Original Article

Temporal Expression of SOX9 and Type II Collagen in Spheno-Occipital Synchondrosis of Mice after Mechanical Tension Stimuli

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ABSTRACT

Objective: To associate the expressions of SOX9 and type II collagen during growth in the synchondrosis with and without tensile stress in order to understand the role of these factors in the growth of cartilage in spheno-occipital synchondrosis.

Materials and Methods: Sixty 1-day-old male BALB/c mice were randomly divided into experimental and control groups. Each group was subdivided again into five different time points which were 6, 24, 48, 72, and 168 hours. Each subgroup consisted of five mice. Each mouse was sacrificed using an overdose of pentobarbitone sodium. The synchondroses were aseptically removed and incubated in a 24-well plate with or without tensile stress in tissue culture. Tissue sections were stained immunohistochemically to quantitatively analyze the expression of SOX9 and type II collagen.

Results: There was a statistically significant increase of 57% ($P < .001$) in the expression of SOX9 between the experimental and control groups at 24 hours, followed by a significant increase of 44.4% ($P < .001$) in the expression of type II collagen at 72 hours.

Conclusions: SOX9 may play an important role for early differentiation of chondrocytes and increase the expression of type II collagen, a major component of the extracellular matrix, during the growth of cartilage in the spheno-occipital synchondrosis.

KEY WORDS: Spheno-occipital synchondrosis; SOX9; Type II collagen; Tensile stress; Cartilage

INTRODUCTION

Cranial base synchondroses are important growth centers of the craniofacial skeleton. This is especially true of the spheno-occipital synchondrosis because of its late ossification and major contribution to post natal cranial base growth. This serves as an important link between the development of the cranial vault and the facial skeleton.[1–4] Disturbance in their development causes abnormalities in the craniofacial region such as Apert syndrome, Crouzon syndrome, Down syndrome, Turner syndrome, craniosynostosis syndromes, cleidocranial dysplasia, and cleft palate.[3]

The synchondrosis develops from a cartilaginous template known as the chondrocranium which eventually is replaced by bone through endochondral ossification. The role of cartilage in endochondral ossification is obvious—it provides the anlagen for bone formation. Rabie and co-workers[5–9] showed that cartilage formation is closely related to bone formation in the mandibular condyle of growing rats. As the cartilage formation increases it will offer more opportunity for more bone to form because cartilage serves as a template onto which bone will form.[5] Corresponding to these results, the growth of the synchondroses must be modulated harmoniously by a cascade of events from various growth factors and regulatory factors that originate internally in the synchondroses. Unlike the long bones, where many regulatory factors have already been identified, only a few studies were performed to investigate the factors regulating the growth of the spheno-occipital synchondrosis.[10,11]

This led us to look for SOX9 expression during growth of the spheno-occipital synchondrosis due to its critical role in governing chondrocyte differentiation.

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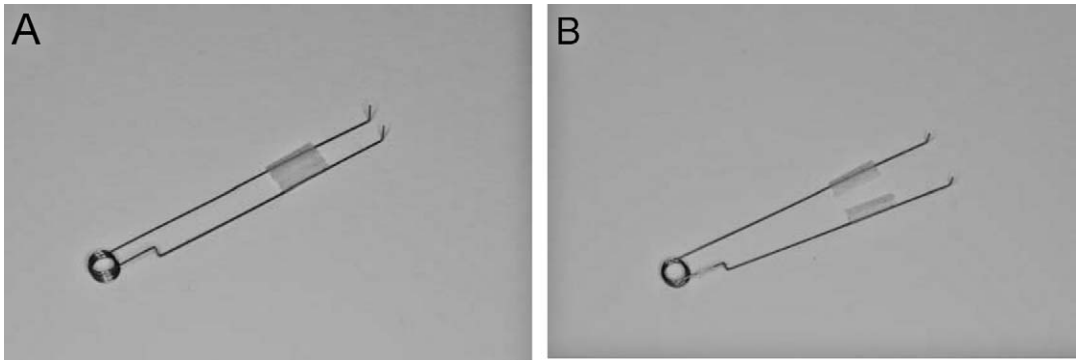

Figure 1. (A) Mechanical spring before activation. (B) Mechanical spring after activation.

