

ACTIVATION OF B-CATENIN SIGNALLING LEADS TO TEMPOROMANDIBULAR JOINT DEFECTS

M. Wang^{1,*}, S. Li², W. Xie^{2,3}, J. Shen¹, H-J. Im², J.D. Holz¹, M. Wang⁴, T.G.H. Diekwisch⁵ and D. Chen^{2,§}

¹Department of Orthopaedics, Center for Musculoskeletal Research, University of Rochester Medical Center, Rochester, NY 14642, USA

²Department of Biochemistry, Rush University Medical Center, Chicago, IL 60612, USA

³Liaoning University of Traditional Chinese Medicine, Liaoning 110847, China

⁴Department of Oral Anatomy and Physiology and TMD, College of Stomatology, Fourth Military Medical University, Xi'an 710032, China

⁵Department of Oral Biology, University of Illinois at Chicago College of Dentistry, Chicago, IL 60612, USA

*Current address: Section of Endocrinology, Department of Internal Medicine, Yale University, New Haven, CT 06520, USA

Abstract

Despite extensive research in knee and hip osteoarthritis (OA), the underlying mechanism of temporomandibular joint (TMJ) disorder remains largely unknown. The purpose of this study was to determine whether the constitutive activation of β -catenin in the middle and deep layers of the articular cartilage can compromise the homeostasis of this tissue in the TMJ. *Col2CreER^{T2}* transgenic mice were bred with *Rosa^{mTmG}* reporter mice to determine Cre recombination efficiency. *Col2CreER^{T2}* mice were then crossed with *β -catenin^{lox(ex3)/+}* mice to generate *β -catenin* conditional activation mice, *β -catenin(ex3)^{Col2ER}*. TMJ samples were harvested when the mice were 1-, 3- or 6-month-old and evaluated using histology, histomorphometry and immunohistochemistry. *β -catenin(ex3)^{Col2ER}* mice were further crossed with *Mmp13^{lox/lox}* and *Adamts5^{-/-}* mice to generate (*β -catenin(ex3)/Mmp13^{Col2ER}* and *β -catenin(ex3)^{Col2ER}/Adamts5^{-/-}* double mutant mice to investigate the role of *Mmp13* and *Adamts5* in the development of TMJ disorder. High levels of Cre-recombination were seen in *Col2CreER^{T2};Rosa^{mTmG}* mice. Progressive TMJ defects developed in 1-, 3- and 6-month-old *β -catenin(ex3)^{Col2ER}* mice, as revealed by histology and histomorphometry. Results further demonstrated that the defects observed in *β -catenin(ex3)^{Col2ER}* mice were significantly decelerated after deletion of the *Mmp13* or *Adamts5* gene in (*β -catenin(ex3)/Mmp13^{Col2ER}* or *β -catenin(ex3)^{Col2ER}/Adamts5^{-/-}* double mutant mice. In summary, we found that β -catenin is a critical gene in the induction of TMJ cartilage degeneration, and over-expressing *β -catenin* in TMJ cartilage leads to defects assembling an OA-like phenotype. Deletion of *Mmp13* and *Adamts5* in *β -catenin(ex3)^{Col2ER}* mice ameliorates the development of TMJ defects. This study suggests that *Mmp13* and *Adamts5* could be potential therapeutic targets for the treatment of TMJ disorders.

Keywords: Temporomandibular joint, osteoarthritis, β -catenin, MMP13, Adamts5.

§Address for correspondence:

Di Chen, MD, PhD
Department of Biochemistry
Rush University Medical Center
1735 West Harrison Street

Cohn Research Building, Suite 508
Chicago, IL 60612, USA

Telephone Number: 1-312-942-5702

FAX Number: 1-312-942-3053

E-mail: di_chen@rush.edu

Introduction

The temporomandibular joint (TMJ) is a hinge and gliding joint that connects the condyle of the mandibular bone with the temporal articular surface composed of a concave, socket-like articular fossa and a convex eminence. Similar to the knee joint, the temporal bone and the condyle are separated and cushioned by an articular disc (Fig. 1A). The TMJ disc separates the joint space into two distinct parts. The superior joint space is bounded by the articular fossa and the articular eminence. The inferior joint space is bounded by the condyle. TMJ is one of the most frequently used joints in the human body. It is reported that about 40-70 % American adults have at least one sign of TMJ disorders and at least 33 % among those have one symptom, such as pain, limited mandibular motion, and TMJ sounds (Scrivani *et al.*, 2008). The American Academy of Orofacial Pain classified TMJ disorder into different subtypes (de Leeuw, 2008). Among these subgroups, TMJ osteoarthritis (OA) is a highly prevalent degenerative disease that affects articular cartilage during pathological conditions, as well as during the aging process. Currently, rest therapy, pain relief, and thermotherapy are the principal clinical treatment methods, and there is no preventative treatment. Moreover, current knowledge of TMJ OA is derived primarily from clinical investigations, and many aspects of TMJ OA, especially the underlying molecular mechanisms, remain largely under-studied (Rando and Waldron, 2012).

Jaw function and movement result in continuous and ever changing mechanical loads exerted on the TMJ, which in turn affect the morphology and composition of its cartilages (Iwasaki *et al.*, 2010). TMJ condylar articular cartilage is an avascular, compressible tissue comprised of dense extracellular collagen fibres and proteoglycans that protect the joint from damage during mechanical loading. The collagen fibres are well organised and aligned in an antero-posterior orientation to optimise resistance to shear stress (Singh and Detamore, 2008). Morphologically,

