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Developmental Expression Pattern

Gene expression analysis reveals that formation of the mouse anterior secondary palate involves recruitment of cells from the posterior side

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ABSTRACT Cleft palate is a common birth defect caused by disruptions in secondary palate development. Anterior-posterior (A-P) regional specification plays a critical role in palate development and fusion. Previous studies have shown that at the molecular level, the anterior palate can be defined by the expression of *Shox-2* and the posterior palate by *Meox-2* expression in certain mouse strains. Here, we have extended previous studies by performing a more detailed analysis of these genes during mouse palate development. We found that the expression patterns of *Shox-2* and *Meox-2* are dynamic during palate development. At embryonic day 12.5 (E12.5), *Shox-2* expression is localized to the anterior end and its expression domain covers less than 25% of the length of the palate shelf. The *Shox-2* expression domain then gradually expands towards the posterior end and ultimately occupies more than 60% of the palate shelf by E14.5. The expansion of the *Shox-2* domain may involve induction of *Shox2* expression in additional cells. Reciprocally, the *Meox-2* expression domain at E12.5 covers a large portion of the palate shelf, a region more than 70% of the entire palate, but then regresses to the posterior 25% by E14.5. This regression is likely caused by the repression of *Meox-2* expression in certain *Meox2* expressing cells, rather than the cessation of cell proliferation. Therefore, certain *Meox-2* positive "primitive posterior cells" are differentiated/converted into *Shox-2* positive "definitive anterior cells" during A-P regional specification.

KEY WORDS: *Shox-2, Meox-2, secondary palate, regional specification, craniofacial development*

During mammalian embryogenesis, the formation of the continuous secondary palate between the oral and nasal cavities involves multiple developmental steps that lead to the fusion of the two bilateral palate shelves along the facial midline (Ferguson 1988; Murray and Schutte, 2004; Nawshad *et al.*, 2004). At the histological level, a developing mouse secondary palate shelf contains a block of neural crest derived mesenchymal cells surrounded by a multi-layered epithelial sheet that originates from the facial ectoderm (Ferguson 1988). In mouse development, the prospective palatal mesenchymal cells begin to be specified in the maxillary processes at embryonic day 11.5 (E11.5) (Murray and Schutte, 2004). The developing palate shelves first grow down vertically along the two sides of the tongue between E12.5 and E13.5. From E14.5, however, the two palatal shelves elevate above the level of the dorsal tongue. The two elevated palatal shelves will continue to grow horizontally and meet each other along the facial midline (Ferguson 1988; Murray and Schutte, 2004; Nawshad *et al.*, 2004). The contact of the two palate shelves at their medial edge epithelium (MEE) regions induces fusion of the two palate shelves and the formation of a continuous palate by E15.5 (Shuler *et al.*, 1991; Shuler *et al.*, 1992; Carette and Ferguson, 1992; Griffith and Hay 1992). The proximal-distal growth of the palate shelf is crucial for midline contact. Following contact, the two medial edge epithelial sheets merge to form the MEE seam that will soon undergo degeneration (Ferguson 1988; Shuler *et al.*, 1991; Shuler *et al.*, 1992; Carette and Ferguson 1992; Griffith and Hay 1992; Murray and Schutte, 2004). This process requires the differentiation of palatal medial edge epithelial cells that are distinct from the epithelial cells on oral and nasal sides (Ferguson *et al.*, 1984). Moreover, tissue recombination experiments revealed that the differentiation of medial epithelial cells is determined by the underlying medial edge mesenchymal cells (Ferguson *et al.*, 1984). In addition to proximal-distal growth and medial-lateral differentiation, the palate shelves

Abbreviations used in this paper: A-P, anterior-posterior; MEE, medial edge epithelium; PS, palate shelves.

