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The Role of Estrogen Receptor α in Growth Plate Cartilage for Longitudinal Bone Growth

Anna E Börjesson,[1] Marie K Lagerquist,[1] Chen Liu,[2] Ruijin Shao,[3] Sara H Windahl,[1] Camilla Karlsson,[4] Klara Sjögren,[1] Sofia Movérare-Skrtic,[1] Maria Christina Antal,[4] Andrée Krust,[5] Subburaman Mohan,[6] Pierre Chambon,[5] Lars Sävendahl,[2] and Claes Ohlsson[1]

[1]Centre for Bone and Arthritis Research, Institute of Medicine, University of Gothenburg, Sahlgrenska Academy, Göteborg, Sweden
[2]Pediatric Endocrinology Unit, Department of Woman's and Children's Health, Karolinska Institutet, Stockholm, Sweden
[3]Department of Physiology/Endocrinology, Institute of Neuroscience and Physiology, Sahlgrenska Academy, Göteborg, Sweden
[4]Institute for Laboratory Medicine, Department of Clinical Chemistry and TransfusionMedicine, Sahlgrenska University Hospital, Gothenburg, Sweden
[5]IGBMC, Illkirch, France
[6]Musculoskeletal Diseases Center, Jerry L Pettis VA Medical Center, Loma Linda, CA, USA

ABSTRACT

Estrogens enhance skeletal growth during early sexual maturation, whereas high estradiol levels during late puberty result in growth plate fusion in humans. Although the growth plates do not fuse directly after sexual maturation in rodents, a reduction in growth plate height is seen by treatment with a high dose of estradiol. It is unknown whether the effects of estrogens on skeletal growth are mediated directly via estrogen receptors (ERs) in growth plate cartilage and/or indirectly via other mechanisms such as the growth hormone/insulin-like growth factor 1 (GH/IGF-1) axis. To determine the role of ERα in growth plate cartilage for skeletal growth, we developed a mouse model with cartilage-specific inactivation of *ERα*. Although mice with total *ERα* inactivation displayed affected longitudinal bone growth associated with alterations in the GH/IGF-1 axis, the skeletal growth was normal during sexual maturation in mice with cartilage-specific *ERα* inactivation. High-dose estradiol treatment of adult mice reduced the growth plate height as a consequence of attenuated proliferation of growth plate chondrocytes in control mice but not in cartilage-specific *ERα*$^{-/-}$ mice. Adult cartilage-specific *ERα*$^{-/-}$ mice continued to grow after 4 months of age, whereas growth was limited in control mice, resulting in increased femur length in 1-year-old cartilage-specific *ERα*$^{-/-}$ mice compared with control mice. We conclude that during early sexual maturation, ERα in growth plate cartilage is not important for skeletal growth. In contrast, it is essential for high-dose estradiol to reduce the growth plate height in adult mice and for reduction of longitudinal bone growth in elderly mice. © 2010 American Society for Bone and Mineral Research.

KEY WORDS: BONE; ESTROGEN RECEPTOR; GROWTH; GROWTH PLATE; CARTILAGE

Introduction

Longitudinal bone growth occurs at the growth plate cartilage through a process called *endochondral ossification*, in which cartilage is formed and then replaced by bone tissue.[1] Low estradiol (E2) levels enhance skeletal growth during early sexual maturation (ie, 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.[1,2] 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.[2]

The key role of estrogens for skeletal growth is demonstrated by the findings that both males and females with estrogen deficiency, caused by a mutation in the *aromatase* gene, display no clear pubertal growth spurt and continue to grow into adulthood owing to a lack of epiphyseal fusion in the long bones, which results in increased adult height.[2–7] One male patient with a point mutation in exon 2 of the estrogen receptor (ER) α had a similar phenotype, demonstrating that ERα is the major ER to mediate the effects of estrogens on longitudinal bone growth.[8] Estrogen therapy resulted in rapid growth plate closure in patients with aromatase deficiency but not in the man with a mutation in the *ERα* gene.[3,4,8] In rodents, the growth plates do not fuse directly after sexual maturation, but high-dose

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Address correspondence to: Claes Ohlsson, Centre for Bone and Arthritis Research, Klin Farm Lab, Vita Stråket 11, Department of Internal Medicine, Sahlgrenska University Hospital, SE-41345 Göteborg, Sweden. E-mail: Claes.Ohlsson@medic.gu.se

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E2 treatment results in a clear reduction of the growth plate height.[9]

The two nuclear ERs, ERα and ERβ, as well as the membrane G-protein-coupled receptor GPR30 proposed to be an ER, are expressed in growth plate cartilage.[10–16] ERα is the main functional ER in the mouse growth plate, although ERβ slightly modulates longitudinal bone growth in female but not in male mice.[17,18] We recently demonstrated that female *GPR30*$^{-/-}$ mice displayed reduced longitudinal bone growth and reduced growth plate height that could not be reduced further by E2 treatment.[12,15] At the current stage of knowledge, it is difficult to evaluate whether GPR30 is a functional ER in the growth plate or whether the growth phenotype of female *GPR30*$^{-/-}$ mice is secondary to other phenotypes.

We recently evaluated the growth plates of elderly female mice with complete *ERα* inactivation and therefore no remaining ERα protein, demonstrating increased growth plate height and prolonged longitudinal bone growth compared with control littermates.[19] Importantly, the increased growth plate height and continued growth in elderly female mice with complete inactivation of *ERα* resemble the lack of growth plate fusion and continued growth seen in the patients with aromatase deficiency and in the man with an inactivating *ERα* mutation.[2–7]

Although ERα is the main ER regulating skeletal growth, it is unknown whether the different effects of estrogens on skeletal growth are mediated via growth plate–located ERα and/or via indirect mechanisms not requiring ERα in growth plate cartilage. Estrogens are crucial regulators of the growth hormone/insulin-like growth factor 1 (GH/IGF-1) axis, and therefore, some of the effects of estrogens on skeletal growth might be indirect via modulation of the GH/IGF-1 axis.[2,20–22] An indirect stimulatory effect of estrogens via effects on the GH/IGF-1 axis is supported by the finding that during sexual maturation, male *ERα*-inactivated mice display reduced longitudinal bone growth associated with reduced serum IGF-1 levels.[17]

Despite the well-known effects of estrogens on longitudinal bone growth, data from in vitro experiments are inconclusive. There are reports of estrogens stimulating,[1,23–25] inhibiting,[26,27] or having no effect[28] on chondrocyte proliferation. In addition, it has not yet been possible to evaluate in vivo the role of ERα in growth plate cartilage. To determine the role of ERα in growth plate cartilage for longitudinal bone growth, a mouse model with cartilage-specific inactivation of *ERα* was developed and evaluated in this study.

