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ABSTRACT

Indian hedgehog (Ihh) is a critical mediator transducing mechanical signals to stimulate chondrocyte proliferation. To clarify the cellular signal transduction pathway that senses and converts mechanical signals into tissue growth in mandibular condyle, we evaluated Ihh expression and its relation to the kinetics of replicating mesenchymal cells in condylar cartilage during natural growth and mandibular advancement. Thirty-five-day-old Sprague-Dawley rats were fitted with functional appliances. Experimental animals with matched controls were doubly labeled with iododeoxyuridine and bromodeoxyuridine so that we could evaluate the cycles of the proliferative mesenchymal cells. Mandibular advancement triggered Ihh expression in condylar cartilage. A higher level of Ihh expression coincided with the increase of the replicating mesenchymal cells' population and the shortening of the turnover time. These findings suggested that Ihh acts as a mediator of mechanotransduction that converts mechanical signals resulting from anterior mandibular displacement to stimulate cellular proliferation in condylar cartilage.

KEY WORDS: Ihh, condylar cartilage, proliferation, mechanical stress, cell cycle.

Indian Hedgehog: A Mechanotransduction Mediator in Condylar Cartilage

INTRODUCTION

The adaptive potential of the condyle in response to mandibular protrusion has been the objective of numerous studies (Charlier *et al.*, 1969; Petrovic *et al.*, 1975; McNamara and Carlson, 1979; Bollen *et al.*, 1989; Haas and Holick, 1996; Kantomaa and Pirttiniemi, 1996). Recently, Rabie and co-workers reported that condylar growth is regulated by a host of orchestrated influences of various growth factors and other regulatory factors that are endogenously expressed by cells in the mandibular condyles (Rabie and Hägg, 2002). This work was the basis against which cellular and molecular changes in the condyles during mandibular advancement were compared (Rabie *et al.*, 2002, 2003b,c,d). Mandibular forward positioning leads to an increase in the number of replicating mesenchymal cells (Rabie *et al.*, 2003d), an increase in the level of expression of PTHrP and Sox9, the factors regulating chondrocyte differentiation and maturation (Rabie *et al.*, 2003b,c), and an increase in the level of expression of VEGF, the central regulator of vascularization (Rabie *et al.*, 2002), thus leading to new bone formation in the condyle (Rabie *et al.*, 2003a).

However, little is known about the underlying mechanisms of mechanotransduction. Mechanotransduction is the process of translating mechanical stimulation into cellular responses. Mechanical stress plays a fundamental role in regulating cellular activities during tissue morphogenesis (Ingber, 1991). Tavernarakis and Driscoll (1997) reported that many developmental processes depend on external mechanical cues that stimulate internal molecules. This brings us to a central question in the field of growth modifications: How would changing the biophysical environment in the condyle, as a result of mandibular advancement, solicit cellular and molecular changes that lead to bone formation?

Indian hedgehog (Ihh), a member of the vertebrate hedgehog morphogen family, was reported to be a most dynamic mechano-responsive factor expressed in chondrocytes (Wu *et al.*, 2001). The hedgehog protein family has been identified as key morphogens during skeletal development and regeneration (McMahon, 2000). Among the hedgehog proteins, Ihh is involved in late limb development by regulating chondrocyte proliferation and differentiation (McMahon, 2000). *In vitro* experiments showed that mechanical stress stimulated Ihh expression in chondrocytes by 18-fold (Wu *et al.*, 2001). Overexpression of Ihh in transgenic mice led to an increase in the number of replicating chondrocytes in the growth plate (Long *et al.*, 2001). In contrast, Ihh null mice encountered severe dwarfism with a reduction in the rate of DNA synthesis (St-Jacques *et al.*, 1999), thus highlighting the importance of Ihh to chondrocyte proliferation during long bone development. It is possible that Ihh has a similar regulatory role in condylar cartilage. To find out whether Ihh is a functional factor that mediates condylar growth in response to mandibular advancement, we designed this study to evaluate Ihh expression and correlate its expression pattern to mesenchymal cells' kinetics in the condyle during natural growth and during forward mandibular positioning.

