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Sex hormones receptors play a crucial role in the control of femoral and mandibular growth in newborn mice

R. A. Márquez Hernández, J. Ohtani, T. Fujita, H. Sunagawa, T. Kawata, M. Kaku, M. Motokawa and K. Tanne

Department of Orthodontics and Craniofacial Developmental Biology, Hiroshima University Graduate School of Biomedical Sciences, Japan

Correspondence to: Dr Rene Arturo Marquez Hernandez, Urbanizacion el bosque, calle Miño #14, Villaviciosa de Odon, Madrid, Spain. E-mail: ranaa2@hotmail.com

SUMMARY Sex hormones are important for bone growth. However, the mechanism by which sex hormone receptors influence bone growth remains unclear. In orthodontic treatment, there is a need to develop an indicator of bone maturity to accurately predict the beginning and end of growth. This indicator might be developed from the screening of sex hormones. The purpose of this study was to investigate the role of each sex hormone receptor on bone growth in newborn mice.

Five-day-old C57BL/6J mice were used in this experiment. Forty mice underwent an orchiectomy (ORX), ovariectomy (OVX), or sham surgery. One week after surgery, the femur and the mandible were resected for immunohistochemical staining. Alternatively, 80 mice were daily injected with antagonist against receptors oestrogen alpha (ERα), beta (ERβ), or androgen receptor (AR). One week after the first injection, radiographs of the femur and mandible were taken and then measured. Analysis of variance and pairwise comparisons (Fisher) were performed to examine the differences in values measured among the groups

In the sham-operated male and female mice, ERβ was found to be more prominent than ERα and AR during all experimental periods. In the ORX and OVX groups, the expressions of all receptors were significantly reduced in comparison with the sham-operated control group throughout the experiment. Moreover, femur and mandibular growth were significantly affected in the group injected with ERβ antagonist.

The deficiency of any sex hormone leads to reduced bone growth. In particular, a disturbance in ERβ produces a greater aberrance in both male and female mice immediately after birth.

Introduction

Bone growth is mediated by various factors, such as hormones, nutrition, and growth hormones. Among these factors, sex hormones are known to be important for the regulation of reproductive functions, but they are also a major determinant of bone growth and its maintenance (Lorenzo, 2003). Their role in bone growth is initiated in the embryonic stage (Ben-Hur *et al.*, 1993) and continues through puberty (Nilsson *et al.*, 2003; Fujita *et al.*, 2004), resulting in the maintenance of homeostasis (Venken *et al.*, 2008). The sex hormones, androgen and oestrogen, are important for bone homeostasis in both genders. Both males and females express oestrogen receptor alpha (ERα), beta (ERβ), and androgen receptor (AR).

In previous reports, it was concluded that the lack of sex hormone production in mice with ovariectomy (OVX) and orchiectomy (ORX) leads to a disturbance of femoral and craniofacial bone growth immediately after birth (Fujita *et al.*, 2004). However, it still remains unclear how sex hormone receptors contribute to the control of bone growth and maintenance of its homeostasis.

In orthodontic treatment, it is important to precisely predict the effective timing of treatment to improve skeletal discrepancy. The growth period is different from patient to patient; therefore, there is a need to develop an indicator of bone maturity, i.e. a hormonal indicator, which would help to accurately predict the beginning and end of growth. The purpose of this study was to investigate how sex hormones act on bone growth immediately after birth using immunohistochemical and morphometric analyses.

Materials and methods

Immunohistochemical analysis

Forty C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine, USA) were used in this experiment. The newborn mice were kept with their progenitors with free access to food and water. The animals were housed under controlled temperature and light conditions (20–22°C, 12 hour light/12 hour dark cycle). The average weight of the mice was 2.20 g at 5 days of age. The weight remained constant and equal in all groups during the experimental period. The mice were treated under the ethical regulations defined by the Ethics Committee of Hiroshima University Faculty of Dentistry.

