RESEARCH REPORTS
Biological

A.B.M. Rabie*, G.H. Tang, H. Xiong, and U. Hägg

Hard Tissue Biology and Repair Research Group and Orthodontics, Faculty of Dentistry, The University of Hong Kong, Prince Philip Dental Hospital, 34 Hospital Road, Hong Kong SAR, China; *corresponding author, rabie@hkusua.hku.hk

*J Dent Res* 82(8):627-631, 2003

# PTHrP Regulates Chondrocyte Maturation in Condylar Cartilage

## ABSTRACT

PTHrP is a key factor regulating the pace of endochondral ossification during skeletal development. Mandibular advancement solicits a cascade of molecular responses in condylar cartilage. However, the pace of cellular maturation and its effects on condylar growth are still unknown. The purpose of this study was to evaluate the pattern of expression of PTHrP and correlate it to cellular dynamics of chondrocytes in condylar cartilage during natural growth and mandibular advancement. We fitted 35-day-old Sprague-Dawley rats with functional appliances. Experimental animals with matched controls were labeled with bromodeoxyuridine 3 days before their death, so that mesenchymal cell differentiation could be traced. Mandibular advancement increased the number of differentiated chondroblasts and subsequently increased the cartilage volume. Higher levels of PTHrP expression in experimental animals coincided with the slowing of chondrocyte hypertrophy. Thus, mandibular advancement promoted mesenchymal cell differentiation and triggered PTHrP expression, which retarded their further maturation to allow for more growth.

**KEY WORDS:** PTHrP, condylar cartilage, chondrocyte, differentiation, maturation.

Received January 20, 2003; Last revision April 20, 2003; Accepted May 15, 2003

A supplemental appendix to this article is published electronically only at http://www.dentalresearch.org.

## INTRODUCTION

Attempts to increase mandibular growth by functional appliances have been a subject of controversy in orthodontic practice (Chen *et al.*, 2002). Nevertheless, evidence from animal experiments is accumulating to demonstrate favorable condylar responses as a result of mandibular forward positioning (McNamara and Carlson, 1979; Petrovic *et al.*, 1981; Salo and Kantomaa, 1993; Kantomaa and Pirttiniemi, 1996). Most of these studies, however, were based on morphological observations until recently, when a concert of growth factors coordinating condylar growth was identified (Rabie and Hagg, 2002; Rabie *et al.*, 2002). The availability of such information facilitated answers to critical questions in the field of growth modifications, such as whether functional appliance therapy accelerates and/or enhances condylar growth. Cells in the proliferative layer of the developing mandibular condyle express Sox9 transcription factor, required for the differentiation of mesenchymal cells to chondroblasts (Rabie and Hagg, 2002). Chondrocytes express Sox9, which regulates the synthesis of type II collagen, the main component of condylar cartilage matrix, thus affecting condylar cartilage formation and subsequently condylar growth (Rabie *et al.*, 2003a). On the one hand, acceleration of condylar growth could mean accelerated entry of mesenchymal cells into the chondrogenic route, which would require that the expression of the factors regulating such processes be accelerated as well. On the other hand, enhancing condylar growth could primarily depend on the amount and rate of chondrogenesis of condylar tissues (Rabie *et al.*, 2003a). Thus, the use of data resulting from quantitative analysis of the levels of expression of Sox9, type II collagen, and the ultimate amount of bone formation during natural growth (Rabie and Hagg, 2002) and during mandibular advancement led us to conclude that functional appliances accelerate and enhance condylar growth (Rabie *et al.*, 2003a).

Yet, the underlying mechanisms regulating cellular dynamics within the condyle during mandibular advancement are still not fully understood. Cells within condylar cartilage are spatially organized. After proliferation, mesenchymal cells differentiate into chondroblasts and chondrocytes, followed by maturation and hypertrophy in addition to synthesis of extracellular matrices (Luder *et al.*, 1988). The cartilage template is eventually replaced by bone (Rabie *et al.*, 2002). As early maturation of the chondrocytes ceases chondrogenesis and induces osteogenesis (Meikle, 1973; Kantomaa and Hall, 1991), the maintenance of the chondroblast layer, where mesenchymal cells stop proliferation and initiate differentiation, is thus a major regulatory point for continuing condylar growth. Mechanical forces not only affect the proliferative activities of the chondroprogenitor cells, but also have a great impact on their further differentiation and maturation (Meikle, 1973; Copray *et al.*, 1983; Rabie *et al.*, 2003b).

