female mice with a cartilage-specific ERα inactivation also have an increased bone length, emphasizing that local ERα is of importance for age-dependent reduction of longitudinal bone growth (2). Our present study together with these previous human and mouse studies strongly suggest that ERα is the dominant ER for the normal age-dependent reduction and cessation of longitudinal bone growth observed in mouse and humans, respectively, and that this effect is mediated via growth plate-located ERα (2, 30, 31).

Growth Plate Closure in ERαAF-1^0 Mice

The young adult but not the prepubertal female ERαAF-1^0 mice show decreased length of the long bones associated with decreased growth plate height, suggesting that ERα AF-1 is crucial for a normal longitudinal bone growth during sexual maturation. Importantly, in the old (16- to 19-mo-old) female ERαAF-1^0 mice, the lengths of the long bones are severely reduced, and the growth plates in the proximal tibiae are closed. Further evaluation of the timing of the growth plate closure revealed that the adult (8-mo-old) ERαAF-1^0 mice were in the process of closing their growth plates. Before growth plate closure had occurred, the height of the proliferative chondrocyte zone of the growth plate was reduced, and the mechanism for growth plate closure in the ERαAF-1^0 mice seems to involve a reduction in chondrocyte proliferation and an increase in chondrocyte apoptosis, reflected by a significantly altered balance between chondrocyte apoptosis and prolifera-

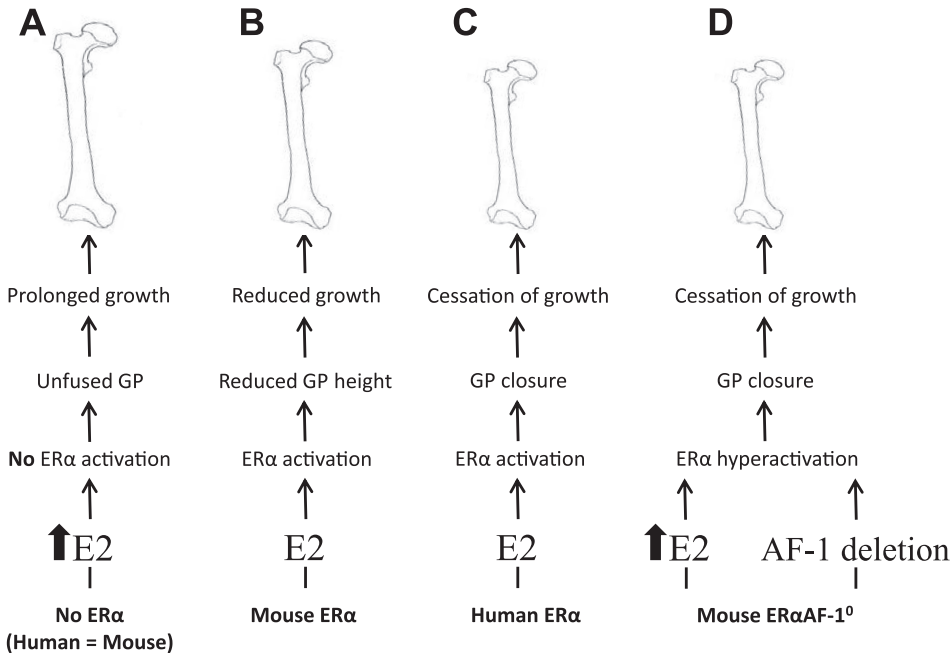

Fig. 7. Proposed role for ERα in the regulation of growth plate closure in mice and humans. Inactivation of ERα with no remaining ERα protein results in prolonged and enhanced longitudinal bone growth associated with wider growth plates in both mice and humans (*A*). In mice, a normal ERα activation results in an age-dependent reduced growth plate height and longitudinal bone growth (*B*), whereas, in humans, a physiological elevation of ERα activation during late puberty results in growth plate closure and thereby cessation of longitudinal bone growth (*C*). In mice, growth plate closure only occurs in mice with specific inactivation of AF-1 in ERα (*D*), resulting in a hyperactive ERα in the growth plate. This hyperactivation of ERα in growth plate cartilage could be a direct effect of the deletion of the AF-1 and/or an indirect effect as a result of the elevated estradiol levels in the ERαAF-1^0 mice (*D*).

tion. In a previous 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 (36). Our present finding is in line with this previous study in rabbits in that the ERα AF-1-deficient mice show growth plate closure that is associated with a disturbed balance between proliferation and apoptosis of the chondrocytes.

