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# NOTCH SIGNALING IN MANDIBULAR CONDYLAR CARTILAGE DEVELOPMEMT

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**Abstract**
*Objective:* The purpose of this study was to investigate the expression pattern of Notch signaling in mandibular condylar cartilage, as a type of secondary cartilage.
*Methods:* Mandibular condyle of ddY mice were fixed from embryonic day 14 (E14) through just after birth (equivalent to E19). Samples were cut into 4 μm serial sections through the central area of the mandibular condyle at the sagittal plane. Serial sections were examined using histological, immunohistochemical (IHC) and *in situ* hybridization (ISH) techniques.
*Results:* At E14, there were no developmental features of mandibular condyle. At the distal upper portion of developmental mandibular bone, mesenchymal cell proliferation and condensation without metacholomatic reaction to toluidine blue (TB) were seen. At E15, mandibular condylar cartilage was clearly evident, as TB metacholomasia. In IHC specimens at E14, expression of Notch1 intracellular domain (NICD) was observed in the nuclei of coagulating mesenchymal cells. After E15, NICD appeared in the nuclei and the cytoplasms of cells. In ISH examination at E14, expressions of Notch1 mRNA appeared in cytoplasm of proliferating chondrocytes. From E15 to E19, Notch1 mRNA was detected throughout almost all cytoplasm in all layers.
*Conclusion:* These IHC and ISH results suggest that Notch signaling plays an essential role for mandibular condylar cartilage morphogenesis and development.

*Key words:* Notch; mandibular condylar cartilage; secondary cartilage; *in situ* hybridization; immunohistochemistry

## INTRODUCTION

Mandibular condylar cartilage differs from the primary cartilage in morphological and biochemical organization [1, 2]. The formation pattern of this type of cartilage attracts many researchers because it suggests large possibilities in the field of orthodontics and embryology.

Various studies have shown that mandibular condylar cartilage is related to morphogenesis regulation factors and their signaling [3-5]. Recently we have reported that Runx2 plays an important role for mandibular condylar cartilage development, in particular that Runx2 is essential for the onset of "secondary cartilage" differentiation [6]. We considered that morphogenesis regulation factors, as well as Runx2, play an important role in morphogenesis during embryonic stage. Notch1 is membrane-bound protein, which regulates the differentiation gene for changing the cell type [7]. However, there have been no reports on mandibular condylar cartilage with the exception of our latest paper [8], although there are some reports on the distribution on articular cartilage [9, 10].

In general, Notch signalling is necessary for cell fate determination, cell proliferation and differentiation [11, 12]. Therefore, we focused on Notch signalling in the developing mouse mandibular condylar cartilage.

## MATERIALS AND METHODS

### ANIMAL EXPERIMENTS

A total of 12 pregnant ddY mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). The mandibular condylar cartilages were removed from the mice under anesthesia with ether. They were sampled at each of the following embryonic days: E14, E15, E16, E17, E18 and just after birth (equivalent to E19). The Matsumoto Dental University Committee for Animal Experimentation approved the study.

### HISTOLOGY

The materials were immediately fixed in 4% paraformaldehyde/0.05M phosphate-buffered solution and decalcified in 10% ethylenediamine tetraacetic acid. The materials were then dehydrated by passage through a series of ethanols and embedded in paraffin. Samples were cut at 4μm serial sections. Serial sections were then collected onto silane-coated slides and examined by histological (toluidine blue [TB] (pH 7.0), immunohistochemistry (IHC) and *in situ* hybridization (ISH) techniques.

### IHC

For IHC, deparaffinized sections were prepared after being pretreated with 0.13% pepsin for 30min at

37 °C. Examination was carried out using a Dako EnVision+Kit-K4006 (Dako, Glostrup, Denmark) and two monoclonal antibodies: anti-human Notch1 intracellular domain (btan20, NICD: 1/10) and anti-rat osteopontin (MPIIIB10, OPN: 1/10). The NICD monoclonal antibody was developed by Atranvanis-Tsakonas[13, 14]. The OPN monoclonal antibody was developed by Solush and Franzen [15]. Both antibodies were obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Science, Iowa City, Iowa, under contract NO1-HD-7-3236 from the National Institute of Child Health and Human Development. Samples were then counterstained with hematoxylin. OPN was used as a positive control. Immunohistochemical staining using phosphate buffered saline in place of the primary antibody was included as a negative control.

