ORIGINAL PAPER

# Gender- and region-specific variations of estrogen receptor α and β expression in the growth plate of spine and limb during development and adulthood

Xin-Feng Li · Shan-Jin Wang · Lei-Sheng Jiang · Li-Yang Dai

**Abstract** Although estrogen action is indispensable for normal bone growth in both genders, the roles of estrogen receptors (ERs) in mediating bone growth are not fully understood. The effects of ER inactivation on bone growth are sex and age dependent, and may differ between the axial and appendicular regions. In this study, the spatial and temporal expression of ERα and β in the tibial and spinal growth plates of the female and male rats during postnatal development was examined to explore the possible mechanisms. The level of mRNA was examined and compared with quantitative real-time PCR. The spatial location was determined by immuno-histochemical analysis. The 1-, 4-, 7-, 12- and 16-week age stages correspond to early life, puberty and early adulthood after puberty, respectively. Gender- and region-specific differences in ERα and β expression were shown in the growth plates. Mainly nuclear staining of ERα and β immunoreactivity was demonstrated in the spinal and tibial growth plate chondrocytes for both genders. Moreover, our study indicated significant effect of gender on temporal ERα and β expression and of region on temporal ERα/ERβ expression ratio. However, spatial differences of region-related ERα and β expression were not observed. Gender-related spatial changes were detected only at 16 weeks of both spine and limb growth plates. ERα and β immunoreactivity was detected in the resting, proliferative and prehypertrophic chondrocytes in the early life stage and during puberty. After puberty, ERα expression was mainly located in the late proliferative and hypertrophic chondrocytes in female, whereas the expression still extended from the resting to hypertrophic chondrocytes in males. Gender- and region-specific expression patterns of ERα and β gene might be one possible reason for differences in sex- and region-related body growth phenotypes. Gender, age and region differences should be taken into consideration when the roles of ERs in the growth plate are investigated.

**Keywords** Estrogen · Estrogen receptor · Growth plate · Spine · Limb · Longitudinal growth

## Introduction

Endochondral bone formation at the growth plate, a highly organized cartilage structure located between the epiphyseal and metaphyseal bone, is responsible for the longitudinal bone growth. This process is tightly regulated by a multitude of endocrine signals that are even more complex during the accelerated growth period of puberty (Kronenberg 2003; Nilsson et al. 2005; Giustina et al. 2008; Chagin and Sävendahl 2009). Among them, estrogen is of crucial importance. The findings of genetic disorders have allowed us to gain insight into the unique role that each sex steroid plays in the growing skeleton. Findings of a man with defective estrogen receptor (ER) α and individuals with aromatase deficiency suggest that estrogen action is indispensable for normal pubertal growth and growth plate fusion in both genders. Estrogen plays a critical role in skeletal growth of males as well as females (Lebovitz and Eisenbarth 1975; Ben-Hur et al. 1993; Smith et al. 1994; Morishima et al. 1995; Bilezikian et al. 1998;

X. -F. Li and S. -J. Wang contributed equally to this paper.

X.-F. Li · S.-J. Wang · L.-S. Jiang · L.-Y. Dai (✉)
Department of Orthopedic Surgery, Xinhua Hospital,
Shanghai Jiaotong University School of Medicine,
1665 Kongjiang Road, 200092 Shanghai, China
e-mail: chinaspine@163.com

X.-F. Li
Department of Orthopedic Surgery, Renji Hospital,
Shanghai Jiaotong University School of Medicine,
Shanghai, China

Carani et al. 1997; Maffei et al. 2004). The timing of the growth spurt in boys and girls also suggests that estrogen is a more potent stimulator for skeletal growth than androgen (Lee and Witchel 1997). In patients with familial male precocious puberty, antiandrogen alone is not sufficient to revert skeletal growth to a prepubertal rate, but the addition of an aromatase inhibitor may be effective, thus indicating that the growth promoting effects of testosterone are mediated via estrogen aromatization (Laue et al. 1989). In androgen insensitivity patients, the growth spurt is near normal (Zachmann et al. 1986), whereas little or no growth spurt occurs in patients with aromatase deficiency (Grumbach 2000). These findings also provide strong suggestive evidence that pubertal growth spurt may be primarily induced by estrogen.

The actions of estrogen on the skeleton mainly occur via interactions with the receptors $ER\alpha$ and $\beta$. Knockout mouse models have been developed to study the regulation of longitudinal growth by estrogen. The effects of ER inactivation on bone growth are sex and age dependent according to the analyses of bone length in these mice (Vidal et al. 1999, 2000; Sims et al. 2002; Chagin et al. 2004; Windahl et al. 1999, 2001; Lindberg et al. 2001; Tozum et al. 2004; Parikka et al. 2005). Gender-dependent effects of 17 $\beta$-estradiol on bone growth were also noted in young mice (Ornoy et al. 1994). Compared with age- and sex-matched wild-type (WT) mice, a significant decrease in the length of femoral bone was observed in $ER\alpha$-knockout (ERKO) females; in contrast, a slight decrease was shown in ERKO males (Parikka et al. 2005; Couse and Korach 1999). More interestingly, the effects of ER on bone growth may differ between axial and appendicular regions (Vidal et al. 1999). The sum of these reports suggests that the developmental pattern of ER may be varied at different age stages of different regions and genders.

Estrogen can regulate bone growth by systemic and direct action. The facts that both $ER\alpha$ and $\beta$ are detected in growth plate chondrocytes during development indicates the direct effects of estrogen on chondrocytes (Braidman et al. 1995; Kusec et al. 1998; Kennedy et al. 1999; Nilsson et al. 1999; Braidman et al. 2001; van der Eerden et al. 2001, 2002; Nilsson et al. 2002, 2003). However, results and conclusions on the expression patterns of $ER\alpha$ and $\beta$ in the growth plates are different and conflicting in these studies. During early life and sexual maturation, the location of ER is involved in the resting and proliferating chondrocytes (Nilsson et al. 2002), late proliferating and early hypertrophic chondrocytes (van der Eerden et al. 2002), hypertrophic zone (Nilsson et al. 1999; Egerbacher et al. 2002), and in all zones (Kusec et al. 1998; Nilsson et al. 2003). In addition, Kennedy et al. 1999 showed that $ER\alpha$ immunostaining disappeared during sexual maturation in rats, and therefore proposed that this might be responsible for the absence of growth-plate closure in the rat. However, the expression of $ER\alpha$ and $\beta$ in the developing

vertebral growth plate has not been established, nor can gender differences in ER expression be detected (van der Eerden et al. 2002; Nilsson et al. 2003).

In the present study, we investigated, for the first time, the gender- and region-specific variations of the developmental pattern for $ER\alpha$ and $\beta$ expression in rat limb and spine growth plates. Quantitative real-time PCR, and immunohistochemistry were used for $ER\alpha$ and $\beta$ examination and comparison in this study. The stages of early life (1 and 4 weeks), puberty (7 weeks), the end of puberty (12 weeks) and after puberty (16 weeks) were chosen for examination at the mRNA and protein level.

## Materials and methods

### Experimental animals

Female and male wistar rats were purchased from Shanghai Laboratory Animal Co. Ltd. (SLAC, Shanghai, China). The rats were maintained on a 12 h light/dark cycle at $23 \pm 2°C$, with food and water freely available. The local ethics committee for animal experiments approved the experimental procedures and protocols. The animals of both genders ($n = 5$) were sacrificed at 1, 4, 7, 12, and 16 weeks of age, of which the tibiae and lumbar spine bone were isolated. The fixation as described by van der Eerden et al. (2002) was used, which was composed of 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 mol/L phosphate buffer supplemented with 75 mmol/L lysine monohydrochloride and 10 mmol/L Na-periodate. Half of the isolated samples were stored in this fixation for 24 h, followed by decalcification for about 4 weeks in 15% ethylenediaminetetraacetic acid (EDTA), including 0.5% paraformaldehyde. For immunohistochemistry study, the samples were dehydrated in a graded series of ethanol and embedded in paraffin. The method described previously (van der Eerden et al. 2000; Gevers et al. 2002; Sampson et al. 2007; Mitani et al. 2006) was used for the dissection of the growth plates of the other half tissue specimens. By cutting away all sides of the cartilage-bone borders to avoid contamination with fibrous tissue or bone tissue, the growth plate material was separated and the obtained fragments were rapidly frozen in liquid nitrogen for RNA preparation. Several studies have shown that the growth plate cartilages were negative for type I collagen (Mundlos and Zabel 1994; Camper et al. 2001; Fukunaga et al. 2003). Therefore, absence of collagen type I mRNA was used for judgment of the removing of contamination with bone tissue.

### RNA isolation and quantitative real-time PCR

By grinding the frozen sample in liquid nitrogen using a mortar and pestle followed by extraction using Trizol

reagent (Invitrogen) according to the manufacturer's protocol, total RNA from each growth plate specimen was isolated. The procedure followed was as described previously (Li et al. 2010, 2011). The quantity and purity of the isolated RNA was measured at OD260 and OD280, and analyzed on a $0.5 \times 5$ TBE (0.045 mol/L Tris borate, 0.001 mol/L EDTA) 1% agarose gel to check the integrity of the RNA. Using the One-Step TaKaRa PrimescriptTM RT Reagent Kit (TaKaRa, Japan), first-strand cDNA was synthesized from 1 μg total RNA.

ERα, β and collagen type I gene expression was measured by real-time PCR. PCR amplification was performed with the SYBR Premix Ex Taq (Takara) in a LightCycler (Roche Diagnostics) according to the manufacturer's instructions. For these reactions, specific oligonucleotide primers to rat sequences were designed by program Oligo 4.0 (National Biosciences, Inc., Plymouth, MN) on the basis of sequences in GenBank. The forward and reverse primer sequences for the amplifications were as follows: ERα: 5′-TGTTAC GAAGTGGGCATGATGAA-3′ and 5′-GCCAAAGGTTG GCAGCTCTC-3′; ERβ: 5′-AGCGACCCATTGCCAATC A-3′ and 5′-CTGGCACAACTGCTCCCACTAA-3′; collagen type I: 5′-GACATGTTCAGCTTTGTGGACCTC-3′ and 5′-GGGACCCTTAGGCCATTGTGTA-3′; β-actin: 5′-GGAGATTACTGCCCTGGCTCCTA-3′ and 5′-GACT CATCGTACTCCTGCTTGCTG-3′. Two steps were included in the reactions: first, the samples were heated to 95°C for 30 s; second, there were 20 cycles consisting of 5 s at 95°C followed by 20 s at 60°C. Finally, the melting curve was analyzed. A standard curve of $C_t$ values versus serial dilutions of cDNA was used to determine the amplification efficiency. In our study, a standard curve was created from serial dilutions spanning six orders of magnitude, yielding a correlation coefficient of at least 0.98 in all experiments. PCR efficiency was close to 1, indicating a doubling of DNA at each PCR cycle, as expected. The amount of ERα and β gene relative to the internal control gene, β-actin, was calculated with the $2^{-\Delta C_t}$ formula. The $C_t$ value of each cDNA sample was defined as the cycle number at which the fluorescence intensity of each target gene was amplified within the linear range of the reaction. The data were analyzed in triplicate.

## Immunohistochemistry

For immunohistochemistry, paraffin sections (5 μm thick) were treated with xylene to remove paraffin and rehydrated in graded alcohol baths followed by three rinses with phosphate-buffered saline (PBS). Slides were immunostained with the streptavidin–biotin peroxidase (SABC) technique according to our previously described procedure (Zhao et al. 2008; Li et al. 2011).