Materials and Methods

Reporter mice

Breeding pairs of transgenic *Col2α1-Cre* mice were generated, as described previously.[29] These mice display a cartilage-specific *Cre* expression and have the capacity to recombine *loxP*-flanked DNA sequences in a cartilage-specific manner.[29,30] Studies using reporter mice have demonstrated that the *Col2α1-Cre* mice cause a specific recombination in cartilage not only in the appendicular but also in the axial skeleton.[29] To analyze the expression pattern of Cre recombinase in *Col2α1-Cre* transgenic mice, we used the *ROSA26-Cre* reporter mouse strain *R26R*.[31] Col2α1-Cre transgenic mice were bred with *R26R* mice. Humerus and liver from 4-week-old mice were stained with X-gal to detect β-galactosidase (β-gal) activity (LacZ Tissue Staining Kit, InvivoGen, San Diego, CA, USA) (Fig. 1*A*, *B*).

Generation of mice with cartilage-specific *ERα* inactivation

Mice in which exon 3 of the *ERα* gene is flanked by the *loxP* sequence (*ERα*$^{flox/flox}$[32]) were crossed with *Col2α1-Cre* mice. Crosses to generate *ERα*$^{flox/flox}$ *Col2α1-Cre* mice (referred to as *Col2α1-ERα*$^{-/-}$ or *cartilage-specific ERα*$^{-/-}$ *mice*) were performed according to the following breeding scheme. *ERα*$^{flox/flox}$ mice were bred to *Col2α1-Cre*$^{+}$ mice to generate *ERα*$^{flox/-}$ *Col2α1-Cre* mice. These mice then were bred to *ERα*$^{flox/flox}$ mice to generate conditional mutants (*ERα*$^{flox/flox}$ *Col2α1-Cre*). The conditional mutants were bred to *ERα*$^{flox/flox}$ mice to generate littermate conditional mutant (*Col2α1-ERα*$^{-/-}$ mice) and control (*ERα*$^{flox/flox}$) offspring that were used for our experiments (Fig. 1*C*). The *ERα*$^{flox/flox}$ mice are inbred C57BL/6 mice, and the *Col2α1-Cre* mice were generated on a B6SJLF1 background and backcrossed 6 times with C57BL/6 mice.

The following primer pairs were used for genotyping the mice: first primer pair (for presence or absence of *loxP* sequence), P1: 5′-TTGCCCGATAACAATAACAT-3′; and P2: 5′-ATTGTCTCTTTCTGA-CAC-3′; second primer pair (to determine the deletion of exon 3), P3: 5′-GGCATTACCATTCTCCTGGGAGTCT-3′; and P4: 5′-TCGCTTTCCTGAAGACCTTTCATAT-3′.[33] The presence of the *Cre* gene was determined by using primer pair P5 (5′-CCAGGCTAAGTGCCTTCTCTACA-3′) and P6 (5′-AATGCTTCTGTCCGTTTGCCGGT-3′).

In experiment 1, the growth pattern was followed during and shortly after sexual maturation until 17 weeks of age. In experiment 2, 12-week-old female and male mice were gonadectomized and then treated with vehicle or E2 (830 ng per mouse and day) for 5 weeks using slow-release pellets (Innovative Research of America, Sarasota, FL, USA). In experiment 3, skeletal growth was followed during aging until 1 year of age in female mice.

Mice with total inactivation of *ERα*

The growth pattern during sexual maturation until 17 weeks of age of male mice with cartilage-specific *ERα*$^{-/-}$ was compared with that of male mice with a total *ERα* deletion. The generation of ERα-deficient mice has been described previously.[32] These mice have a deletion in exon 3 of the *ERα* gene in all tissues (total *ERα*$^{-/-}$), and they have earlier been shown not to express any remaining truncated ERα protein.[32] The total *ERα*$^{-/-}$ mice and control (*ERα*$^{+/+}$) littermates were inbred C57BL/6 mice that were generated by breeding male *ERα*$^{+/-}$ mice with female *ERα*$^{+/-}$ mice.

Chondrocyte cultures

Cartilage was dissected from the femurs and tibias of 2-week-old *Col2α1-ERα*$^{-/-}$ and control mice. Chondrocytes were isolated from the surrounding matrix by collagenase digestion over night (type II clostridial collagenase, 0.8 mg/mL; Worthington Biochemicals, Lakewood, NJ, USA). The isolated cells were seeded

