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ORIGINAL ARTICLE

Alteration of BMP-4 and *Runx2* expression patterns in mouse temporomandibular joint after ovariectomy

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OBJECTIVE: Temporomandibular disorder (TMD) includes a number of clinical conditions involving the masticatory musculature or the temporomandibular joint (TMJ) and associated structures. Previous studies have shown the presence of high-affinity estrogen receptors in the TMJ articular cartilage. The aim of this study was to evaluate the developmental changes in mouse TMJ under estrogen deficiency.

MATERIALS AND METHODS: Four-month-old ovariectomized mice were killed after certain weeks. We examined the significant alterations of the expression patterns of bone morphogenetic protein (BMP)-4, *Runx2*, and bone sialoprotein (BSP) after ovariectomy.

RESULTS: In the control group, BMP-4, *Runx2*, and BSP expressions showed no definite difference at any stage. In the ovariectomy group, the intensity of BMP-4 and *Runx2* expression increased after ovariectomy. BSP immunoreactivity, however, increased slightly at 2 weeks but then decreased gradually.

CONCLUSIONS: Estrogen plays important roles in the metabolism and maintenance of TMJ via regulations of signaling molecules such as BMP-4, *Runx2*, and BSP. Our results suggest that estrogen deficiency is a candidate cause of TMD. This study revealed further osteogenetic properties of estrogen that may be useful in the clinical treatment and prevention of TMD.

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Keywords: temporomandibular joint; temporomandibular disorder; estrogen; BMP-4; *Runx2*; BSP

Introduction

The temporomandibular joint (TMJ) gives raise to the unique mammalian articulation of the tooth-bearing lower jawbone with the temporal bone of skull base. The formation of the temporal and condylar portions of the joint begins around 7–7.5 weeks of gestation in human fetuses (Van der Linden *et al*, 1987). The condyle originates as a condensation of mesenchymal tissue separated from the mandibular body and ramus, inferiorly, and from the temporal region, superiorly. The condylar blastema grows inferiorly to join with the advancing bone front and periosteum of the ramus, and posterolaterally toward the temporal blastema (Perry *et al*, 1985). In mouse TMJ development, bone formation starts at embryonic day 15 (E15) in the tibial cartilage and at E16 in the condylar cartilage. The hypertrophic cell zone in the condylar cartilage extends rapidly during E15 and E16 (Shibata *et al*, 2002).

Temporomandibular disorder (TMD) includes a number of clinical conditions involving the masticatory musculature or the TMJ and associated structures. Although the etiology of this disease is not fully understood, TMD has been assumed to be caused by a combination of factors, such as occlusion, mental stress, strength, and endurance. Although the prevalence of TMD is a matter of debate, that women make up the majority of patients treated for TMD is extensively hypothesized and documented in numerous epidemiologic studies (Campbell *et al*, 1993; Koidis *et al*, 1993; Abubaker *et al*, 1996). This disorder is 1.5–2 times more prevalent in women than in men, and 80% of patients treated for TMD are women (LeResche, 1997). Pain onset tends to occur after puberty and peaks in the reproductive years (Meisler, 1999), with the highest prevalence in women aged 20–40 years (Kuttila *et al*, 1998). The gender and age distribution of TMD suggests a possible link between its pathogenesis and the female reproductive system.

It has been shown that estrogen deficiency has an effect on general bone condition; however, only a few

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studies have been carried out specifically on the mandibular condyle (Tanaka *et al*, 1999). Recently, quantitative computed tomography has been used to measure mandibular condyle bone mineral density. The results demonstrated that condylar bone mineral density decreases quickly after menopausal osteoporosis (Yamada *et al*, 1997). This brings to light the possible clinical consideration that general osteoporosis could alter the structure of the mandibular condyle. In another study investigating the effects of ovariectomy on young rat TMJ, the thickness of the articular soft tissue was reported to increase after ovariectomy, while bone volume decreased (Okuda *et al*, 1996).