The molecular mechanism by which estrogen may accelerate growth plate fusion is not well understood, but it is likely that the estrogenic effect is mediated via ERα. Old ERαAF-1^0 but not WT mice had fused growth plates, indicating an acceleration of the growth plate senescence in the absence of AF-1. In contrast, mice with a complete ERα inactivation displayed wider growth plates, suggesting that they have a reduced senescence of the growth plate. There are several transcriptional cofactors that bind to ERα AF-1 and ERα AF-2; these cofactors are critical determinants of the ability of ERα to regulate gene transcription (23). It is possible that there are cofactors specific for ERα AF-1 that have repressive effects on the ERα-mediated growth plate senescence pathway. When AF-1 is deleted, these cofactors are unable to bind to ERα and the lack of cofactor binding could lead to the result that the repressive effect of AF-1 on the growth plate senescence is lost. The AF-1-deficient ERα could then become hyperactive, via the ERα AF-2; induce growth plate fusion; and thereby cause cessation of longitudinal bone growth. However, in the ERαAF-1^0 mice, the E_2 levels are, due to disturbed negative feedback regulation, elevated, and this could lead to that the threshold level for estrogen-induced growth plate closure is reached and that the AF-1-truncated ERα might have retained the capacity to mediate growth plate closure induced by these supraphysiological E_2 levels.

Our results indicate that the ERαAF-1^0 mice could be used as models for studying E_2-mediated growth plate closure. These results also suggest that it would be interesting to study if some patients with short stature have an inactivating mutation in the AF-1 region in ERα, which could have led to a hyperactive ERα causing premature growth plate closure and thereby reduced final height. A role for ERα for the normal variation in human height is strongly supported by our recent findings using large-scale meta-analyses of genome-wide association studies clearly establishing the ERα locus to be involved in the regulation of adult height (20).

Growth plate closure in appendicular and axial skeleton seems to be regulated in different manners, and our data suggest that the growth plates in the appendicular skeleton fuse faster and are more affected by high E_2 levels than the growth plates in the axial skeleton. This has also been shown in girls subjected to estrogen therapy, to prevent them from being extremely tall, where the leg growth is almost completely stopped while the growth of the trunk is reduced to a lesser extent (3).

Proposed Role for ERα in the Regulation of Growth Plate Closure in Mice and Humans

Our results are summarized in Fig. 7. Inactivation of ERα with no remaining ERα protein results in prolonged and enhanced longitudinal bone growth associated with wider growth plates in both mice and humans (Fig. 7*A*). In mice, a normal ERα activation results in an age-dependent reduced growth plate height and longitudinal bone growth (Fig. 7*B*), whereas, in humans, a physiological elevation of ERα activation during late puberty results in growth plate closure and thereby cessation of longitudinal bone growth (Fig. 7*C*). In mice, growth plate closure only occurs in mice with specific inactivation of AF-1 in ERα (Fig. 7*D*), resulting in a hyperactive ERα in the growth plate. This hyperactivation of ERα in growth plate cartilage could be a direct effect of the deletion of the AF-1 and/or an indirect effect as a result of the elevated E_2 levels in the ERαAF-1^0 mice (Fig. 7*D*).

We conclude that the prolonged substantial longitudinal bone growth observed in elderly ERα$^{-/-}$ mice resembles the continued postpubertal growth seen in humans with ERα inactivation or aromatase deficiency. In contrast, specific inactivation of ERα AF-1 results in a hyperactive ERα, altering the balance between chondrocyte proliferation and apoptosis in the growth plate, leading to growth plate closure. Therefore, this study suggests that *1*) growth plate closure is induced by functions of the ERα that do not require AF-1 and *2*) ERα AF-1 opposes growth plate closure.

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DISCLOSURES

All the authors state that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Author contributions: A.E.B. and C.O. conception and design of research; A.E.B., S.H.W., E.K., E.E., M.K.L., C.E., M.C.A., and A.K. performed experiments; A.E.B., S.H.W., E.K., E.E., M.K.L., C.E., P.C., L.S., and C.O. analyzed data; A.E.B., L.S., and C.O. interpreted results of experiments; A.E.B. and C.O. prepared figures; A.E.B., L.S., and C.O. drafted manuscript; A.E.B. and C.O. edited and revised manuscript; A.E.B., S.H.W., E.K., E.E., M.K.L., C.E., M.C.A., A.K., P.C., L.S., and C.O. approved final version of manuscript.

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