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Biological

G.H. Tang[1,2] and A.B.M. Rabie[1]*

[1]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; and [2]Department of Orthodontics, School of Stomatology, Shanghai Second Medical University, Shanghai, China; *corresponding author, rabie@hkusua.hku.hk

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Runx2 Regulates Endochondral Ossification in Condyle during Mandibular Advancement

ABSTRACT

Runx2 is a transcription factor prerequisite for chondrocyte maturation and osteoblast differentiation. We tested the hypothesis that Runx2 is responsible for signaling chondrocyte maturation and endochondral ossification in the condyle during mandibular advancement. Fifty 35-day-old Sprague-Dawley rats were fitted with functional appliances for 3, 7, 14, 21, and 30 days. Experimental animals with 50 matched controls were labeled with bromodeoxyuridine for evaluation of the invasion of chondroclasts and osteoblasts into condylar cartilage. Mandibular advancement elicited Runx2 expression in condylar cartilage, and subsequently led to an expansion of type X collagen domain in the hypertrophic layer. Stronger Runx2 mRNA signals in subchondral bone corresponded with the increase in the recruitment of osteoblasts and chondroclasts, which preceded the increase of new bone formation in the condyle. Thus, Runx2 mediates chondrocyte terminal maturation and endochondral ossification in the mandibular condyle in response to mandibular advancement.

KEY WORDS: Runx2, mechanical stress, mandibular condyle, chondrocyte, osteoblast.

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INTRODUCTION

Functional strain in bone tissue has long been recognized as a controlling stimulus for adaptive bone remodeling (Lanyon, 1987). More recently, Tavernarakis and Driscoll (1997) reported that many developmental processes depend on external mechanical cues that stimulate internal molecules. Understanding the link between mechanical strain and ultimate bone growth could open new avenues for inducing bone growth as well as manipulating jaw growth at will. A very successful example for manipulating bone growth in response to mechanical strain is growth modification of the jaw. When the mandible is moved forward, with the aid of jaw-protrusion devices, a change in the biophysical environment leads to significant jaw growth (Chayanupatkul *et al.*, 2003). The significant bone increase in the mandibular condyle in response to external mechanical forces was the result of a host of orchestrated influences of various growth factors and other regulatory factors that are endogenously expressed by cells in the mandibular condyle (Rabie and Hagg, 2002; Rabie *et al.*, 2002, 2003a,c; Tang *et al.*, 2004).

The mechanotransduction pathway through which the cells receive and convert mechanical signals into tissue growth is still being elucidated. Changing the biophysical environment of the temporomandibular joint by stretching the condylar attachment in response to moving the mandible forward has been shown to have an impact on mesenchymal cell proliferation (Petrovic *et al.*, 1975; Rabie *et al.*, 2003b), proteoglycan synthesis (Kantomaa *et al.*, 1994), collagen formation (Kantomaa and Pirttiniemi, 1996; Rabie *et al.*, 2003a), and the expression of osteonectin and cyclic nucleotides (Kantomaa and Hall, 1991; Haas and Holick, 1996). However, the key question in the field of growth modification of the mandibular condyle remains unanswered: How could such a mechanical stimulation, as a result of mandibular advancement, further regulate the endochondral bone formation in the condyle?

Runx2, also designated Cbfa1, is a transcription factor that belongs to the Runt-domain gene family (Ogawa *et al.*, 1993; Ducy *et al.*, 1997). Accumulated data documented that Runx2 is a prerequisite for osteoblast differentiation and function by transcriptionally up-regulating all the major osteoblast-specific genes, such as osteocalcin, type I collagen, bone sialoprotein, osteopontin, alkaline phosphatase, and collagenase-3 (Ducy *et al.*, 1997, 1999; Harada *et al.*, 1999; Jimenez *et al.*, 1999; Karsenty *et al.*, 1999). Runx2 also regulates chondrocyte maturation and terminal differentiation during skeletogenesis (Inada *et al.*, 1999; Enomoto *et al.*, 2000; Takeda *et al.*, 2001). Recently, the expression of Runx2 has been identified in the mandibular condyle (Kuboki *et al.*, 2003; Rabie *et al.*, 2004). Beyond its function in embryonic development, Runx2 couples the processes of chondrocyte maturation, extracellular matrix materialization and degradation, as well as osteoblast invasion, during the post-natal growth of the mandibular condyle (Rabie *et al.*, 2004). It has been demonstrated

