

Expression of Sox 9 and type II and X collagens in regenerated condyle

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SUMMARY The present study was designed to examine the expression of Sox 9 and type II and X collagens in regenerated condyle resulting from the use of a functional appliance. Ninety, 3-week-old, mice were divided equally into the following groups: two experimental groups (condylectomy group and condylectomy with functional appliance group) and the corresponding control group. In the condylectomy group, a unilateral condylectomy was performed on the right side. In the condylectomy with appliance group, the mandible was repositioned in a forward direction using a functional appliance after unilateral condylectomy. The expression of Sox 9 and type II and X collagens in the condyle was determined immunohistochemically 4 weeks after surgery.

In mice with a condylectomy, the expression was minimal. On the other hand, these factors were highly expressed in the condylectomized side with the appliance. It is thus speculated that cartilaginous regeneration is due to the expression of chondrogenic factors, such as Sox 9 and type II and X collagens. It is also suggested that condyle regeneration results from an optimal intra-articular environment with appropriate joint spaces achieved by condylar repositioning.

Introduction

The mandibular condyle has an important role in mandibular growth. Therefore, any injuries to the condyle during growth disturb the growth of the mandible. A previous study has shown that mandibular advancement with a functional appliance has the ability to regenerate cartilaginous tissues on an injured condyle and recover the reduced mandibular growth (Nakano *et al.*, 2009). However, it remains unclear if the regenerated condyle has a normal function similar to controls, and furthermore which factors improve the regeneration of the condyle during mandibular advancement.

Regulation factors for the proliferation and differentiation of articular chondrocytes are roughly classified as follows: (1) growth differentiation factors [thyroxine, parathyroid hormone (PTH), PTH-related peptide, sex hormone, vitamin D, basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF-I and II), bone morphogenetic proteins (BMPs), and transforming growth factor- β (TGF- β)], (2) extracellular matrixes (collagen and hyaluronate), and (3) transcription factors (Sox 5, Sox 6, Sox 9, and Runx). Sox 9 is a transcription factor that differentiates mesenchymal cells in the proliferative layer of the condylar cartilage to chondrocytes (Lefebvre and de Crombrughe, 1998). Sox 9 is expressed and activated in chondroblasts and migrates to the nucleus with cartilage transformation and then mediates differentiation to chondrocytes associated with the expression of type II collagen (Rabie and Hägg, 2002). There are various factors

relevant to the differentiation of chondrocytes in addition to Sox 9; however, the latter may be indispensable for the production of matrix protein, as was demonstrated by the fact that mesenchymal cells with Sox 9 deficiency exhibit no differentiation to hypertrophic chondrocytes associated with the expression of type II collagen (Lefebvre and de Crombrughe, 1998). The anomaly of endochondral ossification was reported in type II collagen dominant-negative mice (Rintala *et al.*, 1997). Disturbance in craniofacial growth, including undergrowth of mandibular body length, was found in type II collagen knock out mice (Savontaus *et al.*, 2004). It is thus speculated that type II collagen is also essential for cartilaginous growth of the craniofacial skeleton.

Chondrocytes, differentiated from mesenchymal cells in a proliferative layer, turn into hypertrophic chondrocytes in association with the expression of type X collagen (Ng *et al.*, 1997; Healy *et al.*, 1999). Therefore, hypertrophic chondrocyte synthesizes type X collagen (Salo *et al.*, 1996) and is a specific expression site of type X collagen (Hamada *et al.*, 1999). Moreover, the availability of hypertrophic chondrocytes was shown as a primary parameter for long bone remodelling and endochondral ossification in the healing process of bone fracture (Grant *et al.*, 1987).

A defect of Sox 9 prohibits synthesis of type II collagen, which is an essential protein for bone and a cartilage, and a morphologic anomaly of the femur is found in Sox 9 knock

out mice (Bi *et al.*, 1999). It is assumed that Sox 9 deficiency influences all cartilage-derived structures, leading to systemic skeletal dysplasia (Giordano *et al.*, 2001), and Sox 9 plays an important role in bone and cartilage formation during growth. Although transcription factor Sox 9 strongly influences the expression of type II and X collagens, which are matrix proteins to control cartilage transformation, the influence of Sox 9 on mandibular growth is still unknown.

