Region-specific effects of blocking estrogen receptors on longitudinal bone growth

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Abstract

Estrogen receptors (ERs) regulate the development of the growth plate (GP) by binding to estrogen, a phenomenon that determines the growth of skeletal bone. However, the exact mechanisms underlying the regulatory effects of ERs on axial and appendicular growth plates during puberty remain unclear. In the present study, the strategy of ERβ blocking resulted in increased longitudinal elongation of the appendicular bone (P < 0.01), whereas ERα blocking suppressed appendicular elongation (P < 0.05). Blocking both ERs did not have opposite effects on axial longitudinal growth. The expression of chondrocyte proliferation genes including collagen II, aggrecan, and Sox9 and hypertrophic marker genes including collagen X, MMP13, and Runx2 was significantly increased in the growth plate of female mice treated with ERβ antagonist compared with that in the GP of control mice (P < 0.05). There were no significant differences in local insulin-like growth factor 1 (IGF-1) expression among these groups (P > 0.05), and Indian hedgehog protein (Ihh) and parathyroid-related protein (PTHrP) expressions differed among these groups (P < 0.05). ERs appeared not to affect axial bone growth during puberty in female mice (P > 0.05). Our data show that the blocking of different ER subtypes might have a region-specific influence on longitudinal appendicular and axial growth.

Key Words
- estrogen receptor
- skeletal longitudinal growth
- growth plate
- endochondral formation

Introduction

The formation and growth of the axial and appendicular skeleton depend strictly on endochondral formation, which is initiated in the fetal stage and continues to adolescence (Kronenberg 2003). The growth plate (GP) is located between the epiphyseal and metaphyseal bone and consists of three zones: reserve zone (RZ), proliferative zone (PZ), and hypertrophic zone (HZ) (Hunziker 1994, Abad et al. 2002). Chondrocytes located in the RZ have been reported to possess stem-like potential, which could promote the proliferative effects of chondrocytes in the PZ (Hunziker 1994, Abad et al. 2002, Schrier et al. 2006, Raimann et al. 2017). Chondrocytes in the PZ proliferate rapidly and are aligned in columns, a phenomenon that directly causes the longitudinal elongation of the bone along the vertebral and appendicular axes (Shapiro et al. 2005, Mackie et al. 2008, So et al. 2020). As the dividing chondrocytes begin to grow, they secrete extracellular matrix (ECM) and undergo cell death or transdifferentiate into osteoblasts. These changes result in the transition from the PZ to the HZ (Bahney et al. 2014, Zhou, et al. 2014, Hu et al. 2017). Hypertrophic chondrocytes at the HZ promote the transformation from cartilage to the trabecular bone by inducing vascular invasion and recruiting osteoblasts. This process is called endochondral ossification and is

Puberty is the second growth peak in mammals. Endochondral ossification is regulated by multiple endocrine factors, especially during puberty (Giustina et al. 2008, Chagin & Sävendahl 2009, Albrethsen et al. 2020). Estrogen is a key sex steroid in female mammals and it plays an important role in regulating the longitudinal bone growth via estrogen receptors (ERα and ERβ) (Börjesson et al. 2013). ERs (α and β) have been reported to be expressed in the GP in humans and rodents (Kusec et al. 1998, Nilsson et al. 1999, Nilsson et al. 2002, van der Eerden et al. 2002). Patients with aromatase deficiency or ERα inactivation show abnormal pubertal growth and GP closure (Lebovitz & Eisenbarth 1975, Carani et al. 1997, Maffei et al. 2004, Quaynor et al. 2013), suggesting that estrogen or ERs are indispensable for normal growth plate development. In recent years, various ER knockout (KO) mouse models have been employed to clarify the effects of ERs on skeletal longitudinal growth (Lindberg et al. 2001, Movérane et al. 2003). A global absence of ERβ (ERβ KO) increases appendicular bone growth, while global ERα inactivation (ERα KO) reduces bone growth, suggesting that ERα and ERβ might represent a 'yin-yang' dynamic with respect to bone growth regulation (Vidal et al. 1999, Lindberg et al. 2001, Chagin et al. 2004). There is controversy regarding the effects of both ER subtypes on axial longitudinal growth (Vidal et al. 1999, Chagin et al. 2004). Increased femur length has been observed in female ERβ KO mice, and decreased femur length has been observed in female ERα KO mice; the accompanying variations in the growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis have been proposed as mechanisms underlying the skeletal growth regulatory effects of Ers (Vidal et al. 1999, Lindberg et al. 2003). However, the contribution of circulation vs local IGF-1 to longitudinal growth regulated by different ERs remains unclear (Perry et al. 2008). Thus, the role played by ERs in regulating skeletal development needs to be studied. Our previous study revealed age and regional variations in the expression of both ER subtypes on tibial and spinal GPs in both sexes (Li et al. 2012). Both ER subtypes were more highly expressed during puberty than at any other time, and the ratio of ERα expression to ERβ expression differed between limbs and spine, which could partially explain the controversial roles played by ERs in appendicular and axial GPs. We, therefore, hypothesized that the regulatory effects on pubertal longitudinal bone growth of the various ER subtypes would not only influence IGF-1 circulation but also regulate local IGF-1 expression.

