Overexpression of Indian hedgehog partially rescues short stature homeobox 2-overexpression-associated congenital dysplasia of the temporomandibular joint in mice

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Abstract. The role of short stature homeobox 2 (shox2) in the development and homeostasis of the temporomandibular joint (TMJ) has been well documented. Shox2 is known to be expressed in the progenitor cells and perichondrium of the developing condyle. A previous study by our group reported that overexpression of shox2 leads to congenital dysplasia of the TMJ via downregulation of the Indian hedgehog (Ihh) signaling pathway, which is essential for embryonic disc primordium formation and mandibular condylar growth. To determine whether overexpression of Ihh may rescue the overexpression of shox2 leading to congenital dysplasia of the TMJ, a mouse model in which Ihh and shox2 were overexpressed (Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice) was utilized to assess the consequences of this overexpression on TMJ development during post-natal life. The results showed that the developmental process and expression levels of runt-related transcription factor 2 and sex determining region Y-box 9 in the TMJ of the Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice were similar to those in wild-type mice. Overexpression of Ihh rescued shox2 overexpression-associated reduction of extracellular matrix components. However, overexpression of Ihh did not inhibit the shox2 overexpression-associated increase of matrix metalloproteinases (MMPs) MMP9, MMP13 and apoptosis in the TMJ. These combinatory cellular and molecular defects appeared to account for the observed congenital dysplasia of TMJ, suggesting that overexpression of Ihh partially rescued shox2 overexpression-associated congenital dysplasia of the TMJ in mice.

Introduction

The temporomandibular joint (TMJ), a highly specialized synovial joint, permits movement and function of the mammalian jaw, which consists of a glenoid fossa, a condylar head of the mandible and a fibrocartilaginous disc (1,2). The glenoid fossa as well as the condyle develop from two distinct mesenchymal condensations, the temporal and condylar blastemas, and ossify through specific mechanisms: The glenoid fossa undergoes intramembranous ossification, while the condyle is endochondral in origin (3,4). Although TMJ development during embryonic and post-natal life involves complex processes that are well documented, little information is available regarding the cellular and molecular mechanisms involved in TMJ morphogenesis.

The condyle is a crucial growth site in the mandible and displays similarities with the growth plate of the long bones. It has four distinct zones, including a zone of hypertrophic chondrocytes, a zone of flattened chondrocytes, a progenitor cell layer and a fibrous cell layer (5). One key gene which was previously reported to be expressed in the growth plate of the condylar cartilage is Indian hedgehog (Ihh), which belongs to a family of potent secreted signaling proteins (6). Ihh, in conjunction with PTHrP, is essential for maintaining the growth of adjacent proliferating chondrocytes and has an indirect role in controlling the rate of chondrocyte differentiation by acting in a negative feedback loop with parathyroid-hormone-related protein (PTHrP) in the periarticular perichondrium (7,8). Ihh acts on target cells through the cell-surface receptors Smoothened (Smo) and Patched (Ptc) and in cooperation with primary cilia, and signaling is mediated by the Gli family of transcription factors (10-12). Gli1, a transcriptional activator of Ihh targets, is transcriptionally upregulated by Ihh signaling. In mice, cartilage development begins in the center of the condyle, and Ihh begins to have a significant effect in the condylar cartilage at E15.5 (6,13). In
Ihh-/- mutant mice, the organization of the growth-plate-like zone in the condyle is disrupted, and the articular disc does not form (6). In summary, Ihh is crucial for TMJ formation and development, where it appears to regulate the condylar cartilage phenotype, chondroprogenitor cell function, as well as growth and elongation events.

A previous study by our group showed that downregulation of the Ihh signaling pathway is one of the causes of congenital dysplasia of the TMJ in mice with short stature homeobox 2 (shox2) overexpression (14). Shox2, a member of shox gene family, is found only in vertebrates, suggesting its role in the development of the internal skeleton and its associated structures (4,13,14). The present study was conducted to determine whether overexpression of Ihh may rescue the shox2 overexpression-associated effects on the TMJ phenotype using a mouse model of Ihh and shox2 overexpression (Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice).