Antigen retrieval was performed in 10 mmol/L citrate buffer (pH 6.0) at 95°C for 10 min and allowing them to cool down to room temperature for 20 min. Then sections were incubated in 1% $H_2O_2$ for 15 min for blocking the endogenous peroxidase activity. Sections were incubated overnight at 4°C with mouse polyclonal antibody of the active form of the ERα (Santa Cruz, sc-543, 1:100 dilution) and ERβ (Abcam, ab3576, 1:100 dilution)after preincubation with 5% normal goat serum (Vector, S-1000) for 30 min at room temperature. Then sections were incubated with the corresponding biotinylated goat anti-rabbit IgG (Vector, BA-1000), applied for 30 min at a dilution of 1:200, followed by a triple wash in PBS. Finally, at room temperature, the sections were incubated in ABC complex (Vectastain ABC kit, Vector Cat#PK-6100) for 30 min. Staining was detected with DAB peroxides substrate solution for 3 min, followed by rinsing in distilled water briefly. The slides were dehydrated in graded ethanol, cleared in xylene, and mounted with Permount medium after counterstaining with Gill's hematoxylin solution for 3 min. Control experiments were incubated with the antibody preincubated with a blocking peptide. ERα and β isoforms had been detected in ovary and liver specimens (Nilsson et al. 2002). The primary antibodies with the same concentrations were used to stain positive control tissues (liver and ovary).

## Semi-quantitative analysis of ERα and β staining intensities

Using the following intensity categories: 0, no staining; 1, weak but detectable staining; 2, moderate or distinct staining; 3, intense staining, the intensities of ERα and β immunoreactivity were evaluated semi quantitatively. For each sample, a HSCORE value was derived by summing the percentages of cells that stained at each intensity group and multiplying that by the weighted intensity of the staining. The formula $HSCORE = \sum_i i \times P_i$, where $i$ represents the intensity scores, and $P_i$ is the corresponding percentage of the cells, was used for calculation. For each growth plate sample, three tissue sections from each sample were randomly achieved and five randomly selected areas were evaluated for each tissue slide under the microscope with 200× original magnification. The percentage of the cells at each intensity group within these areas was determined by two investigators blinded to the type of the tissues. The mean score was used.

## Statistical analysis

All data from ERα and β immunohistochemistry scores in the growth plate were normally distributed as tested by Kruskal–Wallis (H) test. Quantitative data regarding ERα

and $\beta$ gene expression and HSCORE analysis are presented as mean $\pm$ SD and analyzed by three-factor (age, gender and region) ANOVA. The Bonferroni test was used for post-hoc comparison. A $P$ value of $< 0.05$ was considered statistically significant. Statistical analysis was performed using the SPSS (SPSS Inc., USA) statistical package.

## Results

### Region-specific changes of ERα and $\beta$ mRNA expression in male and female rat growth plate

We assessed the expression of ERα and $\beta$ in the growth plate of the rat axial and appendicular region of both genders by quantitative real-time PCR. The developmental change curves of ERα and $\beta$ mRNA expression were shown in Figs. 1 and 2.

Variations of ERα and $\beta$ expression in tibial and spinal growth plate of both genders were noted in this study (Figs. 1a, 2a). In female rats, at fast growth stage during early life and just before puberty, there were no significant differences of ERα gene expression between spine and tibia. During and at the end of puberty (7 and 12 weeks), a significant difference of the relative ERα expression occurred in spine and limb growth plate. The expression was higher in spine than in limb. The difference increased during puberty. After puberty (16 weeks), ERα expression in spinal and tibial growth plate returned to a similar level to that during puberty. In contrast, ER$\beta$ expression was lower in spine than in limb.

In male rats, ERα expression in spinal growth plate was significantly lower than that in tibia just before and during puberty (4 and 7 weeks). Whereas, after puberty, growth plate in spine had a significantly higher ERα expression than that in limb (Fig. 1a). As for the ER$\beta$, just before puberty, it was significantly higher in spinal growth plate. During and after puberty, the expression differences between spine and limb were similar to ERα (Fig. 2a).

### Gender-specific variations of ERα and $\beta$ mRNA expression in axial and appendicular growth plate

In tibial growth plate, Fig. 1b showed significantly higher ERα mRNA expression of male rats than that of female rats just before and at puberty (4 and 7 weeks). However, at the end of puberty, a significantly lower ERα expression was indicated in male than in female. With regard to the expression of ER$\beta$, male and female had similar variations at all time intervals except after puberty (16 weeks). After puberty, ER$\beta$ expression was significant higher in female limb than that in male (Fig. 2b).

**Fig. 1** The developmental changes of ERα expression in growth plate of the rat spine and limb of both genders. Total RNA was extracted from the growth plate, reverse transcribed, assessed by quantitative real-time PCR and analyzed by multiple comparisons using the Bonferroni test. **a** Region-specific changes of ERα mRNA expression in growth plate. In male rats, ERα gene expression in spine was significantly lower than that of limb at 4, 7 weeks. After 16 weeks, expression in spinal growth plate was markedly higher than that in limb. In female rats, ERα expression was significantly lower in spine at 7 and 12 weeks. $P < 0.05$ between spine and limb of female (*inverted filled triangles*) and male (*asterisks*) rats. **b** Gender differences of ERα expression. In limb, male rats had significantly higher ERα mRNA expression than female rats at 4 and 7 weeks of age. However, at 12 weeks of age, male rats had lower ERα expression than females. In spine, gender differences mainly occurred at 4, 7, 12 and 16 weeks of age. $P < 0.05$ between female and male limb (*asterisks*) and spine (*stars*) at each development stage. **c–f** Age-related differences of ERα mRNA expression in spine and limb of both genders. Data are presented as fold changes compared to the expression level of 1 week. $^{\#}p < 0.05$ Limb♀ and Spine♀ 7, 12, 16 weeks versus 1 week; 7, 12, 16 versus 4 weeks; 7 versus 12 weeks; 12 versus 16 weeks. Limb♂ 4, 7, 16 weeks versus 1 week; 12, 16 versus 4 weeks; 12, 16 versus 7 weeks. Spine♂ 4, 16 weeks versus 1 week; 7, 12, 16 versus 4 weeks; 16 weeks versus 7 weeks; 12 versus 16 weeks. **g, h** There was hardly any collagen type I mRNA in all spinal and tibial growth plate specimens for judgment of the removing of contamination with bone tissue

In spinal growth plate, no significant gender difference of ERα gene expression was observed only at fast growth stage during early life (1 week). Compared with females, the relative ERα expression level was significantly higher just before and after puberty (4 and 16 weeks), and lower during puberty (7 and 12 weeks) in males (Fig. 1b). No gender-specific variations of ER$\beta$ expression occurred at 1 and 7 weeks. In comparison to female, male spinal growth plate had significantly higher ER$\beta$ expression just before and after puberty, and a significantly lower expression at the end of puberty (Fig. 2b).

### Age-related changes of ERα and $\beta$ gene expression in the growth plate

In the appendicular region of female rats as shown in Figs. 1a, c and 2a, c, expression of ERα and $\beta$ mRNA increased continuously from early life stage to puberty (1, 4 and 7 weeks). Both of them peaked at the end of puberty (12 weeks). The expression of ERα was up-regulated earlier than that of ER$\beta$ during development. ERα gene expression significantly increased from the onset of the puberty. No significant up-regulation was observed for the expression of ERα during early life (1 and 4 weeks) and for the expression of ER$\beta$ from early life stage to puberty (1, 4 and 7 weeks). After sexual maturation (16 week), ERα expression decreased close to that of the puberty again ($P > 0.05$), whereas ER$\beta$ expression was significantly higher than that during the puberty ($P < 0.05$). In the axial skeleton, ERα and $\beta$ expression changes appeared to vary in a similarly
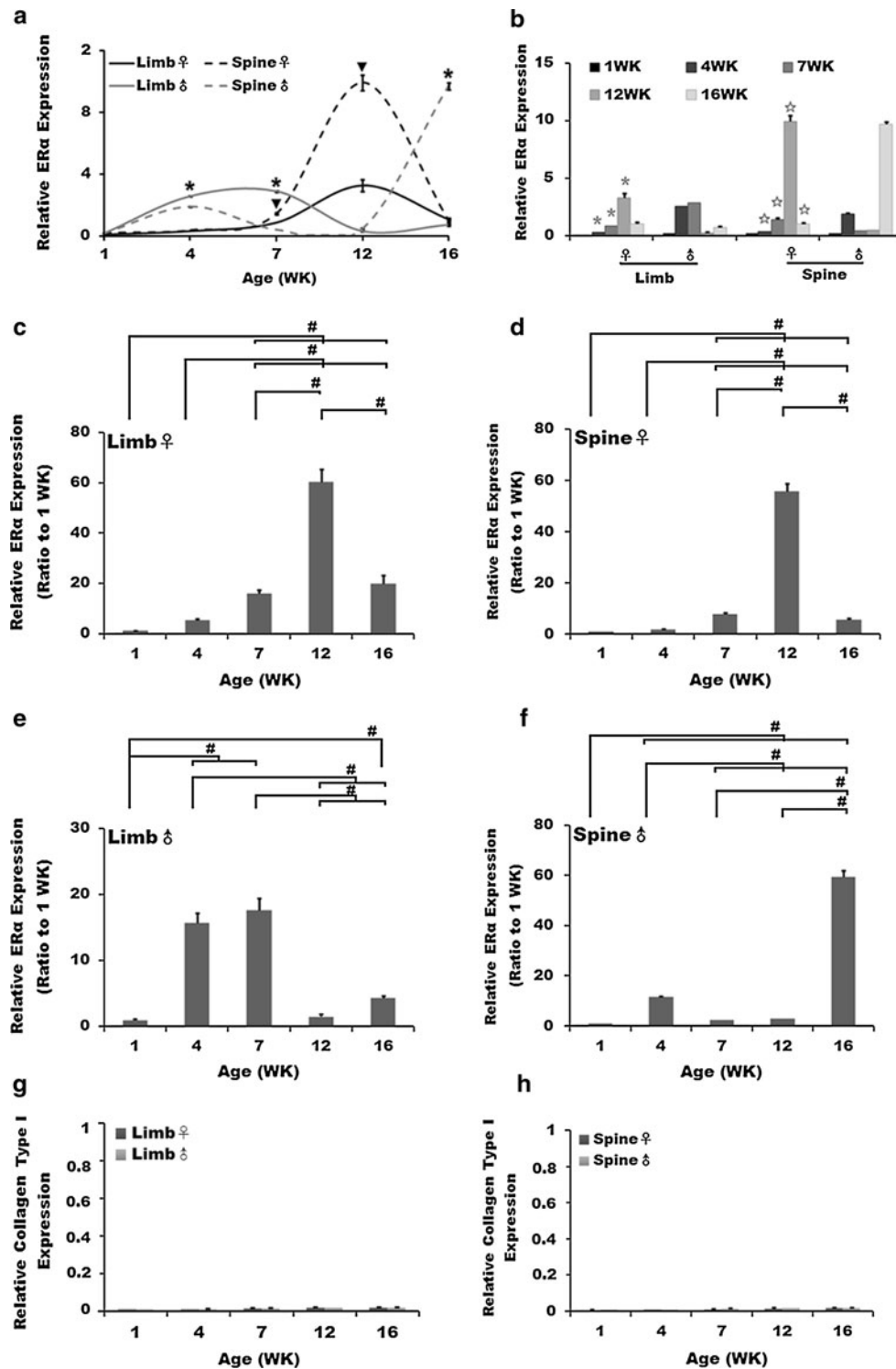
age-related manner as in the appendicular region (Figs. 1a, d, 2a, d). After sexual maturation (16 week), not similar to the limb, ERβ expression was not significantly higher than that during the early life and puberty stages in the axial skeleton.

