

## A histochemical study on condylar cartilage and glenoid fossa during mandibular advancement

Payam Owtad<sup>a</sup>; Zoe Potres<sup>a</sup>; Gang Shen<sup>b</sup>; Peter Petocz<sup>b,d</sup>; M. Ali Darendeliler<sup>c</sup>

### ABSTRACT

**Objective:** To evaluate cellular hypertrophic activities in the mandibular condylar cartilage (MCC) and the glenoid fossa (GF) during mandibular advancement in the temporomandibular joint (TMJ) of Sprague-Dawley rats, as evidenced by fibroblast growth factor 8 (FGF8).

**Methods and Materials:** Fifty-five female 24-day-old Sprague-Dawley rats were randomly divided into four experimental and control groups, with a mandibular advancement appliance on the experimental rats' lower incisors. The rats were euthanized on days 3, 14, 21, and 30 of the study, and their TMJ was prepared for a immunohistochemical staining procedure to detect FGF8.

**Results:** FGF8 expression was significantly higher among the experimental rats ( $P = .002$ ). Patterns of ascension and descension of FGF8 expression were similar in experimental and control samples. The results show an overall enhanced osteogenic transition occurring in both the MCC and the GF in experimental rats in comparison with controls. The level of cellular changes in the MCC is remarkably higher than in the GF.

**Conclusion:** In the MCC and the GF, cellular morphologic and hypertrophic differentiations increase significantly during mandibular advancement. It is also concluded that endochondral ossification in the MCC and intramembranous ossification in the GF occur during adaptive remodeling. (*Angle Orthod.* 2011;81:270–276.)

**KEY WORDS:** Mandibular condylar cartilage; Glenoid fossa; FGF8; Adaptive bone remodeling; Mandibular advancement

### INTRODUCTION

Several studies have discussed mandibular advancement as a functional therapy for skeletal Class II malocclusion<sup>1</sup> and have shown that a fundamental factor in regulating cellular activities during tissue

morphogenesis is mechanical stress. Forward positioning of the mandible is followed by adaptive remodeling in the mandibular condylar cartilage (MCC) and the glenoid fossa (GF).<sup>2–6</sup> Growth modification of the lower jaw during mandibular forward positioning is a successful example of bone remodeling in response to a change in the biophysical environment.<sup>1</sup> Many studies with rats and monkeys have shown that new bone formation in the condyle and the GF occurs in response to mandibular advancement.<sup>6–8</sup>

This remodeling occurs by expression of endogenous regulatory factors of cells in the mandibular condyle through an endochondral ossification process<sup>1–3</sup> and intramembranous ossification in the GF.<sup>4,6,7</sup>

The population size of the mesenchymal cells present in the subperiosteal connective tissues of the MCC directly affects the number of bone-making cells available to engage in the formation of new bone during craniofacial development. Undifferentiated mesenchymal cells in the extracellular matrices (ECMs) give rise to other cell types as the need arises and are present in the ECM of developing bones in the skull, including the temporal bone.<sup>9</sup>

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**Table 1.** Experimental Design

Experiment Day 0	Rats' Age, Days <sup>a</sup>	Study Criteria		
	24 (no sacrifice)	Entry to the Experiment, Random Grouping, and Laboratory Initial Settlements		
	Age at the Day of Sacrifice	Experimental Group (35 rats)	Control Group (15 rats)	Total Sacrificed Samples (55 rats)
3	27	10 rats	5 rats	15 rats
14	38	10 rats	5 rats	15 rats
21	45	10 rats	5 rats	15 rats
30	54	5 rats	5 rats	10 rats

<sup>a</sup> Female Sprague-Dawley rats were collected for the study, as approved by the Westmead Animal Ethics Committee.

The fibroblast growth factors (FGFs) regulate mesenchyme and chondrocyte proliferation in MCC adaptive remodeling.<sup>10</sup> FGF signaling results in a decrease in chondrocyte proliferation and an acceleration of hypertrophic differentiation and morphologic changes in chondrocytes.<sup>5</sup> FGF8 is expressed in highly proliferating, columnar chondrocytes and in early hypertrophic and hypertrophic chondrocytes.<sup>11</sup>

The aim of this study is to evaluate hypertrophic activities in the MCC and the GF during mandibular advancement in Sprague-Dawley rats, as evidenced by FGF8. In this study, FGF8 is used as an indicator of cellular chondrogenic and morphologic differentiation and hypertrophic activity, to demonstrate the histochemical nature of the adaptive response of bone to mandibular protrusion.