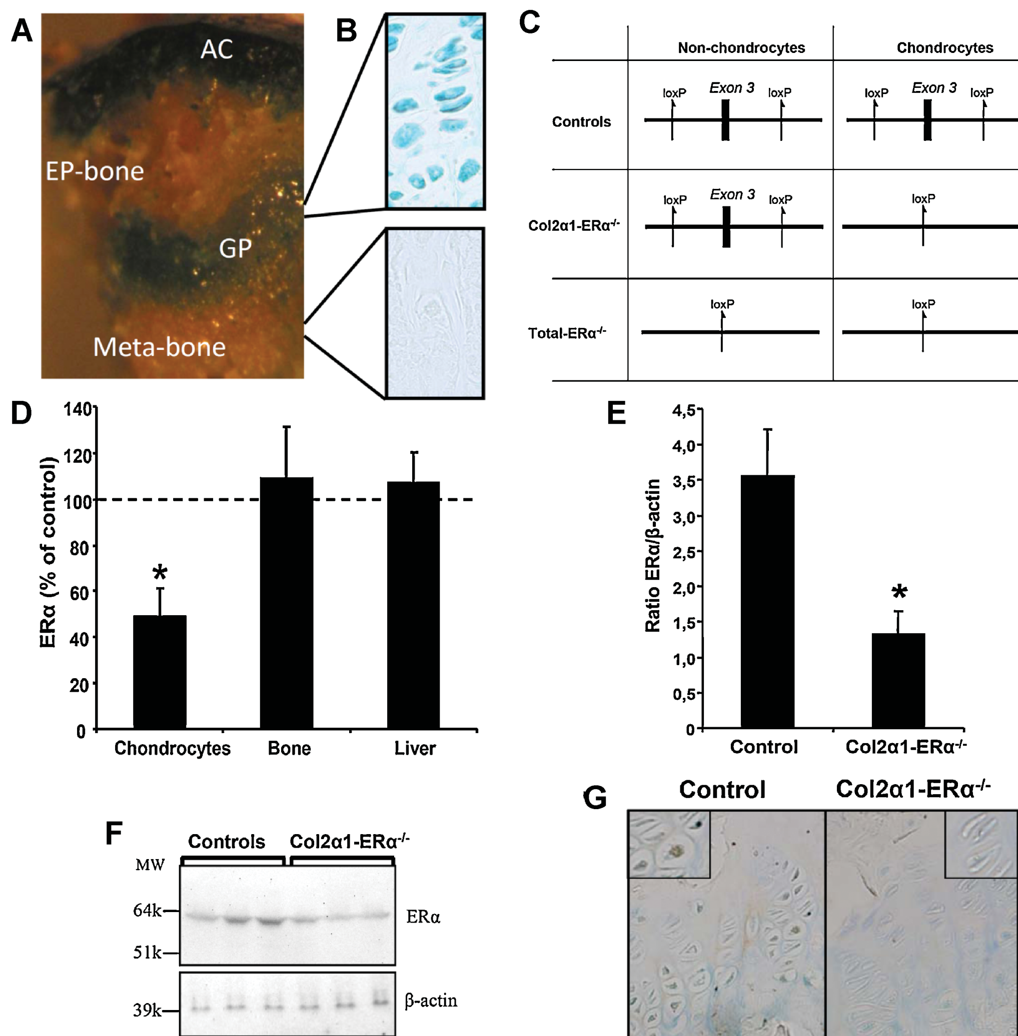

Fig. 1. Cartilage-specific inactivation. (*A*, *B*) Distal humerus stained with X-gal to detect β-galactosidase activity of 4-week-old *Col2α1-Cre* mice mated with the *ROSA26-Cre* reporter mice demonstrating specific β-galactosidase activity in articular cartilage (AC) and growth plate cartilage (GP) but not in the epiphyseal (EP bone) or metaphyseal bone (Meta bone). (*A*) Macroscopic photograph of distal humerus sectioned by a scalpel. (*B*) Histologic sections of growth plate cartilage (*upper panel*) and metaphyseal bone (*lower panel*) in the distal humerus. (*C*) Schematic diagram of targeted deletion of exon 3 of *ERα*. (*D*) *ERα* mRNA levels in cultured chondrocytes, bone (whole femur) and liver of *Col2α1-ERα*$^{-/-}$ mice expressed as percent of control mice. (*E*, *F*) Western blot analyses of ERα expression in cartilage of *Col2α1-ERα*$^{-/-}$ mice. Signal intensities of the mouse ERα protein were normalized to β-actin as ratios to produce arbitrary densitometric units of relative abundance. Values are given as mean ± SEM. $^{*}p < .05$. (*G*) Positive ERα immunostaining in the distal femur growth plate of control mice but not of *Col2α1-ERα*$^{-/-}$ mice.

at 10^4 cells/cm^2 in multiwell plates (BD Biosciences, Bedford, MA, USA) in expansion medium consisting of DMEM/F12 (Invitrogen, Paisley, UK) supplemented with L-ascorbic acid (0.025 mg/mL; Apotekets Production Unit, Umeå, Sweden), gentamicin sulfate (50 mg/L; Gibco, Eggenstein, Germany), amphotericin B (250 mg/mL; Gibco), L-glutamine (2 mM; Gibco), and 10% fetal calf serum (Gibco). Primary chondrocytes were harvested for RNA isolation when the cells reached 80% confluence.

Quantitative real-time PCR analysis

Total RNA was prepared using TriZol Reagent [bone (whole femur) and cultured chondrocytes; Life Technologies, Carlsbad, CA, USA) or RNeasy Kit (liver, Qiagen, Valencia, CA, USA) according to the manufacturers' instructions. The RT-PCR analysis was performed using the ABI Prism 7000 Sequence Detection System (PE Applied Biosystems, Carlsbad, CA, USA). The mRNA abundance of each gene was calculated using the standard curve method (User Bulletin 2, PE Applied Biosystems) and adjusted for the expression of 18S. We used predesigned RT-PCR assays from Applied Biosystems for analysis of *ERα* (covering parts of exon 2 and 3 of *ERα*; PE Applied Biosystems, Mm00433147_m1) and *MUP* (covering parts of exon 3 and 4 of *MUP*; PE Applied Biosystems; Mm01702819_m1) mRNA levels.

Western blot analyses

Cartilage was homogenized in a pellet pestle mixer (Merck KGaA, Darmstadt, Germany) using the following ice-cold lysis buffer: 50 mM Tris-HCl, 0.5 M NaCl, 0.5% Nonidet-P40, 50 mM NaF, 0.5 mM Na_3VO_4, and 20 mM $Na_4P_2O_7 \cdot 10H_2O$, pH 7.4, and a cocktail of protease inhibitors (2 mM EDTA, 1 mM PMSF,

10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 1 mM dithiothreitol). Lysates were kept on ice for 30 minutes and centrifuged at 12,000*g* for 30 minutes at 4°C to remove solid material. The supernatants were collected, and their protein concentrations were determined via a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as the standard. Immunoblot analyses were performed using standard procedures to evaluate the abundance of ERα, and β-actin.[34] Equivalent amounts of protein were directly electrophoresced on 4% to 12% Bis-Tris gels (Novex, San Diego, CA, USA). Blots generated with these extracts were probed with primary antibodies. The immunosignal-CDP-Star substrate for the alkaline phosphatase system (Tropix, Bedford, MA, USA) was used to visualize protein bands. Immunoblotted signals were visualized using a LAS-1000 cooled charge-coupled device camera (Fujifilm, Tokyo, Japan). Individual bands were quantified directly from membranes by densitometry using Image Gauge software (Fujifilm). Signal intensities of the mouse ERα protein were normalized to β-actin as ratios to produce arbitrary densitometric units (ADUs) of relative abundance. All steps were carried out at room temperature unless otherwise stated.

Measurement of serum hormone levels

Commercially available radioimmunoassay (RIA) kits were used to assess serum concentrations of testosterone (ICN Biomedicals, Costa Mesa, CA, USA), E2 (Siemens Medical Solutions Diagnostics, Los Angeles, CA,USE), luteinizing hormone (LH; Immunodiagnostic Systems, Liege, Belgium), and IGF-1 (double-antibody IGF-binding protein-blocked RIA; Mediagnost, Tubingen, Germany).

Dual-energy X-ray absorptiometry (DXA)

Analyses of total-body bone mineral density (BMD), spine BMD, and total-body fat percent were performed by DXA using the Lunar PIXImus mouse densitometer (Wipro GE Healthcare, Madison, WI, USA).

