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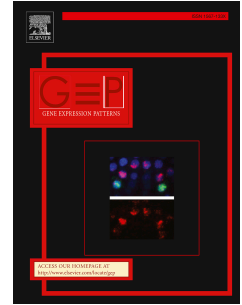
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# Accepted Manuscript

Spatio-temporal expression of *Sox* genes in murine palatogenesis

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## Spatio-temporal expression of *Sox* genes in murine palatogenesis

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**SUMMARY**

Members of the *Sox* gene family play critical roles in many biological processes including organogenesis. We carried out comparative *in situ* hybridization analysis of seventeen *Sox* genes (*Sox1-14, 17, 18* and *21*) during murine palatogenesis from initiation to fusion of the palatal shelves above the dorsal side of the tongue. At palatal shelf initiation (E12.5), the localized expression of six *Sox* genes (*Sox2, 5, 6, 9, 12* and *13*) was observed in the shelves, whereas *Sox4* and *Sox11* showed ubiquitous expression. During the down-growth of palatal shelves (E13.5), *Sox4, Sox5,* and *Sox9* exhibited restricted expression to the interior side of the palatal shelves facing the tongue. Following elevation of the palatal shelves (E14.5), *Sox2, Sox11* and *Sox21* expression was present in the midline epithelial seam. We thus identify dynamic spatio-temporal expression of *Sox* gene family during the process of palatogenesis.

## INTRODUCTION

Cleft palate is one of the most common congenital birth defects in humans, suggesting that palatogenesis is easily perturbed by genetic and environmental factors (Gritli-Linde 2007, Dixon et al., 2011, Iwata et al., 2011). Many molecules have been identified in palatogenesis, which requires fine tuning in terms of the timing, location and size of their expression for normal palate development (Murray and Schutte 2004, Smith et al., 2013, Meng et al., 2009, Bush and Jiang 2012, Gritli-Linde 2007, Iwata et al., 2011, Dixon et al., 2011). The secondary palate initiates as the formation of palatal shelves that emerge from the internal side of the maxillary arch. The palatal shelves are composed of mesenchyme derived mainly from the neural crest and a thin layer of oral epithelium (Ito et al., 2003). The secondary palate develops through sequential and reciprocal interactions between the epithelium and mesenchyme, and involves multiple developmental events such as growth, elevation and fusion (Murray and Schutte 2004, Smith et al., 2013, Meng et al., 2009, Bush and Jiang 2012, Gritli-Linde 2007, Iwata et al., 2011, Dixon et al., 2011). The growing bilateral palatal shelves extend downward beside the developing tongue, and then elevate above the dorsal side of the tongue. Following elevation, the paired palatal shelves grow horizontally towards each other to meet along the midline. The midline epithelial seam (MES) is formed at this junction, and disappears allowing the fusion of the palatal shelves with mesenchymal confluence. It has been shown that many genes are differentially expressed along the anterior-posterior axis in the developing palate (Li and Ding 2007, Bush and Jiang 2012). Distinct shapes have also been reported in developing palatal shelves along the same axis (Yu and Ornitz 2011). This suggests that distinct molecular mechanisms are present in palatogenesis along the anterior-posterior axis.

Sox proteins are characterized by a highly conserved DNA binding motif and high mobility group (HMG) domain. Twenty *Sox* genes have been identified in mice. Members of the *Sox* gene family show dynamic and diverse expression patterns during development. Mutation analyses in mice provide evidence that they play multiple roles during development (Pevny and Lovell-Badge 1997, She and Yang 2015). Although mutation in *Sox2*, *Sox5*, *Sox9* and *Sox11* has been shown to result in cleft palate in human and/or mouse, the expression of the *Sox* family members – including *Sox2*, *Sox5*, *Sox9* and *Sox11* – in palate development remains unclear (Male et al., 2002, Langer et al., 2014, Sock et al., 2004; Langer et al., 2014, Bi et al., 2001, Mori-Akiyama et al., 2003, Smits et al., 2001).

We therefore carried out comparative *in situ* hybridization analysis of seventeen *Sox* genes (*Sox1-14*, *17*, *18* and *21*) during murine palatogenesis. Here, we identify the dynamic spatio-temporal expression of *Sox* genes in palatal development.

## RESULTS

Since distinct molecular mechanisms are present in palatogenesis along the anterior-posterior axis, we examined expression of the *Sox* gene family in the palatal tissues of the anterior, middle and posterior regions.

