Expressing human SHOX in Shox2\(^{SHOX\,K I/K I}\) mice leads to congenital osteoarthritis-like disease of the temporomandibular joint in postnatal mice

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Abstract. The temporomandibular joint (TMJ), a unique synovial joint whose development differs from that of other synovial joints, develops from two distinct mesenchymal condensations that grow toward each other and ossify through different mechanisms. The short stature homeobox 2 (Shox2) gene serves an important role in TMJ development and previous studies have demonstrated that Shox2\(^{SHOX\,K I/K I}\) mice display a TMJ defective phenotype, congenital dysplasia and premature eroding of the articular disc, which is clinically defined as a TMJ disorder. In the present study, Shox2\(^{SHOX\,K I/K I}\) mouse models were used to investigate the mechanisms of congenital osteoarthritis (OA)-like disease during postnatal TMJ growth. Shox2\(^{SHOX\,K I/K I}\) mice were observed to develop a severe muscle wasting syndrome from day 7 postnatal. Histological examination indicated that the condyle and glenoid fossa of Shox2\(^{SHOX\,K I/K I}\) mice was reduced in size in the second week after birth. The condyles of Shox2\(^{SHOX\,K I/K I}\) mice exhibited reduced expression levels of collagen type II and Indian hedgehog, and increased expression of collagen type I. A marked increase in matrix metalloproteinase 9 (MMP9) and MMP13 in the condyles was also observed. These cellular and molecular defects may contribute to the observed (OA)-like phenotype of Shox2\(^{SHOX\,K I/K I}\) mouse TMJs.

Introduction

The temporomandibular joint (TMJ) is a complex structure that consists of the condyle, fibrocartilaginous disc and glenoid fossa, which is essential for jaw movement and is only found in mammals (1,2). Osteoarthritis (OA) is a TMJ disorder that affects 8-16% of the human population, and can lead to chewing difficulties and chronic myofacial pains (3). Degradation of the condyle cartilage is one of the main factors that lead to TMJ OA (4). Condyle structure and function are affected extracellular matrix (ECM) components, including collagens, glycosaminoglycans (GAGs) and proteoglycans (PGS) (5,6). Investigation of the alterations that may occur in condyle composition is important for understanding the basis of the pathological process of TMJ OA (7).

The ECM components, which are critical for resistance to compressive forces and for maintaining tensile properties, are altered in several cartilage-related pathologies and at different stages of the same pathological process (8). Additionally, cartilage degradation results from an imbalance between anabolic and catabolic cytokine signaling pathways, which can lead to an increase in matrix-degrading proteases and a decrease in matrix synthesis (9,10). These alterations are characterized by the significant upregulation of matrix metalloproteinases (MMPs), which are responsible for the significant degradation of cartilage ECM proteins (11). MMPs function to cleave aggrecan and collagens, the two most abundant ECM components of skeletal tissue (12). MMP9, an MMP subtype implicated in the degradation of cartilage ECM proteins, has been observed to play a central role in connective tissue remodeling and basement membrane turnover (2,5). MMP13, a member of the MMP family of neutral endopeptidases, is highly overexpressed in chondrocytes and synovial cells during OA (13,14).

The short stature homeobox 2 (Shox2) gene is important for the development of all long bones that undergoes endochondral ossification (15,16). A previous study has demonstrated that mice expressing human SHOX (Shox2\(^{SHOX\,K I/K I}\) mice) do not exhibit TMJ dysplasia and ankylosis at birth, but display a postnatal, prematurely eroded articular disc (2). This suggests...
that, although human SHOX can exert similar functions to mouse Shox2 in the regulation of early TMJ development, the human gene has a distinct function in regulating TMJ growth in postnatal mice (2). Therefore, the cellular and molecular alterations that contribute to the congenital OA-like disease of the TMJ in Shox2\textsuperscript{SHOX KI/KI} mice were investigated in the present study.