Materials and methods

Mouse embryo collection. The animal procedure used in the present study was approved by the Institutional Animal Care and Use Committee (IACUC) of Fujian University of Traditional Chinese Medicine (Fuzhou, China). A total of 72 Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice, generated by crossing Wnt1-Cre; pMes-stop shox2 mice (14) with pMes-stop Ihh mice, and a total of 72 wild-type mice were provided by the laboratory of Dr Yiping Chen (Department of Cell and Molecular Biology, Tulane University, New Orleans, LA, USA). The mice were exposed to a 12 h light/dark cycle in a temperature (22±1°C) and humidity (56±1%) controlled environment. They were maintained on a 0.3% sodium diet and were housed five/cage. The mice were sacrificed with CO2; and the embryos were extracted in phosphate-buffered saline (PBS; pH 7.4; GE Healthcare Life Sciences; Logan, UT, USA) at 4°C. When the presence of a vaginal plug was found in the morning, the age of the embryo was defined as embryonic day 0.5 (E0.5). Embryonic heads were fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich, St Louis, MO, USA)/PBS at 4°C overnight. The heads of post-natal day 0 (P0), P7, P14 and P21 mice were fixed and decalcified in Surgipath Decalcifier I (Leica Biosystems Richmond Inc., Richmond, CA, USA) for different times depending on the age of the mouse according to the manufacturer's instructions (4).

Histological analyses. Paraffin-embedded heads were sectioned at a thickness of 10 µm with a microtome. For histological analysis of the TMJ, the serial sections were stained with azon red/anilin blue staining (Sigma-Aldrich) according to standard procedures. Briefly, paraffin sections were deparaffinized in xylene (Sigma-Aldrich) and rehydrated through a gradient series of ethanol concentrations (Merck & Co., Inc., Kenilworth, NJ, USA). They were then stained with 0.5% azon red (Sigma-Aldrich) for 2 h at 56°C, rinsed in 5% phosphotungstic acid (Sigma-Aldrich) for 3 h at room temperature, stained with 0.5% anilin blue (Sigma-Aldrich) for 2 h, then were dehydrated and mounted on slides. The morphology of TMJ was observed under an Olympus BH-2 light microscope (Olympus, Tokyo, Japan).

Immunohistochemistry. Paraffin-embedded heads were sectioned at a thickness of 8 µm for immunohistochemistry. Subsequent to blocking with goat serum (1:10; Invitrogen Life Technologies, Carlsbad, CA, USA) and incubation for 15 min at room temperature, the sections were incubated with polyclonal antibodies against runt-related transcription factor 2 (Runx2; 1:1,000; ab76956), sex determining region Y-box 9 (Sox9; 1:500; ab26414), collagen type I (col I) (1:500; ab34710), collagen type II (col II) (1:200; ab53047), aggrecan (1:500; ab36861), matrix metalloproteinase 9 (MMP9) (1:300; ab38898), MMP13 (1:50; ab75606) and Ihh (1:200; ab39634) from Abcam (Cambridge, MA, USA) overnight at 4°C. The slides were then washed three times with PBS and were incubated with a biotinylated horseradish peroxidase goat anti-rabbit secondary antibody (1:1,000 dilution; Invitrogen Life Technologies) for 20 min at 37°C. The slides were then washed three times with the secondary antibody using PBS. Immunolabelling was visualized with 0.05% diaminobenzidine (Invitrogen Life Technologies) in PBS for 5 min at room temperature, and the slides were then rinsed for 10 min under running tap water. The TMJ immunohistochemical staining was analyzed under the Olympus BH-2 light microscope.