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also grow and differentiate in the anterior-posterior (A-P) direction. The A-P differentiation at the morphological and cellular levels has long been recognized as the palate shelf displays distinct characterizations along its A-P axis; In particular, the anterior palate is committed to be the bony hard palate, whereas the posterior region will form the soft palate that is composed primarily of smooth muscle (Cui *et al*., 2005). Only recently, however, has the importance of A-P regional specification in palate fusion been appreciated at the molecular level (Hilliard *et al*., 2005). The mouse homeobox gene *Msx1* is expressed only in the anterior palatal mesenchymal cells (Zhang *et al*., 2002; Hilliard *et al*., 2005) and the loss of *Msx1* function in mice results in a complete cleft palate rather than only an anterior cleft, indicating the importance of anterior palate development in palate fusion (Satokata and Maas, 1994; Zhang *et al*., 2002; Hilliard *et al*., 2005). Furthermore, the cleft palate caused by *Msx1* inactivation can be rescued by trans-expression of the *BMP4* gene (Zhang *et al*., 2002; Hilliard *et al*., 2005). Interestingly, *in vitro* explant culture showed that only the anterior, but not the posterior, palatal mesenchymal cells can proliferate in response to the addition of BMP (Zhang *et al*., 2002; Hilliard *et al*., 2005). The expression of *Pax9* is restricted to the posterior region during palate development and disruption of this gene in mice results in cleft palate (Peters *et al*., 1998; Hilliard *et al*., 2005). All of these experimental results indicate that A-P differentiation and regional specification play critical roles in palate development and fusion. However, our current understanding of the A-P regional specification at the molecular level is limited and several fundamental issues remain to be addressed. For example, the chronology of palatal A-P regional specification has not been examined in detail and it is assumed that the anterior and posterior palates initiate simultaneously and develop synchronously.

In this study, we analyzed three markers, *Msx1*, *Shox-2* and *Meox-2*, to examine the A-P regional specification during palate development. Mouse *Shox-2* gene belongs to a recently identified paired-related homeobox gene family that also found in humans and chicken (Blaschke *et al*., 1998; Semina *et al*., 1998; Clement-Jones *et al*., 2000; Cobb *et al.,* 2006; Tiecke *et al*., 2006). The founding member of this family, human *SHOX* gene, is associated with bone related short-stature disorders including Turner syndrome and Leri-Weill syndromes (Clement-Jones *et al*., 2000). Human *SHOX2* gene shares 80% similarity with human *SHOX* gene at amino acid level in the homeodomain and C-terminal regions (Blaschke *et al*., 1998; Semina *et al*., 1998). In mouse, only one *Shox* family member, *Shox-2*, has been identified to date (Yu *et al*., 2005; Cobb *et al*., 2006). The amino acid sequences between human and mouse Shox-2 proteins are 99% identical (Blaschke *et al*., 1998; Semina *et al*., 1998). Unlike SHOX, the association of *SHOX-2* gene with human disease has not been reported. However, studies with mouse embryos indicate that *Shox-2* function is also required for bone growth, at least, in limb system development (Cobb *et al*., 2006). Inactivation of *Shox-2* specifically in mouse limb buds leads to severe chondrogenesis defects in long-bone development (Cobb *et al*., 2006). Further analysis

