Wnt5a regulates directional cell migration and cell proliferation via Ror2-mediated noncanonical pathway in mammalian palate development

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Tissue and molecular heterogeneities are present in the developing secondary palate along the anteroposterior (AP) axis in mice. Here, we show that Wnt5a and its receptor Ror2 are expressed in a graded manner along the AP axis of the palate. Wnt5a deficiency leads to a complete cleft of the secondary palate, which exhibits distinct phenotypic alterations at histological, cellular and molecular levels in the anterior and posterior regions of the palate. We demonstrate that there is directional cell migration within the developing palate. In the absence of Wnt5a, this directional cell migration does not occur. Genetic studies and in vitro organ culture assays further demonstrate a role for Ror2 in mediating Wnt5a signaling in the regulation of cell proliferation and migration during palate development. Our results reveal distinct regulatory roles for Wnt5a in gene expression and cell proliferation along the AP axis of the developing palate, and an essential role for Wnt5a in the regulation of directional cell migration.

KEY WORDS: Wnt5a, Ror2, Palatogenesis, Cleft palate, Non-canonical Wnt pathway, Cell migration

INTRODUCTION

Cleft palate is a congenital disorder arising from a failure in the highly regulated multiple-step process of the secondary palate development. In mice, the palatal shelves arise from the outgrowing maxillary prominences at embryonic day 11.5 (E11.5), and then grow vertically down on the side of the tongue (E12.5-E13.5), followed by rapid elevation to a horizontal position around E14.0. Subsequently, two palatal shelves meet at midline and fuse to establish the mesenchymal continuity of the definitive palate. Recent studies have demonstrated that the developing palate is not a homogeneous organ (Hilliard et al., 2005; Okano et al., 2006; Gritli-Linde, 2007). Many genes involved in palate development exhibit regional differential expression along the anteroposterior (AP) axis. The anterior and posterior palatal mesenchyme also shows different molecular and cellular responses to growth factors. This tissue and molecular heterogeneity could lead to differential cell behaviors, such as cell proliferation, production of different extracellular matrix and cell migration, in the palate along the AP axis. However, despite rapid progress in the field, the exact cellular and molecular mechanisms that are involved in morphological changes during palate development remain unknown.

The Wnt family genes encode a large and diverse group of signaling molecules in the patterning, proliferation and differentiation of a variety of organs and cell types (Cadigan and Nusse, 1997). Wnt ligand binds to its receptor Frizzled (Fz) and Lrp5/6 to activate the canonical Wnt/β-catenin signaling pathway, or functions through β-catenin-independent pathways, the noncanonical pathways, including the planar cell polarity (PCP) and Wnt/Ca2+ pathways (Veeman et al., 2003). Wnt ligands are typically classified into canonical and noncanonical Wnts by the pathways they work through. Wnt5a is a representative of the noncanonical Wnt class. One function of the noncanonical pathway is its inhibition of the canonical Wnt signaling. In Xenopus, overexpression of Wnt5a blocks secondary axis induction by Wnt8 (Torres et al., 1996). In the developing limbs, Wnt5a blocks canonical Wnt pathway by promoting degradation of β-catenin in a GSK3-independent way (Topol et al., 2003). Noncanonical Wnt signaling also regulates, via the PCP pathway, convergent extension movement, an essential cell migration process during vertebrate gastrulation (Mlodzik, 2002). Altered Wnt5a function leads to dysregulated convergent extension movement in vertebrates (Moon et al., 1993; Kilian et al., 2003). It was recently reported that an alternative Wnt receptor, Ror2, an orphan tyrosine kinase, mediates Wnt5a-initiated noncanonical signaling and is required for the Wnt5a-mediated inhibition of the canonical signaling (Oishi et al., 2003; Mikels and Nusse, 2006). Interestingly, Wnt5a is also capable of activating Wnt/β-catenin signaling in the presence of Fz4 and Lrp5 (Mikels and Nusse, 2006). In addition, the orphan receptor Ryk could also bind to Wnt5a besides to other Wnt ligands (Lu et al., 2004; Keeble et al., 2006).

Wnt signaling was initially implicated in palatogenesis based on the findings that WNT3 mutations in humans and Wnt9b targeted inactivation in mice cause a cleft palate phenotype with an incomplete penetrance (Niemann et al., 2004; Carroll et al., 2005). Mapping of the chromosomal translocation in individuals with non-syndromic cleft lip and palate identified a region containing WNT5A (Blanton et al., 2004). Although β-catenin expression was reported in the developing palate (Martinez-Alvarez et al., 2000; Tudela et al., 2002; Nawshad and Hay, 2003), specific ablation of β-catenin in the epithelial-derived tissues by a K14-Cre transgenic allele appeared not to affect palate formation (Huelsken et al., 2001).
RESULTS
Expression of \( Wnt5a \) and its receptors in the developing palate.