In both appendicular and axial skeleton of male rats (Figs. 1a, e, f, 2a, e, f), ERα and β expression showed a significant increase just before puberty (4 weeks), which was earlier than that in females. In the appendicular skeleton, ERα expression reached the peak value at 4 weeks, with no significant expression difference between 4 and 7 weeks, followed by decline. ERβ expression peaked during puberty (7 weeks). At the end of puberty, the expression of both

**Fig. 2** The developmental changes of ERβ expression in growth plate of the rat spine and limb of both genders. The data were analyzed by multiple comparisons using the Bonferroni test. **a** Region differences of ERβ expression mainly occurred at 12 and 16 weeks in female rats and 4, 7 and 16 weeks in males. $P < 0.05$ between spine and limb of female (*inverted filled triangles*) and male (*asterisks*) rats. **b** Gender differences of ERβ expression mainly presented from 4 to 16 weeks in limb and at 4, 12, and 16 weeks in spine. $P < 0.05$ between female and male limb (*asterisks*) and spine (*stars*) at each development stage. **c–f** Age-related differences of ERβ mRNA expression in spine and limb of both genders. Data are presented as fold changes compared to the expression level of 1 week. $^{#}p < 0.05$ Limb♀ 12, 16 weeks versus 1 week; 12, 16 weeks versus 4 weeks; 12, 16 weeks versus 7 weeks; 12 weeks versus 16 weeks. Spine♀ 1, 4, 7 weeks versus 12 weeks; 12 weeks versus 16 weeks. Limb♂ 4, 7 weeks versus 1 week; 7, 12, 16 weeks versus 4 weeks; 12, 16 weeks versus 7 weeks. Spine♂ 4, 16 weeks versus 1 week; 7, 12 weeks versus 4 weeks; 12 versus 16 weeks
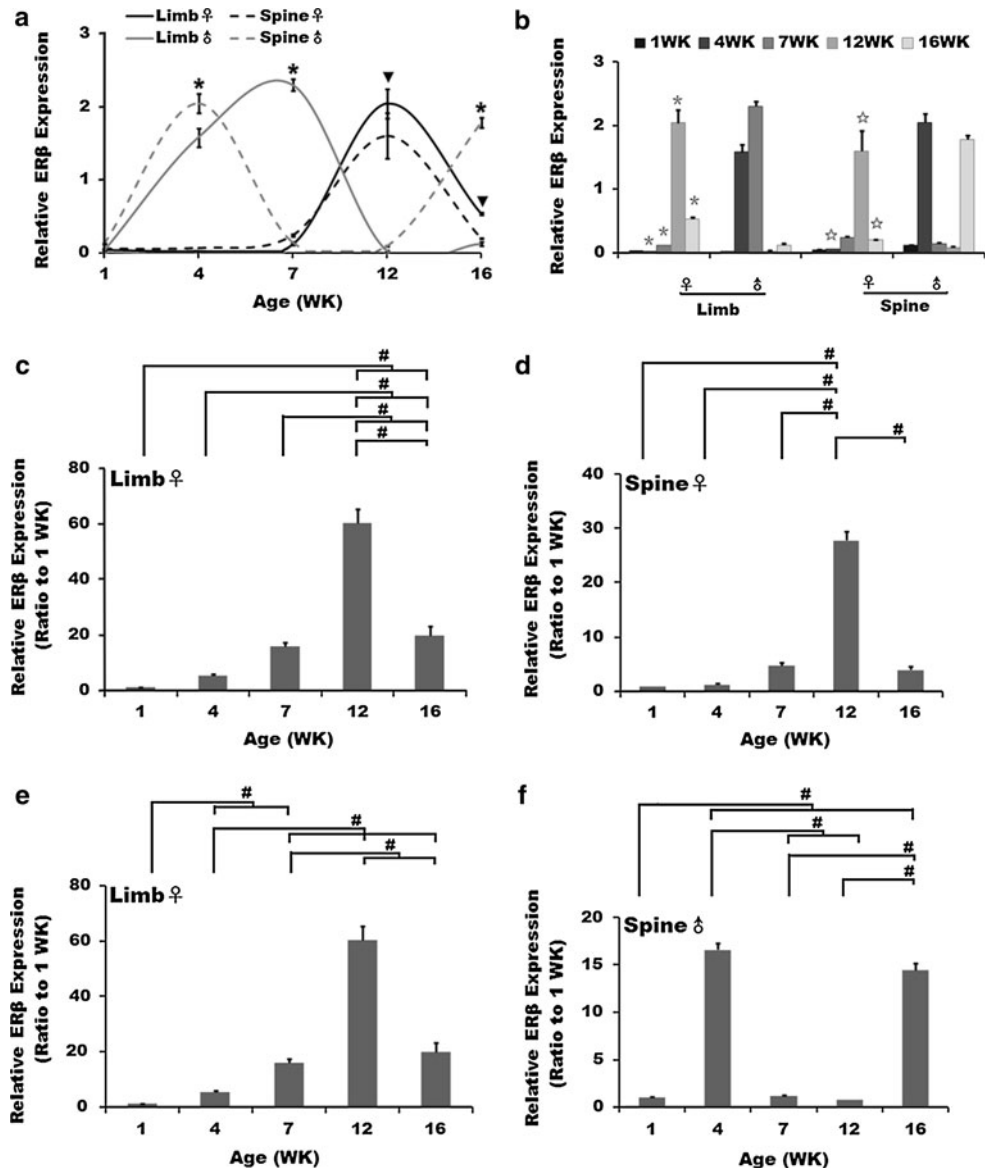


receptors approached the level of early life stage (1 week, $P > 0.05$). After sexual maturation (16 weeks), although an up-regulation of both ERα and β gene expression was observed, compared with the expression level of each fast growth stage during early life (1 week), only ERα gene had a significant increase ($P < 0.05$). In the axial skeleton of male rats, ERα and β expression increased before puberty, and decreased during puberty. Then a significant up-regulation presented after puberty in the spinal growth plate. Unlike the appendicular region, the peak value of both ERα and β expression was just before puberty (4 weeks) in spinal growth plate of male rats. After puberty (16 weeks), the ratio to 1 week of ERα was higher (about fourfold) than that of ERβ in spine. Before sex maturation, the peak value of ERβ expression ratio to 1 week in limb growth plate was about sixfold of that in spine.

## Age-, region- and gender-related changes of the ratio of ERα/ERβ expression in growth plate

Figure 3a showed that female spinal growth plate had lower ERα/ERβ expression ratio than limb in 1 week of early life. Just before and during puberty (4 and 7 weeks), the expression ratio was higher in female limb than that in spine. At the end of and after puberty, the ratio in spine increased, significantly higher than that in limb. In male, significant expression ratio differences between limb and spine were only demonstrated in 1 and 12 weeks, whereas the differences were contrary to that in female. The effect of skeleton site on the change curves of temporal ERα/ERβ expression ratio might be more significant than that of gender.

There are gender-related variations of ERα/ERβ expression ratio in all observed developing stages in limb

**Fig. 3** The developmental changes of ERα/ERβ expression ratio in growth plate of the rat spine and limb of both genders. **a** Region-specific differences of ERα/ERβ ratio presented at all development stages in females and 1 and 12 weeks in males. The effect of skeleton site on the change curves of temporal ERα/ERβ expression ratio might be more significant than that of gender. $P < 0.05$ between spine and limb of female (*inverted filled triangles*) and male (*asterisks*) rats. **b** Gender differences of ERα/ERβ mainly were revealed at 1, 4 and 7 weeks of age in spine and at all development age in limb. $P < 0.05$ between female and male limb (*asterisks*) and spine (*stars*) at each development stage. **c–f** Age-related differences of ERα/ERβ ratio in spine and limb of both genders. Data are presented as fold changes compared to the expression level of 1 week. [#]$p < 0.05$ Limb♀ 4, 7 weeks versus 1 week; 7, 12, 16 weeks versus 4 weeks; 12, 16 weeks versus 7 weeks. Spine♀ 4, 7, 12 weeks versus 1 week. Limb♂ 4, 7, 12 weeks versus 1 week; 12, 16 weeks versus 4 weeks; 12, 16 weeks versus 7 weeks; 12 weeks versus 16 weeks. Spine♂ 12, 16 weeks versus 1 week; 7, 12, 16 weeks versus 4 weeks; 12, 16 weeks versus 7 weeks
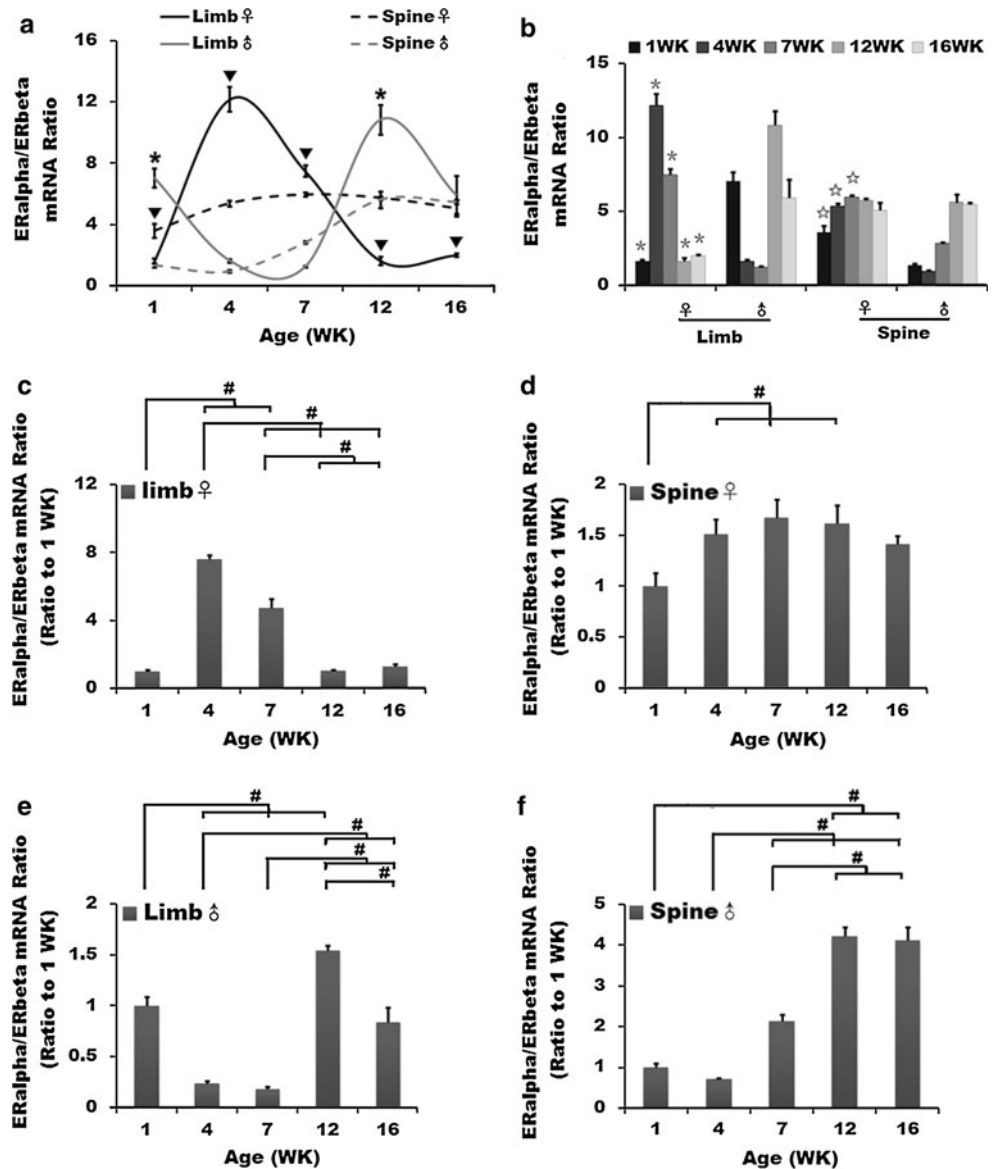


growth plate (Fig. 3b). However, in spinal growth plate, variations were found only in early life and puberty (1, 4 and 7 weeks). Just before and during puberty (4 and 7 weeks), the ratio was significantly low in limb and spine in both genders.