In order to understand the mechanism of action and effect of estrogen in these cases, the expression patterns of bone formation markers were investigated in the TMJ after ovariectomy. *Runx2* is a transcriptional factor that belongs to the runt-domain gene family. Each Runx family protein has a unique function. *Runx1* is essential for definitive hematopoiesis (Komori, 2003), *Runx2* plays important roles in the multiple steps of skeletal development and *Runx3* is a major growth regulator of gastric epithelial cells (Li *et al*, 2002). *Runx2* is important in osteoblast differentiation from multi-potent mesenchymal cells. It enhances osteoblast differentiation at an early stage, and inhibits osteoblast differentiation at a late stage (Liu *et al*, 2001). *Runx2* is up-regulated by BMP-4, and this interaction between *Runx2* and BMP-4 starts from common signaling molecules called Sma and Mad (Smads) (Yoshiaki and Kohei, 2003). BMP-4 induces the formation of both cartilage and bone in cell proliferation, apoptosis, differentiation, and morphogenesis (Reddi, 2001). Bone sialoprotein (BSP) is one of the non-collagenous proteins, and a relative of acidic Ca-binding proteins such as bone Gla protein (BGP), osteopontin, osteonectin, etc. While other proteins are also present in non-mineralized tissues, BSP is present at sites of bone formation (Fujisawa and Kuboki, 1998).

In this study, to clarify the relationship between estrogen and TMD, 4-month-old mice were examined for BMP-4, *Runx2*, and BSP expression patterns between control and ovariectomized groups. Morphological changes in the condyle during development were studied using hematoxylin and eosin (HE) and Alcian blue staining of embryonic day 18 (E18), postnatal (PN) 2 and 6 (PN2) and (PN6) mice. This study focused mainly on molecular expression patterns as markers of osteogenic capacity to define the causative factor for TMD, but not on gross morphologic changes such as bone volume and soft tissue change. These results would be helpful to understand the clinical considerations of TMD associated with estrogen deficiency.

Materials and methods

All experiments were performed according to the guidelines of the Intramural Animal Use and Care Committee, College of Dentistry, Yonsei University.

Experimental design

Four-month-old female ICR mice weighing 35–40 g were used for this study. The animals were randomly divided into two groups [control and ovariectomized (OVX)]. The animals from each group were killed by cervical dislocation at 1, 4 and 8 weeks post-ovariectomy. The heads were separated immediately and the TMJ dissected bilaterally. Mouse embryos were obtained from time-mated pregnant mice. The day that the presence of a vaginal plug was confirmed was designated as embryonic day 0 (E0). E18, postnatal (PN) 2 and 6 mice were used. Animals were housed by group, and received a standard equilibrated diet and tap water. Room temperature and humidity were maintained at 22 ± 2°C and 55 ± 5%, respectively, and the room was artificially illuminated (lights on from 05:00 to 17:00 hours).

Surgical procedure

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (2.5 mg/100 g of body weight). Bilateral ovariectomies were performed by the ventral approach, with an incision made 1 cm above the vaginal orifice. In order to control bleeding, the ligaments were tied and cut.

Hematoxylin–eosin and Alcian blue staining

Four-month-old female ICR mice were perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). The TMJ with surrounding tissues were dissected and further fixed in 4% PFA in 0.1 M PBS overnight at 4°C. The tissues were decalcified with 0.5 M ethylenediaminetetraacetic acid (EDTA) for 8 weeks at 4°C. They were then passaged through gradations of ethanol and xylene, and embedded in paraffin; 8-μm serial sections were cut through the central area of the mandibular condyle along the sagittal plane, mounted on coated slides that electrostatically attract the sections and stored at room temperature. The sections were stained with Alcian blue for 30 min and washed with running tap water for 2 min. After rinsing, they were counterstained in nuclear fast red solution for 5 min. They were washed again and dehydrated. After clearing with xylene, they were mounted with a resinous mounting medium.