The aim of the present study was, therefore, to investigate the roles of ERs in longitudinal bone growth using methyl-piperidino-pyrazole (MPP, an ERα selective antagonist) and 4-(2-phenyl-5,7-bis(trifluoromethyl)pyrazololo(1,5-a) pyrimidin-3-yl) phenol (PHTPP, an ERβ selective antagonist) in female mice during puberty. The findings of this study could help refine the hypothesis pertaining to the effects of ERs on longitudinal bone growth during puberty in female mice.

Methods and materials

Animals

Three-week-old female C57BL/6 mice (n= 6/group) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. and housed under 12-h light:12-h darkness conditions with standard mice feed and sterile water. All experiment protocols were approved by the Animal Care and Use Committee of Peking University People’s Hospital.

Experimental procedures

Twenty-four mice were randomly divided into the following three groups: control (Ctrl), MPP treated mice (MPP), and PHTPP treated mice (PHTPP). After 1 week of adaptive feeding, the female mice were treated with different kinds of ER antagonists during puberty (4 to 12 weeks). Control group mice were subcutaneously injected with 100 μL saline solution. The MPP group mice and PHTPP group mice were intraperitoneally injected with 0.3 mg/kg/day, dissolved in 1% DMSO and PHTPP (0.3 mg/kg/day, dissolved in 1% DMSO), respectively. All groups were administered injections 5 days/week for 8 weeks.

Detection of serum growth hormone (GH) and insulin-like growth factor-1 (IGF-1) by ELISA

At the end of the 8-week period, blood samples were collected by ventricular puncture from all animals before sacrificing. The blood sample was clotted at room temperature and centrifuged for serum isolation, and serum samples were stored at –80°C before the test. The GH and IGF-1 detection ELISA kits were used to determine the serum levels of GH and IGF-1 (Shanghai Institute of Biological Product, Shanghai, China), and the testing steps were performed according to the manufacturer’s instructions.
Tissue sample collection and length measurements

After the mice were sacrificed, their femurs were collected to record the length of appendicular bone growth. The lumbar spine (L1-L6 vertebrae) was collected in its entirety to record the length of axial bone growth. The length of these axial and appendicular bones was measured ex vivo using a vernier caliper. The right femurs and lumbar L5 vertebrae were fixed in 4% paraformaldehyde for at least 24 h. 10% EDTA was used for decalcification of these bone tissue at least 4 weeks. After decalcification, femur and spine were embedded in paraffin for future histological analysis.

Quantitative histology of axial and appendicular growth plate using HE staining

Hematoxylin and eosin (HE) staining was performed according to previous studies (Jin et al. 2018). Paraffin of femur and lumbar (L5 vertebrae) tissue was incised into 4 μm thick sections, and then deparaffinized with xylene, followed by staining with HE according to the kit manual protocol. According to the previous study (Iravani et al. 2017), the quantitative analysis was carried out by measuring the growth plate under the 10× magnification using a Nikon Eclipse microscope (Nikon). Two non-consecutive sections per mouse were used for the HE staining, and the height of whole femur GP and vertebral GP was recorded by the mean values of 20 measurements per section. The height of the proliferative and hypertrophic zones was analyzed by 20 columns in each GP section, and the mean values of PZ or HZ height were recorded for each section. ImageJ (NIH) was used to measure the height of femur and lumbar GP height in a blind method.

