ORTHODONTICS & CRANIOFACIAL RESEARCH

ORIGINAL ARTICLE

DOI: 10.1111/j.1601-6343.2011.01509.x

Mandibular and femoral growth alteration after sex hormone disruption in growing mice

RA Márquez Hernández
J Ohtani
T Fujita
H Sunagawa
E Ishikawa
N Tsubamoto
T Kawata
M Kaku
M Motokawa
K Tanne

Authors' affiliation:
R.A. Márquez Hernández, J. Ohtani, T. Fujita, H. Sunagawa, E. Ishikawa, N. Tsubamoto, T. Kawata, M. Kaku, M. Motokawa, K. Tanne, Department of Orthodontics and Craniofacial Developmental Biology, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan

Correspondence to:
Dr. Rene Arturo Márquez Hernández
Department of Orthodontics
Hiroshima University
1-2-3 Kasumi
Minami-ku
Hiroshima city
Japan
E-mail: ranaa2@hotmail.com

Dates:
Accepted 5 February 2011

To cite this article:
Márquez Hernández RA, Ohtani J, Fujita T, Sunagawa H, Ishikawa E, Tsubamoto N, Kawata T, Kaku M, Motokawa M, Tanne K:
Mandibular and femoral growth alteration after sex hormone disruption in growing mice
Orthod Craniofac Res 2011;**14**:63–69

Structured Abstract

Authors – Márquez Hernández RA, Ohtani J, Fujita T, Sunagawa H, Ishikawa E, Tsubamoto N, Kawata T, Kaku M, Motokawa M, Tanne K

Objectives – To investigate how mandibular and femoral growth is affected when sex hormone– specific receptor antagonist is administered in growing mice.

Materials and methods – Forty C57BL/6J mice were used in this experiment. At 5 days of age, the mice received daily injection of estrogen receptor alpha (ERα), beta (ERβ), or androgen receptor (AR) antagonists, and their body weight was assessed every 4 days. One, four and eight weeks after the initial injection, radiographs of the mandible and femur were taken and measured. Analyses of variance and pairwise comparisons (Fisher) were performed to examine the differences in values measured among the groups.

Results – Mandibular growth was affected by ERβ antagonist injection in male mice at 4 and 8 weeks. In female mice, the growth was affected during all the experimental period, when ERβ was administered. Moreover, at 8 weeks, mandibular growth was also affected in male and female mice injected with ERα antagonist and in male mice injected with AR antagonist. Femoral growth was affected during all the experimental period in male and female mice injected with ERβ antagonist. Moreover, at 8 weeks, the growth was affected in male and female mice injected with ERα antagonist and in male mice injected with AR antagonist.

Conclusions – Growth of the mandible and femur in mice, in part, is induced in response to the stimulation of ERβ in chondrocytes before and during early puberty. In late and after puberty, the growth is induced by the stimulation of ERα in male and female mice and that of AR in male mice.

Key words: androgen; estrogen; growth alteration; growth prediction; sex hormones

Introduction

Hormones play a crucial role in regulating most body functions such as the metabolism, growth and development, reproduction, water and electrolyte balance and behavior (1). Their actions depend on the concentrations

according to the stage of life which the individual is experiencing. The sex hormones are well known to be important and essential for the regulation of reproductive functions. In addition, they also exert effects on the nervous and cardiovascular systems and are indispensable for the development and the structural integrity of the skeleton (2). The sex hormones, androgen and estrogen, are believed to exert their effects on bone irrespective of gender (3). Androgen interacts with the androgen receptor (AR) and estrogen with the estrogen receptor alpha (ERα) and beta (ERβ); thus, they regulate together the different bone envelopes during bone growth and the maintenance in both genders (4).

Irregularities of sex hormones production in post-menopausal women cause a reduction in bone mineral density (BMD) (5). In adult men following orchiectomy (ORX) because of prostate cancer, as a consequence of the treatment procedure, the levels of sex hormones are reduced, producing a reduction in the BMD (6). In growing mice, ovariectomy (OVX) and ORX lead to a disturbance of craniofacial and femoral bone growth immediately after birth and during bone growth and development (7), and also a lower expression of sex hormone receptors on chondrocytes (8). In a previous study, screening of the expression of sex hormone receptors showed a substantial reactivity of ERβ in chondrocytes of growing mandibular condyle and femur in mice (8). However, it still remains unclear how sex hormone receptors contribute to the control of bone growth and the maintenance of its homeostasis.

