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ORIGINAL ARTICLE

Sox9 mRNA expression in the developing palate and craniofacial muscles and skeletons

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Abstract

Background. *Sox9* is a critical transcription factor for chondrogenesis and sex determination. Haploinsufficiency mutations of *SOX9* in humans lead to campomelic dysplasia. Inactivation of *Sox9* in the craniofacial region of mice results in an absence of endochondral bones and in malformation of other structures. This suggests that *Sox9* plays multiple roles in craniofacial development and these remain to be elucidated. In order to study the functions of *Sox9* in craniofacial development, a preliminary expression examination was performed. ***Material and Methods.*** To detect the expression of *Sox9* mRNA, antisense riboprobe was synthesized by *in vitro* transcription. Radioactive *in situ* hybridization was performed on sagittal and coronal sections of mice head from organogenesis to the early postnatal stage. ***Results.*** It was found that *Sox9* was expressed in multiple stages and distinct processes. Besides the expression in cartilage, it was seen in the fusing stage of palatogenesis. *Sox9* was also present during differentiation and maturation of craniofacial muscles. In addition, it was observed in intramembranous skeletal elements at restricted sites and stage. ***Conclusions.*** The expression pattern suggests that *Sox9* serves broad roles in craniofacial development.

Key Words: *Development and growth, head, palatogenesis, signaling, Sox9*

Introduction

SOX9 is a critical transcription factor for skeletogenesis and sex determination [1,2]. Haploinsufficiency of *SOX9* in humans leads to campomelic dysplasia (CD), a severe dwarfism syndrome characterized by sex reversal and the skeletal malformation of endochondral bones (OMIM, 114290). It has recently been found that *SOX9* serves other roles in various developmental processes, but these remain to be defined [3–6]. *SOX9* is also an important participant in craniofacial development. Mammal craniofacial development is generated from the neural crest layer, which gives rise to most of the craniofacial structures. *Sox9* is required for the induction and cell lineage specification of the neural crest cells [3,7]. Inactivation of *Sox9* from mouse neural crest leads to multiple skeletal defects in the basicranium, jaw, and palate [7].

Craniofacial bones are formed by two parallel mechanisms: intramembranous and endochondral ossification. The basicranium is a major site of endochondral ossification in the craniofacial area, while other bones are formed predominantly through intramembranous ossification. Nasal and ear cartilages, on the other hand, exist as permanent cartilages that remain cartilaginous throughout life. After initial development, cartilaginous or fibrous structures are formed among craniofacial bones to act as growth centers for further growth. In the calvaria, open fibrous sutures act as growth centers, whereas in the basicranium cartilage structures similar to long bone growth plate, termed synchondroses, are formed as growth sites [8]. Recent studies suggest that *Sox9*, besides having critical roles in chondrogenesis, is also important for growth center regulation in craniofacial development [9,10].

Deficiency of *Sox9* is concomitant with cleft palate [7,11], implying a critical role in palatogenesis. Palatogenesis is a multi-step process that includes palatal shelf outgrowth, elevation, fusion, and transdifferentiation or apoptosis of the medial epithelial seam. Throughout this process, the mesenchymal cells actively proliferate, which is a crucial event for palatogenesis. Development of the palate is regulated differently along the anterior-posterior axis.

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In the anterior part of the secondary palate, mesenchymal cells proliferate and differentiate into osteoblasts and form palatine bones, whereas mesenchymal cells in the posterior part mainly undergo myogenic differentiation and form muscles of the soft palate. Cleft palate comprises a major portion of craniofacial developmental anomalies. The results of gene manipulation in mice imply that *Sox9* is a critical signaling gene within the genetic network of palatogenesis [7,11]. However, it is still unclear which process *Sox9* regulates in palatogenesis. Furthermore, detailed cellular localization of *Sox9* in many craniofacial elements is still lacking. In this study, I examined the expression pattern of *Sox9* during craniofacial development, while focusing on developing palate and skeletal and muscular elements.