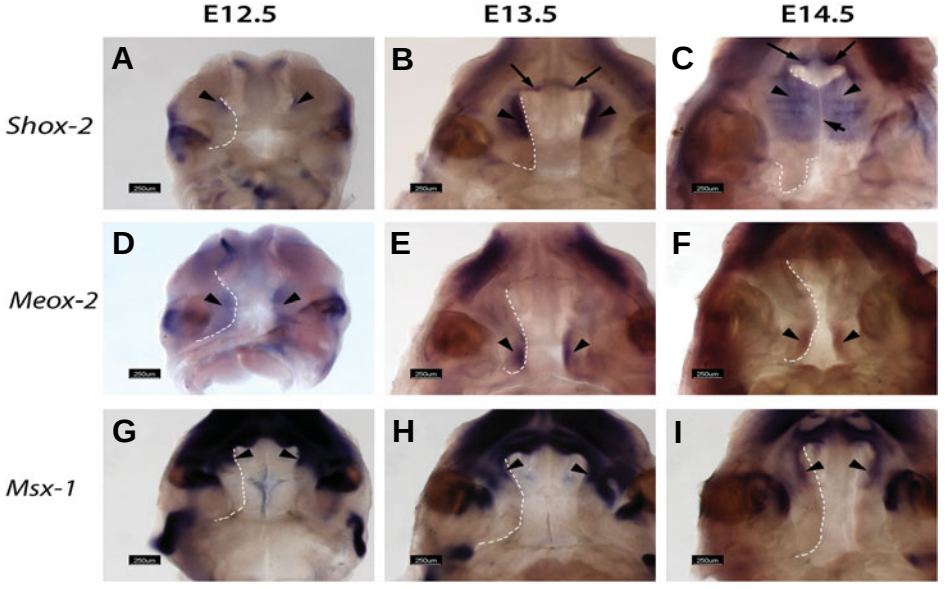

Fig. 1 (Left). Whole mount *in situ* hybridization showing the expression of *Shox-2, Meox-2* and *Msx1* during mouse secondary palate development from E12.5 to E14.5. (A-C) *Expression of* Shox2 *in the secondary palate shelves (arrowheads) and primary palate (arrows) at* **(A)** *E12.5,* **(B)** *E13.5 and* **(C)** *E14.5. The expression is absent from the medial edge epithelium (short arrow) at* **(C)** *E14.5.* **(D-F)** *Expression of* Meox-2 *in the secondary palate shelves (arrowheads) at* **(D)** *E12.5,* **(E)** *E13.5 and* **(F)** *E14.5.* **(G-I)** *Expression of* Msx1 *in the secondary palate shelves (arrowheads) at* **(G)** *E12.5,* **(H)** *E13.5 and* **(I)** *E14.5. Scale bars represent 250 μm. The white dotted lines indicate the edge of the palate shelf.*

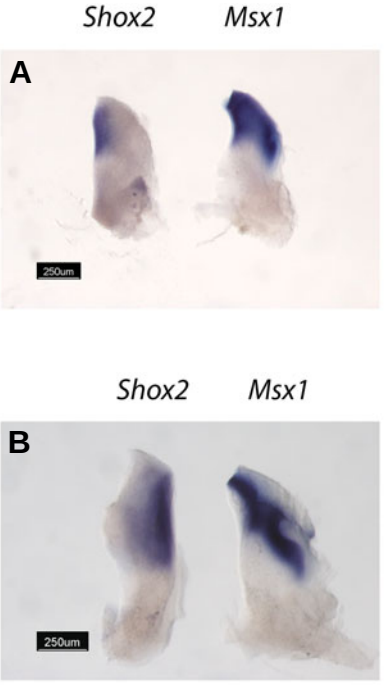

Fig. 2 (Right). Comparison of *Shox-2* and *Msx1* expression domains in palate shelves. (A) *Whole-mount views of dissected E12.5 palate shelves showing that the* Msx1 *expression domain is larger than the* Shox-2 *expression domain at this stage.* ***(B)*** *Whole-mount views of dissected E13.5 palate shelves showing that the expanded* Shox-2 *domain covers more area than the* Msx1 *expression domain. Scale bars represent 250 μm.*

indicated that *Shox-2* functions upstream of *Runx2* during chondrogenesis (Cobb *et al*., 2006). In addition to the limb, *Shox-2* is also highly expressed in craniofacial region including the secondary palate (Hilliard *et al*., 2005; Yu *et al*., 2005). Interestingly, the expression of *Shox-2* in the palate is restricted only to the anterior region (Hilliard *et al*., 2005; Yu *et al*., 2005). Loss of *Shox2* function alters FGF signaling and leads to cleft only in the anterior palate (Yu *et al*., 2005).