Peripheral quantitative computed tomography (pQCT)

Computed tomographic scans were performed with the pQCT XCT RESEARCH M (Version 4.5B, Norland, Fort Atkinson, WI, USA) operating at a resolution of 70 μm, as described previously.[18] Trabecular volumetric BMD (vBMD) was determined ex vivo with a metaphyseal pQCT scan of the proximal tibia. The scan was positioned in the metaphysis at a distance distal from the proximal growth plate corresponding to 3% of the total length of the tibia, and the trabecular bone region was defined as the inner 45% of the total cross-sectional area. Cortical bone parameters (cortical vBMD and cortical thickness) were analyzed in the mid-diaphyseal region of the tibia.[17]

Quantitative histology of growth plates

Distal femur was 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 (Tokyo, Japan) connected to a Hamamatsu digital camera C4742-95 (Hamamatsu City, Japan) and a computer. All histologic 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 20 measurements per growth plate. Hypertrophic chondrocytes were defined by positive collagen type X immunostaining (see below). The height of the terminal hypertrophic chondrocyte, the cell in the last intact lacuna, was measured in 24 different columns per growth plate and averaged.

Immunohistostaining of type X collagen and ERα

Immunodetection of type X collagen was performed as described previously[35] with the following modification: Prior to suppressing endogenous peroxidase activity, antigen retrieval was carried out in citrate buffer (0.1 M) at +80°C in a water bath for 1 hour and then remained in the citrate buffer overnight, cooling down to reach room temperature. Immunostaining of ERα was performed based on a standard protocol for immunohistochemistry. After deparaffinization and rehydration in descending grades of ethanol, antigen retrieval was performed as mentioned earlier. Nonspecific antibody binding was eliminated with 3% serum in PBS at room temperature for 45 minutes prior to addition of the primary antibody. Rabbit monoclonal primary antibody (RM-9101, 1:200, NeoMarkers, Inc, Freemont, CA, USA) was applied overnight, followed by biotinylated goat antirabbit IgG (1:200, DakoCytomation A/S, Glostrup, Denmark) for 1 hour at room temperature. The signals were enhanced with avidin-biotin complex (Vectastain ABC kit, PK-4001, Vector Laboratories, Burlingame, CA, USA) and visualized by diaminobenzidine. The slides then were counter-stained with alcian blue, dehydrated, and mounted. As the negative control, primary antibody was omitted.

Evaluation of cell proliferation and apoptosis

At the end of the experiment, animals were injected with BrdU solution (50 mg/kg; RPN 20, Amersham Biosciences UK, Buckinghamshire, UK) 19 and 3 hours prior to death. The bones then were fixed in 4% formaldehyde and decalcified in 10% EDTA, and 5-μm-thick paraffin-embedded sections were obtained. BrdU$^+$ chondrocytes were detected by using a cell proliferation kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. The rate of chondrocyte proliferation in the growth plate was expressed as the number of BrdU$^+$ cells per unit area. 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.[35]

Results

Cartilage-specific inactivation of *ERα*

We and others have shown that the *Col2α1-Cre* mouse strain, used in this study, has the capacity to specifically inactivate *loxP*-flanked genes in cartilage/chondrocytes.[29,30] To validate that

the *Col2α1-Cre* mouse strain has the capacity to specifically recombine DNA in chondrocytes postnatally, we mated *Col2α1-Cre* mice with *ROSA26-Cre* reporter mice. Humerus and liver of 4-week-old offspring were stained with X-gal to detect β-galactosidase activity. Specific β-galactosidase activity, as a result of Cre recombinase action, was found in articular and growth plate cartilage, whereas no β-galactosidase activity was seen in the epiphyseal or metaphyseal bone or in the liver (Fig. 1*A*, *B*). Mice with a specific *ERα* inactivation in chondrocytes had a reduction of *ERa* mRNA levels in chondrocytes but not in bone or liver compared with control mice (Fig. 1*C*, *D*). Western blot analyses of ERα protein levels in cartilage demonstrated a 62% reduction in *Col2α1-ERα*$^{-/-}$ mice compared with control mice (Fig. 1*E*, *F*). Immunohistochemistry of the growth plate cartilage demonstrated ERα immunoreactivity in growth plate chondrocytes of control mice, but this was almost completely abolished in *Col2α1-ERα*$^{-/-}$ mice (Fig. 1*G*). Serum E2, LH, and testosterone levels, as well as the uterine weight, were normal in *Col2α1-ERα*$^{-/-}$ mice (Table 1), demonstrating that neither serum sex steroids nor the negative-feedback regulation of serum sex steroids were affected in the *Col2α1-ERα*$^{-/-}$ mice.

Normal skeletal growth during sexual maturation in cartilage-specific *ERα*$^{-/-}$ mice

The body weight, femur length (indicator of appendicular skeleton), and crown-rump length (indicator of axial skeleton) were followed during growth and development until 17 weeks of age in *Col2α1-ERα*$^{-/-}$ and control mice (Fig. 2). As expected, male mice developed higher body weight and increased femur and crown-rump lengths compared with female mice. However, both male and female *Col2α1-ERα*$^{-/-}$ mice displayed normal body weight gain and skeletal growth compared with control mice (Fig. 2). This should be compared with the reduced femur and crown-rump lengths seen in male mice with total *ERα* inactivation (Fig. 3). These findings demonstrate that total but not cartilage-specific *ERα* inactivation results in affected skeletal growth during sexual maturation. The reduced femur and crown-rump lengths in the 17-week-old male total *ERα*$^{-/-}$ mice were associated with a significant reduction of serum IGF-1 levels (–20% ± 6% versus control littermates, $p < .01$), whereas serum IGF-1 levels were unchanged in *Col2α1-ERα*$^{-/-}$ mice (14% ± 7% versus control littermates, nonsignificant). Liver expression of major urinary protein (MUP) is strongly regulated by the GH secretory pattern and is normally higher in male than in female mice.[21] Male mice with total *ERα* inactivation had reduced *MUP* mRNA levels in the liver (−24% ± 18% versus male control mice, $p < .05$), but there was no change in male *Col2α1-ERα*$^{-/-}$ mice (−11% ± 9% versus male control mice, nonsignificant). Fat mass and BMD were unaffected in the *Col2α1-ERα*$^{-/-}$ mice compared with controls (Table 1).