Materials and methods

Mouse details. Animal procedures used in the present study were approved by the Institutional Animal Care and Use Committee of the Fujian University of Traditional Chinese Medicine (Fuzhou, China). The generation of Shox2\textsuperscript{SHOX KI/KI} mice from the lab of Dr Yiping Chen (Department of Cell and Molecular Biology, Tulane University, New Orleans, LA, USA) was conducted as described previously (2,17). A total of 96 mice (48 male and 48 female; 5/cage) were bred on a C57BL/6J background (42 wild-type Shox2\textsuperscript{+/+} mice, 12 Shox2\textsuperscript{SHOX KI/+} mice and 42 Shox2\textsuperscript{SHOX KI/KI} mice). All mice were exposed to a 12-h light/dark cycle, in a temperature (22±1°C) and humidity (56±5%)-controlled environment and maintained on a 0.3% sodium diet. Mice were sacrificed using carbon dioxide (CO\textsubscript{2}) and the heads from postnatal day 0 (P0), P7, P14 and P21 mice were fixed and decalcified in Surgipath’s Decalcifier I\textsuperscript{a} (Leica Biosystems GmbH, Wetzlar, Germany) for a variable time-period (2-7 days) that was dependent on the age of the mice (2,5).

Histological analyses. A total of 36 mouse heads were dehydrated using a graded ethanol series, cleared with xylene (Sigma-Aldrich, St. Louis, MO, USA), paraffin-embedded and sectioned at 10 µm using a microtome. Histological analysis of TMJ sections was performed as previously described using standard hematoxylin-eosin (HE) staining (18), Safranin O-Fast Green staining (19) and azon red/anilin blue (Sigma-Aldrich) staining (2,16).

Bone staining. A total of 6 P21 wild-type Shox2\textsuperscript{+/+} and Shox2\textsuperscript{SHOX KI/KI} mice were fixed in 90% ethanol for a minimum of 1 week. Prior to fixation, the skins, adipose tissues, eyeballs and thoracoabdominal organs were removed. Specimens were placed in 85% ethanol containing 1.5% potassium hydroxide (KOH) and 1% Alizarin Red S (Sigma-Aldrich) staining (Fig. 1A and B). Body weight analysis at P0, P7, P14, and P21 demonstrated that wild-type Shox2\textsuperscript{SHOX KI/+}, Shox2\textsuperscript{SHOX KI/+} and Shox2\textsuperscript{SHOX KI/KI} mice displayed a similar body weight at birth (P0); however, Shox2\textsuperscript{SHOX KI/KI} mice displayed a significant reduction in body weight at P7 (P<0.0001), P14 (P<0.0001) and P21 (P<0.0001) compared with wild-type Shox2\textsuperscript{SHOX KI/+} mice (Fig. 1C).