In female rats, the ratio of ERα/ERβ expression peaked just before puberty in both appendicular and axial skeleton (no significant differences in axial skeleton between 4 and 7 weeks). During puberty, the ERα/ERβ expression ratio had a significant down-regulation in limb, whereas no significant changes occurred in spine (Fig. 3a, c, d). In male rats, in the stages of early life (1 and 4 weeks), a significant decrease of the ERα/ERβ expression ratio was indicated in appendicular skeleton. No significant change was observed in axial region. In both regions of male rats, a

significant up-regulation of the ratio was observed during the entire puberty (7 and 12 weeks), and followed by a significant decrease in both regions after puberty (Fig. 3a, e, f).

### ERα and β immunoreactivity in control tissues

To investigate the sensitivity and specificity of the active form of ERα and β immunostaining, positive control experiment was performed using liver and ovary specimens from rats. Immunohistochemistry revealed nuclear ERα immunoreactivity in hepatocytes and strong nuclear ERβ immunostaining in granulosa cells in the ovary (Fig. 4a, b). When the antibody was preincubated with a blocking peptide, no staining was detected (Fig. 4c, d).

**Fig. 4** Control experiments for immunohistochemistry. Positive immunoreactivity produced a brown coloration. Localization of ERα and β protein in sections of liver and ovary from Wistar rats. **a** Predominantly nuclear staining of ERα immunoreactivity was mainly detected in hepatocytes. **b** Nuclear ERβ immunostaining was found in granulosa cells in ovary. **c, d** No staining could be observed when the antibody was preincubated with a blocking peptide. *Bar* represents 50 μm
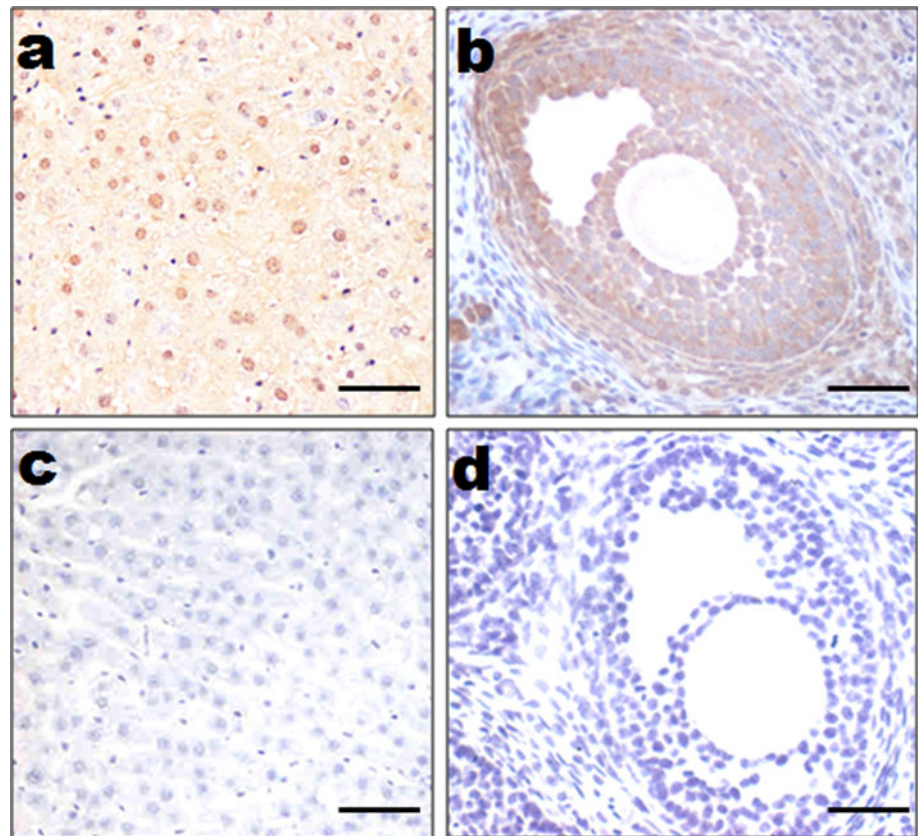


## Expression of ERα and β protein in the growth plate of the male and female rat axial and appendicular region

Distribution of ERα and β protein was detected on the sections of tibial (Figs. 5, 6) and spinal (Figs. 7, 8) growth plate from female and male rats of 1, 4, 7, 12, and 16 weeks. Both cytoplasmic and nuclear staining of ERα and β immunoreactivity in growth plate chondrocytes was detected in the spinal and tibial growth plate of both genders. Abundant nuclear staining of ERα and β immunoreactivity was observed in chondrocytes. Compared with each nuclear staining, cytoplasmic staining of ERα seemed lesser than that of ERβ. The results of semiquantified analysis using the HSCORE were shown in Figs. 9 and 10.

For the spatial organization of the tibial and spinal growth plates of 1-, 4-, 7- and 12-week-old, both genders had similar distribution of the ERα protein expression. At 1 and 4 weeks of age, ERα immunoreactivity was detected in the resting, proliferative and prehypertrophic chondrocytes. Staining in the hypertrophic chondrocytes appeared to be less intense compared with the resting and proliferative region. During puberty, 7 and 12 weeks of age, ERα immunoreactivity was extended from the resting to the hypertrophic chondrocytes in limb and spine. At slow growth stages after puberty (16 weeks of age), ERα expression was mainly located in the late proliferative and

hypertrophic chondrocytes in female limb and spine, whereas the expression still extended from the resting to hypertrophic chondrocytes in males. The spatial expression patterns for ERβ were similar to that of ERα in different zones of growth plate in limb and spine of both genders.

ERα and β HSCORE data showed age-related variations in tibial growth plate of both genders (Figs. 9a, b, 10a, b). The values of ERα and β increased with age during early life and sexual maturation, and peaked at 12 weeks for female limb. After sexual maturation (16 week), the values decreased close to that of 1 week again ($P > 0.05$), whereas, in male limb, the values peaked at 4 and 7 weeks, followed by down-regulation ($P < 0.05$) at 12 weeks, and then up-regulated at 16 weeks.

According to the HSCORE analysis, ERα and β in the spinal growth plate showed a similar age-related variation to that in the appendicular region of female (Figs. 9c, d, 10c, d). In agreement with the expression results of female spine by quantitative real-time PCR, the positive staining HSCORE value was significantly low in 16 weeks compared with that in 1, 4, 7 and 12 weeks ($P < 0.05$). In male rats, ERα and β HSCORE values increased from 1 to 4 weeks, and was then down-regulated at 12 weeks and finally up-regulated at 16 weeks in the spinal growth plate. The peak value appeared at 16 weeks for ERα, and at 4 and 16 weeks for ERβ.
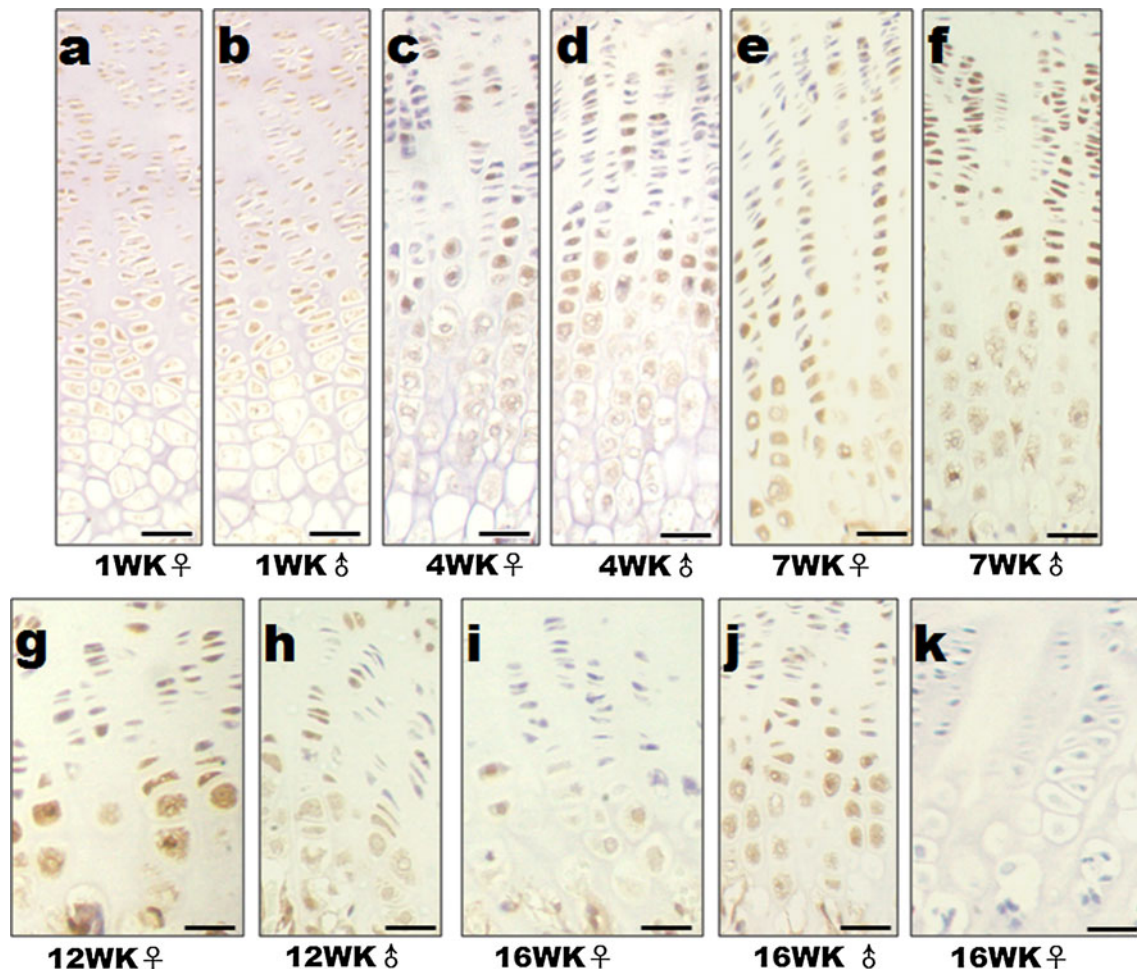
**Fig. 5** Immunohistochemistry of ERα in tibial growth plates during development of female and male rats. **a–d** At fast growth stage during childhood and just before puberty (1 and 4 week), ERα immunoreactivity was detected in the resting, proliferative and prehypertrophic chondrocytes. Staining in the hypertrophic chondrocytes appeared to be less intense compared with the resting and proliferative region. **e–h** During puberty (7 and 12 weeks), ERα mainly located in the proliferative and hypertrophic chondrocytes. **i**, **j** After puberty (16 weeks), ERα expression was mainly located in the late proliferative and hypertrophic chondrocytes in female rats, whereas, the expression still extended from the resting to hypertrophic chondrocytes in males. **k** Absence of staining was noted when the antibody was preincubated with a blocking peptide. *Bar* represents 50 μm

In addition, HSCORE analysis of the ERα and $\beta$ immunolabeling also revealed gender- and region- specific changes in the growth plate (Figs. 9e, f, 10e, f). Significant region-specific differences were shown at 7, 12, 16 weeks for ERα and 4, 7, 16 weeks for ER$\beta$ in males and at 12, 16 weeks for ER$\beta$ in females ($P < 0.05$) (Figs. 9e, 10e). Significant gender-related differences were mainly noted at 4 and 12 weeks for ERα and 4, 7, 12 and 16 weeks for ER$\beta$ in the limb; in the spine, were noted at 4, 7, 12 and 16 weeks for ERα and 4, 12 and 16 weeks, respectively ($P < 0.05$) (Figs. 9f, 10f).