Parathyroid-hormone-related protein (PTHrP) belongs to the parathyroid hormone (PTH) family (Strewler, 2000). In marked contrast to PTH, which is

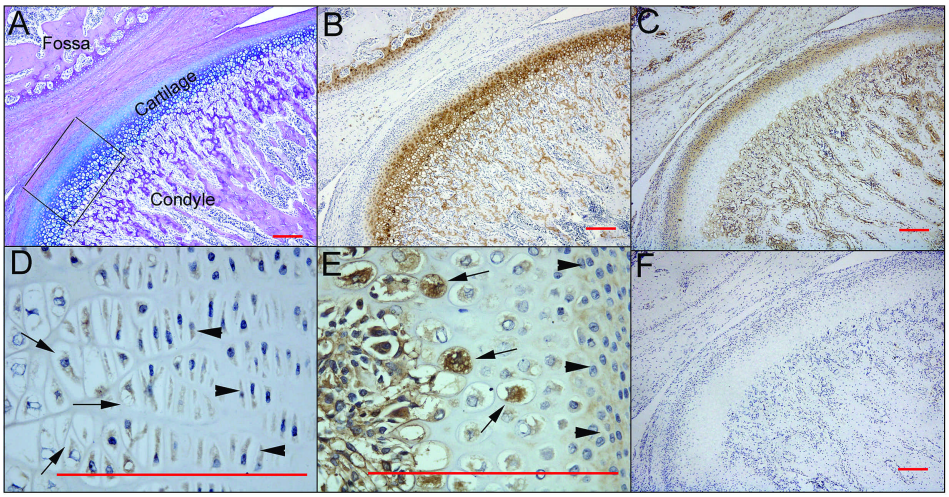


**Figure 1.** An overview of the temporomandibular joint of a 42-day-old rat showing histological staining of alcian blue-PAS **(A)**, with a measurement frame in the posterior condyle. Immunostainings of type II collagen **(B)** and PTHrP **(C,D,E)** are demonstrated compared with the negative control **(F)**. High magnification shows PTHrP expression in chondroblast cells (arrowheads) and hypertrophic chondrocytes (arrows) in the tibia growth plate (D) and mandibular condyle (E). Scale bars: 200 μm.