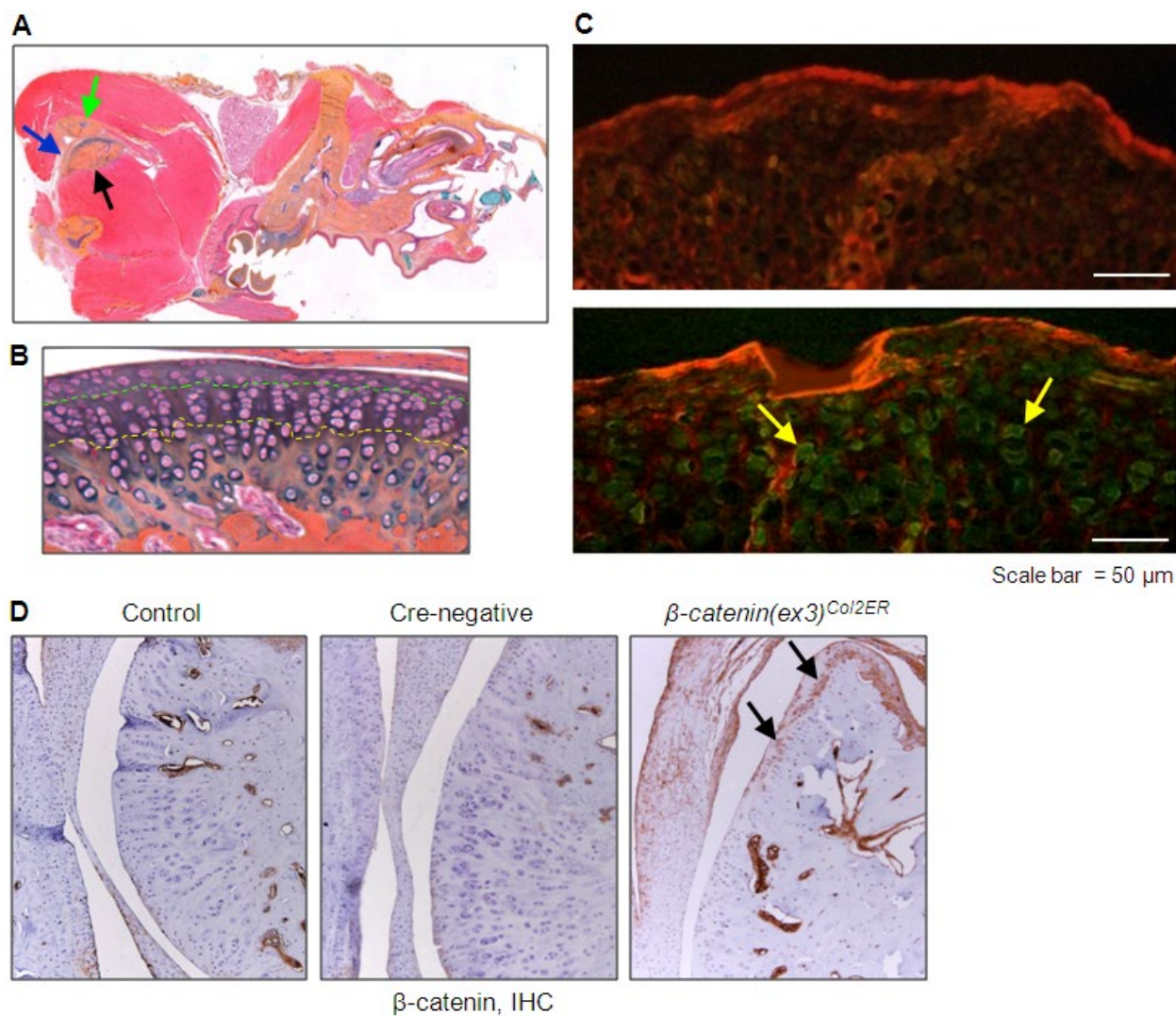


Fig. 1. *Col2CreER²* mice efficiently target TMJ cartilage tissue. (A) Alcian blue/H&E stained sagittal skull section of adult wild-type mouse. The temporal bone (green arrow) and the mandible condyle (black arrow) are separated by an articular disc (blue arrow). TMJ condylar cartilage was stained with Alcian blue. (B) Normal TMJ cartilage can be divided into three layers: superficial layer (above green dashed line), middle layer (between green and yellow dashed lines), and deep layer (below yellow dashed line). (C) *Col2CreER²* mice were bred with *Rosa^{mT/mG}* reporter mice. Tamoxifen was administered when the mice were 2-week-old (0.1 mg/g body weight, i.p., daily for 5 d). TMJ samples were harvested at 4-week-old. Fluorescent images showed red mT labelling in Cre-negative control mouse (upper panel) and green mG labelled chondrocytes in Cre-positive mouse (yellow arrows) (lower panel). (D) β -catenin IHC was performed and the result showed that β -catenin expression was up-regulated in the TMJ tissue of *β -catenin(ex3)^{Col2ER}* mice.

the condylar cartilage is divided into the superficial, middle, and deep layers (Fig. 1B) (Luder *et al.*, 1988; Mizoguchi *et al.*, 1996). Above the middle layer the tissue is fibrocartilage-like and beneath the middle layer is hyaline-like (Kuroda *et al.* 2009). The principal component of the superficial layer is type I collagen (Col1), and the principal component of the middle and deep layer is type II collagen (Col2) (Hirschmann and Shuttleworth, 1976). Mandibular condylar articular chondrocytes are the only cell type embedded in TMJ cartilage, and their function includes maintenance of the cartilaginous matrix.

The pathological progression of TMJ OA parallels that of knee and hip OA and is considered to be a similar

disease (Kuroda *et al.*, 2009; de Bont *et al.*, 1993). During the initiation stage of TMJ OA, the cartilage surface remains intact. At the cellular level, articular chondrocytes with low metabolism undergo hypertrophy, followed by apoptosis. These cellular effects result in cartilage fibrillation and subsequent progressive cartilage loss at the tissue level. Subchondral bone sclerosis and osteophyte formation happen in late-stage OA (Wang *et al.*, 2011). At the molecular level, primary cartilage matrix proteins, such as Col2 and aggrecan, start to decrease, concomitant with gradual increases in the expression of marker genes of chondrocyte hypertrophy, such as *Runx2* and *Col10*, as well as the genes coding for primary enzymes responsible for cartilage degeneration, such as

Mmp13, *Adamts4* and *Adamts5* (Neuhold *et al.*, 2001). Decreasing anabolic components, increasing metabolic components and proteolytic enzymes in the cartilage eventually tip the balance away from matrix maintenance toward degradation, resulting in complete cartilage erosion over time.

β -catenin is a key molecule in the canonical Wnt signalling pathway, which plays a critical role in bone, cartilage and joint formation, and development (Yang, 2003). Dysregulation of β -catenin causes cartilage degeneration and OA. In previous studies, we have reported that β -catenin expression was significantly up-regulated in articular cartilage in patients with knee OA (Zhu *et al.*, 2009) and in the intervertebral disc (IVD) tissues from patients with IVD degeneration (Wang *et al.*, 2012). Furthermore, *Col2-CreER^{T2}* driven cartilage-specific β -catenin overexpression in both knee joint and the IVD tissue leads to progressive cartilage loss and a degenerative phenotype in this mouse model (Zhu *et al.*, 2009; Wang *et al.*, 2012). These findings suggest that β -catenin is a critical molecule in OA pathogenesis. MMP13 and Adamts5 are key enzymes that target collagens and aggrecans for proteolytic degradation in cartilage (Glasson *et al.*, 2005; Knäuper *et al.*, 1996; Reboul *et al.*, 1996; Roach *et al.*, 2005; Wang *et al.*, 2012, 2013). In the present study, we investigated whether the constitutive activation of β -catenin in the middle and deep layers of the articular cartilage can compromise the homeostasis of this tissue in the TMJ using a β -catenin(*ex3*)^{Col2ER} mouse model.

Materials and Methods

Animals

Rosa^{mTmG} reporter mice, *Mmp13^{lox/lox}* mice, and *Adamts5^{-/-}* mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) (Muzumdar *et al.*, 2007; Stickens *et al.*, 2004; McCulloch *et al.*, 2009a, 2009b). *Col2CreER^{T2}* transgenic mice were generated as previously described (Chen *et al.*, 2007; Zhu *et al.*, 2008; Jin *et al.*, 2011; Wang *et al.*, 2012; 2013). β -catenin(*ex3*)^{lox/lox} mice were originally reported by Harada *et al.* (1999), and we have used these mice in our previous studies (Zhu *et al.*, 2009; Wang *et al.*, 2012, 2014). β -catenin(*ex3*)^{Col2ER} mice, (β -catenin(*ex3*)/*Mmp13*)^{Col2ER} and β -catenin(*ex3*)^{Col2ER}/*Adamts5^{-/-}* double mutant mice, and their Cre-negative littermate control mice were generated as shown in Tables 1-3. Tamoxifen (Sigma, St. Louis, MO, USA) was administered into 2-week-old mice by intraperitoneal (i.p.) injection (1 mg/10 g body weight for 5 d), $n = 5$ in each group. Animal protocols were approved by the IACUC of the University of Rochester.

Histology and histomorphometry

We dissected skulls from β -catenin(*ex3*)^{Col2ER} mice, (β -catenin(*ex3*)/*Mmp13*)^{Col2ER} mice, β -catenin(*ex3*)^{Col2ER}/*Adamts5^{-/-}* mice and their corresponding Cre-negative control mice. Samples were fixed in 10 % neutral buffered formalin (VWR, Radnor, PA, USA) for 3 d, then decalcified with formic acid (Decal Chemical Corp., Suffern, NY, USA) for 7 d. After neutralising with

Table 1. Breeding and production of β -catenin(*ex3*)^{Col2ER} mice.

Breeding	Desired progeny
a) β -catenin(<i>ex3</i>) ^{lox/+} x β -catenin(<i>ex3</i>) ^{lox/+}	β -catenin(<i>ex3</i>) ^{lox/lox}
b) <i>Col2CreER^{T2}</i> x β -catenin(<i>ex3</i>) ^{lox/lox}	β -catenin(<i>ex3</i>) ^{Col2ER} and β -catenin(<i>ex3</i>) ^{lox/+}

Table 2. Breeding and production of (β -catenin(*ex3*)/*Mmp13*)^{Col2ER} mice.