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MATERIALS & METHODS

Experimental Animals and Cell Cycle Study

One hundred female SD rats at 35 days of age were randomly allotted into 5 control and 5 experimental groups ($n = 10$). The experimental animals were fitted with functional appliances which positioned the mandible forward (Rabie *et al.*, 2001). Rats were killed after 3, 7, 14, 21, and 30 days. To evaluate the cell cycle of the proliferating mesenchymal cells in condylar cartilage, we applied a double-labeling technique with two thymidine analogues (Vanky *et al.*, 1998). Five rats in each group were first given an intraperitoneal injection of iododeoxyuridine (IdU, Sigma, St. Louis, MO, USA) at 2.5 mg/100 g body weight, followed 1 hr later by another injection of bromodeoxyuridine (BrdU, Sigma, St. Louis, MO, USA) with the same dosage. Animals were killed 1 hr after the last administration. The injections were given to each rat at the same time of the day (9:00 a.m.

~ 11:00 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.

Immunohistochemistry

After condyles were harvested and paraffin-embedded, 3- μ m sections were made mid-sagittally. A standard immunohistochemical staining was carried out with a three-step avidin-biotin complex method (Rabie *et al.*, 2003d). Two specific monoclonal primary antibodies (IU-4 and Br-3, Caltag Lab, Burlingame, CA, USA) were used to visualize the proliferating cells incorporated with BrdU and/or IdU. IU-4 recognized both BrdU and IdU, while Br-3 reacted with BrdU only. Three neighboring sections mounted on one slide were applied with either IU-4, Br-3, or non-immune serum (negative control) for the immunostaining. Ihh expression was identified with a polyclonal rabbit antiserum raised against human Ihh (Santa Cruz Bio. Inc., Santa Cruz, CA, USA). Its reaction with rats' tissues by immunohistochemistry has been reported elsewhere (van der Eerden *et al.*, 2000). Specimens from the proximal tibia growth plate served as positive controls.

Quantitative and Statistical Analysis

A true-color computer-assisted image-analyzing system with a digital camera (Leica DC 300 V 2.0, Leica, Wetzlar, Germany) and software (Qwin V2.4, Leica, Cambridge, UK) was used for the quantitative analysis (Rabie *et al.*, 2003c). Measurements were carried out in a frame of 550 X 200 μ m in the posterior region of the condyle (Fig. 1A), where the most prominent cellular responses and highest amount of bone formation were documented in response to mandibular advancement (Rabie *et al.*, 2002, 2003a,b,d). The whole mesenchymal cell population within the

