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Replacing *Shox2* with human *SHOX* leads to congenital disc degeneration of the temporomandibular joint in mice

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Abstract

The temporomandibular joint (TMJ) consists of the glenoid fossa arising from the otic capsule through intramembranous ossification, the fibrocartilaginous disc and the condyle, derived from the secondary cartilage by endochondral ossification. We have reported previously that cranial neural crest-specific inactivation of the homeobox gene *Shox2*, which is expressed in the mesenchymal cells of maxilla-mandibular junction and later in the progenitor cells and perichondrium of the developing chondyle, led to dysplasia and ankylosis of the TMJ, and replacement of the mouse *Shox2* with the human *SHOX* gene rescued the dysplastic and ankylosis phenotypes but developed a prematurely worn out articular disc. In this study, we investigated the molecular and cellular bases for the premature wear out articular disc in the TMJ of mice carrying the human *SHOX* replacement allele in the *Shox2* locus (referred as $Shox2^{SHOX\text{-}KI/KI}$). We found that the developmental process and expression of several key genes in the TMJ of $Shox2^{SHOX\text{-}KI/KI}$ mice appeared similar to the controls. However, the disc of the $Shox2^{SHOX\text{-}KI/KI}$ TMJ exhibited a reduced level of Col I and Aggrecan, accompanied by increased activities of matrix metalloproteinases (MMPs) and a down-regulation of *Ihh* expression. Dramatically increased cell apoptosis in the disc was also observed. These combinatory cellular and molecular defects appear to contribute to the observed disc phenotype, suggesting that while the human *SHOX* can exert similar function as the mouse *Shox2* in regulating early TMJ development, it apparently has a distinct function in the regulation of those molecules that are involved in tissue homeostasis.

Keywords

Shox2; temporomandibular joint; articular disc; extracellular matrix; matrix metalloproteinases

Introduction

The temporomandibular joint (TMJ), a complex structure that is essential for jaw movement and found only in mammals, consists of the glenoid fossa of the temporal bone, the condylar head of the mandible, and a fibrocartilaginous disc located between these two bones and associated muscles and tendons (Gu and Chen, 2013). In mice, TMJ development starts with two distinct and separate mesenchymal condensations, the temporal and condylar blastemas, at embryonic day 13.5 (E13.5). The condylar blastema rapidly grows toward the temporal

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blastema. At E15.5, the forming glenoid fossa and condyle as well as the anlage of the articular disc are clearly visible. At E16.5, the glenoid fossa and condyle assume their position and complementary shape, with the articular disc beginning to form. Meanwhile, the upper synovial cavity becomes discernible. At E17.5, all the major components of TMJ are well-formed, with the fossa and condyle in complementary shapes with the articular disc dividing the joint cavity into two compartments (Gu et al., 2008; Michikami et al., 2012).

The articular disc is an important component of TMJ function, buffering compressing stroke from the joint and allowing rotational and translational movement (Herring, 2003; Sperber et al., 2010). It initiates with the formation of a flat mesenchymal condensation separated from the apex of the developing condyle and located between the temporal bone and the condylar head (Frommer, 1964). As embryo develops, the anlage of articular disc grows rapidly and acquires a characteristic compacted organization, especially in its medial portion. The flanking upper and lower cavities form subsequently and become filled with joint-lubricating fluids, and the disc eventually matures into a definitive fibrocartilage structure (Frommer., 1964; Ochiai et al., 2010; Bhaskar., 1953). The Hedgehog (Hh) signaling pathway has been implicated in the initial formation and separation of the articular disc from the apex of condyle, as evidenced by the absence of the articular disc formation in *Ihh*$^{-/-}$ and *Gli2*$^{-/-}$ mutant mice (Shibukawa et al., 2007; Purcell et al., 2009). In addition, Hh signaling may also play a role in maintaining the proper structure and tissue homeostasis of the TMJ, because ablation of *Ihh* in the cartilages of neonatal mice leads to TMJ dysplasia and partial disc ankylosis (Ochiai et al., 2010).

SHOX and *SHOX2*, the members of the short stature homeobox gene family, are found only in vertebrates, implicating their role in the development of the internal skeleton and its related structures (Clement-Jones et al., 2000). In humans, mutations in *SHOX* have been associated with a series of short-stature conditions, including Turner syndrome, Leri-Weill dyschondrosteosis, and Langer dysplasia that exhibit abnormalities in the skeletal development (Bobick and Cobb., 2012; Hirschfeldova et al., 2012). While *SHOX2* expression has been observed in the developing limbs in a complementary pattern to that of *SHOX* and in the developing heart of human embryo (Clement-Jones et al., 2000; Liu et al, 2011; 2012), there is no any known syndrome that has been linked to *SHOX2* mutations thus far. In mice, targeted inactivation of *Shox2* leads to severe defects in a number of developing organs including the limb, heart, palate, as well as the TMJ that exhibits dysplasia and ankylosis (Yu et al., 2005; 2007; Cobb et al., 2006; Blaschke et al., 2007; Gu et al., 2008).