Material and methods

Preparation of tissues

All the procedures involving mouse use were approved by the Animal Welfare Committee of the University of Bergen. Mice (NMRI) were killed by cervical dislocation and decapitation. Embryonic (E) and postnatal (P) mice (E9 to E18, P0, P3, P5, and P9) were dissected in PBS and fixed in 4% PFA in PBS overnight at 4°C. Mice embryos harvested at E14 or older were decalcified with 12.5%EDTA–2.5%PFA in PBS. They were then dehydrated in a serial of ethanol and embedded in paraffin. Sagittal sections of 7 μm from the midline of mouse heads were cut and mounted, dried overnight at 37°C, and stored at 4°C. Coronal sections were limited to E13 and E14.

Preparation of hybridization probes

Preparation of hybridization probes has been described previously [9]. Briefly, fragments of *Sox9* cDNA was generated by RT-PCR and subcloned in a pGEM-T easy vector (Promega). [^{35}S]-labeled riboprobes are made through *in vitro* transcription from the cDNA-containing vectors using [^{35}S] uridine 5′ triphosphate (Promega). Following transcription, cDNA templates were digested with RNase-free DNase I. Riboprobes were quantified in a liquid scintillation analyzer.

In situ hybridization

Briefly, sections were deparaffined in xylene, rehydrated through serial ethanol, washed in PBS, treated with proteinase K, post-fixed in 4% PFA, and followed by PBS washing. The sections were then acetylated with freshly prepared acetic anhydride in triethanolamine-HCl (pH 8) for 10 min, followed by water washes. The sections were dehydrated in series of ethanol solutions and air-dried. Probe solutions were pipetted onto the sections. Hybridization was carried out for about 15 h at 55°C in a humid chamber. Following hybridization, the sections were washed under high stringent conditions with 20 mM dithiothreitol (DTT) in 50% formamide and 2 × saline sodium citrate buffer (SSC) for 1 h at 65°C and then in 0.1 × SSC for 1 h at 55°C. They were dehydrated in 70% ethanol and air-dried. The sections were then dipped in NTB-2 emulsion (Eastman Kodak, New Haven, Ct., USA) and stored in desiccated containers for autoradiography. After exposure for 2 to 3 weeks at 4°C, they were developed using Kodak D-19 developer and rapid fixer, counterstained with hematoxylin, and mounted. No specific signal was detected in sections hybridized with the control sense probes.

Image processing

Images were taken with a SPOT Insight digital camera (Diagnostic Instruments, Sterling Heights, Mich., USA) mounted on a Zeiss Axioskop2 microscope (Carl Zeiss Jena GmbH, Jena, Germany). The bright-field and dark-field images of each section were digitized separately and processed with Adobe Photoshop 7.0 software (Adobe Systems, San Jose, Calif., USA).

Results

Sox9 expression in the secondary palate

In mouse, the palatal shelves start to elevate at E13 and fuse at E14. *Sox9* was already seen in the mesenchyme of the elevating secondary palatal shelves at E13 (Figure 1A, B). This expression was intensified in the midline mesenchyme of the fusing palatal shelves at E14 (Figure 1C–F). In the anterior part, the palatal shelves were fusing; *Sox9* was highly expressed in the mesenchyme adjacent to the medial epithelial edge. Posteriorly, palatal shelves had fused; *Sox9* expression was high in the mesenchyme, but weak or absent in the medial epithelial seam. In the sagittal section, it was observed that *Sox9* was concentrated on the anterior and middle parts of the palatal shelves, and became weak in the posterior tip (Figure 1G, H). This expression of *Sox9* was found remarkably similar to that of *Bmp2* (Figure 2A–D). In subsequent development, *Sox9* was downregulated (Figure 1I, J). From the end of embryogenesis, *Sox9* was undetectable in the anterior palate, but showed an expression in the soft palate muscles (Figure 3A, B).