**MATERIALS AND METHODS**

Fifty-five female Sprague-Dawley rats at the age of 24 days were randomly divided into the experimental group (n = 35) and the control group (n = 20); the study was approved by the Westmead Animal Ethical Committee (Protocol No: 4113.06-08). All rats were kept in the same well-controlled temperature and humidity environment. They were fed a soft palate diet and had uninhibited access to water 24 hours a day throughout the entire experimental period (Table 1).

The animals were sedated and a crown former was positioned on their lower anterior incisors in such a way that it caused mandibular forward-downward positioning during the rats' rest and functional bite (Figure 1B). Animals in the control groups were not fitted with an appliance and were untreated (Figure 1A). Body weight was monitored throughout the experiment.

**Euthanasia and Tissue Preparation**

The animals in each subgroup were euthanized, respectively, by carbon dioxide gas (Aligal 2, Air Liquide Australia Ltd, Fairfield NSW, Australia) on the 27th, 38th, 45th, and 54th days of the rats' age.

Immediately after death, the heads were removed and were fixed in 4% paraformaldehyde for 24 hours. The heads were then decalcified in 20% ethylenediaminetetraacetic acid (EDTA), pH 7 to 7.4, at 4°C to 8°C, for 4 to 6 weeks.

The temporomandibular joint (TMJ) was dissected, and surrounding soft tissues were removed until the TMJ was exposed. Excess tissues were removed, and specimens with the buccal surface of the ramus parallel to the surface of the block were embedded in paraffin. Serial sections 5 µ thick were cut through the TMJ at the parasagittal plane using a rotary microtome (Leitz 1516, Leica Microsystems, Wetzlar, Germany); sectioning was continued until the approximate middle of the condyle was reached. At this level, a few sections were floated onto glass slides coated with poly-L-lysine.

Individual variations occurred in TMJ orientation in the skull. Thus, to make a reliable comparison, the plane of each section throughout each of these anatomic variables was adjusted as identically as possible between samples. Sections cut from each sample were assigned to immunohistochemical staining for fibroblast growth factor 8 (FGF8).

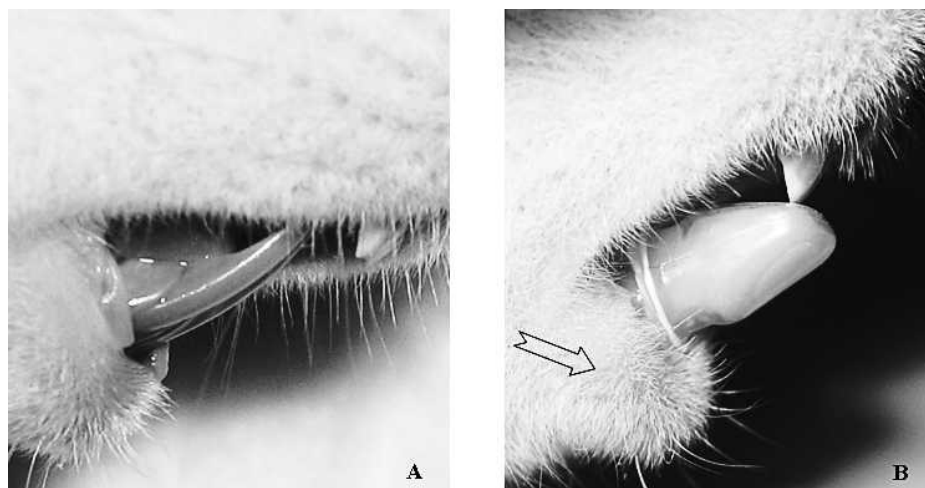
**Immunohistochemical Examinations**

The specific primary antibody used was FGF8 goat polyclonal antibody collagen (N-19, Cat #SC 6958, Lot #E300, 200 µg/mL; Santa Cruz Biotechnology Inc, Santa Cruz, Calif). The secondary antibody was rabbit antigoat immunoglobulin (Ig)G (HRP, Code No. P0449; Dako A/S, Glostrup, Denmark).

Immunohistochemistry was carried out using a method in which the sections were dewaxed and rehydrated and were treated with glycine, 3% hydrogen peroxide, horse serum, the primary antibody, and the secondary antibody.