Immunohistochemistry

Eight-micrometer sections were blocked in 0.3% hydrogen peroxide for 15 min. The tissue sections were boiled in 10 mM citrate buffer (pH 6.0) for 10 min and cooled at room temperature for 20 min. The slides were incubated in rabbit polyclonal antibody against bone sialoprotein (BSP) (Cat. no. AB1854; Chemicon, Temecula, CA USA), mouse monoclonal antibody against bone morphogenic protein (BMP)-4 (Cat. no. SC-12721; Santa Cruz, CA, USA) and mouse monoclonal antibody against collagen type X (Cat. no. V10200; Biomeda Corp., Foster City, CA USA) at 4°C overnight. After washing with PBS, the specimens were made to reacted, with two consecutive incubations, with the biotinylated goat anti-rabbit secondary antibody, anti-mouse secondary antibody and streptavidin peroxidase at room temperature

for 10 min each. Finally, the specimens were visualized using a diaminobenzidine (DAB) reagent kit (Cat. no. 00-2014; Zymed, San Francisco, CA, USA). The immunostained sections were counterstained with hematoxylin.

In situ *hybridization*

In situ hybridization on wax sections was performed as previously described (Eblaghie *et al*, 2004). To construct DIG-labeled RNA probes, a 400-bp fragment of murine *Runx2* cDNA was subcloned into pBluescript KS (Cat. no. 212207, Stratagene La Jolla, CA, USA). The *Runx2* plasmid was kindly gifted by Dr. H.-M. Ryoo. The sense probe was constructed by digestion with *Sac*I and T3 RNA polymerase transcription.

Results

Developing TMJ morphology at E18, PN2, and PN6

In this study, we examined the detailed morphological changes in TMJ development using HE and skeletal staining. These developmental observations in TMJ formation are helpful to understand the precise alterations of TMJ after ovariectomy. To understand the precise morphogenesis of early mouse TMJ development, serial histological sections of the lateral head region were studied in detail. The articular cartilage is composed of fibrous, prehypertrophic, hypertrophic and calcified cartilage layers in E18, PN2, and PN6 mice (Figure 1). In general, histological sections show similar morphological features in all three stages, but distinct differences are seen during detailed observations. In E18 mice, the articular cartilage overlies the mandibular condyle (Figure 1A–C), the TMJ articular cartilage was thicker with a large hypertrophic chondrocyte zone (Figure 1A, asterisks). The morphology of the condylar neck had become prominent by PN6. In the TMJ, endochondral bone formations gradually progressed from E18 to PN6. Figure 1D–F shows histological sections stained with Alcian blue. Strongly acidic mucosubstances (cartilage) stained blue, nuclei stained pink to red, and cytoplasm stained pale pink. In E18 (Figure 1D) the cartilage had an irregular border that was thicker than in PN2 (Figure 1E) and PN6 (Figure 1F) cartilages. In addition, in E18 the undifferentiated fibroblast-like cell layer was covered by a thin layer of cartilage (Figure 1D, arrowheads), unlike in PN2 and PN6. In all three stages, endochondral bone formation was progressively more evident.

Expression patterns of BMP-4, Runx2*, BSP, and collagen type X in TMJ*

To clarify the effect of estrogen deficiency on general bone condition, the expression patterns of key signalling molecules (BMP-4, *Runx2*, and BSP) were examined in the TMJ. The expression intensities of BMP-4, *Runx2*, and BSP are summarized in Table 1. Our results showed that BMP-4 and *Runx2* expression was more dominant in the OVX group than in the control group. BSP, however, showed weaker expression in the OVX group than in the control group.

Figure 2A shows the TMJ articular cartilage in 4-month-old female mice. The cartilage was relatively thick with a large hypertrophic chondrocyte zone. This