Neither *Sox1* nor *Sox3* expression could be detected in palatal shelves from embryonic day (E) 12.5 to E14.5 (data not shown).

In mice, the secondary palate initiates as palatal shelves that emerge at E12.5 from the internal side of maxillary arch. *Sox2* showed strong expression in the epithelium of the palatal shelves (Fig. 1A-C). *Sox4* was ubiquitously expressed in the maxillary arch, while its expression was slightly weaker in palatal shelf mesenchyme (Fig. 1D-F). *Sox5* exhibited restricted expression in the presumptive maxillary bone region (Fig. 1G-I). *Sox6* was expressed in the epithelium of the palatal shelves, while it also showed restricted expression in the presumptive maxillary bone region (Fig. 1J-L). *Sox8* expression could not be detected in the palatal shelves at E12.5 (Fig. 1M-O). *Sox9* exhibited restricted expression in the presumptive maxillary bone region (Fig. 2A-C). *Sox11* showed ubiquitous expression in the maxillary arch (Fig. 2D-F). No expression of *Sox12* was observed in developing palatal shelves (Fig. 2G-I). Weak expression of *Sox13* was observed in epithelium of palatal shelves at the anterior region and the middle region, whereas *Sox13* expression could not be detected in developing palatal shelves at the posterior region (Fig. 2J-L). No expression of *Sox14* or *Sox21* could be detected in palatal shelves at E12.5 (Fig. 2M-R). Additionally, there were no significant differences in expression of *Sox2*, *Sox4*, *Sox5*, *Sox6*, *Sox9* and *Sox11* in the palatal shelves between the regions of the anterior-posterior axis.

In mice, the bilateral palatal shelves extend downward beside the developing tongue at E13.5. *Sox2* exhibited restricted strong expression in the epithelium of palatal shelves (Fig. 3A-C; Langer et al., 2014). *Sox4* expression was restricted to the mesenchyme of the interior side of the palatal shelves facing the tongue (Fig. 3D-F). The expression of *Sox4* in the palatal shelves at the anterior region was stronger than those at the posterior region. *Sox5* was weakly expressed in the mesenchyme of the palatal shelves facing the tongue at the middle region, whereas no expression could be detected in the palatal shelves at other regions (Fig. 3G-I). *Sox6* showed expression in the epithelium of the palatal shelves and presumptive maxillary bone region (Fig. 3J-L). Weak expression of *Sox8* was observed in the epithelium of the palatal shelves at the anterior and the middle regions (Fig. 3M-O). *Sox9* expression was restricted to the mesenchyme at the tip of the palatal shelves at the anterior region, and to the mesenchyme of the interior side of the palatal shelves facing the tongue at the middle and posterior regions (Fig. 4A-C). *Sox11* was strongly expressed in both the epithelium and mesenchyme of the palatal shelves at the anterior region, while expression in the mesenchyme became significantly weaker at the middle and the posterior regions (Fig. 4D-F). Weak *Sox12* expression was observed in the epithelium of the developing palatal shelves at E13.5 (Fig. 4G-I). No expression of *Sox13* or *Sox14* could be detected in the palatal shelves at E13.5 (Fig. 4J-O). *Sox21* was expressed in epithelium of palatal shelves at the anterior region (Fig. 4P-R).

At E14.5, the bilateral palatal shelves elevate horizontally and grow towards the midline to make contact with each other, forming the midline epithelial seam (MES). Weak expression of *Sox2* was observed in the MES, while *Sox2* was strongly expressed in epithelium facing the oral cavity (Fig. 5A-C; Langer et al., 2014). *Sox4* showed expression in palatal epithelium, whilst it was weakly expressed in the mesenchyme (Fig. 5D-F). *Sox5* expression was

observed in the mesenchyme around the MES at the anterior and the middle regions, whilst it was weakly expressed in the mesenchyme at the posterior region (Fig. 5G-I). *Sox6* was expressed in the epithelium of the palate, excluding the MES, whilst it showed expression in the maxillary bone (Fig. 5J-L). Weak expression of *Sox8* was observed in the MES at the posterior region, whereas other regions showed no *Sox8* expression in the MES (Fig. 5M-O). *Sox9* showed restricted expression in the mesenchyme around the MES for all regions (Fig. 6A-C). *Sox11* was expressed in the MES, whilst it was weakly expressed in the epithelium facing the oral cavity (Fig. 6D-F). Weak expression of *Sox12* was observed in the epithelium and maxillary bone (Fig. 6G-I). No expression of *Sox13* or *Sox14* could be detected in the palatal shelves at E14.5 (Fig. 6J-O). *Sox21* was weakly expressed in the epithelium including the MES at the posterior region, whereas no expression of *Sox21* was observed at the either anterior or middle regions (Fig. 6P-R).