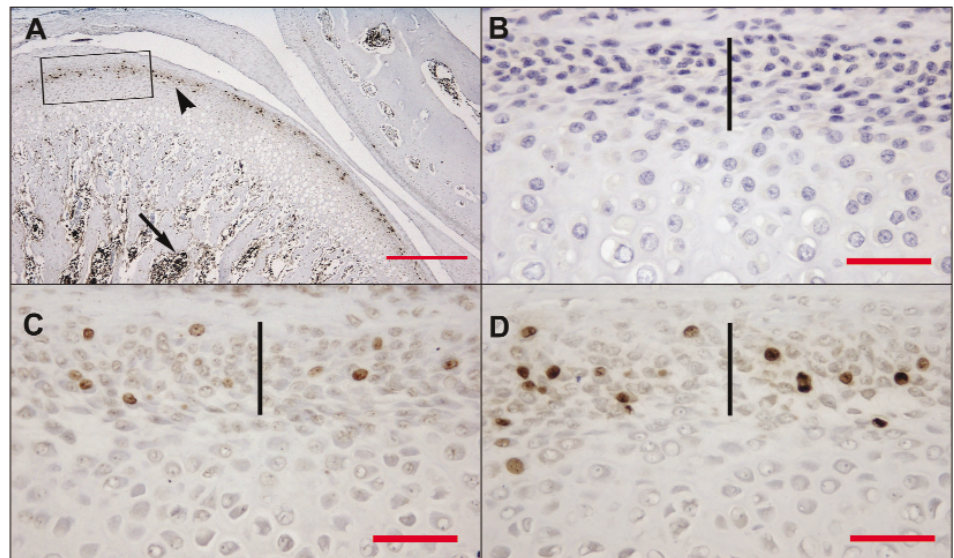


Figure 1. Immunostaining of IdU showed replicating cells in the proliferative layer (arrowhead) and blood vessels (arrow) in the mandibular condyle of 42-day-old rats during natural growth (A). Quantitative analysis was carried out in a measurement frame in the posterior condyle. Three neighboring sections (B,C,D) were used to evaluate the cell cycle of the replicating mesenchymal cells. Negative control without primary antibody was used to quantify the total mesenchymal cells' population (B). Cells labeled with BrdU were detected by Br-3 primary antibody (C). Cells labeled with both BrdU and IdU were detected by IU-4 primary antibody (D). Vertical bars denoted the proliferative layer. Scale bars: 400 μ m for A; 50 μ m for B, C, and D.

proliferative layer was quantified on the negative sections (Fig. 1B). We used the computer to count the number of labeled cells by identifying the positive cells stained brown (at least 80 pixels, Figs. 1C, 1D). The number of labeled cells over the total mesenchymal cell population was referred to as the labeling index (LI). We determined the time for DNA synthesis (S phase) by identifying the labeled cells that had left the S phase during the interval between the two injections (1 hr), using the formula described by Shibui *et al.* (1989):

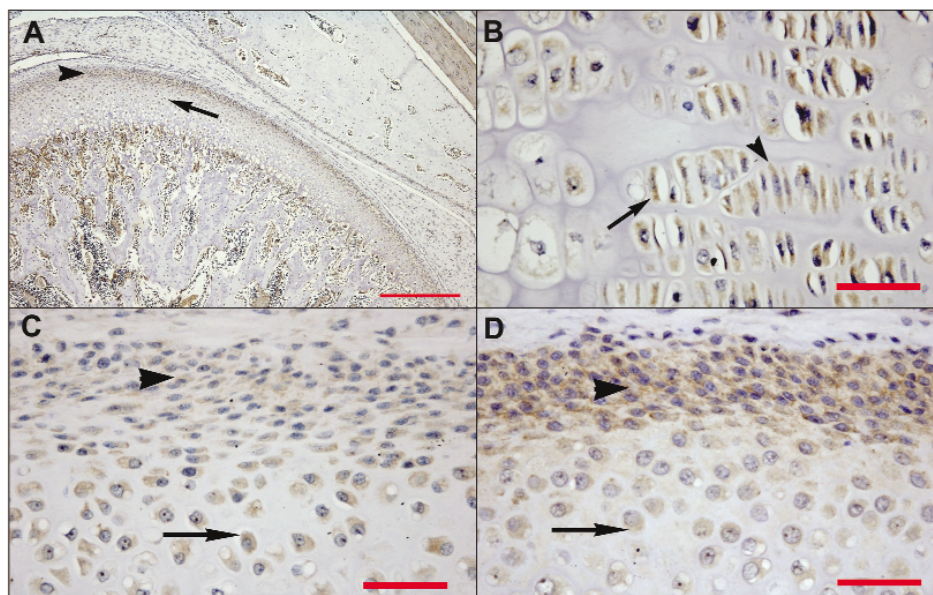
$$S \text{ phase} = \frac{\text{BrdULI}}{\text{IdULI} - \text{BrdULI}} \times 1 \text{ hour} \quad \text{and}$$