At 5 days of age, the mice underwent ORX ($n = 10$), OVX ($n = 10$), or sham surgery (male $n = 10$ and female $n = 10$).

Surgery was undertaken at 5 days old in order to minimize mortality. One week after surgery, the animals were sacrificed under general anaesthesia with pentobarbital sodium (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan) and the femur and mandible were resected. The specimens were immediately fixed in 70 per cent ethanol, decalcified in ethylenediaminetetraacetic acid for 20 days, dehydrated, and then embedded in paraffin. The femur and the mandible were cut into sagittal and coronal sections 6 μm thick, mounted on histological glass slides, and then prepared for immunohistochemical staining. Rabbit polyclonal antibody with affinity to ERα (PA1-21018; Affinity Bio Reagents, Golden, Colorado, USA), ERβ (PA1-310B; Affinity Bio Reagents), and AR (PA1-110; Affinity Bio Reagents) were used to detect the expression of the receptors. Staining was realized following the Vectastain® Elite® ABC kit protocol (PK-6101; Vector Laboratories, Burlingame, California, USA) and the expression was revealed by ImmPACT™ diaminobenzidine (DAB) substrate (SK-4105; Vector Laboratories). After staining the tissues with DAB, they were counter-stained with methyl green, dehydratated, and glass mounted. The number of reactive cells was counted manually at ×400 magnification under a light microscope (BX50F4; Olympus, Tokyo, Japan). The reactive cells were interpreted as delineated brown-stained chondrocytes in an area located at the dorsal part of the proximal growth plate of the femur, and the central part of the top of the mandibular condyle. All reactive chondrocytes in the undifferentiated, proliferating, and hypertrophic cartilage layers seen in this area were counted.

Morphometric analysis

Eighty C57BL/6J mice (Jackson Laboratory), divided into four groups, were used in this part of the experiment. At 5 days of age, the mice were subjected to daily subcutaneous injection with ERα (male $n = 10$ and female $n = 10$), ERβ (male $n = 10$ and female $n = 10$), AR (male $n = 10$ and female $n = 10$) antagonists, or saline (male $n = 10$ and female $n = 10$). A selective ERα antagonist methyl-piperidino-pyrazole (Tocris Bioscience, Bristol, UK), dissolved in a saline solution, was administered at a dose of 2 mg/kg (Sun *et al.*, 2002). A selective ERβ antagonist 4-[2-phenyl-5, 7-bis (trifluoromethyl) pyrazolo [1,5-a]pyrimidin-3-yl] phenol (Tocris Bioscience), dissolved in 70 per cent dimethyl sulphoxide and 30 per cent saline, was administered at a dose of 4.7 mg/kg (Compton *et al.*, 2004). A selective AR antagonist, Flutamide (F9397, Sigma Aldrich, Tokyo, Japan), was dissolved in ethanol/oil and administered at a dose of 24 mg/kg.

Twelve days after birth, the femur and mandible were subjected to radiographic ray exposure in a micro-FX1000 system (Fuji Film Inc., Tokyo, Japan). Digital radiographic films were exposed to an electric current of 45 KVp and 10 μA with an exposure time of 15 seconds. The longitudinal length of the femur was measured on the radiograph as the distance from the most protrusive point at the top of the mesial epiphysis convexity to the most protrusive point at the bottom convexity of the distal epiphysis along a perpendicular line running from the centre of the femur (Figure 1). Cephalometric analysis of the mandible was performed on a lateral cephalogram according to a previously reported method (Kiliaridis *et al.*, 1985). Only three measurements from that method were employed in order to avoid enmascaration from growth of bones other than the mandible and to express linear changes in the mandible produced mainly by endochondreal ossification (Figure 1).

The radiographs were blindly and randomly measured by one investigator (RAMH). The measurements were checked twice, with a 1 week interval, and the mean of these measurements was recorded.

