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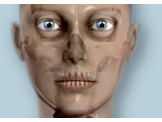

ORIGINAL ARTICLE

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Histological study of the nasal septal cartilage in BALB/c-*bm/bm* mouse which spontaneously induces malocclusion

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Structured Abstract

Objectives – The BALB/c-*bm/bm* mouse is characterized by short limbs and short tail attributed to undersulfated glycosaminoglycans. Anterior transverse crossbite sometimes spontaneously appears in BALB/c-*bm/bm* mice. The BALB/c-*bm/bm* mouse shows a short nose and cranium. The reason for hypo-growth of anterior craniofacial structures has not been clarified, although the nasal septal cartilage might be related to the growth of anterior craniofacial structures. Therefore, the purpose of this study was to evaluate histological findings of the nasal septal cartilage at the border region of the ethmoid and sphenoid bone in BALB/c-*bm/bm* mice.

Materials and Methods – BALB/c mice (wild type) and BALB/c-*bm/bm* mice with normal occlusion (*bm/bm*) were used. Sagittal sections of female mice aged 2, 4, and 8 weeks were stained with hematoxylin and eosin for histological analysis.

Results – At the border region between the nasal septal cartilage and the ethmoid bone in *bm/bm*, the area of proliferative zone was significantly smaller than that in wild type. At the border regions between the nasal septal cartilage and both the ethmoid and sphenoid bones, the number of proliferative chondrocytes was significantly smaller. Normal endochondral ossification was not observed at the border region between the nasal septal cartilage and the sphenoid bone in *bm/bm*.

Conclusion – The findings suggest that disorder of endochondral ossification in the nasal septal cartilage contributes to the hypo-growth of anterior craniofacial structures in *bm/bm*.

Key words: animal model; nasal septal cartilage; proliferative chondrocyte

Introduction

BALB/c-*bm/bm* (*bm* homozygotes) mice are derived from BALB/c mice, which are bred C57BL-*bm/bm* mice (1). The dwarfism caused by the phenotype of *bm*, which is autosomal recessively inherited, has been shown to be attributed to undersulfation of matrix glycosaminoglycans in the cartilage because of a missense mutation in the 3′-phosphoadenosine 5′-phosphosulfate (PAPS) synthase 2 gene (*Papss2*) (2).

Malocclusion, incisal lateral (and sometimes anteroposterior) crossbite, spontaneously occurs in about 10% of BALB/c-*bm/bm* mice (3). However, anterior lateral crossbite does not occur in mice with the same *bm* mutation as that in C57BL-*bm/bm* mice. This crossbite also does not occur in non-brachymorphic mice such as BALB/c-+/+ and BALB/c-*bm/*+ (*bm* heterozygotes) mice.

Malocclusions are attributed to various factors including prenatal and postnatal factors, but the reason for the occurrence of malocclusions has not yet been clarified. Many animal studies have been performed to clarify the cause of malocclusion. However, in the most of those studies, appliances were applied to animal to induce malocclusion (4–6). Whereas, as regards the animals in which the malocclusions occur spontaneously, there is only a report using the cleft palate mouse (7) except for the BALB/c-*bm/bm* mouse. Therefore, the BALB/c-*bm/bm* mouse is an important animal model for investigating the etiology of malocclusion.

Malocclusion spontaneously occurs in BALB/c-*bm/bm* mice at 2–3 weeks after birth. It has been shown that endochondral growth was disturbed in the condylar cartilage (8) and in the spheno-occipital synchondrosis (SOS) (9) of BALB/c-*bm/bm* mice compared with that in BALB/c and BALB/c-*bm/*+ mice. The disturbance of endochondral growth has been thought to be one of the factors responsible for malocclusion in BALB/c-*bm/bm* mice. This means that an inherited feature of having *bm* homozygotes is a primary cause of malocclusion, and it is thought that a genetic factor is involved in the generation of malocclusion. On the other hand, the amount of lateral displacement of the maxillary alveolus and mandibular alveolus (lateral crossbite) was significantly decreased if the incisors were trimmed to eliminate occlusal interference just after anterior lateral crossbite had occurred (10), suggesting that environment factors are also involved in the generation of malocclusion.