E2 treatment reduces growth plate height in gonadectomized control but not cartilage-specific *ERα*$^{-/-}$ mice

To evaluate the ability of a high E2 dose to reduce the growth plate height in *Col2α1-ERα*$^{-/-}$ mice, adult ovariectomized mice were treated with either vehicle or a slightly supraphysiologic E2 dose (830 ng/mouse/day). This E2 treatment resulted in a clear estrogenic response in bone, reflected by increased total-body BMD, spine BMD, trabecular vBMD, cortical vBMD, and cortical thickness of control mice, and the magnitude of this response was similar in the *Col2α1-ERα*$^{-/-}$ mice (Table 2). Also, other estrogen-sensitive nonskeletal tissues responded normally to the E2 treatment in *Col2α1-ERα*$^{-/-}$ mice, reflected by reduced

Table 1. Organ Weights and Bone Mineral Density in 17-Week-Old *Col2α1-ERα*$^{-/-}$ and Control Mice

	Female		Male	
	Control	*Col2α1-ERα*$^{-/-}$	Control	*Col2α1-ERα*$^{-/-}$
Body and tissue weights				
Body weight (g)	27.8 ± 1.3	28.7 ± 1.0	37.3 ± 2.0	39.2 ± 2.0
Uterus (% of body weight)	0.354 ± 0.033	0.292 ± 0.026	NA	NA
Gonadal fat (% of body weight)	4.32 ± 0.55	4.70 ± 0.59	3.18 ± 0.42	3.67 ± 0.36
DXA				
Total-body BMD (mg/cm^2)	54.3 ± 0.6	53.1 ± 0.8	59.9 ± 0.6	60.6 ± 0.7
Spine BMD (mg/cm^2)	61.9 ± 1.6	60.4 ± 1.9	66.2 ± 1.5	65.6 ± 1.5
Fat (%)	24.3 ± 2.2	24.7 ± 1.8	20.4 ± 2.7	23.3 ± 2.6
pQCT				
Trabecular vBMD (mg/mm^3)	232 ± 9	234 ± 14	ND	ND
Cortical vBMD (mg/cm^3)	1157 ± 7	1157 ± 5	ND	ND
Cortical thickness (μm)	223 ± 4	225 ± 4	ND	ND
Serum parameters				
Estradiol (pg/mL)	11.56 ± 1.51	9.96 ± 1.26	ND	ND
Testosterone (ng/mL)	BSA	BSA	6.00 ± 0.74	5.65 ± 0.96
LH (ng/mL)	0.69 ± 0.06	0.62 ± 0.05	0.57 ± 0.06	0.69 ± 0.08

Values are given as mean ± SEM (n = 10 to 12). DXA = dual energy X-ray absorptiometry; pQCT = peripheral quantitative computer tomography; BMD = bone mineral density; vBMD = volumetric BMD; ND = not determined; NA = not applicable; BSA = below sensitivity of assay (sensitivity for testosterone assay = 0.1 ng/mL).

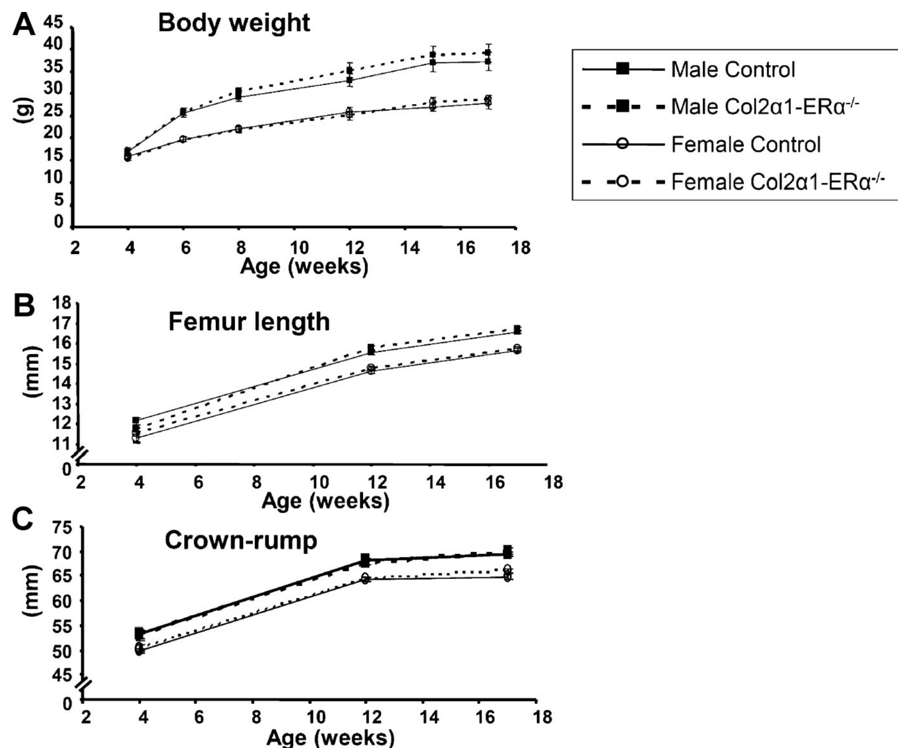

Fig. 2. Normal skeletal growth during sexual maturation in cartilage-specific *ERα*$^{-/-}$ mice. The body weight, femur length, and crown-rump length followed during growth and development until 17 weeks of age in *Col2α1-ERα*$^{-/-}$ and control mice. Values are given as mean ± SEM ($n = 9$ to 45).

thymus weight, increased uterine weight, and reduced fat mass (Table 2). In contrast, E2 treatment resulted in reduced growth plate height in the distal femur of control mice but not of *Col2α1-ERα*$^{-/-}$ mice (Fig. 4*A*, *B*). To understand the mechanism by which E2 affected the growth plate in control mice but not in *Col2α1-ERα*$^{-/-}$ mice, quantitative morphologic studies were performed on the distal femur growth plate. These studies revealed that E2 significantly reduced the height of the proliferative zone (Fig. 4*D*), whereas no significant effect was seen on the height of the hypertrophic zone or the size of the terminal hypertrophic chondrocytes (Fig. 4*E*; Table 2). E2 treatment reduced chondrocyte proliferation significantly in the growth plate of control mice but not of *Col2α1-ERα*$^{-/-}$ mice (Table 2). The number of apoptotic chondrocytes was not affected by E2 treatment in control or *Col2α1-ERα*$^{-/-}$ mice (Table 2).

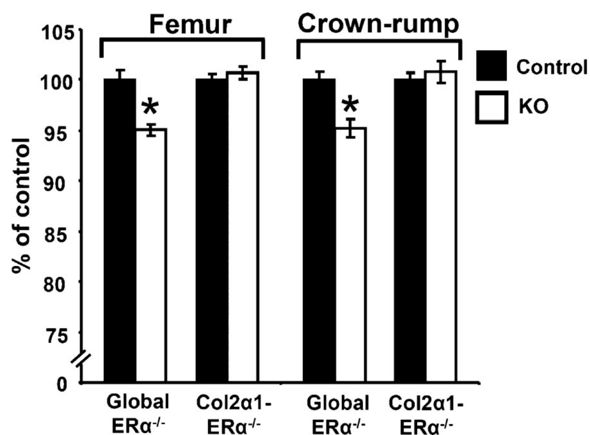

Fig. 3. Femur and crown-rump lengths of male total and cartilage-specific *ERα*$^{-/-}$ mice. Values are expressed as percent of control mice and given as mean ± SEM ($n = 9$ to 12).