Meox-1 and *Meox-2* define a subfamily of murine homeobox genes with mesoderm and mesenchyme-specific expression during development (Candia *et al*., 1992). Gene targeting experiments have demonstrated that *Meox-2* is required for mouse limb muscle development and that *Meox-1* and *Meox-2* play an overlapping and essential role during early skeletal morphogenesis (Mankoo *et al*., 1999; Mankoo *et al*., 2003). We have previously reported that *Meox-2* expression in mouse secondary palate is restricted to the posterior end in the MF1 and C57 BL/6 strains (Jin and Ding, 2006).

The specific expression of *Shox2* and *Msx1* in the anterior palate and *Meox-2* in the posterior palate prompted us to use these markers to examine the A-P regional specification during mouse palate development.

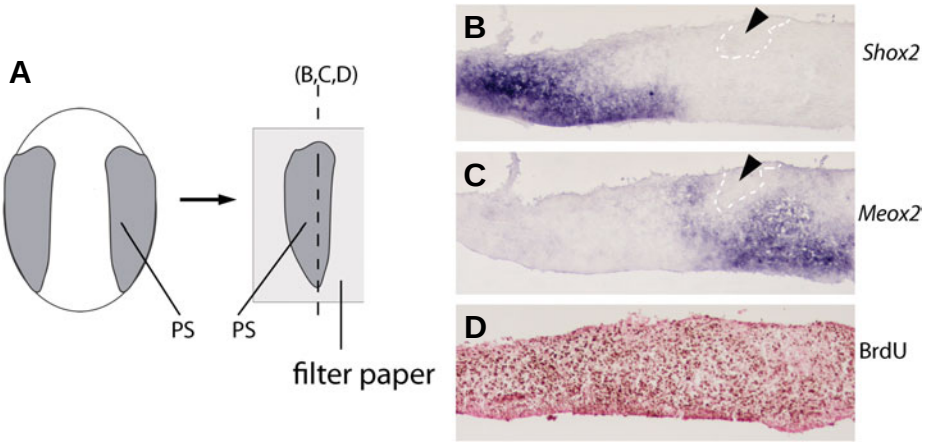

Fig. 3. Cell proliferation assay showing similar intensities of BrdU incorporation in *Shox-2* and *Meox*-2 expression areas. (A) *A diagram showing the preparation of sagittal sections of palate shelves (PS).* **(B-D)** *Adjacent sagittal sections of palate shelves on E13.25 and processed for* in situ *hybridization with* **(B)** Shox-2 *probe,* **(C)** Meox-2 *probe and* **(D)** *BrdU incorporation assay. The white dotted lines indicate the edge of extra non-palatal tissue (arrowhead).*

Results and Discussion

Regional specific expression of Shox-2*,* Meox-2 *and* Msx1 *in mouse secondary palate development*

Previous studies reported the expression of *Meox-2* (Jin and Ding, 2006) and *Shox2* (Yu *et al*., 2005) during mouse palate development. In the current study, we analyzed the expression of these two genes by whole mount *in situ* hybridization in C57BL/6 mouse embryonic heads from E12.5 to E14.5, focusing on the sizes of the expression domains at different stages. As shown in Fig. 1, both *Shox2* and *Meox-2* display dynamic expression patterns during palate development. At E12.5, the expression of *Shox2* is localized within a small anterior region, an area less than 25% of the entire palate (Fig.1A). However, the *Shox2* expression domain undergoes a dramatic expansion towards the posterior direction at E13.5 and occupies the anterior one-half of the palate (Fig. 1B). This directional expansion of the *Shox2* expression domain continues into E14.5 and results in the expression of *Shox2* in more than 60% of the palate (Fig.1C). The expression is predominately in mesenchymal cells (see Fig. 3B). The expression is absent in the MEE region at E14.5 (Fig. 1C). In addition to the secondary palate, *Shox2* expression is also found in the primary palate (Fig.1B and C). In marked contrast to *Shox2* expression, the expression of *Meox-2* at E12.5 covers a large portion (over 70%) of the palate shelf with only a small area in the anterior region that is negative for *Meox-2* expression (Fig.1D). At E13.5, however, the expression of *Meox-2* significantly regresses to the posterior end and occupies only 40% of the palate in this region (Fig. 1E). The regression of the *Meox-2* expression domain continues into E14.5 and the size of the *Meox-2* expression domain shrinks to about 25% of the palate, a region corresponding to the future soft palate (Fig. 1F). The expression is strictly mesenchyme-specific (Jin and Ding, 2006).