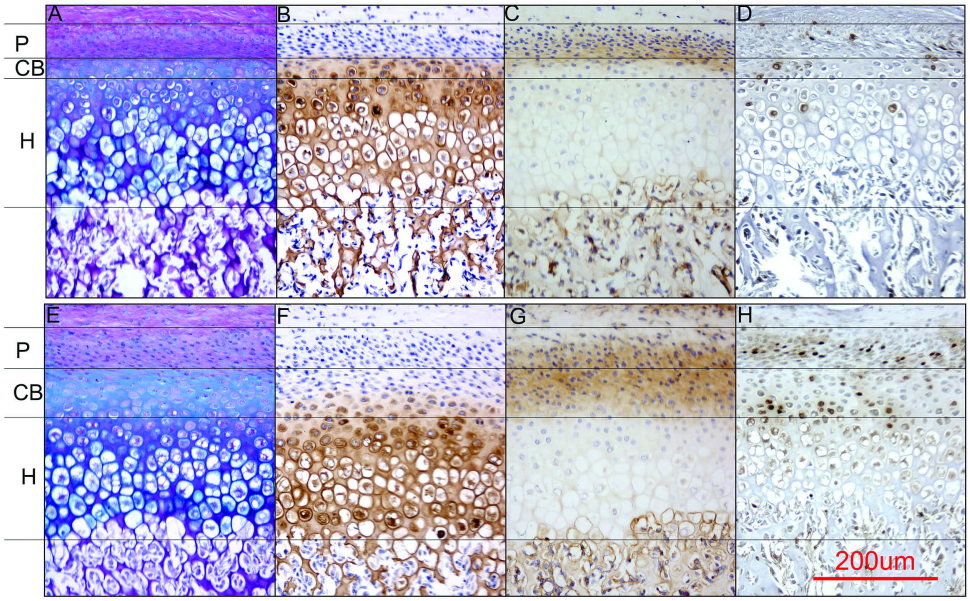


**Figure 2.** Immunohistochemistry shows PTHrP expression (C,G) and mesenchymal cell migration (BrdU labeling, D,H) in posterior condylar cartilage during natural growth (**A,B,C,D:** 42-day-old rats) and mandibular advancement (**E,F,G,H:** 7 days after the experiment). Cartilage layers are demarcated with alcian blue-PAS staining (A,E) and type II collagen immunostaining (B,F). The proliferative layer (P) contains densely packed mesenchymal cells. The chondroblast layer (CB) is stained with alcian blue only. The hypertrophic layer (H) with strong type II collagen signals is both alcian-blue- and PAS-positive.

a circulating hormone, PTHrP is a local messenger with multiple functions in many tissues (Strewler, 2000). During skeletal genesis, the physiologic action of PTHrP in cartilage is to regulate endochondral bone formation by controlling the pace of chondrocyte differentiation and maturation (Karaplis *et al.*, 1994; Amling *et al.*, 1997). Thus, the purpose of this study is to investigate the potential role of PTHrP during post-natal growth of mandibular condyle by identifying: (1) the expression of PTHrP in the mandibular condyle during natural growth and during mandibular forward positioning; (2) the correlation of the temporal patterns between PTHrP expression and cellular dynamics; and (3) the correlation of PTHrP expression with cartilage formation.

## MATERIALS & METHODS

### Experimental Animals and Cell Dynamics Study

We randomly assigned 100 female Sprague-Dawley rats (35 days old) to 5 control and 5 experimental groups (n = 10). Experimental animals were fitted with appliances which positioned the mandible forward (Rabie *et al.*, 2001). Rats were killed after 3, 7, 14, 21, and 30 days. Three days before death, 5 rats in each group were given an intraperitoneal injection of bromodeoxyuridine (BrdU, Sigma, St. Louis, MO, USA) at the dosage of 5 mg/100 g body weight. The injection was administered to each rat at the same time of day (9:00-10:00 a.m.). The experiment was approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong.

### Histological and Immunohistochemical Staining

Condyles were harvested, and paraffin sections were cut mid-sagittally. Cartilage layers were identified by combined alcian blue and PAS (Periodic acid and Schiff reagent) staining (Cook, 1996). Immunohistochemistry was carried out with a three-step avidin-biotin complex method as described (Rabie *et al.*, 2003b). We used a monoclonal anti-BrdU antibody (Sigma, St. Louis, MO, USA) to visualize the BrdU-labeled cells. The expression of PTHrP was evaluated by a polyclonal rabbit antiserum raised against human PTHrP at the amino-terminus of 1-34 (IDS Ltd., Boldon, UK). This antibody was murine-reactive and has no cross-reactivity with PTH (Yamazaki *et al.*, 1999). Type II collagen expression was also examined in parallel with the corresponding antibody (Santa Cruz Bio. Inc., Santa Cruz, CA, USA). For negative controls, non-immune serum was applied instead of the primary antibodies. Specimens from proximal tibia growth plate of a 14-day-old rat served as positive controls.