The aim of this study was to assess the expression of Sox 9 and type II and X collagens on regenerated condyle in response to mandibular advancement resulting from the use of a functional appliance.

Materials and methods

This study was approved by the Ethics Committee of Hiroshima University.

Experimental animals

The animal model used in this study has already been described previously (Nakano *et al.*, 2009). Ninety, 3-week-old, C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine, USA) were divided equally into the following groups: two experimental groups (a condylectomy group and a condylectomy with appliance group) and the corresponding control group that underwent sham surgery. In the condylectomy group, a unilateral condylectomy was performed on the right side, and the mandible was allowed to function. In the condylectomy with appliance group, the mandible was repositioned in a forward direction with a functional appliance after unilateral condylectomy. The control mice underwent a sham procedure in the condylar area on the right side without the use of any appliance.

The animals in all the groups were fed a granulated diet for the first 4 days and a solid diet (CE-2; CLEA, Tokyo, Japan) after surgery.

Unilateral condylectomy

The condylectomy was performed under general anaesthesia with pentobarbital. Through a pre-articular approach, the condyle was totally exposed and the excision was performed carefully so as not to damage the surrounding structure. Using a stereoscopic microscope (SZX9; Olympus Optical, Tokyo, Japan) the condylar head was cut 0.5 mm below the condylar neck. Trauma from the external dermal incision was kept to a minimum and the incision area was carefully sutured. Analgesic buprenorphine (Lepetan; Otsuka Pharmaceutical, Tokyo, Japan) was given to the mice immediately after surgery. These procedures were performed under sterilized conditions.

Functional appliance

The mandible was repositioned in the forward direction with a functional appliance consisting of a 0.016×0.022 inch

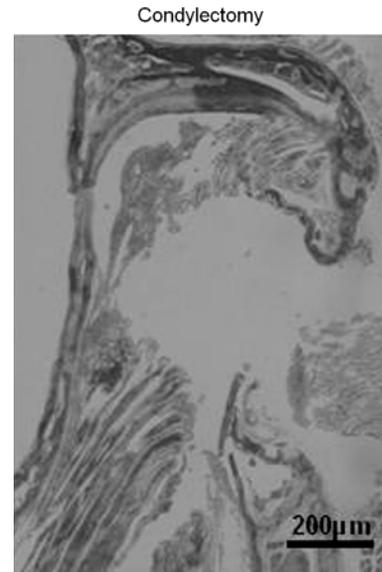


Figure 1 Photomicrograph of the condyle, stained with azocarmine-aniline blue, in the condylectomized mice.

Co-Cr wire (3M Unitek, Monrovia, California, USA). The appliance was fixed to the palate by ligating the maxilla with the wire to reposition the mandible anteriorly in an edge-to-edge occlusion in the intercuspal position. Lateral cephalograms were then obtained to determine if the lower anterior teeth were moved forward approximately 0.5 mm by the functional appliance. During a series of experiments, it was confirmed that the animals could masticate without any disturbance while wearing the appliance for 24 hours.

Histologic and immunohistochemical examination

Four weeks after the beginning of the experiment, the mice were killed with an overdose of sodium pentobarbital (Nembutal; Daiippon Sumitomo Pharma, Osaka, Japan) and the mandibular condyle was removed. The specimens were fixed in 4 per cent formaldehyde, decalcified in citric-formic acid for 1 week, dehydrated in an ascending ethanol series (70, 80, 90, 95, 99, and 100 per cent), embedded in paraffin, and cut into frontal sections (5 µm thick). The sections were first used for histologic examination of the mandibular condyle with and without the application of a functional appliance (Figure 1).

Expression of Sox 9 and type II and X collagens on the condyle was then examined on these sections immunohistochemically stained with human Sox 9 (SC-20095; Santa Cruz Biotechnology, Santa Cruz, California, USA), bovine type II collagen (LB-1297; LSL, Tokyo, Japan), and rat type X collagen (LB-0092; LSL) by means of a Vectastain ABC-GO Kit (Vector Laboratories, Burlingame, California, USA) and counterstained with methyl green. Normal rabbit IgG (Santa Cruz Biotechnology) was used as the control for the polyclonal antibody.