Then the slides were dipped in 3,3'-diaminobenzidine (DAB) in chromogen solution (Dako Liquid DAB + Substrate Chromogen System, Code K3467, Dako A/S) and finally were counterstained with Mayer's hematoxylin for background staining. Negative controls were included, in which the primary antibody was

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**Figure 1.** Bite jumping appliance. Normal incisal relationship in control rats. The bite jumping appliance is adjusted on the lower incisors of experimental rats to move their mandible into a forward position during rest and function.

replaced by FGF8-blocking peptide (N-19 P, Cat #SC 6958 P, Lot #F268, 100  $\mu$ g/0.5 mL, Santa Cruz Biotechnology) to ascertain the specificity of the immunostaining.

After the clearing protocol, slides were covered by mounting medium (Fisher Scientific Permount SO-P-15, 500 mL, 1.1 pt; Fisher Scientific, Fair Lawn, NJ) and a coverslip for long-term storage of slides and additional microscopic studies.

### Quantitative Imaging

Digital images were taken from stained tissues with a Leica digital imaging microscope and its software (Leica Application Suite Software; Leica Microsystems, Bannockburn, Ill) at 10 $\times$ , 20 $\times$ , and 60 $\times$  magnification.

Expressions of FGF8 were quantified by manually counting the cells of positive reacted immunostaining signals on the computer screen from 20 $\times$  magnified images. Cells were counted from the middle quarter of the MCC and the distal third of the GF, where the most prominent cellular responses to mandibular repositioning occur.<sup>2,12</sup> Cells that were stained with certain intensity were counted, and those that were weakly stained were excluded.

### Statistical Analysis

After the first counting, data were collected again 4 weeks later by the same observer, and the method of error (ME) was tested. No statistically significant difference was noted among the registrations. Data were analyzed using a statistical package (Statistical Package for the Social Sciences [SPSS] for Windows, version 16.0, SPSS Inc, Chicago, Ill).

### RESULTS

The cytoplasm of early hypertrophic and hypertrophic cells, beneath the layer of cell proliferation and above the erosive zone, were positively stained for FGF8 (Figure 2D,G). FGF8 is located mainly in the cytoplasm of osteochondroprogenitor cells, chondroblasts, and chondrocytes before their degeneration, which is shown by extra magnification of a typical immunopositive cell for FGF8 from the hypertrophic layer of the mandibular condylar cartilage of a 38-day-old experimental sample (Figure 2).