Msx1 is another gene reported to display anterior specific expression during palate development (Zhang *et al*., 2002; Hilliard *et al*., 2005). In contrast to *Shox2* expression, our whole mount *in situ* hybridization data shows that *Msx1* expression undergoes modest changes from E12.5 to E14.5 with respect to the A-P axis of the palate (Fig. 1G-I). At E12.5, the expression of *Msx1* covers roughly 25% of the palate and is restricted to the anterior region (Fig.1G). The expression domain increases to about 30% of the length of the palate on E13.5 and E14.5 (Fig. 1H and I). The actual size of the *Msx1* expression domain does increase, but the increase roughly fits the growth of palate. It is worth noting that previous studies have shown that *Meox-2* and *Msx1* are expressed only in the mesenchymal cells (Zhang *et al*., 2002; Hilliard *et al*., 2005; Jin and Ding, 2006), whereas the expression of *Shox2* is initially (up to E12.5) only in mesenchymal cells and then extends to epithelial cells from E13.5 (Hilliard *et al*., 2005; Yu *et al*., 2005).

On E12.5, the area of the *Msx1* expression domain is at least equal to the area of the *Shox2* expression domain, if not larger (Fig.2 A). However, as a result of the *Shox2* expansion, the *Shox2* expression domain is larger than the *Msx1* expression domain on E13.5 (Fig. 2B).

The shifts of Shox2 *and* Meox-2 *expression domains are not due to differential cell proliferation rates between the anterior and posterior palate*

There are at least two possible interpretations for the dynamic expression patterns of *Shox2* and *Meox-2* during palate development: 1) the anterior palate has a significant growth advantage over the posterior palate which leads to the expansion of anterior palate and the relative shrinkage of posterior

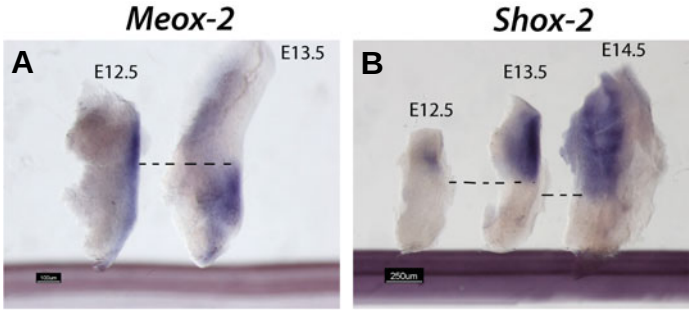

Fig. 4. Repression of *Meox-2* expression and induction of *Shox-2* expression during palate development. (A,B) *Palate shelves are arranged with the anterior side facing up.* **(A)** *The actual size of the* Meox-2 *expression domain at E13.5 is shorter than that at E12.5.* **(B)** *The actual size of the non-*shox-2 *expressing region is reduced during palate development. Scale bars represent (A) 100 μm and (B) 250 μm.*

palate; 2) The anterior and posterior palates have a similar growth rate, but a portion of *Meox-2* expressing cells are switched to be *Shox2* expressing cells during palate development.