There have been several studies on cartilage in *bm* mice. Wikstrom et al. (11) carried out a morphologic study on the epiphyseal growth zone, and Wezeman and Bollnow (12) studied tibial growth plate articular cartilage in other *bm* mice (C57Bl/6J × C3He). Kajii et al. (8) and Tsukamoto et al. (9) studied condylar cartilage and the SOS of BALB/c-*bm/bm* mice, respectively.

Radiographic (3) and three-dimensional microcomputed tomography (13) studies showed that the anteroposterior length of the cranium of BALB/c-*bm/bm* mice was significantly shorter than that of normal BALB/c mice. According to Tsukamoto et al. (9), abnormal endochondral ossification in the SOS of BALB/c-*bm/bm* mice induced inhibition of growth of the posterior cranial base. However, factors related to inferior growth of the anterior cranial base and the nose have not been determined.

Much attention has been paid to the role of the nasal septal cartilage in the growth of the front region of the cranium in mammals. Scott (14, 15) reported that the cartilaginous nasal septum is primarily responsible for translation of the facial bones, permitting growth of the midfacial region to proceed in a downward and forward direction by the mechanism of surface deposition of new bone matrix. It was also revealed in *in vitro* studies that growth potential of the nasal septal cartilage might be equivalent to that of epiphyseal growth plate cartilage (16, 17).

Therefore, the aim of this study was to obtain histological findings of the nasal septal cartilage in BALB/c-*bm/bm* mice that give an insight into the cause of hypo-growth of the cranio-maxillary complex of the mouse.

Material and methods

Material

BALB/c mice were purchased from Nippon Clea (Tokyo, Japan). By outbreeding between a *bm/bm*

mouse of the C57BL strain and a normal mouse of the BALB/c strain, the *bm* gene was successfully transferred to BALB/c strain mouse (*bm* heterozygotes). BALB/c-*bm* homozygote mice were generated by crossbreeding between BALB/c-*bm* heterozygote mice (1).

The mice were housed under standard conditions with temperature of 25°C, 50% humidity, and a 12-h (from 08:00 to 20:00) light cycle. They were supplied with tap water and food pellets.

Female mice were divided into two groups: BALB/c mice (wild type, n = 15) and BALB/c-*bm/bm* mice with normal occlusion (*bm/bm*, n = 15). After body weights of the mice in the two groups had been measured, the mice in both groups were killed at 2, 4, and 8 weeks of age (n = 5 at each age). Histological evaluation of the nasal septal cartilage was carried out.

The procedures were reviewed and approved by the Animal Care and Use Committee of Hokkaido University.

Methods

All of the mice in the two groups, wild type and *bm/bm*, were anesthetized by diethyl ether inhalation and perfused with 10% formalin neutral buffer via the left ventricle. After removing soft tissues and the mandible, the skull was dissected and then incubated in freshly prepared fixative at 4°C for 3 days. Each specimen was decalcified in 5% ethylenediamine tetraacetic acid solution at 4°C for 4 weeks and routinely embedded in paraffin wax.

Sagittal sections of 5 μm in thickness including the nasal septal cartilage were made. The sections were stained with hematoxylin and eosin for histological observation. The central region of the nasal septal cartilage and the middle part of border regions of the nasal septal cartilage at the ethmoid and sphenoid bones were observed using a light microscope (BX50; Olympus, Tokyo, Japan) (Fig. 1).