Sox9 expression in the craniofacial muscles

Sox9 was present during myogenic differentiation and maturation, but was weak and restricted in early myogenesis. The expression started to be clearly observed and widespread from the end of

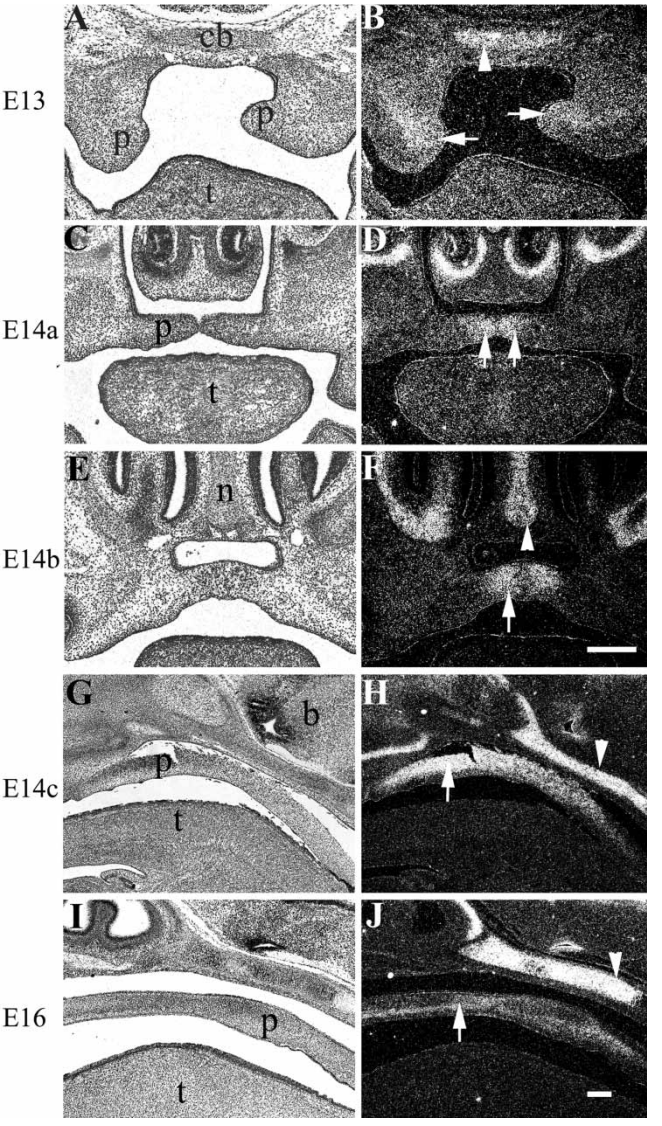

Figure 1. *Sox9* expression in palatogenesis. At E13, *Sox9* was seen in the mesenchyme of the elevating palatal shelves (A, B; *arrows*). One day later, when the palatal shelves were fusing, *Sox9* expression was intensified in the midline mesenchyme of the palatal shelves (C–H; *arrows*). At this time, the palatal shelves had started to fuse in the anterior part (C, D) and had already fused in the posterior part (E, F). In sagittal section, its expression was concentrated on the anterior part of the secondary palate (G, H; *arrow*). The expression was downregulated in subsequent development (I, J; *arrow*). Arrows and arrowheads indicate the expression. Scale bar: 200 μm. Scale bar in F applies to A, B, C, D, E, G, H; scale bar in J applies to I. b: brain; cb: cranial base; n: nasal cartilage; p: palatal shelves, t: tongue. E14a: coronal section of the anterior part of the palatal shelves at E14; E14b: coronal section of the middle part of the palatal shelves at E14; E14c: sagittal section at E14.

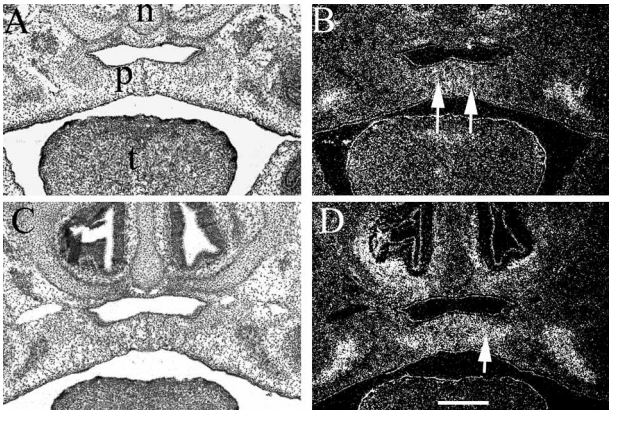

Figure 2. *Bmp2* expression in the fusing palate. *Bmp2* was expressed in the mesenchyme of the fusing palate (*arrow*) overlapping with *Sox9*. Scale bar: 200 μm. n: nasal cartilage; p: palate; t: tongue.