Statistical analysis

Analysis of variances and pairwise comparisons (Fisher) were performed to examine the differences in values measured among each group with a confidence level greater than 95 per cent. The calculated data were normally distributed and expressed as the mean ± the standard deviation (SD). $P < 0.05$ was considered statistically significant.

Results

Immunoreactivity to ERα, ERβ, and AR in the femoral growth plate

In the male and female control groups, immunoreactivity to ERβ and AR was 71.3 and 67.7 per cent, respectively, significantly higher than that to ERα. Immunoreactivity to ERα was higher in the female control group than in the

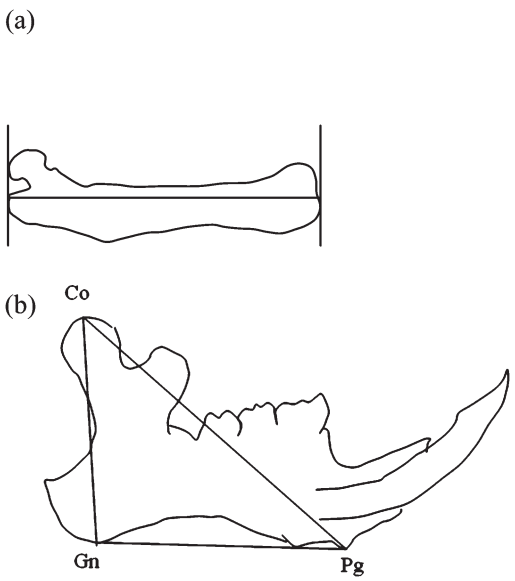

Figure 1 Linear measurement of the femur (a) and the mandible (b). Co: the most postero-superior point on the mandibular condyle. Gn: the most inferior point on the contour of the angular process of the mandible. Pg: the most inferior point on the contour of the lower border of the mandible, adjacent to the incisors.

male control group (Figure 2a). When the male and female mice were compared, both genders showed similar reactive receptors, indicating no gender preference for a specific receptor. In OVX and ORX groups, the reactivities to all the antibodies were significantly lower ($P < 0.01$) when compared with the corresponding control groups (males: ERα 74.5, ERβ 97.6, and AR 100 per cent lower and females: ERα 64.1, ERβ 88, and AR 98.7 per cent lower).

Immunoreactivity to ERα, ERβ, and AR in the condylar cartilage

In the female control group, immunoreactivity to ERβ was 32.6 per cent higher than to ERα, whereas the reactivities of ERα and ERβ were 53.3 and 68.5 per cent, respectively, significantly higher than that of AR. In the male controls, the expression of all receptors was similar, although the expression of ERβ was more prominent. Comparing the male and female controls, ERβ was similarly expressed while ERα expression was higher in the male than in the female group. AR showed a significantly higher expression in the males than in the females (Figure 2b). In the OVX and ORX groups, the expression of all the receptors was significantly reduced ($P < 0.01$) when compared with the corresponding control group, except for ERβ in the ORX group, which showed no significant difference (males: ERα 94.7, ERβ 18.2, and AR 76.6 per cent lower and females: ERα 75.5, ERβ 82, and AR 57.1 per cent lower).

Changes in the size of the femur

The femoral length in both male and female mice was significantly smaller in the ERβ antagonist injection group than in the corresponding control groups (males 15.8 and females 8.1 per cent smaller). The remaining groups were within the normal range of the corresponding control groups (Figure 3a).

Changes in the size of the mandible

Condylar height was significantly smaller in the ERβ antagonist injection groups than in the male and female control groups (males 22 and females 11.5 per cent smaller). Mandibular length was significantly smaller in the male and female ERβ antagonist injection groups than in the corresponding control groups (males 12.5 and females 8.2 per cent smaller). The mandibular body length was within the normal range of the controls for both genders (Figure 3b).