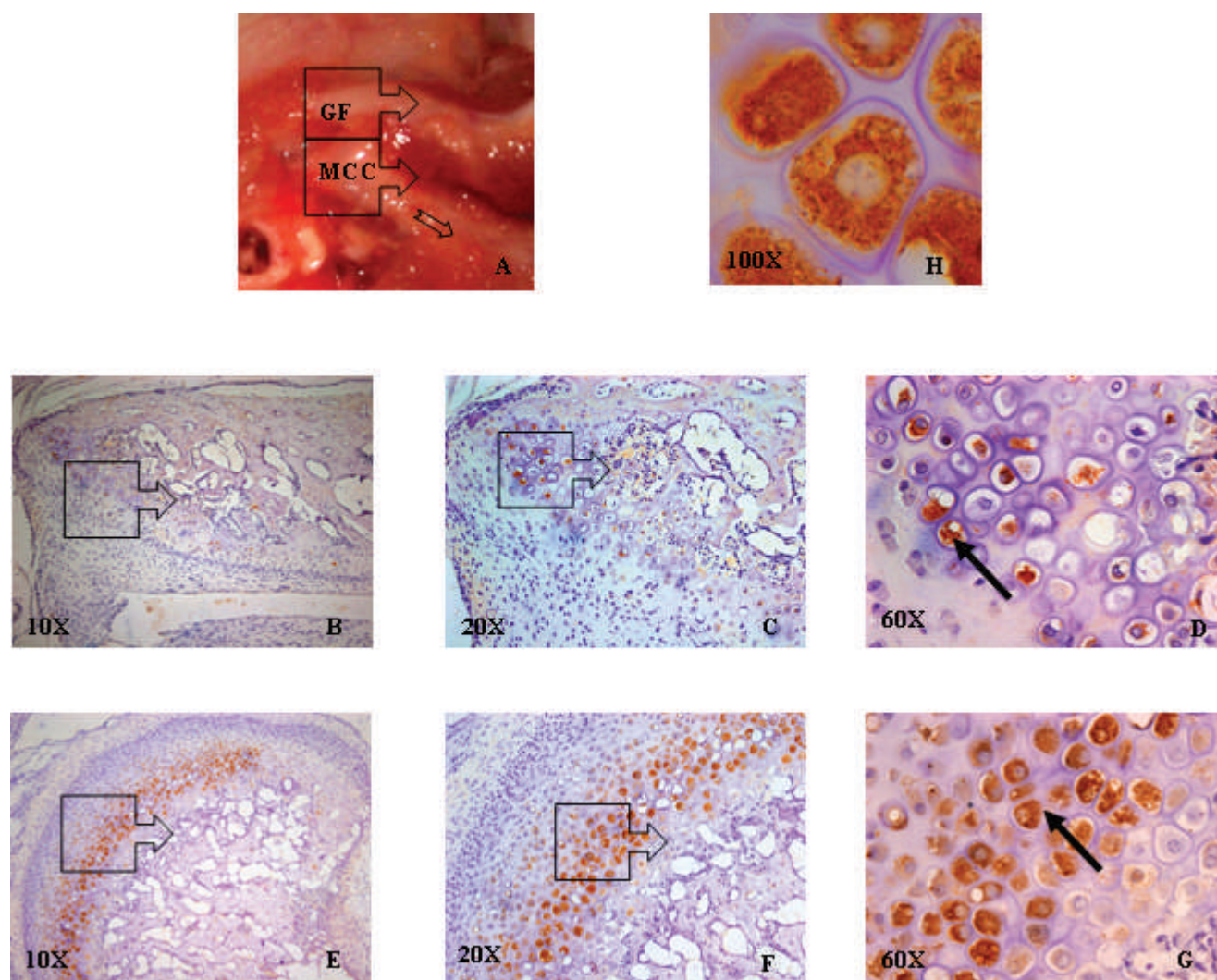
The level of FGF8 expression in the condyle and glenoid fossa of experimental rats generally is significantly higher than in relevant control samples. Furthermore, it is clear that the amount of cellular activity in the mandibular condylar cartilage is greatly higher than in the glenoid fossa in both controls and experimental rats at different stages of growth and development. The effect of the appliance on FGF8 expression is generally significant in the MCC and the GF (FGF8c,  $P = .002$ ; FGF8gf,  $P = .002$ ). The level and pattern of FGF8 expression are shown in Table 2 and Figure 3.

### DISCUSSION

The histologic structures of the rats' TMJ are similar to those of humans, with morphologic differences.<sup>13</sup> Because of this similarity and the possibility of a histochemical study on rats based on previous studies,<sup>14,15</sup> 55 Sprague-Dawley rats were used in this experiment, as in other histologic and biochemical investigations.<sup>3,4,8,12-14,16,17</sup>

Even though some studies on monkeys indicate that such adaptive responses are nonexistent and negligi-





**Figure 2.** FGF8 in the MCC and the GF. Photograph of a female Sprague-Dawley rat's TMJ (A) shows the anatomic relationship of the condyle (A-MCC) and the articular fossa (A-GF), and the arrow (A) shows the direction of forward-downward displacement of the condyle during mandibular advancement. Photomicrographs show immunostaining for FGF8 expressed in the glenoid fossa of another experimental sample (27-day-old rat, wearing bite jumping appliance for 3 days) (B, C, and D) and the mandibular condylar cartilage of an experimental sample (38-day-old rat, wearing bite jumping appliance for 14 days) (E, F, and G).

ble,<sup>18</sup> several other findings, such as those of the current study, indicate positive significant TMJ adaptation in response to mandibular advancement.<sup>2,3,16,19-22</sup> However, the pattern of this adaptive response in the MCC is different from that in the GF.

The adaptive response of the MCC-GF complex could be described by the growth relativity hypothesis and the functional matrix theory. The mandible is displaced to a forward position, viscoelastic forces are applied on the MCC-GF complex at the same time in reverse directions, and the forces are transduced by being radiated beneath the articular layers of both the MCC and the GF.<sup>23-25</sup>

The role of the MCC in the process of growth and development of the TMJ and its adaptive response to

mandibular advancement are remarkably greater, particularly during the period of the present study.