Immunohistochemical analyses. A total of 54 mouse heads were dehydrated using a graded ethanol series, cleared with xylene, paraffin-embedded and sectioned at 8 µm for immunohistochemical analysis. Immunohistochemical staining was performed as previously described (2). Paraffin sections were de-paraffinized and rehydrated in a series of alcohol dilutions, incubated for 20 min at 100°C in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval and then cooled to room temperature. Sections were blocked using goat serum (1:10, Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated for 15 min at room temperature. This was followed by overnight incubation at 4°C with polyclonal antibodies against runt-related transcription factor 2 (1:1,000; ab76956), sex determining region Y-box 9 (1:500; ab26414), collagen type I (col I; 1:500; ab34710), collagen type II (col II; 1:200; ab53047), aggrecan (1:500; ab36861), MMP9 (1:300; ab38898), MMP13 (1:50; ab75606) and Indian hedgehog (Ihh; 1:200; ab39634). Antibodies were obtained from Abcam (Cambridge, MA, USA). Slides were then washed 3 times using phosphate-buffered saline (PBS), followed by incubation with a biotinylated horseradish peroxidase goat anti-rabbit secondary antibody (1:1,000; Thermo Fisher Scientific, Inc.) for 20 min at 37°C. Slides were then washed 3 times using PBS. Immunolabeled samples were visualized by incubating in 0.05% diaminobenzidine (Invitrogen; Thermo Fisher Scientific, Inc.) diluted in PBS for 5 min at room temperature, followed by rinsing for 10 min under a running water tap. Immunohistochemically stained mouse TMJs were visualized using an Olympus BH-2 light microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis. Experiments were repeated a minimum of 3 times and presented as the mean ± standard deviation. Statistical analysis of the data was achieved using one-way analysis of variance with SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Shox2\textsuperscript{SHOX KI/KI} mice develop a severe muscle wasting syndrome. The majority of Shox2\textsuperscript{SHOX KI/KI} mice survived beyond 7 days after birth but gradually developed a severe muscle wasting syndrome (Fig. 1). At P21, 5/20 (25%) Shox2\textsuperscript{SHOX KI/KI} mice survived following weaning, but were notably reduced in size compared with Shox2\textsuperscript{SHOX KI/+} and wild-type Shox2\textsuperscript{SHOX KI/+} mice (Fig. 1A and B). Body weight analysis at P0, P7, P14, and P21 demonstrated that wild-type Shox2\textsuperscript{SHOX KI/+}, Shox2\textsuperscript{SHOX KI/+} and Shox2\textsuperscript{SHOX KI/KI} mice displayed a similar body weight at birth (P0); however, Shox2\textsuperscript{SHOX KI/KI} mice displayed a significant reduction in body weight at P7 (P<0.0001), P14 (P<0.0001) and P21 (P<0.0001) compared with wild-type Shox2\textsuperscript{SHOX KI/+} mice (Fig. 1C).

Shox2\textsuperscript{SHOX KI/KI} mice exhibit an abnormal TMJ. Histological analysis of TMJ development in postnatal mice using HE staining (Fig. 2A), Safranin O-Fast green staining (Fig. 2B) and azon red/anilin blue staining (Fig. 2C), demonstrated that Shox2\textsuperscript{SHOX KI/KI} mice displayed congenital cartilage degradation from P7. To investigate the Shox2\textsuperscript{SHOX KI/KI} TMJ phenotype further, changes in condyle and glenoid fossa width, starting from the region that displayed the most significant change, was investigated at P0, P7, P14 and P21 time points. The average glenoid fossa and condyle width in wild-type Shox2\textsuperscript{SHOX KI/+} mice at each time point was defined as 100%. The glenoid fossa and condyle width was similar in wild-type Shox2\textsuperscript{SHOX KI/+}, Shox2\textsuperscript{SHOX KI/+} and Shox2\textsuperscript{SHOX KI/KI} mice at P0 (Fig. 2D and E). However, the glenoid fossa and condyle width in Shox2\textsuperscript{SHOX KI/KI} mice was significantly reduced when compared with wild-type Shox2\textsuperscript{SHOX KI/+} and Shox2\textsuperscript{SHOX KI/KI} mice at P14 (P<0.0001) and P21 (P<0.0001) stages (Fig. 2D and E). Notably, these developmental alterations of the TMJ, led to restrained jaw movement and eating and drinking difficulties, which is clinically defined as TMJ OA.
Figure 1. Shox2<sub>SHOX KI/KI</sub> mice develop a severe muscle wasting syndrome. (A) Photograph of a wild-type Shox2<sup>+/+</sup>, Shox2<sub>SHOX KI/+</sub> and Shox2<sub>SHOX KI/KI</sub> mice at P21. (B) Bone staining of a wild-type Shox2<sup>+/+</sup> and Shox2<sub>SHOX KI/KI</sub> mice at P21. (C) Comparison of wild-type Shox2<sup>+/+</sup>, Shox2<sub>SHOX KI/+</sub> and Shox2<sub>SHOX KI/KI</sub> mice body weights at P0, P7, P14 and P21 time points. Error bars represent the standard deviation. *P<0.01 vs. wild-type Shox2<sup>+/+</sup> mice. Shox, short stature homeobox; P21, postnatal day 21.