## Discussion

Although the effects of estrogen on bone growth are well known, the roles of estrogen receptors (ERs) in mediating bone growth are not fully understood. Recently, several researchers have demonstrated that both male and female rat growth plate cartilage cells possess estrogen receptors. In the present study, for the first time, the gender-, region- and age-related expression of the ERα and $\beta$ mRNA and protein in the growth plate of the rat spine and limb was surveyed during development. The developmental stages of 1, 4, 7, 12, and 16 weeks of age in rats are compatible with the representative time points of longitudinal growth in humans (Hughes and Tanner 1970). These time points correspond to fast growth during childhood, just before puberty, puberty, the end of puberty, and after puberty (van der Eerden et al. 2000). Quantitative measurement of temporal expression pattern of ERα and $\beta$ gene suggested gender- and region-specific variations in the axial and appendicular growth plate. In our immunohistochemistry
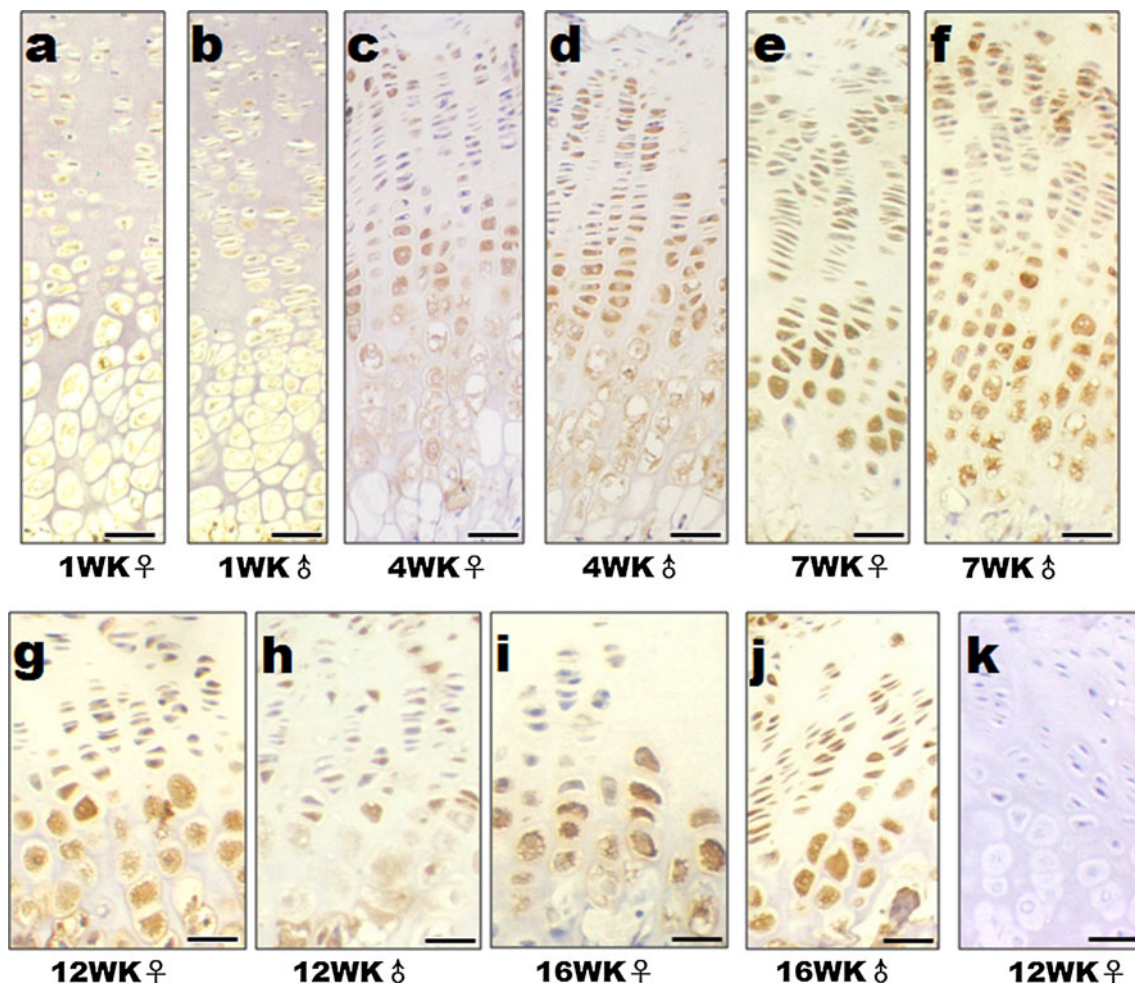
**Fig. 6** Spatial expression patterns for ERβ were similar to that of ERα in different zones of growth plate in limb of both genders. Similar to ERα expression, abundant nuclear staining of and ERβ immunoreactivity was observed in chondrocytes. **a–j** Spatial ERβ expression patterns at 1–16 weeks of female and male rats. **k** No staining could be observed when the antibody was preincubated with a blocking peptide. *Bar* represents 50 μm

study, gender-related spatial changes of ERα and β expression were detected only at 16 weeks of both spine and limb growth plate. However, region-specific spatial changes of ERα and β expression were not detected in our present study.

In the previous studies, only male (Nilsson et al. 2002) or both gender (Kennedy et al. 1999; van der Eerden et al. 2002) rats were used. Age range of the studied rats extended from the stages of early life to the puberty (Kennedy et al. 1999; Nilsson et al. 2002) or to the post puberty stages (van der Eerden et al. 2002). No related research on the vertebral growth plate was reported. In the present study on the growth plate of the rat, gender-, region- and age-related expression patterns of ERα and β gene and protein were all included.

Kennedy et al. (1999) acclaimed that the absence of growth plate fusion at sexual maturation in rats might be caused by the disappearance of ERα expression in the

growth plate cartilage. Whereas, as reported in the literature (van der Eerden et al. 2002; Nilsson et al. 2002), the present study demonstrated that ERα and β were detected in the limb growth plate throughout postnatal development. This difference may be due to methodological differences or the antibody they used. Specific ER isoforms, such as shorter isoforms, are not recognized by all antibodies, may also express in growth plate chondrocytes during sexual maturation. Frozen sections were used for immunofluorescent staining in their study, whereas Nilsson et al., van der Eerden et al. and we used a widely used method, paraffin-embedded tissues with antigen retrieval, for immunodetection of nuclear receptors (van der Eerden et al. 2002; Nilsson et al. 2002).

An immunohistochemistry study indicated that ERα and β expression zone was minimal at early ages, increasing only just before epiphyseal fusion (Nilsson et al. 2002). Quantitative assessment in our study also indicated that the level of
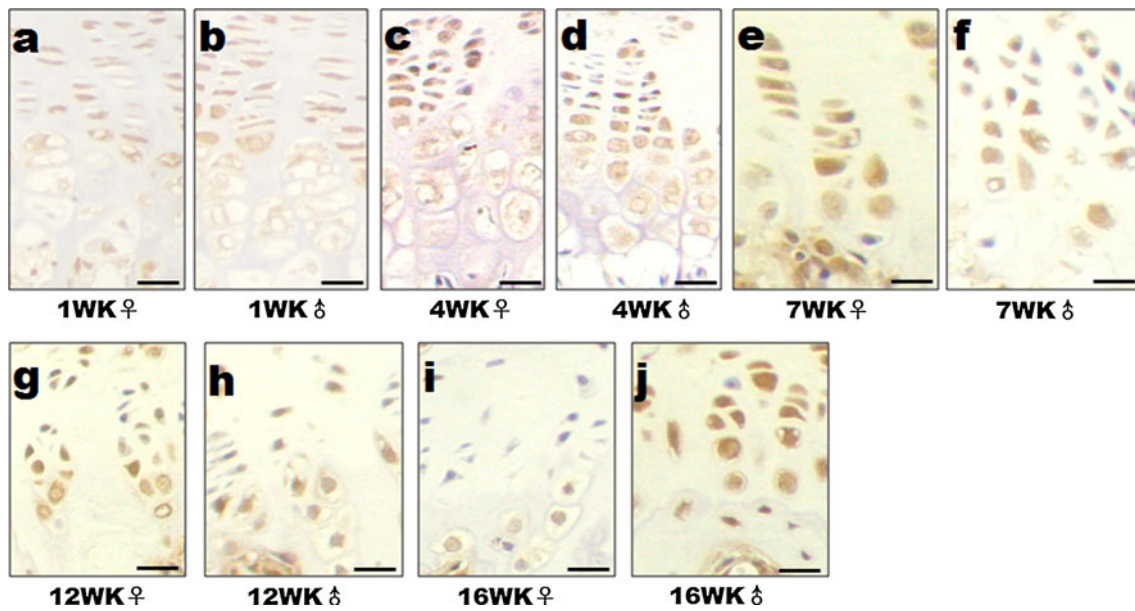
**Fig. 7** Immunohistochemical localization of ERα in spinal growth plates of female and male rats during development. **a–h** Before and during puberty (1, 4, 7 and 12 weeks of age), the expression pattern of ERα in the spinal growth plate was extended from the resting to the hypertrophic chondrocytes. **i, j** After puberty (16 weeks of age), ERα protein expression was confined to the late proliferative and hypertrophic chondrocytes in female, while extended from the resting to hypertrophic chondrocytes in males. *Bar* represents 50 μm
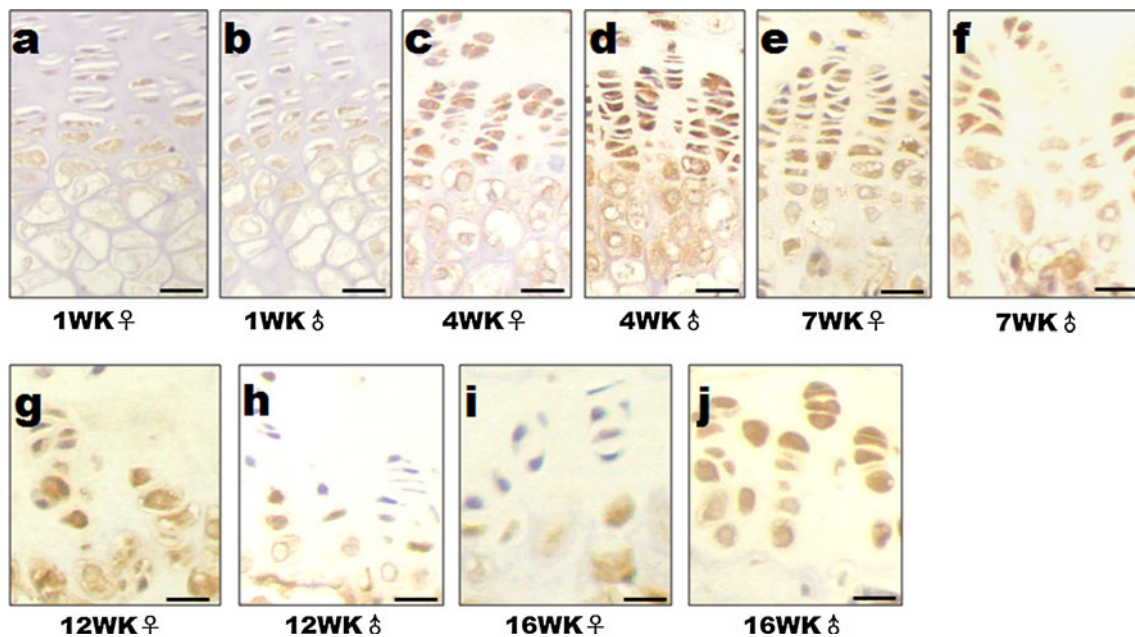