Analyses of 17-week-old orchidectomized mice demonstrated that also for male mice, high-dose E2 treatment (830 ng/mouse/day) reduced the distal femur growth plate height in control mice but not in *Col2α1-ERα*$^{-/-}$ mice (Fig. 4*C*).

Prolonged longitudinal bone growth in cartilage-specific *ERα*$^{-/-}$ mice

To evaluate the role of growth plate-located ERα for age-dependent reduction in longitudinal bone growth and growth plate height, the skeletal growth was followed for a prolonged period of time after sexual maturation in female mice. Adult female cartilage-specific *ERα*$^{-/-}$ mice continued to grow after 4 months of age, whereas very little growth was seen in female control mice, resulting in increased femur length in 1-year-old cartilage-specific *ERα*-inactivated mice compared with control mice (Fig. 5). Serum IGF-1 levels were not affected in 1-year-old female cartilage-specific *ERα*$^{-/-}$ mice compared with control mice (–3.0% ± 6.5% versus control littermates, nonsignificant). Axial skeletal growth, analyzed as the increase in crown-rump length, was not significantly affected in the 1-year-old cartilage-specific *ERα*$^{-/-}$ mice (crown-rump length 2.1% ± 1.7% versus control littermates, nonsignificant).

Discussion

Estrogens acting via ERα regulate longitudinal bone growth in both genders. The effects of estrogens on bone growth generally are believed to be mediated either indirectly via regulation of the

Table 2. Estrogenic Response in Ovariectomized Mice

	Control		Col2α1-ERα$^{-/-}$	
	Vehicle	E2	Vehicle	E2
Growth plate				
Height of terminal hypertrophic chondrocyte (μm)	10.5 ± 0.3	9.6 ± 0.8	10.5 ± 0.3	10.5 ± 0.4
Proliferation index (BrdU incorporation; cells/mm^2)	770 ± 67	485 ± 95*	795 ± 84	554 ± 90
Apoptosis (%)	1.29 ± 0.25	1.09 ± 0.21	0.73 ± 0.17	1.04 ± 0.19
Body and tissue weights				
Body weight (g)	29.2 ± 1.3	25.8 ± 0.8*	30.7 ± 1.4	26.4 ± 2.6*
Uterus (% of body weight)	0.072 ± 0.012	0.624 ± 0.076*	0.054 ± 0.003	0.623 ± 0.070*
Thymus (% of body weight)	0.263 ± 0.024	0.132 ± 0.004*	0.246 ± 0.013	0.167 ± 0.015*
Gonadal fat (% of body weight)	4.81 ± 0.50	2.08 ± 0.18*	4.88 ± 0.40	2.09 ± 0.19*
DXA				
Total-body BMD (mg/cm^2)	52.1 ± 0.4	59.7 ± 1.2*	51.9 ± 0.6	58.0 ± 1.2*
Spine BMD (mg/cm^2)	58.6 ± 2.0	75.1 ± 2.8*	54.4 ± 2.2	73.2 ± 2.6*
Fat (%)	27.8 ± 2.6	16.1 ± 0.9*	28.9 ± 2.6	16.2 ± 1.1*
pQCT				
Trabecular vBMD (mg/mm^3)	181 ± 5	671 ± 79*	173 ± 7	581 ± 62*
Cortical vBMD (mg/cm^3)	1134 ± 6	1221 ± 8*	1136 ± 8	1203 ± 5*
Cortical thickness (μm)	223 ± 5	261 ± 6*	224 ± 4	261 ± 4*

Values are given as mean ± SEM (n = 8 to 16). DXA = dual energy X-ray absorptiometry; pQCT = peripheral quantitative computer tomography; BMD = bone mineral density; vBMD = volumetric BMD.

*$p < .05$, Student's t test versus vehicle.

GH/IGF-1 axis or via direct effects in growth plate cartilage. Owing to lack of an adequate animal model, it has not been possible to evaluate the relative role of these two pathways for the effects of estrogens on skeletal growth. We herein developed a mouse model with specific inactivation of *ERα* in cartilage and demonstrated that ERα in growth plate cartilage is not required for skeletal growth during early sexual maturation, whereas the direct effect of ERα in growth plate cartilage is essential for high-dose E2 to reduce growth plate height in adult mice and for reduction of longitudinal bone growth in elderly mice.

Estrogens stimulate longitudinal bone growth during early puberty.[2] The mechanism behind this effect is not clear, although low-dose estrogen treatment increases serum GH and IGF-1, which may contribute to the pubertal growth spurt. An effect via the GH/IGF-1 axis is supported by the fact that ER blockade downregulates the GH/IGF-1 axis.[2,36] To determine the possible role of ERα in growth plate cartilage for skeletal growth during sexual maturation, mice with cartilage-specific *ERα* inactivation were evaluated in this study. These mice displayed normal skeletal growth during sexual maturation, demonstrating that ERα in growth plate cartilage is not required for normal bone growth during sexual maturation. In contrast, male mice with inactivation of *ERα* in all tissues had a reduced skeletal growth during sexual maturation associated with reduced serum IGF-1 levels and disturbed GH secretion, as indicated by reduced MUP expression in the liver.[17] These findings suggest that indirect, probably GH/IGF-1-mediated effects not requiring ERα in growth plate cartilage are responsible for the role of ERα in modulating skeletal growth during early sexual maturation. An indirect effect via the GH/IGF-1 axis during early sexual maturation is supported by findings from children with idiopathic precocious puberty of central origin (CPP). These children are characterized by an early marked increase in growth velocity, GH secretion, and IGF-1 concentrations owing to elevated E2 concentrations.[2]

In humans, estrogens induce growth plate fusion after sexual maturation as a result of the prolonged exposure to increased serum E2 levels.[36,37] The timing of growth plate fusion can be manipulated by different pharmacologic treatments, which all alter the timing of the peak of estrogenic activity.[38,39] In rodents, it seems as if this threshold level for estrogen-induced growth plate fusion is not normally reached, but it is clear that it can be reached by supraphysiologic E2 treatment.[9,40] This study evaluated the capacity of a high E2 dose to reduce the growth plate height in adult cartilage-specific *ERα*$^{-/-}$ mice. In both sexes, E2 treatment reduced the growth plate height in control mice but not in cartilage-specific *ERα*$^{-/-}$ mice, demonstrating an essential role of cartilage-located ERα for the effect of high dose E2 to reduce growth plate height. Further analyses of the growth plates revealed that E2 treatment reduced growth plate height mainly by reducing the size of the proliferative zone and the frequency of proliferating chondrocytes, whereas apoptosis was unaffected. In contrast to the loss of estrogenic response in the growth plate, several other estrogen-responsive tissues, including fat (reduced weight), bone (increased bone mass), thymus (reduced weight), and uterus (increased weight), responded normally to E2 treatment in the cartilage-specific *ERα*$^{-/-}$ mice compared with control mice, supporting a cartilage-specific inactivation of *ERα*.

