Effects of Mechanical Stress and Deficiency of Dihydrotestosterone or 17β-Estradiol on Temporomandibular Joint Osteoarthritis in Mice

Abstracts

Objective: To observe and analyze the interaction between excessive mechanical stress (MS) and decreased sex hormones on Temporomandibular Joint Osteoarthritis (TMJ-OA), and to discover TMJ-OA disease susceptibility genes by molecular biological analysis to elucidate part of the mechanism of TMJ-OA onset.

Design: For experimental groups, orchiectomy (ORX) or ovariectomy (OVX) was performed on sexually mature 8-week-old mice. A metal plate was attached to the posterior surface of the maxillary incisors to apply excessive MS on mandibular condyles. Male mice were divided into control, ORX, MS, and ORX+MS groups, while female mice were divided into control, OVX, MS, and OVX+MS groups. Mandibular condyles were evaluated by histology and molecular biology.

Results: Histomorphometric analysis of the TMJ in ORX+MS and OVX+MS groups revealed the thinnest chondrocyte layers, highest modified Mankin scores, and significant increases in the number of osteoclasts. Gene expression analysis indicated upregulation of *Angptl7* and *Car1* genes in the mandibular condyles of mice subjected to the combined effects of excessive MS and reduced sex hormones. In vitro analysis suggested that cartilage-like cells overexpressing *Angptl7* enhanced calcification, and osteoblast-like cells overexpression *Car1* suppressed cell proliferation and calcification.

Conclusions: A severe TMJ-OA mouse model was successfully developed by applying

excessive MS on the mandibular condyle of male and female mice with reduced sex hormones. Disease-susceptibility genes *Angptl7* and *Car1* were newly discovered in the experimental groups, suggesting their involvement in the onset mechanism of TMJ-OA.

Introduction

Temporomandibular Joint Osteoarthritis (TMJ-OA) is characterized by progressive cartilage degradation, subchondral bone erosion, and chronic pain, which in severe cases significantly reduces the patients' quality of life. Extensive research has been conducted on TMJ-OA, but its etiology remains unknown.

Age, hormonal factors, and excessive mechanical stress (MS) on the TMJ are proposed risk factors for TMJ-OA[1]. Mandibular condylar cartilage (MCC) is a type of fibrocartilage derived from periosteal tissue. Unlike other skeletal joints composed of hyaline cartilage, the size and characteristics of tissues in fibrocartilage adjust to changes in load[2]. In a decreased occlusal-loading mouse model, a reduction in MCC thickness was observed[3]. However, overload of MS on the TMJ resulted in rapid degradation of cartilage due to imbalanced remodeling of extracellular matrix components [4,5].

The number of patients with TMD seeking treatment was reportedly three to nine times higher in females compared to males, suggesting a correlation between sex hormones and TMD[6]. Severe TMJ-OA has been reported in young females whose blood estrogen levels were medically reduced[7]. The World Health Organization reported that 80.7% of women (mean age of 20.9 years) experienced irregular menstrual cycles[8]. In men, the prevalence of hypogonadism is very low: approximately 5% between the ages of 20–29[9], and total serum testosterone levels are reportedly stable until the age of 70[10]. Therefore, differences in sex hormone levels between men and women may be associated with TMJ-OA prevalence.

In a recent study, the combination of excessive MS and estrogen elicited more severe TMJ-OA than either single factor alone [11]. Mice with reduced sex hormone levels due to orchiectomy (ORX) and ovariectomy (OVX) showed lower overall bone density in both males and females, leading to high-turnover osteoporosis[12]. Thus, if male and female mice were subjected to ORX and OVX to decrease the level of sex hormones, severe TMJ-OA was hypothesized to develop in both genders when the mandibular condyle was subjected to excessive MS. However, few reports have examined the combined effects of sex hormones and MS on TMJ-OA.

In this study, an animal model was developed where excessive MS was applied to the mandibular condyles of mice with systemically lowered levels of sex hormones. The first purpose of this study was to observe and analyze the interaction between excessive MS and reduced sex hormones on TMJ-OA. The second purpose was to discover the TMJ-OA disease susceptibility gene by molecular biological analysis to elucidate a part of the mechanism of TMJ-OA onset.

Materials and methods

2.1 Mice

Eight-week-old male and female C57BL/6J mice from Sankyo Lab Service (Tokyo, Japan) were used. The experimental protocol is shown in Fig. 1(A). Male and female mice were divided into four groups (n = 13 for each group: n = 5 for Histology/micro-CT/ELISA, n = 5 for qRT-PCR and n=3 for Microarray): control, gonadectomy (ORX and OVX), MS, and gonadectomy with MS (ORX+MS and OVX+MS) (Table I). All

mouse breeding and animal experiments were carried out at the animal facilities of Tokyo Dental College (Tokyo, Japan) with the approval of the Institutional Animal Care and Use Committee of Tokyo Dental College (approval number 193106). Hard pellets and water were provided for *ad libitum* consumption.

Gonadectomy was performed under anesthesia for mice in ORX, ORX+MS, OVX, and OVX+MS groups. Sham surgeries were performed on mice in the control and MS groups. After a 1-week recovery period, mice were anesthetized, and resin composite was used to attach a metal plate (TOMY International Inc., Tokyo, Japan) onto the posterior surface of the maxillary incisors in MS, ORX+MS, and OVX+MS groups [Fig.1(B)], as previously reported[13]. No plate was attached to mice after anesthesia in control or gonadectomy-only groups. The mice were weighed weekly to ensure that MS and gonadectomy did not affect body weight.

All mice were sacrificed 4-weeks into the experiment by administering 150 mg/kg of pentobarbital sodium into the abdominal cavity after induction of general anesthesia using sevoflurane inhalation.