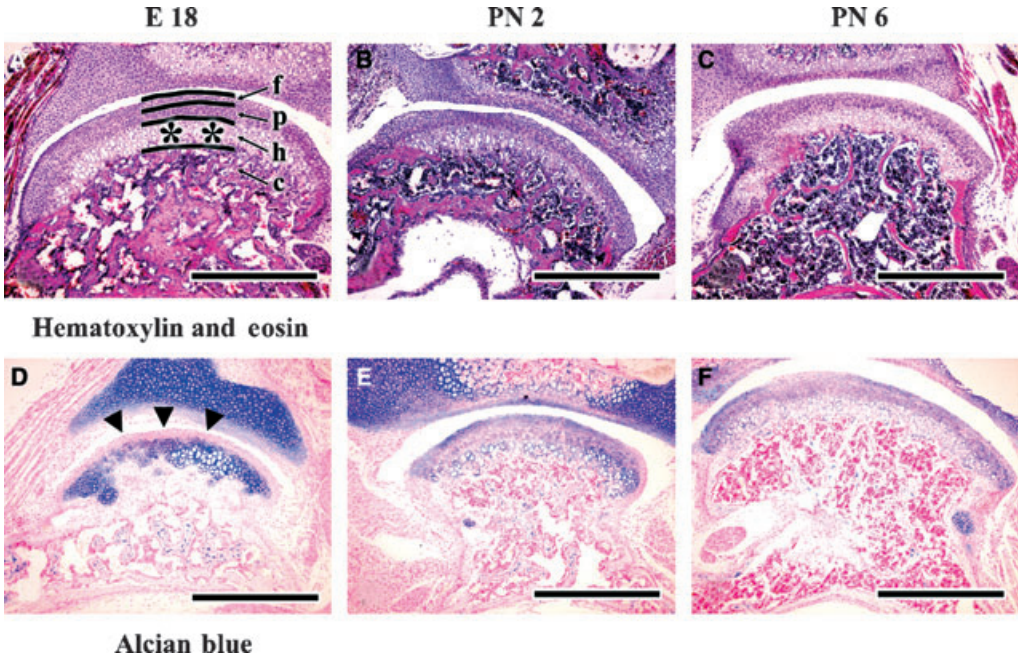

Figure 1 HE and Alcian blue staining of E18, PN2, and PN6. (A) At E18, the mandibular condyle is shown, and the hyaline cartilage is overlying the condyle. HE staining shows the (f) fibrous, (p) prehypertrophic, (h) hypertrophic (asterisks) and (c) cartilage layers of the articular cartilage. (A, B, C) From E18 to PN6, the condylar neck morphology becomes prominent. As seen by Alcian blue staining, the cartilage gets thinner. (D) At E18, unlike PN2 and PN6, undifferentiated fibroblast-like cells are covering the cartilage (arrowheads). (D, E, F) From E18 to PN6, subcondylar endochondral bone formation is more evident

Table 1 Expression results of BMP-4, *Runx2* and BSP in TMJ to control group and ovariectomy group

		Control			*Ovariectomy*		
Layer	*Stage*	*BMP4*	*Runx2*	*BSP*	*BMP4*	*Runx2*	*BSP*
A	1 week	±	+	+ +	±	+	+ +
B	Control (n = 7)	±	+	+ +	+	+	+ +
C	Ovaricctomy (n = 7)	+	+	+ +	+	+	+ +
A	4 weeks	±	+	+ +	±	+ +	+
B	Control (n = 7)	±	+	+ +	+	+ + +	+
C	Ovariectomy (n = 7)	+	±	+ +	+	±	+
A	8 weeks	±	+	+	±	+ +	+
B	Control (n = 7)	±	+	+	±	+ +	+
C	Ovariectomy (n = 7)	±	±	+	±	±	+

The expressions of BMP-4, *Runx2*, and BSP were referred from our previous study in mouse TMJ (Figure 2) after ovariectomy and their expression degree is classified.
−, No staining; ±, weak staining; +, moderate staining; + +, strong staining; + + +, very strong staining.
A, Prehypertrophic layer; B, Hypertrophichy chondrocyte layer; C, Subchondral bone; n, the number of animals.

indicates the presence of relatively high endochondral ossification. BMP-4 is slightly expressed in the prehypertrophic and hypertrophic chondrocytes and in the subchondral bone area. The latter area showed terminal chondrocyte differentiation that eventually resulted in blood vessel ingrowth, recruitment of osteoblast precursors and ossification (Figure 2A,E). In the control group, BMP-4 was examined at 1 and 4 weeks (Figure 2B,C). BMP-4 expression was greatly decreased by 8 weeks when compared with the earlier time points (Figure 2D). BMP-4 was detected mainly in the cytoplasm of cells lining the bone marrow. In the OVX group (Figure 2F–H) at 1 week (Figure 2F), BMP-4 expressed strongly throughout all regions of the TMJ, namely, the cartilage layer and the subcondylar bone area. The strength of the staining decreased with time, until at 8 weeks (Figure 2H) no difference could be detected between the control and OVX groups.