As quantitative measurement, the area of proliferative zone of the cartilage at the border region between the ethmoid bone and the cartilage in wild type and *bm/bm* was measured. The proliferative zone area was defined as the layer in which

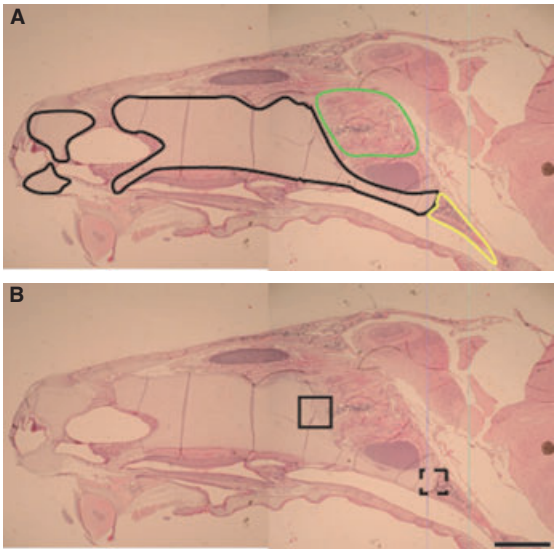

Fig. 1. Low-power images of a sagittal section slice including the center region of the nasal septal cartilage and the ethmoid and sphenoid bones in a BALB/c mouse at 2 weeks of age. In the upper image, the part surrounded by a black line indicates the nasal septal cartilage, the green line indicates the ethmoid bone, and the yellow line indicates the sphenoid bone. In the lower image, the part surrounded by a solid line indicates the border region of the nasal septal cartilage and the ethmoid bone. The dotted line indicates the border region of the nasal septal cartilage and the sphenoid bone. Scale bars: 1000 μm.

proliferative chondrocytes were aligned closely, and the area was measured using NIH image within the range of 250 × 400 μm including the resting and hypertrophic zone (Fig. 2).

The number of proliferative chondrocytes, judged to be flat cells, at the border region between the ethmoid or sphenoid bone and the cartilage in wild type and *bm/bm* was also measured in the same range of 250 × 400 μm including the resting and hypertrophic zone. The slide that was considered to be the closest to the midsagittal section of mouse and also both the next ones were used for quantitative measurement. Therefore, three sections per each mouse were analyzed. To avoid the examiner bias, all sections of wild type and *bm/bm* were randomly selected to measure. The same area or number was measured on two different occasions with a time interval of 2 weeks by the same investigator (K.T.).

The area of proliferative zone and the number of proliferative chondrocytes were statistically analyzed. The chronological significant difference in each group was evaluated by one-way analysis of variance (ANOVA). Significant differences between wild type and *bm/bm* were evaluated by