Results

Histomorphometric findings

Regeneration of the condyle was demonstrated in all mice in the condylectomy and condylectomy with appliance groups. Although the size of the condyle was 2-fold larger when compared with the controls, its shape was irregular in the condylectomy group. Four layers of the condylar cartilage, observed in the controls, were not detected in the condylectomy group. Furthermore, various tissues, such as bone, muscle, and cartilage, were irregularly arranged in this group (Figures 2 A, B).

On the other hand, the size and shape of the condyle in all the mice in the condylectomy and appliance group were exactly equivalent to the controls. Four layers of the condylar cartilage were also clearly detected in the condylectomy and appliance group, although the proliferative layer was thinner and the hypertrophic layer was slightly thicker than in the controls (Figures 2 A, B).

Immunohistochemical findings

In the control group, Sox 9 was highly expressed only in the proliferative layer of the condylar cartilage covered with fibrous connective tissues, and above the pre-hypertrophic layer. The cytoplasm and nucleus of chondroblasts were also intensively stained. Type II collagen exhibited an intensive expression in the extracellular matrix of the pre-hypertrophic layer, while type X collagen was observed only in the post-hypertrophic layer (Figure 2A). On the condylectomized side, a slight expression of Sox 9 was observed in the scattered chondrocytes. Type II collagen was rarely observed although the surrounding substrate of the scattered chondrocyte was lightly stained. Expression of type X collagen was rarely observed on the condylectomized side, similar to Sox 9 and type II collagen (Figure 2B).

In the condylectomized group with a functional appliance, expression of Sox 9 was observed in the proliferative layer of the condylar cartilage covered with fibrous connective tissues and above the pre-hypertrophic layer. The cytoplasm and nucleus of the chondroblasts were stained similarly to the control group. Intensive expression of type II and X collagens was observed in the extracellular matrix of the pre- and post-hypertrophic layers, respectively (Figure 2C).

Discussion

Sox 9 was expressed in the nucleus of chondroblasts, which are undifferentiated mesenchymal cells in a proliferative layer. The upper zone of the pre-hypertrophic cartilage layer also exhibited an intensive appearance of Sox 9 in the control and appliance groups of this study. This result is in agreement with that of Lefebvre and de Crombrugge (1998), who reported a high expression of Sox 9 on