Immunohistochemistry (IHC)

For IHC analysis, the traverse sections were baked at 60°C for 50 min and were deparaffinized using 100% xylene, then rehydrated in graded ethanol to finally wash with water, 5 min in each step. Sections were incubated in 3% H₂O₂ in order to eliminate endogenous peroxidase activity, then using PBS to wash. Then, the sections were immersed in antigen repair liquid (EDTA antigen repair buffer, pH 8.0) and heated in a microwave oven too, next the sections were blocked with 1% goat serum for 30 min. Finally, these sections incubated with rabbit antibody of anti-Collagen II (1:200, Abcam), anti-Collagen X (1:250, Affinity, USA), anti-Aggrecan (1:250, Affinity, USA), anti-Runx2 (1:250, Affinity, USA), anti-Sox9 (1:250, Affinity, USA), anti-MMP13 (1:250, Affinity, USA), anti-IHH (1:200, Proteintech, China), anti-PTHrP (1:250, Affinity, USA), and anti-PCNA (1:200, Abcam) at 4°C overnight, respectively. After washing with PBS triple times, these slides were incubated with peroxidase-conjugated secondary antibody for 2 h at room temperature. Finally, these slides were incubated in ABC complex (Vectastain ABC kit) for 30 min. Staining was detected with DAB peroxides substrate solution for 3 min, followed by rinsing in distilled water. The sections were dehydrated by graded ethanol, cleared in xylene, and mounted with permount medium after counter staining with Gill’s hematoxylin solution for 3 min. The results were analyzed and images were captured using a digital microscope (Nikon). Additionally, to identify the specificity of antibodies, an identical manner was used to treat control samples, but these control slides were incubated with primary antibody pre-incubated with blocking-peptide according to our previous study (Li et al. 2012).

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxy UTP nick-end labelling (TUNEL) assay (apoptosis analysis)

The TUNEL assay was performed according to the manufacturer’s protocol (Roche). Briefly, apoptotic GP cells were identified by TUNEL. The DAB method was used to detect staining.

Quantification of IHC staining

The semiquantitative analysis was carried out to evaluate the intensity of these immunoreactivity according to the HSCORE method (Budwit-Novotn et al. 1986, Song et al. 2021). The intensity of collagen II, aggrecan, collagen X, Mmp13, IGF-1, Ihh, and PTHrP immunoreactivity were evaluated semi-quantitatively using the score of staining: no staining, 0; weak but detectable staining, 1; moderate staining, 2; intense, 3. Briefly, the slides were examined under a 40× objective, and five randomly selected areas were evaluated. In each field, the percentage of staining area was assessed, and the calculation formula was as follows: HSCORE=Σi×Pi, where i represents the intensity scores, and Pi is the corresponding percentage of the areas.

In addition, given that the nuclear expression of Sox9, Runx2, PCNA, and TUNEL, counterstaining by hematoxylin for the sections may influence the quantification using the HSCORE method. Here, we use the positive cell counting method to measure these nuclei-expressed markers according to previous reports.
(Khalaj et al. 2013, Bu et al. 2017, Tang et al. 2020). Briefly, for each staining marker, three tissue sections were randomly observed under the 40× magnification microscope, and five randomly selected areas were evaluated for each slide. Two investigators were blinded to the type of the slide to gain the intensity/positive cell number, and the average score was used.

The femur GP tissue harvest and RNA extraction

The distal femur was median incised using a surgical blade, and the growth plate can be seen under the field of a microscope. Micro-forcep was used to harvest the growth plate tissue. The harvested GP tissues were immediately placed in TRIzol reagent (Life Technologies). A homogenizer was used to make the homogenized GP. Then, the total RNA was extracted according to our previous studies (Li et al. 2012, 2013). NanoDrop spectrophotometer was used to quantify RNA, and 0.6 μg was reverse transcribed into cDNA.