that Runx2 is a molecular linker of mechanical signals and osteoblast differentiation (Ziros *et al.*, 2002). To test the hypothesis that Runx2 is responsible for signaling chondrocyte maturation and endochondral ossification in the condyle during mandibular advancement, we designed this study to investigate the temporal and spatial patterns of the expression of Runx2 in condylar cartilage in response to mandibular advancement, and to correlate Runx2 expression with the pattern of expression of type X collagen, the recruitment of chondroclasts and osteoblasts, and the amount of new bone formation in the condyle.

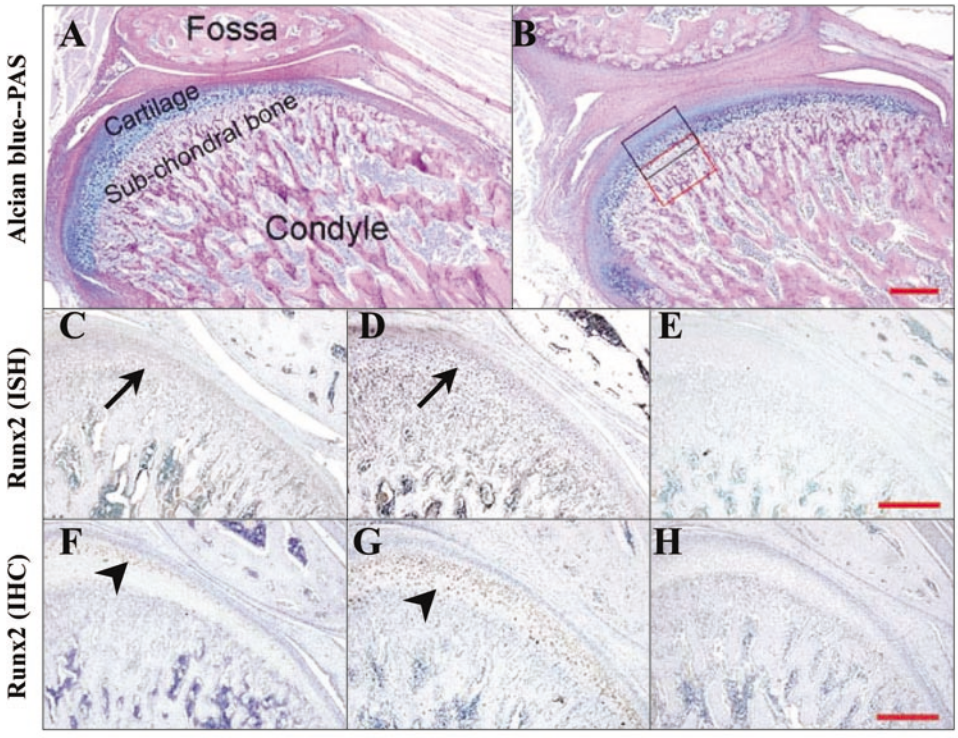

Figure 1. Alcian blue and PAS staining showed an overview of the rat's temporomandibular joint during natural growth (**A**: 42 days old) and during mandibular advancement (**B**: 7 days after the experiment; note the forward positioning of the condyle). Two measurement frames that overlapped at the erosive front in the posterior condyle were used for quantitative analysis (B). *In situ* hybridization (ISH, **C** and **D**, arrows) and immunohistochemical staining (IHC, **F** and **G**, arrowheads) showed Runx2 expression in the rat's condylar cartilage during natural growth (C and F: 49 days old) and mandibular advancement (D and G: 14 days after the experiment). *In situ* hybridization with sense probe **(E)** and immunostaining omitting primary antibody **(H)** served as negative control. Scale bars: 100 μm.