In orthodontic treatment, it is of a great importance to precisely predict the effective timing to improve skeletal discrepancy. Maximum growth period varies from patient to patient; therefore, it is highly anticipated to develop an accurate indicator of bone maturity, i.e. a hormonal indicator to predict the beginning and end of growth, depicted as a velocity curve. The purpose of this study was to investigate how mandibular and femoral growth is affected when sex hormone-specific receptor antagonist is administered in growing mice.

Materials and methods

Experimental animals

Forty C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) were used in this experiment. The new born mice were kept with its progenitors with free access to food and water. The animals were housed under controlled temperature and photoperiods conditions (20–22°C, 12-h light/12-h dark cycle). The average weight of the mice was 2.20 g at 5 days of age. Mice were treated under the ethical regulations defined by the Ethics Committee, Hiroshima University Faculty of Dentistry.

Experimental method

Mice were divided into four groups according to the sex hormone antagonist administered and the control group. At 5 days of age, mice were subjected to daily subcutaneous injection with either ERα (males, n = 5 and females, n = 5), ERβ (males, n = 5 and females, n = 5), AR (males, n = 5 and females, n = 5) antagonists, or saline (males, n = 5 and females, n = 5). A selective ERα antagonist methyl-piperidino-pyrazole (MPP) (Cat. No: 1991, Tocris Bioscience, Bristol, UK) was administered at a dose of 2 mg/kg (9, 10). A selective ERβ antagonist 4-[2-phenyl-5, 7-bis (trifluoromethyl) pyrazolo [1,5-a]pyrimidin-3-yl] phenol (PHTPP) (Cat. No: 2662, Tocris Bioscience) was administered at a dose of 4.7 mg/kg (10, 11). A selective AR antagonist, Flutamide (F9397, Sigma-Aldrich, Tokyo, Japan), was administered at a dose of 24 mg/kg (12).

The body weight was assessed every 4 days throughout the experiment. One, 4 and 8 weeks after initial injection, the mandible and femur were subjected to X-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 s. The magnification error was assessed by using a 10-mm radiopaque metal rod during the radiographic exposure, and later the radiograph magnification correction was accounted.

Cephalometric analysis was performed for the mandible on a lateral cephalogram according to a previously reported method (13). Only three measurements from that method were employed to avoid enmascarated values from growth of bones other than the mandible and to express linear changes in the mandible produced mainly by endochondral ossification (Fig. 1):

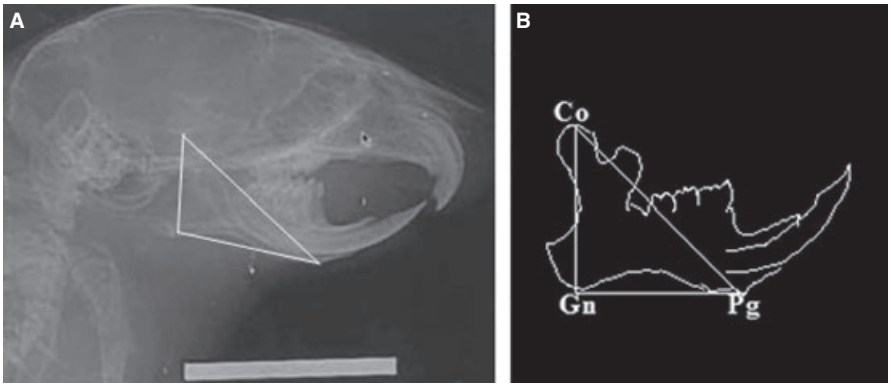

Fig. 1. Measurement items of the mandible. (A) Radiographic image. (B) Schematic representation of the measurement items. Co: the most posterior-superior point on the mandibular condyle. Gn: the most inferior point on the contour of the angular process of the mandible. Pg: the most inferior point on the contour of the lower border of the mandible, adjacent to the incisors. Co–Pg: mandibular length, Co–Gn: condylar height, Gn–pg: mandibular body length.