### Quantitative and Statistical Analysis

A true-color computer-assisted image-analyzing system with a digital camera (Leica DC 300 V2.0, Leica, Wetzlar, Germany) and software (Qwin V2.4, Leica, Cambridge, UK) was used for quantitative analysis (Rabie *et al.*, 2001). Measurements were carried out in a frame of 550 x 400 μm in the posterior region of the condyle (Fig. 1A), where the most prominent cellular responses were documented in response to mandibular advancement (Rabie *et al.*, 2002, 2003a,b). Images were captured at a total magnification of 360x, with the cell layers parallel to the measurement frame (Fig. 2). The demarcation of 3 cartilage layers (proliferative, chondroblast, and hypertrophic) was based on alcian blue-PAS staining and type II collagen immunostaining (Figs. 1A, 1B; Fig. 2). The thickness of each layer was determined as the mean of the measurements at 3 equally divided sites in the frame. PTHrP expression in the proliferative and chondroblast layers was quantified automatically as the percentage of the positive-staining areas (brown; Figs. 2C, 2G) in the measurement frame. The number of BrdU-labeled cells (at least 80 pixels) within the proliferative layer and in the cell layers underneath (chondroblast and hypertrophic) was counted separately by the computer (Figs. 2D, 2H). The data were collected again 4 wks later by the same observer. Statistical analysis was processed with GraphPad InStat (Version 3.00, GraphPad Software Inc., San Diego, CA, USA) for ANOVA with the Bonferroni multiple-comparison test.

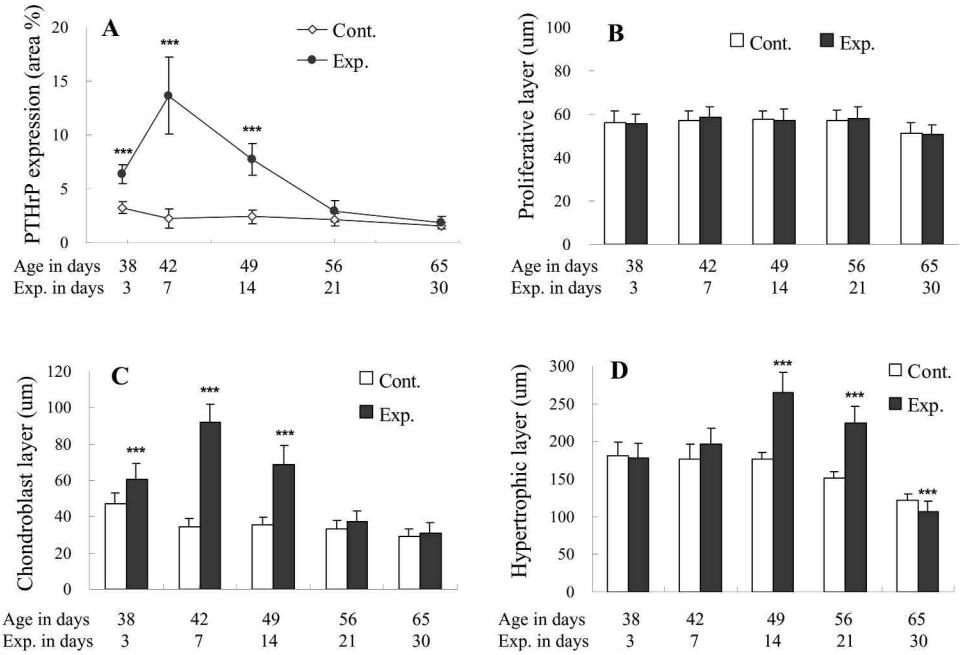


**Figure 3.** The temporal pattern of PTHrP expression **(A)** and the thickness of the proliferative layer **(B)**, chondroblast layer **(C)**, and hypertrophic layer **(D)** during natural growth (Cont) and mandibular advancement (Exp). Values are mean ± SD (n = 10). Significant differences between control and experimental animals are marked with asterisks (***p < 0.001).

## RESULTS

We used the growth plate as the positive control (Fig. 1D), and expression of PTHrP was detected by immunohistochemistry in rat condylar cartilage (Figs. 1C, 1E; Figs. 2C, 2G). PTHrP was predominantly localized at the intersection of the proliferative and chondroblast layers (Figs. 2C, 2G). Strong PTHrP immunoactivity was consistently expressed in the erosive zone (Figs. 1C, 1E). Occasionally, the protein appeared in hypertrophic chondrocytes in the anterior part of the condyle (Fig. 1E). Quantitative analysis demonstrated a significant increase in PTHrP expression after mandibular advancement, with a peak on day 7 (499% increase) (Figs. 2C, 2G; Fig. 3A; Appendix Table, www.dentalresearch.org).