MATERIALS & METHODS

Experimental Animals and BrdU Labeling

One hundred female Sprague-Dawley rats at 35 days of age were randomly allotted to 5 control and 5 experimental groups (n = 10). 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 study the cell kinetics of the skeletal progenitors, we labeled animals with bromodeoxyuridine (BrdU, Sigma, St. Louis, MO, USA) by intraperitoneal injection. Half of the rats in each group received the drug 1 hr before death, at a dosage of 2.5 mg/100 g body weight. The other half of the rats were labeled with BrdU 3 days before death, at a dosage of 5 mg/100 g. The injections were given at the same time of day (9:00-11:00 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.

Histochemical Staining and *in situ* Hybridization

Condyles were harvested, and paraffin sections were cut mid-sagittally. We used PAS (periodic acid and Schiff's reagent) staining and Alcian blue-PAS staining to identify the new bone formation and the histological structure of the condyle (Rabie and Hagg, 2002; Rabie *et al.*, 2003c). Chondroclasts with the characteristics of TRAP (tartrate-resistant acid phosphatase) activities were identified with the Leukocyte Acid Phosphatase Kit (Sigma, St. Louis, MO, USA) (Rabie *et al.*, 2004).

In situ hybridization and immunohistochemical staining were carried out as described in detail previously (Rabie *et al.*, 2004). The sense and antisense digoxigenin 11 UTP-labeled RNA probes were transcribed from a 640-base-pair fragment in exon 8 of mouse Runx2 (Rabie *et al.*, 2004). The primary antibody for Runx2 was a polyclonal antiserum raised against a peptide mapping at the carboxyl terminus of human Runx2 (Santa Cruz Bio. Inc., Santa Cruz, CA, USA). This antibody was found to react with Runx2 isoform II of mouse, rat, and human origin (Rabie *et al.*, 2004). The primary antibody for type X collagen was a monoclonal mouse IgG (Quartett, Berlin, Germany). To visualize the BrdU labeled cells, we used a monoclonal anti-BrdU antibody (Sigma, St. Louis, MO, USA). To ascertain the specificity of the immuonostaining, we included negative controls in which the primary antibodies were replaced by non-immune serum.

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 applied for quantitative analysis (Rabie *et al.*, 2003c). Measurements were carried out in the superior-posterior region of the condyle, where the cartilage layer runs parallel to the articular surface, and appeared to be uniform among animals. Two measurement frames of 550 x 400 μm were applied, with one in the cartilage layer and the other in the subchondral bone layer (Fig. 1). Images were captured inside the frame with a magnification of 360X.

The numbers of cells with positive immunostaining of Runx2 and BrdU (more than 80 pixels) were counted by the computer in the upper and lower measurement frames, respectively. The depth of the hypertrophic layer stained with type X collagen was determined as the mean of the measurements