Real-time quantitative PCR (qPCR)

Gene expression was evaluated by qPCR using a Roche LightCycler® 480II (Roche Diagnostics). PCR amplification was performed with TB Green Premix Ex Taq (Takara) according to the manufacturer's instructions. The forward and reverse primer sequences are listed in Table 1. The progress of qPCR contained two steps: first, an initial 30 s of heating to 95°C, then 20 cycles consisting of 5 s at 95°C followed by 20 s at 60°C. The ΔΔCt method was used to evaluate the transcript levels, and data were normalized for input based on β-actin and expressed relative to mice in the control group.

Statistical analysis

All data are presented as mean ± s.d., and the differences between the control group and treated groups were analyzed by one-way ANOVA methods and post-hoc analysis was carried out with Bonferroni test. A P value < 0.05 was considered as statistically significant. Statistical analysis was performed using SPSS (IBM, 22.0, USA) software.

Results

Effects of selective estrogen receptor α and β antagonists on appendicular and axial growth plate

To determine the role of ER in longitudinal bone growth (appendicular and axial) during puberty, the mice were administered an ER-selective antagonist (MPP and PHTPP) from 4 (pre-puberty) to 12 weeks (end of puberty). Femur length was considered to be the measurement of appendicular growth, while the lumbar length was used as a measurement of axial growth. Longitudinal growth of the femur increased with PHTPP treatment (compared with control group, Fig. 1A, P < 0.01), indicating that ERβ blocking promoted appendicular bone growth during puberty in mice. The femur length was decreased by MPP treatment (compared with the control or PHTPP treatment), suggesting that ERα blocking inhibited the longitudinal growth of appendicular bone (Fig. 1A, P < 0.01). There was no difference in the lumbar spine length among these groups (Fig. 1B), demonstrating that ER antagonists might not affect axial longitudinal growth during puberty in female mice.

Effects of selective estrogen receptor α and β antagonists on appendicular and axial growth plate

We performed HE staining of the femoral and vertebral sections to investigate the effects of non-selective and selective ER antagonists on the GP cartilage. The femur growth plate height was increased by PHTPP treatment compared with the control group (Fig. 2A and B, P < 0.01), whereas MPP decreased the femur GP height compared with the control group (Fig. 2A and B, P < 0.01). Additional histological analysis of the proliferative zone of the femur GP showed that both proliferative zones were increased.
Effects of ERα and ERβ antagonists on femur and spine length. The study groups included control, ERα antagonist MPP, ERβ antagonist PHTPP. Six mice were contained in each group. Values are means ± s.d. ****P < 0.01. Femur length (A, n = 12) and lumbar spine length (B, n = 6). A full color version of this figure is available at https://doi.org/10.1530/JOE-21-0049.

Effects of selective estrogen receptor α and β antagonists on chondrocyte proliferation and hypertrophic genes

The above results showed that the mice treated with different ER antagonists had different longitudinal bone growth and GP heights. To investigate the role of ER antagonists in chondrocyte proliferation and hypertrophy, we performed IHC staining with proliferative markers (anti-collagen II, anti-Runx2, anti-aggrecan), and hypertrophic markers (anti-MMP13, anti-collagen X, and anti-Sox9) on the femur GP. Collagen II, aggrecan and Sox9, which were mostly expressed in the proliferative and pre-hypertrophic chondrocytes of the GP, were increased in the PHTPP group compared with the control group (Fig. 4 A, B and C). There were no differences between the MPP and control group in the collagen II and aggrecan protein levels; however, the Sox9 protein level was lower in the MPP group than in the control group. The expression of collagen X, MMP13, and Runx2, which are markers of hypertrophic and terminal stages of hypertrophic chondrocytes, was also increased in the PHTPP group. In contrast, the mice treated with MPP showed a decreased intensity of these hypertrophic markers compared with the control group (Fig. 4D and E).