2.2 Measurement of sex hormones in serum

All mice were fasted and given no water for 3 hours before collecting blood to standardize the results. Blood was collected using a 5 mm animal lancet (MEDIpoint, Mineola, NY) and a BD Microtina blood collection tube (Becton Dickinson, Franklin Lakes, NJ). Blood was centrifuged at 20,000 × g for 10 minutes, and the collected serum was stored at -20°C. 17β-estradiol (E2) and dihydrotestosterone (DHT) levels in serum were measured using a 17β-estradiol-sensitive enzyme-linked immunosorbent

assay (ELISA) kit (Enzo Biochem, Farmingdale, NY) and a DHT ELISA kit (BioVendor, Brno, Czech Republic) (n = 5).

2.3 Microcomputed tomography (Micro-CT) analysis

The right femur was fixed with 4% paraformaldehyde (Wako, Osaka) for 2 days and then placed in 70% ethanol. Micro-CT (HMX225-ACTIS+4; Tesco, Japan) was used to acquire the femur's three-dimensional (3D) images. Image analysis software (TRI/3D-BON; Ratoc System Engineering, Japan) was used to perform bone structure analysis. Micro-CT images were taken with a slice width of 0.01mm. Trabecular parameters were analyzed in the secondary trabecular region 200 μm away from the proximal end of the femoral proximal growth plate. Bone volume per tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular spacing (Tb.Spac) were measured (n = 5).

2.4 Histomorphometry and immunohistochemistry

The mandibular head was sectioned and fixed with 4% paraformaldehyde for 2 days after removing soft tissues. Specimens were decalcified with 10% EDTA solution (Muto Pure Chemicals, Tokyo, Japan) at 4°C for 4 weeks and embedded in paraffin. The right TMJ was sectioned vertically (5-μm thick).

Cartilage thickness was analyzed from H&E-stained sections. The average thickness of cartilage at the mid-coronal portion of the MCC was measured. Averages were calculated for each group (n = 5).

TRAP staining was performed using a TRAP staining kit (Sigma-Aldrich, St. Louis, MO, USA) to evaluate osteoclast differentiation in the subchondral bone. The

total number of osteoclasts per square millimeter (mm^2) was counted (n = 5). TRAP-positive cells with more than three nuclei were counted as osteoclasts.

Safranin O-stained sections were used to examine changes in cartilage breakdown, amounts of proteoglycan, and numbers of chondrocytes in the MCC. Safranin O-positive areas in MCC were determined using ImageJ (National Institutes of Health, Bethesda, MD). The Safranin O-positive area (mm^2) at the mid-coronal portion of the mandibular condylar head was measured, and averages were calculated for each group (n = 5).

Modified Mankin scores were evaluated as previously described (n = 5)[14].

For immunohistochemical staining, tissue sections were deparaffinized and then placed in 95°C water containing an Immunosaver Antigen Retriever (Electron Microscopic Stains, Hatfield, PA) for 45 minutes for antigen activation.

All sections were blocked with PBS supplemented with 1% bovine serum albumin (BSA, Sigma-Aldrich) to reduce nonspecific binding of antigens to the primary antibody. Sections were incubated with the primary antibody (diluted in PBS supplemented with 1% BSA) according to procedures recommended by the manufacturer, as follows: anti-carbonic anhydrase 1 (CA1; Abcam EPR5193, 1:200 dilution), anti-angiopoietin-like 7 (ANGPTL7; Proteintech 10396-1-AP, 1:50 dilution), anti-MMP13 (Proteintech 18165-1-AP, 1:50 dilution).

After incubating with primary antibody overnight at 4°C, the sections were washed three times with PBS. Alexa Fluor 546 Donkey Anti-Rabbit IgG (A1004; Invitrogen, Carlsbad, CA) diluted 1:200 in PBS supplemented with 1% BSA was used

for secondary antibody staining. Next, sections were incubated for 2 hours with Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA) to stain cell nuclei and Phalloidin CruzFluor™ 488 Conjugate (Santa Cruz Biotechnology, Dallas, TX) to stain actin. After staining, sections were washed three times with PBS and mounted. Images were acquired using a confocal laser microscope (LSM 880 with Airyscan, Zeiss, Oberkochen, Germany).

The groups were separated blindly and two researchers evaluated all scores.

2.5 mRNA extraction and Microarray Analysis

As previously described[15], the mandibular head with cartilage/subchondral bone was collected from mice 4 weeks after initiating the experiment. All soft tissues were removed and frozen with liquid nitrogen, then stored at –80°C until RNA extraction with TRIzol reagent (Invitrogen). The frozen TMJ was placed in a mortar containing liquid nitrogen and crushed with a pestle. Crushed bone was homogenized with TRIzol, then incubated and centrifuged. The supernatant was removed, RNA was separated, and total RNA was dissolved in RNase-free water.

Microarray analysis was conducted using a Clariom S Assay (Mouse, Thermo Fisher Scientific) to analyze related genes comprehensively. Gene expression data were normalized using the Global normalization method. The signal value for each probe set and scaling data with the average signal value of the "array" corrected to 500 were calculated using GeneChip Operating Software 1.4 (MAS5.0; Affymetrix, Santa Clara, CA). Based on the result, the expression ratio was calculated from the signal value of

the group to be compared. Genes with an expression ratio of two or more were extracted for each sex, and heatmaps were performed on the top ten genes.

Quantitative RT-PCR (qRT-PCR) was performed to quantify genes that appeared to be highly expressed in both males and females based on the results of microarray analysis. Reverse transcription was performed on total RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Life Science, Osaka, Japan). THUNDERBIRD® SYBR® qPCR Mix (Toyobo Life Science) was used for qRT-PCR. The expression intensity ratio of each target gene was determined relative to endogenous control β-actin (*Actb*) using the $\Delta\Delta C_T$ method. Quantitative RT-PCR was performed using a primer set of *Car1*, *Col1a1*, *Spp1*, *Bglap*, *Angptl7*, *Col2a1*, *Actb* (Supplemental data C).