**Fig. 8** Spatial expression patterns for ERβ were similar to that of ERα in spinal growth plates of female and male rats during development. **a–j** ERβ expression patterns from 1 to 16 weeks of female and male rats. *Bar* represents 50 μm

ERα and β gene expression was low at early ages, while around puberty, the expression level was markedly high in limb and spine of both genders. Another in situ hybridization study did not show any differences in expression of ERs between genders, apart from a relatively higher ER gene expression in males after sexual maturation (van der Eerden et al. 2002). However, in our study, gender differences were detected in several specific life stages in both limb and spine. Nilsson et al. (2002) observed that both the pattern of expression and the intensity of ERα and β immunostaining appeared to be similar during development of male rats. Another study also suggests that ERα and β protein staining
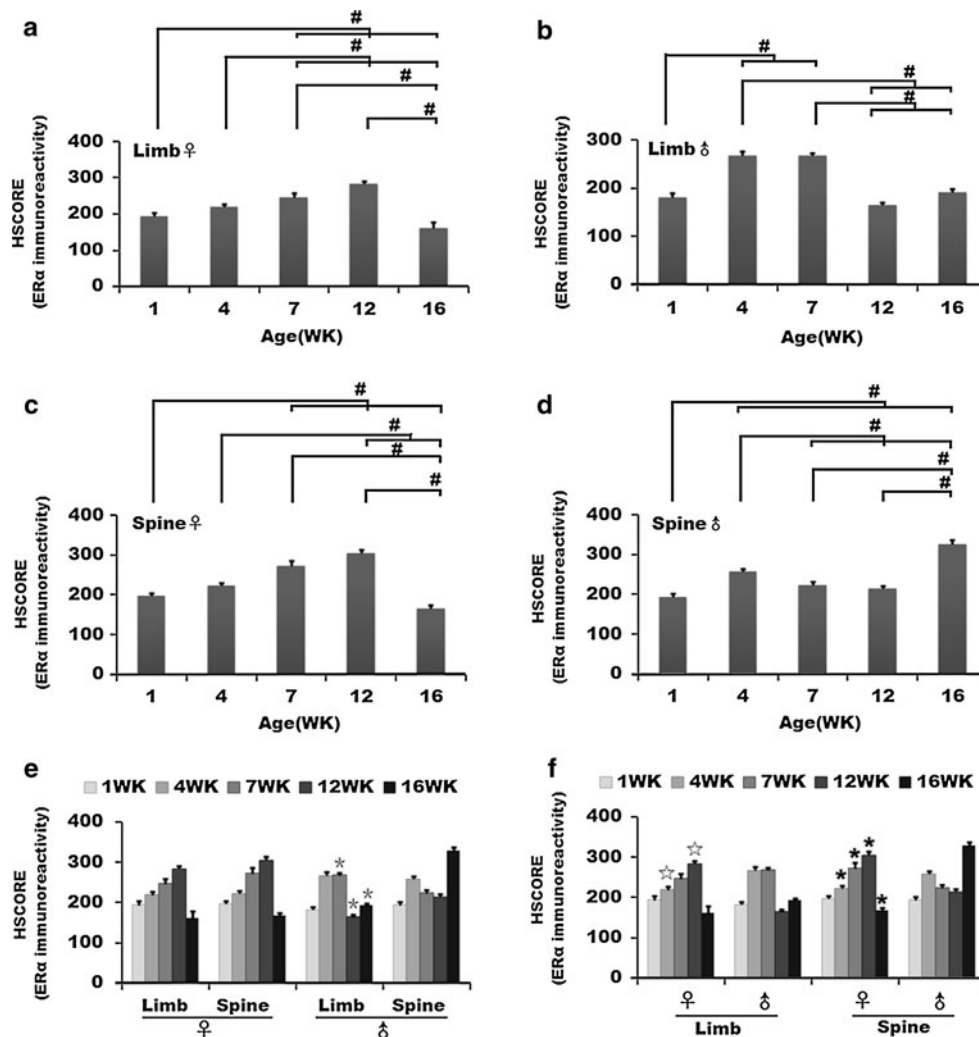
**Fig. 9** HSCORE analysis of the ERα immunostaining intensities in the growth plate of rat spine and limb of both genders. All samples exhibited immunoreactivity for ERα throughout the development. **a–d** Age-related changes of the HSCORE values in spine and limb of both genders. $^{#}p < 0.05$ Limb♀ 7, 12, 16 weeks versus 1 week; 7, 12, 16 versus 4 weeks; 16 weeks versus 7 weeks; 12 versus 16 weeks. Limb♂ 4, 7 weeks versus 1 week; 12, 16 versus 4 weeks; 12, 16 weeks versus 7 weeks. Spine♀7, 12, 16 weeks versus 1 week; 12, 16 versus 4 weeks; 16 weeks versus 7 weeks; 12 versus 16 weeks. Spine♂ 4, 7, 16 weeks versus 1 week; 7, 12, 16 versus 4 weeks; 16 weeks versus 7 weeks; 12 versus 16 weeks. **e** Region-specific

changes in the growth plate. In male rats, the values in the spine were significantly lower than that in the limb at 7 weeks. At 12 and 16 weeks, values in the spinal growth plate were significantly higher than that in the limb. $P < 0.05$ between the spine and limb of male (*asterisks*) rats. **f** Gender differences of the HSCORE values. In the limb, compared with females, male rats had significantly lower values at 12 weeks and higher data at 4 weeks of age. In spine, gender differences mainly presented at 4, 7, 12 and 16 weeks of age. $P < 0.05$ between female and male limb (*stars*) and spine (*asterisks*) at each development stage

patterns in male and female rats were identical at all ages (van der Eerden et al. 2002). Whereas, in our study, spatial expression differences of ERα and β protein between genders were only detected after puberty (16 weeks). In situ hybridization study demonstrated that expression area of ERβ mRNA was more extensive than that of ERα gene in female rats only during sexual maturation (van der Eerden et al. 2002). In our study, variations of ERα/ERβ mRNA expression ratio were indicated in all observed developing stages in both spine and limb growth plate. The different

expression patterns of ERα and β in growth plate could be due to the non-quantitative nature of immunohistochemistry and in situ hybridization. Immunohistochemistry and in situ hybridization have the advantage of the detection of target proteins in situ, however, calcified hard tissues remain difficult to analyze for the hardness of tissue samples. Decalcification had an effect on the degree of staining (Matthews 1982; Hosoya et al. 2005; Neves et al. 2011). Quantitative real-time PCR was used for gene detection of tissue without decalcification in the present study. This method can make a
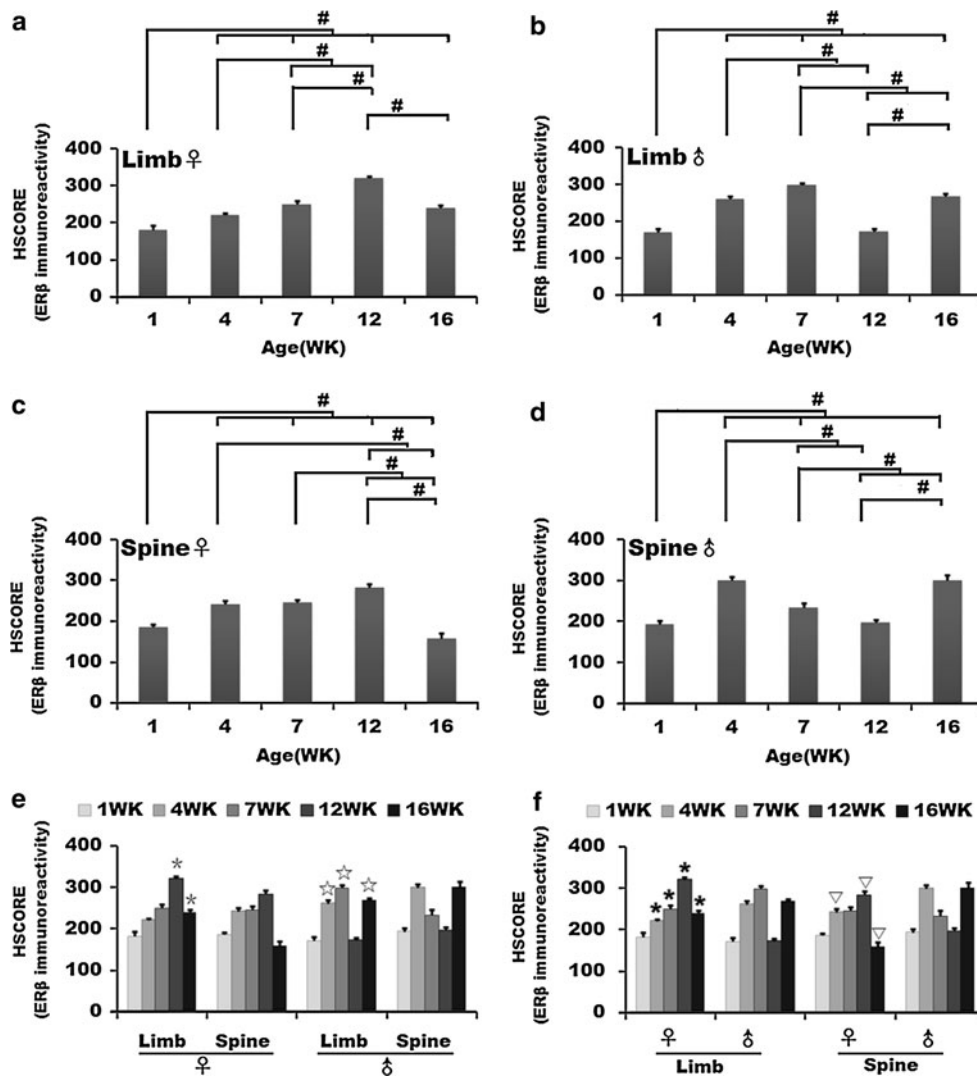
**Fig. 10** HSCORE analysis of the ER$\beta$ immunostaining intensities in the growth plate of rat spine and limb of both genders. All samples exhibited immunoreactivity for ER$\beta$ throughout the development. **a–d** Age-related changes of the HSCORE values in spine and limb of both genders. $^{\#}p < 0.05$ Limb♀ 4, 7, 12, 16 weeks versus 1 week; 7, 12 versus 4 weeks; 12 versus 7 weeks; 12 versus 16 weeks. Limb♂ 4, 7, 16 weeks versus 1 week; 7, 12 versus 4 weeks; 12, 16 versus 7 weeks; 12 versus 16 weeks. Spine♀4, 7, 12, 16 weeks versus 1 week; 12, 16 versus 4 weeks; 12, 16 versus 7 weeks; 12 versus 16 weeks. Spine♂ 4, 7, 16 weeks versus 1 week; 7, 12 versus 4 weeks; 12, 16 weeks versus 7 weeks; 12 versus 16 weeks. **e** Region-specific changes in the growth plate. In female rats, the

HSCORE values were significantly lower in spine at 12 and 16 weeks. In male rats, the values in the spine were significantly lower than that in the limb at 4 and 16 weeks. At 7 weeks, values in the spinal growth plate were significantly higher than that in the limb. $P < 0.05$ between the spine and limb of female (*asterisks*) and male (*stars*) rats. **f** Gender differences of the HSCORE values. In the limb, compared with females, male rats had significantly lower values at 12 weeks and higher data at 4, 7, 16 weeks of age. In spine, gender differences mainly presented at 4, 12 and 16 weeks of age. $P < 0.05$ between female and male limb (*asterisks*) and spine (*inverted open triangles*) at each development stage

rapid and accurate assessment of changes in gene expression and also can gain insight into potential changes in protein level and function.