$$\text{turnover time} = \frac{S \text{ phase}}{\text{BrdULI}}$$

Turnover time is the time required for a given number of cells to replace their original population. The Ihh expression was quantified as the percentage of the areas staining positive in the same measurement frame. The data were collected again 4 wks later by the same observer. The difference between experimental and control groups was tested by unpaired *t* test with GraphPad InStat (Version 3.00, GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Cells labeled with BrdU or IdU were mainly limited to the proliferative layer of the condylar cartilage and also in the blood vessels (Fig. 1A). No signal was observed in the negative



natural growth (Fig. 3B). Mandibular advancement, however, led to a significant increase of replicating cell population, with the peak identified on day 7 and dropping to control levels after 14 days (Fig. 3B). As far as the duration of the cell cycle was concerned, neither the age nor the advancement statistically changed the DNA synthesis time (Table). However, the turnover time was dramatically shortened in experimental groups for 14 days (Table).

DISCUSSION

The present study demonstrated that Ihh is the mechanotransduction mediator in mandibular condyles that leads to cellular proliferation. A close correlation existed between Ihh and cellular turnover and cell renewal capacity in the condyles.

Cell kinetics studies provide a better understanding of the proliferation characteristics of individual cell lineage.

Incorporation of tritiated thymidine during DNA synthesis has been intensively used to study proliferative activity as well as cellular dynamics (Folke and Stallard, 1967; Petrovic *et al.*, 1975; Luder *et al.*, 1988). It is time-consuming, however, and subject to radiation hazard. BrdU and IdU are also thymidine analogues and are more convenient to use when combined with immunostaining (Gratzner, 1982; Rabie *et al.*, 2003d). Shibui *et al.* (1989) introduced a feasible method to estimate the S phase and turnover time of the cell cycle with BrdU and IdU double-labeling. It has been successfully used *in vivo* to evaluate epiphyseal cartilage growth and was proved to be a reliable approach (Vanky *et al.*, 1998, 2000).

For mesenchymal cells in the mandibular condyle, Folke and Stallard (1967) reported a seven-hour S phase with 100 hrs of cell cycle in four-week-old rats. Coincidentally, Luder *et al.* (1988) estimated a turnover time of 3 days in 20-day-old rats. In this study, we calculated that the S phase varied from 6.9 to 7.2 hrs without statistical difference between ages or after functional alteration (Table). This is in line with the fact that the duration of the S phase was fairly constant in mammalian cells (Folke and Stallard, 1967). However, mandibular advancement significantly shortened the turnover time, while it was prolonged with age during

Figure 2. Immunostaining showed Ihh expression in the proliferative layer (arrowhead) and the chondroblast layer (arrow) in the mandibular condylar cartilage (A) and tibia growth plate (B) of 42-day-old rats during natural growth. A significantly higher level of Ihh expression was detected in the proliferative layer of condylar cartilage during mandibular advancement (D, 7 days after experiment) than that of during natural growth (C, 42-day-old rats). Scale bars: 400 μ m for A; 50 μ m for B, C, and D.

control, where the primary antibodies were replaced by non-immune serum (Fig. 1B). More proliferating cells were labeled during the interval between the two injections of BrdU and IdU (Figs. 1C, 1D). Ihh expression was detected by immunohistochemistry in the proliferative layer and the chondroblast layer of condylar cartilage (Fig. 2A) and growth plate (Fig. 2B). The signal diminished progressively in the hypertrophic layer. Strong positive staining was also found in the erosive zone (Fig. 2A). Quantitative analysis demonstrated a significantly higher level of Ihh expression in the proliferative layer during mandibular advancement on day 3 and day 7 (Figs. 2C, 2D). The amounts of increase were 63% and 76%, respectively (Fig. 3A).