Three days after being labeled with BrdU, half of the replicated mesenchymal cells remained in the proliferative layer, while the other half (43-53%, Fig. 4) moved into the subjacent chondroblast and hypertrophic layers (Figs. 2D, 2H). Mandibular advancement significantly increased the number of labeled cells in proliferative layers and also in the subjacent layers for up to 14 days (Fig. 4). The labeled cells reached the upper hypertrophic layer during natural growth (Fig. 2D), but gathered in the chondroblast layer in experimental animals (Fig. 2H).

## DISCUSSION

BrdU is a thymidine analogue that can be incorporated into replicating cells during DNA synthesis (Wynford-Thomas and Williams, 1986). This provides a method whereby cell migration and differentiation can be traced, and adds a third dimension to the histological interpretation of cellular dynamics. In young rat condylar cartilage, mesenchymal cells have a life span of 5 to 7 days before they leave for the medullary cavity (Folke and Stallard, 1967; Luder *et al.*, 1988). The cell cycle is around 100 hrs, and the duration from DNA synthesis to mitosis is 10 hrs (Folke and Stallard, 1967). Theoretically, these proliferative cells should just have completed their mitosis once and doubled their population in 3 days. Thus, half of the BrdU-labeled cells would be the newly recruited cells. Our results showed that from 48% to 53% of the labeled cells migrated into the chondrocyte layer in the animals from age 38 days to 49 days (Fig. 4), indicating that nearly all of the newly recruited mesenchymal cells differentiated into chondrocytes. Thus, the increased replicating mesenchymal cell numbers, in response to mandibular advancement, led directly to an increase in chondrocyte population (Fig. 4). This confirmed early reports of a close correlation between increased replicating mesenchymal cells and bone formation as a result of mandibular advancement (Petrovic *et al.*, 1981; Rabie *et al.*, 2003b). The lesser contribution of replicated mesenchymal cells to chondrocytes in 65-day-old rats (43%) could explain the slowed condylar growth with age (Fig. 4).

It has been pointed out that the most rewarding aspect of regulating condylar growth was not cell proliferation alone, but rather the differentiation and maturation rate of cartilage cells (Kantomaa and Pirttiniemi, 1996). Meikle (1973) stated that extrinsic mechanical stress was essential for chondroblast differentiation in the mandibular condyle. Mechanical forces were