1. From the most posterior-superior point on the mandibular condyle (Co) to the most inferior point on the contour of the lower border of the mandible, adjacent to the incisors (Pg), defined as the mandibular length.
2. From Co to the most inferior point on the contour of the angular process of the mandible (Gn), defined as the condylar height.
3. From Gn to Pg, defined as the mandibular body length.

The 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 center of the femur (Fig. 2).

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

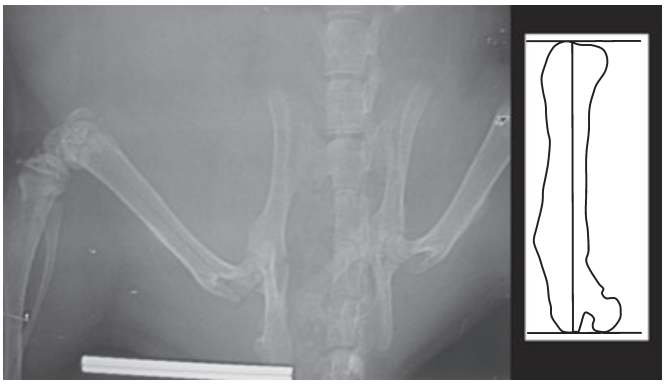

Fig. 2. Radiograph and design of the femur showing the method used to measure the longitudinal length of the femur. Measurements were performed from the most protruded point in the mesial epiphysis convexity to the most protruded point in the distal epiphysis along a perpendicular line running in the center of the femur.

Statistical analysis

Analysis of variance (ANOVA) and pairwise comparisons (Fisher) were performed to examine the differences in measured values among the four groups with a confidence level greater than 95%. The calculated data were normally distributed and expressed as the mean ± standard deviation. $p < 0.05$ was considered statistically significant.

Results

Changes in body weight

In respect to the body weight of mice, male and female mice showed no significant differences between the experimental and the corresponding control groups throughout the experiment (Fig. not shown).

Changes in the size of the mandible

Male mice, at 1 week after initial injection, exhibited a significantly smaller mandibular length by 13% and condylar height by 22% in the group injected with ERβ antagonist than in the control group. At 4 weeks after initial injection, mice exhibited a significantly 10% smaller condylar height in the group injected with ERβ antagonist than in the control group. At 8 weeks after initial injection, male mice exhibited a significantly smaller condylar height by 13%, mandibular body length by 10% and mandibular length by 12% in the group injected with ERα antagonist than in the control group. In addition, at 8 weeks after initial injection, male mice exhibited a significantly 5% smaller mandibular length also in the group injected with AR antagonist than in the control group (Table 1).

Female mice, at 1 week after initial injection, exhibited a significantly smaller mandibular length by 8% and condylar height by 12% in the group injected with ERβ antagonist than in the control group. At 4 weeks after initial injection, mice exhibited a significantly 4% smaller condylar height in the group injected with ERβ antagonist than in the control group. At 8 weeks after initial injection, female mice exhibited a significantly smaller condylar height by 6% and mandibular length by 9% in the group injected with ERα than in the control group. In addition, mice exhibited a significantly smaller condylar height by 11% and mandibular length by 9% in the group injected with ERβ antagonist than in the control group (Table 2).

Changes in the size of femur

The femur of male mice at 1 week after initial injection was significantly smaller by 16% in the group injected with ERβ antagonist than in the control group. At 4 weeks, the femurs were significantly smaller by 6% in the groups injected with AR antagonist and by 12% in the group injected with ERβ antagonist when compared to the control group. Eight weeks after, the femurs were all significantly smaller in the groups injected with AR, ERα and ERβ antagonists than in the control group by 3, 8, and 7%, respectively (Table 3).

The femur of female mice at 1 and 8 weeks after initial injection was significantly smaller in the group injected with ERβ antagonist than in the control group by 12 and 3%, respectively. Moreover, at 8 weeks after initial injection, the femur was also significantly smaller in the group injected with ERα antagonist than in the control group by 6% (Table 4).

Discussion

Growth pattern in mice is similar to that in humans. In mice, three separate postnatal growth cycles occur. The first cycle exhibits its maximum velocity around 7 days after birth and culminates at 14 days. The second cycle exhibits its maximum velocity from 21 to 23 days and reaches the peak immediately after 28th day. The third cycle exhibits its maximum velocity at about 6 weeks and thereafter decreases in velocity continuously but very slowly, so that the growth still occurs up to the 50th and 60th weeks (14). In this experiment, we evaluated the growth of mice at 12, 33, and 61 days after birth, which correspond to each growth cycle in mice. Moreover, these periods in mice correspond to three growth periods in humans such as immediately after birth, peak of puberty and maturation.