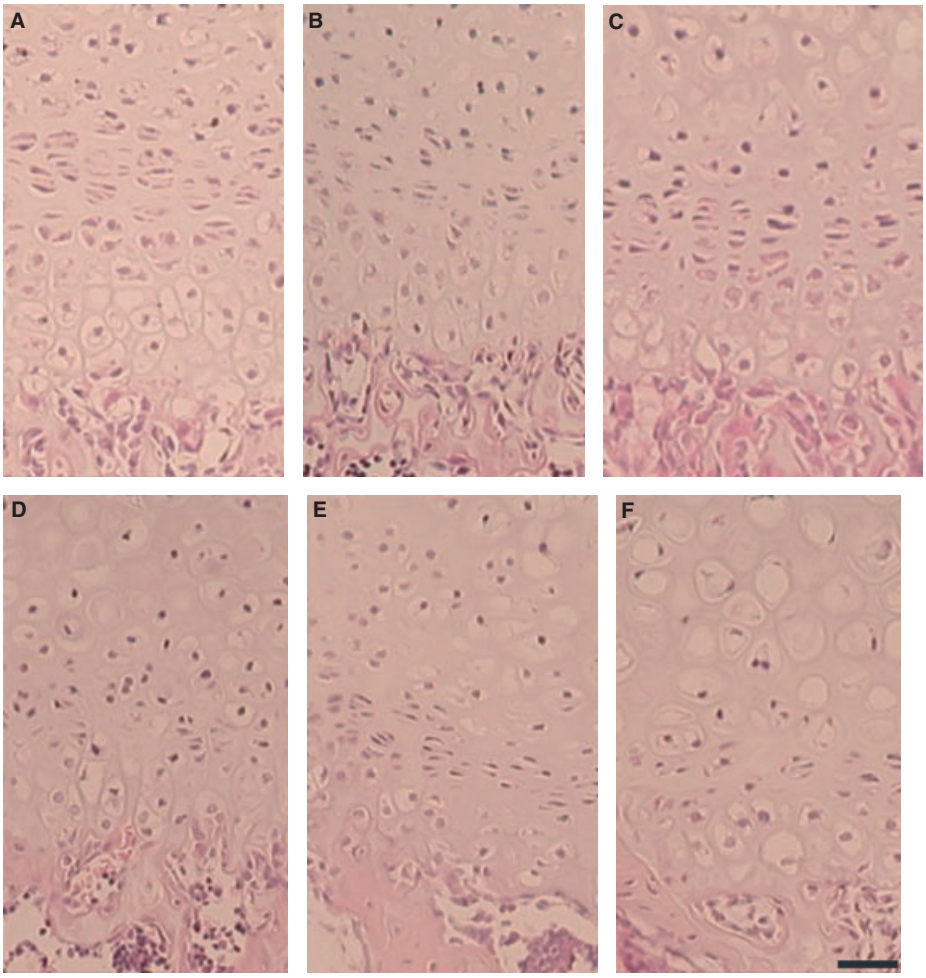

Fig. 2. High-power images at the border region between the nasal septal cartilage and the ethmoid bone. (A) wild type at 2 weeks of age; (B) *bm/bm* at 2 weeks of age; (C) wild type at 4 weeks of age; (D) *bm/bm* at 4 weeks of age; (E) wild type at 8 weeks of age; (F) *bm/bm* at 8 weeks of age. Scale bar: 50 μm.

the unpaired *t*-test. These analyses were performed by using the SPSS statistical package (version 14.0; SPSS Inc, Chicago, IL, USA) with a probability level of $p < 0.05$ being considered statistically significant.

Results

Mean body weights of wild type at the ages of 2, 4, and 8 weeks were 5.1, 14.6, and 25.0 g, respectively, and mean weights of *bm/bm* at ages of 2, 4, and 8 weeks were 3.1, 10.2, and 16.5 g, respectively. At the ages of 4 and 8 weeks, there were significant differences in body weights between the two groups. Histological examination of the central region of the nasal septal cartilage showed that mature hyaline cartilage had spread from the nasal tip to border of the ethmoid and sphenoid bones in both groups.

Histological images of the border region between the nasal septal cartilage and ethmoid bone in wild type are shown in Fig. 2A, C, E. The cartilage consisted of three distinguishable zones, resting, proliferative, and hypertrophic zones, so that the image seemed to be associated with normal endochondral ossification. Chondrocytes were irregular in arrangement and size in *bm/bm* at the ages of 4 and 8 weeks (Fig. 2B, D, F). At all ages, intergroup comparison showed that the area of proliferative zone at the border region in *bm/bm* was significantly smaller than that in wild type (Table 1), and the number of proliferative chondrocytes at the border region in *bm/bm* was significantly smaller than that in wild type (Table 2).

The area of proliferative zone was significantly smaller (Table 1), and the number of proliferative chondrocytes was significantly smaller (Table 2) at the border region in older mice both groups.