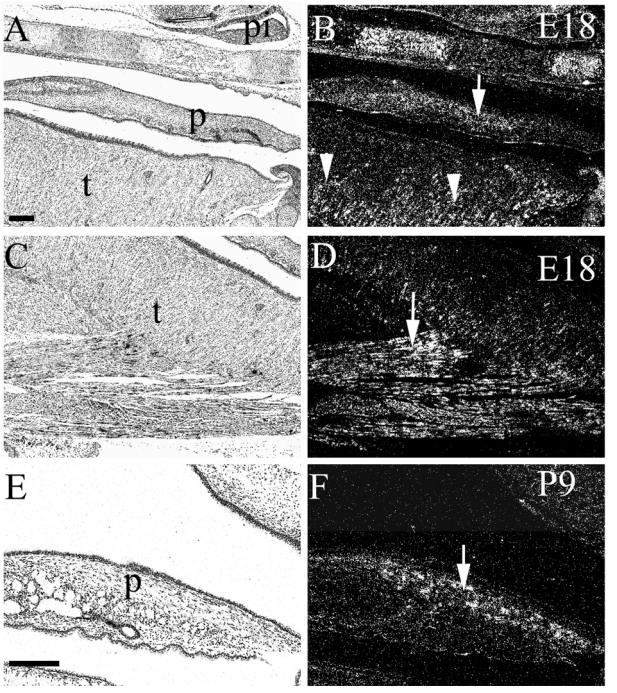

Figure 3. *Sox9* expression in the craniofacial muscles. *Sox9* was expressed in craniofacial muscles from the late embryonic stage. Transcripts were clustered in the palatal and tongue muscles (A, B, C, D). This expression was maintained and upregulated in the postnatal stage (E, F; *arrow*). Arrows and arrowheads indicate the expression. Scale bar: 200 μm. Scale bar in A applies to B, C, D; scale bar in E applies to F. p: palate; pi: pituitary gland; t: tongue.

embryogenesis (Figure 3A–D) and was maintained and upregulated in postnatal stages (Figure 3E, F). This was most obvious in the soft palate and tongue, which are basically muscular organs.

Sox9 expression in the craniofacial skeleton

At E9, prior to mesenchyme condensation, *Sox9* was seen in all the initial mesenchymal cells that were destined to form cartilages of facial primordia. This was obvious in the ventral and lateral sides of the brain, where the chondrocranium would be formed (Figure 4A, B). One day later, *Sox9* was present in all the condensations of prospective cartilages in craniofacial region. In sagittal section, high expression was seen in the presumptive chondrocranium and the posterior portion of the mandibular arch, where the epiglottis would be formed (Figure 4C, D). In subsequent development, *Sox9* was intensely expressed in all developing cartilages at their early stage, i.e. the Meckel's cartilage, chondrocranium, and nasal cartilages (Figure 4E, F). It was also observed in the mesenchyme condensation of the anterior part of mandible in the labial side (Figure 4G, H). In subsequent development, its expression disappeared from the differentiated cartilage, but continued in the permanent cartilage in the nasal cavity and synchondroses of the basicranium (Figure 4I, G). In the endochondral basicranium, its expression was found to be remarkably related to that of *Cbfa1*. They overlapped in early chondrocranium, with the absence of *Cbfa1* in the synchondroses (Figure 5A, B). Following differentiation, expression of *Sox9* and *Cbfa1* was dissociated. *Sox9* was downregulated from the differentiated chondrocytes, whereas *Cbfa1* was upregulated in these cells (Figure 5C, D). *Cbfa1* was never expressed in the synchondroses, where *Sox9* persisted (Figure 5 E, F).