Figure 2. Postnatal Shox2<sub>SHOX KI/KI</sub> mice demonstrate congenital cartilage degradation in the TMJ. Coronal sections of the TMJ in wild-type Shox2<sup>+/+</sup>, Shox2<sub>SHOX KI/+</sub> and Shox2<sub>SHOX KI/KI</sub> mice at P0, P7, P14 and P21 with (A) hematoxylin-eosin, (B) Safranin O-Fast Green and (C) azon red/anilin blue staining. Comparison of TMJ (D) glenoid fossa and (E) condyle width in Shox2<sub>SHOX KI/+</sub> and Shox2<sub>SHOX KI/KI</sub> mice relative to wild-type Shox2<sup>+/+</sup> mice (100%). Green and blue lines in P0 wild-type Shox2<sup>+/+</sup> mice in (A) indicate the positions at which glenoid fossa and condyle widths were measured on tissue sections. Error bars represent the standard deviation. *P<0.01 vs. wild-type Shox2<sup>+/+</sup> mice. TMJ, temporomandibular joint; Shox, short stature homeobox; P0, postnatal day 0.
Figure 3. Altered expression patterns of extracellular matrix proteins and Ihh in the TMJ of Shox^{2SHOX KI/KI} mice. Immunohistochemical staining of (A-D) collagen type I, (E-H) collagen type II and (I-L) aggrecan in the coronal sections of TMJs in postnatal wild-type Shox^{+/+} and Shox^{2SHOX KI/KI} mice at P7 and P14. Arrows indicate regions of the condyle and glenoid fossa where protein expression is altered. Ihh, Indian hedgehog; TMJ, temporomandibular joint; Shox, short stature homeobox; P7, postnatal day 7.
Upregulation of col I and downregulation of col II and Ihh in the condyle of the Shox2SHOX KI/KI mice. The ECM of the condyle is composed of collagens, PGS, GAGs and aggrecan (20). ECM components serve an important role in maintaining tensile properties and resistance to compressive forces. Alterations in ECM components are associated with TMJ cartilage degradation (8). Therefore, the expression levels of the matrix proteins col I, col II, aggrecan and Ihh were investigated in the present study, in order to determine an association between the observed TMJ growth alterations in postnatal Shox2SHOX KI/KI mice and changes in ECM components. Immunohistochemical analysis demonstrated an increase in col I expression levels in the condyles of Shox2SHOX KI/KI mice compared with wild-type Shox2SHOX+/+ mice at P7 and P14, whereas col II expression levels were reduced (Fig. 3A-H). However, no difference in the expression levels of aggrecan in the condyles of Shox2SHOX KI/KI mice and wild-type Shox2SHOX+/+ mice was observed (Fig. 3I-L). The expression levels of Ihh were determined using immunohistochemical analysis at P7. The results demonstrated that Ihh expression levels in Shox2SHOX KI/KI mouse condyles were significantly downregulated compared with wild-type Shox2SHOX+/+ mice (Fig. 3M and N). Therefore, upregulation of col I and downregulation of col II and Ihh in the condyles may be responsible for the observed congenital cartilage degradation in Shox2SHOX KI/KI mice.