The developing secondary palate displays tissue heterogeneity as well as differential gene expression along the AP axis (Hilliard et al., 2005). In an effort to identify genes that exhibit such an expression pattern in the palate, we compared the gene expression profiles from the anterior (anterior to the first molar) versus posterior (posterior to the first molar) region of the E13.5 palatal shelves by Affimetrix chip microarray assays. Several genes that encode components of Wnt signaling pathway, including \( Wnt5a \), frizzled 4 (\( Fzd4 \)), \( Ror2 \) and \( \beta\)-catenin (\( Catnb \)), showed differential expression levels between the anterior and posterior palate, which was subsequently confirmed by in situ hybridization assays. \( Wnt5a \) expression was detected in the palatal mesenchyme, in a graded fashion along the AP axis (higher levels in the anterior region) (Fig. 1A,E). A gradient expression for \( Wnt5a \) along the nasal-oral axis was also observed (Fig. 1A,E). \( Fzd4 \) and \( Ror2 \) were also expressed, in a similar AP gradient fashion, in the epithelium and mesenchyme of the palate, with a restricted mesenchymal domain adjacent to the medial edge epithelium (MEE) (Fig. 1B,C,F,G). A graded expression of \( \beta\)-catenin, mainly in the MEE, was also detected along the AP axis of the palate (Fig. 1D,H).

\( Wnt5a \) mutant mice exhibit complete cleft secondary palate.

The expression of \( Wnt5a \) and its receptors \( Fzd4 \) and \( Ror2 \) suggests a role for these genes in palatogenesis. The fact that \( Ror2 \) knockout mice exhibit a cleft palate phenotype (Schwabe et al., 2004) and that individuals with non-syndromic cleft lip and palate have been linked to chromosomal translocation containing \( WNT5A \) locus (Blanton et al., 2004) promoted us to examine a cleft palate phenotype in \( Wnt5a \)–/– mice. Gross morphological and histological examinations revealed a complete cleft palate phenotype in \( Wnt5a \)–/– mice (Fig. 2A,B). In the mutants, the deformed palatal shelves were distinguishable as early as E12.5 but became pronounced by E13.5. At E13.5, when the palatal shelves in the wild type take the vertical position on both sides of the tongue, the anterior region of the mutant palatal shelves remained in the horizontal position (Fig. 2C,D). By contrast, the posterior portion of the mutant palatal shelves grew in a vertical growth position (Fig. 2E,F). Morphologically, the mutant anterior palatal shelf appeared round, shortened and blunt-ended, lacking the ventrolateral indentation of the shelf (Fig. 2D), whereas the posterior shelf was shortened, broadened and wedge-shaped (Fig. 2F). At E14.5, the wild-type palatal shelves have elevated above the tongue and fused at the midline (Fig. 2G,I). In \( Wnt5a \) mutant, the anterior region of the palatal shelves remained in horizontal level but was too short to make a contact (Fig. 2H). However, the posterior palatal shelves failed to elevate (Fig. 2J). The lack of contact between the palatal shelf and tongue/mandible suggests an intrinsic defect in elevation. The gradient expression of \( Wnt5a \) along the AP axis of the developing palate appears to account for the distinct morphological defects found in the anterior and posterior palate of the mutant. In addition, \( Wnt5a \) mutants also displayed a short and small tongue, and a deformed oral cavity, which may contribute, to some extent, to the cleft palate defect.

\( Wnt5a \) mutant exhibits an impaired palatal growth.

To reveal the cellular mechanisms responsible for the palate deformation in \( Wnt5a \)–/– mice, we carried out cell proliferation and apoptosis assays as well as in vitro palate fusion assay. We
determined cell proliferation rate in the wild type and $Wnt5a^{+/−}$ palates at E13.5 by BrdU labeling. As controls, BrdU-positive cells were also counted in the maxillary mesenchyme lateral to the palate where $Wnt5a$ is not expressed. An altered cell proliferation rate was found in the mutant palatal mesenchyme (Fig. 3). Intriguingly, a significantly elevated cell proliferation level was seen in the anterior palate, whereas a decreased rate occurred in the posterior palate. This differential alteration of cell proliferation rate in the $Wnt5a^{+/−}$ palate along the AP axis implicates dual roles for $Wnt5a$ in the regulation of cell proliferation. It is interesting to note that, in the anterior palate of the mutant, the mesenchymal cells on the future nasal side were much more mitotic than cells on the future oral side. This dysregulated cell proliferation level in the future nasal aspect apparently accounts for, at least partially, the malformed shape of the mutant palatal shelves and a failure in change of palate growth direction from the horizontal to vertical position. However, TUNEL assays revealed a comparable level of cell apoptosis in the wild-type and $Wnt5a^{+/−}$ palatal shelves at E13.5 (data not shown). To determine whether the formation of a cleft palate in $Wnt5a^{−/−}$ mutants could also be attributed to a failure in palate fusion, we employed an in vitro organ culture system to examine palate fusion. The results demonstrated that pairs of palatal shelves from E13.5 $Wnt5a^{−/−}$ embryos, when placed in contact with the MEE and facing one another, were able to fuse, in a way identically to the wild-type controls (data not shown). The dysregulated cell proliferation in the palatal mesenchyme therefore represents a major cellular process that causes a cleft palate formation in $Wnt5a^{−/−}$ mutants.