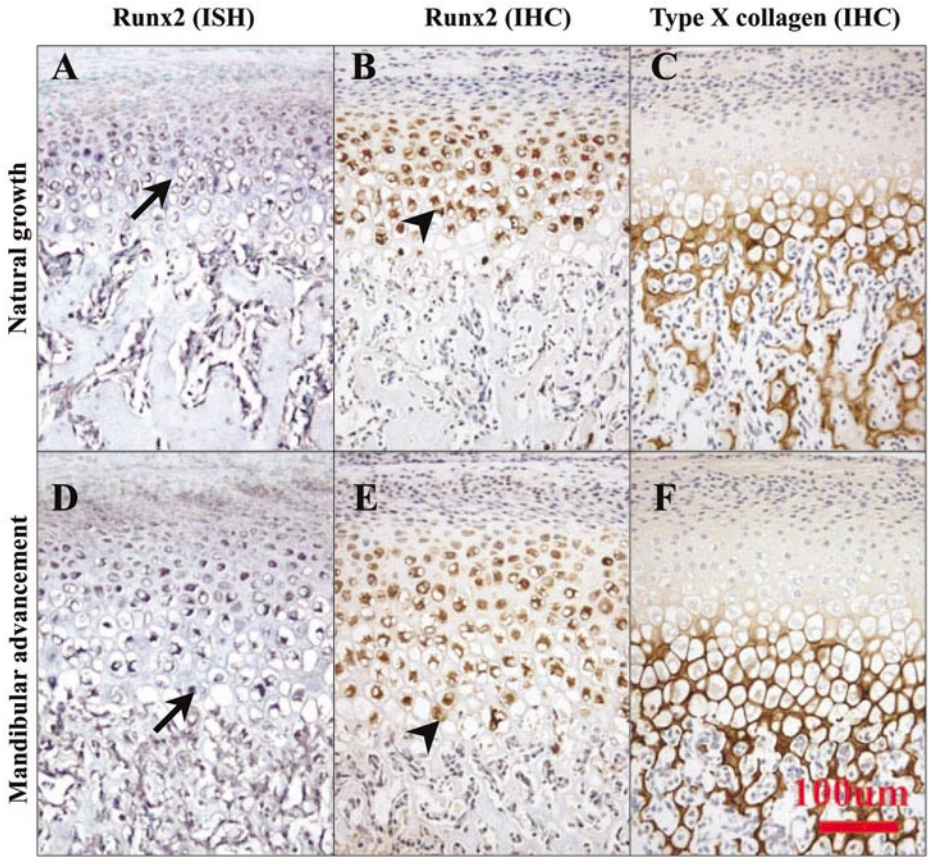

Figure 2. High magnification showed the expression of Runx2 (**A** and **D**, *in situ* hybridization, arrows; **B** and **E**, immunohistochemical staining, arrowheads) and type X collagen (**C** and **F**: immunohistochemical staining) in the rat's condylar cartilage during natural growth (A,B,C: 56 days old) and during mandibular advancement (D,E,F: 21 days after the experiment).

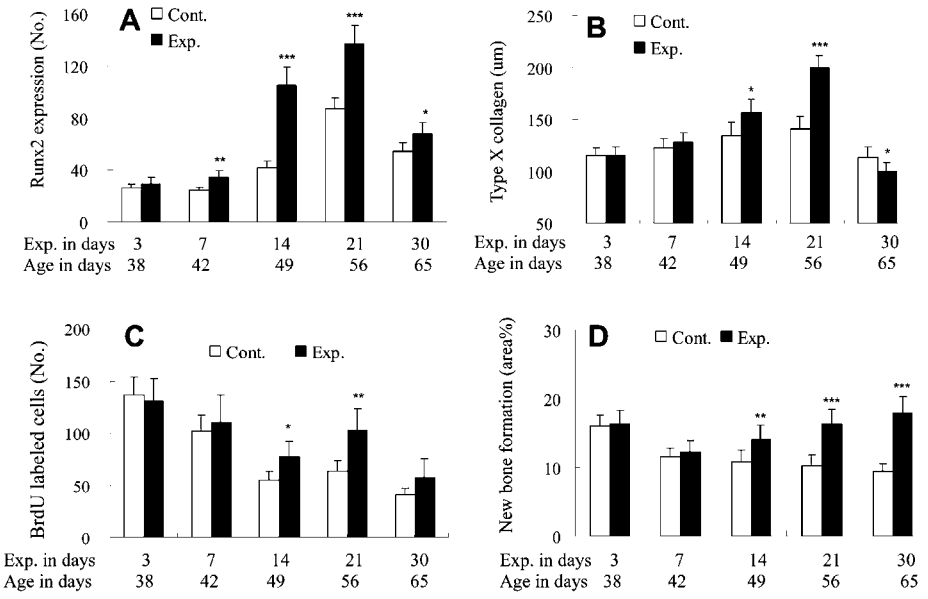

Figure 3. The temporal pattern of the expression of Runx2 (**A**, n = 10), type X collagen (**B**, n = 10), BrdU-labeled cells in the erosive zone (**C**, n = 5), and the amount of new bone formation (**D**, n = 10) in the mandibular condyle during natural growth (Cont.) and during mandibular advancement (Exp.). Values were mean ± SD. Significant difference between control and experimental animals was marked with asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