SOX9 has essential, nonredundant roles in specifying the commitment and differentiation of mesenchymal cells toward the chondrogenic lineage in all developing skeletal elements.[12] Furthermore, SOX9 directly activates gene expression of type II collagen, a major component of cartilaginous extracellular matrix that acts as an early and abundant main marker of the chondrocytes and forms the framework of cartilage matrix.[13,14] Therefore, we hypothesized that SOX9 and type II collagen expression could be induced by mechanical stimulation (tensile stress). Thus, SOX9 may play a role in the early differentiation of chondrocytes and increase the expression of type II collagen,[12,13] a major component of extracellular matrix, during cartilage growth in the spheno-occipital synchondrosis.

The objectives of this study were to:

- Identify and quantify the temporal expression of SOX9 transcription factor in the spheno-occipital synchondrosis with and without mechanical stimulation (tensile stress).
- Identify and quantify the temporal expression of type II collagen in the spheno-occipital synchondrosis with and without mechanical stimulation (tensile stress).
- Associate the expression of SOX9 and type II collagen events occurring during growth of the synchondrosis in order to understand the role these factors play in the growth of the cartilage in the spheno-occipital synchondrosis.

MATERIALS AND METHODS

This experiment was approved by the committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong (CULATR 1106-05). Sixty 1-day-old male BALB/c mice were randomly divided into experimental and control groups. Each group was subdivided again into five different time points which were 6, 24, 48, 72, and 168 hours. Each subgroup consisted of five mice.

Surgical Explants of Cranial Base

Each mouse was sacrificed by injecting intra peritoneal (i/p) an overdose of pentobarbitone sodium (150–200 mg/kg) according to the euthanasia guidelines of Rodent Feti and Neonates from the Laboratory Animal Unit, The University of Hong Kong. An incision was made across the cranium to remove the brain from the cranial base, then the mandible was dissected and removed. An excision was started from the baso-occiptal synchondrosis and ran to the posterior part of the hard palate. The spheno-occipital synchondrosis was aseptically removed and incubated in a 24-well plate with or without mechanical stimulation (tensile stress) in medium culture at 37°C and 5% CO_2.

Medium Culture

The serum free protocol was modified from Shum et al,[14] but modified using BGJb medium (GIBCO, Invitrogen) supplemented with 2 mg/mL bovine serum albumin (BSA; Sigma A-9647), 100 μg/mL sodium ascorbate (Sigma A-4034), 1 mM beta-glycerophosphate (Fluka 50020), antibiotics and antimycotics (GIBCO, Invitrogen). The medium culture was changed every 24 hours.

Mechanical Spring

The mechanical spring was made according to the model of Ikegame et al[15] and was adjusted to deliver 0.2 gram tensile stress when the distance at the top of each arm (length 4 cm) was set at 5 mm and decreased at a rate of 0.01 g/mm (Figure 1). For the control, the springs were maintained at 5 mm by means of adhesive tape, hence giving 0 gram of tensile stress (Figure 1).

Immunohistochemistry

The methods of tissue preparation, sectioning and techniques of immunohistochemistry of SOX9 and

type II collagen were the same as those described by Rabie et al.[7] Immunohistochemistry of the transcription factor SOX9 and type II collagen was performed in order to localize their expression in the spheno-occipital synchondrosis after incubation in medium culture with different time intervals. The specificity of the antibody was ascertained against an epitope corresponding to amino acids 407–496 of SOX9 of human origin and epitope mapping near the C-terminus of collagen α1 type II of human origin. For negative controls, nonimmune serum was applied instead of the primary antibodies. Specimens from the tibia served as positive controls.