**Altered gene expression in the Wnt5a$^{−/−}$ palate**

We next examined the expression of a number of genes in the palatal shelves of $Wnt5a^{−/−}$ mutants. They are either potential $Wnt5a$ downstream genes or genes that exhibit an overlapped expression domain in the palate with $Wnt5a$, including $Shh$, $Ptc1$, $Bmp4$, $Bmp2$, $Mx1$, $Fgf10$, $Fgfr2$, $Shox2$ and $Sox9$. In E13.5 $Wnt5a$ mutants, $Bmp4$ was downregulated in the anterior palate (Fig. 4C,D), but displayed an ectopic expression domain in the posterior palate mesenchyme on the nasal side, whereas $Bmp4$ expression was not normally expressed (Fig. 4G,H). Consistent with the fact that mesenchymally expressed $Bmp4$ positively regulates $Shh$ in the palatal epithelium (Zhang et al., 2002), $Shh$ in the MEE of the anterior palate was downregulated (Fig. 4A,B), whereas an ectopic $Shh$ expression was observed in the nasal side epithelium of the posterior palate, immediately adjacent to the ectopically expressed $Bmp4$ domain in the palatal mesenchyme (Fig. 4F,H). $Ptc1$, a downstream target and receptor of $Shh$, displayed altered expression pattern accordingly along the AP axis (Fig. 4I,J,M,N). However, as the mutant palatal shelves are so malformed, the exact position where $Shh$ is ectopically activated in the palate remains to be determined. Nevertheless, these observations place $Wnt5a$ upstream of $Shh$ and $Bmp4$, consistent with the previous report in the developing lung (Li et al., 2002; 2005). $Mx1$, a downstream target of $Bmp4$, was partially downregulated in the anterior palatal mesenchyme of the $Wnt5a^{−/−}$ palate (Fig. 4K, Fig. 5L). Although the expression of $Bmp2$, a downstream target of $Shh$ that mediates the mitotic effect of $Shh$ in the anterior palate (Zhang et al., 2002), remained unchanged in the anterior palate of the mutant (data not shown), a significant downregulation of $Bmp2$ was observed in the posterior palatal mesenchyme (Fig. 4O,P). It appears that other upstream factors besides $Shh$ are also involved in regulating $Bmp2$ expression.
**Fig. 3. BrdU labeling of cells in the developing palate.**

(A-D) Coronal sections through the anterior (A,B) and posterior (C,D) palates of an E13.5 wild-type control (A,C) and an E13.5 Wnt5a mutant (B,D) showing BrdU-labeled cells. Boxes in each panel indicate the area where the labeled cells were counted and compared.

(E) Comparison of percentage of BrdU-labeled cells in the fixed areas of the palate in the wild-type control and mutant embryos. Standard deviation values are indicated as the error bars. *P<0.05.

By contrast, Fgf10 and its receptor Fgfr2 exhibited comparable expression level and pattern in both wild-type and mutant palate (data not shown). These results demonstrate that Wnt5a acts differentially, in term of gene expression regulation, in the anterior and posterior palate.