2.6 Overexpression experiments in vitro

An ATDC5 (Sigma-Aldrich) cell line that overexpressed *Angptl7* and an MC3T3-e1 (ATCC CRL-2594) cell line that overexpressed *Car1* were established. For gene transfer, the plasmids of *Angptl7* and *Car1* expression vector map shown in [supplemental data A, B] were used, and the genes were transferred into the target cells by electroporation. To confirm that the *Car1* and *Angptl7* genes were overexpressed, quantitative evaluation of mRNA by qRT-PCR and protein expression by immunohistochemical staining were performed.

2.7 Cell culture

Osteoblast-like cells (MC3T3-E1) and chondrocyte-like cells (ATDC5) were seeded into 6-well plate / 1×10^6 cells and 48-well plate / 1×10^5 cells, respectively. MC3T3-E1 cells were cultured in α-MEM (Gibco, Sigma-Aldrich) containing 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco) as a cell proliferation medium. When the medium reached 80% confluence, it was replaced with an ossification medium (OCM, 100 mM ascorbic acid, 5 mM b-glycerophosphate, and 10 nM dexamethasone). ALP staining was performed on the 7th day, and alizarin red staining was performed on the 21st day to measure extracellular calcification. ATDC5 cells were three-dimensionally 3D cultured using Matrigel (Corning®) to confirm proteoglycan secretion. After adding the mixture of Matrigel matrix to cartilage medium (DMEM-F12 + 10%FBS + 5%Pen /Strep, Gibco) to 5 mg/ml on ice, 1000 μl/well on a 6-well plate and 100 μl on a 48-well plate, it was coated with a gel layer by incubating at 37℃ for 30 minutes. Ascorbic acid and β-glycerophosphate, with concentrations of 50 ng/mL and 10 mM, respectively, were added to accelerate chondrogenic differentiation and produce a physiologically mineralized extracellular matrix. On reaching confluence, cells were stimulated with 10 μg/mL insulin, 5.5 μg/mL transferrin, and 6.7 ng/mL sodium selenite (ITS, Gibco). After culturing for 21 days, the color change by staining was observed, the total RNA was extracted, and then qRT-PCR was performed. The cells were maintained at 37℃ in an atmosphere containing 5%CO_2, and the medium was changed every 2 days.

2.8 Cell proliferation assay

To measure cell proliferation, ATDC5 and MC3T3-E1 cells were seeded on a 96-well plate at 1×10^5 / well and cultured in 100 μl of the medium. 10 μl of WST-1

(Roche, Mannheim) was added to each well one day after cell seeding. The absorbance was measured 2 hours after the addition of WST-1 at a wavelength of 450 nm using a microplate reader. The control wavelength was set to 650 nm.

2.9 Alkaline phosphatase (ALP) and Alizarin red staining

ALP staining was performed to confirm ALP activity, and alizarin red staining was performed to measure extracellular calcification. On the 7^{th} day after cell differentiation, ALP staining was performed using 1-step NBT (Thermo Scientific), and color changes were observed. On the 21^{st} day after cell differentiation, Alizarin red staining was performed using alizarin red solution (1%, Sigma-Aldrich) after fixing the cells with 10% formalin. Cells were washed with distilled water before capturing the images. Alizarin red dye was eluted with 5% formic acid, then 100 µl eluate was collected from each well, and absorbance was measured at a wavelength of 425 nm using a microplate reader.

2.10 Alcian blue and Safranin O Staining

Alcian blue and Safranin O staining were used to evaluate cartilage extracellular matrix components (proteoglycans and glycosaminoglycans) on samples collected at the end of the experiment (21 days of culture). Alcian blue (0.1%, Wako) staining was performed by incubating 3D cultured samples for 30 minutes. The Safranin O staining consisted of staining the cells with Weigert's iron hematoxylin working solution for 7 minutes and 0.1% Safranin O for 5 minutes. Samples were washed after each staining step, left to air dry, and rinsed in absolute alcohol.

2.11 Statistical analysis

All data were statistically analyzed using SPSS (IBM, Armonk, NY). Two two-way ANOVA models stratified by gender were used for multiple comparisons. A p-value less than 0.05 was considered statistically significant. The two-way ANOVA models were tested on the assumptions of "normality" and "homoscedasticity." The sample size was calculated with the statistical power of 80%, significance level of 5%, and effect size of 1.8.

Results

Experimental groups and sample sizes are listed in (Table I). Neither MS nor gonadectomy had a significant effect on body weight.

Reduction of serum sex hormones by ORX and OVX

Significant decreases in DHT and E2 were observed in groups that underwent ORX and OVX, respectively [Fig. 1(C, D)]. Bone structure analysis of the femur is shown in [Supplemental data D, E]. ORX and OVX groups exhibited significantly reduced BV/TV (%), trabecular number (1/mm), and trabecular thickness (μm), and significantly increased trabecular spacing (μm) compared with the control group.

TMJ-OA induced by sex hormone (DTH or E2) deficiency and excessive MS

In H&E staining [Fig. 2(A–D), Fig. 3(A–D)], cartilage thickness was significantly reduced in MS, ORX+MS, and OVX+MS groups compared with their respective controls [Fig. 2(Q), Fig. 3(Q)]. In TRAP staining [Fig. 2(E–H)], the number of osteoclasts was significantly higher in all experimental groups compared with controls.

In particular, ORX+MS and OVX+MS groups exhibited a four-fold increase in the number of osteoclasts compared with controls [Fig. 2(R), Fig. 3(R)].