Discussion

In a previous study, it was shown that the lack of either oestrogen or androgen, stimulated by gonadectomy, resulted

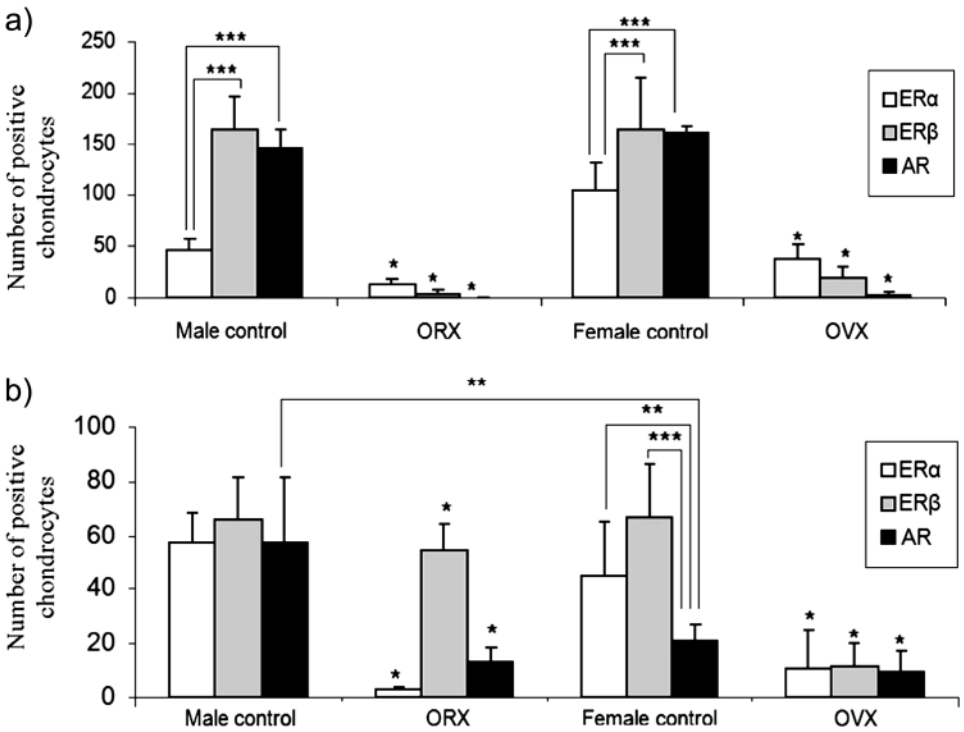

Figure 2 Number of positive reactive cells in control male/female and ovariectomized (OVX)/orchiectomized (ORX) mice in the femoral growth plate (a) and condylar cartilage (b) at 12 days of age. Single asterisk indicates significant difference in the number of positive reactive cells of gonadectomized mice when compared with the corresponding control group (*$P < 0.01$). Double and triple asterisks indicate significant difference between the number of positive reactive cells of the receptors in the control groups (**$P < 0.05$, ***$P < 0.01$). ERα, oestrogen receptor alpha; ERβ, oestrogen receptor beta; AR, androgen receptor.