Rodents do not have a *SHOX* ortholog in its genome, and the mouse Shox2 shares 99% identity at the amino acid level with its human counterpart. In an effort to study functional redundancy between *SHOX* and *SHOX2* genes during embryogenesis, we created a knock-in mouse line with a replacement of mouse *Shox2* by the human *SHOX* coding sequence (referred as *Shox2*$^{\text{SHOX-KI/KI}}$) (Liu et al., 2011). We have reported that although *Shox2*$^{\text{SHOX-KI/KI}}$ mice did not exhibit TMJ dysplasia and ankylosis at the birth, a phenotype observed in mice carrying inactivation of *Shox2* in the cranial neural crest lineage, the mice did develop a different TMJ defect, a premature wear out articular disc postnatally, clinically defined as TMJ disc disorders (Gu et al., 2008; Liu et al., 2011). In this study, we used *Shox2*$^{\text{SHOX-KI/KI}}$ mice as a model to investigate the cellular and molecular alterations that may contribute to this congenital degeneration of the TMJ disc.

Materials and Methods

Mice and mouse embryo collection

The generation of *Shox2*$^{\text{SHOX-KI/KI}}$ mice has been described previously (Liu et al., 2011), and the genotype of the mice was determined by a PCR-based method using tail DNA. The

age of the embryo was defined as the embryonic day 0.5 (E0.5) when the vaginal plug was found in the morning of the day. Animals and procedures used in this study were approved by the IACUC of Tulane University.

Histological analyses

Mouse heads were collected in ice-cold phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde (PFA)/PBS at 4°C overnight. The heads from postnatal day 0 (P0), P7, P14, and P21 mice were fixed and decalcified in Surgipath Decalcifier I for various days depending on the age of the mouse. Samples were then dehydrated through a graded ethanol series, cleared with xylene, embedded in paraffin, and coronal-sectioned at 10-μm thickness. For histological analysis of TMJ, serial sections were subjected to standard Azon red/Anilin blue staining (Presnell and Schreibman, 1997). Three heads of each genotype at each time points were used for histological and statistical analyses.

In situ hybridization and Immunohistochemistry

Paraffin-embedded samples were coronal-sectioned at 10-μm for in situ hybridization and at 8-μm for immunohistochemistry. Non-radioactive riboprobes were synthesized by an in vitro transcription labeling kit using Digoxigenin-UTP according to the manufacturer's instructions (Boehringer Mannheim). Section in situ hybridization was conducted as described previously (St Amand et al., 2000). Immunohistochemical staining was performed using polyclonal antibody against Runx2 (1:1000, ab76956), Sox9 (1:500, ab26414), Collagen I (1:500, ab34710), Collagen II (1:200, ab53047), Aggrecan (1:500, ab36861), MMP9 (1:300, ab38898), MMP13 (1:50, ab75606), and Ihh (1:200, ab39634) from Abcam according to the manufacturer's instruction. The secondary antibody were used the biotinylated anti-rabbit IG (vector laboratories), and immunohistochemistry staining was performed using the DAB™ LSAB + System-HRP Kit (invitrogen) following the procedure recommended by the manufacturer. Slides were briefly counterstained in hematoxylin and dehydrated, and mounted with Permount. In situ hybridization and immunohistochemical assays were repeated at least 3 times for each probe or antibody.

In situ zymography

P0 mouse heads were immersed in ZBF fixative at room temperature for 2 hr, dehydrated through 15% and 30% sucrose at 4°C overnight, frozen in OCT compound by liquid nitrogen, and then sectioned at 10-μm thickness using a cryostat. DQ-gelatin (1 mg/ml; Molecular Probes, E12055) was used as substrate with 1:10 dilution in the in situ zymography buffer according to the manufacturer's instruction. In situ zymography was performed according to the protocols reported previously (Sakakura et al., 2007; Pessoa et al., 2013; Mazzoni et al., 2012). Briefly, 100 μl of the mixture was applied onto the sections and covered with a coverslip. The sections were incubated at 37°C for 2 hrs in a dark humid chamber. The gelatinolytic activity was observed as green fluorescence with a fluorescence microscope. Three samples of control and transgenic animals were subjected for zymography analysis.