Table 1. **Mean measurements of the mandible of male mice injected with sex hormone receptor antagonists and control groups at 1, 4, and 8 weeks after initial injection. $p < 0.05$ indicates a significant difference between the experimental and control group**

	Control		ERα antagonist			ERβ antagonist			AR antagonist		
Age	Mean	SD	Mean	SD	Significance	Mean	SD	Significance	Mean	SD	Significance
1 week											
Co–Gn	3.28	0.33	3.53	0.29	NS	2.56	0.18	$p < 0.05$	3.13	0.41	NS
Gn–Pg	5.16	0.67	5.35	0.33	NS	4.94	0.40	NS	5.54	0.60	NS
Co–Pg	6.33	0.43	6.43	0.34	NS	5.54	0.50	$p < 0.05$	6.04	0.32	NS
4 weeks											
Co–Gn	5.07	0.43	4.74	0.32	NS	4.54	0.41	$p < 0.05$	4.77	0.23	NS
Gn–Pg	7.07	0.43	6.76	0.16	NS	6.98	0.50	NS	6.91	0.32	NS
Co–Pg	8.73	0.78	8.38	0.33	NS	8.50	0.57	NS	8.23	0.37	NS
8 weeks											
Co–Gn	5.74	0.21	4.99	0.26	$p < 0.05$	5.61	0.32	NS	5.51	0.34	NS
Gn–Pg	8.14	0.25	7.36	0.36	$p < 0.05$	8.13	0.44	NS	7.80	0.14	NS
Co–Pg	9.99	0.26	8.70	0.45	$p < 0.05$	9.63	0.39	NS	9.50	0.16	$p < 0.05$

AR, androgen receptor.

Table 2. **Mean measurements of the mandible of female mice injected with sex hormone receptor antagonists and control groups at 1, 4, and 8 weeks after initial injection. $p < 0.05$ indicates a significant difference between the experimental and control group**

	Control		ERα antagonist			ERβ antagonist			AR antagonist		
Age	Mean	SD	Mean	SD	Significance	Mean	SD	Significance	Mean	SD	Significance
1 week											
Co–Gn	3.50	0.26	3.43	0.24	NS	3.10	0.26	$p < 0.05$	3.55	0.26	NS
Gn–Pg	5.46	0.62	5.29	0.24	NS	5.52	0.33	NS	5.48	0.06	NS
Co–Pg	6.89	0.19	6.58	0.26	NS	6.33	0.36	$p < 0.05$	6.74	0.11	NS
4 weeks											
Co–Gn	4.78	0.17	4.68	0.32	NS	4.59	0.22	*$p < 0.05$*	4.72	0.41	NS
Gn–Pg	6.82	0.47	6.88	0.19	NS	6.99	0.39	NS	6.55	0.25	NS
Co–Pg	8.58	0.23	8.62	0.28	NS	8.49	0.41	NS	8.25	0.15	NS
8 weeks											
Co–Gn	5.55	0.29	5.20	0.21	$p < 0.05$	4.90	0.62	$p < 0.05$	5.50	0.10	NS
Gn–Pg	7.90	0.37	7.41	0.67	NS	7.69	0.17	NS	7.79	0.19	NS
Co–Pg	9.81	0.35	8.95	0.27	NS	8.95	0.54	NS	9.48	0.28	NS

AR, androgen receptor.

Table 3. **Mean measurements of the femur of male mice injected with sex hormone receptor antagonists and control groups at 1, 4, and 8 weeks after initial injection. $p < 0.05$ indicates a significant difference between the experimental and control group**

	Control		ERα antagonist			ERβ antagonist			AR antagonist		
Age	Mean	SD	Mean	SD	Significance	Mean	SD	Significance	Mean	SD	Significance
1 week	6.09	0.22	6.04	0.08	NS	5.13	0.46	$p < 0.05$	5.79	0.36	NS
4 weeks	10.73	0.66	10.24	0.82	NS	9.44	0.83	$p < 0.05$	10.04	0.44	$p < 0.05$
8 weeks	12.95	0.32	11.86	0.49	$p < 0.05$	12.02	0.23	$p < 0.05$	12.56	0.51	$p < 0.05$

AR, androgen receptor.