The proliferative activity of the mesenchymal cells, as indicated by the labeling index, decreased with age during

Table. IdU Labeling Index, Estimated S Phase Time, and Turnover Time of the Replicating Mesenchymal Cells in Mandibular Condylar Cartilage during Natural Growth (N.G.) and during Mandibular Advancement (Adv.)

Experimental Days		3	7	14	21	30
IdU (%)	N.G.	55.5 \pm 6.0 ^a	45.3 \pm 3.2	31.0 \pm 3.8	28.5 \pm 2.3	27.7 \pm 2.1
	Adv.	76.6 \pm 14.7*	85.4 \pm 15.4***	46.1 \pm 5.5**	28.9 \pm 2.6	28.5 \pm 3.0
S phase (hrs)	N.G.	7.2 \pm 0.4	7.0 \pm 0.5	7.1 \pm 0.7	7.2 \pm 1.0	7.0 \pm 1.0
	Adv.	6.9 \pm 0.5	7.0 \pm 0.5	6.9 \pm 0.4	6.9 \pm 1.0	7.2 \pm 1.0
Turnover time (hrs)	N.G.	149.0 \pm 16.4	177.4 \pm 22.8	263.8 \pm 22.3	287.7 \pm 37.9	290.4 \pm 33.1
	Adv.	106.3 \pm 24.7*	95.7 \pm 14.4***	173.4 \pm 24.3***	275.0 \pm 41.7	288.3 \pm 34.9

^a Values were mean \pm SD (n = 5). Significant difference between natural growth and mandibular advancement is marked with asterisks (*p < 0.05, **p < 0.01, ***p < 0.001).

natural growth (Table). Although the turnover time is a theoretical value, based on the assumption that all the cells have the same capacity of replication (Shibui *et al.*, 1989), it is still of the utmost importance, since it reflects cell renewal and thus controls tissue expansion. Therefore, the present result showed that cell replication in the condyle slows with age (Fig. 3B), indicating a decrease in cell renewal and thereby explaining the slowed growth of the condyle with age. In contrast, cell renewal in response to mandibular advancement was enhanced significantly, and this directly influences the growth potential of the condyle (Table). Analysis of the current data further supports our recent report, where the increase in the number of replicating mesenchymal cells was closely correlated with the increase in the amount of bone formation within the condyle in response to forward mandibular positioning (Rabie *et al.*, 2003d). The acceleration in cell renewal reported here could be a function of the duration of some cell-cycle phases changing more than others. Since the G2 plus M interval is relatively short (3 hrs) and varies only slightly (Folke and Stallard, 1967), such an acceleration of turnover time after mandibular advancement can be ascribed to either a lessening of the G1 phase, or the promoting of more G0 cells into the cell cycle. In fact, both the G1 and G0 phases are more sensitive to systemic and local controls (Wilsman *et al.*, 1996; Vanky *et al.*, 2000). In other words, the increased condylar growth by functional appliance therapy could be initiated by a constant number of mesenchymal cells replicating more rapidly or by an increase in the cells participating in the proliferative pool.

Thus far, we have presented only that a significant increase in cellular replication occurred in response to mechanical stimulation, but we have not discussed how mechanical stress would stimulate such a cellular response. In the present study, we identified the pattern of expression of Ihh, a key regulator of chondrocyte proliferation during skeletal genesis, and we correlated its expression pattern to that of the replicating mesenchymal cells in the condyle during natural growth and during mandibular advancement. In the mandibular condyle, cells in the proliferative layer as well as chondroblasts express Ihh (Figs. 2A, 2C). The same patterns were observed in the growth plate (Fig. 2B), as previously reported (St-Jacques *et al.*, 1999). Quantitative analysis showed a significant increase of Ihh expression in the proliferative layer immediately after mandibular advancement (Figs. 2C, 2D, 3A). This indicated that Ihh is a dynamic factor sensitive to mechanical stresses applied to the condyles.