### ISH

Digoxigenin (DIG)-labeled single strand RNA probes of Notch1 and OPN were prepared using a DIG Labeling Kit (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) according to the manufacturer's instructions. Sense and anti-sense DIG-11-UTP-labeled RNA probes were constructed. For Notch1, cDNA fragments (Notch1 ca. 0.72 kb 5475-6201) was obtained by RT-PCR and subcloned into pCR21 (Invitrogen, Tokyo, Japan). For OPN, OPN ca. 1.2 kb was kindly provided by Associate Professor Shintaro Nomura of Osaka University School of Medicine. For ISH, the 4µm sections were deparaffinized in xylene, rehydrated in ethanol and incubated with 3mg/ml of proteinase K (Roche Diagnostic GmbH, Penzberg, Germany) in 10mM Tris-HCl (pH 8.0) and 1mM EDTA for 15min at 37 °C. Acetylation of the sections was performed by incubation with freshly-prepared 0.25% acidic anhydride in 0.1M triethanolamine-HCl buffer (pH 8.0) for 10min at room temperature. The hybridization solution contained 50% deionized formamide, 10% dextran sulfate, 1xDehardt's solution, 600mM NaCl, 0.25% SDS, 250 mg/ml of *Escherichia coli* tRNA (proteinases treated) 10mM dithiothreitol, and 0.1 to 2.0 mg/ml of DIG-UTP labeled RNA probe. The probe was placed on the sections and covered by parafilm and incubated at 50 °C overnight in a moist chamber. After hybridization, the slides were washed with a series of SSC at 50 °C and then incubated with 1.5% blocking reagent in DIG 1 buffer for 60 min. Anti-DIG-AP Fab fragment (1:800) in DIG 1 buffer was applied to the sections and incubated for 1hr at room temperature. Coloring solution containing 337.5mg/ml of nitro blue tetrazolium and 165mg/ml of 5-bromo-4-chloro-3-indolyl phosphate in DIG 3 buffer (100mM tris-HCl, pH 9.5, 100mM NaCl, 50 mM MgCl2) was mounted on the sections and incubated at 37 °C until the signal-noise ratio was maximum. The slides were mounted after counterstaining with methyl green. The negative controls included hybridization with sense (mRNA) probe. OPN was used as a positive control.

## Results

### Histology

At the distal upper portion of developmental mandibular bone, mesenchymal cell proliferation and condensation without metacholomatic reaction to TB were seen at E14 (Fig. 1). At E15, mandibular condylar cartilage was clearly evident, as TB metacholomasia (Fig. 2). At E17, perichondral ossification has already started at the periphery of the chondrocytes (Fig. 3). At E18, endochondral ossification progressed and the mandibular condyle increased in volume (Fig. 4).

### IHC

At E14, expression of NICD peptide was observed in the nuclei of coagulating mesenchymal cells (Fig. 5). At E15, the proliferating cells had positive products of NICD in their cytoplasms, cell membrane and nuclei of almost all coagulating cells (Fig. 6). At E16, NICD positive reactions were detected in cells of the proliferative, maturative and hypertrophic layers, and weakly labeled in cells of fibrous layer. The pattern of distribution and intensity of expression of NICD were not uniform. At E17, cytoplasmic and nuclear reactions of NICD factors appeared strongly in the cells just inside of the condylar cartilage sheath. At E18, NICD immunohistochemical positive products were observed in almost all cells of the layers, and they were mostly distinct in the sheath of the condyle. At just after birth, NICD was observed in a portion of almost all layer cells in their cytoplasm, nucleus and membrane. After E17 and up to birth, the pattern of distribution and intensity of expression of NICD peptides were not uniform.

Proliferating chondrocytes showed positive reactions to OPN-antigen through the examination periods, particularly in the cytoplasms of the proliferating chondrocytes (Fig. 7).

### ISH

At E14, expressions of Notch1 mRNA appeared in cytoplasms of proliferating chondrocytes. Their distribution intensity was not uniform. At E15, cytoplasmic and membranous reactions of Notch1 gene appeared in the cells of chondrocytes (Fig. 8). After E16 and up to just after birth, cytoplasmic positivities for Notch1 mRNA were detected throughout almost all condylar cells (Fig. 9). However, positive signals for Notch1 mRNA were reduced at the rim of condyle. Furthermore, their distribution pattern was not uniform.

OPN mRNA was detected in almost all cells of all layers at E15 and E17 (Fig. 10). Signals were localized in their cytoplasms. At just after birth, OPN signals were restricted in cytoplasm of maturative and proliferative layers.