Table 4. **Mean measurements of the femur of female mice injected with sex hormone receptor antagonists and control groups at 1, 4, and 8 weeks after initial injection. $p < 0.05$ indicates a significant difference between the experimental and control group**

	Control		ERα antagonist			Erβ antagonist			AR antagonist		
Age	Mean	SD	Mean	SD	Significance	Mean	SD	Significance	Mean	SD	Significance
1 week	5.93	0.25	6.48	0.19	NS	5.45	0.63	$p < 0.05$	5.88	0.33	NS
4 weeks	10.60	0.09	10.39	0.37	NS	10.15	0.09	NS	10.61	0.37	NS
8 weeks	12.74	0.26	12.01	0.26	$p < 0.05$	12.36	0.42	$p < 0.05$	12.66	0.16	NS

The mechanism of action of bone growth may be different from that of bone maintenance in adults (15). Bone maintenance in adults is believed to be principally mediated via ERα (16). In contrast, in the present study, the administration of ERα antagonist caused smaller mandible and femur size length 8 weeks after initial injection.

Immediately after birth, the functions regulated by ERα seem to be of less significance when compared to the functions regulated by ERβ. This lead us to think

that different receptors are involved during bone growth and bone maintenance; it is thus speculated that ERβ receptor is involved in bone growth immediately after birth. In contrast, the administration of ERβ antagonist at 1, 4, and 8 weeks created a shorter femur in male and at 1 and 8 weeks in female. In addition, the administration of AR antagonist in male and ERα antagonist in male and female also caused a shorter femur at 8 weeks. It is demonstrated that ERβ is involved in long bone growth in both genders before and during puberty, and AR and ERα are also involved in bone growth and maintenance during late puberty and maturation. From these results, it would be reasonably assumed that immediately after birth, ERβ is the main pathway by which sex hormones stimulate bone growth.

The administration of ERβ antagonist at 1 and 4 weeks in both genders and at 8 weeks in female caused a smaller mandible. In addition, the administration of AR and ERα antagonists produced a smaller mandible at 8 weeks. These measurements were affected in male by AR antagonist and in male and female by ERα antagonist. These evidences emphasize that ERβ is a major receptor involved in bone growth after birth and during early puberty. However, the growth alteration seen in the group administered with ERβ appears to catch up after initial decline. This might be because of a higher stimulatory action of ERα in counteraction of non-functional ERβ (17), as also the action of AR in male mice. Moreover, for mandibular growth, both endochondral and perichondral activities are important, although endochondral bone formation seems to exceed the perichondral bone formation (18). This might be the reason why the condylar height and mandibular length were mainly affected by sex hormones antagonists in both genders.

In this research, we used the selective estrogen receptors (ERs) antagonists MPP and PHTPP. In the past, it was difficult to investigate the individual effects of the ERs because of an inexistence of selective antagonists. Lately, MPP and PHTPP have been introduced as selective ERs antagonists, even though extensive findings about them are not available in the literature. *In vitro* assays characterized MPP as ERα antagonist (9); however, emerging evidence suggests that it may more accurately resemble a selective estrogen receptor modulator (SERM) with mixed agonist/antagonist properties depending on the tissue they influence (19). For the present results, we have characterized them as specific antagonists of ERα and ERβ, although further studies have to be performed.

Conclusions

From these results, it is assumed that the reduced bone growth found in the mandible and femur is a result of the inability of sex hormones to especially stimulate ERβ in male and female before and during early puberty. In late and after puberty, the discrepancy is caused by the inefficacy to stimulate ERα, ERβ, and AR in male and ERα and ERβ in female.

In this era of the growing presence of genetics in diagnosis, it would become practical to create normal indices based on genetic information. In the future, the variations in the expression of sex hormone receptors throughout puberty and maturation might become reliable indicators of bone maturity, which would provide an insight into scientific diagnosis for orthodontic treatment.

