Regional divergence of palate medial edge epithelium along the anterior to posterior axis

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ABSTRACT Recent studies have shown that mouse palatal mesenchymal cells undergo regional specification along the anterior-posterior (A-P) axis defined by anterior Shox2 and Msx1 expression and posterior Meox2 expression. A-P regional specification of the medial edge epithelium, which is directly responsible for palate fusion, has long been proposed, but it has not yet been demonstrated due to the lack of regional specific markers. In this study, we have demonstrated that the palate medial edge epithelium is regionalized along the A-P axis, similar to that for the underlying mesenchyme. Mmp13, a medial edge epithelium specific marker, was uniformly expressed from anterior to posterior in wild-type mouse palatal shelves. Previous studies demonstrated that medial edge epithelium expression of Mmp13 was regulated by TGF-β3. We have found that the changes in Mmp13 expression in TGF-β3 knockouts varied along the A-P axis, and can be broken down into three distinct regions. These regions correlated with regional specification of the underlying medial edge mesenchymal cells and timing of palate fusion. Mouse palate medial edge epithelium along the A-P axis can be divided into different regions according to the differential response to the loss of TGF-β3.

KEY WORDS: Mmp13, TGF-β3, mouse secondary palate, regional specification

Mammalian palatogenesis is a complex developmental process in which the bilateral palate shelves fuse along the facial midline to form the continuous palate that separates the oral and nasal cavities (Bush and Jiang, 2012, Ferguson, 1988, Murray and Schutte, 2004, Nawshad et al., 2004). Each nascent palatal shelf is made up of a core of neural crest-derived mesenchymal cells enclosed by multiple layers of ectoderm-derived epithelial sheets (Bush and Jiang, 2012, Chai and Maxson, 2006, Ferguson, 1988). In mice, between embryonic day 12.5 and 13.5 (E12.5-E13.5) the two developing palatal shelves first grow in a vertical direction lateral to the tongue. On E14.5, however, the vertical palatal shelves re-orient to form horizontal shelves above the dorsal side of the tongue. The two horizontal palatal shelves continue to grow until they meet each other at their medial edge epithelium (MEE) areas (Bush and Jiang, 2012, Chai and Maxson, 2006, Ferguson, 1988). MEE contact induces a series of cellular events that culminates in the elimination of the epithelial seam formed from the union of the palatal shelves. This medial edge seam (MES) disappears by E15.5 leading to the mesenchymal confluence of the definitive palate (Carette and Ferguson, 1992, Griffith and Hay, 1992, Shuler et al., 1992). MEE cells are critical players in the process of fusion and their differentiation is determined in part by signals from the underlying medial edge mesenchymal cells as demonstrated by tissue recombination experiments (Ferguson et al., 1984). In parallel with vertical and horizontal growth, the palatal shelves also extend along the A-P axis as the head develops (Li and Ding, 2007, Welsh and O’Brien, 2009). Moreover, the palatal shelf exhibits A-P regional differentiation as well as A-P growth. The morphological, cellular and molecular differences between anterior and posterior palatal mesenchymal cells are quite evident and therefore are better understood than those of the MEE. Anterior palate mesenchymal cells will form the bony palate through ossification and most of the posterior mesenchymal cells will become smooth muscle cells and form the soft palate (Cui et al., 2005, Ferguson, 1988). Recently, A-P regional specification of palatal mesenchymal cells has been studied at the molecular level (Hilliard et al., 2005). Mouse homeobox gene Msx1 is expressed only in anterior palatal mesenchymal cells (Hilliard et al., 2005, Zhang et al., 2002), and is required for palate growth because loss of Msx1 function in mice

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leads to cleft palate due to a growth defect (Satokata and Maas, 1994, Zhang et al., 2002). The cleft palate defect in Msex1 mutant embryos can be rescued by trans-expression of the Bmp4 gene, indicating BMP4 functions downstream of MSX1 in palate growth control (Zhang et al., 2002). Moreover, the proliferation of anterior, but not the posterior, palatal mesenchymal cells are responsive to BMP treatment (Zhang et al., 2002). Similar to Msex1, Shox2 is another homeobox gene expressed only in the anterior palate, and loss of its expression in mice leads to an anterior cleft palate (Yu et al., 2005).

Unlike Msex1 and Shox2, the expression of Meox2, another homeobox gene, displays expression specifically in the posterior palate and 23% of Meox2 mutant embryos have posterior cleft palate due to a post-fusion defect (Jin and Ding, 2006, Li and Ding, 2007).