In order to determine the association between osteogenesis and estrogen, *Runx2* expression was observed in the TMJ in both the control and ovariectomy groups. In 4-month-old mice, *Runx2* was broadly localized in the cartilage and ossification areas (Figure 2I,M). In the control group (Figure 2J–L), *Runx2* expression was detected at all time points with similar signal intensity. *Runx2* expression was limited in the prehypertrophic and hypertrophic chondrocyte layers and weak in the subcondylar bone area. In the OVX group (Figure 2N–P), *Runx2* expression was more evident than in the control group. Staining progressively increased from 1 to 4 weeks, peaked at 4 weeks and slightly declined at 8 weeks. *Runx2* expression was visualized mainly in the prehypertropic and hypertrophic chondrocyte layers, although faint staining was seen in the subcondylar bone area.

The numbers of noncollagenous cells in the control and OVX groups were visualized by staining with BSP antibody. BSP is the major noncollagenous protein in collagen-based mineralized tissues, and has been implicated in mineral deposition, cell–matrix and matrix–matrix interactions during root development (Bosshardt and Nanci, 1998). In the control group (Figure 2Q–U), staining increased from 1 to 2 weeks, but returned to baseline by 8 weeks (Figure 2Q,T,U). In the OVX group (Figure 2V–X), many cells in the cartilage and subcondylar bone exhibited strong BSP expression at 1 week (Figure 2V). From 2 to 8 weeks, the number of positive cells decreased gradually. At 8 weeks, we could not find any difference between the control and OVX groups.

Immunolocalization of the collagen type X apparently distinguished the fibrous layer, such as the 'articular zone,' 'articular layer,' and 'resting zone,' from the hypertrophic zone. To identify the hypertrophic zone of the developing condyles, we examined the localization of collagen type X, a marker for the hypertrophic zone (Figure 2a–h). Interestingly, the ratio of the collagen type X-positive hypertrophic zone in the entire condyle seemed higher in the ovariectomy groups (Figure 2f–h), but was attenuated in the control groups (Figure 2a–e).

Discussion

TMD in connection with estrogen

The gender and age distribution of TMD suggests a possible link between its pathogenesis and the female reproductive system. In general, the effects of estrogen deficiency on bone conditions have been studied; however, only a few studies have specifically dealt with the mandibular condyle (Tanaka *et al*, 1999). Recently, computed tomography has been used to measure mandibular condyle bone mineral density. These measurements have demonstrated that condylar bone mineral density decreases quickly after menopausal osteoporosis (Yamada *et al*, 1997). This indicates the clinically important possibility that general osteoporosis could alter the structure of the mandibular condyle. It has also been reported that the mandibular condyles of adult ovariectomized rats showed significantly lower bone volume 1 month after ovariectomy (Tanaka *et al*, 2000). Previous studies have shown the presence of high-affinity estrogen receptors in human TMJ articular cartilage, articular disk, and synovial membrane (Abubaker *et al*, 1993). Because these hormones exert their influence on target cells through sex steroid receptors, it can be assumed that TMJ is an estrogen target organ. The fact that estrogen receptors are found in the articular disk implies that an estrogen deficiency can induce both bone-related changes and disk problems. In a study using castrated rats, steroid sex hormones were found to have an effect on TMJ disk collagen and protein content (Abubaker *et al*, 1996). Another study concluded that estrogen deficiency has the potential to cause disk disease (Ng *et al*, 1999). Hormones have been suggested to be a major factor defining the host-adaptive capacity of the TMJ (Arnett *et al*, 1996). According to research diagnostic criteria for TMD, TMJ osteoarthritis, which is one of the entities of TMD, can be defined as an inflammatory condition