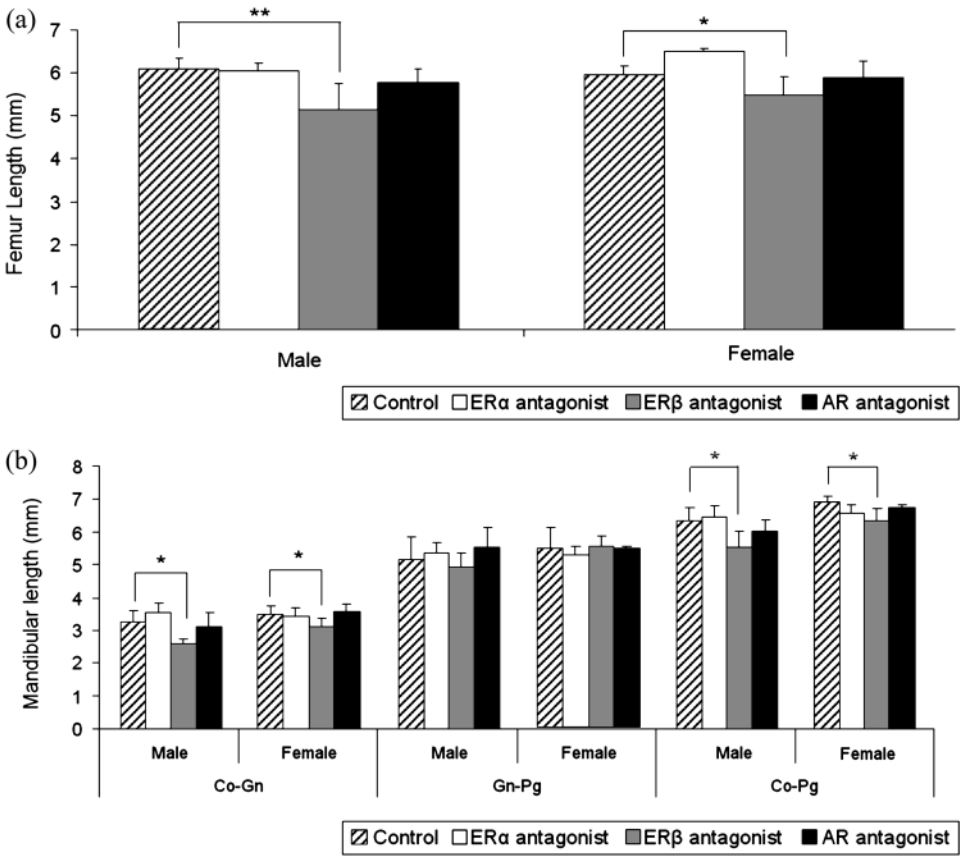

Figure 3 Femoral lengths (a) and mandibular (b) lengths in 12-day-old mice injected with sex hormone antagonists. Asterisks indicate a significant difference in length in the experimental groups when compared with the control groups $*P < 0.05$ and $**P < 0.01$. ERα, oestrogen receptor alpha; ERβ, oestrogen receptor beta; AR, androgen receptor.

in reduced bone growth. This deficiency was observed from the first week after birth to the end of pubertal growth (Fujita *et al.*, 2004). It has been demonstrated in this study that OVX and ORX mice 12 days after birth exhibit a significantly lower expression of sex hormone receptors on chondrocytes than the corresponding control groups, indicating that sex hormones directly affect bone growth immediately after birth.

It is still unclear which receptor among the three is mainly involved in bone growth. Various conclusions have been hypothesized, i.e. *in vivo* (Jansson *et al.*, 1983; Ren *et al.*, 1989) and *in vitro* (Carrascosa *et al.*, 1990) studies of androgen showed that this hormone stimulates longitudinal bone growth through a direct effect on the growth plate of male mice. It has also been reported that androgen is able to prevent bone loss in OVX rodents (Coxam *et al.*, 1996). In addition, the effect of ERα alone was evaluated in males with natural mutations of the ERα gene, demonstrating low bone mineral density (BMD) and failure to establish peak bone mass (Smith *et al.*, 1994). Furthermore, ER gene knockout mice exhibited lower BMD in both genders (Korach, 1994). ERα knockout mice (ERKO) but not ERβ knockout mice also developed severe osteoporosis (Couse and Korach, 1999; Lindberg *et al.*, 2001, 2002, 2003). Nevertheless, although there is a reduction of BMD in ERKO, OVX produces more profound bone loss (Pan *et al.*, 1997). This additional bone loss caused by OVX in ERKO suggests that ERβ might also be as important as ERα for oestrogen action. In the present study, the effect of ERα appeared to be of less significance when compared with that of ERβ. The above findings lead to the conclusion that as different receptors are involved during bone growth and bone maintenance there is a strong possibility that ERβ receptors are involved in bone growth immediately after birth.