at 3 equally divided sites in the upper frame. The amount of new bone formation was quantified as the percentage of the positive-PAS-staining areas in the lower frame (Rabie *et al.*, 2001). The data were collected again 4 wks later by the same observer. The difference between experimental and control groups at each time point was tested by unpaired *t* test with GraphPad InStat (Version 3.00, GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Upon mandibular advancement, stronger expression of Runx2 mRNAs was observed in condylar cartilage and the subchondral bone (Figs. 1A-1D, 2A, 2D). Mechanical loading also elevated Runx2 protein synthesis, especially in hypertrophic chondrocytes (Figs. 1F, 1G, 2B, 2E). Quantitative analysis demonstrated significantly higher levels of Runx2 expression in condylar cartilage in response to mandibular advancement (Fig. 3A). Type X collagen was limited to the hypertrophic layer and to the cartilage islands in the subchondral bone (Figs. 2C, 2F). Significant expansion of the type X collagen domain was recorded on days 14 and 21 during mandibular advancement (Figs. 2C, 2F, 3B). At the end of the experiment on day 30, however, type X collagen staining dropped below the control level (Fig. 3B).

When BrdU was injected 1 hr before death, only bone marrow cells and the chondroclasts were labeled (Figs. 4A, 4C). Osteoblasts lining the trabecular bone lacked positive staining (Fig. 4A). Alternatively, when BrdU was administered three days before death, the osteoblasts and the progenitor cells approaching the bone surface were labeled (Fig. 4B). Chondroclasts with strong TRAP activity remained positively stained (Figs. 4C, 4D). Thus, the three-day BrdU-labeled cells detected in the erosive zone of the cartilage were skeletal cells, either osteoblasts or chondroclasts (Figs. 4E, 4F). Quantitative measurements showed an increase in the number of labeled skeletal cells on days 14 and 21 during mandibular advancement (Fig. 3C).

DISCUSSION

The mandibular condyle undergoes endochondral bone formation, where it necessitates tight control of chondrogenesis, cartilage degradation, blood vessel invasion, and osteoblast recruitment (Rabie and Hagg, 2002). In the present study, we showed that the transcription factor Runx2 is in charge of regulating these processes during growth modification of the condyle induced by forward mandibular positioning.

Runx2 has been well-documented to regulate chondrocyte hypertrophy during chondrogenesis in long bones. Runx2-deficient mice showed impaired chondrocyte differentiation with no hypertrophic chondrocytes (Inada *et al.*, 1999). Overexpression of Runx2 caused acceleration of endochondral ossification due to precocious chondrocyte maturation (Takeda *et al.*, 2001). An *in vitro* study suggested that Runx2 is a target of mechanical signals (Ziros *et al.*, 2002). In the mandibular condyle, Runx2 was expressed in pre-hypertrophic and hypertrophic chondrocytes during natural growth (Rabie *et al.*, 2004). Forward mandibular positioning causes pull in the posterior condyle, which creates a strain alignment of the mesenchymal cells and other cells in the extracellular matrix of condylar cartilage (Rabie *et al.*, 2001; Tang *et al.*, 2004). Analysis of our present data demonstrated that stretching the condylar tissues enhanced Runx2 expression in condylar cartilage, on both transcriptional and translational levels (Figs. 1, 2). To reveal if the induced Runx2 expression promoted chondrocyte hypertrophy in condylar cartilage, we evaluated type X collagen expression. Expression of type X collagen is the trait of hypertrophic chondrocytes, and it precedes the onset of endochondral ossification in the mandibular condyle (Rabie and Hagg, 2002). In the present study, an expansion of type X collagen domain in the hypertrophic layer was identified on days 14 and 21 in response to mechanical strain (Figs. 2C, 2F, 3B). It was important to note that the increase of Runx2 expression was first identified on day 7 (Fig. 3A), which preceded that of the type X collagen (Fig. 3B). Moreover, the increase of Runx2 expression under mechanical strain was most evident on day 14 (152%), which preceded the maximum increase of type X collagen expression on day 21 (56%) (Figs. 3A, 3B). It was suggested that the expression of type X collagen is not a direct target of Runx2, but rather is secondary to the appearance of hypertrophic chondrocytes regulated by Runx2 (Enomoto *et al.*, 2000). Thus, mandibular advancement elevated Runx2 expression in condylar cartilage. Runx2 subsequently promoted chondrocyte hypertrophy and terminal maturation, leading to an increase in the synthesis of type X collagen and an expansion of the cartilage template.