*Sox7*, *Sox17* and *Sox18* belong to group F of the *Sox* gene family. SoxF members have been shown to be implicated in regulating blood and lymphatic vascular development (Francois et al., 2010, 2011; Morini and Dejana 2014). A punctate expression pattern of *Sox7*, *Sox17* and *Sox18* was seen throughout the mesenchyme of the palatal shelves from E12.5 to E14.5, and probably represents vascularization of the tissue (data not shown). *Sox10* has been reported to play a critical role in neural development (Bondurand and Sham 2013, Weider et al., 2013). *Sox10* also shows a punctate expression pattern in the palatal shelves from E12.5 to E14.5, and is likely to be related to nerve development (data not shown).

## DISCUSSION

Members of the *Sox* gene family show dynamic and diverse expression patterns during development of many organs, and analysis of mutations in mice suggest that the *Sox* gene family plays multiple roles during development (Pevny and Lovell-Badge 1997, She and Yang 2015). Our results show dynamic spatio-temporal expression of *Sox* genes in the developing palatal shelves, through which they may play a critical role in palate development.

*Sox2* mutation has been shown to result in the failure of fusion of the palatal shelves in both human and mouse (Male et al., 2002, Langer et al., 2014). Expression of *Sox2* was observed in the epithelium of the palatal shelves including the MES, which disappears through apoptosis to allow the fusion of the palatal shelves, with mesenchymal confluence. It has previously been shown that *Sox2* is involved in regulating apoptosis (Chen et al., 2014, Feng et al., 2013, Lin et al., 2012). It is thus likely that *Sox2* is essential for palatogenesis through its regulation of apoptosis of the MES region.

It was originally believed that the tip of the vertical palatal shelf beside the tongue at E13.5 corresponded to the medial edge region in the horizontal palatal shelf at E14.5. This has recently been shown not to be the case. It is in fact the interior side of the palatal shelves facing the tongue that forms the prospective medial edge of the palatal shelves (Jin et al., 2010). In theory, this region grows horizontally to a position above the dorsal side of the tongue, rather than the palatal shelves undergoing physical rotation. Indeed, expression of several genes including *Gooseoid* were observed in both the interior side facing the tongue before elevation and the medial edge of the palatal shelves after elevation (Jin et al., 2010). Our results also revealed that *Sox5* and *Sox9* were expressed in the interior side of the palatal

shelves facing the tongue before elevation and the medial edge of the palatal shelves after elevation. *Sox9* mutation has been shown to lead to cleft palate (Bi et al., 2001, Mori-Akiyama et al., 2003). *Sox5* mutants also exhibit cleft palate due to failure of palatal shelf elevation (Smits et al., 2001). It is possible that the interior side of the palatal shelves is a key region for the elevation process.

*Sox11* mutant mice show failure of palatal shelf formation (Sock et al., 2004). Our results revealed that *Sox11* was expressed in both the mesenchyme and epithelium of the palatal shelves at E12.5 when palatal shelves begin forming. It is possible that *Sox11* is an essential molecule for the initiation of palate development.

Histological differences are known in palatal shelves along the anterior-posterior axis (Yu and Ornittz 2011). For example, shelf elevation in the anterior region occurs at a later stage in comparison with those of more posterior region (Yu and Ornittz 2011). In addition to histological differences, many genes are differentially expressed in the developing palate along the anterior-posterior axis (Li and Ding 2007, Bush and Jiang 2012). Our results also revealed that *Sox4*, *Sox5*, *Sox9*, *Sox11*, *Sox13* and *Sox21* showed different expression patterns in the palatal shelves along the anterior-posterior axis. It is conceivable that they have different roles in palatogenesis between the regions of the palatal shelves.

Epithelial-mesenchymal interaction plays a critical role in palate development. *Sox2*, *Sox12*, *Sox13* and *Sox21* showed restricted expression in the epithelium, whereas *Sox5* and *Sox9* were expressed in the mesenchyme. It is possible that *Sox* family members are relevant to epithelial-mesenchymal interactions during palatogenesis.