Safranin O staining indicated a decrease in cell number from the surface to mature cartilage layers and an uneven surface (indicated by red arrows) in MS, ORX+MS, and OVX+MS groups [Fig. 2(J, L), Fig. 3(J, L)]. These groups also exhibited significant decreases in Safranin O-positive areas [Fig. 2(S), Fig. 3(S)] and significant increases in modified Mankin scores [Fig. 2(T), Fig. 3(T)]. MMP13, a major enzyme involved in cartilage destruction associated with osteoarthritis, was also strongly expressed by chondrocytes [Fig. 2(N, P), Fig. 3(N, P)].

Differential gene expression in condylar cartilage and subchondral bone

Microarray analysis was performed to identify potential new genes involved in the early stages of TMJ-OA. The top 10 genes with the highest expression in both sexes are shown in Table II[16-33], and a heat map was created to visualize the results [Fig. 4(A), Fig. 5(A)]. *Angptl7* showed maximum upregulation, and *Car1* showed triple upregulation in both sexes.

To confirm the results of microarray analysis, expression of *Angptl7* and *Car1* genes, which were upregulated in the male and female experimental groups, were quantitatively compared by qRT-PCR [Fig. 4(B, C), Fig. 5(B, C)]. As a result, *Angptl7* was upregulated approximately four-fold in ORX+MS and OVX+MS groups compared to male and female controls, respectively. In addition, *Car1* was upregulated four-fold in the ORX+MS group and five-fold in the OVX+MS group compared to male and

female controls, respectively. Values were greatest in the groups with reduced sex hormones and excessive MS.

Localization of ANGPTL7 and CA1

Based on the results of the microarray analysis, immunohistological staining of mandibular condyles was performed to examine the expression and localization of ANGPTL7 (encoded by *Angptl7*) and CA1 (encoded by *Car1*), which were upregulated in the male and female experimental groups. ANGPTL7 showed strong localization in the anterior condylar head cartilage of males and females in the ORX+MS and OVX+MS groups, respectively [Fig. 4(D–K), Fig. 5(D–K)]. CA1 also showed strong localization along the trabecula of subchondral bone in the ORX+MS and OVX+MS groups [Fig. 4(L–S), Fig. 5(L–S)].

Overexpression of *Car1* in osteoblast-like cells

A remarkable increase in CA1 shown in red in immunohistochemical staining and a significant increase in *Car1* mRNA level was observed in qRT-PCR, confirming successful overexpression of *Car1* in MC3T3-E1 [Fig. 6 (A, D)]. ALP and Alizarin red S staining showed decreased ALP activity and calcification matrix formation due to *Car1* overexpression [Fig. 6 (B, H)]. Overexpression of *Car1* in MC3T3-E1 reduced cell proliferation [Fig. 6 (C)]. Compared with the control, *Car1* overexpression showed a marked decrease in the mRNA levels of *Col1a1*, *Spp1*, and *Bglap* involved in bone formation by qRT-PCR [Fig. 6 (E-G)].

Overexpression of *Angptl7* in chondrocyte-like cells

It was confirmed that the overexpression of *Angptl7* was successful in ATDC5 because a marked increase in ANGPTL7 shown in red was observed in immunohistochemical staining, and a significant increase in mRNA level of *Angptl7* was observed in qRT-PCR [Fig. 6 (I, L)]. Alcian blue and Safranin O staining showed no remarkable difference between control and *Angptl7* overexpression [Fig. 6 (J)]. Overexpression of *Angptl7* in ATDC5 increased cell proliferation [Fig. 6 (K)]. In qRT-PCR, the mRNA level of *Col2a1* involved in chondrogenesis was unchanged compared to the control, but the mRNA levels of *Spp1* and *Bglap* involved in bone formation were significantly increased [Fig. 6 (M-O)].

Discussion

In this study, an animal model was developed to evaluate the interaction between excessive MS and reduced sex hormones on TMJ-OA. This model revealed three new major findings. First, a TMJ-OA mouse model was successfully developed with the combined effects of excessive MS and reduced sex hormones on male and female mice. Second, the understanding of the pathogenesis of TMJ-OA by excessive MS and reduced sex hormones was improved. Third, *Angptl7* and *Car1* were discovered as candidates of disease-susceptible genes that may be key to understanding the pathogenesis of TMJ-OA.

TMJ-OA mouse model by combined effects of excessive MS and reduced sex hormones

In this study, the existing method[11,13] of applying excessive MS on the mandibular central incisor by attaching a resin device to the posterior surface of the maxillary central incisor to cause occlusal imbalance was improved by replacing resin with metal. The metal device had excellent wear resistance; therefore, the plate could be made thin, allowing the mice to consume a hard pellet diet for 3 weeks. Prolonged intake of hard foods may be effective in causing TMJ-OA; therefore, it was assumed that this device would cause uneven occlusion and add MS to the MCC.

Histomorphometric results showed that the group with the device had decreased cartilage thickness, safranin O staining, and chondrocyte number and showed pathological changes such as articular cartilage erosion. The modified Mankin score was also significantly higher, and the subchondral bone also showed a significant increase in osteoclasts, showing bone resorption. Due to these factors, it was confirmed that excess MS induced by the metal plate resulted in the mice developing TMJ-OA.

From the femoral bone structure analysis and ELISA results, it was confirmed that gonadectomy caused the development of high-turnover osteoporosis in mice.

TMJ-OA was observed in all experimental groups; however, the most severe symptoms were observed in groups with reduced sex hormones and MS (ORX+MS and OVS+MS). These results indicated the successful development of a new TMJ-OA mouse model with the combined effects of excess MS and reduced sex hormones.

Pathogenesis of TMJ-OA caused by excessive MS and reduced sex hormones

In this study, MMP13 expression and cartilage destruction were increased in groups subjected to MS. Excessive stress on MCC induces VEGF expression[4]. Furthermore, VEGF increases MMP expression while suppressing TIMP expression, resulting in imbalanced remodeling of extracellular matrix components, ultimately destroying cartilage[5]. VEGF also acts on subchondral bone and induces endothelial cells and osteoclasts to promote bone resorption[34]. In this study, low-cellularity and thinning of cartilage were observed in groups subjected to MS. These results are consistent with previous studies reporting that MS causes chondrocyte apoptosis and cartilage thinning[35].