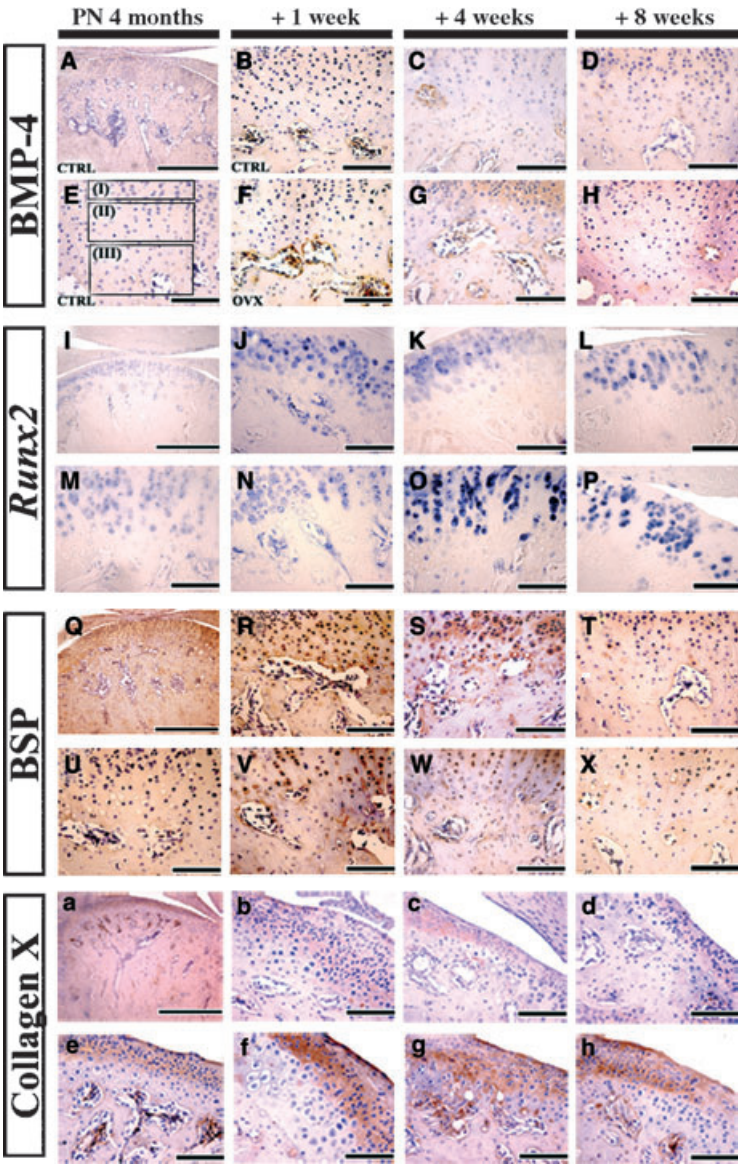

Figure 2 BMP-4, *Runx2*, BSP and collagen type X expression patterns in the TMJ.
(A–H) BMP-4 expression as seen by immunohistochemistry at 1, 4 and 8 weeks in the control and OVX groups. (A, E) The TMJ articular cartilage of 4-month-old female mice ('E' is higher magnification of 'A'). The (I) prehypertrophic (II) hypertrophic chondrocytes and (III) subchondral bone layers can be seen. In the control group, BMP-4 expression is detected at 1 and 4 weeks (B, C), but is faint at 8 weeks (D). BMP-4 expression is seen mainly in the cytoplasm of the bone marrow lining. (F, G, H) In the OVX group, BMP-4 is strongly expressed throughout the cartilage layer and subcondylar bone area at 1 week (F). BMP-4 expression decreases from 1 to 8 weeks. (H) Finally, at 8 weeks no difference can be detected between the control and OVX groups.
(I–P) *Runx2* expression with *in situ* hybridization at 1 week, 4 weeks and 8 weeks in the control and OVX groups. (I, M) In 4-month-old mice, *Runx2* was broadly localized in the cartilage and ossification area ('M' is higher magnification of 'I'). (J, K, L) In the control group, similar signal intensity is noted, except the 1-week group. *Runx2* expression was detected in all groups and similar signal intensity was noted. *Runx2* expression was limited in the prehypertrophic and hypertrophic chondrocyte layers. Faint expression was detected in the subcondylar bone area. (N, O, P) In the OVX group, more progressively intensive staining is noted from 1 week group (N) to 8 weeks group (P). More progressively, intensive staining was noted from the 1-week group to the 4-week group, and reached to peak at the 4-week group, but slightly declined in the 8-week group.
(Q–X) BSP expression as visualized by immunohistochemistry at 1, 4 and 8 weeks in the control and OVX groups. (Q, U) BSP expression was noted at all stages ('U' is higher magnification of 'Q'). (R, S, T) In the control group, BSP expression increased slightly from 1 to 2 weeks (data not shown). (S) At 4 weeks and (T) 8 weeks, BSP expression returned to baseline. (V, W, X) In the OVX group, expression increased slightly at 1 week, and then decreased with time. All control groups expressed more strongly than the OVX groups. CTRL denotes the control group and OVX denotes the ovariectomy group.
(a–h) Collagen type X localization was visualized by immunohistochemistry at 1, 4 and 8 weeks in the control and OVX groups ('e' is higher magnification of 'a'). The collagen type X expression was significantly increased in the ovariectomy groups (f, g and h), whereas its levels were extremely decreased in the control groups' hypertrophic zone (a–e)