Upregulation of MMP9 and MMP13 in the condyle of the Shox2SHOX KI/KI mice. MMP9 and MMP13 are zinc-dependent endopeptidases that function to degrade ECM collagens, and have been observed to be elevated in TMJ OA (21). Therefore, the next aim of the present study was to investigate whether the observed reduction in col II expression levels in Shox2SHOX KI/KI mouse TMJs was caused by increased MMP9 and MMP13 activity. To test this hypothesis, immunohistochemical analysis was conducted to determine MMP9 and MMP13 protein expression levels in Shox2SHOX KI/KI and wild-type Shox2SHOX+/+ mouse TMJs. As presented in Fig. 4D, MMP9 and MMP13 exhibited increased expression levels in Shox2SHOX KI/KI mouse TMJs at P7 compared with the wild-type Shox2SHOX+/+ mice. These results indicate that increased MMP9 and MMP13 activity may be responsible for the observed reduction in col II expression levels and the
congenital cartilage degradation in Shox2\textsuperscript{2SHOX KI/KI} mouse TMJs.

Discussion

Conditional inactivation of Shox2 has been observed to result in dysplasia of the condyle and glenoid fossa and articular disc ankylosis (16). Additionally, Shox2\textsuperscript{2SHOX KI/KI} mice exhibit premature erosion of the articular disc (2). These results support the putative functional role of Shox2 in TMJ formation and development. In the present study, alterations in TMJ growth in postnatal Shox2\textsuperscript{2SHOX KI/KI} mice were observed. The results demonstrate that the downregulation of col II and Ihh, and upregulation of col I, MMP9 and MMP13 may contribute to the congenital OA-like phenotype observed in postnatal Shox2\textsuperscript{2SHOX KI/KI} mouse TMJs.

To further investigate the mechanism of the congenital OA-like disease, the present study focused on the changes in ECM composition, Ihh and MMPs in the postnatal Shox2\textsuperscript{2SHOX KI/KI} mouse TMJs. Understanding the alterations that occur in the ECM composition of TMJ are necessary to elucidate the pathological process of TMJ OA (6). Cartilage destruction, mediated by the loss of col II and PGS, is a characteristic feature of OA (22,23). The initial loss of col II occurs in the superficial and upper-middle zones of the TMJ and extends into the deeper zones, with increasing destruction accompanied by the pathological process of OA (24). In normal hyaline cartilage, the predominant collagen type is col II, which is produced by chondrocytes. However, col I is also involved in the pathogenesis of OA; the relative concentrations of carboxyterminal telopeptides of col I and II may therefore act as a marker of cartilage degradation (25). The cleavage of fibrillar collagens by MMPs contributes to the substantial degradation of tissues during OA. MMP9 and MMP13 demonstrate a preference for degrading fibrillar collagen substrates, such as col II, however, also possess the ability to degrade the large hydrodynamic cartilage proteoglycan aggregate (26,27). Ihh, a regulator of chondrocyte and chondroprogenitor proliferation as well as chondrocyte maturation, has been observed to be essential for embryonic and postnatal TMJ development (28-30). Therefore, in order to investigate the mechanisms of congenital OA-like disease development in Shox2\textsuperscript{2SHOX KI/KI} mice in the present study, the composition of ECM proteins (col I, col II and aggrecan), Ihh, MMP9 and MMP13 protein expression levels in postnatal Shox2\textsuperscript{2SHOX KI/KI} mouse TMJs were determined. Using immunohistochemical staining, the results demonstrated decreased expression of col II and Ihh, and increased expression of col I, MMP9 and MMP13 in Shox2\textsuperscript{2SHOX KI/KI} condyles compared with wild-type Shox2\textsuperscript{+/-} mice at stages P7 and P14. This provides additional evidence that OA-like disease occurred in Shox2\textsuperscript{2SHOX KI/KI} mouse TMJs. It is currently unknown whether the observed reduction in Ihh expression levels confers elevated MMP activity and loss of col II in postnatal Shox2\textsuperscript{2SHOX KI/KI} mouse TMJs. However, the Ihh signaling pathway serves a significant role in regulating tissue structure homeostasis during TMJ development and requires further investigation.

In conclusion, postnatal Shox2\textsuperscript{2SHOX KI/KI} mouse TMJs were observed to develop congenital OA-like disease, which provides a novel in vivo model for studying the molecular and cellular mechanisms of TMJ OA.

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