Tunel assay and BrdU labeling

Apoptosis was measured by using In Situ Cell Death Detection Kit from Roche. Samples were sectioned at 5-μm thickness, and subjected to immunodetection according to the manufacturer's instruction. Cell proliferation rate was determined by intraperitoneal BrdU injection into pregnant mice at a dose of 1.5 ml labeling reagent/100 g body weight using the BrdU labeling and Detection Kit II from Roche. Two hrs after BrdU injection, mice were sacrificed, and the heads of E18.5 mice were fixed in Carnoy's fixative, ethanol-dehydrated, paraffin-embedded, and sectioned at 5 μm. The sections were subjected to immunodetection

according to the manufacturer. Three independent experiments (one control and one transgenic sample per experiment) were conducted for Tunel or BrdU labeling assay.

Results and Discussion

Shox2$^{SHOX\text{-}KI/KI}$ mice exhibit premature wear out defect of the articular disc from neonatal stage

We have reported previously that *Shox2*$^{SHOX\text{-}KI/KI}$ mice developed a severe wasting syndrome mostly likely attributed to the premature wear out defect of the articular disc in the TMJ (Liu et al., 2011). To investigate the cellular and molecular alternations that may contribute to this non-pathological disc degeneration, we started with time course analysis of changes in thickness of the articular disc in the TMJ. We focused on the central part of the disc where the defect appears most significant. The average thickness of the wild type disc at each time point (N = 3 for each time point) is defined as 100 percent. We found that at P0, the thickness of the disc appeared comparable between wild type control and *Shox2*$^{SHOX\text{-}KI/KI}$ mice (N = 3) (Fig. 1a, 1b, 1g). However, at P7, the thickness of the *Shox2*$^{SHOX\text{-}KI/KI}$ articular disc (N = 3) was reduced more than 50%, as compared to the controls (Fig. 1c, 1d, 1g). At P14, the *Shox2*$^{SHOX\text{-}KI/KI}$ disc (N = 3) was further reduced to about 30% compared to the controls (Fig. 1e, 1f, 1g). These results indicate that the TMJ disc degeneration begins after the birth and occurs primarily in the first week after the birth.

Shox2$^{SHOX\text{-}KI/KI}$ mice display normal gene expression in the developing TMJ

We have shown previously that tissue-specific inactivation of *Shox2* in the cranial neural crest cells leads to TMJ dysplasia and ankylosis, accompanied by significantly down-regulation of *Sox9, Runx2,* and *Ihh* (Gu et al., 2008). We sought to determine if the expression of these genes was altered in the developing TMJ of *Shox2*$^{SHOX\text{-}KI/KI}$ mice. Our in situ hybridization and immunohistochemical assays demonstrate comparable expression patterns and levels of Sox9 and Runx2 in the *Shox2*$^{SHOX\text{-}KI/KI}$ and control TMJ at E14.5 and E16.5 (Fig. 2a–h). In addition, comparable *Ihh* expression level was also retained in the *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ at E15.5 (Fig. 2i, 2j). These observations suggest that human *SHOX* can substitute for mouse *Shox2* to regulate early TMJ development.

Reduction of extracellular matrix proteins in the articular disc the *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ

The articular disc is composed of fibrocartilage and extracellular matrix (ECM) components including collagens, glycosaminoglycans (GAGs) and proteoglycans (PGS), with collagens making up 50% of the wet volume (Berkovitz and Robertshaw, 1993; Gu et al., 2003; Willard et al., 2012). These ECM components play critical role in resistance to compressive forces and in maintaining the tensile properties of the tissue, and alterations in the ECM components are associated with synovial joint diseases (Garnero et al., 2000). We wondered if changes in the ECM components would account for the articular disc degeneration in the *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ. We examined the expression of several ECM components including Collagen type I (Col I), Collagen type II (Col II), and Aggrecan that have been shown to be present in the TMJ articular disc (Takahashi and Sato., 2001; Gu et al., 2003; Kondoh et al., 2003; Ahn et al., 2007; Natiella et al., 2009). For easy comparison, we used P0 mice, because at this time the thickness of the articular disc is comparable between wild type and the knock-in mice (Fig. 1). Immunohistochemcal analyses showed that the expression of Col I, which is by far the most prevalent component of the TMJ disc's ECM (Mills et al., 1994; Landesberg et al., 1996), was reduced in the disc but not in the glenoid fossa in the TMJ of *Shox2*$^{SHOX\ KI/KI}$ mice (Fig. 3a, 3b). However, obvious alteration in Col II expression level was not detectable in the articular disc the *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ, despite a slight reduction in the chondrocytes of the condyle (Fig. 3c, 3d). In addition, Aggrecan level was reduced in both the disc and condyle of *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ as compared to the controls (Fig. 3e, 3f).