within the joint resulting from a degenerative condition of the joint structures. The combined influence of estrogen and inflammation may contribute to the profound gender difference observed in the frequency, duration, and severity of TMD pain in humans (Flake *et al*, 2005).

Early TMJ development

In E18 mice, the condylar cartilage is composed of fibrous, proliferating, hypertrophic, and calcified cartilage layers. The hypertrophic cell layer was much more enlarged in E18 than in PN2 or PN6 mice. These findings are consistent with a previous report (Shibata *et al*, 2002). The increase of the hypertrophic chondrocyte layer implicates the active forming phase of the endochondral bone. Subchondral bone formation also tends to increase with time. In Figure 1D, the arrowheads indicate the layer not stained by Alcian blue. This layer becomes thinner and more even from E18 to PN6. This layer indicates a very primitive developmental stage and that TMJ has a different developmental origin than other joints. While the TMJ is synovial, it develops via intramembranous bone formation and is characterized by an independent blastema and secondary cartilage formation. Therefore, it can be speculated that the fibrous connective tissue cells lining the TMJ articular surface do not have the same features as the hyaline articular cartilage.

Expression patterns of signal molecules in TMJ

Bone morphogenetic proteins (BMP) are the members of the TGF-β superfamily and have been implicated in tissue growth and remodeling. BMPs were initially identified by the ability of bone extracts to induce bone formation at extra-skeletal sites (Wozney, 1989). BMPs induce the formation of both cartilage and bone (Reddi, 1998). BMP-4 was expressed in regenerating cartilage and bone cells (Onishi *et al*, 1998). The steady BMP-4 expression seen in the control group is consistent with the findings of Koike *et al* (1995). They found that the bone mass increase of the subchondral and central regions in a sham group corresponds with the age-related increase (Koike *et al*, 1995). In the OVX group, BMP-4 was highly expressed throughout the TMJ. Tanaka *et al* (1999) found that the mandibular condyle, under intermittent loading by occlusion, frequently stimulated bone formation. Furthermore, this counteracted the accelerated bone resorption induced by estrogen deficiency (Tanaka *et al*, 1999).