Discussion

During embryogenesis, *Sox9* is essential for endochondral bone formation and sex determination [1,2,12]. Recently, it has been found to serve broader roles in embryogenesis, involved in heart, pancreas, and neural crest formation [3–6,13]. In this study, it was found that, besides its presence in chondrogenesis, *Sox9* is also involved in a number of other developmental processes in the craniofacial area.

Sox9 is essential for palatogenesis, as demonstrated by the gene targeting approach [7,11]. However, it is still unclear which process *Sox9* regulates. This study shows that *Sox9* is expressed in the mesenchyme of palatal shelves prior to and during palatal fusion, whereas it is downregulated in subsequent development. This expression implies that *Sox9* might be essential for mesenchyme proliferation and thus the outgrowth and fusion process. This hypothesis is also supported by *Sox9* deficiency mice, in which development of the palatal shelves is arrested after an initial budding [7]. I also observed remarkable parallel expression of *Sox9* and *Bmp2* in

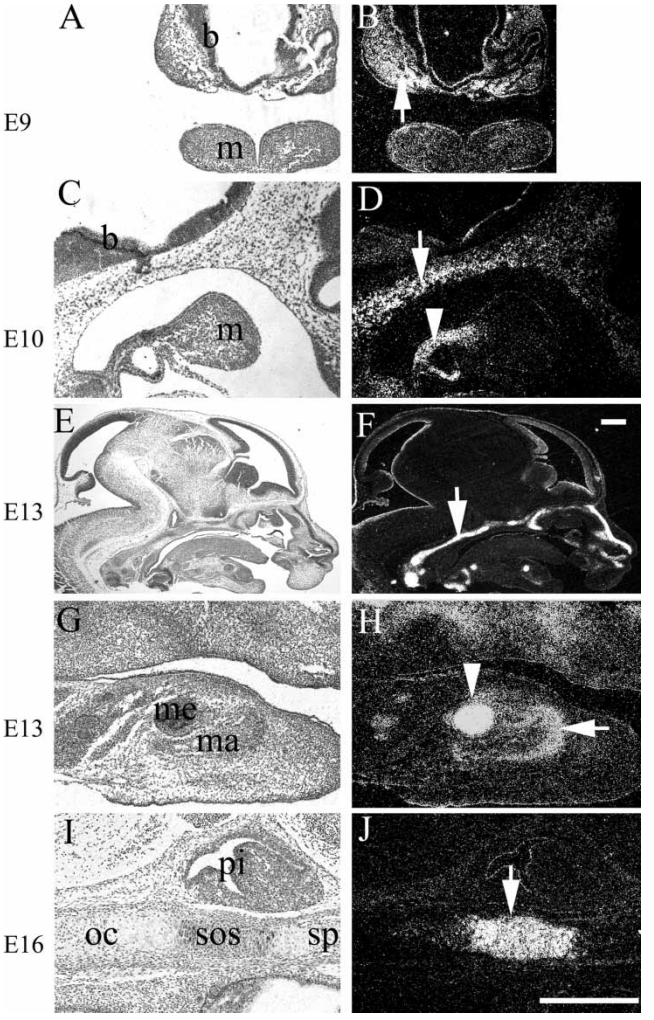

Figure 4. *Sox9* expression in the craniofacial skeletons. At E9, *Sox9* was seen in the mesenchyme of the ventral and lateral sides of the neural brain (A, B; *arrow*). At E10, it was clearly seen at the posterior presumptive chondrocranium (*arrow*) and the cartilage of pharyngeal arches (C, D). Later it was highly expressed in the chondrocranium, nasal cartilage (E, F), and Meckel's cartilage (G, H, *arrowhead*). It was also localized in the mesenchyme condensation of the mandible in the anterior part (G, H; *arrow*). Later, *Sox9* was maintained in the synchondroses and permanent cartilages (I, J; *arrow*). Arrows and arrowheads indicate the expression. Scale bar represents 200 μm. Scale bar in F applies to E; Scale bar in J applies to A, B, C, D, G, H, I. b: brain; ma: mandibular arch; me: Meckel's cartilage; oc: occipital bone; sp: sphenoid bone; sos: sphenoid-occipital synchondrosis.