Table 1. **Area of proliferative zone of cartilage at the border region between the nasal septal cartilage and the ethmoid bone**

	2 week	4 week	8 week	*p*-value
Wild type	$4.64 \times 10^4 \pm 0.40 \times 10^4$	$3.39 \times 10^4 \pm 0.24 \times 10^4$	$2.95 \times 10^4 \pm 0.14 \times 10^4$	<0.001***
bm/bm	$2.90 \times 10^4 \pm 0.14 \times 10^4$	$2.72 \times 10^4 \pm 0.05 \times 10^4$	$1.90 \times 10^4 \pm 0.25 \times 10^4$	<0.001***
p-value	0.001**	<0.001***	0.006**	

Mean values and standard deviation are shown. unit: μm^2.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 2. **Number of proliferative chondrocytes at the border region between the nasal septal cartilage and the ethmoid bone**

	2 week	4 week	8 week	*p*-value
Wild type	64.8 ± 2.2	48.8 ± 3.7	35.2 ± 4.2	<0.001***
bm/bm	38.2 ± 4.4	29.2 ± 3.3	12.0 ± 3.0	<0.001***
p-value	<0.001***	<0.001***	0.02*	

Mean values and standard deviation are shown.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Histological images of the border region between the nasal septal cartilage and sphenoid bone in wild type are shown in Fig. 3A, C, E. The cartilage also consisted of three distinguishable zones: resting, proliferative, and hypertrophic zones, so that the image seemed to be associated with normal endochondral ossification. Chondrocytes in *bm/bm* at the ages of 2, 4, and 8 weeks were irregular in arrangement and size (Fig. 3B, D, F). Moreover, normal endochondral ossifica-

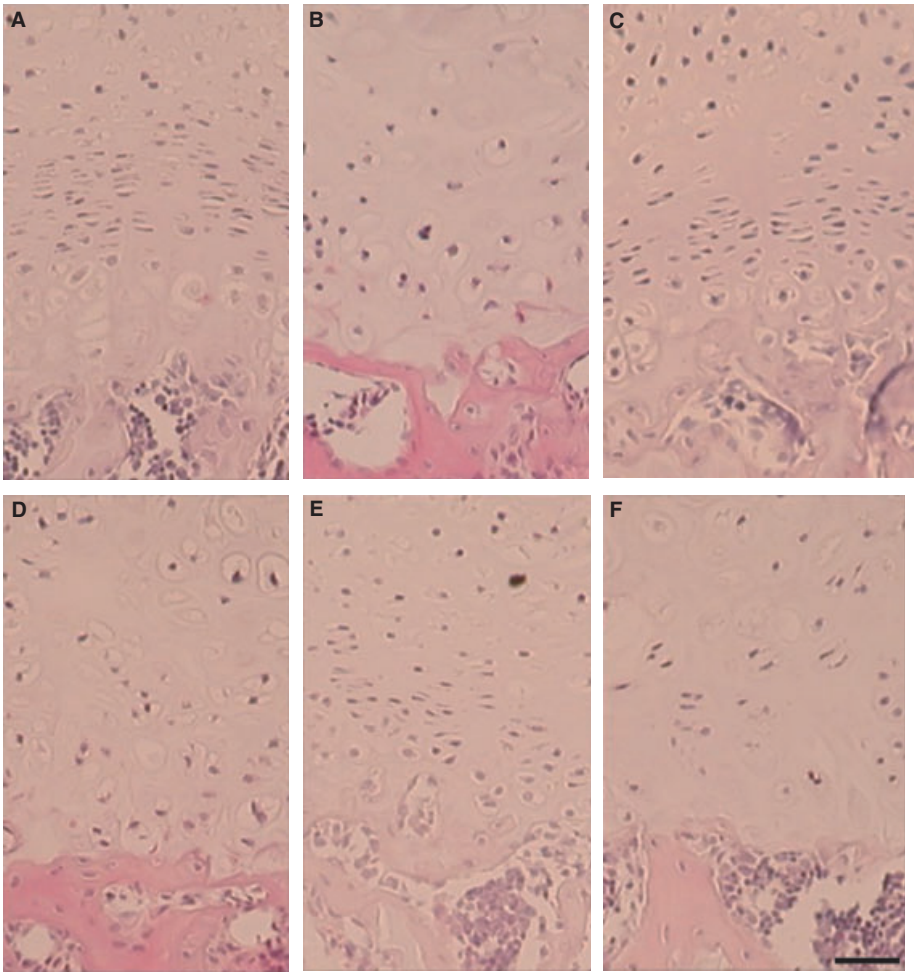

Fig. 3. High-power images at the border region between the nasal septal cartilage and the sphenoid bone. (A) wild type at 2 weeks of age; (B) *bm/bm* at 2 weeks of age; (C) wild type at 4 weeks of age; (D) *bm/bm* at 4 weeks of age; (E) wild type at 8 weeks of age; (F) *bm/bm* at 8 weeks of age. Scale bar: 50 μm.