## Temporal expression pattern for ER$\alpha$ and $\beta$: gender differences

Estrogen is the critical hormone in controlling growth plate acceleration and fusion in both genders. The effect of

estrogen differs between genders (Kaludjerovic and Ward 2008). Sex-related changes of temporal ER$\alpha$ and $\beta$ mRNA expression in growth plate were shown in the present study using quantitative real-time PCR. The HSCORE analysis supported the data obtained from quantitative real-time PCR. The effect of gender on the change curves of temporal ER$\alpha$ and $\beta$ expression might be more significant than that of skeleton site (Figs. 1a, 2a). In female spine and limb growth plate, ER$\alpha$ and $\beta$ gene expression was increased

during puberty, peaked at the end of sexual maturity and followed by declined expression. In contrast, in male growth plate, a significant increase of ERα and β expression was detected earlier (4 weeks).

Windahl et al. (1999) observed no significant difference of bone growth between prepubertal WT females and males. Consistently, gender-related differences of ERα and β were not noted in early life stages (1 week) in the present study. Chagin et al. (2004) demonstrated a differential effect between ERα and β on the growth plate, suggesting that ERβ is possibly more important in regulating female epiphyseal fusion. ERβ might be a physiological inhibitor of skeletal growth in female mice. Our study indicates that ERβ expression was still higher in female limb than that in male at slow growth stages after puberty (16 weeks of age). The different expression patterns of ERβ might partly contribute to the different limb length in females and males.

It has been suggested that the effects of ER inactivation on bone growth are sex dependent. Compared with females, in males, decreased longitudinal skeletal growth was only presented in ERα-knockout and double ERα/β-knockout (DERKO) mice, but not in ERβ-knockout (BERKO) mice. Such findings suggested that ERα, but not ERβ, mediates important effects of estrogen in the regulation of the male skeleton during growth and maturation (Vidal et al. 2000). The effects of ERα on bone growth and fusion in female mice might be more important than that in males (Parikka et al. 2005). In the tibiae of aged ERKO females, the growth plates were totally absent, whereas, in WT females as well as in ERKO and wild-type males, the growth plate cartilages were detectable (Parikka et al. 2005). Our study showed that ERα expression rate was higher than that of ERβ in limb and spine in nearly all developmental stages of both genders. ERα/ERβ expression ratio also presented as age, gender and region specific.

New trabecular bone is formed through endochondral ossification at the growth plate (Buie et al. 2008). The integration of the structural transformation from cartilage to bone is crucial in achieving longitudinal growth and peak bone mass (Byers et al. 2000). Trabecular BMD starts declining with aging in adulthood (Yuen et al. 2010). The age-related deterioration in trabecular bone begins in young adulthood and continues throughout life, with changes more pronounced in females than in males (Khosla et al. 2006; Glatt et al. 2007) Another observation from normal Japanese subjects also indicated that bone mass decreased with aging in females but not in males after puberty (Tsunenari et al. 1993). In the present study, after sexual maturation (16 weeks), an up-regulation of both ERα and β expression was shown in male rats, especially in spinal growth plate. The gender-specific expression patterns of ERα and β might partly contribute to the changes

in trabecular bone. Other growth-related molecular signaling indirectly controlled by ER might also be responsible. In the future, attempts should be made to unravel gender- and region-related actions of ER to further characterize their roles in the regulation of longitudinal growth of the bone.

Temporal expression pattern for ERα and β: region differences

The disproportional body growth phenotypes in limb and spine related to ER inactivation have been reported. In the female rat, gonadectomy also resulted in a bigger femur, but there was little change in spine (Seeman 2001). In growing female rats, estrogen deficiency leads to an increase in the length of appendicular bones, while growth was normal at spine (Zhang et al. 1999). Our study showed no region-specific expression patterns of ERα and β gene in both genders at fast growth period during early life stages (1 week) (Figs. 1b, 2b). In females, differences between spine and limb were revealed at 7 and 12 weeks for ERα, and at 12 and 16 weeks for ERβ. In males, differences of both ERα and β expression presented before, during and after puberty (4, 7 and 16 weeks). The HSCORE analysis results confirmed the data obtained from quantitative real-time PCR. Therefore, our study suggested that region-specific changes were also most prominent around puberty. The effect of skeleton site on the change curves of temporal ERα/ERβ expression ratio might be more significant than that of gender (Fig. 3a).

ERα and β might have differential effects on the growth of appendicular and axial skeletal site. A decreased femur/CR (crown-rump length) ratio was displayed for ERKO mice, while an increased ratio was displayed for BERKO mice. Compared with WT controls, the axial skeletal growth increase was bigger than that of the appendicular in BERKO female mice (Lindberg et al. 2001). Female ERKO mice demonstrate a disproportional growth phenotype: decreased appendicular skeletal growth with normal axial skeletal growth (Vidal et al. 1999). Whereas, a man with an inactivating point mutation in the ERα gene resulted in tall stature, predominantly due to increased appendicular growth (Smith et al. 1994). Different effect of ERα on the regulation of the growth of the appendicular skeleton in man and female mice might be due to gender or species specific reasons. In another study, however, a significant decrease in length of lumbar vertebrae was demonstrated in ERKO male and female mice (Parikka et al. 2005). Therefore, the differential effects of ER inactivation on bone growth between axial and appendicular regions should be studied further. Gender differences should be considered when the phenotypes in bones of the limb and spine were investigated.

## Region- and age-related spatial expression patterns of ERα and β in rat growth plate

Our immunohistochemistry study demonstrated that the region-related differences of ERα and β spatial expression did not occurred in the tibial and spinal growth plate of both genders at fast growth stage in early life, just before, during and after puberty. Spatial expression differences of ERα and β between genders were only detected after puberty (16 weeks). More extensive distribution (from the resting to hypertrophic chondrocytes) of ERα and β expression in male growth plate might also partly contribute to the different limb length in females and males.

By affecting the proliferation, hypertrophy, and calcification of chondrocytes through ERα and β, estrogen has a direct effect on longitudinal growth. In our study, ERα and β immunostaining were mainly revealed in the nuclei of chondrocytes in the tibial and spinal growth plate. Low staining in cytoplasm of chondrocytes was also detected. These results indicated the main target of estrogen in the chondrocytes at different stages and layers of growth plate.

In the present study, more intense staining of ERα and β signaling was shown in resting and proliferating zone at fast growth stage in early life and just before puberty, and in late proliferating and hypertrophic chondrocytes after puberty. It suggested that estrogen might have higher effect on proliferative chondrocytes than on hypertrophic zone during fast growth period in early life. In contrast, there might be more impact of estrogen for hypertrophic chondrocytes than for proliferating chondrocytes after puberty.

In the human growth plate, expression of ERα and β has also been detected. The regulation of growth in rats is comparable to that in humans, except that at the end of sexual maturation. In human, epiphyseal fusion occurs at the time of sexual maturation. However, most growth plates of rat bone do not close until very late in life (Hughes and Tanner 1970). This might limit the present study toward human physiology.

An immunohistochemistry study demonstrated that ERα and β were expressed in the human growth plate through childhood and adolescence with identical distribution and the hypertrophic chondrocyte was the main target cell for estrogen action within the growth plate (Egerbacher et al. 2002). Bord et al. (2001) observed that ERα and β immunoreactivity were located in proliferative and prehypertrophic chondrocytes in the neonatal human rib growth plate, with lower levels of expression in the late hypertrophic zone. ERα and β protein were also expressed in the human proximal tibial growth plate throughout pubertal development, with a greater frequency in the resting and proliferative zones than in the hypertrophic zone (Nilsson et al. 2003). No difference between the sexes was detected. The staining intensity of ERα was similar at all Tanner

pubertal stages, whereas ERβ had a slight decrease in the proliferative zone during pubertal development (Nilsson et al. 2003). The above data indicated that ERα and β expression patterns in the human growth plate might be similar to that in the rats. Gender-specific growth parameters (Yilmaz et al. 2005) and region-specific growth tempo (Bass et al. 1999; Bradney et al. 2000) were also indicated in the literature. Therefore, the quantitative comparison study of age-, gender- and region-specific ERα and β expression patterns in the human growth plate should be performed further in the future.

In summary, our quantitative real-time PCR and immunohistochemical studies suggest that ERα and β are expressed in the limb and spine growth plate during development in both male and female rats. Gender- and region-specific differences are observed, for the first time, in ERα and β gene expression in axial and appendicular regions. Moreover, our study shows significant effect of gender on temporal ERα and β expression and region on temporal ERα/ERβ expression ratio. Staining of ERα and β signaling was more intense in resting and proliferating zone at fast growth stage in early life and just before puberty, whereas darker in late proliferating and hypertrophic chondrocytes after puberty. No significant spatial differences of ERα and β expression are found between regions. Gender-related spatial differences were only detected after puberty. The effect of estrogen on growth plate should be studied further taking into account sex, age and region differences.

## References

Bass S, Delmas PD, Pearce G, Hendrich E, Tabensky A, Seeman E (1999) The differing tempo of growth in bone size, mass, and density in girls is region-specific. J Clin Invest 104:795–804

Ben-Hur H, Mor G, Blickstein I, Likhman I, Kohen F, Dgani R, Insler V, Yaffe P, Ornoy A (1993) Localization of estrogen receptors in long bones and vertebrae of human fetuses. Calcif Tissue Int 53:91–96

Bilezikian JP, Morishima A, Bell J, Grumbach MM (1998) Increased bone mass as a result of estrogen therapy in a man with aromatase deficiency. N Engl J Med 339:599–603

Bord S, Horner A, Beavan S, Compston J (2001) Estrogen receptors alpha and beta are differentially expressed in developing human bone. J Clin Endocrinol Metab 86:2309–2314

Bradney M, Karlsson MK, Duan Y, Stuckey S, Bass S, Seeman E (2000) Heterogeneity in the growth of the axial and appendicular skeleton in boys: implications for the pathogenesis of bone fragility in men. J Bone Miner Res 15:1871–1878

Braidman IP, Davenport LK, Carter DH, Selby PL, Mawer EB, Freemont AJ (1995) Preliminary in situ identification of estrogen target cells in bone. J Bone Miner Res 10:74–80

Braidman IP, Hainey L, Batra G, Selby PB, Saunders PT, Hoyland JA (2001) Localization of estrogen receptor protein expression in adult human bone. J Bone Miner Res 16:214–220

Buie HR, Moore CP, Boyd SK (2008) Postpubertal architectural developmental patterns differ between the L3 vertebra and proximal tibia in three inbred strains of mice. J Bone Miner Res 23:2048–2059

Byers S, Moore AJ, Byard RW, Fazzalari NL (2000) Quantitative histomorphometric analysis of the human growth plate from birth to adolescence. Bone 27:495–501