The patients with aromatase deficiency and the man with an inactivating mutation in *ERα* continued to grow after sexual maturation as a result of unfused growth plates.[2–8] We have found recently that elderly female mice with complete inactivation of *ERα* and no remaining ERα protein also continue

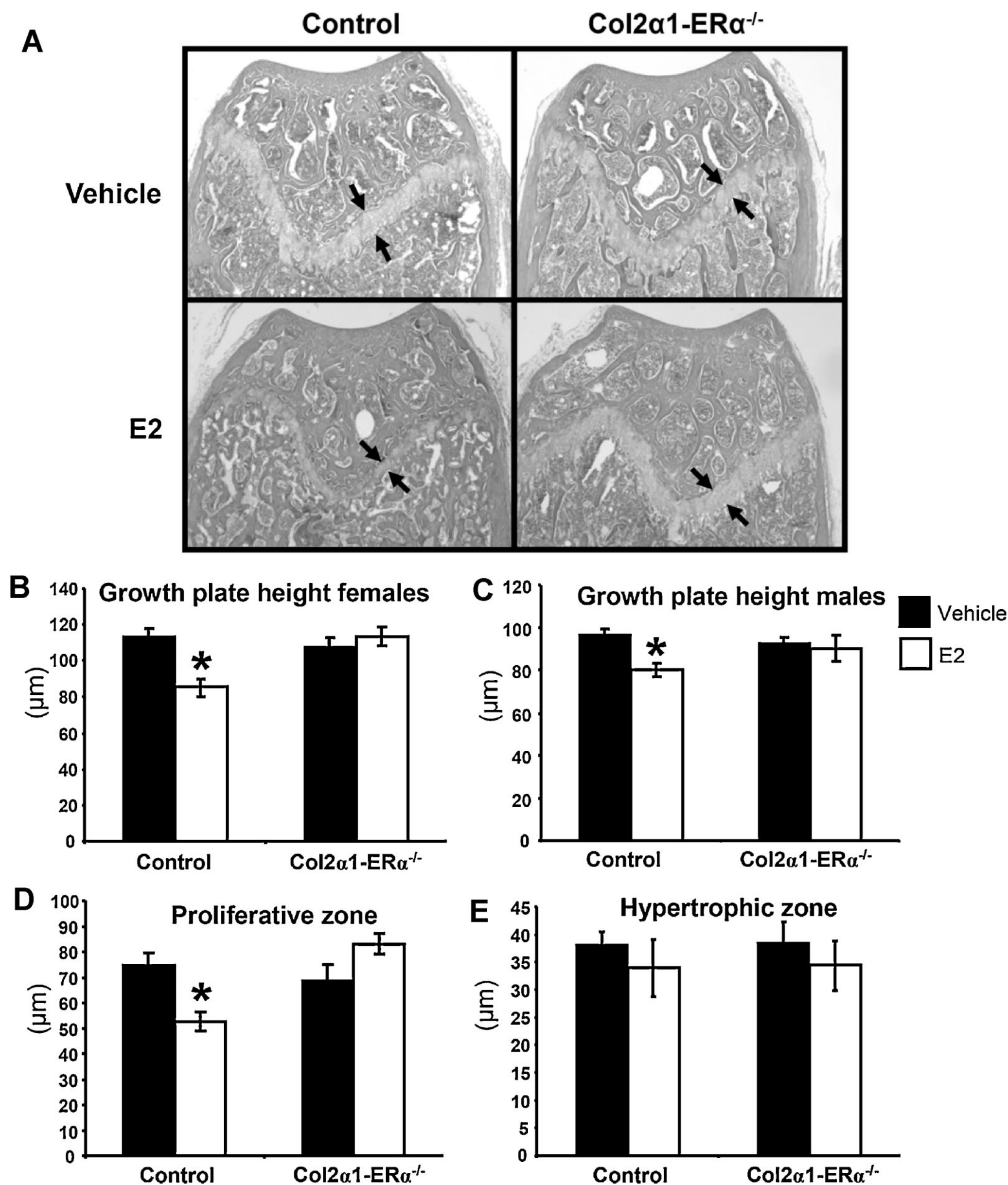

Fig. 4. E2 treatment reduces growth plate height in gonadectomized control but not cartilage-specific *ERα*$^{-/-}$ mice. E2 treatment reduced growth plate height in both female (*A*, *B*) and male (*C*) control mice but not in *Col2α1-ERα*$^{-/-}$ mice. Growth plate height is indicated by the distance between arrows in the histologic sections. E2 reduced the height of the proliferative zone (*D*) but not the hypertrophic zone (*E*) of female control mice. Values are given as mean $\pm$ SEM ($n = 8$ to 18).

to grow for a much longer time than their control littermates, reflected by increased femur length in elderly female *ERα*$^{-/-}$ mice compared with control littermates.[19] To evaluate the role of growth plate cartilage-located ERα in growth during adulthood, we followed the skeletal growth of female mice with cartilage-specific *ERα* inactivation up to 1 year of age. Similar to what is seen in humans with aromatase deficiency or *ERα* mutation and in female mice with total *ERα* inactivation, the mice with cartilage-specific *ERα* inactivation continued to grow throughout adulthood, as reflected by an increase in bone length compared with control mice. This finding demonstrates that ERα in growth plate cartilage is required for the reduction of longitudinal bone growth normally seen in elderly mice. In contrast, the axial skeletal growth was not significantly affected in the elderly cartilage-specific *ERα*$^{-/-}$ mice. The increased appendicular but not axial skeletal growth in the elderly female cartilage-specific *ERα*$^{-/-}$ mice resembles the eunuchoid habitus seen in patients with aromatase deficiency and in the man with an inactivating *ERα* mutation.[2–8]

Although there are clear species differences between humans and mice, these findings of the importance of ERα in growth plate cartilage both for high-dose E2 to reduce growth plate height in adult mice and for age-dependent reduction of longitudinal bone growth in adult mice suggest that growth plate–located ERα also may be essential for growth plate fusion and cessation of longitudinal bone growth in humans.