The marker gene levels based on qPCR displayed similar trends to the IHC results (Fig. 5). In total, these data demonstrated that ERβ blocking might increase the GP length by enhancing the expression levels of differentiation and hypertrophy genes, and ERα blocking might decrease the length of GP by downregulating hypertrophic genes.
Effects of selective estrogen receptor α and β antagonists on serum levels of GH and IGF-1 and local IGF-1.

The longitudinal bone growth was often associated with an altered GH/IGF-1 axis. The serum levels of GH were increased in the PHTPP group but decreased in the MPP group compared with control group mice (Fig. 6A). Serum levels of IGF-1 were also increased in the PHTPP group, while they were decreased in MPP group compared with the control group (Fig. 6B). IHC staining of IGF-1 in the femur GP and qPCR results of IGF-1 revealed no differences among these groups (Fig. 6C, D and E).

Effects of selective estrogen receptor α and β antagonists on the IHH/PTHrP signaling

PTHrP is a local autocrine/paracrine hormone, and Ihh is a member of the hedgehog family of secreted proteins. Ihh regulates the transition zone between proliferation and the pre-hypertrophic zone, while PTHrP delays the rate of transition of PZ differentiation to HZ. To investigate the effects of selective ER α and β antagonists on the IHH/PTHrP signaling, we performed IHC staining with anti-PTHrP and anti-Ihh markers and performed qPCR on...
femur GP (Fig. 7). A high PTHrP intensity and a low Ihh intensity were shown in the PHTPP group compared with the control group. While the PTHrP intensity in the MPP group was lower than that of the control group. There was no difference in Ihh intensity between MPP and control group (Fig. 7A, B, C and D). The qPCR results showed same trends for PTHrP and Ihh gene transcription (Fig. 7E and F). Collectively, these data suggest that ERα and β play different roles in regulating the Ihh/PTHrP signaling.

Discussion

Although the role of ERs in longitudinal bone growth is well known, the effects of ERs (α and β) on the GP matrix and the regulatory mechanisms of the vertebral and appendicular skeleton during puberty in female mice are not fully understood. The present study revealed that in female mice during puberty, treatment with PHTPP (a selective ERβ antagonist) increased longitudinal femur, while treatment with MPP (a selective ERα antagonist) decreased the femur longitudinal bone growth. However, there were no differences in the effects of ERα or β antagonists on the longitudinal lumbar growth. The roles of the ERs differed in appendicular and axial bone growth during puberty in the female mice. In the IHC study, ERβ blocking promoted the expression of proliferous and hypertrophic markers in the femur GP, whereas ERα blocking downregulated the expression of these markers. PHTPP also increased PTHrP expression and decreased Ihh expression in the femur GP compared with control and MPP groups. Although the serum GH and IGF-1 levels were higher in the PHTPP group than in the control and MPP groups, the results of the IHC staining and qPCR of IGF-1 did not differ among these groups. Collectively, these data suggest that ERβ blocking can promote appendicular bone growth via the Ihh/PTHrP signaling pathway rather than the GH/IGF-1 pathway in situ and that ERα antagonism plays a suppressive role in bone growth during puberty in female mice, results in line with those of previous studies on longitudinal bone growth in global ERα and β knockout mice (Vidal et al. 1999, Lindberg et al. 2001, Chagin et al. 2004).