Thus, the reduction in Col I and Aggrecan levels could alter the composition of the disc ECM and contribute to a fragile articular disc in *Shox2*$^{SHOX\text{-}KI/KI}$ mice.

Enhanced matrix metalloproteinase activity and apoptosis in the disc of *Shox2*$^{SHOX\ KI/KI}$ TMJ

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases capable of degrading extracellular matrix, including collagens and Aggrecan. We asked if the reduced level of Col I and Aggrecan in the articular disc of *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ is a consequence of enhanced MMP activities. To test this possibility, we first conducted in situ zymography to determine total MMP activity in P0 control and *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ disc. As shown in Fig. 4a, while strong MMP activity was found in the glenoid fossa and an above background level of MMP activity was also seen in the condyle, MMP activity was not detected at all in the articular disc of the wild type TMJ. In contrast, we detected ectopic MMP activity in the articular disc and enhanced MMP activity in the condyle of *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ (Fig. 4b). Consistent with this enhanced total MMP activity, we found that MMP9 and MMP13, which have been shown to be elevated in the articular disc of TMJ with osteoarthritis and are capable of degrading fibrillar collagens, including Col I and Col 2, and Aggrecan (Kubota et al., 1998; Tanaka et al., 2001; Burrage et al. 2006; Malemud, 2006; Leonardi et al., 2008; Zhang et al., 2010), exhibited ectopic expression in the articular disc of the *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ (Fig. 4c–f), consistent with the reduction of Col I and Aggrecan. Since Col II, the primary component in hyaline cartilage, is present only in a small amount in the TMJ disc (Mills et al., 1994; Landesberg et al., 1996; Fig. 3c), a visible reduction, as assessed by immunohistochemical staining, may not be detectable in the transgenic animals. Nevertheless, these results indicate that enhanced MMP activities are responsible for the reduction of ECM components, which may ultimately lead to the degeneration of the articular disc in *Shox2*$^{SHOX\text{-}KI/KI}$ mice. In addition, despite that *Ihh* expression remained altered in the early developing condyle of the *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ (Fig. 2), we found that Ihh protein level was reduced significantly in the condyle and was absent in the disc of the *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ, as compared to the controls (Fig. 4g, 4h). Although whether or not this reduction in Ihh expression represents a causative of the elevated MMP activity in the postnatal *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ is current unknown, it is consistent with a role for Ihh signaling in maintaining the proper structure and tissue homeostasis of the TMJ in postnatal mice (Ochiai et al., 2010), and warrants future investigation.

We further examined if there exist cellular defects that could potentially contribute to the disc phenotype in the *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ. Apoptosis is known to be responsible for disc tissue degeneration correlated with the severity of disc pathologic processes (Caltabiano, et al., 2013; Leonardi et al.,2010; Loreto et al., 2011). We therefore conducted Tunel assay to determine if there is any alteration in cell apoptosis in the disc. Indeed, our results demonstrate a dramatically increased number of apoptotic cells in the articular disc of *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ at P0 as compared to that in the wild-type controls (Fig. 4i, 4j), suggesting a contribution of cell apoptosis to the disc degeneration in *Shox2*$^{SHOX\text{-}KI/KI}$ mice. In contrast, BrdU labeling assay revealed unaltered cell proliferation rate in the articular disc of the *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ compared to the control at E18.5 (data not shown). We have reported previously that lack of *Shox2* leads to altered expression of *Bmp4* and *Fgf10* in the developing limb and the palatal shelves, respectively (Yu et al., 2005; 2007). Since these two growth factors are involved in the regulation of cell proliferation and surviving, we wondered if the elevated cell apoptosis in the articular disc of *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ is a consequence of altered expression of these two factors. We conducted in situ hybridization experiments to examine the expression of *Bmp4* and *Fgf10* in the TMJ of controls and transgenic animals at P0. However, the results show no

expression of *Bmp4* and *Fgf10* in the articular disc of TMJ in both control and *Shox2*$^{SHOX\text{-}KI/KI}$ mice (data not shown).

Although classified as synovial joint, the developmental processes of the TMJ appear distinct from other synovial joints. The TMJ develops from two distinct mesenchymal condensations, the condylar and glenoid fossa blastemas, each of which undergoes a different ossification process. In addition, the TMJ is unique because of the presence of the articular disc that divides the joint cavity into upper and lower joint cavities and allows shock absorption and jaw movement (Gu and Chen, 2013). Osteoarthritis of the TMJ, a common subtype of TMJ disorders in humans, causes progressive degradation of TMJ tissues, disc degeneration (thinning), leading to difficulty in eating, drinking, and speaking (Broussard, 2005; Guarda-Nardini et al., 2012; Krisjane et al., 2012). Since the causes of TMJ disc disorders remain largely unknown, current clinical therapy focuses mainly on pain reduction and tissue resection, but its resection could eventually lead to degradation of the joint as a whole (Tanaka et al., 2008).