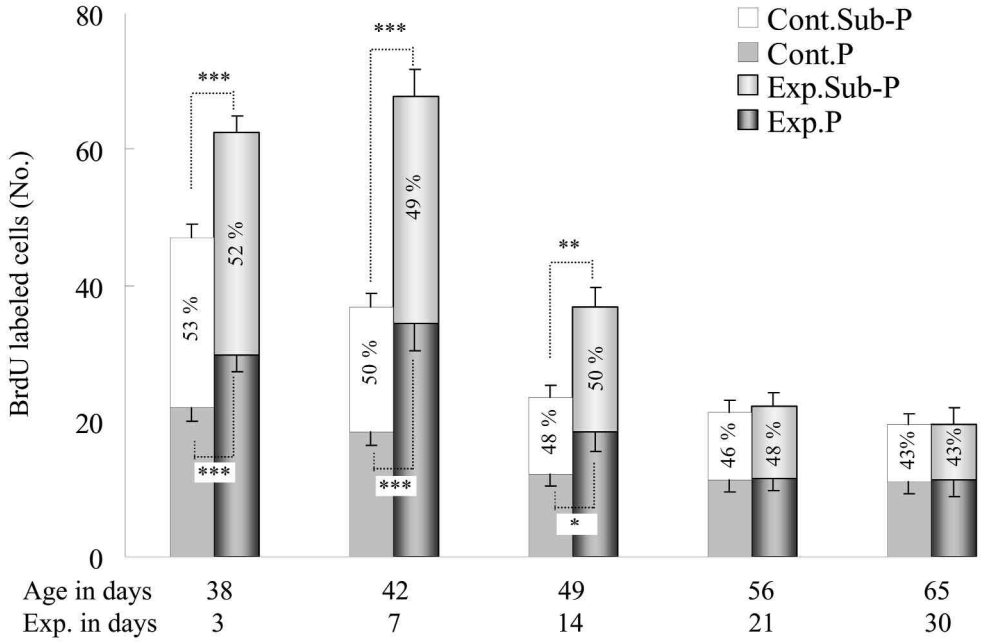


**Figure 4.** BrdU-labeled cells in the proliferative layer (P), and the subjacent chondroblast and hypertrophic layers (Sub-P) during natural growth (Cont) and mandibular advancement (Exp). The proportion of labeled cells that moved out of the proliferative layer over the total labeled cells is indicated. Values are mean ± SD (n = 5). Significant differences between control and experimental animals are marked with asterisks (*P < 0.05, **p < 0.01, ***p < 0.001).

also shown to influence chondrocyte maturation (Kantomaa *et al.*, 1994). Thus, it was essential to examine the expression of PTHrP, a key regulator of chondrocyte differentiation and maturation, in response to mandibular advancement. In this study, PTHrP immunoactivities were detected in condylar cartilage during post-natal growth. The pattern corresponded to the previous reports of PTHrP expression in growth plate and embryonic condylar cartilage (Yamazaki *et al.*, 1999; van der Eerden *et al.*, 2000). We did not detect significant variations in PTHrP expression during natural growth. Mandibular advancement, however, triggered a five-fold increase in PTHrP level on day 7 (Fig. 3A). It is important to note that the increased PTHrP expression was associated with the increase of new chondrocyte populations after mandibular advancement (Figs. 3A, 4). It was documented that PTHrP up-regulates Sox9 transcription (Huang *et al.*, 2000, 2001), which has been shown to promote the differentiation of mesenchymal cells into chondroblasts in the mandibular condyle (Rabie *et al.*, 2003a). Thus, PTHrP may have acted upon the mesenchymal cells and induced their differentiation through the Sox9 pathway. Our data further supported the recent *in vitro* findings that PTHrP treatment increased the cartilage nodule number in chicken mandibular mesenchyme culture (Zhao *et al.*, 2002). The lower quantity of PTHrP in the control animals could be due to a slow pace of cellular differentiation occurring during the slow period of condylar growth that follows the growth spurt. Growth spurts in rats exist on day 31.5 (Luder, 1996), and the rats used in this study were between the age of 35 and 65 days.

Alcian blue has been used to stain aggrecan, a chondroblast marker which has been detected in chondrogenic cells, and its expression preceded that of type II collagen (Fukada *et al.*, 1999). With alcian blue-PAS staining, we found an expansion in the chondroblast layer after mandibular advancement (Fig. 3C). Furthermore, we showed that new replicated cells after mandibular advancement accumulated in the chondroblast layer, which was coincident with the higher PTHrP level (Figs. 2H, 3A). It is important to note that these cells have already undergone hypertrophy during natural growth, where PTHrP signals were much lower (Figs. 2D, 3A). The current results are in agreement with earlier reports where mice with overexpressed PTHrP showed an accumulation of pre-hypertrophic chondrocytes (Amling *et al.*, 1997). These findings implied that PTHrP plays a similar role in the condyle, where it inhibits further chondroblast maturation. The accumulation of chondroblasts induced by PTHrP expression thus holds great growth potential, because it would enable chondrogenesis to continue (Kantomaa and Hall, 1991). Therefore, it is important to consider the modality of treatment in the field of growth modification in light of the current data and other recent reports. In the clinic, mechanical strain produced by mandibular advancement leads to changes in the biophysical environment of the joint, which solicits cellular and molecular responses (Rabie *et al.*, 2001, 2002, 2003a,b). Among mandibular responses, increased expression of PTHrP by the cells of the condyle retards the chondroblast maturation, thus allowing for more replication of proliferative mesenchymal cells (Fig. 4). Recently, we demonstrated a close correlation between the population size of the replicating mesenchymal cells in the temporomandibular joint and growth potential during mandibular advancement (Rabie *et al.*, 2003b). Here, we verified that the hypertrophic layer, along with type II collagen, the framework of cartilage, increased on days 14 and 21 of advancement (Fig. 3D). This echoes our previous finding, that the more cartilage matrix formed in the condyle, the greater the amount of new bone formation (Rabie *et al.*, 2003a).