To investigate the basis of *Shox2* expansion and *Meox-2* regression, we carried out a bromodeoxyuridine (BrdU) incorporation assay to examine the cell proliferation status along the A-P axis of the mouse palate in sagittal sections. Since the palate shelf prior to E14.5 is growing vertically, we dissected E13.25 mouse palates and placed them on filter paper in order to obtain sagittal sections with the correct orientation (Fig. 3A). Adjacent sections were cut and processed for the BrdU assay and for *in situ* hybridizations with *Shox2* and *Meox-2* antisense RNA probes. As shown in figure 3, the *Shox2* and *Meox-2* expressing regions gave similar intensities of BrdU signals (Fig. 3B-D), suggesting the cell proliferation rates in *Shox2* and *Meox-2* expressing regions are similar. To further determine the relative cell proliferation rates in the two regions, we went to determine the density of BrdU positive cells in the two regions by normalizing the BrdU positive cell number to size. It appeared that the densities of BrdU positive cells in the two regions are almost identical. Nevertheless, the

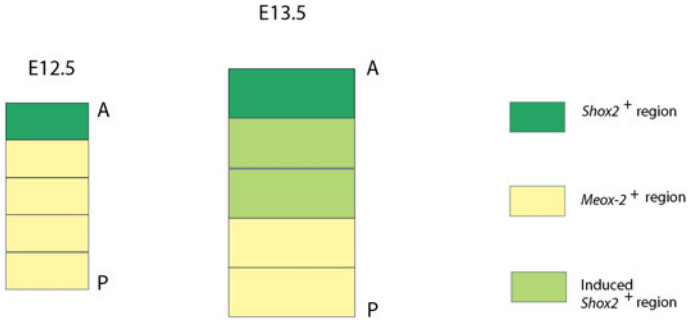

Fig. 5. A schematic representation of the expansion of anterior palate. *On E12.5, most of the palatal mesenchymal cells are considered to be posterior cells as defined by the expression of* Meox-2, *a posterior marker and the absence of* Shox-2, *an anterior marker. From E12.5 to E13.5, the expansion of the anterior palate occurs, during which a portion of original* Meox-2 *expressing posterior cells convert to the anterior cells by expressing* Shox-2. *Abbreviations: A, anterior; P, posterior.*

cells in cells the *Meox-2* expressing area are highly proliferating. Therefore, the actual size of *Meox-2* expression domain should, in principle, increase unless a percentage of *Meox-2* expressing cells lose their expression and vice versa, the actual size of non-*Shox2* expression domain should also increase unless some of them are differentiated into *Shox2* expressing cells.

We next directly compared the *Shox2* and *Meox-2* expression domains in palates among different stages. As shown in figure 4, the actual size of the *Meox-2* expression domain at E13.5 is shorter compared to E12.5 palates (Fig.4A), although the palatal cells are highly proliferative at this stage as shown by the BrdU assay and the E13.5 palate is longer than the E12.5 palate. This result indicates that a certain portion of *Meox-2* expressing cells undergo gradual repression of *Meox2* expression during palate development. Similarly, the actual size of the non-*Shox2* expressing region at E14.5 is shorter than the *Shox2* negative regions on E13.5 and E12.5 (Fig. 4B), indicating that some additional cells acquire *Shox2* expression during palate development.

It has long been recognized by morphology and histology that a well developed mammalian secondary palate consists of an anterior bony palate and posterior muscular soft palate (Ferguson 1988; Cui *et al.*, 2005; Hilliard *et al.*, 2005). Our current study revealed that the anterior and posterior palate regions change dynamically during development at least by molecular measurement. From E12.5 and E13.5, for example, a portion of *Meox-2*$^+$ / *Shox2*$^-$ cells are induced or converted to be *Shox2*$^+$ / *Meox-2*$^-$ cells (Fig. 5). We propose that at early stages (prior to E12.5), the majority of palatal mesenchymal cells are more like posterior cells at the molecular level since they are positive for *Meox-2*, a posterior marker and negative for the anterior markers, *Shox2* and *Msx1*. Here, we refer to those early *Meox-2*$^+$/*Shox2*$^-$ cells as "primitive posterior cells". During the expansion of the anterior palate, a portion of original *Meox-2* expressing "primitive posterior cells" differentiate to become definitive anterior palatal cells by switching from *Meox-2* expression to *Shox2* expression. Thus, the formation of the anterior palate may involve two steps: 1) establishment of A-P polarity; and 2) extend further anterior differentiation posteriorly.