Breeding	Desired progeny
a) <i>Col2CreER^{T2}</i> x <i>Mmp13^{lox/lox}</i>	<i>Col2CreER^{T2}</i> ; <i>Mmp13^{lox/+}</i>
b) <i>Col2CreER^{T2}</i> ; <i>Mmp13^{lox/+}</i> x <i>Mmp13^{lox/lox}</i>	<i>Mmp13^{Col2ER}</i>
c) <i>Mmp13^{lox/lox}</i> x β -catenin(<i>ex3</i>) ^{lox/lox}	β -catenin(<i>ex3</i>) ^{lox/+} ; <i>Mmp13^{lox/+}</i>
d) β -catenin(<i>ex3</i>) ^{lox/+} ; <i>Mmp13^{lox/+}</i> x β -catenin(<i>ex3</i>) ^{lox/+} ; <i>Mmp13^{lox/+}</i>	β -catenin(<i>ex3</i>) ^{lox/lox} ; <i>Mmp13^{lox/lox}</i>
e) <i>Col2CreER^{T2}</i> ; <i>Mmp13^{lox/lox}</i> x β -catenin(<i>ex3</i>) ^{lox/lox} ; <i>Mmp13^{lox/lox}</i>	(β -catenin(<i>ex3</i>)/ <i>Mmp13</i>) ^{Col2ER} and β -catenin(<i>ex3</i>) ^{lox/+} ; <i>Mmp13^{lox/lox}</i>

Table 3. Breeding and production of β -catenin(*ex3*)^{Col2ER}/*Adamts5^{-/-}* mice.

Breeding	Desired progeny
a) <i>Col2CreER^{T2}</i> x <i>Adamts5^{-/-}</i>	<i>Col2CreER^{T2}</i> ; <i>Adamts5^{+/-}</i>
b) <i>Col2CreER^{T2}</i> ; <i>Adamts5^{+/-}</i> x <i>Adamts5^{-/-}</i>	<i>Col2CreER^{T2}</i> ; <i>Adamts5^{-/-}</i>
c) <i>Adamts5^{-/-}</i> x β -catenin(<i>ex3</i>) ^{lox/lox}	β -catenin(<i>ex3</i>) ^{lox/+} ; <i>Adamts5^{+/-}</i>
d) β -catenin(<i>ex3</i>) ^{lox/+} ; <i>Adamts5^{+/-}</i> x β -catenin(<i>ex3</i>) ^{lox/+} ; <i>Adamts5^{+/-}</i>	β -catenin(<i>ex3</i>) ^{lox/lox} ; <i>Adamts5^{-/-}</i>
e) <i>Col2CreER^{T2}</i> ; <i>Adamts5^{-/-}</i> x β -catenin(<i>ex3</i>) ^{lox/lox} ; <i>Adamts5^{-/-}</i>	β -catenin(<i>ex3</i>) ^{Col2ER} / <i>Adamts5^{-/-}</i> and β -catenin(<i>ex3</i>) ^{lox/+} / <i>Adamts5^{-/-}</i>

Cal-arrest (Decal Chemical Corp., Suffern, NY, USA), samples were processed and embedded in paraffin. Three μm thick mid-sagittal sections at 3 different levels (50 μm apart) were cut from the medial compartment of the TMJ. The sections were stained with Alcian blue/H&E (AB/H&E) and Safranin O/Fast green (SO/FG). Quantitative histomorphometric measurement, using ImagePro 4.5 (Leeds Precision Instruments, Minneapolis, MN, USA), was used to collect data. Alcian blue-positive staining areas were outlined on projected images of each histologic section to determine articular cartilage area and thickness. Three slides per mouse, 5 mice per group were analysed in the experiment.

Microcomputed tomography (μCT)

Prior to histological processing, we evaluated formalin-fixed TMJ tissues by μCT . We used a Scanco *vivaCT40* cone-beam scanner (SCANCO Medical, Brüttisellen, Switzerland) with 55 kVp source and 142 μA current. We scanned the TMJ tissues at a resolution of 10.5 μm . The scanned images from each group were evaluated at the same thresholds to allow 3-dimensional structural rendering of each sample.

Cre-recombination efficiency

Rosa^{mTmG} (membrane-Tomato/membrane-Green) mice contain two loxP sites on either side of the mT cassette. Mice express red fluorescence in all cell types and tissues before Cre-recombination and green fluorescence following Cre-recombination (Muzumdar *et al.*, 2007). *Col2CreER^{T2}* mice were bred with *Rosa^{mTmG}* mice to generate *Col2CreER^{T2}; Rosa^{mTmG}* mice. Tamoxifen was administered into 2-week-old mice by i.p. injection (1 mg/10 g body weight for 5 d). Skulls were dissected, fixed in 0.2 % glutaraldehyde at 4 °C for 4 d, followed by washing three times with phosphate buffered saline (PBS). Samples were decalcified in 14 % EDTA at 4 °C for 3 weeks, cryo-protected in 30 % sucrose at 4 °C for 3 d, and then embedded and processed for frozen sections. 3 μm -thick sections were imaged with a fluorescence microscope.

Immunohistochemistry

3 μm -thick paraffin sections were baked at 60 °C overnight. Slides were then deparaffinised, and rehydrated. Antigen retrieval of collagen II and collagen X was performed using 0.2 g pepsin in 50 mL of 0.01 N HCl at 37 °C for 25 min. Antigen retrieval of Runx2 was performed using a 0.01 M citrate buffer pH 6.0 at 95 °C for 2 h. Dako endogenous blocking reagent (S2003, Dako, Carpinteria, CA, USA)

was then used to quench endogenous peroxidase for 10 min. Non-specific binding sites were blocked with 1:20 normal horse/goat serum (S-2000, Vector Laboratories, Burlingame, CA, USA) for 20 min at room temperature. 1:80 dilution (2.5 $\mu\text{g}/\text{mL}$) of collagen II (MS-235-P, Thermo Scientific, Rockford, IL, USA), 1:50 dilution of collagen X (2031501005, Quartett, Berlin, Germany), or 1:100 dilution (10 $\mu\text{g}/\text{mL}$) of Runx2 (D130-3, MBL International, Woburn, MA, USA) primary antibodies were added and the slides were incubated at 4 °C overnight. Secondary biotinylated horse anti-mouse antibody (BA-2000, Vector Laboratories) or goat anti-mouse antibody (BA-9200, Vector Laboratories) at the dilution of 1:200 was added for 30 min on the second day, followed by incubation with 1:250 streptavidin (21130, Pierce, Rockford, IL, USA) for 30 min. Positive staining was detected by Romulin AEC Chromagen (Biocare Medical RAEC810L, Concord, CA, USA).

Cell proliferation and apoptosis assays

We dissected TMJs from *β -catenin(ex3)^{Col2ER}* mice and Cre-negative control mice. Samples were fixed in 10 % formalin, decalcified, and embedded in paraffin. The condyles were sectioned into serial sections at 3 μm thick in an anterior-posterior direction. Cell proliferation was carried out using anti-PCNA antibody at the dilution of 1:200 (ab18197, ABCAM®, Cambridge, UK). Apoptosis assay was carried out using a TUNEL assay kit according to the manufacturer's instructions (G3250, Promega, Madison, WI, USA).

Real-time PCR analysis

1-month-old *β -catenin(ex3)^{Col2ER}* mice and their Cre-negative control mice were sacrificed. TMJ cartilage was collected from freshly sacrificed mice to obtain total RNA. After washing with sterile PBS 3 times, the TMJ cartilage tissue was digested in 1 % pronase (Roche, Basel, Switzerland) and dissolved in 1x PBS for 30 min in a 37 °C water bath with continuous shaking. This was followed by washing with serum-free Dulbecco's modified Eagle medium (DMEM) 3 times. The TMJ cartilage tissue was then digested in 1 % Collagenase A (Roche) dissolved in serum-free DMEM for 3 h in a 37 °C water bath with continuous shaking. DMEM with 10 % foetal bovine serum (FBS) was then added to stop digestion and a 40 μm cell strainer was used to filter the digestion solution. Cells were collected and RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. 1 μg aliquots of RNA were

Table 4. Name and sequences of PCR primers.

Primer Name	Sequence
Runx2 (forward)	5'-GAGGGCACAAGT TCTATCTGGA-3'
Runx2 (reverse)	5'-GGTGGT CCGCGATGATCTC-3'
Col10a1 (forward)	5'-ACCCCAAGGACCTAAAGGAA-3'
Col10a1 (reverse)	5'-CCCCAGGATACCCTGTTTTT-3'
Mmp13 (forward)	5'-TTTGAGAACACGGGGGAAGA-3'
Mmp13 (reverse)	5'-ACTTTGTTGCCAATTCCAGG-3'
Adamts4 (forward)	5'-GGCAGATGACAAGATGGCAGCATT-3'
Adamts4 (reverse)	5'-AGACGAGTCAACCACCAAGTTGACA-3'
Adamts5 (forward)	5'-CCAAATGCACCTTCAGCCACGATCA-3'
Adamts5 (reverse)	5'-AATGTCAAGTTGCACTGCTGGGTG-3'

reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Real time PCR was performed using the Rotor Gene (RG 6000, Rotor Gene, Hilden, Germany) real time DNA amplification system (Corbett Research, Westborough, MA, USA) according to the manufacturer's instructions. Reactions were performed in a 20 μ L final volume containing cDNA samples, 50 μ M primers of interest, and SYBR green master mix (Applied Biosystems, Foster City, CA, USA) for PCR amplification. The name and sequence of PCR primers are listed in Table 4.