Clinical relevance

In the past, the sex hormones were considered mostly important for their effects in sexual differentiation in humans. However, at present, these hormones are a theme of study for other important processes in which they are involved. The present study is the beginning of many other studies to come, relating these hormones and its receptors with the stages of bone growth. It is our vision that this research contributes to the development of an accurate bone growth curve normality in humans for diagnostic purposes.

Acknowledgements: This investigation was supported in part by a Grant-in aid (No.19890139) from the Ministry of Education, Science, Sports and Culture in Japan.

References

1. Guyton A, Hall J. Introduction to endocrinology. In: WB Saunders editors. *Textbook of Medical Physiology*, 11th edn. Pennsylvania: Elsevier; 2006. pp. 906–7.
2. Lorenzo J. A new hypothesis for how sex steroid hormones regulate bone mass. *J Clin Invest* 2003;111:1641–3.
3. Manolagas SC, Kousteni S, Jilka RL. Sex steroids and bone. *Recent Prog Horm Res* 2002;57:385–409.
4. Venken K, Callewaert F, Boonen S, Vanderschueren D. Sex hormones, their receptors and bone health. *Osteoporos Int* 2008;11:1517–25.
5. Riggs L. Bone turnover in age-related osteoporosis: early contributions of Pierre Delmas. *Osteoporos Int* 2009;20:1289–90.
6. Eriksson S, Eriksson A, Steger R, Carlstrom K. Bone mineral density in patients with prostatic cancer treated with orchidectomy and with estrogen. *Calcif Tissue Int* 1995;57:97–9.
7. Fujita T, Ohtani J, Shigekawa M, Kawata T, Kaku M, Kohno S et al. Effects of sex hormone disturbances on craniofacial growth in newborn mice. *J Dent Res* 2004;83:250–4.
8. Márquez Hernández RA, Ohtani J, Fujita T, Sunagawa H, Kawata T, Kaku M et al. Sex hormone receptors play a crucial role in the control of femoral and mandibular growth in newborn mice. *Eur J Orthod*. 2011; doi: 10.1093/ejo/cjq124.
9. Sun J, Huang YR, Harrington WR, Sheng S, Katzenellenbogen JA, Katzenellenbogen BS. Antagonists selective for estrogen receptor α. *Endocrinology* 2002;143:941–7.
10. Valsecchi AE, Franchi S, Panerai AE, Sacerdote P, Trovato AE, Colleoni M. Genistein, a natural phytoestrogen from soy, relieves neuropathic pain following chronic constriction sciatic nerve injury in mice: anti-inflammatory and antioxidant activity. *J Neurochem* 2008;107:230–40.
11. Compton DR, Sheng S, Carlson KE, Rebacz NA, Lee IY, Katzenellenbogen BS et al. Pyrazolo[1,5-a]pyrimidines: estrogen receptor ligands possessing estrogen beta antagonist activity. *J Med Chem* 2004;47:5872–93.
12. Imperato-McGinley J, Sanchez RS, Spencer JR, Yee B, Vaughan ED. Comparison of the effects of the 5 alpha-reductase inhibitor finasteride and the antiandrogen flutamide on prostate and genital differentiation: dose-response studies. *Endocrinology* 1992;3:1149–56.
13. Kiliaridis S, Engstorm C, Thilander B. The relationship between the masticatory function and craniofacial morphology. I. A cephalometric longitudinal analysis in the growing rat fed a soft diet. *Eur J Orthod* 1985;7:273–83.
14. Robertson TB. Experimental studies on growth. II. The normal growth of the white mouse. *J Biol Chem* 1916;24:363–83.
15. Vanderschueren D, Bouillon R. Androgens and bone. *Calcif Tissue Int* 1995;56:341–6.
16. Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocrinology* 1999;20:358–417.
17. Lau HHL, Ho AYY, Luk KDK, Kung AWC. Estrogen receptor β gene polymorphisms are associated with higher bone mineral density in premenopausal, but not postmenopausal southern Chinese women. *Bone* 2002;31:276–81.
18. Luder HU. Perichondrial and endochondral components of mandibular condylar growth: morphometric and autoradiographic quantitation in rats. *J Anat* 1994;185:587–98.
19. Santollo J, Eckel LA. Effect of a putative ERα antagonist, MPP, on food intake in cycling and ovariectomized rats. *Physiol Behav* 2008;9:193–8.