The conversion of cartilage to bone requires a precise coordination of the activities of chondroclasts and osteoblasts to remove the cartilage and to deposit the osteoid. Runx2 was first identified as a crucial transcription factor for osteoblast differentiation (Ducy *et al.*, 1997, 1999; Harada *et al.*, 1999; Jimenez *et al.*, 1999; Karsenty *et al.*, 1999). Under mechanical stress, more Runx2 mRNA expression was identified in the subchondral bone area of the mandibular condyle (Figs. 1D,

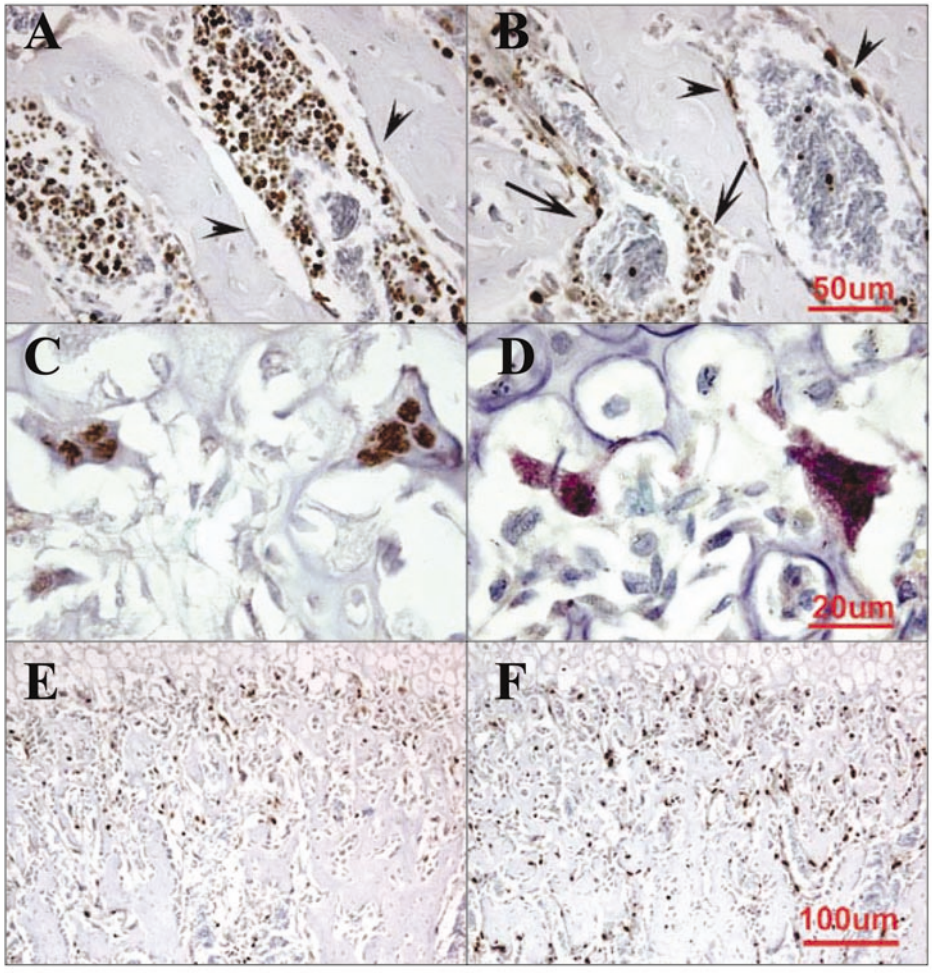

Figure 4. BrdU labeling in 56-day-old rats showed the differentiation of osteoblasts. When BrdU was administered 1 hr before death **(A)**, the labeled cells were accumulated in the center of the blood vessels. The disc-shaped osteoblasts lining the subchondral bone surface were not labeled (A, arrowheads). When BrdU was administered 3 days before death **(B)**, almost all the labeled cells in the marrow cavity were skeletal progenitor cells migrating toward the cavity wall (B, arrows). The differentiated osteoblasts on the bone surface were labeled (B, arrowheads). The labeled cells in the erosive front were multinuclear chondroclasts **(C)** with strong TRAP activities **(D)**. Three-day BrdU labeling showed more osteoblasts and chondroclasts in the erosive zone during mandibular advancement (**F**: 14 days after the experiment) than during natural growth (E: 49 days old).

1G). To find out if osteoblast transportation was also increased, we counted the BrdU-labeled cells in the erosive front of the condylar cartilage (Figs. 4E, 4F). The TRAP-positive chondroclasts adjacent to eroding calcified cartilage were constantly labeled from 1 hr to 3 days after BrdU administration (Figs. 4C, 4D). The differentiated osteoblastic cells, however, were observed only 3 days after the labeling (Fig. 4B). This was in line with earlier reports that the formation of chondroclasts could be detected as early as 5 min after [^{3}H]thymidine administration (Heeley *et al.*, 1983), while the proliferation and differentiation of precursors into osteoblasts required 72 hrs (Turner *et al.