In situ zymography. Heads of P0 mice were fixed in zinc-based fixative (ZBF; 36.7 mM ZnCl, 27.3 mM ZnAc, x H2O and 0.63 mM CaAc in 0.1 mM Tris pH 7.4; all from Sigma-Aldrich) and frozen in optimum cutting temperature compound (Tissue-Tek; Sakura Finetek USA, Inc., Torrance, CA, USA) compound using liquid nitrogen. According to the manufacturer's instructions, 10-µm sections were incubated at 37°C for 2 h in a dark humidified chamber with 1 mg/ml dye-quenched gelatin (100 µl; E1205; Molecular Probes Europe BV, Leiden, The Netherlands), which was applied on top of the sections and was covered with a coverslip (14-17). The gelatinolytic activity was observed as green fluorescence under a fluorescence microscope (Axioskop 50; CarlZeiss, Oberkochen, Germany).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and bromodeoxyuridine (BrdU) labeling assay. Apoptosis was tested using the In Situ Cell Death Detection kit, alkaline phosphatase (Roche, Basel, Switzerland). Paraffin-embedded heads were sectioned at 5 µm and subjected to immunodetection according to the manufacturer's instructions (4,14). At E14.5 or E16.5, pregnant mice were injected with 1.5 ml labeling reagent/100 g using the BrdU Labeling and Detection Kit II (Roche) for 2 h. The heads of embryonic mice were fixed in Carnoy's fixative [6:3:1 ratio of ethanol (Merck & Co., Inc.), chloroform (Sigma-Aldrich) and glacial acetic acid (Merck & Co., Inc.)], ethanol-dehydrated, paraffin-embedded and sectioned at 5 µm. The sections were subjected to immunodetection for analysis of cell proliferation according to the manufacturer's instructions (13).

Statistical analysis. All experiments were repeated at least three times, and values are expressed as the mean ± standard deviation. Statistical analyses of the data were performed using the Student's t-test with SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.
Results

Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice exhibit TMJ dysplasia. Histological analyses of TMJs did not indicate any differences between wild-type mice and Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice at P0 and P7, while TMJ dysplasia was observed at P14. To investigate the phenotype of the TMJs, a time-course analysis of alternation in the width of the glenoid fossa and condyle of the TMJ was performed. The observation was focused on the widest part of the glenoid fossa and condyle where the defect was most significant. The average width (obtained from three
measurements of different animals) of the glenoid fossa and condyle in the wild-type mice at each time-point was defined as 100%. That the width of the glenoid fossa and condyle appeared to be similar between wild-type mice and Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice at P0 and P7 (Fig. 1a-d). However, at P14 and P21, the width of the condyle and glenoid fossa in the Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice was decreased compared with that in the wild-type mice (P<0.05) (Fig. 1e-j). These results suggested that the TMJ dysplasia occurred primarily in the second week following birth.

To assess the effect of the TMJ dysplasia on the body weight in the Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice, the body weight was determined at different developmental stages (P0, P7, P14 and P21) (Fig. 1k). Mice stopped suckling and started eating solid food at P21. The results demonstrated that the body weight between wild-type mice and Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice at P0 and P7 (Fig. 1a-d). However, at P14 and P21, the width of the condyle and glenoid fossa in the Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice was decreased compared with that in the wild-type mice (P<0.05) (Fig. 1e-j). These results suggested that the TMJ dysplasia occurred primarily in the second week following birth.

To assess the effect of the TMJ dysplasia on the body weight in the Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice, the body weight was determined at different developmental stages (P0, P7, P14 and P21) (Fig. 1k). Mice stopped suckling and started eating solid food at P21. The results demonstrated that the body weight between wild-type mice and Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice was similar at P0 and P7; however, it was significantly different at P14 and P21 (P<0.05), indicating that the observed changes of the TMJ in Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice may have affected the function of the TMJ, leading to a reduction in body weight and the development of a wasting syndrome.

Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice display normal expression of Runx2 and Sox9 in the developing TMJ. To determine whether the protein levels of Runx2 and Sox9 were altered in the developing TMJ of Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice, immunohistochemical analysis was performed. There were no differences in Runx2 and Sox9 protein levels in the TMJ between wild-type mice and Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice at E14.5 and E16.5 (Fig. 2a-h), suggesting that overexpression of Ihh did not affect the formation and development of the TMJ during the embryonic stage.

Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice show no abnormalities in the extracellular matrix (ECM) composition. The ECM, including collagens, proteoglycans and glycosaminoglycans, is the main component of articular cartilage (4). It is essential for the resistance to compressive forces and maintaining the tensile properties of the cartilage. A previous study by our group showed that overexpression of shox2 led to a reduction of col I and col II expression (14). To account for the dysplasia of TMJ in the Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice, the expression of several key components of the ECM, including col I, col II and aggrecan was assessed at P0 and P7 (Fig. 3a-l). Immunohistochemical analysis showed
that the protein levels of col I and col II were similar between wild-type and Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice at P0 and P7. However, the protein levels of aggrecan were increased in the condyle in the TMJ of Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice. These results suggested that overexpression of Ihh rescued the ECM composition in the Wnt1-Cre; pMes-stop shox2 mice.

Ihh is overexpressed in the TMJ of Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice. To determine whether the expression of Ihh was significantly upregulated in the developing TMJ of Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice, the protein levels of Ihh were assessed using immunohistochemistry at P0 and P7 (Fig. 3m-p). The protein levels of Ihh were increased in the condyle of Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice compared with those in the wild-type mice, supporting that Ihh was upregulated in the TMJ.

MMPs activity is upregulated in the TMJ of Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice. The previous study by our group showed that overexpression of shox2 resulted in enhanced MMP activity (14). In the present study, it was therefore assessed whether overexpression of Ihh was able to inhibit the enhancement of MMP activity. First, in situ zymography was performed to determine total MMP activity in the TMJs of wild-type mice and Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice at P0. As shown in Fig. 4a and b, enhanced MMP activity was identified in the glenoid fossa; however, above-background levels of MMP activity were also observed in the condyle. Consistent with this enhanced total MMP activity, the expression of MMP9 and MMP13 in the TMJs of Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice was found to be enhanced compared with that in the wild-type mice at P0 and P7 (Fig. 4c-j). These results indicated that enhanced MMP activity is associated with dysplasia of the TMJ, and that the shox2 overexpression-associated increases in MMP activity was not sufficiently rescued by overexpression of Ihh.

Apoptosis is enhanced in the glenoid fossa of the Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice. The present study further examined whether the overexpression of Ihh was able to decrease the apoptosis that potentially contributed to the TMJ phenotype of the Wnt1-Cre; pMes-stop shox2 mice. The results of the TUNEL assay showed a significantly enhanced number of apoptotic cells in the glenoid fossa of Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice compared to that in the wild-type mice at P0 (P<0.05) (Fig. 5a-c), indicating that overexpression of Ihh did not inhibit shox2 overexpression-associated apoptosis of glenoid fossa. In addition, a BrdU labeling assay revealed there was no alteration in the cell proliferation rate in the condyle of the wild-type mice and Wnt1-Cre; pMes-stop shox2; pMes-stop Ihh mice at E14.5 and E16.5 (Fig. 6a-f).

Discussion

The results of the present study provided solid evidence that Ihh is required for the formation and development of the TMJ. Compared with the results of a previous study by our group (14),
the results of the present study demonstrated that overexpression of Ihh rescued the shox2 overexpression-associated reduction of ECM components; however, shox2-associated increases of MMP activity, MMP9 and MMP13 levels as well as apoptosis in the TMJ were not sufficiently attenuated, suggesting that overexpression of Ihh partially rescued shox2-associated congenital dysplasia of the TMJ in mice.

In the present study, detailed histological analysis of the TMJ indicated changes in the width of glenoid fossa and condyle between post-natal wild-type mice and Wnt1-Cre; pMes-stop Ihh mice. Values are expressed as the mean ± standard deviation (n=3). *P<0.05 vs. wild-type. TMJ, temporomandibular joint; P, post-natal day; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.
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