In the present study, the expression of sex hormones receptors on the chondrocytes in the sham-operated control group showed that ERβ is an important component during this developmental stage since its expression was as high in males as in females. The administration of ERβ antagonist resulted in a shorter femur in both male and female mice, demonstrating that ERβ affects long bone growth. Immunohistochemical staining showed that ERα was moderately highly expressed in the condylar cartilage, but the size of the mandible was not significantly affected in the experimental animals treated with ERα antagonists. From

these results, it may be speculated that immediately after birth, ERβ is the main pathway through which sex hormones stimulate bone growth.

For growth of the mandible, both endochondral and perichondral activities are important, although endochondral bone formation seems to exceed perichondral bone formation (Luder, 1994). This might be the reason why only mandibular height and length were affected by ERβ antagonist at 12 days after birth in both genders. The principal mechanism of mandibular growth is endochondral bone formation. Immediately after birth, endochondral bone formation in the condyle should be more affected (Luder, 1994); therefore, it is assumed that the mandibular condyle is affected more than the mandibular body since condylar height is mainly controlled by endochondral bone formation. Moreover, differences in the relationship of chondrocytes reacting to sex hormones were found between the mandible and femur. This difference might be due to their cellular origin. The epiphyseal growth plate is a primary cartilage and the condylar cartilage a secondary cartilage (Beresford, 1981). The primary and secondary cartilages are different in the cellular organization, growth pattern (Copray *et al.*, 1986; Silbermann *et al.*, 1987), and some biochemical features such as the types of collagen (Silbermann *et al.*, 1987; Mizoguchi *et al.*, 1996), which are postulated to produce different reactions to sex hormones.

Comparing the expression patterns of sex hormone receptors and the effects of sex hormone antagonists in mice, no exact correlations were found. This might be due to the stimulation pathway by which sex hormones interact with their receptors. Sex hormones can affect cellular function via a classic or non-classic pathway. In the classic pathway, sex hormones interact with intracellular receptors in the cytoplasm or in the nucleus (Heinlein and Chang, 2002), while in the non-classic pathway, they elicit their effects through the ligand-binding domain of sex hormone receptors in the plasma membrane (Manolagas *et al.*, 2002). In this study, the receptor evaluated was in the nucleus and cytoplasm, thus the classic pathway could be reasonably speculated as the mechanism. Some authors believe that in non-reproductive sites of action, the non-classic pathway exerts a more important role (Manolagas *et al.*, 2002), thus by evaluating only the classic pathway, it might be that the principal pathway was overlooked and further studies of need to be performed.

Conclusions

Immediately after birth, sex hormone deficiency reduces the expression of sex hormone receptors on chondrocytes in the femoral growth plate and mandibular cartilage, thus causing growth disturbance. This disturbance seems to be caused by the inability to activate ERβ both in males and females. These findings suggest that in chondrocytes, sex hormones stimulate bone growth via ERβ before pubertal growth.

Gene expression of ERβ in blood might be a valuable tool for growth assessment by creating a normality index during the growth period. For this purpose, further investigations should be conducted examining this receptor behaviour from birth until the end of growth.

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References

Ben-Hur H *et al.* 1993 Localization of estrogen receptors in long bones and vertebrae of human fetuses. Calcified Tissue International 53: 91–96

Beresford W A 1981 Secondary cartilages I and II. In: Chondroid bone, secondary cartilage and metaplasia. Urban & Schwarzenberg, Baltimore, pp. 23–65

Carrascosa A, Audi L, Ferrandez M A, Ballabriga A 1990 Biological effects of androgens and identification of specific dihydrotestosterone binding sites in cultured human fetal epiphyseal chondrocytes. Journal of Clinical Endocrinology and Metabolism 70: 134–140

Compton D R *et al.* 2004 Pyrazolo[1,5-a]pyrimidines: estrogen receptor ligands possessing estrogen beta antagonist activity. Journal of Medicinal Chemistry 47: 5872–5893