A most interesting finding in the present study is that the accumulation of Ihh expression in the mesenchymal cells during mandibular advancement corresponded to the significant increase of the number of proliferating cells (Figs. 3A, 3B). It was demonstrated that Ihh up-regulates the expression of cyclin D1, a kinase that controls the "pass" of restriction point in the G1 phase by up-regulating a group of regulatory factors necessary for the transition from the G1 to the S phase (Twyman, 1998; Long *et al.*, 2001). This is in line with our results showing that increased Ihh expression during mandibular advancement shortened the turnover time of the replicating mesenchymal cells. These findings point out that mechanotransduction and cell proliferation in the condyle are connected by Ihh.

Wu *et al.* (2001) showed that mechanical stress led to matrix deformation, which in turn induced Ihh expression. It is

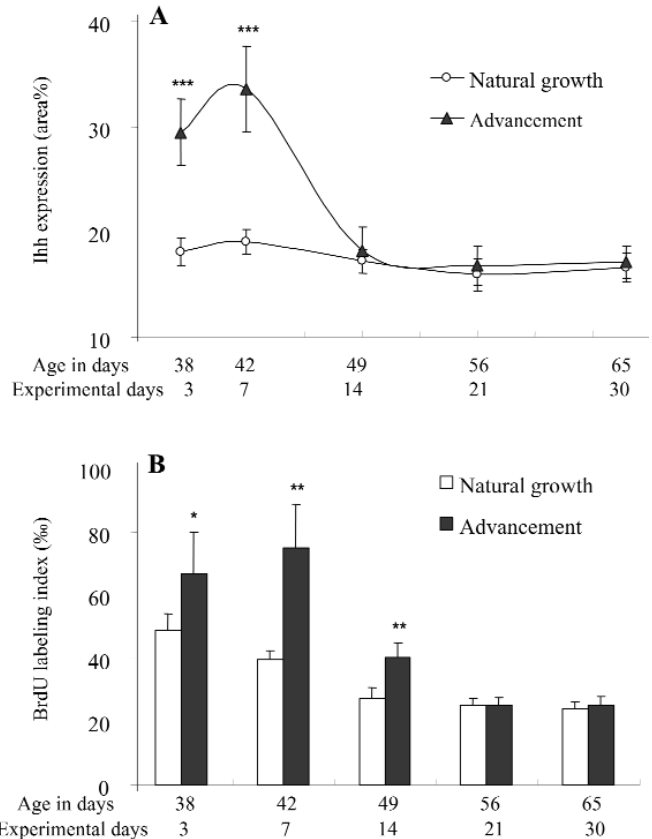


Figure 3. The temporal pattern of Ihh expression (A, $n = 10$) and the replicating mesenchymal cells' population (B, BrdU labeling index, $n = 5$) in condylar cartilage during natural growth and mandibular advancement. Values were mean \pm SD. Significant difference between two groups is marked with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

conceivable, then, that, upon mandibular advancement, deformation of the mesenchymal cells and other cells in the extracellular matrix creates a strain alignment that causes a deformation of the cytoskeleton of these cells (Rabie *et al.*, 2001), which subsequently triggers Ihh expression. Thus, the extra-expression of Ihh elicited by functional appliance treatment promotes mesenchymal cell proliferation and subsequently initiates a series of cellular and molecular responses that lead to bone formation in the condyles.

In conclusion, mandibular advancement triggered Ihh expression in condylar cartilage. Ihh is a mechanotransduction mediator that converts mechanical signals resulting from forward mandibular positioning to stimulate cellular proliferation in the condyle.