Statistical analysis

The values are presented as mean \pm standard error, and error bars represent standard error. For comparison of two groups of data, unpaired Student's *t*-test was performed. For comparison of multiple groups of data, one-way analysis of variance (ANOVA) was performed followed by Dunnett's test. **p* < 0.05 was considered as significant difference between groups.

Results

High Cre-recombination efficiency in TMJ cartilage

To evaluate *Col2-Cre* expression and recombination efficiency in the TMJ cartilage, *Col2CreER^{T2}* mice were bred with *Rosa^{mT/mG}* reporter mice to generate *Col2CreER^{T2}; Rosa^{mT/mG}* mice. Tamoxifen was administered when the mice were 2-week-old and TMJ samples were harvested at 1-month-old. The red fluorescent image of TMJ cartilage in Cre-negative control mice revealed absence of *Col2Cre*-expressing cells (Fig. 1C, upper panel), and the green-labelled chondrocytes in *Col2CreER^{T2}* mice showed *Col2Cre*-expressing cells (Fig. 1C, lower panel). IHC assay showed that β -catenin overexpression was detected at the edges of TMJ cartilage (Fig. 1D). These results demonstrate that TMJ chondrocytes were targeted by *Col2CreER^{T2}* mice with high efficiency.

Conditional activation of β -catenin induces TMJ cartilage defects

β -catenin(ex3)^{Col2ER} mice were generated by crossing *Col2CreER^{T2}* mice with *β -catenin(ex3)^{flox/flox}* mice (Table 1) and the role of β -catenin in TMJ tissue function was investigated. Tamoxifen was administered to 2-week-old mice and TMJ samples were harvested from these mice at 1, 3 and 6 months of age. One-month-old control mice showed thick and intact TMJ cartilage, which stained positive with Alcian blue. Furthermore, the chondrocytes in the control mice were well-organised in the three layers: smaller and round cells in the top superficial layer; medium-sized cells sitting in the lacuna were present in large numbers in the middle layer; and fewer, bigger, hypertrophic, mature cells in the deep layer (Fig. 2A). In contrast, 1-month-old *β -catenin(ex3)^{Col2ER}* mice already presented early signs of TMJ OA: decreased chondrocyte numbers in the superficial layer accompanied with less Alcian blue staining in this area, cells in the middle and deep layers illustrated increased hypertrophy, and the cartilage was thinner. At 3 months of age, articular

chondrocyte numbers in the superficial and middle layers of TMJ cartilage of *β -catenin(ex3)^{Col2ER}* mice were drastically reduced; hypertrophic cells were still present in the deep layer. However, total TMJ cartilage exhibited visibly reduced Alcian blue staining, demonstrating a decrease in proteoglycan levels (Fig. 2A). At 6 months of age, increased severity of defects in TMJ tissue was observed in *β -catenin(ex3)^{Col2ER}* mice. In addition to decreased cellularity of the middle and deep layers of cartilage, decreased cartilage area and subchondral bone sclerosis were also observed (Fig. 2A). Compared to middle and deep layers, the superficial area of TMJ cartilage is relatively normal. This is probably because the superficial layer of TMJ cartilage was not targeted by *Col2*-directed β -catenin expression. These results were further confirmed by μ CT. Significant reduction in TMJ joint space was observed in 3- and 6-month-old *β -catenin(ex3)^{Col2ER}* mice (Fig. 2B). Histomorphometric analysis revealed that the total cartilage area and total cartilage thickness were significantly decreased in *β -catenin(ex3)^{Col2ER}* mice compared to age-matched Cre-negative control mice. The total cartilage area decreased 22 % in *β -catenin(ex3)^{Col2ER}* mice compared to their Cre-negative littermate controls at 1 month of age, and decreased 41 % and 48 % in 3- and 6-month-old *β -catenin(ex3)^{Col2ER}* mice, respectively (Fig. 2C). These results were consistent with the quantification of total cartilage thickness. Cartilage thickness decreased 21 %, 39 % and 40 % in *β -catenin(ex3)^{Col2ER}* mice at 1, 3 and 6 months of age, respectively, compared to Cre-negative controls (Fig. 2D).

Histology and IHC analyses of β -catenin(ex3)^{Col2ER} mice

To further investigate OA related pathological alterations in *β -catenin(ex3)^{Col2ER}* mice, Safranin O/Fast green (SO/FG) staining was performed to assess changes in aggrecan levels in 3-month-old mice. Although the integrity of the TMJ articular surface was maintained, aggrecan expression as indicated by red staining was reduced in *β -catenin(ex3)^{Col2ER}* mice, compared to Cre-negative controls (Fig. 3A). Immunohistochemistry (IHC) revealed decreased *Col2* expression in the *β -catenin(ex3)^{Col2ER}* mice. In addition, IHC results also showed significantly increased *Runx2* and *Col10* expression in *β -catenin(ex3)^{Col2ER}* mice compared to controls, indicating the chondrocytes underwent hypertrophy at this stage (Fig. 3A). The result of PCNA staining showed that there were no significant changes in PCNA staining in TMJ tissues of *β -catenin(ex3)^{Col2ER}* mice, suggesting that overexpression of β -catenin in TMJ tissue did not affect TMJ cell proliferation (Fig. 3B). Data of TUNEL staining demonstrated an increase in numbers of apoptotic cells compared to that in control mice, but did not reach statistical significance between *β -catenin(ex3)^{Col2ER}* and control mice (Fig. 3C). Taken in concert, these results indicate that conditional activation of β -catenin in the TMJ induces degenerative defects that may not be due to changes in cell proliferation and apoptosis.

Gene expression analysis of β -catenin(ex3)^{Col2ER} mice

To determine changes in gene expression in the TMJ cartilage, cells were isolated from TMJ cartilage of