Copray J C, Jansen H W, Duterloo H S 1986 Growth and growth pressure of mandibular condylar and some primary cartilages of the rat *in vitro*. American Journal of Orthodontics and Dentofacial Orthopedics 90: 19–28

Couse J F, Korach K S 1999 Estrogen receptor null mice: what have we learned and where will they lead us? Endocrinology 20: 358–417

Coxam V, Bowman B M, Mecham M, Roth C M, Miller M A, Miller S C 1996 Effects of dihydrotestosterone alone and combined with estrogen on bone mineral density, bone growth, and formation rates in ovariectomized rats. Bone 19: 107–114

Fujita T *et al.* 2004 Effects of sex hormone disturbances on craniofacial growth in newborn mice. Journal of Dental Research 83: 250–254

Heinlein C, Chang C 2002 Androgen receptor (AR) coregulators: an overview. Endocrine Reviews 23: 175–200

Jansson J O, Eden S, Isaksson O 1983 Sites of action of testosterone and estradiol on longitudinal bone growth. American Journal of Physiology 244: 135–140

Kiliaridis S, Engstöm C, Thilander B 1985 The relationship between masticatory function and craniofacial morphology. I. A cephalometric longitudinal analysis in the growing rat fed a soft diet. European Journal of Orthodontics 7: 273–283

Korach K S 1994 Insights from the study of animals lacking functional estrogen receptor. Science 266: 1524–1527

Lindberg M K, Alatalo S L, Halleen J M, Mohan S, Gustafsson J A, Ohlsson C 2001 Estrogen receptor specificity in the regulation of the skeleton in female mice. Journal of Endocrinology 71: 229–236

Lindberg M K *et al.* 2002 Estrogen receptor specificity for the effects of estrogen in ovariectomized mice. Journal of Endocrinology 174: 167–178

Lindberg M K *et al.* 2003 Estrogen receptor (ER)-β reduces ERα regulated gene transcription, supporting a 'ying yang' relationship between ERα and ERβ in mice. Molecular Endocrinology 17: 203–208

Lorenzo J 2003 A new hypothesis for how sex steroid hormones regulate bone mass. The Journal of Clinical Investigation 111: 1641–1643

Luder H U 1994 Perichondrial and endochondral components of mandibular condylar growth: morphometric and autoradiographic quantitation in rats. Journal of Anatomy 185: 587–598

Manolagas S C, Kousteni S, Jilka R L 2002 Sex steroids and bone. Recent Progress in Hormone Research 57: 385–409

Mizoguchi I, Takahashi I, Nakamura M 1996 An immunohistochemical study of regional differences in the distribution of type I and type II collagens in rat mandibular condylar cartilage. Archives of Oral Biology 41: 863–869

Nilsson O *et al.* 2003 Localization of estrogen receptors α and β and androgen receptor in the human growth plate at different pubertal stages. Journal of Endocrinology 177: 319–326

Pan L C *et al.* 1997 Estrogen receptor-alpha knockout (ERKO) mice lose trabecular and cortical bone following ovariectomy. Journal of Bone and Mineral Research 12: S134 (abstract)

Ren S G, Malozowski S, Sanchez P, Sweet D E, Loriaux D L, Cassorla F 1989 Direct administration of testosterone increases rat tibial epiphyseal growth plate width. Acta Endocrinologica (Copenhagen) 121: 401–405

Silbermann M, Reddi A H, Hand A R, Leapman R D, Franzen A 1987 Further characterization of the extracellular matrix in the mandibular condyle in neonatal mice. Journal of Anatomy 151: 169–188

Smith E P *et al.* 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. New England Journal of Medicine 331: 1056–1061

Sun J, Huang Y R, Harrington W R, Sheng S, Katzenellenbogen J A, Katzenellenbogen B S 2002 Antagonists selective for estrogen receptor α. Endocrinology 143: 941–947

Venken K, Callewaert F, Boonen S, Vanderschueren D 2008 Sex hormones, their receptors and bone health. Osteoporosis International 11: 1517–1525