Results and Discussion

Previous studies have shown that Tgf-β3 is highly expressed in palate epithelium and is required for MEE differentiation and fusion (Fitzpatrick et al., 1990, Kaartinen et al., 1995, Pelton et al., 1990, Proetzel et al., 1995). Mmp13 is expressed during mouse palate development, exclusively in MEE cells on E14.5 and E15.5 (Blavier et al., 2001, Jin et al., 2008, Jin et al., 2010). This expression is regulated by TGF-β3 because Mmp13 expression in Tgf-β3 mutant MEE cells is significantly decreased (Blavier et al., 2001). To investigate the A-P regional specification of the MEE during mouse palate development, we first examined the expression of Tgf-β3 and Mmp13 in mouse palate shelves by whole-mount in situ hybridization and found that both mRNAs were uniformly expressed in the MEE from anterior to posterior on E14.5 and E15.5. As shown in figure 1, on E14.5, the Tgf-β3 expression domain covers the entire A-P axis of the palatal shelf (Fig. 1A) from the anterior (arrowheads in Fig. 1A) to the posterior aspect (arrows in Fig. 1A). Correspondingly, the expression domain of Mmp13 also covers the
entire A-P axis from anterior end (arrowhead in Fig. 1B) to the soft palate (arrows in Fig. 1B). On E15.0, the expression of Mmp13 further extends into the anterior margin (arrowheads in Fig. 1C), and later in the primary palate on E15.5 (arrowheads in Fig. 1D). Section in situ hybridization also confirmed that the expression of Mmp13 in the MEE of wild type embryo was equally strong from the anterior to posterior aspect (Fig. 2). In Zeb1 mutant embryos, the two palatal shelves do not contact because of a delay in re-orientation (Jin et al., 2008, Jin et al., 2010). However, Mmp13 was still highly expressed in the MEE from anterior end to posterior soft palate indicating that its expression was independent of palatal shelf contact (Fig. 1E) (Jin et al., 2008, Jin et al., 2010). As mentioned above previous studies demonstrated that Mmp13 expression is decreased in Tgf-β3 mutant palatal shelves (Blavier et al., 2001). We therefore determined the expression of Mmp13 in Tgf-β3 mutant embryos by whole-mount in situ hybridization. We observed that the change of Mmp13 expression in response to the loss of TGF-β3 was dependent upon the specific location along the A-P axis (Fig. 1F). We have divided the MEE into three regions, based on Mmp13 expression in E14.5 Tgf-β3 mutants (Fig. 1F): region I was negative for Mmp13 expression; region II shows weak expression of Mmp13; region III, corresponding to the soft palate, was negative for Mmp13 expression. Section in situ hybridization also confirmed that Mmp13 expression is present only in the middle region (Fig. 3C), but absent in the anterior and posterior regions in Tgf-β3 mutant palate shelves (Fig. 3 B and D, respectively).

To determine these regions more precisely, we determined Mmp13 expression in Tgf-β3 mutant palatal shelves in relation to the position of the rugae, as determined by Shh expression (Fig. 4). E14.5 Tgf-β3 mutant embryonic heads were bisected and one palatal shelf subjected to whole-mount in situ hybridization for Mmp13 expression and the other for Shh expression. The rugae were numbered based on their positions along the A-P axis according to the published report, in which the most anterior one is designated as ruga 1 (Economou et al., 2012). By comparing Mmp13 expression with Shh expression, we determined that region I, the anterior Mmp13 negative area, was located between rugae 1 and 2, region II, the middle Mmp13 positive area, was located between rugae 2 and 8, and region III, the posterior Mmp13 negative area, corresponded to the entire soft palate (Fig. 5A and Fig. 6). On E15.5, region I of Tgf-β3 mutant palatal shelves expressed low level Mmp13 similar to region II, whereas region III was devoid of Mmp13 expression (Fig. 5B).

Region III has the highest sensitivity to the loss of TGF-β3, since it is negative for Mmp13 expression in Tgf-β3 mutants on both E14.5 and E15.5 (Fig. 5 A and B). Region II, however, is the least sensitive to the loss of TGF-β3, because it retains weak Mmp13 expression even in the absence of TGF-β3 (Fig. 5 A,B).