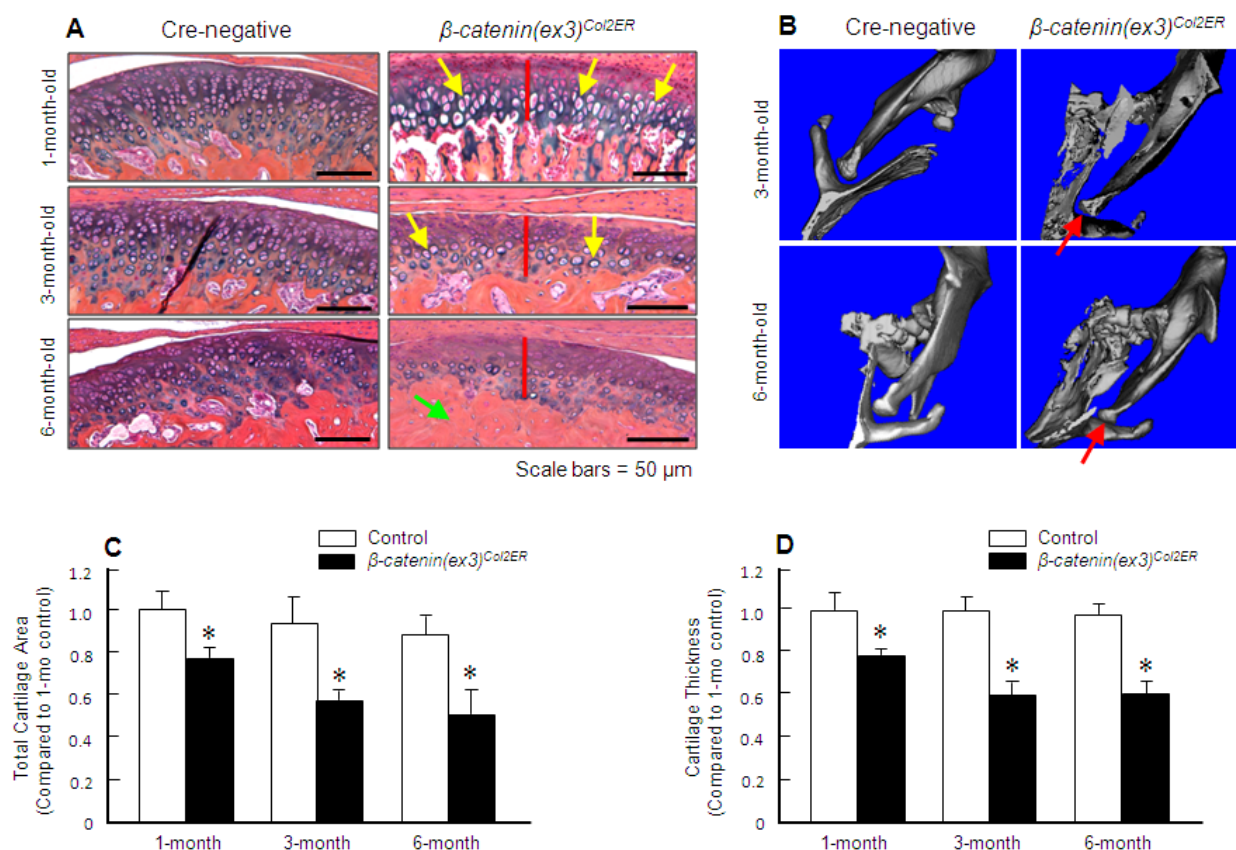


Fig. 2. Activation of β -catenin induces a TMJ OA-like phenotype. (A) Tamoxifen was administered into 2-week-old β -catenin(ex3)^{Col2ER} mice and Cre-negative control littermates (1 mg/10 g body weight, i.p., daily for 5 d). TMJ samples were harvested when the mice were 1-, 3- or 6-month-old and Alcian blue/H&E staining was performed. Histology results showed increased cartilage degradation in 1-, 3- and 6-month-old β -catenin(ex3)^{Col2ER} mice compared to Cre-negative control mice. Large numbers of hypertrophic chondrocytes were identified in 1- and 3-month-old β -catenin(ex3)^{Col2ER} mice (yellow arrows). Severe subchondral sclerosis was found in 6-month-old β -catenin(ex3)^{Col2ER} mice (green arrow). Reduction in cartilage thickness in 1-, 3- and 6-month-old β -catenin(ex3)^{Col2ER} mice is indicated by red bars. (B) μ CT images of 3- and 6-month-old β -catenin(ex3)^{Col2ER} mice showed decreased disc space in β -catenin(ex3)^{Col2ER} mice (red arrows) compared to control mice. (C) Total TMJ cartilage area was quantified by tracing the Alcian blue-positive area. Cartilage area decreased 22 %, 41 % and 48 % in 1-, 3- and 6- month-old β -catenin(ex3)^{Col2ER} mice compared to the same aged control mice (* p < 0.05, unpaired Student t -test, n = 5). (D) Total cartilage thickness was quantified by tracing the Alcian blue-positive thickness in the centre of the TMJ cartilage. Total cartilage thickness decreased 21 %, 39 % and 40 % in 1-, 3- and 6-month-old β -catenin(ex3)^{Col2ER} mice compared to the same aged control mice (* p < 0.05, unpaired Student t -test, n = 5).

1-month-old mice. RT-PCR results were consistent with IHC findings. *Runx2* (Fig. 4A) and *Col10* (Fig. 4B) expression were increased 2.8 and 4.1 fold, respectively, in β -catenin(ex3)^{Col2ER} mice compared to control mice. Furthermore, in β -catenin(ex3)^{Col2ER} mice, the expression of genes encoding cartilage degrading enzymes, such as *Mmp13* (Fig. 4C), *Adamts4* (Fig. 4D), and *Adamts5* (Fig. 4E) was increased 4.5, 2.7 and 4.0 fold, respectively. These results suggest that the activation of β -catenin signalling could lead to chondrocyte hypertrophy and degenerative defects.

Ameliorated TMJ defects by deletion of the *Mmp13* or *Adamts5* gene

To assess the roles of MMP13 and Adamts5 in the development of TMJ defects, (β -catenin(ex3)/*Mmp13*)

Col2ER and β -catenin(ex3)^{Col2ER}/*Adamts5*^{-/-} double mutant mice were generated (Tables 2 and 3). Tamoxifen was administered when the mice were 2-week-old and histology analysis was performed. In 3-month-old mice, TMJ defect progression was markedly decelerated in both (β -catenin(ex3)/*Mmp13*)^{Col2ER} and β -catenin(ex3)^{Col2ER}/*Adamts5*^{-/-} double mutant mice (Figs. 5A and B) compared to β -catenin(ex3)^{Col2ER} mice (Fig. 2A). Histology results showed a greater amount of intact cartilage and more pronounced Alcian blue and SO/FG staining in the double mutant mice, indicating higher collagen and aggrecan content in the cartilage. Results of histomorphometry also showed that TMJ defects were decelerated after deletion of either the *Mmp13* or *Adamts5* gene in β -catenin(ex3)^{Col2ER} mice (Figs. 5C and D). Compared to the 3-month-old β -catenin(ex3)^{Col2ER}