the fusing palatal shelves. *Bmp* signaling is essential for palatogenesis; block of the *Bmp* signal pathway by receptor inactivation in the craniofacial region of mice results in cleft palate [14,15]. In further analysis, it was found that the cleft is due to insufficiency of mesenchyme proliferation, whereas the palatal shelves are capable of fusion when placed together [14,15]. Furthermore, craniofacial deletion of *Bmp4*, a critical ligand in putative facial epithelia [16], only leads to cleft lips, whereas the palate fuses normally [15]. So far, no other members of the *Bmp* family have been implicated to be directly involved in this process. Therefore, *Bmp2* appears to be the critical ligand for palatogenesis. *Bmp2* and *Sox9* signal loop have been identified in many developing processes [17–19]. The observation of high expression of *Bmp2* and *Sox9* in the mesenchyme of fusing palate implies that these two signal pathways may converge in palatogenesis.

A major finding of this study is the widespread expression of *Sox9* in the developing muscles. Craniofacial myogenesis is distinct from that of other sites by its early expression of myogenic regulation factors (*MRFs*), and thus early maturation [20,21]. The tongue is basically a muscular organ and a good model for studying myogenesis in the craniofacial region. Tongue development first undergoes a rapid expansion process and then myogenic differentiation at E15 [20–22]. *Sox9* expression was weak and restricted in the initial expanding process, but was high in the differentiating muscles, suggesting a role in regulating myogenic differentiation. This holds true in the soft palate. *Sox9* has also been detected in a myoblastic cell line [23]. So far, the function of *Sox9* in the developing craniofacial muscles is unknown. Further studies are warranted to elucidate the roles of *Sox9* in muscle development.

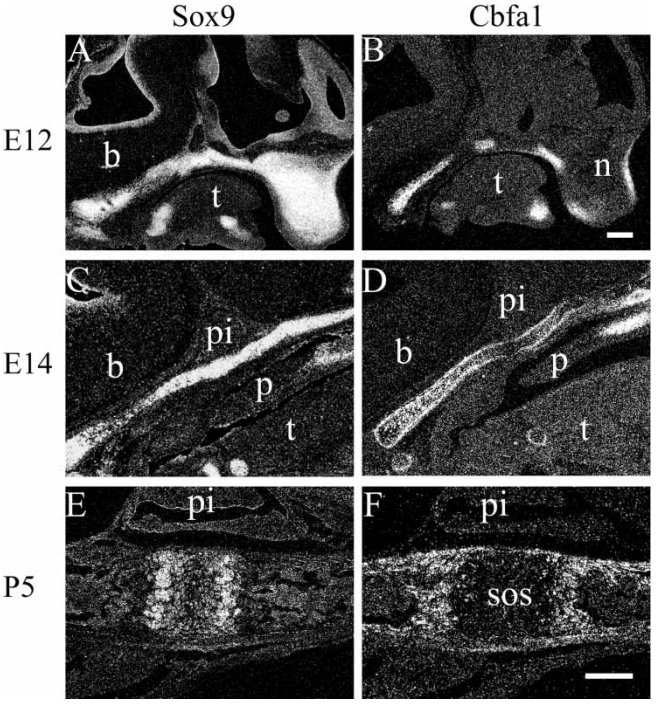

Figure 5. Comparison of *Sox9* and *Cbfa1* expression in the developing basicranium. In early development, *Sox9* was expressed throughout the chondrocranium, while *Cbfa1* was absent from the presumptive synchondrosis (A, B). Subsequently, their expression dissociated from the differentiating cartilage. *Sox9* disappeared from the differentiated cartilage, where *Cbfa1* was upregulated (C, D). Later, *Sox9* and *Cbfa1* displayed mutual exclusive expression domains (E, F). Scale bar: 200 μm. b: brain; t: tongue; n: nose; pi: pituitary gland; p: palate; sos: sphenoid-occipital synchondrosis.