The changes in the ECM composition of the articular disc are associated with pathological processes of TMJ disorders. Cartilage degeneration, mediated by the loss of collagens and proteoglycans, is a characteristic feature of osteoarthritis (Hollander et al., 1995; Bank et al., 1997). It is well established that elevated MMP activities are responsible for such degradation of ECM in the progress of cartilage degeneration in TMJ osteoarthritis (Kubota et al., 1998; Kanyama et al., 2000; Gepstein et al., 2003). Our results show that the premature disc wear out phenotype, a congenital degenerative defect, is associated with reduced amounts of Col I and Aggrecan in the *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ. The fact that ectopic/enhanced MMP activity was observed in the articular disc of the *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ suggests a similar mechanism for this congenital degenerative process. Most incidents of TMJ disorder in humans occur after an infection or a trauma, but congenital cases, although rare, have been reported (Ferraz et al., 2012; Gu et al., 2008; Mortazavi and Motamedi, 2007). While the congenital TMJ ankylosis has been reported to a patient with a short stature phenotype (Komorowska, 1997), genetic alterations that cause congenital articular disc degeneration of the TMJ are completely unknown. Our studies demonstrate an important role for *Shox2* in TMJ development and function and provide evidence for a genetic association with the congenital articular disc degeneration, suggesting that *SHOX2* could represent a susceptible locus for osteoarthritis of the TMJ. Nevertheless, the *Shox2*$^{SHOX\text{-}KI/KI}$ mice provide a unique model for studying molecular and cellular mechanisms of TMJ disorders including articular disc degeneration.

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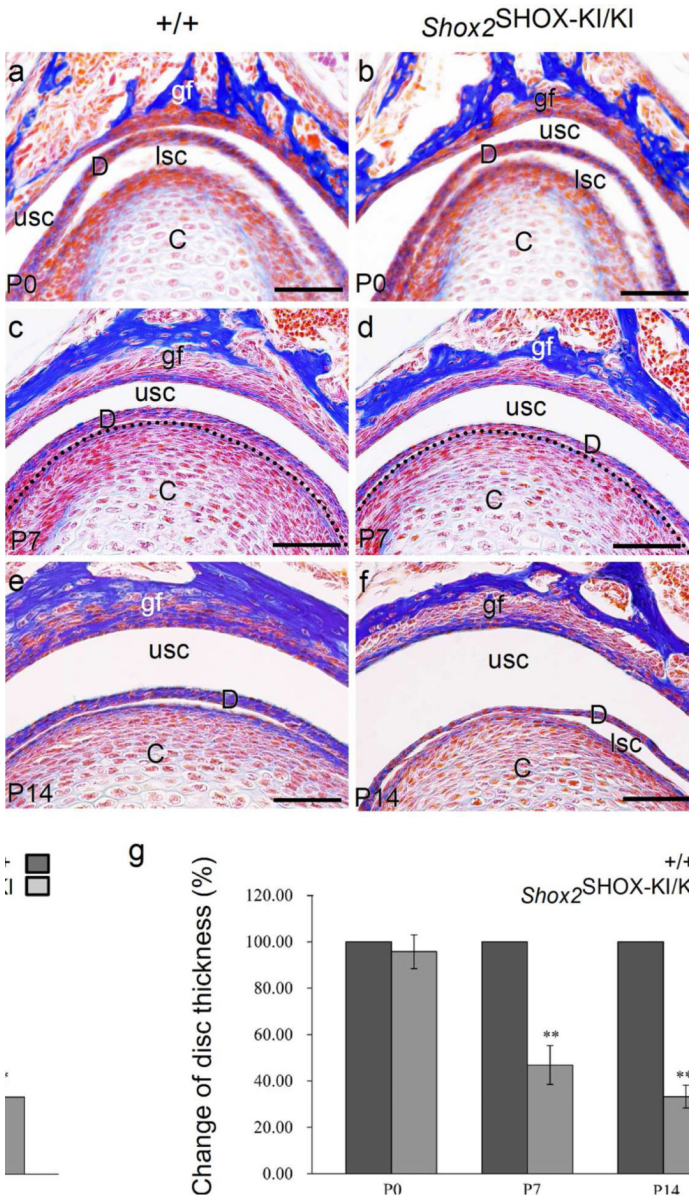

Figure 1. Histological analyses of disc degeneration in the TMJ of *Shox2*$^{\text{SHOX-KI/KI}}$ mice

(a, c, e) Coronal sections of the TMJ in wild type mice at P0 (a), P7 (c), and P14 (e). (b, d, f) coronal sections of the TMJ in *Shox2*$^{SHOX\ KI/KI}$ mice at P0 (b), P7 (d), and P14 (f). (g) Comparison of disc thickness of the TMJ in wild type controls and *Shox2*$^{SHOX\ KI/KI}$ mice Standard deviation is shown as error bars. **: $P < 0.01$. C, condyle; d, disc; gf, glenoid fossa; lsc, lower synovial cavity; usc, upper synovial cavity. Bars = 200 μm.