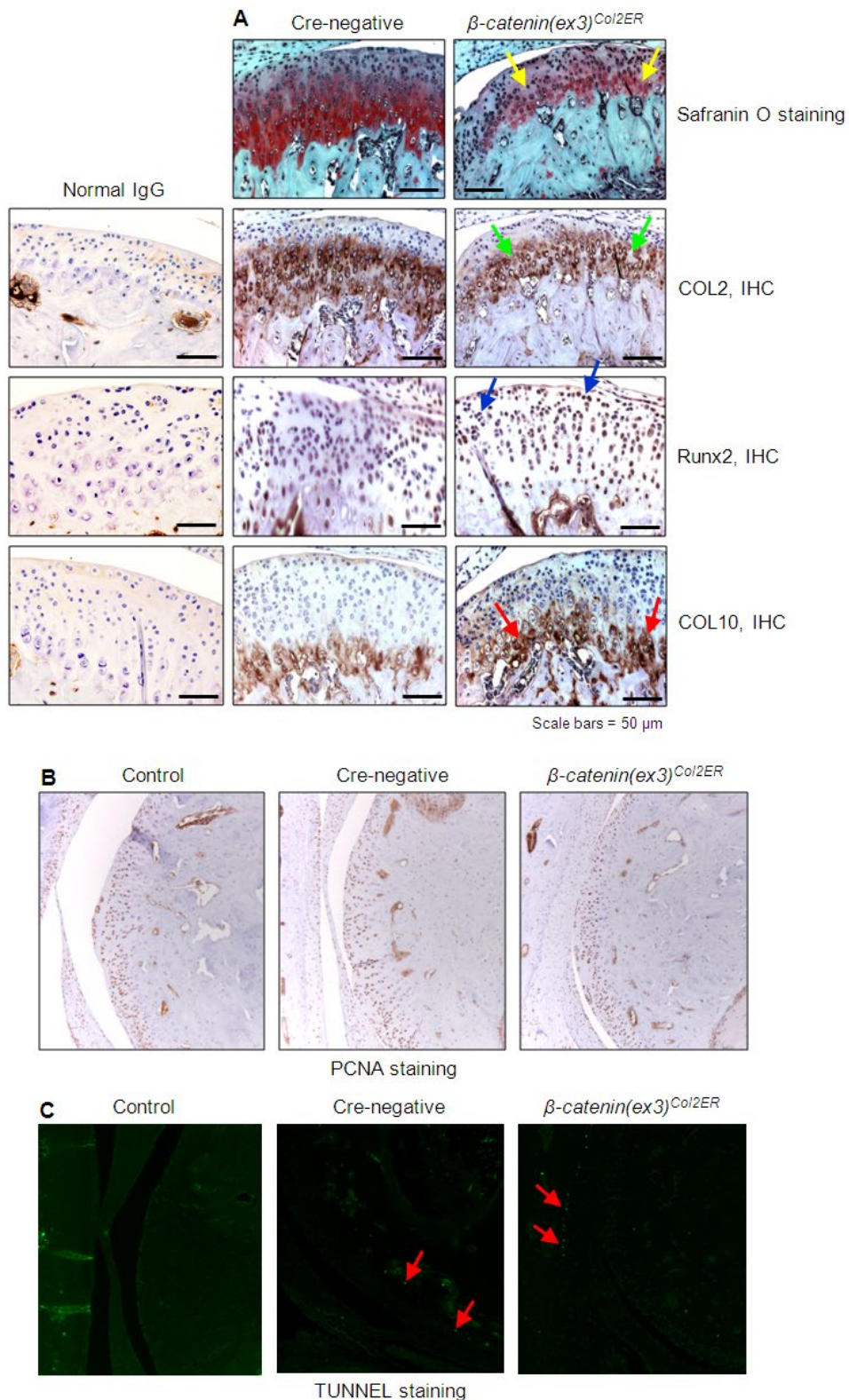


Fig. 3. Alterations in matrix protein expression, cell proliferation and apoptosis in β -catenin(ex3)^{Col2ER} mice. Tamoxifen was administered when β -catenin(ex3)^{Col2ER} and Cre-negative control mice were 2-week-old (1 mg/10 g body weight, i.p., daily for 5 d). TMJ cartilage samples were harvested at 3-month-old. (A) Safranin O/fast green staining was performed to evaluate aggrecan levels. Results showed that aggrecan levels were significantly reduced in β -catenin(ex3)^{Col2ER} mice (yellow arrows). IHC was performed to detect changes in COL2, Runx2 and COL10 protein expression in β -catenin(ex3)^{Col2ER} mice. IHC results showed that COL2 expression was significantly decreased (green arrows), and expression of Runx2 (blue arrows) and COL10 (red arrows) was significantly increased in β -catenin(ex3)^{Col2ER} mice. PCNA (B) and TUNNEL (C) staining was performed in TMJ tissues derived from Cre-negative control and β -catenin(ex3)^{Col2ER} mice. Results showed that there were no significant changes in cell proliferation (PCNA staining) and apoptosis (TUNNEL staining) in β -catenin(ex3)^{Col2ER} mice.

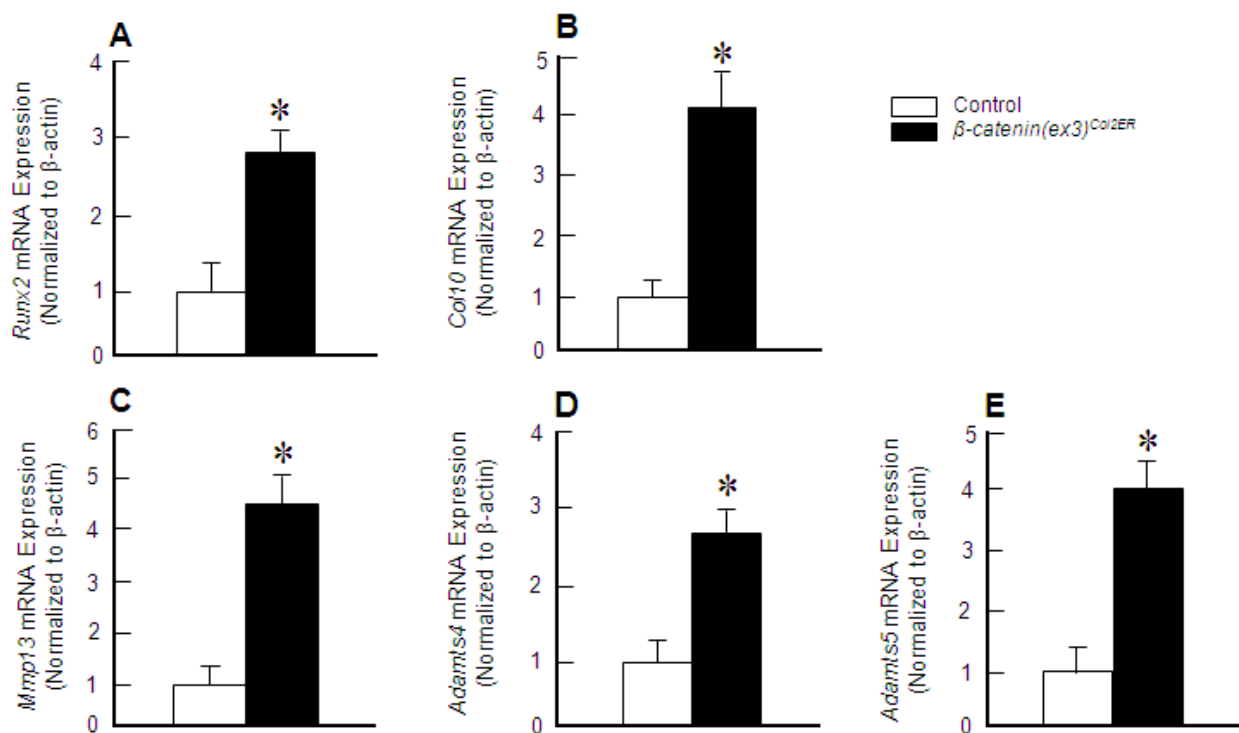


Fig. 4. Alterations in gene expression in β -catenin(ex3)^{Col2ER} mice. Total RNA was extracted from TMJ tissues from 4-week-old β -catenin(ex3)^{Col2ER} mice and Cre-negative control mice (pooled from 4 mice) and real-time PCR analysis was performed. The results showed that expression of *Runx2* (A), *Col10* (B), *Mmp13* (C), *Adamts4* (D), and *Adamts5* (E) was significantly increased 2.8, 4.1, 4.5, 2.7 and 4.0 fold, respectively, in TMJ tissues derived from β -catenin(ex3)^{Col2ER} mice (* $p < 0.05$, unpaired Student *t*-test, $n = 3$).

Table 5. Deletion of the *Mmp13* or *Adamts5* gene reverses the TMJ OA phenotype observed in 3- and 6-month-old β -catenin(ex3)^{Col2ER} mice.

Mouse group	Cartilage area*	% Area recovery [†]	Cartilage thickness*	% Thickness recovery [†]
β -catenin(ex3) ^{Col2ER} (3-month-old)	59 %		61 %	
$[\beta$ -catenin(ex3)/ <i>Mmp13</i>] ^{Col2ER} (3-month-old)	92 %	33 %	94 %	33 %
β -catenin(ex3) ^{Col2ER} / <i>Adamts5</i> ^{-/-} (3-month-old)	87 %	28 %	75 %	14 %
β -catenin(ex3) ^{Col2ER} (6-month-old)	52 %		60 %	
$[\beta$ -catenin(ex3)/ <i>Mmp13</i>] ^{Col2ER} (6-month-old)	78 %	26 %	70 %	10 %
β -catenin(ex3) ^{Col2ER} / <i>Adamts5</i> ^{-/-} (6-month-old)	73 %	21 %	66 %	6 %

* Compared to the Cre-negative littermate controls.

[†] Compared to β -catenin(ex3)^{Col2ER} mice.

mice, the loss of cartilage area was reversed 33 and 28 % in (β -catenin(ex3)/*Mmp13*)^{Col2ER} and β -catenin(ex3)^{Col2ER}/*Adamts5*^{-/-} double mutant mice. Compared to the 6-month-old β -catenin(ex3)^{Col2ER} mice, the loss of cartilage area was reversed 26 and 21 % in these double mutant mice (Table 5). Detailed quantitative analyses revealed that there was a 41 % cartilage loss in 3-month-old β -catenin(ex3)^{Col2ER} mice. However, only a 8 and 13 % cartilage area decrease was observed in 3-month-old (β -catenin(ex3)/*Mmp13*)^{Col2ER} and β -catenin(ex3)^{Col2ER}/*Adamts5*^{-/-} double mutant mice compared to Cre-negative controls (Fig. 5C and Table 5). In 6-month-old mice, there was a 48 % cartilage

loss in β -catenin(ex3)^{Col2ER} mice. In contrast, only 22 and 27 % of the cartilage area decreased in (β -catenin(ex3)/*Mmp13*)^{Col2ER} and β -catenin(ex3)^{Col2ER}/*Adamts5*^{-/-} double mutant mice compared to Cre-negative controls (Fig. 5C and Table 5). These results demonstrated that the deletion of either *Mmp13* or *Adamts5* gene can significantly reverse β -catenin activation-induced TMJ cartilage loss. Quantification of cartilage thickness showed similar results (Fig. 5D). There was a 39 % decrease in cartilage thickness in β -catenin(ex3)^{Col2ER} mice. However, only a 6 and 25 % decrease in cartilage thickness was observed in 3-month-old double mutant mice. In 6-month-old mice, there was