Previous studies have shown that both *Meox-2* and *Shox2* expression domains cover the whole palate region from proximal to distal from E12.5 (Yu *et al.*, 2005; Jin and Ding, 2006). The expansion of *Shox2* and *Meox2* expression domains in the proximal-distal direction is likely due to cell proliferation.

Since little is known about the down stream target genes for *Shox2* and *Meox2* at this moment, further studies are required to uncover the molecular mechanism underlying the switch from *Meox-2* expressing to *Shox2* expressing. It is possible, however, that the anterior genes such as *Shox2* encode transcription repressors that suppress the posterior gene expression.

Although our explanation for the regression of *Meox2* expression is repression of *Meox-2* expression in certain *Meox-2* expressing cells, it is also possible that the regression is caused by posterior migrations or movements of *Meox-2* expressing cells. However, the cell migration and movement of palate mesenchymal cells have not yet been demonstrated experimentally. Since no apoptosis has been observed in palate

mesenchymal cells from E12.5 to E14.5, the regression of the *Meox-2* expression domain is not likely due to apoptosis.

Experimental Procedures

Mice

As reported in our previous study, the regional specific expression of *Meox-2* was observed in C57BL/6 and MF1 mice. The *Meox-2* expression does not show regional specificity in certain mouse strains such as Swiss Webster. Therefore, all the mice used in this study are C57BL/6 from Taconic.

Examination of gene expression and the size of expression domains

C57BL/6 mouse embryos were dissected in cold PBS on E12.5, 13.5 and 14.5 (the day when vaginal plugs were observed was designated as day E0.5.). The lower jaw and a piece of brain tissue were cut off and the remaining tissue was processed for non-radioactive whole mount *in situ* hybridization using digoxygenin-labeled antisense riboprobes as previously described (Jin and Ding, 2001). The results were examined under a Nikon SMZ1500 dissecting microscope and photographed using a Nikon DXM1200F digital camera. To measure the size of expression domain, we imported the image into Adobe Photoshop and used its digital ruler to determine the relative size of the expression domains and its percentage of the entire palate. For section *in situ* hybridization, the palatal shelves were dissected and sagittal sections were prepared as described below. 12μm cryosections were cut and air-dried overnight before the *in situ* hybridization was carried out (Jin and Ding, 2006). The results of section *in situ* hybridization were scored under a Nikon E600 microscope.

Preparation of palatal sagittal sections

To obtain palatal sagittal sections with correct orientation prior to E14.5, palatal shelves at E13.25 were dissected out and briefly placed on filter paper before fixation (Fig. 3A). The fixed palate shelves were dehydrated in 30% sucrose in PBS overnight before O.C.T embedding. The filter papers were removed during the embedding.

Cell proliferation assay

Cell proliferation was determined by a bromodeoxyuridine (BrdU) incorporation assay. Briefly, pregnant female mice were injected with BrdU (Sigma) at 200mg/kg and euthanized 2 hours later and embryos were collected. The palatal shelves were dissected and prepared for sagittal cryo-section as described above. Incorporation of BrdU was determined by immunocytochemistry using an anti-BrdU antibody from BD Bioscience (Cat # 347580). To determine the density of BrdU positive cells, the numbers of BrdU positive cells in Shox-2 and Meox-2 expressing areas were counted separately and the size of each region was measured in Adobe Photoshop.

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