In PTHrP knockout mice, the abnormalities of endochondral ossification differed according to the features of different cartilages (Karaplis *et al.*, 1994; Ishii-Suzuki *et al.*, 1999; Suda *et al.*, 1999). Chondrocytes in the growth plate and posterior cranial base encountered accelerated hypertrophy and premature mineralization (Karaplis *et al.*, 1994; Ishii-Suzuki *et al.*, 1999). In contrast, cartilage in the mandibular condyle showed proportional reduction of type II and type X collagen domains (Ishii-Suzuki *et al.*, 1999), and this was due to decreased proliferative activity of chondrocytes in both the flattened and hypertrophic layers (Suda *et al.*, 1999). In response to mandibular advancement, we showed expansion of the chondroblast layer with higher PTHrP expression (Figs. 3A, 3C), which was subsequently followed by enlargement of the hypertrophic layer (Fig. 3D). This clearly

points to the important role that PTHrP plays in regulating the pace of chondrocyte maturation in mandibular condylar cartilage.

In conclusion, mandibular advancement triggered PTHrP expression in condylar cartilage, which promoted the differentiation of mesenchymal cells into chondroblasts, but retarded their further maturation. This endows the condyle with more potential to build up the cartilage frame for future endochondral bone formation.

## ACKNOWLEDGMENT

This research was supported by CRCG grant 10203770.22311.08003.323.01 from the University of Hong Kong.

## REFERENCES

Amling M, Neff L, Tanaka S, Inoue D, Kuida K, Weir E, *et al.* (1997). Bcl-2 lies downstream of parathyroid hormone-related peptide in a signaling pathway that regulates chondrocyte maturation during skeletal development. *J Cell Biol* 136:205-213.

Chen JY, Will LA, Niederman R (2002). Analysis of efficacy of functional appliances on mandibular growth. *Am J Orthod Dentofacial Orthop* 122:470-476.

Cook HC (1996). Carbohydrates. In: Theory and practice of histological techniques. Bancroft J, Stevens A, Turner D, editors. New York: Churchill Livingstone, pp. 193-194.

Copray JC, Jansen HW, Duterloo HS (1983). Growth of the mandibular condylar cartilage of the rat in serum-free organ culture. *Arch Oral Biol* 28:967-974.

Folke LE, Stallard RE (1967). Cellular kinetics within the mandibular joint. *Acta Odontol Scand* 25:469-489.

Fukada K, Shibata S, Suzuki S, Ohya K, Kuroda T (1999). In situ hybridisation study of type I, II, X collagens and aggrecan mRNas in the developing condylar cartilage of fetal mouse mandible. *J Anat* 195:321-329.

Huang W, Zhou X, Lefebvre V, de Crombrugghe B (2000). Phosphorylation of SOX9 by cyclic AMP-dependent protein kinase A enhances SOX9's ability to transactivate a Col2a1 chondrocyte-specific enhancer. *Mol Cell Biol* 20:4149-4158.

Huang W, Chung UI, Kronenberg HM, de Crombrugghe B (2001). The chondrogenic transcription factor Sox9 is a target of signaling by the parathyroid hormone-related peptide in the growth plate of endochondral bones. *Proc Natl Acad Sci USA* 98:160-165.

Ishii-Suzuki M, Suda N, Yamazaki K, Kuroda T, Senior PV, Beck F, *et al.* (1999). Differential responses to parathyroid hormone-related protein (PTHrP) deficiency in the various craniofacial cartilages. *Anat Rec* 255:452-457.

Kantomaa T, Hall BK (1991). On the importance of cAMP and Ca++ in mandibular condylar growth and adaptation. *Am J Orthod Dentofacial Orthop* 99:418-426.