This lower amount of activity in the GF might show that adaptation of the whole TMJ occurs mainly by ossification and relocation of the MCC and relocation of the GF, as a harmonized biologic response to mandibular protrusion. Otherwise, it is possible that the GF is not significantly relocated from its initial position, which could be a reason for future relapses of successful functional mandibular treatment. If the GF does not remarkably remodel or relocate, then the soft tissue attachments pull the condyle back to its initial relationship with the GF. For clearer and more detailed information in this regard, these possibilities should be precisely studied and evaluated by a combination of

**Table 2.** FGF8c and FGF8gf: Experimental Rats vs Controls on Different Experiment Days<sup>a</sup>

	Group <sup>b,c</sup>	Day 3	Day 14	Day 21	Day 30
Condylar cartilage	Exp <sup>d</sup>	21.676 <sup>b</sup> ± 3.798	72.719 <sup>b</sup> ± 1.494	52.209 <sup>b</sup> ± 1.738	25.203 <sup>b</sup> ± 2.686
	95% CI	14.017, 29.336	69.706, 75.732	48.705, 55.713	19.786, 30.619
	Cont	16.182 <sup>b</sup> ± 3.521	61.337 <sup>b</sup> ± 2.456	44.012 <sup>b</sup> ± 2.737	26.951 <sup>b</sup> ± 4.400
	95% CI	9.081, 23.283	56.384, 66.290	38.494, 49.531	18.078, 35.823
Glenoid fossa	Exp <sup>c</sup>	17.503 <sup>b</sup> ± 1.526	2.903 <sup>b</sup> ± 0.607	0.348 <sup>b</sup> ± 0.749	0.626 <sup>b</sup> ± 1.098
	95% CI	14.424, 20.583	1.678, 4.128	-1.162, 1.859	-1.590, 2.842
	Cont	10.758 <sup>b</sup> ± 1.405	1.010 <sup>b</sup> ± 0.889	0.278 <sup>b</sup> ± 1.207	0.308 <sup>b</sup> ± 1.803
	95% CI	7.923, 13.592	-0.785, 2.804	-2.157, 2.713	-3.330, 3.945

<sup>a</sup> Quantitative analysis of FGF8 expression in condylar cartilage (FGF8c) and glenoid fossa (FGF8gf). The number of FGF8-immunopositive cells is considered for calculating the values and for performing statistical analyses of experimental samples vs controls on different experiment days. This indicates the effect of the bite jumping appliance on the level of FGF8 expression at different stages of rat growth.

<sup>b</sup> Data were presented as M ± SE (standard error) (No.). CI: confidence interval—lower, upper.

<sup>c</sup> Covariates appearing in the model were evaluated at the following values: wts = 148.077 (c) and 147.824 (gf).

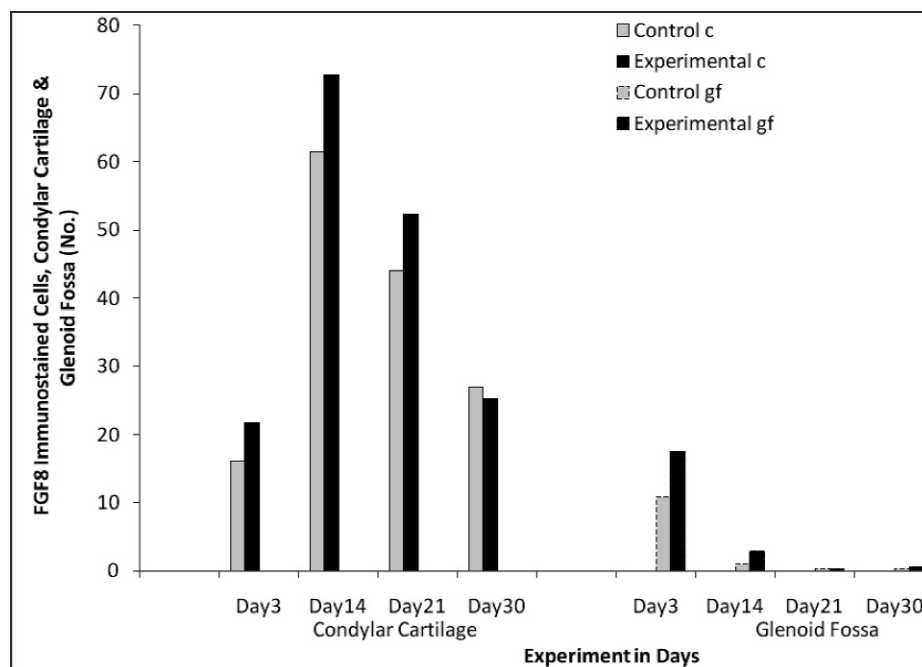
<sup>d</sup> *P* = .002. The effect of the appliance was statistically significant for FGF8c and FGF8gf.