There are two subtypes of estrogen receptors (ER), ERα and ERβ, involved in both cartilage protection and destruction. Specifically, ERα promotes the maturation of chondrocytes[2] and promotes the formation of condylar fibrocartilage[36]. In contrast, ERβ has a negative effect on cartilage formation by inhibiting chondrocyte maturation[2]. In this study, homeostasis was maintained by the opposite action of these two ERs, suggesting that severe cartilage destruction did not occur in the OVX group.

Estrogen induces an increase in Sema3A and has a bone-protecting effect by promoting osteoblast differentiation and suppressing osteoclast differentiation[37]. In this study, the reduced estrogen group did not have this Sema3A-mediated osteoprotective effect, which may have resulted in severe destruction of subchondral bone.

There are two known pathways for androgen action: a direct action of the androgen itself and an indirect action whereby testosterone is metabolized by aromatase to become estrogen. Since the direct action of androgen on cartilage has both protective

and destructive effects [38-41], MMP13 did not increase in the ORX alone group. Perhaps the combination of ORX and MS significantly increased the expression of MMP13. Decreased femoral bone density and destruction of subchondral bone were observed in both reduced sex hormone groups because of the suppression of estrogen conversion by aromatase.

The results described above indicate that excessive MS and reduced sex hormones cause destructive MCC and subchondral bone changes. Moreover, a combination of these factors results in loss of homeostasis and the development of more severe TMJ-OA.

Disease-susceptibility genes

Microarray analysis was performed to screen for disease-susceptibility genes, which are crucial to determining the onset mechanism of TMJ-OA. Compared with their respective control groups, a significant increase in *Angptl7* and *Car1* expression was observed in groups with severe TMJ-OA because of the combined effects of MS and reduced sex hormones.

ANGPTL7 is upregulated specifically by hypoxia to promote angiogenesis[16]. Cartilage is an avascular tissue adapted to hypoxic environments. However, when inflammation occurs in the joint, the partial pressure of oxygen in cartilage is reduced to even lower levels than normal, increasing the expression of inflammatory factors and angiogenic factors (VEGF), resulting in devastating changes in MCC [42].

In this study, *Angptl7* expression was four times stronger in groups with excessive MS and reduced sex hormones than in the control group. In addition, strong localization of ANGPTL7 was observed in the MCC. It has been reported that vascular repair and angiogenesis are reduced in ER knockout mice[43]. Results suggested that the synergistic effect of reduced angiogenesis due to estrogen deficiency and tissue compression due to excessive MS created a marked hypoxic environment in TMJ and caused a destructive change in condylar cartilage.

Car1 exhibited the highest gene expression in groups with MS and reduced sex hormones and showed strong localization along the trabecular bone of subchondral bone. In addition, *Car1* has been implicated in both the promotion of apoptosis in endothelial cells[19] and inappropriate bone calcification resulting from $CaCO_3$ formation[18]. These are all possible causes for the onset of TMJ-OA; however, as the detailed mechanism by which MS or reduced sex hormones increase *Car1* expression has not been clarified, additional research is necessary.

The results of in vitro target gene overexpression analysis showed that *Car1* overexpression may promote bone destruction. The results of *Angptl7* overexpression suggest that *Angptl7* overexpression contributes to chondrocyte proliferation but promotes chondrocyte calcification rather than chondrocyte layer formation. Therefore, one part of the new mechanism of exacerbating TMJ-OA was shown from the present results of suppression of osteoblast proliferation and bone formation by *Car1* and calcification of cartilage by *Angptl7*.

Conclusions

A severe TMJ-OA mouse model was successfully developed in mice by applying excessive MS on the mandibular condyle of male and female mice with reduced sex hormones (DHT and E2, respectively). Disease-susceptibility genes *Angptl7* and *Car1* were newly discovered in the experimental groups, suggesting their involvement in the mechanism of TMJ-OA onset.

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Contribution

Tomohisa Ootake: research planning, sample preparation and data collection, preparation of master thesis.

Takenobu Ishii: Project chief

Kenji Sueishi: Provided information on clinical problems associated with TMJ-OA.

Akira Watanabe: Provided surgical techniques.

Yoichi Ishizuka: Provided tissue sectioning techniques.

Katsuhiko Amano: Provided gene analysis technology.

Masashi Nagao: Provided information on TMJ-OA model creation.

Kazuaki Nishimura: Provided immunological analysis technology.

Yasushi Nishii: Provided morphological measurement technology for 3D images.

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Competing Interests

The authors declare no conflicts of interest associated with this manuscript.

