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Fetal jaw movement affects Ihh signaling in mandibular condylar cartilage development: The possible role of Ihh as mechanotransduction mediator

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ABSTRACT

Objective: Jaw movement is an important mechanical factor for prenatal development of the condylar cartilage of mandible. Fetal jaw movement restriction has been shown to cause deformity of the mandibular condyle. We hypothesized that this treatment affects the expression of mechanosensitive molecules, namely Indian hedgehog (*Ihh*) and Parathyroid hormone related protein (*PTHrP*) in the condyle.

Experimental methods: We restrained jaw movement by suturing the jaw of E15.5 mouse embryos and allowed them to develop until E18.5 using *exo utero* system, and analyzed them by immunohistochemistry and *in situ hybridization* methods.

Results: Morphological, histomorphometric and immunohistochemical study showed that the mandibular condylar cartilage was reduced and deformed, the volume and total cell numbers in the condylar cartilage were also reduced, and number and/or distribution of 5-bromo-2′-deoxyuridine-positive cells, Ihh-positive cells in the mesenchymal and pre-hypertrophic zones were significantly and correspondingly decreased in the sutured group. Using *in situ hybridization*, reduced expression of *Ihh*, *PTHrP* and their related receptors were observed in condylar cartilage of the sutured embryos.

Conclusions: Our results revealed that the altered mechanical stress induced by prenatal jaw movement restriction decreased proliferating cells, the amount of cartilage, and altered expression of the *Ihh* and *PTHrP*, suggesting that *Ihh* act as mechanotransduction mediators in the development of mandibular condylar cartilage.

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1. Introduction

Mechanical stress plays a fundamental role in regulating cellular activities during tissue morphogenesis.[1,2] It has also been shown that many developmental processes depend on external mechanical cues and internal molecules that sense mechanical signals.[3–5]

Previous studies have shown that chondrocyte proliferation and thickness of condylar cartilage in the mandible are modulated by mechanical factors associated with temporomandibular joint (TMJ) movement in the postnatal period,[6–11] and that decreased loading, promoted by cutting incisive teeth and/or a soft diet, decreases cell proliferation, extracellular matrix production,[12] and bone formation[13] in the mandibular condyle.

We have shown that prenatal jaw movement is an important mechanical factor in endochondral bone formation of the mandibular condyle, articular disc and temporalis muscle,[14–16] and Keith has suggested a possible clinical significance.[17] The morphological analyses in these studies and the further mathematical analysis[18] showed that fetal jaw movement restriction leads to mandibular condyle deformity, but it remains unknown which molecules are affected by mechanical strain due to the restriction of jaw movement.

The hedgehog family proteins have been identified as key morphogens during skeletal development and regeneration, and among them, Indian hedgehog (*Ihh*) plays roles in late limb development by regulating chondrocyte proliferation and differentiation.[19]

Ihh has been shown to be an essential mediator that connects mechanical stress to proliferation of cultured chondrocytes from chick embryonic sternum,[20] and a mechanotransduction mediator that converts mechanical signals resulting from forward mandibular positioning to stimulate cellular proliferation in the condyle of adult rats.[21] Subsequent studies on $Ihh^{-/-}$ mice established that *Ihh* and its receptor Patched1 (*Ptc1*) signaling plays roles in growth of the mandibular condyle in mouse fetuses.[22] Moreover, a close relationship has been suggested between *Ihh* and *PTHrP* in the signaling induced by mechanical strain in cartilage and bone development.[22] *Ihh* and *PTHrP* are two of the major members of the feedback loop which regulate chondrocyte proliferation and the timing of the decision to exit the proliferative pool and differentiate,[23–25] however, it is still unclear whether the fetal jaw movement restriction affects the molecular cascade and the feedback loop involving by *Ihh*, *PTHrP* and their respective receptors (*Ptc1*, *PTHrP-R*) in the prenatal development of mandibular condylar cartilage.

Therefore, to elucidate the mechanism of the effects of mechanical stimulation on the fetal mandibular condylar cartilage, we examined the effect of restriction of prenatal jaw movement on the expression of *Ihh* and *PTHrP* using an *exo utero* surgery system.

2. Materials and methods

2.1. Animals

Female Jcl:ICR mice aged 8–20 weeks (CLEA, Tokyo, Japan) were used. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Graduate School of Medicine, Shimane University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Shimane University (Permit Number: IZ24-113). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. Jaw movement restriction by exo utero surgery

A female mouse was housed with a potent male overnight, and noon of the day when the vaginal plug was found was designated as the E0.5. *Exo utero* surgery was performed at E15.5 as described previously (for general procedures see,[26–28] for restrictions of fetal jaw movement see[14–16]). Intrauterine jaw movement starts on E14 in mice.[29] E14 mouse embryos were too small for manipulation (jaw movement restriction by suture) and after jaw movement restriction at E14, the survival rate of the embryos was very low. At E15.5, the survival rate of the embryos was still low but much better than E14. Briefly, at E15.5, the pregnant dams were anesthetized with 50 mg/kg body weight (BW) of pentobarbital. The abdominal wall was incised longitudinally at the midline from beneath the xiphoid process to the lower abdomen. Then detached the skin from the abdominal muscles, and a longitudinal incision of the anterior abdominal muscles was made. Then, we pulled gently the intact right (or left) uterine horn out of the abdomen. Longitudinal incision was made on the myometrium at the counter side of the placenta, exposing the fetuses covered by the embryonic membrane. The umbilical cords of unnecessary fetuses were cut with scissors and the fetuses were removed. We left three or four fetuses in each horn of the uterus for manipulation. One sided (left or right) fetuses' mandible and maxilla were fixed by an 8-0 nylon suture and designed as the sutured group. We also performed a sham operation by passing the needle from the mandible through the maxilla of opposite sided fetuses without making a knot, designed them as the sham-operated group. After the operation, the fetuses together with the uterus were placed back in the abdominal cavity of the dam, and the incision on the abdominal muscle was sutured closed with 3- or 4-0 silk line; incision of the abdominal skin was closed by auto suture (MikRon 9 mm Autoclip Applier). The embryos were allowed to develop *exo utero* till E18.5.[14–16]

We also prepared E18.5 *in utero* control group embryos with absolutely no procedure done to compare with the sham-operated group. Since we found no significant difference between them,[14–16] (data not shown), we here show only data from the sham-operated and sutured groups.

2.3. CT scan and 3-D analysis of the mandible, and bone staining

The dams were killed at E18.5 by cervical dislocation under anesthesia with pentobarbital. After confirmation by measuring BW and crown rump length (CRL) that there was no general systemic growth retardation[14–16,26] (data not shown), we performed CT scan and 3-dimensional (3-D) analyses for observing deformity in the sutured mandibles[18] (Fig. 1A–D). We compared between the sutured and sham-operated