histochemical, cephalometric, and electromyographic methods over a longer period of time.

Signaling molecules of the FGF family regulate endochondral ossification at several levels<sup>26</sup>; therefore results of the current study are consistent with the fact that the mandibular condyle is a growth site and is ossified through endochondral ossification.<sup>22</sup> However, endochondral ossification in the GF is observed only during initial stages of rats' growth (27 days of rats' age), as indicated by detection of FGF8 at that age and not after that age. This could indicate that osteogenesis generally slows down in rats' GF at this age, or

that intramembranous ossification is the dominant ossification process in the GF afterward, and no cartilage tissue is found in the GF during later growth and development. Intramembranous ossification in the GF is similarly reported in other studies.<sup>27</sup>

Immunopositive cells for FGF8 molecules are detected in the cytoplasm of chondrogenic cells in the deep columnar proliferative layer and in early hypertrophic and hypertrophic zones.<sup>11,26</sup> The location of FGF8 expression is consistent with other reports, which suggests that FGF8 could be known as an indicator for osteogenesis through an endochondral



**Figure 3.** FGF8c and FGFgf: diagram for experimental rats vs controls on different experiment days. In this diagram, the means of the numbers of FGF8-immunopositive cells in the mandibular condylar cartilage (FGF8c) and in the glenoid fossa (FGF8gf) are compared in experimental and control rats on different experiment days.

ossification process by patterning and regulating chondrocytes' proliferation and their hypertrophic morphologic differentiation.<sup>11,28,29</sup>

This indicates that FGF8 is more involved in hypertrophic activities than in chondrocyte proliferation. FGF8 plays a role in cellular chondrogenic differentiation and in creating morphologic changes from mesenchymal cells to chondroblasts and from chondroblasts to bone-making cells. This has also been reported by Minina et al.<sup>28</sup> On the molecular level, FGF signaling reduces chondrocyte proliferation and induces hypertrophic differentiation of chondrocytes.<sup>28</sup>

The higher amount of FGF8 expression in experimental samples, in comparison with control samples, generally shows enhanced osteogenic transition occurring in both the MCC and the GF, with the exception of FGF8 excretion in the MCC on experiment day 30, during which the level of expression in control animals was slightly, although not significantly, higher than in the experimental rats. Additional long-term studies are required to find out the reason for the lower molecular and cellular activities in the GF than in the MCC.

Evaluating the expression of FGF8 in experimental rats versus controls suggests that mandibular advancement does not change the pattern of molecular activity, but just increases the level of activity. The pattern of FGF8 expression follows the pattern of normal growth and development of the TMJ, in reference to controls.

The FGF8 pick of expression is at approximately the same level in experimental and control rats. Considering this evidence and the fact that FGF8 in the condyle is slightly higher in controls on day 30, it is possible that stepwise advancement may generate more changes in the MCC-GF complex than one-step advancement. This is similarly suggested by other researchers.<sup>16,17</sup>

The future direction would be to design and perform studies covering longer periods of the rats' lives and comparing the MCC and the GF with other growth centers and growth sites on each sample, such as epiphyseal plates, synchondroses (eg, spheno-occipital synchondrosis), maxillary sutures (eg, intermaxillary suture), cranial sutures, and mandibular symphyses at earlier stages of growth. This could be done with cephalometric evaluations, in addition to histochemical evaluations, performed during the period of the experiment to measure the level and direction of growth and development.

## CONCLUSION

- Structural and molecular adaptations occurred in the MCC and the GF of the experimental animals. Hypertrophic differentiations were significantly in-

creased in both parts, which could enhance bone formation during the adaptive response. Therefore, mandibular growth modification takes place as an end result of extracellular morphologic differentiations and hypertrophic changes in the both the MCC and the GF.

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