Therefore, the MEE cells are indeed divergent along the A-P axis. Since tissue recombination experiments revealed that the MEE cells received signals from the underlying mesenchymal cells, we hypothesized that these mesenchymal cells were also divergent.

**Fig. 3.** Section in situ hybridization showing Mmp13 is expressed in the palate medial edge epithelium (MEE, arrows) only in the middle region (C), but not in the anterior (B) and posterior (D) regions in E14.5 Tgf-β3 mutant embryos. The dashed lines in (A) indicate the positions of panels (B,C,D). Scale bars, 100 μm.

**Fig. 4 (Left).** Diagram showing the method to determine the Mmp13 expression domain in Tgf-β3 mutant palatal shelves in relation to rugae as marked by Shh expression.

**Fig. 5 (Right).** Regional specification of medial edge epithelium in relation to the rugae and subjacent mesenchymal cells. The anterior end of the Mmp13 expression domain in the Tgf-β3 mutant palatal shelf at E14.5 (A) resides approximately at ruga 2, see bi-arrowheads in (A) and the posterior end corresponded to ruga 8, see arrow in (A). At E15.5 (B), the anterior end of the Mmp13 expression domain extended to ruga 1, see bi-arrowheads in (B) and the posterior end stayed at the position of ruga 8. Rugae 1-8 is coincident with the anterior end, see arrowhead in (C) and posterior end of the Gsc expression domain (C). Scale bars represent 500 μm.
We determined the expression of Goosecoid (Gsc), a marker for the cells underlying the medial edge (Jin et al., 2010, Proetzel et al., 1995) and found that it was present roughly in regions I and II, but not in region III based on the characteristic morphology of posterior soft palate (Fig. 5C), suggesting that the medial edge mesenchymal cells adjacent to the MEE along the A-P axis were indeed different than cells located deeper in the mesenchyme and that this could determine MEE regional specification along A-P axis.

It is interesting that MEE regional specification along the A-P axis correlates with the timing of palatal shelf fusion. Initial fusion of the palatal shelves begins within region II. Fusion then proceeds from this point both anteriorly and posteriorly (regions I and III, respectively). In addition, Tgf-β3 mutant mice in certain background such as CF-1 strain display posterior cleft palate (Proetzel et al., 1995), the area corresponding to the region III, which is the most sensitive region to TGF-β3 loss.

In summary, these data demonstrate that not only is there gene-expression along the anterior-posterior axis identified by Goosecoid, but also for the medial edge epithelial cells suggesting a dynamic interaction between these two cell types that will likely play a role in fusion of the palatal shelves, perturbations of which will lead to clefts of the secondary palate.

Materials and Methods

Mice

We obtained the Tgf-β3 mutant line on C57BL/6J background from the Jackson Laboratory, Maine, USA (stock number 002619), that was originally generated by Dr. Thomas Doetschman (Proetzel et al., 1995). Tgf-β3-/- mice have a complete anterior to posterior cleft palate (Proetzel et al., 1995). The Zeb1 mutant line has been previously reported by Dr. Yujiro Higashi (Takagi et al., 1998). Further analysis of the palate defects in this mutant were also described by Jin et al., (Jin et al., 2008, Jin et al., 2010).

Examination of gene expression

Whole mount in situ hybridization was carried out according to Shen (Shen, 2001). Briefly, C57BL/6 wild type mouse embryos and Tgf-β3 and Zeb1 mutant embryos were dissected in cold PBS at the desired embryonic stages (the day when vaginal plugs were observed was designated as E0.5). Heads were separated and the lower jaws removed and the remaining tissue was fixed in 4% paraformaldehyde in PBS overnight at 4°C followed by dehydration through 25%, 50% and 75% methanol in PBS containing 0.1% Tween 20. The samples were stored in 100% methanol at -20°C until used for in situ hybridization. The treated embryonic tissues were processed for non-radioactive whole mount in situ hybridization using digoxigenin-labeled antisense riboprobes as described by Shen (Shen, 2001). The hybridized embryos underwent post-hybridization fixation and were maintained in 80% glycerol for photography.

For the experiment described in figures 4 and 5, more than 5 embryos at each stage were tested, and each embryo was marked by unique cuttings after bissection in order to be paired correspondingly after in situ hybridization.

Digoxigenin based section in situ hybridizations were carried out on cryo-sections according to Shen (Shen, 2001), followed by count staining with nuclear fast red (Vector Laboratory, Cat# H-3403).

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References


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