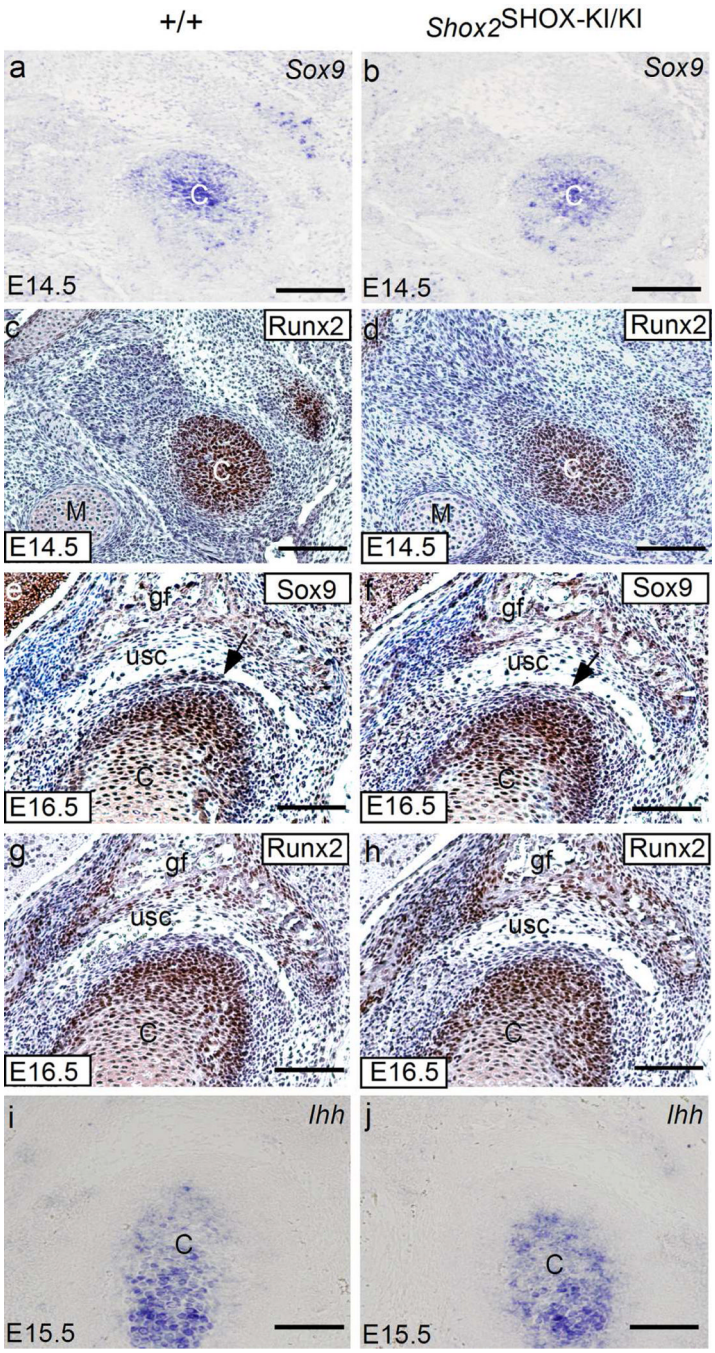

Figure 2. Expression of *Sox9, Runx2,* and *Ihh* in the developing TMJ of *Shox2*$^{\text{SHOX-KI/KI}}$ mice

(a, b) In situ hybridization shows *Sox9* expression in the developing condyle of E14.5 wild type (a) and *Shox2*$^{\text{SHOX-KI/KI}}$ embryo (b). (c, d) Immunohistochemical staining shows Runx2 expression in the developing condyle of E14.5 wild type (c) and *Shox2*$^{\text{SHOX-KI/KI}}$ embryo (d). (e–h) Immunohistochemical staining show Sox9 (e, f) and Runx2 (g, h) expression in the developing TMJ of E16.5 wild type (e, g) and *Shox2*$^{\text{SHOX-KI/KI}}$ embryo (f, h). Arrows in (e, f) point to the disc anlage. (i, j) In situ hybridization shows *Ihh* expression in the developing condyle of E15.5 wild type (i) and *Shox2*$^{\text{SHOX-KI/KI}}$ embryo (j). C, condyle; gf, glenoid fossa; usc, upper synovial cavity. Bars = 100 μm.