Sox9 is a master transcription factor for chondrogenesis regulating the expression of *Col2a1* gene, a chondrocyte-specific enhancer element [24]. *Sox9* null cells are excluded from chondrogenic condensation [25]. In craniofacial development, *Sox9* acts from neural crest formation and specification [3]. Later, *Sox9* is mainly seen in the early chondrocranium and the permanent cartilages, whereas it disappears from terminally differentiated chondrocytes. The expression, along with other functional studies [26,27], suggests a role for *Sox9* in preventing the cartilage from being terminally differentiated. This role is essential for the existence of permanent cartilages. It has been suggested that expression of *Sox9* and *Cbfa1* determines whether skeletal tissues form as bone, persistent cartilage, or replacement cartilage [26]. In support of this, inactivation of *Sox9* in the neural crest results in osteogenic marker expression of nasal cartilages [7]. In this study, the author observed mutually exclusive expression domains of *Sox9* and *Cbfa1* in the endochondral basicranium, in which *Cbfa1* was seen in differentiation chondrocytes, whereas *Sox9* decreased from those cells. In general, *Sox9* shows an expression pattern in craniofacial endochondral skeletons parallel to that of other sites, implying an equivalent role of *Sox9* in this type of bone.

Interestingly, *Sox9* is observed in intramembranous skeletal elements, exemplified by the anterior part of the mandible. The anterior palate, where *Sox9* is expressed, is also a site of prospective intramembranous ossification. This expression suggests a possible role in intramembranous bone formation. In support of this, *Sox9* was found in osteogenic cells in the craniofacial bone and bone collar of long bone [28]. Very recently, *Sox9* was found to control the fusion of the posterior frontal suture in the calvaria, which is composed of intramembranous skeletal elements [10]. The regulatory role in the cranial growth centers indicates that *Sox9*, besides regulating early development, is also an important signal for later growth. Moreover, mammal development and growth are sensitive to *Sox9* dosage; mutation of only one copy of *Sox9* gene is enough to cause severe developmental defects. It is therefore logical to speculate that disturbance of *Sox9* function might be a possible mechanism of growth insufficiency in the cranium, as well as other facial bones.

In summary, *Sox9* is expressed in distinct processes during craniofacial development, such as skeletogenesis and myogenesis in a number of organs including palate, tongue, and craniofacial skeletons. The expression suggests important roles in craniofacial development. Although roles of *Sox9* in skeletogenesis in general have been extensively investigated using appendicular bones, its specific functions in craniofacial skeletal development remain to be analysed, particularly its roles and mechanisms in control of the skeletal growth centers. Its expression in developing palate points to a novel role in regulating palatogenesis. This study and other

functional analyses suggest that *Sox9* is a critical signaling within the genetic network of palatogenesis. Therefore, unraveling of the Sox9 signaling pathway will undoubtedly extend our understanding of craniofacial development, as well as pathological mechanisms. Further studies are warranted to elucidate its functions and mechanisms in these specific developmental processes during craniofacial development.

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References

[1] Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, et al. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. Cell 1994;79:1111–20.

[2] Foster JW, Dominguez-Steglich MA, Guioli S, Kowk G, Weller PA, Stevanovic M, et al. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. Nature 1994;372:525–30.

[3] Cheung M, Briscoe J. Neural crest development is regulated by the transcription factor Sox9. Development 2003;130: 5681–93.

[4] Lioubinski O, Muller M, Wegner M, Sander M. Expression of Sox transcription factors in the developing mouse pancreas. Dev Dyn 2003;227:402–8.

[5] Lee YH, Saint-Jeannet JP. Sox9, a novel pancreatic marker in Xenopus. Int J Dev Biol 2003;47:459–62.

[6] Rahkonen O, Savontaus M, Abdelwahid E, Vuorio E, Jokinen E. Expression patterns of cartilage collagens and Sox9 during mouse heart development. Histochem Cell Biol 2003;120:103–10.

[7] Mori-Akiyama Y, Akiyama H, Rowitch DH, de Crombrugghe B. Sox9 is required for determination of the chondrogenic cell lineage in the cranial neural crest. Proc Natl Acad Sci USA 2003;100:9360–5.