References

1. Tanaka E, Detamore MS, Mercuri LG. Degenerative Disorders of the Temporomandibular Joint: Etiology, Diagnosis, and Treatment. J Dent Res. 2008; 87:296-307.
2. Robinson JL, Cass K, Aronson R, Choi T, Xu M, Buttenbaum R, et al. Sex Differences in the Estrogen-Dependent Regulation of Temporomandibular Joint Remodeling in Altered Loading. Osteoarthritis Cartilage. 2017; 25:533-43.
3. Chen J, Sorensen KP, Gupta T, Kilts T, Young M, Wadhwa S. Altered functional loading causes differential effects in the subchondral bone and condylar cartilage in the temporomandibular joint from young mice. Osteoarthritis Cartilage. 2009; 17:354-61.
4. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, et al. Activation of Vascular Endothelial Growth Factor Gene Transcription by Hypoxia-Inducible Factor 1. Mol Cell Biol. 1996; 16:4604-13.
5. Pufe T, Harde V, Petersen W, Goldring MB, Tillmann B, Mentlein R. Vascular endothelial growth factor (VEGF) induces matrix metalloproteinase expression in immortalized chondrocytes. J Pathol. 2004; 202:367-74.
6. Steven J, David A, Leonard B. Temporomandibular disorders. Dentistry today 2000; 19:78-87
7. Gunson MJ, Arnett GW, Formby B, Falzone C, Mathur R, Alexander C. Oral contraceptive pill use and abnormal menstrual cycles in women with severe condylar resorption: A case for low serum 17β-estradiol as a major factor in progressive condylar resorption. Am J Orthod Dentofacial Orthop. 2009; 136:772-9.
8. Karout N, Hawai SM, Altuwaijri S. Prevalence and pattern of menstrual disorders among Lebanese nursing students. East Mediterr Health J. 2012; 18:346-52.
9. Harman SM, Metter EJ, Tobin JD, Pearson J, Blackman MR. Longitudinal Effects of Aging on Serum Total and Free Testosterone Levels in Healthy Men. J Clin Endocrinol Metab. 2001; 86:724-31.
10. Fabbri E, An Y, Gonzalez-Freire M, Zoli M, Maggio M, Studenski SA, et al. Bioavailable Testosterone Linearly Declines Over A Wide Age Spectrum in Men and Women From The Baltimore Longitudinal Study of Aging. J Gerontol A Biol Sci Med Sci. 2016; 71:1202-9.
11. Wu Y, Kadota-Watanabe C, Ogawa T, Moriyama K. Combination of estrogen deficiency and excessive mechanical stress aggravates temporomandibular joint osteoarthritis in vivo. Arch Oral Biol. 2019; 102:39-46.
12. Reim NS, Breig B, Stahr K, Eberle J, Hoeflich A, Wolf E, et al. Cortical Bone Loss in Androgen-Deficient Aged Male Rats Is Mainly Caused by Increased Endocortical Bone Remodeling. J Bone

Miner Res. 2008; 23:694-704.

13. Shirakura M, Tanimoto K, Eguchi H, Miyauchi M, Nakamura H, Hiyama K, et al. Activation of the hypoxia-inducible factor-1 in overloaded temporomandibular joint, and induction of osteoclastogenesis. Biochem Biophys Res Commun. 2010; 393:800-5.
14. Thomas CM, Fuller CJ, Whittles CE, Sharif M. Chondrocyte death by apoptosis is associated with cartilage matrix degradation1. Osteoarthritis Cartilage. 2007; 15:27-34.
15. Embree M, Ono M, Kilts T, Walker D, Langguth J, Mao J, et al. Role of subchondral bone during early-stage experimental TMJ osteoarthritis. J Dent Res. 2011; 90:1331-8.
16. Parri M, Pietrovito L, Grandi A, Campagnoli S, De Camilli E, Bianchini F, et al. Angiopoietin-like 7, a novel pro-angiogenetic factor over-expressed in cancer. Angiogenesis. 2014; 17:881-96.
17. Suzuki K, Yoshida K, Ueha T, Kaneshiro K, Nakai A, Hashimoto N, et al. Methotrexate upregulates circadian transcriptional factors PAR bZIP to induce apoptosis on rheumatoid arthritis synovial fibroblasts. Arthritis Res Ther. 2018; 20:55.
18. Chang X, Han J, Zhao Y, Yan X, Sun S, Cui Y. Increased expression of carbonic anhydrase I in the synovium of patients with ankylosing spondylitis. BMC Musculoskelet Disord. 2010; 11:279.
19. Torella D, Ellison GM, Torella M, Vicinanza C, Aquila I, Iaconetti C, et al. Carbonic Anhydrase Activation Is Associated With Worsened Pathological Remodeling in Human Ischemic Diabetic Cardiomyopathy. J Am Heart Assoc. 2014;3: e000434.
20. Mantila Roosa SM, Liu Y, Turner CH. Gene Expression Patterns in Bone Following Mechanical Loading. J Bone Miner Res. 2011; 26:100-12.
21. Koyama M, Ishihara K, Karasuyama H, Cordell JL, Iwamoto A, Nakamura T. CD79 alpha/CD79 beta heterodimers are expressed on pro-B cell surfaces without associated mu heavy chain. Int Immunol. 1997; 9:1767-72.
22. Yang HJ, Ju F, Guo XX, Ma SP, Wang L, Cheng BF, et al. RNA-binding protein RBM3 prevents NO-induced apoptosis in human neuroblastoma cells by modulating p38 signaling and miR-143. Sci Rep. 2017; 7:41738.
23. Liu J, Zhang M, Niu C, Luo Z, Dai J, Wang L, et al. Dexamethasone inhibits repair of human airway epithelial cells mediated by glucocorticoid-induced leucine zipper (GILZ). PLoS One. 2013;8: e60705.
24. Kaminski WE, Wenzel JJ, Piehler A, Langmann T, Schmitz G. ABCA6, a novel a subclass ABC transporter. Biochem Biophys Res Commun. 2001; 285:1295-301.
25. Beard NA, Laver DR, Dulhunty AF. Calsequestrin and the calcium release channel of skeletal and cardiac muscle. Prog Biophys Mol Biol. 2004; 85:33-69.