Table 3. **Number of proliferative chondrocytes at the border region between the nasal septal cartilage and the sphenoid bone**

	2 week	4 week	8 week	*p*-value
Wild type	67.6 ± 8.0	42.6 ± 4.0	38.5 ± 2.1	<0.001***
bm/bm	7.0 ± 1.4	8.0 ± 1.7	9.0 ± 2.6	0.06
p-value	<0.001***	<0.001***	0.001**	

Mean values and standard deviation are shown.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 4. **Number of proliferative chondrocytes at the border region between the nasal septal cartilage and the ethmoid or sphenoid bone of *bm/bm***

	2 week	4 week	8 week
The ethmoid bone	38.2 ± 4.4	29.2 ± 3.3	12.0 ± 3.0
The sphenoid bone	7.0 ± 1.4	8.0 ± 1.7	9.0 ± 2.6
p-value	<0.001***	<0.001***	0.16

Mean values and standard deviation are shown.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

tion was not observed in *bm/bm*, and the area of proliferative zone could therefore not be measured. While layered structure about proliferative chondrocytes was not observed, proliferative chondrocyte itself was observed as a flat cell, so the number of proliferative chondrocytes could be measured. At all ages, the number of proliferative chondrocytes at the border region between the nasal septal cartilage and sphenoid bone in *bm/bm* was significantly smaller than that in wild type as was also found at the border region between the nasal septal cartilage and ethmoid bone (Table 3). The number of proliferative chondrocytes was significantly smaller in order wild type, although there was no significant difference in the number of proliferative chondrocytes between *bm/bm* of different ages.

Histological findings at the border regions of the ethmoid and sphenoid bones were not noticeably different in wild type, but proliferative and hypertrophic zones of the nasal septal cartilage at the border region of the sphenoid bone were much more irregular than these at the border region of the ethmoid bone in *bm/bm* (Figs 2 and 3). In *bm/bm* at the ages of 2 and 4 weeks, the number of proliferative chondrocytes in the border region of the sphenoid bone was significantly smaller than that in the border region of the ethmoid bone (Table 4).

Discussion

Histological examination in a previous study showed that each zone of the epiphyseal growth plates was shorter and columns of the chondrocytes were arranged more irregularly in brachymorphic mice (18). On the other hand, the basic structure of the nasal septal cartilage at the border region between the cartilage and the bone seems to be similar to that of epiphyseal growth plates in mice. It has been reported that the nasal septal cartilage contributes to enlargement of the facial skeleton by displacing facial bone. The primary mechanism is septal interstitial growth, and endochondral ossification of the caudal septum plays a secondary role (19–24). There has been no histological examination of the nasal septal cartilage in brachymorphic mice, especially in *bm/bm* mice. Results of histological analysis in the present study showed that the number of proliferative chondrocytes in the nasal septal cartilage of *bm/bm* was significantly smaller in the border region between the cartilage and the ethmoid or sphenoid bone. In the border region between the cartilage and ethmoid bone of *bm/bm*, the area of proliferative zone was significantly smaller, and in the border region between the cartilage and sphenoid bone of *bm/bm*, proliferative and hypertrophic zones were much more irregular. In this study, proliferative chondrocytes was judged to be flat cells, although using histological marker such as BrdU for proliferative chondrocytes will be better approach to count the proliferative chondrocytes.