[8] Nie X. Cranial base in craniofacial development: developmental features, influence on facial growth, anomaly, and molecular basis. Acta Odontol Scand 2005;63:127–35.

[9] Nie X, Luukko K, Kvinnsland I, Kettunen P. Developmentally regulated expression of *Shh* and *Ihh* in the developing mouse cranial base: comparison with *Sox9* expression. Anat Rec A Discov Mol Cell Evol Biol 2005;286:891–8.

[10] Sahar DE, Longaker MT, Quarto N. Sox9 neural crest determinant gene controls patterning and closure of the posterior frontal cranial suture. Dev Biol 2005;280: 344–61.

[11] Bi W, Huang W, Whitworth DJ, Deng JM, Zhang Z, Behringer RR. Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization. Proc Natl Acad Sci USA 2001;98:6698–703.

[12] Wright E, Hargrave MR, Christiansen J, Cooper L, Kun J, Evans T, et al. The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos. Nat Genet 1995; 9:15–20.

[13] Akiyama H, Chaboissier MC, Behringer RR, Rowitch DH, Schedl A, Epstein JA. Essential role of Sox9 in the pathway that controls formation of cardiac valves and septa. Proc Natl Acad Sci USA 2004;101:6502–7.

[14] Dudas M, Sridurongrit S, Nagy A, Okazaki K, Kaartinen V. Craniofacial defects in mice lacking BMP type I receptor Alk2 in neural crest cells. Mech Dev 2004;121:173–82.

[15] Liu W, Sun X, Braut A, Mishina Y, Behringer RR, Mina M, et al. Distinct functions for Bmp signaling in lip and palate fusion in mice. Development 2005;132:1453–61.

[16] Gong SG, Guo C. Bmp4 gene is expressed at the putative site of fusion in the midfacial region. Differentiation 2003; 71:228–36.

[17] Healy C, Uwanogho D, Sharpe PT. Regulation and role of Sox9 in cartilage formation. Dev Dyn 1999;215: 69–78.

[18] Theodosiou NA, Tabin CJ. Sox9 and Nk $\times$2.5 determine the pyloric sphincter epithelium under the control of BMP signaling. Dev Biol 2005;279:481–90.

[19] Zehentner BK, Dony C, Burtscher H. The transcription factor Sox9 is involved in BMP-2 signaling. J Bone Miner Res 1999;14:1734–41.

[20] Shuler CF, Dalrymple KR. Molecular regulation of tongue and craniofacial muscle differentiation. Crit Rev Oral Biol Med 2001;12:3–17.

[21] Yamane A, Mayo M, Shuler C, Crowe D, Ohnuki Y, Dalrymple K. Expression of myogenic regulatory factors during the development of mouse tongue striated muscle. Arch Oral Biol 2000;45:71–8.

[22] Nie X. Apoptosis, proliferation, and gene expression patterns in mouse developing tongue. Anat Embryol (Berl) 2005;210:125–32.

[23] Matsushita T, Matsui N, Fujioka H, Kubo S, Kuroda R, Kurosaka M, et al. Expression of transcription factor Sox9 in rat L6 myoblastic cells. Connect Tissue Res 2004;45:164–73.

[24] Bell DM, Leung KK, Wheatley SC, Ng LJ, Zhou S, Ling KW, et al. SOX9 directly regulates the type-II collagen gene. Nat Genet 1997;16:174–8.

[25] Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B. Sox9 is required for cartilage formation. Nat Genet 1999; 22:85–9.

[26] Eames BF, Sharpe PT, Helms JA. Hierarchy revealed in the specification of three skeletal fates by Sox9 and Runx2. Dev Biol 2004;274:188–200.

[27] Akiyama H, Chaboissier MC, Martin JF, Schedl A, de Crombrugghe B. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev 2002;16:2813–28.

[28] Yamashiro T, Wang XP, Li Z, Oya S, Aberg T, Fukunaga T, et al. Possible roles of Runx1 and Sox9 in incipient intramembranous ossification. J Bone Miner Res 2004;19: 1671–7.