Puberty is the second growth peak in mammals. In addition to the development of secondary sexual characteristics, longitudinal body growth is an important marker of puberty (Albrethsen et al. 2020). Estrogen is a key sex hormone in female puberty and is an important factor that affects the longitudinal bone growth process (Ağirdil 2020). The regulatory effects of estrogen are dose-dependent; low estrogen levels stimulate bone growth, while high estrogen levels lead to GP fusion in late puberty (Juul 2001). The cellular response to estrogen is mediated...
by ERs, and previous studies using a different global ER KO mouse model have suggested that ERα and ERβ have opposing effects on longitudinal bone growth in female mice (Vidal et al. 1999, Lindberg et al. 2001, Chagin et al. 2004). ERβ KO in adult mice increases axial skeletal length (crown-rump length, CR) compared with that in WT mice (Lindberg et al. 2001, Chagin et al. 2004). ERα KO in adult mice decreases the appendicular skeletal growth but retains normal axial skeletal growth (Vidal et al. 1999). Our data suggest that ERβ blocking promotes appendicular skeletal growth, while ERα blocking has a negative role in regulating long bone growth (Fig. 1), ultimately aligning with the results from global ER knockout female mice. Furthermore, the ER selective antagonist had no significant influence on the axial length (Fig. 1B), suggesting that the effects of ERs on axial bone growth might not play a major role during puberty. The longitudinal skeletal growth was based on the endochondral formation of the GPs (Kronenberg 2003). GP thickness is an important histological marker of longitudinal bone growth. In this study, the ERβ antagonist increased the femoral GP cartilage thickness and PZ height. However, ERα blocking decreased the femur GP cartilage

Figure 6
(A) Effects of ERα and ERβ antagonists on serum level of growth hormone (GH), and (B) IGF-1. (C) Effects of ERα and ERβ antagonists on IGF-1 in femur growth plate using IHC staining. (D) Quantification of IGF-1 intensity. (G) IGF-1 gene expression in femur growth plate. n = 5–6. Values are means ± s.d., *p < 0.05, ****p < 0.0001, and ns denoted not significant. A full color version of this figure is available at https://doi.org/10.1530/JOE-21-0049.
height and PZ height. We hypothesized that the increase in GP might be due to a higher proliferation index and a lower apoptosis index. We observed a significant increase in the intensity of PCNA staining of the femur GP in the PHTPP group compared with the control group, while the PCNA intensity in the femur GP in the MPP group was lower than that of the control and PHTPP groups. However, there was no difference in the apoptosis index among these groups, despite a slightly lower apoptosis index in the MPP group than in the other groups. Recent studies have found no differences in apoptosis with inactivation/activation of different Ers (Börjesson et al. 2012, Iravani et al. 2017). Taken together, these data demonstrated that a higher level of PCNA staining supported thicker GP and PZ zones. However, the molecular mechanism of ER-mediated GP proliferation and apoptosis remains to be identified.

Although disproportional axial and appendicular body growth related to ER inactivation have been reported, the effects of ER on longitudinal axial growth are still complex. Lindberg et al. (2001) showed that the longitudinal axial bone growth was greater in ERβ knockout female mice than in WT mice. However, Vidal et al. (1999) reported normal axial skeletal growth in ERα-deficient female mice. Parikka et al. (2005) showed that ERα-deficient mice had a significant decrease in the lumbar vertebral length. Chagin et al. (2004) demonstrated that ERβ-deficient female mice have a longer crown-rump length at 4 months but no differences at 18 months compared with WT mice, ERα/−/−, or ERα and β double knockout female mice. The height of the vertebral GP was similar in these groups at both time points. In the present study, the blocking of different Ers did not influence longitudinal axial growth during puberty in the female mice. One explanation for the different longitudinal axial growth results is that, in the previous reports, the mice were either adults or aged females, unlike the female mice used in the present study that were at puberty. Based on our previous study (Li et al. 2012), another explanation is that the ERα/ERβ expression ratio was significantly downregulated in the appendicular GP, while no significant changes occurred in vertebral GP.
During puberty in female mice. The difference in ERα/ERβ expression ratio in axial and appendicular GP might play a role in regulating longitudinal bone growth and determining the disproportional growth between the spine and limbs growth (Li et al. 2012). In addition, ERα antagonists can inhibit the proliferation of appendicular GP chondrocytes in vitro compared with axial GP chondrocytes, while ERβ antagonists had no significant effects on the axial and appendicular GP chondrocytes in vitro (Shi et al. 2017). Collectively, the biological effects of ERα and ERβ on the differential growth phenotypes of axial and appendicular bones might be region-specific during the pubertal period of female mice, and the underlying mechanisms of ERs on regulating the skeletal growth are still not well understood.