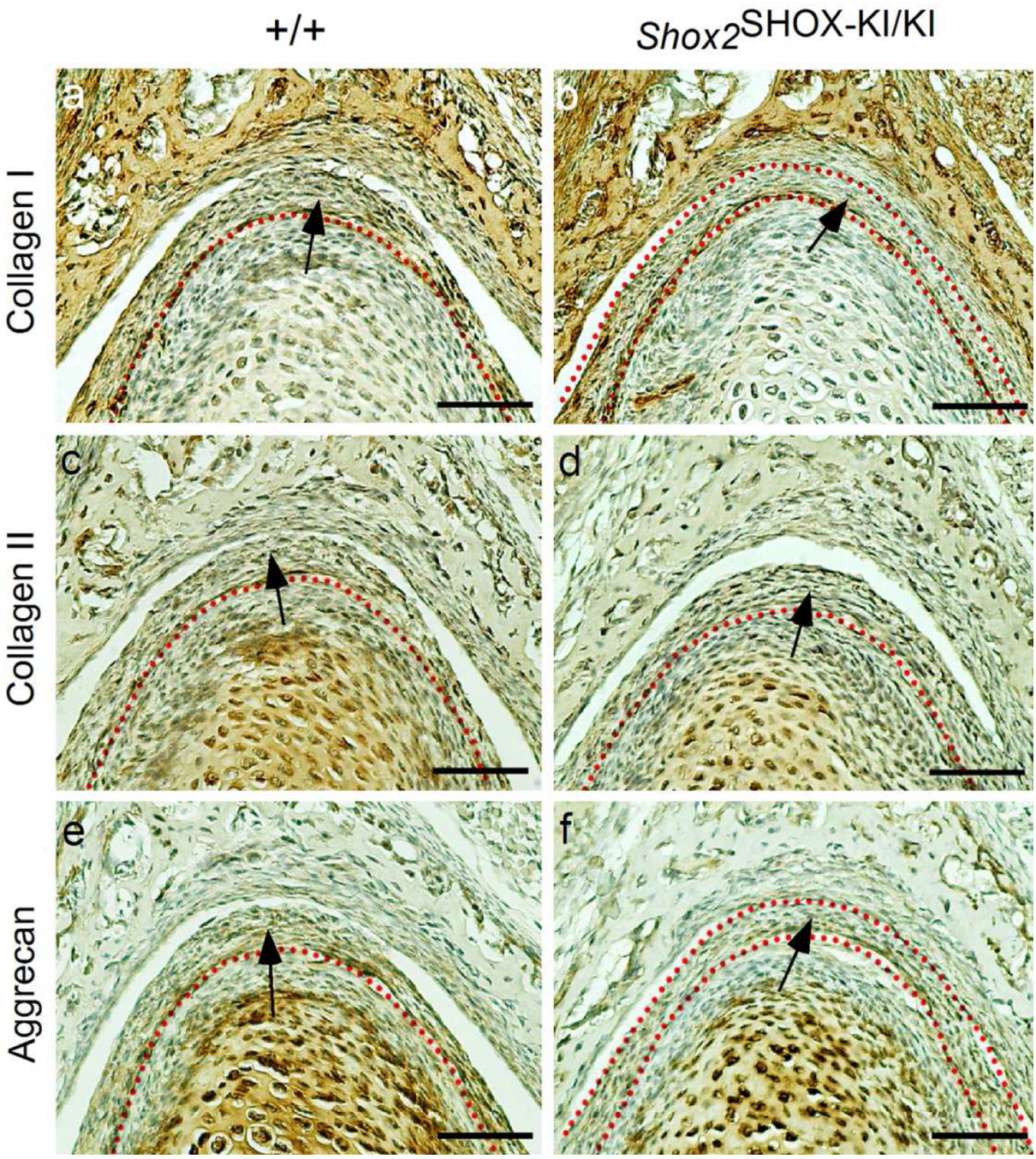

Figure 3. Expression of extracellular matrix in the postnatal TMJ of *Shox2*$^{\text{SHOX-KI/KI}}$ mice

Expression of Col I (a, b), Col II (c, d), and Aggrecan (e, f) in the TMJ of P0 wild type (a, c, e) and *Shox2*$^{\text{SHOX-KI/KI}}$ (b, d, c). Arrows point to the disc where expression is altered. Bars = 200 μm.