*, 1998). Analysis of our data showed a decrease in the recruitment of osteoblasts and chondroclasts in the condyle during natural growth (Fig. 3C). This could explain the decrease in the amount of new bone formation in the condyle with age (Fig. 3D). Mandibular advancement, however, significantly increased the formation of these cells on days 14 and 21, which followed the elevation of Runx2 expression first identified on day 7 (Figs. 3A, 3C). This suggested that, in the condyle, Runx2 might play a role in osteoblast differentiation and function similar to that demonstrated in long bones. Large increases in osteoblastic and chondroblastic invasion on day 21 might account for the decrease in type X collagen domain as it is being replaced by bone, and therefore a significant increase in new bone formation on day 30 is noted (Figs. 3B, 3D).

Runx2 has been stated to up-regulate VEGF (vascular endothelial growth factor) expression (Zelzer *et al.*, 2001). Thus, analysis of the present data further supports our previous findings that mandibular forward positioning solicits an increase in the expression of VEGF, the key regulator of neovascularization (Rabie *et al.*, 2002). The significant increase in the invasion of new blood vessels, with their perivascular sites rich in mesenchymal cells, thus contributes to a significant increase in the number of skeletal progenitors in the erosive zone. The increase in Runx2 expression triggered by mechanical stimulation could induce mesenchymal cell differentiation into osteoblasts, while the hematopoietic cells differentiated into chondroclasts. The increase in the recruitment of osteoblasts and chondroclasts thus directly contributed to the removal of cartilage and the deposition of bony tissues, leading to enhanced endochondral ossification in the condyle.

Elucidating the signaling pathway that regulates the growth modification of the mandibular condyle under biomechanical stimulation is essential for the practicing clinician to be able to base his/her clinical treatment modalities on sound scientific concepts. Our present findings reiterated the importance of Runx2 during endochondral bone formation in mandibular condyles. Mandibular advancement elicited Runx2 expression, which up-regulated the expression of VEGF and elevated type X collagen expression. The cooperation of these factors promoted chondrocyte terminal maturation and also induced chondroclast invasion and osteoblast formation, resulting in more new bone formation in the condyle in response to forward mandibular positioning.

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