Camper L, Holmvall K, Wängnerud C, Aszódi A, Lundgren-Akerlund E (2001) Distribution of the collagen-binding integrin alpha10beta1 during mouse development. Cell Tissue Res 306:107–116

Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER (1997) Effect of testosterone and estradiol in a man with aromatase deficiency. N Engl J Med 337:91–95

Chagin AS, Sävendahl L (2009) Genes of importance in the hormonal regulation of growth plate cartilage. Horm Res 71(Suppl 2):41–47

Chagin AS, Lindberg MK, Andersson N, Moverare S, Gustafsson JA, Sävendahl L, Ohlsson C (2004) Estrogen receptor-$\beta$ inhibits skeletal growth and has the capacity to mediate growth plate fusion in female mice. J Bone Miner Res 19:72–77

Couse JF, Korach KS (1999) Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 20:358–417

Egerbacher M, Helmreich M, Rossmanith W, Haeusler G (2002) Estrogen receptor-$\alpha$ and estrogen receptor-$\beta$ are present in the human growth plate in childhood and adolescence, in identical distribution. Horm Res 58:99–103

Fukunaga T, Yamashiro T, Oya S, Takeshita N, Takigawa M, Takano-Yamamoto T (2003) Connective tissue growth factor mRNA expression pattern in cartilages is associated with their type I collagen expression. Bone 33:911–918

Gevers EF, van der Eerden BC, Karperien M, Raap AK, Robinson IC, Wit JM (2002) Localization and regulation of the growth hormone receptor and growth hormone-binding protein in the rat growth plate. J Bone Miner Res 17:1408–1419

Giustina A, Mazziotti G, Canalis E (2008) Growth hormone, insulin-like growth factors, and the skeleton. Endocr Rev 29:535–559

Glatt V, Canalis E, Stadmeyer L, Bouxsein ML (2007) Age-related changes in trabecular architecture differ in female and male C57BL/6J mice. J Bone Miner Res 22:1197–1207

Grumbach MM (2000) Estrogen, bone, growth and sex: a sea change in conventional wisdom. J Pediatr Endocrinol Metab 13(Suppl 6):1439–1455

Hosoya A, Hoshi K, Sahara N, Ninomiya T, Akahane S, Kawamoto T, Ozawa H (2005) Effects of fixation and decalcification on the immunohistochemical localization of bone matrix proteins in fresh-frozen bone sections. Histochem Cell Biol 123:639–646

Hughes PC, Tanner JM (1970) The assessment of skeletal maturity in the growing rat. J Anat 106:371–402

Kaludjerovic J, Ward WE (2008) Diethylstilbesterol has gender-specific effects on weight gain and bone development in mice. J Toxicol Environ Health A 71:1032–1042

Kennedy J, Baris C, Hoyland JA, Selby PL, Freemont AJ, Braidman IP (1999) Immunofluorescent localization of estrogen receptor-$\alpha$ in growth plates of rabbits, but not in rats, at sexual maturity. Bone 24:9–16

Khosla S, Riggs BL, Atkinson EJ, Oberg AL, McDaniel LJ, Holets M, Peterson JM, Melton LJ III (2006) Effects of sex and age on bone microstructure at the ultradistal radius: a population-based noninvasive in vivo assessment. J Bone Miner Res 21:124–131

Kronenberg HM (2003) Developmental regulation of the growth plate. Nature 423:332–336

Kusec V, Virdi AS, Prince R, Triffitt JT (1998) Localization of estrogen receptor- in human and rabbit skeletal tissues. J Clin Endocrinol Metab 83:2421–2428

Laue L, Kenigsberg D, Pescovitz OH, Hench KD, Barnes KM, Loriaux DL, Cutler GB Jr (1989) Treatment of familial male precocious puberty with spironolactone and testolactone. N Engl J Med 320:496–502

Lebovitz HE, Eisenbarth GS (1975) Hormonal regulation of cartilage growth and metabolism. Vitam Horm 33:575–648

Lee PA, Witchel SF (1997) The influence of estrogen on growth. Curr Opin Pediatr 9:431–436

Li H, Jiang LS, Dai LY (2010) High glucose potentiates collagen synthesis and bone morphogenetic protein-2-induced early osteoblast gene expression in rat spinal ligament cells. Endocrinology 151:63–74

Li XF, Yan J, Jiang LS, Dai LY (2011) Age-related variations of leptin receptor expression in the growth plate of spine and limb: gender- and region-specific changes. Histochem Cell Biol 135:487–497

Lindberg MK, Alatalo SL, Halleen JM, Mohan S, Gustafsson JA, Ohlsson C (2001) Estrogen receptor specificity in the regulation of the skeleton in female mice. J Endocrinol 171:229–236

Maffei L, Murata Y, Rochira V, Tubert G, Aranda C, Vazquez M, Clyne CD, Davis S, Simpson ER, Carani C (2004) Dysmetabolic syndrome in a man with a novel mutation of the aromatase gene: effects of testosterone, alendronate, and estradiol treatment. J Clin Endocrinol Metab 89:61–70

Matthews JB (1982) Influence of decalcification on immunohisto-chemical staining of formalin-fixed paraffin-embedded tissue. J Clin Pathol 35:1392–1394

Mitani H, Takahashi I, Onodera K, Bae JW, Sato T, Takahashi N, Sasano Y, Igarashi K, Mitani H (2006) Comparison of age-dependent expression of aggrecan and ADAMTSs in mandibular condylar cartilage, tibial growth plate, and articular cartilage in rats. Histochem Cell Biol 126:371–380

Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K (1995) Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J Clin Endocrinol Metab 80:3689–3698

Mundlos S, Zabel B (1994) Developmental expression of human cartilage matrix protein. Dev Dyn 199:241–252

Neves JD, Omar NF, Narvaes EA, Gomes JR, Novaes PD (2011) Influence of different decalcifying agents on EGF and EGFR immunostaining. Acta Histochem 113:484–488

Nilsson LO, Boman A, Sävendahl L, Grigelioniene G, Ohlsson C, Ritzén EM, Wroblewski J (1999) Demonstration of estrogen receptor-$\beta$ immunoreactivity in human growth plate cartilage. J Clin Endocrinol Metab 84:370–373

Nilsson O, Abad V, Chrysis D, Ritzén EM, Sävendahl L, Baron J (2002) Estrogen receptor-$\alpha$ and -$\beta$ are expressed throughout postnatal development in the rat and rabbit growth plate. J Endocrinol 173:407–414

Nilsson O, Chrysis D, Pajulo O, Boman A, Holst M, Rubinstein J, Martin Ritzén E, Sävendahl L (2003) Localization of estrogen receptors-$\alpha$ and -$\beta$ and androgen receptor in the human growth plate at different pubertal stages. J Endocrinol 177:319–326

Nilsson O, Marino R, De Luca F, Phillip M, Baron J (2005) Endocrine regulation of the growth plate. Horm Res 64:157–165

Ornoy A, Giron S, Aner R, Goldstein M, Boyan BD et al (1994) Gender dependent effects of testosterone and 17 $\beta$-estradiol on bone growth and modelling in young mice. Bone Miner 24:43–58

Parikka V, Peng Z, Hentunen T, Risteli J, Elo T, Väänänen HK, Härkönen P (2005) Estrogen responsiveness of bone formation in vitro and altered bone phenotype in aged estrogen receptor-$\alpha$ deficient male and female mice. Eur J Endocrinol 152:301–314

Sampson HW, Dearman AC, Akintola AD, Zimmer WE, Parrish AR (2007) Immunohistochemical localization of cadherin and catenin adhesion molecules in the murine growth plate. J Histochem Cytochem 55:845–852

Seeman E (2001) Unresolved issues in osteoporosis in men. Rev Endocr Metab Disord 2:45–64

Sims NA, Dupont S, Krust A, Clement-Lacroix P, Minet D, Resche-Rigon M, Gaillard-Kelly M, Baron R (2002) Deletion of estrogen receptors reveals a regulatory role for estrogen receptors-$\beta$ in bone remodeling in females but not in males. Bone 30:18–25

Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS (1994) Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 331:1056–1061

Tozum TF, Oppenlander ME, Koh-Paige AJ, Robins DM, McCauley LK (2004) Effects of sex steroid receptor specificity in the regulation of skeletal metabolism. Calcif Tissue Int 75:60–70

Tsunenari T, Tsutsumi M, Ohno K, Yamamoto Y, Kawakatsu M, Shimogaki K, Negishi H, Sugimoto T, Fukase M, Fujita T (1993) Age- and gender-related changes in body composition in Japanese subjects. J Bone Miner Res 8:397–402

van der Eerden BC, Karperien M, Gevers EF, Löwik CW, Wit JM (2000) Expression of Indian hedgehog, parathyroid hormone-related protein, and their receptors in the postnatal growth plate of the rat: evidence for a locally acting growth restraining feedback loop after birth. J Bone Miner Res 15:1045–1055

van der Eerden BCJ, Karperien M, Wit JM (2001) The estrogen receptor in the growth plate: implications for pubertal growth. J Pediatr Endocrinol Metab 14:1527–1533

van der Eerden BCJ, Gevers EF, Lowik CW, Karperien M, Wit JM (2002) Expression of estrogen receptor and in the epiphyseal plate of the rat. Bone 30:478–485

Vidal O, Lindberg M, Sävendahl L, Lubahn DB, Ritzen EM, Gustafsson JA, Ohlsson C (1999) Disproportional body growth in female estrogen receptor-$\alpha$-inactivated mice. Biochem Biophys Res Commun 265:569–571

Vidal O, Lindberg MK, Hollberg K, Baylink DJ, Andersson G, Lubahn DB, Mohan S, Gustafsson JA, Ohlsson C (2000) Estrogen receptor specificity in the regulation of skeletal growth and maturation in male mice. Proc Natl Acad Sci USA 97:5474–5479

Windahl SH, Vidal O, Andersson G, Gustafsson JA, Ohlsson C (1999) Increased cortical bone mineral content but unchanged trabecular bone mineral density in female ER$\beta$ (−/−) mice. J Clin Invest 104:895–901

Windahl SH, Hollberg K, Vidal O, Gustafsson JA, Ohlsson C, Andersson G (2001) Female estrogen receptor $\beta^{-/-}$ mice are partially protected against age-related trabecular bone loss. J Bone Miner Res 16:1388–1398

Yilmaz D, Ersoy B, Bilgin E, Gümüşer G, Onur E, Pinar ED (2005) Bone mineral density in girls and boys at different pubertal stages: relation with gonadal steroids, bone formation markers, and growth parameters. J Bone Miner Metab 23:476–482

Yuen KW, Kwok TC, Qin L, Leung JC, Chan DC, Kwok AW, Woo J, Leung PC (2010) Characteristics of age-related changes in bone compared between male and female reference Chinese populations in Hong Kong: a pQCT study. J Bone Miner Metab 28:672–681

Zachmann M, Prader A, Sobel EH, Crigler JF Jr, Ritzén EM, Atarés M, Ferrandez A (1986) Pubertal growth in patients with androgen insensitivity: indirect evidence for the importance of estrogens in pubertal growth of girls. J Pediatr 108:694–697

Zhang XZ, Kalu DN, Erbas B, Hopper JL, Seeman E (1999) The effects of gonadectomy on bone size, mass, and volumetric density in growing rats are gender-, site-, and growth hormone-specific. J Bone Miner Res 14:802–809

Zhao CQ, Liu D, Li H, Jiang LS, Dai LY (2008) Expression of leptin and its functional receptor on disc cells: contribution to cell proliferation. Spine 33:E858–E864