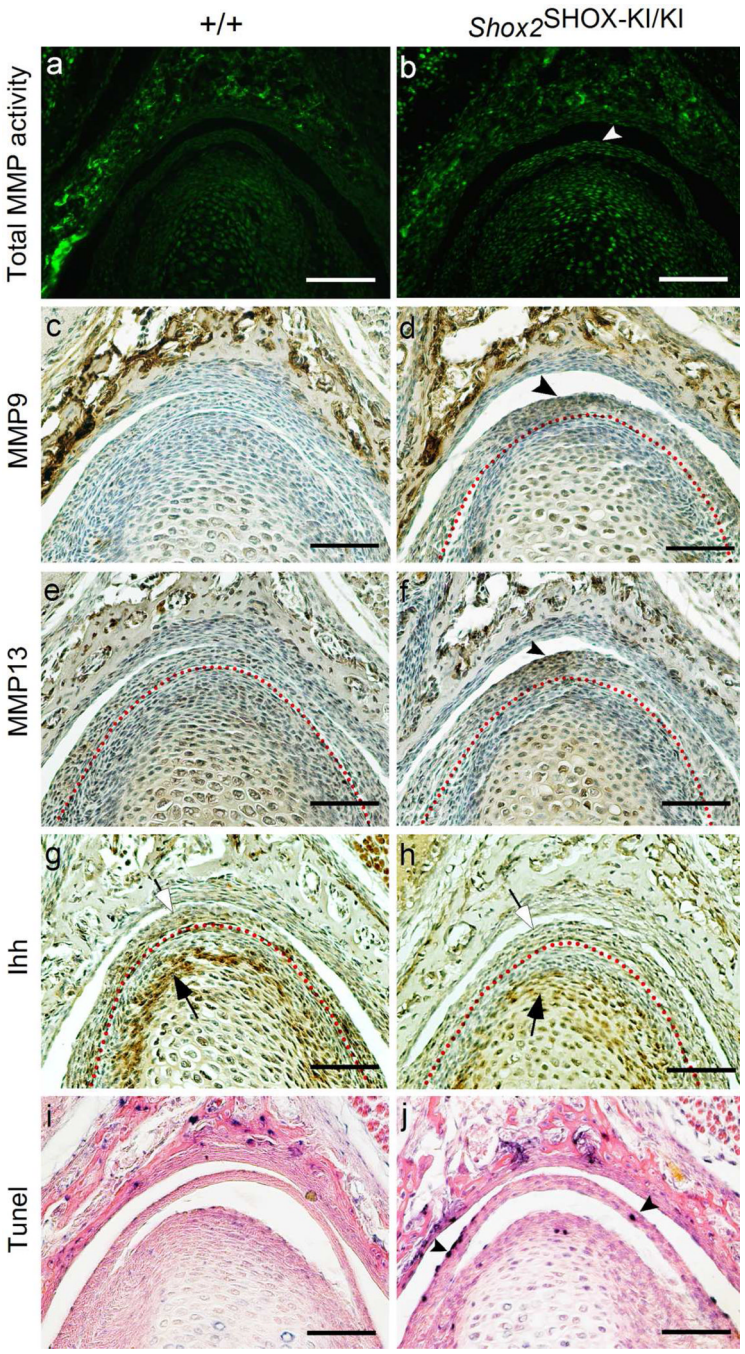

Figure 4. Altered expression of MMPs and Ihh and enhanced apoptosis in the postnatal TMJ of *Shox2*$^{SHOX\text{-}KI/KI}$ mice

(a, b) In situ zymography on coronal sections of P0 wild type (a) and *Shox2*$^{SHOX\text{-}KI/KI}$ (b) TMJ show MMP activity. Note the ectopic MMP activity in the disc (arrowhead) of the *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ. (c–f) Immunohistochemical staining on coronal sections of P0 wild type (c, e) and *Shox2*$^{SHOX\text{-}KI/KI}$ (d, f) shows expression of MMP9 (c, d) and MMP-13 (e, f). Arrowheads in (d) and (f) point to the disc where elevated expression is significant. (g, h) Immunohistochemical staining on coronal sections of P0 wild type (g) and *Shox2*$^{SHOX\text{-}KI/KI}$ (h) TMJ shows Ihh protein expression and distribution. Open arrows point to the disc, and the black arrows point to Ihh expression domain in the condyle. (i, j) TUNEL assay on

coronal sections of P0 wild type (i) and *Shox2*$^{SHOX\text{-}KI/KI}$ (j) TMJ shows increased apoptotic cells in the articular disc of the *Shox2*$^{SHOX\text{-}KI/KI}$ TMJ. Arrowheads in (j) point to the apoptotic cells in the disc. Bar = 200 μm.