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REFERENCES

- Bollen AM, Makinen KK, Makinen PL, Carlson DS (1989). Collagenolytic and phosphatase activity in the rat mandible after

- functional protrusion. *Arch Oral Biol* 34:267-273.
- Charlier JP, Petrovic A, Herrmann-Stutzmann J (1969). Effects of mandibular hyperpropulsion on the prechondroblastic zone of young rat condyle. *Am J Orthod* 55:71-74.
- Folke LE, Stallard RE (1967). Cellular kinetics within the mandibular joint. *Acta Odontol Scand* 25:469-489.
- Gratzner HG (1982). Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science* 218:474-475.
- Haas DW, Holick MF (1996). Enhanced osteonectin expression in the chondroid matrix of the unloaded mandibular condyle. *Calcif Tissue Int* 59:200-206.
- Ingber D (1991). Integrins as mechanochemical transducers. *Curr Opin Cell Biol* 3:841-848.
- 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.
- Long F, Zhang XM, Karp S, Yang Y, McMahon AP (2001). Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. *Development* 128:5099-5108.
- 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.
- McMahon AP (2000). More surprises in the Hedgehog signaling pathway. *Cell* 100:185-188.
- McNamara JA Jr, Carlson DS (1979). Quantitative analysis of temporomandibular joint adaptations to protrusive function. *Am J Orthod* 76:593-611.
- Petrovic AG, Stutzmann JJ, Oudet CL (1975). Control processes in the postnatal growth of the condylar cartilage of the mandible. In: Determinants of mandibular form and growth. Craniofacial growth series. Monograph No. 4. McNamara JA, editor. Ann Arbor, MI, USA: Center for Human Growth and Development, University of Michigan, pp. 101-127.
- Rabie AB, Hägg U (2002). Factors regulating mandibular condylar growth. *Am J Orthod Dentofacial Orthop* 122:401-409.
- Rabie AB, Zhao Z, Shen G, Hägg U, 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, Hägg U (2002). The correlation between neovascularization and bone formation in the condyle during forward mandibular positioning. *Angle Orthod* 72:431-438.
- Rabie AB, Chayanupatkul A, Hägg U (2003a). Stepwise advancement using fixed functional appliances: experimental perspective. *Semin Orthod* 9:41-46.
- Rabie AB, She TT, Hägg U (2003b). Functional appliance therapy accelerates and enhances condylar growth. *Am J Orthod Dentofacial Orthop* 123:40-48.
- Rabie AB, Tang GH, Xiong H, Hägg U (2003c). PTHrP regulates chondrocyte maturation in condylar cartilage. *J Dent Res* 82:627-631.
- Rabie AB, Wong L, Tsai M (2003d). Replicating mesenchymal cells in the condyle and glenoid fossa during mandibular forward positioning. *Am J Orthod Dentofacial Orthop* 123:49-57.
- Shibui S, Hoshino T, Vanderlaan M, Gray JW (1989). Double labeling with iodo- and bromodeoxyuridine for cell kinetics studies. *J Histochem Cytochem* 37:1007-1011.
- St-Jacques B, Hammerschmidt M, McMahon AP (1999). Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev* 13:2072-2086.
- Tavernarakis N, Driscoll M (1997). Molecular modeling of mechanotransduction in the nematode *Caenorhabditis elegans*. *Annu Rev Physiol* 59:659-689.
- Twyman RM (1998). Advanced molecular biology. Trowbridge: BIOS Scientific Publishers Limited.
- 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.
- Vanky P, Brockstedt U, Hjerpe A, Wikstrom B (1998). Kinetic studies on epiphyseal growth cartilage in the normal mouse. *Bone* 22:331-339.
- Vanky P, Brockstedt U, Nurminen M, Wikstrom B, Hjerpe A (2000). Growth parameters in the epiphyseal cartilage of brachymorphic (bm/bm) mice. *Calcif Tissue Int* 66:355-362.
- Wilsman NJ, Farnum CE, Green EM, Lieferman EM, Clayton MK (1996). Cell cycle analysis of proliferative zone chondrocytes in growth plates elongating at different rates. *J Orthop Res* 14:562-572.
- Wu Q, Zhang Y, Chen Q (2001). Indian hedgehog is an essential component of mechanotransduction complex to stimulate chondrocyte proliferation. *J Biol Chem* 276:35290-35296.