Kantomaa T, Pirttiniemi P (1996). Differences in biologic response of the mandibular condyle to forward traction or opening of the mandible. An experimental study in the rat. *Acta Odontol Scand* 54:138-144.

Kantomaa T, Tuominen M, Pirttiniemi P (1994). Effect of mechanical forces on chondrocyte maturation and differentiation in the mandibular condyle of the rat. *J Dent Res* 73:1150-1156.

Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VL, Kronenberg HM, *et al.* (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev* 8:277-289.

Luder HU (1996). Comparative skeletal maturation, somatic growth, and ageing. In: Postnatal development, ageing, and degeneration of the temporomandibular joint in humans, monkeys, and rats. Luder HU, editor. Ann Arbor: The Center for Human Growth & Development, The University of Michigan, pp. 111-132.

Luder HU, Leblond CP, von der Mark K (1988). Cellular stages in cartilage formation as revealed by morphometry, radioautography and type II collagen immunostaining of the mandibular condyle from weanling rats. *Am J Anat* 182:197-214.

McNamara JA Jr, Carlson DS (1979). Quantitative analysis of temporomandibular joint adaptations to protrusive function. *Am J Orthod* 76:593-611.

Meikle MC (1973). In vivo transplantation of the mandibular joint of the rat; an autoradiographic investigation into cellular changes at the condyle. *Arch Oral Biol* 18:1011-1020.

Petrovic AG, Stutzmann J, Gasson N (1981). The final length of the mandible: is it genetically determined? In: Craniofacial biology. Craniofacial growth series. Monograph No. 10. Carlson DS, editor. Ann Arbor, MI: Center for Human Growth & Development, The University of Michigan, pp. 105-126.

Rabie AB, Hagg U (2002). Factors regulating mandibular condylar growth. *Am J Orthod Dentofacial Orthop* 122:401-409.

Rabie AB, Zhao Z, Shen G, Hagg EU, Robinson W (2001). Osteogenesis in the glenoid fossa in response to mandibular advancement. *Am J Orthod Dentofacial Orthop* 119:390-400.

Rabie AB, Leung FY, Chayanupatkul A, Hagg U (2002). The correlation between neovascularization and bone formation in the condyle during forward mandibular positioning. *Angle Orthod* 72:431-438.

Rabie AB, She TT, Hagg U (2003a). Functional appliance therapy accelerates and enhances condylar growth. *Am J Orthod Dentofacial Orthop* 123:40-48.

Rabie AB, Wong L, Tsai MJ (2003b). Replicating mesenchymal cells in the condyle and the glenoid fossa during mandibular forward positioning. *Am J Orthod Dentofacial Orthop* 123:49-57.

Salo L, Kantomaa T (1993). Type II collagen expression in the mandibular condyle during growth adaptation: an experimental study in the rabbit. *Calcif Tissue Int* 52:465-469.

Strewler GJ (2000). The physiology of parathyroid hormone-related protein. *N Engl J Med* 342:177-185.

Suda N, Shibata S, Yamazaki K, Kuroda T, Senior PV, Beck F, *et al.* (1999). Parathyroid hormone-related protein regulates proliferation of condylar hypertrophic chondrocytes. *J Bone Miner Res* 14:1838-1847.

van der Eerden BC, Karperien M, Gevers EF, Lowik 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.

Wynford-Thomas D, Williams ED (1986). Use of bromodeoxyuridine for cell kinetic studies in intact animals. *Cell Tissue Kinet* 19:179-182.

Yamazaki K, Suda N, Kuroda T (1999). Distribution of parathyroid hormone-related protein (PTHrP) and type I parathyroid hormone (PTH) PTHrP receptor in developing mouse mandibular condylar cartilage. *Arch Oral Biol* 44:853-860.

Zhao Q, Brauer PR, Xiao L, McGuire MH, Yee JA (2002). Expression of parathyroid hormone-related peptide (PthrP) and its receptor (PTH1R) during the histogenesis of cartilage and bone in the chicken mandibular process. *J Anat* 201:137-151.