After the sections were dewaxed and rehydrated, antigenic sites were exposed by digestion with 0.1 M citric acid (pH 3) for 30 minutes at 37°C and nonspecific binding was reduced by treating in 3% H_2O_2 for 10 minutes followed by incubation 3% BSA (Sigma A-9085) blocking serum. The sections were then incubated with polyclonal rabbit-anti-human SOX9 (Santa Cruz Bio Inc, Santa Cruz, Calif; sc-20095), dilution 1:100, and secondary biotin-conjugated goat anti-rabbit for SOX9 (Dako E0432), dilution 1:200, preadsorbed with ABC reagent (Strept ABComplex/HRP, Dako K0377) successively each for 1 hour at 37°C. The sections were washed with tris buffered saline (TBS) between each step.

After the sections in the type II collagen groups were dewaxed, rehydrated, and treated in 3% H_2O_2 and protease digestion (Proteinase K, Sigma P-6556, 10 μg/mL), they were incubated 3% BSA (Sigma A-9085) blocking serum with goat polyclonal antibody type II collagen (Santa Cruz Bio; sc-7763), dilution 1:200 and secondary biotin-conjugated rabbit anti-goat for type II collagen (Dako 0466) dilution 1:200, preadsorbed with ABC reagent successively each for 1 hour at 37°C. The sections of both SOX9 and type II collagen were developed in 0.05% 3,3-diaminobenzidine (Sigma D-5637) for 2 minutes and counterstained with Mayer hematoxylin for 3 minutes, cleaned, and mounted with coverslips.

Quantitative and Statistical Analysis

Brown staining (at least 100 pixel) that localizes the expression of SOX9 and type II collagen in the spheno-occipital synchondrosis was measured with a true-color RGB (red-green-blue) computer-assisted image analyzing system with a digital camera (Leica DC 300 V2.0, Wetzlar, Germany) and with software (Leica Qwin Pro, version 2.6; Leica Microsystems Imaging Solutions) following the method of Rabie et al.[7] This system acquires high-definition digital images of the specimen, and features from the acquired images are selected by the operator. The amount of positive staining (Figure 2) is recognized and quantified by the computer software according to the color, shade, and contrast of the feature selected. The sections were quantified under a fixed measurement frame 165 μm^2 at 40$\times$ magnification (Leitz Orthoplan, Wetzler, Germany). Each section was examined and confirmed to be located in the midsagittal plane consistently by comparing the width of the spheno-occipital synchondrosis. For each subject, three sections were measured and a total of 240 sections were quantified. The amount of staining for all sections was quantified and evaluated by one examiner. The difference between the experimental and control groups was tested by unpaired *t*-test with SPSS for Windows (version 13.0, SPSS Inc, Chicago, Ill).

Method Error

Ten randomly drawn SOX9 and type II collagen specimens were quantified on two separate occasions about 1 month apart to calculate the method error. Method error in measuring the areas of the staining was calculated by the formula:

$$\mathbf{ME} = \sqrt{\frac{\sum d^2}{2n}}$$

where d is the difference between two registrations, and n is the number of double registrations. The method error (mm^2) for the measurements of SOX9 and type II collagen was found to be 0.002 and 0.005, respectively. A P value larger than .05 was used to indicate that there were no statistically significant differences between two measurements.

RESULTS

SOX9

Histologically, SOX9 was expressed by the cells in the proliferative and prehypertrophic zones of the spheno-occipital synchondrosis as shown in Figure 2A. Mechanical tension stimuli led to earlier higher expression of SOX9 starting at 6 hours in the experimental groups in all regions when compared to the expression at 6 hours in the control groups ($P < .001$). The maximum expression peaked at 24 hours, while at 24 hours in the control groups the expression declined (Figure 3). When the expression was followed until 168 hours, the experimental group SOX9 maintained a higher level of expression during mechanical tension stimuli than it did in the control groups. There was a statistically significant increase ($P < .001$) of 57% in the expression of SOX9 between control and experimental groups at 24 hours.