## Discussion

Mandibular condylar cartilage is regulated by a number of morphogenesis regulation factors and/or their signaling, such as fibroblast growth factor receptor,

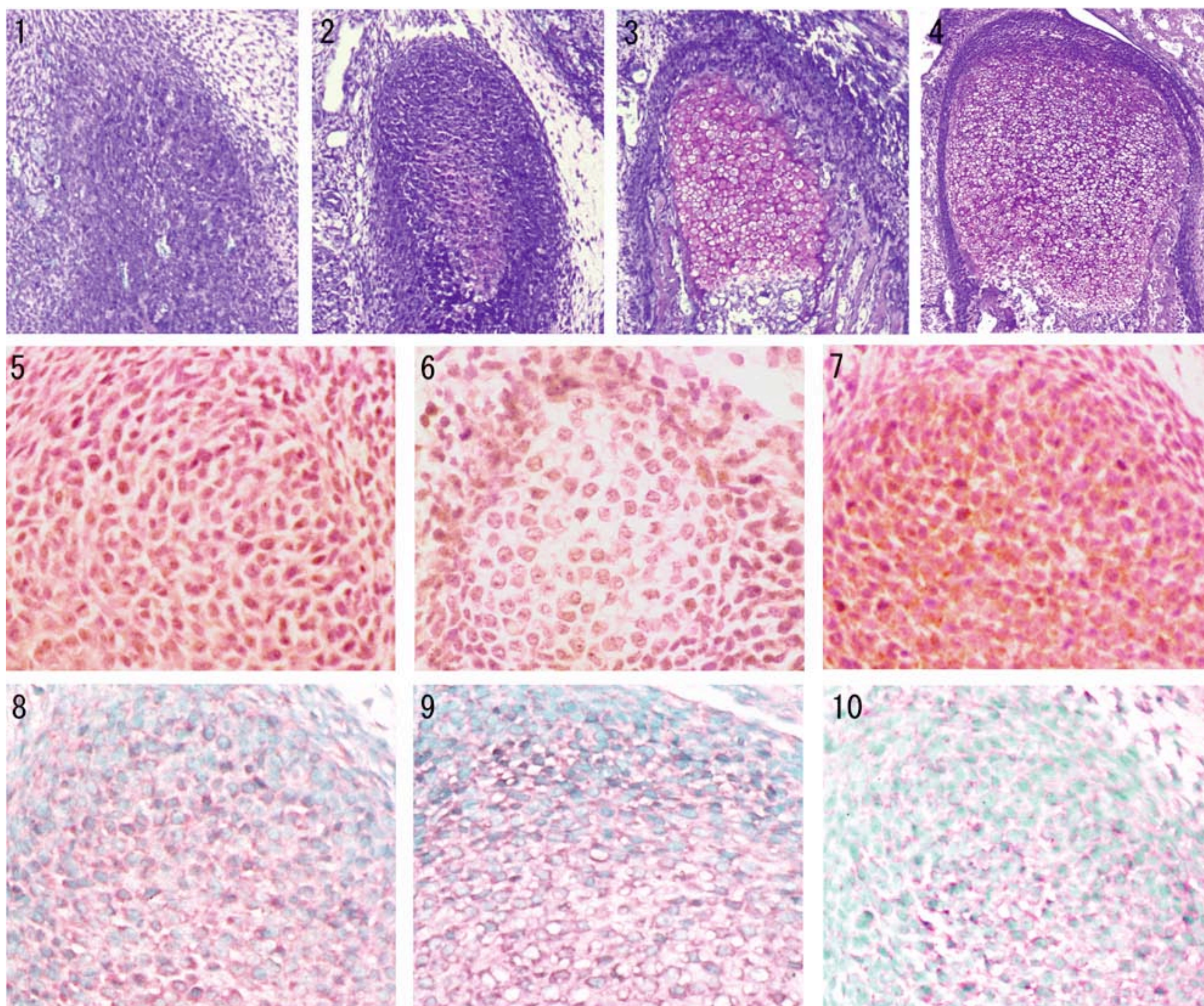


*Fig. 1*. Cells are coagulating at the upper portion of the mandible body with no metacholomatic reaction (E14, TB magnification x50).

*Fig. 2*. Mandibular condylar cartilage is clearly evident. The middle portion shows metachromasia (E15, TB magnification x50).

*Fig. 3*. Direct bone formation occurs (E17, TB magnification x50).

*Fig. 4*. Mandibular condyle increases in volume (E 18, TB magnification x40).

*Fig. 5*. Expression of NICD is visible in the coagulating cells (E14, IHC magnification x430).

*Fig. 6*. Proliferating cells have NICD positive products (E15, IHC magnification x430).

*Fig. 7*. Positive reaction to OPN is detected in almost all chondrocytes (E15, IHC magnification x430).