Western blot analyses of ERα protein levels in cartilage demonstrated a 62% reduction in *Col2α1-ERα*$^{-/-}$ mice compared with control mice. The remaining ERα expression in the cartilage of *Col2α1-ERα*$^{-/-}$ mice might be due to either contamination

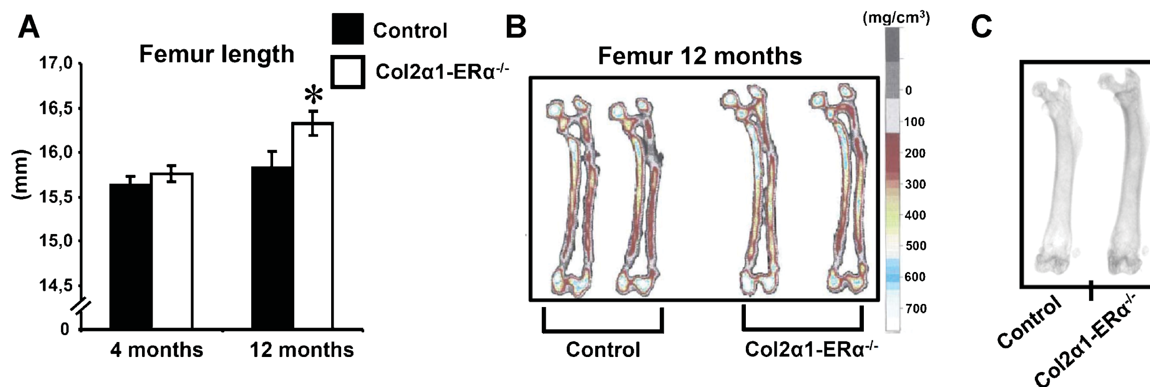

Fig. 5. Prolonged longitudinal bone growth in cartilage-specific $ER\alpha^{-/-}$ mice. (*A*) Adult female cartilage-specific $ER\alpha^{-/-}$ mice continued to grow after 4 months of age, whereas limited growth was seen in female control mice, resulting in increased femur length in the 1-year-old cartilage-specific *ERα* inactivated mice compared with control mice. Values are given as mean ± SEM ($n = 9$ to 12). Further X-ray analyses using pQCT (*B*) and micro–computed tomography (μCT) (*C*) of femurs from 1-year-old female mice illustrate that bone length was increased, whereas bone mineral density and cortical bone dimensions were unaffected in *Col2α1-ERα*$^{-/-}$ mice compared with control mice.

with nonchondrocytes or incomplete recombination in chondrocytes. One should emphasize the possibility that incomplete deletion of ERα in the chondrocytes could contribute to the normal skeletal growth during early sexual maturation in the *Col2α1-ERα*$^{-/-}$ mice. However, the absence of high-dose E2 to reduce growth plate height in adult *Col2α1-ERα*$^{-/-}$ mice and the continued growth in elderly *Col2α1-ERα*$^{-/-}$ mice support the notion that the degree of *ERα* inactivation was of physiologic significance.

In summary, we demonstrate that indirect, probably GH/IGF-1-mediated effects (Fig. 6*A*) not requiring ERα in growth plate cartilage are responsible for the role of ERα in modulating skeletal growth during early sexual maturation associated with low serum E2 levels. In contrast, direct effects (Fig. 6*B*) of ERα in

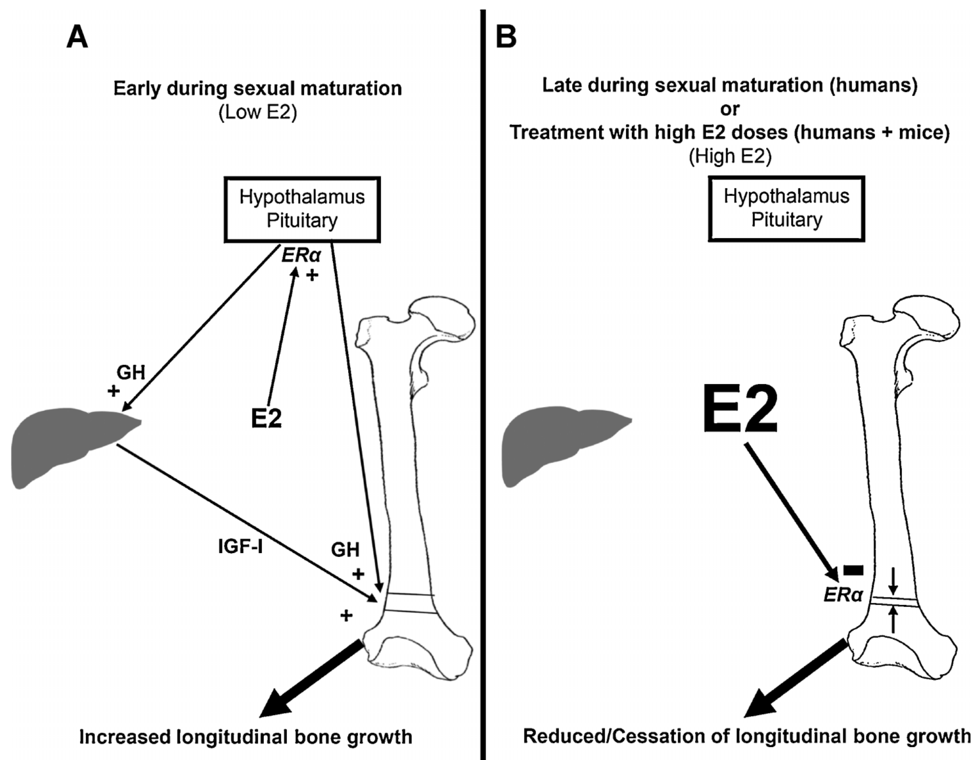

Fig. 6. Proposed role of ERα in longitudinal bone growth. Our findings demonstrate that (*A*) indirect, probably GH/IGF-1-mediated effects, not requiring ERα in growth plate cartilage, are responsible for the role of ERα in modulating skeletal growth during early sexual maturation associated with low serum E2 levels, whereas (*B*) direct effects of ERα in growth plate cartilage is required for the effect of a high E2 dose in reducing 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-1 axis, whereas the higher E2 levels during late puberty (in humans) or after high-dose E2 treatment (in humans and mice) reduce bone growth via a direct effect on ERα in growth plate cartilage. + = stimulatory action; − = inhibitory action.

growth plate cartilage are required for a high E2 dose to reduce 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-1 axis, whereas the higher E2 levels during late puberty (in humans) or after E2 treatment (in humans and mice) reduce bone growth via a direct effect on ERα in growth plate cartilage.

Disclosures

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

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