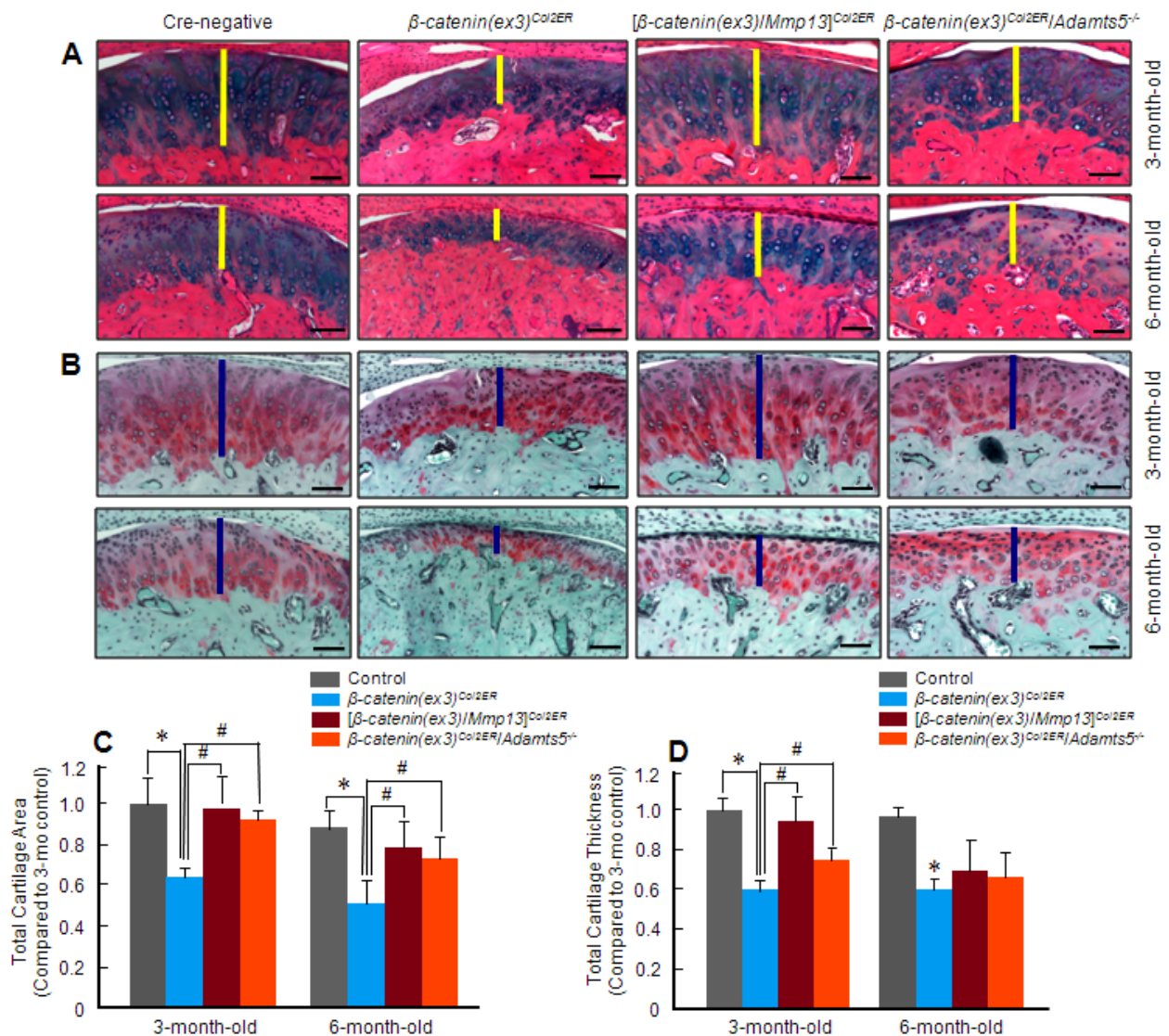


Fig. 5. OA-like phenotype was decelerated when either the *Mmp13* or the *Adamts5* gene was deleted in β -catenin(ex3)^{Col2ER} mice. Tamoxifen was administered into 2-week-old mice (0.1 mg/g body weight, i.p., daily for 5 d). TMJ samples were harvested from 3- or 6-month-old mice and Alcian blue/H&E and SO/FG staining were performed. Alcian blue/H&E staining (A) and SO/FG staining (B) showed decelerated TMJ defect progression and more aggrecan contents in double mutant mice compared to those in β -catenin(ex3)^{Col2ER} mice. (C) Total TMJ cartilage area was quantified by tracing the Alcian blue-positive area. Cartilage areas were 8 and 13 % decreased in 3-month-old $[\beta$ -catenin(ex3)/Mmp13]^{Col2ER} and β -catenin(ex3)^{Col2ER}/Adamts5^{-/-} double mutant mice, and were 22 and 27 % decreased in 6-month-old $[\beta$ -catenin(ex3)/Mmp13]^{Col2ER} mice and β -catenin(ex3)^{Col2ER}/Adamts5^{-/-} mice compared to the same age control mice (**p* < 0.05, compared to Cre-negative control group; ***p* < 0.05, compared to β -catenin(ex3)^{Col2ER} group; one-way ANOVA followed by Dunnett's test, *n* = 5). (D) Total cartilage thickness was quantified by tracing the Alcian blue-positive thickness in the centre of the TMJ cartilage. Total cartilage thickness was decreased 6 and 25 % in 3-month-old double mutant mice and 30 and 34 % decreased in 6-month-old double mutant mice compared to the same age control mice (**p* < 0.05, compared to Cre-negative control group; ***p* < 0.05, compared to β -catenin(ex3)^{Col2ER} group; one-way ANOVA followed by Dunnett's test, *n* = 5).

a 40 % decrease in cartilage thickness in β -catenin(ex3)^{Col2ER} mice. In contrast, only a 30 and 34 % decrease in cartilage thickness was observed in 6-month-old double mutant mice (Fig. 5D and Table 5). Statistical analysis results demonstrated a significant decrease in cartilage thickness in 3-month-old β -catenin(ex3)^{Col2ER} mice. Compared to β -catenin(ex3)^{Col2ER} mice, the reduction of cartilage thickness was significantly reversed in 3-month-old double mutant mice (Fig. 5D).

Discussion

Although TMJ OA is a common degenerative joint disease, the pathological mechanisms of this disease remain largely unknown. In this study, we generated a genetic mouse model and demonstrated that overexpression of β -catenin in TMJ chondrocytes leads to degenerative defects resembling an OA-like phenotype in the TMJ cartilage. Furthermore, deletion of the *Mmp13* or *Adamts5* gene

in β -catenin(*ex3*)^{Col2^{ER}} mice significantly and partially rescued TMJ defects observed in β -catenin(*ex3*)^{Col2^{ER}} mice.

It has been shown that β -catenin protein expression was highly-upregulated in articular cartilage tissues from patients with knee OA (Zhu *et al.*, 2009) and in intervertebral disc tissues from patients with degenerative disc disease (Wang *et al.*, 2012). Col2-driven cartilage-specific activation of β -catenin in mice leads to a knee joint OA-like phenotype and defects in intervertebral disc tissues (Zhu *et al.*, 2009; Wang *et al.*, 2012). Here, we further demonstrated that Col2-driven β -catenin(*ex3*)^{Col2^{ER}} mice also showed TMJ degenerative defects. Even though articular cartilage in the knee joint is hyaline cartilage, and IVD tissue and TMJ cartilage are composed of both hyaline cartilage and fibrocartilage, the mechanisms of degeneration in these tissues are very similar. Moreover, in our study we specifically determine the role of β -catenin signalling in *Col2-Cre* targeting cells. Among articular cartilage, TMJ cartilage and disc tissues, it seems that knee joint articular cartilage was most efficiently targeted by *Col2-Cre* due to high *Col2* expression in this tissue compared to IVD tissue and TMJ cartilage (Chen *et al.*, 2007; Zhu *et al.*, 2008; Jin *et al.*, 2011; Fig. 1).