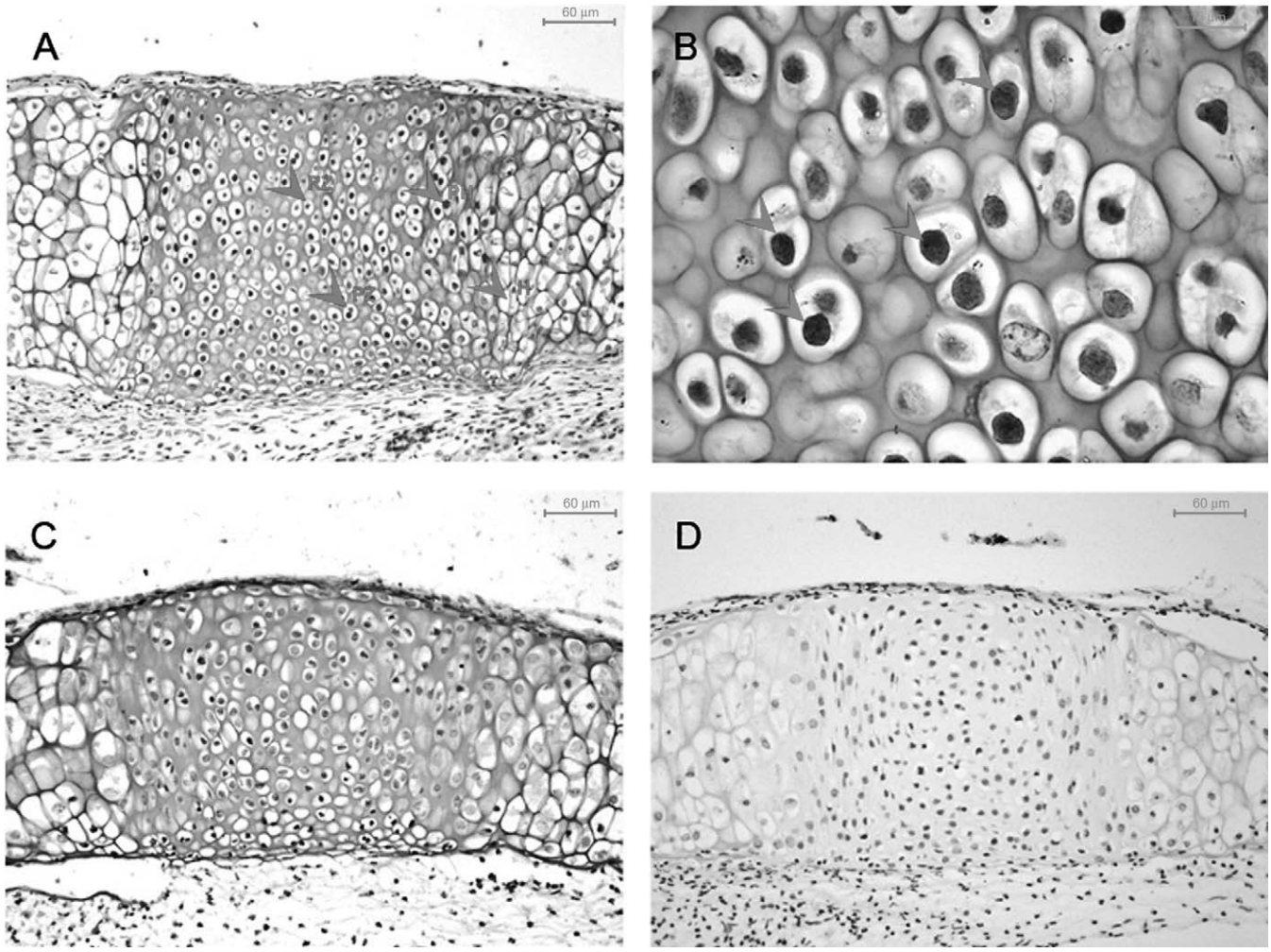

Figure 2. (A) Immunostaining of SOX9 at 24 hours of experimental groups with different zones (marked with arrows): proliferative zone (PZ), prehypertrophic zone (PH), and hypertrophic zone (HZ). (B) Photomicrograph in high magnification (100×) showing a positive immunostaining of SOX9 indicated by brown stains (marked with arrows). (C) Immunostaining of type II collagen. (D) Negative control.

Type II Collagen

Histologically, type II collagen was expressed by the cells in the proliferative, prehypertrophic, and hypertrophic zones of the spheno-occipital synchondrosis as shown in Figure 2. There was a statistically significant increase ($P < .01$) in the expression of type II collagen between the control groups and experimental groups at 48 hours, and maximum expression peaked at 72 hours compared to the control groups at the same time. Regarding the 72-hour experimental group, the expression of type II collagen had a significant increase of 44.4% ($P < .001$) compared to the control groups (Figure 3).

DISCUSSION

In the present study we applied a tensile stress across the spheno-occipital synchondrosis and examined the temporal pattern of SOX9 and type II collagen expression. This thus demonstrated that SOX9 is