26. Huang W, Liang J, Yuan CC, Kazmierczak K, Zhou Z, Morales A, et al. Novel Familial Dilated Cardiomyopathy Mutation in MYL2 Affects the Structure and Function of Myosin Regulatory Light Chain. FEBS J. 2015; 282:2379-93.
27. Kojic S, Medeot E, Guccione E, Krmac H, Zara I, Martinelli V, et al. The Ankrd2 Protein, a Link Between the Sarcomere and the Nucleus in Skeletal Muscle. J Mol Biol. 2004; 339:313-25.
28. Chellaiah MA, Soga N, Swanson S, McAllister S, Alvarez U, Wang D, et al. Rho-A Is Critical for Osteoclast Podosome Organization, Motility, and Bone Resorption. J Biol Chem. 2000; 275:11993-2002.
29. Frey N, Barrientos T, Shelton JM, Frank D, Rütten H, Gehring D, et al. Mice lacking calsarcin-1 are sensitized to calcineurin signaling and show accelerated cardiomyopathy in response to pathological biomechanical stress. Nat Med. 2004; 10:1336-43.
30. Goto T, Yamaza T, Tanaka T. Cathepsins in the osteoclast. J Electron Microsc (Tokyo). 2003; 52:551-8.
31. Sanchez C, Bay-Jensen AC, Pap T, Dvir-Ginzberg M, Quasnichka H, Barrett-Jolley R, et al. Chondrocyte secretome: a source of novel insights and exploratory biomarkers of osteoarthritis. Osteoarthritis Cartilage. 2017; 25:1199-209.
32. Yu XT, Wang PY, Shi ZM, Dong K, Feng P, Wang HX, et al. Up-regulation of urotensin II and its receptor contributes to human hepatocellular carcinoma growth via activation of the PKC, ERK1/2, and p38 MAPK signaling pathways. Molecules. 2014; 19:20768-79
33. Alshbool FZ, Mohan S. Emerging Multifunctional Roles of Claudin Tight Junction Proteins in Bone. Endocrinology. 2014; 155:2363-76.
34. Tanaka E, Aoyama J, Miyauchi M, Takata T, Hanaoka K, Iwabe T, et al. Vascular endothelial growth factor plays an important autocrine/paracrine role in the progression of osteoarthritis. Histochem Cell Biol. 2005; 123:275-81.
35. Li H, Zhang XY, Wu TJ, Cheng W, Liu X, Jiang TT, et al. Endoplasmic Reticulum Stress Regulates Rat Mandibular Cartilage Thinning under Compressive Mechanical Stress. J Biol Chem. 2013; 288:18172-83.
36. Robinson JL, Soria P, Xu M, Vrana M, Luchetti J, Lu HH, et al. Estrogen Promotes Mandibular Condylar Fibrocartilage Chondrogenesis and Inhibits Degeneration via Estrogen Receptor Alpha in Female Mice. Sci Rep. 2018; 8:8527.
37. Hayashi M, Nakashima T, Yoshimura N, Okamoto K, Tanaka S, Takayanagi H. Autoregulation of Osteocyte Sema3A Orchestrates Estrogen Action and Counteracts Bone Aging. Cell Metab. 2019; 29:627-37.

38. Irie T, Aizawa T, Kokubun S. The role of sex hormones in the kinetics of chondrocytes in the growth plate. J Bone Joint Surg Br. 2005; 87:1278-84.
39. Pang S, Flores-Morales A, Skoog L, Chuan Y, Nordstedt G, Pousette A Regulation of matrix metalloproteinase 13 expression by androgen in prostate cancer. Oncol Rep 2004; 11: 1187-92.
40. Danielpour D Function and regulation of transforming growth factor-beta (TGF-β) in the prostate. Eur J Cancer 2005; 41: 846-57.
41. Uria JA, Jimenez MG, Balbin M, Freije JMP, Lopez-Otin C Differential effects of transforming growth factor-β on the expression of collagenase-1 and collagenase-3 in human fibroblasts. J Biol Chem 1998; 273: 9769-77.
42. Bonnet CS, Walsh DA. Osteoarthritis, angiogenesis and inflammation. Rheumatology (Oxford). 2005; 44:7-16.
43. Andrea L George, Shilpi Rajoria, Robert Suriano, Abraham Mittleman and Raj K Tiwari, Hypoxia and estrogen are functionally equivalent in breast cancer-endothelial cell interdependence. Molecular Cancer 2012; 11:80

Figure legend

Fig. 1. Experimental protocol and device. (A) Experimental protocol: ORX and OVX were performed on sexually mature 8-week-old mice. After a 1-week recovery period, a metal place was attached to the posterior surface of the anterior incisors to create an unbalanced occlusion. Male mice were divided into four groups (control, ORX, MS, and ORX+MS) and female mice were divided into four groups (control, OVX, MS, and OVX+MS), for a total of eight groups. (B) Device: To induce an imbalanced occlusion, a metal plate was attached to the posterior surface of the maxillary incisors using a resin composite so that the base thickness was 2 mm. Changes in hormone levels in serum and bone structure analysis of femur. (C) Measurement of serum DHT concentration using ELISA. * $P < 0.05$, ** $P < 0.01$. (D) Measurement of serum E2 concentration using ELISA. * $P < 0.05$, ** $P < 0.01$.

Fig. 2. Histomorphometry and immunohistochemistry of male mouse condyle. (A–P) Right condylar heads of male mice were vertically sectioned (5-μm thickness) before staining with H&E (A–D, scale bar = 100 μm), TRAP (E–H, scale bar = 100 μm), Safranin O (I–L, scale bar = 50 μm), or immunohistochemical staining for MMP13 (M–P; scale bar = 100 μm; red: MMP13, green: actin, blue: nuclei). Safranin O staining indicated a decrease in cell number from surface to the mature cartilage layers and an uneven surface (shown by red arrows) in MS and ORX+MS groups. (Q) Cartilage thickness of H&E stained sections. * $P < 0.05$, ** $P < 0.01$. (R) Measurement of osteoclasts (mm^2) in TRAP-stained sections. TRAP-positive cells with three or more

nuclei were counted as osteoclasts. * $P < 0.05$, ** $P < 0.01$. (S) Measurement of proteoglycan area in safranin O stained sections. * $P < 0.05$, ** $P < 0.01$. (T) Measurement of modified Mankin score for OA severity assessment. * $P < 0.05$, ** $P < 0.01$.