To determine changes in gene expression in TMJ tissues with degenerative defects in β -catenin(*ex3*)^{Col2^{ER}} mice, we isolated TMJ cartilage tissues. We found that RT-PCR results are consistent with the SO/FG and IHC results. *Runx2* and *Col10* are two hypertrophic markers in cartilage during OA development, and their expression levels were increased in β -catenin(*ex3*)^{Col2^{ER}} mice. The RT-PCR results also revealed increases in the expression of genes encoding collagenase (MMP13) and aggrecanases (*Adamts4* and *Adamts5*) in β -catenin(*ex3*)^{Col2^{ER}} mice. It has been shown that MMP13 and *Adamts5* are the primary enzymes leading to cartilage degeneration (Neuhold *et al.*, 2001; Glasson *et al.*, 2005; Wang *et al.*, 2013), and *Adamts5* may play a more crucial role in cartilage degradation compared to *Adamts4* (Glasson *et al.*, 2004). We thus generated (β -catenin(*ex3*)/*Mmp13*)^{Col2^{ER}} and β -catenin(*ex3*)^{Col2^{ER}}/*Adamts5*^{-/-} double mutant mice to determine roles of *Mmp13* and *Adamts5* in β -catenin signalling during TMJ tissue degeneration. Our results showed decelerated TMJ defects in both of these double mutant mice. Although deletion of the *Mmp13* or *Adamts5* gene significantly decreased TMJ defects observed in β -catenin(*ex3*)^{Col2^{ER}} mice, normal cartilage phenotype was not completely restored by deletion of either *Mmp13* or *Adamts5*. There was still a significant loss of proteoglycans in these double mutant mice compared to Cre-negative controls. The possible reason to account for this could be that *Mmp13*, *Adamts4* and *Adamts5*, as well as other catabolic factors, contribute to OA development (Stanton *et al.*, 2005; Glasson *et al.*, 2005; Majumdar *et al.*, 2007), and deletion of only one of them may not be able to completely inhibit cartilage degeneration.

Moreover, our results indicate that the deletion of the *Mmp13* gene in the β -catenin(*ex3*)^{Col2^{ER}} background seems more effective in preventing OA progression than deletion of the *Adamts5* gene. This is probably because MMP13 preferentially cleaves the proteolysis of Col2, which is the most abundant matrix protein

in the TMJ cartilage. MMP13 also targets aggrecan, types IV and IX collagens, gelatin, osteonectin and perlecan in cartilage for degeneration (Shiomi *et al.*, 2010). In contrast, *Adamts5* is an aggrecanase that is mainly responsible for aggrecan degradation. Therefore, deletion of the *Mmp13* gene is likely to prevent the degradation of more substrates responsible for cartilage degeneration compared to deletion of *Adamts5*.

Recent reports demonstrated that β -catenin signalling is responsive to mechanical loading (Lin *et al.*, 2009; Premaraj *et al.*, 2013; Javaheri *et al.*, 2014). Abnormal occlusion could lead to OA development in TMJ tissue (Zhang *et al.*, 2013; Liu *et al.*, 2014). We hypothesise that one of the major upstream regulatory factors for β -catenin signalling in TMJ tissue is mechanical loading. In the future, we will examine if abnormal occlusion (causing mechanical changes in TMJ tissue) will induce β -catenin signalling activation in TMJ tissue. In addition to mechanical loading, growth factors involved in inflammation, such as tumour necrosis factor- α (TNF- α) and interleukin 1 β (IL-1 β) could be potentially important upstream regulators for β -catenin signalling in TMJ tissues. It has been reported that TNF- α and IL-1 β regulate β -catenin signalling in other tissues (Kitazawa *et al.*, 2011; Raymond *et al.*, 2012; Gong *et al.*, 2014). Regarding the mechanism of β -catenin overexpression-induced defects in TMJ tissue, we propose that one of the major downstream mediators for β -catenin signalling in the TMJ tissue is *Runx2*. Activation of β -catenin causes up-regulation of *Runx2*, which subsequently promotes *Mmp13* and *Adamts5* expression in TMJ tissues (Figs. 3 and 4; Wang *et al.*, 2012).

It has been shown that conditional activation of β -catenin in knee cartilage and intervertebral disc cartilage induce knee OA and disc tissue degeneration. β -catenin plays a key role in the activation of downstream target genes, such as *Mmp13*, *Adamts4* and *Adamts5* in cartilage, leading to the development of knee OA and disc degeneration (Wang *et al.*, 2012). The current study is the first to demonstrate that the same signalling mechanism can also induce TMJ cartilage degradation. The potential mechanisms for β -catenin activation in patients with TMJ OA may include activating mutations of the β -catenin gene (Morin *et al.*, 1997; Simon *et al.*, 2005; Jamieson *et al.*, 2004; Derksen *et al.*, 2004), mechanical loading (Hens *et al.*, 2005; Robinson *et al.*, 2006; Sen *et al.*, 2009) and inflammation (Johnson, 2009; Kaler *et al.*, 2009). In summary, our studies suggest that β -catenin may play a role in TMJ cartilage function and may be a mediator in TMJ OA development. *Mmp13* and *Adamts5* are important downstream targets of β -catenin signalling in TMJ chondrocytes during OA development.

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had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Discussion with Reviewers

Reviewer I: Can the authors provide an explanation on why they decided to use the *Col2CreER* mice and not the *Col1CreER* mice, as they stated in the introduction that Col1 is more expressed in the superficial layer of the articular cartilage, the site first affected during the development of OA. Recent literature showed that beta catenin expression is very important for cartilage

homeostasis in this layer of cells (Yasuhara *et al.*, 2011); therefore, the effect of the overexpression of beta catenin in this location of the tissue might have a different effect.

Authors: The reason that we used *Col2-CreER^{T2}* mice is because we found degenerative defects in articular cartilage and disc tissue in β -catenin(ex3)^{Col2ER} in which β -catenin expression was specifically targeted using the *Col2-CreER^{T2}* transgenic mice (Zhu *et al.*, 2009; Wang *et al.*, 2012). We agree with the reviewer that targeting β -catenin in the superficial layer using *Col1-Cre* or *Col1-CreER* mice is an important approach to determine the role of β -catenin in the superficial layer of TMJ cartilage. As a future direction, we plan to express β -catenin in Col1-expressing cells in TMJ tissue and compare phenotypic difference between β -catenin(ex3)^{Col2ER} and β -catenin(ex3)^{Col1ER} mice.

Reviewer II: Do the authors suppose that TMJ cartilage behaves more like hyaline or fibrocartilage, and why do they suppose the cartilages react in their presumed way?

Authors: We have extracted RNA from TMJ tissues of β -catenin(ex3)^{Col2ER} mice and examined changes in gene expression in these tissues and found that expression of Runx2, Col10, Mmp13, Adamts4 and Adamts5 was significantly increased in TMJ tissues of β -catenin(ex3)^{Col2ER} mice compared to that of Cre-negative control mice.

Reviewer III: The authors could comment on why the surface of the joint remains smooth and is not eroded in β -catenin(ex3)^{Col2ER} mice.

Authors: This is probably because we have used *Col2-CreERT2* mice targeting the middle and deep layers of TMJ cartilage but not the superficial layer. Col1 is the major protein expressed in the superficial layer of TMJ cartilage.

Reviewer III: How is the claimed up-regulation of *Adamts5* and *Mmp13* in β -catenin(ex3)^{Col2ER} mice regulated by stabilised β -catenin?

Authors: We propose that overexpression of β -catenin in TMJ tissue causes up-regulation of *Runx2*, which subsequently promotes *Mmp13* and *Adamts5* expression in β -catenin(ex3)^{Col2ER} mice. This signalling mechanism was demonstrated in intervertebral disc tissues in our previous studies (Wang *et al.*, 2012).

Additional Reference

Yasuhara R, Ohta Y, Yuasa T, Kondo N, Hoang T, Addya S, Fortina P, Pacifici M, Iwamoto M, Enomoto-Iwamoto M (2011) Roles of β -catenin signaling in phenotypic expression and proliferation of articular cartilage superficial zone cells. *Lab Invest* **91**:1739-1752.