Our data indicates that eleven members of the *Sox* family showed expression during palatal development. So far, only *Sox2*, *Sox5*, *Sox9* and *Sox11* mutation have been reported to lead to cleft palate (Male et al., 2002, Langer et al., 2014, Sock et al., 2004; Langer et al., 2014, Bi et al., 2001, Mori-Akiyama et al., 2003, Smits et al., 2001). *Sox* genes are classified into nine subgroups according to homology within the HMG domain and other structural motifs, as well as functional assays (Pevny and Lovell-Badge 1997, Wegner 1999). Only members belonging to Group F of the *Sox* family exhibited identical expression patterns during palate development. It has been shown that there is the redundancy between different *Sox* group members in organogenesis (Ito 2010, Kawasaki et al., 2015). In palatogenesis, *Sox4* expression was found to be similar to those of *Sox11* at E12.5 and those of *Sox5* at E13.5. It is therefore possible that redundancy also occurs between members of the *Sox* gene family during palate development.

## Materials and Methods

### *Production and analysis of transgenic mice*

CD1 mice were used for this study. The day on which vaginal plugs were found was considered as embryonic day (E) 0.5. To accurately assess the age of embryos, somite pairs were counted and the stage confirmed using morphological criteria – such as relative size of maxillary and mandibular primordia, extent of nasal placode invagination, and the size of limb buds. Mouse heads were fixed in 4% paraformaldehyde, embedded and serially sectioned at 8  $\mu\text{m}$ .

### *In situ hybridization*

Radioactive *in situ* hybridization with  $^{35}\text{S}$ -UTP-radiolabelled riboprobes was performed as described previously by Ohazama et al., 2008.

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ACCEPTED MANUSCRIPT

**Figure Legends****Figure 1. The expression of *Sox* (2, 4, 5, 6, 8) genes in rodent palatal development at E12.5**

*In situ* hybridisation of *Sox2*, *Sox4*, *Sox5*, *Sox6* and *Sox8* on anterior (A,D,G,J,M), middle (B,E,H,K,N) and posterior (C,F,I,L,O) frontal head sections at E12.5. Scale bars: 500µm.

**Figure 2. The expression of *Sox* (9, 11, 12, 13, 14, 21) genes in rodent palatal development at E12.5**

*In situ* hybridisation of *Sox9*, *Sox11*, *Sox12*, *Sox13*, *Sox14* and *Sox21* on anterior (A,D,G,J,M,P), middle (B,E,H,K,N,Q) and posterior (C,F,I,L,O,R) frontal head sections at E12.5. Scale bars: 500µm.

**Figure 3. The expression of *Sox* (2, 4, 5, 6, 8) genes in rodent palatal development at E13.5**

*In situ* hybridisation of *Sox2*, *Sox4*, *Sox5*, *Sox6* and *Sox8* on anterior (A,D,G,J,M), middle (B,E,H,K,N) and posterior (C,F,I,L,O) frontal head sections at E13.5. Scale bars: 500µm.

**Figure 4. The expression of *Sox* (9, 11, 12, 13, 14, 21) genes in rodent palatal development at E13.5**

*In situ* hybridisation of *Sox9*, *Sox11*, *Sox12*, *Sox13*, *Sox14* and *Sox21* on anterior (A,D,G,J,M,P), middle (B,E,H,K,N,Q) and posterior (C,F,I,L,O,R) frontal head sections at E14.5. Scale bars: 500µm.

**Figure 5. The expression of *Sox* (2, 4, 5, 6, 8) genes in rodent palatal development at E14.5**

*In situ* hybridisation of *Sox2*, *Sox4*, *Sox5*, *Sox6* and *Sox8* on anterior (A,D,G,J,M), middle (B,E,H,K,N) and posterior (C,F,I,L,O) frontal head sections at E14.5. Scale bars: 500µm.

**Figure 6. The expression of *Sox* (9, 11, 12, 13, 14, 21) genes in rodent palatal development at E14.5**

*In situ* hybridisation of *Sox9*, *Sox11*, *Sox12*, *Sox13*, *Sox14* and *Sox21* on anterior (A,D,G,J,M,P), middle (B,E,H,K,N,Q) and posterior (C,F,I,L,O,R) frontal head sections at E14.5. Scale bars: 500µm.