an important factor for early cell differentiation and cartilage formation in the spheno-occipital synchondrosis. Tensile stress produced by a mechanical spring led to an increase of SOX9 expression in the experimental groups compared to the control groups. Increases in SOX9 expression indicated more mesenchymal cells differentiate into the chondrogenic pathway and that will produce more cartilage formation. This will lead to an increase in bone formation and hence growth.[7]

The important role of SOX9 as a regulatory factor was demonstrated in patients who had campomelic dysplasia as described by Foster et al[16] and Wagner et al.[17] They described campomelic dysplasia as a rare genetic disease or severe human dwarfism syndrome due to the lack expression of SOX9. This clearly showed the importance of SOX9 in skeletal development. A study of SOX9 inactivation in limb bud using

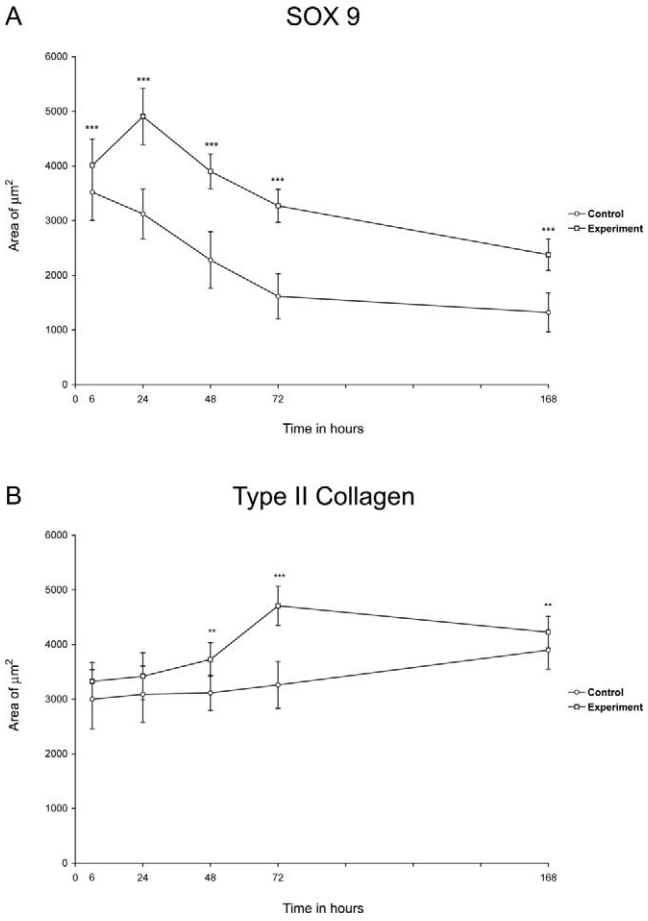

Figure 3. (A) The temporal pattern of SOX9 expression during mechanical tension stimuli. (B) The temporal pattern of type II collagen expression during mechanical tension stimuli. Mean and SD (n = 20). Significant differences between control and experimental groups are marked with asterisks (* $P < .05$; ** $P < .01$; *** $P < .001$).