Fig. 3. Histomorphometry and immunohistochemistry of female mouse condyle. (A–P) Right condylar heads of female mice were vertically sectioned (5-μm thickness) before staining with H&E (A–D, scale bar = 100 μm), TRAP (E–H, scale bar = 100 μm), Safranin O (I–L, scale bar = 50 μm), or immunohistochemical staining for MMP13 (M–P; scale bar = 100 μm; red: MMP13, green: actin, blue: nuclei). Safranin O staining indicated erosion on the cartilage surface (shown by red arrows) in MS and OVX+MS groups. (Q) Cartilage thickness of H&E stained sections. * $P < 0.05$, ** $P < 0.01$. (R) Measurement of osteoclasts (mm^2) in TRAP-stained sections. TRAP-positive cells with three or more nuclei were counted as osteoclasts. * $P < 0.05$, ** $P < 0.01$. (S) Measurement of proteoglycan area in Safranin O-stained sections. * $P < 0.05$, ** $P < 0.01$. (T) Measurement of modified Mankin scores for OA severity assessment. * $P < 0.05$, ** $P < 0.01$.

Fig. 4. Comparison of gene expression in the mandibular condyle of male mice and localization of ANGPTL7 and CA1 by immunohistochemical staining. (A) Comparison of gene expression between control and ORX + MS groups by microarray analysis. Heatmap was created for the top ten genes whose gene expression increased

significantly in the ORX + MS group. (B) Comparison of *Angptl7* gene expression by qRT-PCR * P < 0.05, ** P < 0.01. (C) Comparison of *Car1* gene expression by qRT-PCR * P < 0.05, ** P < 0.01. (D–G) Immunohistochemical staining of ANGPTL7 with low magnification of the entire condyle. Scale bar = 100 μm. Red: ANGPTL7, green: actin, blue: nuclei. (H–K) Immunohistochemical of ANGPTL7 with high magnification of anterior condylar cartilage. Scale bar = 100 μm. red: ANGPTL7, green: actin, blue: nuclei. ANGPTL7 showed strong localization in the anterior condylar head cartilage of males in the ORX+MS group. (L–O) Immunohistochemical staining of CA1 with low magnification of the entire condyle. Scale bar = 100 μm. Red: CA1, green: actin, blue: nuclei. (P–S) Immunohistochemical staining of CA1 with high magnification of anterior condylar cartilage. Scale bar 100 = μm. Red: CA1, green: actin, blue: nuclei. CA1 showed strong localization along the trabecula of subchondral bone in the ORX+MS group.

Fig. 5. Comparison of gene expression in the mandibular condyle of female mice, and localization of ANGPTL7 and CA1 by immunohistochemical staining. (A) Comparison of gene expression between control and OVX + MS groups by microarray analysis. Heatmap was created for the top ten genes whose gene expression increased significantly in the OVX + MS group. (B) Comparison of *Angptl7* gene expression by qRT-PCR * P < 0.05, ** P < 0.01. (C) Comparison of *Car1* gene expression by qRT-PCR * P < 0.05, ** P < 0.01. (D–G) Immunohistochemical staining of ANGPTL7 (low magnification shows the entire condyle. Scale bar = 100 μm. Red: ANGPTL7, green:

actin, blue: nuclei. (H–K) Immunohistochemical staining of ANGPTL7 with high magnification of anterior condylar cartilage. Scale bar = 100 μm. Red: ANGPTL7, green: actin, blue: nuclei. ANGPTL7 showed strong localization in the anterior condylar head cartilage of females in the OVX+MS group. (L–O) Immunohistochemical staining of CA1 with low magnification of the entire condyle. Scale bar = 100 μm. Red: CA1, green: actin, blue: nuclei. (P–S) Immunohistochemical staining of CA1 with high magnification of anterior condylar cartilage, scale bar = 100 μm. Red: CA1, green: actin, blue: nuclei). CA1 showed strong localization along the trabecula of subchondral bone in the OVX+MS group.

Fig.6. In-vitro *Car1* and *Angptl7* overexpression experiments of osteoblast-like and chondrocyte-like cell lines, respectively. (A) Immunohistochemical staining of CA1 in MC3T3-E1 control and MC3T3-E1 *Car1* overexpressing cells. Red: CA1, green: actin, blue: nuclei (B) Comparison of ALP staining and Alizarin red S staining in control and *Car1* overexpressing cells (n = 5). (C) Comparison of cell proliferation assay absorbances in controls and *Car1* overexpressing cells (A450-A690 nm) (n = 6). (D-G) Comparison of *Car1, Col1a1, Spp1, Bglap* gene expression by qRT-PCR (n = 5). (H) Comparison of alizarin red S staining absorbance (425 nm) in control and *Car1* overexpressing cells. (n = 5) (I) Immunohistochemical staining of ANGPTL7 in ATDC5 control and ATDC5 *Angptl7* overexpressing cells. Red: ANGPTL7, green: actin, blue: nuclei (J) Comparison of Alcian Blue staining and Safranine O staining in control and *Angptl7* overexpressing cells (n = 5). (K) Absorbance comparison of cell

proliferation assay in control and *Angptl7* overexpressing cells (A450-A690 nm) (n = 6). (L-O) Comparison of *Angptl7, Col2a1, Spp1, Bglap* gene expression by qRT-PCR (n = 5). The graph values denote the average ± SD, * P <0.05 and ** P <0.01, estimated by one-way ANOVA.

Supplemental data. (A) *Angptl7* Expression vector map (B) *Car1* expression vector map (C) Primers sequences used in qRT-PCR. (D) Three-dimensional (3D) images and bone structure analysis using CT images of male mouse femur. Bone structure analysis: bone volume/tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp). * P < 0.05, ** P < 0.01. (E) 3D images and bone structure analysis using CT images of female mouse femur. Bone structure analysis included BV/TV, Tb.N, Tb.Th, and Tb.Sp. * P < 0.05, ** P < 0.01.