Endochondral ossification is the major process contributing to longitudinal skeletal growth. During this procedural transition from cartilage to bone, the potential for chondrocyte proliferation and hypertrophy are important for GP thickness and are responsible for longitudinal bone growth (Mackie et al. 2008). The chondrocytes in different GP phases secrete unique matrix proteins. Collagen II, aggrecan, and Sox9, which are predominantly expressed in RZ, PZ, and pre-HZ zones, were used as markers of GP cellular proliferation (Wuellling & Vortkamp 2011, Samsa et al. 2017, Ağirdil 2020). Collagen X, MMP13, and Runx2, which are mainly expressed in the HZ zone, were the typical features of GP hypertrophy (Wuellling & Vortkamp 2011, Samsa et al. 2017, Ağirdil 2020). The results of the present study demonstrated that ERβ blocking could promote cellular proliferation and increase the extracellular matrix expression of appendicular GPs, while ERα blocking mildly decreased the ECM expression of appendicular GPs. These results might partially support the differing femoral GP thicknesses among the groups. Our previous in vitro study showed that estrogen could regulate the extracellular matrix (collagen II and X) and stimulate the maturation of appendicular GP chondrocytes through Erα (Shi et al. 2017). Taken together, ERα and ERβ might have different effects on proliferation and hypertrophic matrix metabolism in the femur GPs during puberty in female mice.

Numerous signaling pathways contribute to the GP development, such as GH/IGF-1, Ihh/PTHrP; bone morphogenetic proteins, fibroblast growth factors and Wnts (Kronenberg 2003, Giustina et al. 2008, Ağirdil 2020). GH can initiate and promote GP development by stimulating IGF-1 levels, which leads to chondrocyte hypertrophy and the ECM production (Giustina et al. 2008, Ağirdil 2020). Previous studies have shown that the inactivation of ERα or ERβ leads to differing serum IGF-1 levels. ERα inactivation in female mice decreased the IGF-1 level, while ERβ deficiency led to increased serum IGF-1 levels (Vidal et al. 1999, Windahl, et al. 1999, Lindberg et al. 2001). Furthermore, both ERα- and ERβ-deficient female mice had an intermediate IGF-1 level between ERα KO and ERβ KO mice (Lindberg et al. 2001). In previous studies, IGF-1 serum levels were positively correlated with the femur length (Vidal et al. 1999, Lindberg et al. 2001). The data from this study are consistent with these results. To confirm the contribution of local IGF-1 to GP growth, IHC staining and qPCR were conducted to determine the IGF-1 levels in appendicular GPs. However, the results present no significant differences among the groups, which suggest that GH/IGF-1 axis may not play a major role in regulating local GP metabolism. PTHrP (also called PTHLH) is a member of the parathyroid hormone family that supports chondrocyte proliferation and differentiation and maintains GP thickness (Martin 2016). The Ihh protein affects the progress of chondrocyte differentiation, calcification, and ossification together with PTHrP in a negative feedback loop (Amano et al. 2015, Ağirdil 2020). Runx2 and SOX9 have been shown to regulate chondrocyte metabolism by interacting with Ihh/PTHrP axis (Komori 2011, Ağirdil 2020). In the current study, ERβ blocking leads to the increased expression of PTHrP and decreased Ihh expression in the appendicular GPs compared with the control group. In contrast, ERα blocking leads to the opposite result. These results suggest that ERs might mediate GP development via the Ihh/PTHrP signaling pathway rather than GH/IGF-1 pathway in situ. Considering this pathway’s complex regulatory network, the exact roles of ERs in regulating the Ihh/PTHrP axis require further study.

In summary, this study demonstrates that ER blocking during puberty in female mice results in region-specific effects on longitudinal bone growth. ERβ blocking might play a role in promoting longitudinal appendicular bone growth, while ERα antagonism might play a role in suppressing long bone development in female mice during puberty. ERs might not have a major role in regulating vertebral GP development during puberty. Furthermore, the GH/IGF-1 axis might not play a major role in regulating local GP metabolism during puberty, and ER blocking does play a fundamental role in regulating pubertal longitudinal bone growth, partly by regulating the Ihh/PTHrP pathway.
Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-21-0049.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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