The area of proliferative zone was significantly smaller, and the number of proliferative chondrocytes was significantly smaller at the border region between the nasal septal cartilage and ethmoid bone in both wild type and *bm/bm* of older age. These findings are similar to the results of previous studies using normal mice (20) and rats (25). It is thought that the anterior portion of the maxillofacial complex grows actively through

the nasal septum cartilage at 2, 4, and 8 weeks of age, though anteroposterior growth rate decreases with advance of age. On the other hand, in *bm/bm*, the area of proliferative zone and the number of proliferative chondrocytes at the border region between the nasal septal cartilage and ethmoid bone were already decreased at 2 weeks of age. At the border region between the nasal septal cartilage and sphenoid bone, the number of proliferative chondrocytes in *bm/bm* was significantly smaller and the normal endochondral ossification was not observed. This finding is related to the fact that the anterior portion of the maxillofacial complex in *bm/bm* is almost the same size as that in wild type at birth but is smaller at 2 weeks of age (1).

Tsukamoto et al. (9) reported that a bipolar column of chondrocytes was not seen and that normal endochondral growth was disturbed at 2 weeks of age in the SOS of the cranial base in *bm/bm*. This finding is similar to the results obtained for the nasal septal cartilage of *bm/bm*. Both the SOS and the nasal septal cartilage of *bm/bm* have not only undersulfation of cartilage matrix but also irregular arrangement of columns of chondrocytes, especially in the proliferative zone. Anteroposterior growth in the craniofacial region of *bm/bm* is thought to be inhibited because of the abnormal endochondral growth. At the border region between the nasal septal cartilage and sphenoid bone, columns of chondrocytes in proliferative and hypertrophic zones were much irregular and the normal endochondral ossification was hardly observed compared with those in the border region of the ethmoid bone. Further detailed examination of this difference is needed.

The observation of abnormality of endochondral ossification in *bm/bm* suggested that the amount of bone produced at the border region between the nasal septal cartilage and the ethmoid or sphenoid bone was less than that in wild type. Anterior lateral crossbites spontaneously occur in about 10% of inbred *bm/bm* (3). Poor anteroposterior growth of the anterior portion of the maxillofacial region in *bm/bm* is thought to be due to abnormality of endochondral ossification, particularly decrease in the number of proliferative chondrocytes. It is therefore speculated that decrease in overjet because of the difference in the amount of anteroposterior growth between maxillary bone and mandibular bone induces occlusal interference and subsequent anterior transverse crossbite. Malocclusion spontaneously occurs in *bm/bm*, but it does not spontaneously occur in other brachymorphic mice. It is thought that there is specificity in the amount and period of maxillary and mandibular growth in BALB/c-*bm/bm*, although we have not examined this yet.

In the future, we will investigate the presence and the level of undersulfation at the border region between the nasal septal cartilage and the ethmoid or sphenoid bone, and we will elucidate the site of action of abnormality in cartilage differentiation using *bm/bm* mice with malocclusion.

Conclusions

The nasal septal cartilage of BALB/c-*bm/bm* mice (*bm/bm*) was examined by histological analysis. At all ages, the area of proliferative zone at the border region between the nasal septal cartilage and the ethmoid bone was significantly smaller and the number of proliferative chondrocytes was significantly smaller at both border regions of the ethmoid and sphenoid bones in *bm/bm* mice. Moreover, chondrocytes were irregular in arrangement and size, and normal endochondral ossification was not observed at the border region between the nasal septal cartilage and the sphenoid bone in *bm/bm*. The findings suggest that disorder of the normal endochondral ossification at the nasal septal cartilage contributes to the hypo-growth of anterior craniofacial structures in *bm/bm*.

Clinical relevance

Results obtained in *bm/bm* mice suggested that anteroposterior maxillofacial growth may be inhibited because of abnormal endochondral ossification. From these results, one of the factors causing the characteristic facial morphology in

patients with a disorder of endochondral ossification such as achondroplasia could be clarified.

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