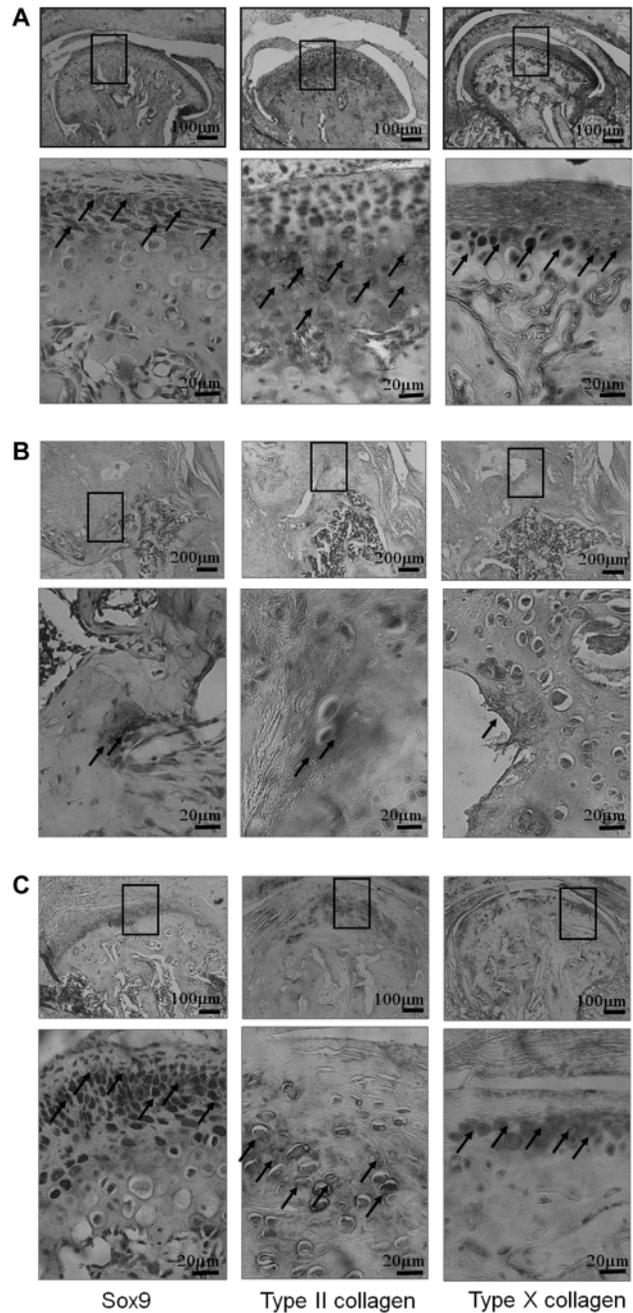


Figure 2 Immunostaining showing Sox 9 and type II and X collagens expression (arrow) in the mandibular condylar cartilage of (A) control mice, (B) condylectomized mice and (C) condylectomized mice with functional appliance.

sufficiently differentiated chondrocytes during the prenatal growth of mice. Since new bone formation from a membranous ossification is initiated 2 or 3 days after bone fracture in mice (Einhorn, 1998) and thereafter endochondral ossification continues for about 4 weeks, histologic examination was designed in this study 4 weeks after the condylectomy. In fact, it is assumed that endochondral ossification in a fracture healing process may be completed

around this period. A previous study, which observed the restoration process in the partially defective femurs of mice, found that Sox 9 was highly expressed in the healing process of bone fractures and the restoration of the fracture was promoted by endochondral ossification (Uusitalo *et al.*, 2005). Moreover, the expression of Sox 9 and type II and X collagens, useful markers for endochondral ossification during membranous ossification and fracture healing process of long bone (Grant *et al.*, 1987), was rarely observed in the condylectomy group as compared with the control group. From this finding, it is understood that endochondral ossification after condylectomy was not successful.

Rabie *et al.* (2003) examined the level of Sox 9 in growing rat condyles and reported that the maximum expression level of Sox 9 was on day 5 after insertion of a functional appliance, but on day 9 in the control group. Moreover, although the expression of Sox 9 was significantly lower in the appliance group as compared with the control group up to experimental day 14, the significant difference in Sox 9 expression between both groups was not found thereafter. Immunohistochemical staining in that study was performed 4 weeks after insertion of a functional appliance. It is assumed from the present study that the maximum expression of Sox 9 in the normal condyle might have been completed during this period.

Sox 9 knock out mice have a defect in the shape of the femur, such as flexure and cartilage transformation (Bi *et al.*, 1999). However, it is unclear how Sox 9 in the condylar cartilage influences mandibular growth. The expression of Sox 9 in the condylectomized area was significantly decreased and the regenerated condyle exhibited an irregular shape. However, as a result of mandibular repositioning with use of a functional appliance, the expression of Sox 9 was increased and the shape of the condyle was improved. It is speculated that the expression of Sox 9 in the condylar cartilage also influences morphogenesis of the condyle due to cartilaginous growth, whereas a reduction may cause an anomaly in the shape of the condyle. Other related factors for cartilaginous growth, Indian Hedgehog (Tang *et al.*, 2004), Runx2 (Tang and Rabie, 2005) and Cbfa1 (Lam and Rabie, 2005), have been reported. These factors are also expressed by cells in the condyle in response to mandibular advancement.

Conclusion

In the regenerated condyle in the appliance group, the endochondral ossification achieved was almost normal and had the function of growth cartilage, because Sox 9 and type II and X collagens were expressed to the same extent as in the control group. Thus, the growth ability of the condyle was activated and the reduction of mandibular growth by the condylectomy was compensated for by

condyle regeneration resulting from the use of a functional appliance.

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