the Cre recombinase/loxP recombination system before chondrogenic mesenchymal condensation resulted in the complete absence of mesenchymal condensation and of subsequent cartilage and bone formation.[18] So far no other transcription factors have been identified that might control early chondrogenic cell fate and differentiation upstream or in the same steps as SOX9 in all developing cartilage elements.[10] An increase in SOX9 expression will increase the number of mesenchymal cells differentiating into chondrocytes.[6] Since chondrocytes will synthesize cartilage, and cartilage is the structural template for bone growth, identifying the temporal pattern of expression of SOX9 transcription factor will be an excellent marker for monitoring acceleration of growth.

In control groups, the SOX9 reached a maximum level of expression at 6 hours and showed a decrease thereafter as shown in Figure 3. During mechanical tension stimuli, however, SOX9 reached a level of expression at 6 hours that was higher than that of the control group, and reached a maximum level of expression at 24 hours. This indicated an increase in the differentiation of mesenchymal cells into chondrocytes, thus possibly increasing the population of chondrocytes potentially available for chondrogenesis. The maximum expression of SOX9 achieved was 57% higher compared to the control groups. Throughout the experiment, SOX9 still maintained a higher level of expression compared to the control groups.

To further investigate the role of SOX9 in chondrogenesis of the synchondrosis, we also monitored the level of type II collagen expression since SOX9 expression regulates type II collagen by directly binding to 48-bp Col2a1 enhancer segments. Transcription of type II collagen gene starts to be expressed following mesenchymal cell condensation that precedes cartilage formation, thus it represents an early marker of chondrocyte differentiation.[19] Its essential structural role in cartilage is most clearly demonstrated by the abnormal skeletal phenotypes displayed by humans and mice carrying mutant pro1(II) collagen chains.[20] The important role of type II collagen mutation in craniofacial development and growth has been confirmed using transgenic Del 1 mice, in which the mutation is characterized by overall retardation of chondrogenesis and osteogenesis such as a reduced anteroposterior length, a smaller size of the mandible, a palatal cleft, and a downward bending snout.[21]

In the control groups, the maximum level of type II collagen expression was reached at 168 hours, but in experimental groups the maximum expression was reached at 72 hours and still maintained a higher level of expression until 168 hours (Figure 3B). This indicated an earlier differentiation of chondrocytes and enhanced level of matrix synthesis resulting in earlier and more cartilage formation. The maximum expression of type II collagen was 44% higher compared to control groups.

The increased expression of SOX9 clearly preceded the deposition of cartilage in the spheno-occipital synchondrosis, indicating that SOX9 is a determinant factor for chondrocyte differentiation rather than a consequence. Proliferating mesenchymal cells subjected to the SOX9 expression will differentiate into chondrocytes, thus increasing the synchondrosis growth potential. Furthermore, SOX9 acts upon these cells to enhance type II collagen synthesis, hence increasing the cartilage matrix formation. The increase in cartilage matrix offers a bigger template to accommodate more bone at a later stage.

CONCLUSIONS

- Mechanical tension stimuli (tensile stress) increases SOX9 and type II collagen expression in the spheno-occipital synchondrosis.

- SOX9 may play a role for early differentiation of chondrocytes and increase the expression of type II collagen, a major component of extracellular matrix, during the growth of cartilage in the spheno-occipital synchondrosis.

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