Runx2 plays an important role in multiple steps of skeletal development including enhancing osteoblast differentiation at the early stage and inhibiting osteoblast differentiation at the late stage. Therefore, *Runx2* expression is seen in all stages in both the control and OVX groups. *Runx2* is highly expressed especially in the prehypertrophic and hypertrophic chondrocyte layers. It is faint, however, in the subchondral bone area. This coincides with Komori's (2000) findings that *Runx2* regulates chondrocyte hypertrophy, osteoblast differentiation, and vascular invasion. In the control group, *Runx2*, like BMP-4, showed persistent expression. These findings suggest that the TMJ continuously undergoes remodeling. In the OVX group, however, *Runx2* expression increased with time. Furthermore, the *Runx2* expression is more evident in the OVX group than in the control group. This expression pattern suggests that the effort to form bone continues during estrogen deficiency.

An immunopositive reaction to BSP was noted at all stages in the control group. In the OVX group, many cells in the cartilage and subcondylar bone area showed strong immunopositive reaction especially at 1 week. This staining, however, decreased with time and by 8 weeks, only faint staining was seen.

Regulation of related bone markers in the TMJ

Steroids significantly affect skeletal integrity. For example, bone mass decreases if glucocorticoids are in excess or when estrogen levels decrease after menopause. Other studies have found that steroid hormones can significantly enhance the transcription potential of *Runx2* in isolated osteoblasts. This correlates with the formation of a physical complex between *Runx2* and hormone-activated ERα. Another study reported that *Runx2* is up-regulated by BMPs (Ducy *et al*, 1997). In addition, *Runx2* acts as a positive regulator of BSP expression and is a direct regulator of BSP in osteoblasts (Roca *et al*, 2005). We focused our study on defining the functional and physical interaction between steroids, *Runx2*, and the related osteogenic markers such as BMP-4 and BSP. There is evidence suggesting mutual interactions among these molecules, but no specific details about how they are regulated in ovariectomized mice have been presented. We speculated that the mice with an estrogen deficiency make significant modifications to keep osteoblast activity at wild-type levels. The bone induction markers (BMP-4 or *Runx2*) were more dominant in the OVX group than in the control group. Moreover, the highest levels of localization of collagen type X were present on the TMJ of the OVX groups. Previous report suggested that the ratio of the collagen type X-positive hypertrophic zone in the entire condyle seemed higher in the early stages but decreased in the later stages (Hossain *et al*, 2005). These findings indicate that osteoblast activity changes in the estrogen deficiency environment of the TMJ of adult mouse that leads to bone formation. The bone formation marker (BSP) expression, however, was weaker in the OVX group than the control group (Figure 3). These results suggest that bone formation induced by occlusal loading was not sufficient to completely turn off OVX-induced bone resorption, because bone volume in the OVX group was significantly lower than that of the sham group at 60 days. Ovariectomy has also been previously shown to clearly inhibit a gain in bone mass (Tanaka *et al*, 1999). So we suggest that ovariectomy in mice could mimic the menopausal and puberty condition in humans. In conclusion, estrogen plays an important role in the metabolism and maintenance of the TMJ by regulating molecules such as BMP-4, *Runx2* and BSP.

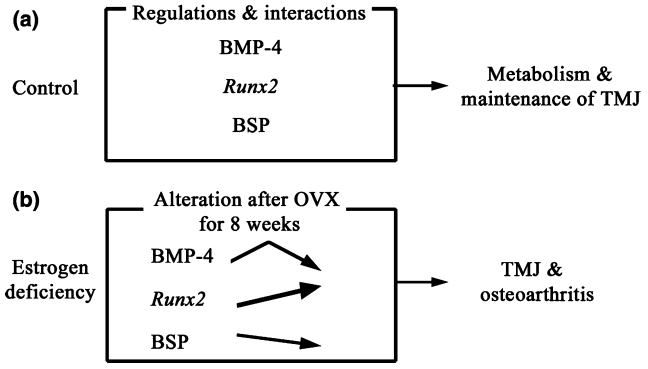

Figure 3 Schematic diagrams regulation of key molecules in TMJ (a) Control: estrogen plays an important role in the metabolism and maintenance of the TMJ by regulating molecules such as BMP-4, *Runx2* and BSP. (b) Estrogen deficiency: the expressions of bone induction markers (BMP-4 or *Runx2*) were more dominant in the OVX group than in the control group. The bone formation marker (BSP) expression was weaker in the OVX group than in the control group

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