*Fig. 8*. Proliferating chondrocytes express Notch1 gene in their cytoplasm (E15, ISH magnification x430).

*Fig. 9*. Notch1 gene expression is seen throughout all layers (E16, ISH magnification x430).

*Fig. 10*. Positive reaction to OPN mRNA is detected in the cytoplasm of most cells of all layers (E15, ISH magnification x430).

platete-derived growth factor receptor, and Runx2 [3, 4, 6, 16, 17].

Generally, Notch1 is an important regulation factor of morphogenesis. It has been reported that Notch1 is a transmembrane protein necessary for cell fate determination, cell proliferation and differentiation [7]. Disruption of Notch ligands and receptors as well as downstream signaling components of the Notch pathway have been implicated in many developmental defects and pathological conditions [18, 19]. Notch functions in all progenitor cells give rise to the limb bone, cartilage, muscles and progenitor [20]. It has also been reported that Notch1 is not required to form the limb skeleton, musculature, or vasculature [21]. Furthermore, the Notch family is a highly-conserved family of cell surface signaling molecules [22]. In the developmental stage, Notch1 takes part in the representative three major phenomena, namely lateral inhibition, induction and asymmetric cell division [23, 24]. Notch signaling has been implicated in bone development and it is expressed in osteoblasts [22, 25]. With reference to cartilage, the Delta-Notch signaling pathway has been reported to be important in regulating the progression of prehypertrophic chondrocytes to hy-

pertrophic chondrocytes in examinations using chicks [26]. Furthermore, Hayes et al. [9] described that Notch signalling is closely related to the formation of articular cartilage and allows for co-oriented ossification in the growth plate in mice experiments.

Concerning our recent paper [8], the Notch1 reactions were localized only in the hypertrophic cells, and these expression patterns were different from each other in the articular cartilage. Math1 is also a regulation factor of morphogenesis [27]. It was distributed mainly in the hypertrophic layer and partially in the proliferative layer [8]. These results suggest that regulation factors of morphogenesis-Notch1 and Math1-may play an essential role in mandibular condylar cartilage development. Moreover Hayes *et al.* has reported that NICD expression becomes restricted to deeper layers of articular cartilage after birth in the mouse [9]. As a result, the inconstant distribution of Notch1 factors suggests that the generation of the condylar cartilage does not correspond with morphogene mechanism in articular cartilage. It is strongly suggested that mandibular condylar and articular cartilage differ slightly from physiological articular cartilage. In this examination, we investigated only mandibular condyle cartilage for a short period. In the future, we hope to investigate further for longer periods after birth.

In this examination, expression of NICD at the cell membrane might relate to cell-to-cell intercommunication from E15 up to just after birth. NICD translocates from the cell membrane to the nucleus, which act as a transcriptional activator and regulating gene expression through the examination period. At E14, Notch1 expression was detected by means of IHC and ISH examination, which indicates that Notch1 expression leads to mesenchymal cells further differentiating into chondrocytes as a secondary cartilage. We have previously reported that Runx2 is essential for the onset of mandibular condylar cartilage development, with examination of type II collagen by IHC and TB stain [6]. We expected NICD also to be essential for the onset of mandibular condylar cartilage development. At E17 and E18, NICD expression appeared in hypertrophic cartilage in IHC specimens. This agrees with our past research which explained that the Runx2 expression of IHC and ISH is present in the hypertrophic layer and also takes part in the endochondral ossification mode [6]. Yasui *et al.* have reported that three modes of ossification during distraction osteogenesis in the rats [28, 29], and have been suggested that some hypertrophic chondrocytes undergo further differentiation into osteobalast-like cells and participate in initial bone formation. The results of the present study support that hypertrophic chondrocytes further differentiate into osteoblasts. At E17 and E18, Our examination results showed the distribution of NICD expression for IHC at the cartilage inside of the sheath of mandibular condylar cartilage development, where direct bone formation occurs. NICD expression might relate to perichondral ossification in which mesemchymal cells differentiate into osteoblasts. On the other hand, the Notch1 gene was expressed at weak levels in the cartilage cytoplasm inside the sheath of mandibular condylar cartilage. Finally, Notch1 gene was continuously expressed in the cartilage cytoplasm in ISH specimens throughout the examination period. Notch1 was detected by means of IHC and ISH examination, which indicates that the Notch1 expression leads to secondary chondrocyte differentiation, especially in morphogenesis during embryonic stage. Furthermore Notch1 distribution intensity is not uniform, thus it is suggested that there is some reason at mandible condylar cartilage development. The examination of the expression of these factors in developing mandibular condylar cartilage in embryonic mice is now progressing. We will report the results in the near future.

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