**Wnt5a and Ror2 exert an epistatic effect on palate development**

As cell proliferation was increased in the anterior palatal mesenchyme lacking Wnt5a and as the canonical Wnt signaling is known to control proliferation, it is possible that Wnt5a normally antagonizes the canonical pathway in the developing palate. To determine this possibility, we examined the expression of TOPGAL, a transgenic reporter of activation of the canonical Wnt signaling pathway, in the wild type and Wnt5a<sup>−/−</sup> background (DasGupta and Fuchs, 1999). We failed to detect TOPGAL expression in the developing palate of both wild type and Wnt5a mutants (data not shown), suggesting that the canonical Wnt signaling pathway may not be involved in palate development. The fact that mice lacking Fz4, the receptor that is expressed in the developing palate and mediates the canonical signaling of Wnt5a, survive to adulthood without a defective palate (Hsieh et al., 2005), implies an involvement of noncanonical pathway. The fact that both Ror2<sup>−/−</sup> and Wnt5a<sup>−/−</sup> mice exhibit a cleft palate phenotype strongly implicates an overlapping role for these two molecules. We therefore examined the cleft palate defect in Ror2<sup>−/−</sup> embryos. Similar to Wnt5a mutant, we found a downregulated Shh expression in the MEE and an enhanced cell proliferation rate in the anterior palatal mesenchyme of Ror2<sup>−/−</sup> embryos (Fig. 5). We subsequently tested a genetic interaction between Wnt5a and Ror2 by compounding Wnt5a<sup>−/−</sup> and Ror2<sup>−/−</sup> to generate double heterozygous mice. Wnt5a<sup>−/−</sup>;Ror2<sup>−/−</sup> mice indeed exhibited cleft palate defects that phenocopy that of Ror2<sup>−/−</sup> mutants, including downregulated Shh expression in the MEE and upregulated cell proliferation rate in the mesenchyme of...
the anterior palate, particularly in the nasal aspect (Fig. 5). These results demonstrate an epistatic effect of these two genes on palatogenesis. However, the posterior palatal shelves of Ror2+/– and Wnt5a+/–;Ror2+/– mice did not show an altered cell proliferation rate, consistent with an extremely low level of Ror2 expression in this region. As the palatal phenotypes in E13.5 wild-type, Wnt5a (0.5 mg/ml) were implanted, respectively, onto explanted implantation experiments. Beads soaked with BSA (1 mg/ml) or Wnt5a and palatogenesis is not the only receptor mediating the function of Wnt5a during palatogenesis.

We further confirm a role for Ror2 in mediating Wnt5a signaling in the regulation of cell proliferation by bead implantation experiments. Beads soaked with BSA (1 mg/ml) or Wnt5a (0.5 mg/ml) were implanted, respectively, onto explanted anterior palatal tissues from E13.5 wild-type, Wnt5a+/– and Ror2+/– embryos. Samples were cultured for 24 hours and pulsed with BrdU prior to fixation. Consistent with the results by in vivo BrdU labeling assays, elevated cell proliferation levels were observed in the Wnt5a+/– and Ror2+/– palatal tissues implanted with BSA beads, when compared with that in the wild-type controls (Fig. 6A,C,E). A significantly decreased rate of cell proliferation was seen in the wild-type palatal tissues implanted with Wnt5a beads (Fig. 6B, 6G), indicating that overdosed exogenous Wnt5a protein represses cell proliferation. Furthermore, a dramatically reduced level of cell proliferation was found in the Wnt5a+/– palate implanted with Wnt5a bead (Fig. 6B, 6D, 6G), demonstrating a rescue of the altered cell proliferation phenotype in the mutants. By contrast, Wnt5a beads failed to rescue cell proliferation defect in the Ror2+/– palatal tissues (Fig. 6F, G).

Wnt5a-deficiency leads to altered cell migration in the palatal mesenchyme

In the assays for gene expression, we made an interesting observation about Sox9 expression, a HMG box gene whose mutation also leads to cleft palate formation (Bi et al., 1999; Mori-Akiyama et al., 2003). Sox9 is expressed and regulated by Shox2 in the anterior palatal mesenchyme (Fig. 4Q) (Yu et al., 2005; Gu et al., 2008). Consistent with an unaltered Shox2 expression (Fig. 4S, T), Sox9 expression was maintained in the anterior palatal mesenchyme of Wnt5a mutant (Fig. 4R). However, a close examination identified a shifted expression domain of Sox9. In the controls, Sox9 expression was restricted to the distal region (the MEE side) of the anterior palate (Fig. 4Q). In the mutant, however, Sox9 expression domain was shifted to the future nasal side (Fig. 4R), which could reflect a positional change of the Sox9-expressing cells in the palate. Based on this assumption, we
reasoned that a constant cell migration exists in the developing palatal mesenchyme. Aberrant cell migration could lead to a change in position of the mesenchymal cells within the \textit{Wnt5a}–/– palate.

To test these hypotheses, we first determined if constant cell migration does exist in the developing palate. E12.5 palatal shelves were isolated and placed with the AP axis marked in organ culture. The anterior and posterior palatal mesenchymal tissues from E12.5 \textit{Actinβ-Egfp} mice were grafted into the explanted palatal shelves in their original positions, respectively. Cell migration was subsequently monitored at 24-hour intervals. As shown in Fig. 7, the \textit{Egfp}-expressing cells underwent an active cell migration within the host tissue environment. Surprisingly, the cell migration was not random but directional, indicating the existence of a chemoattractant gradient. When the \textit{Egfp}-expressing posterior palatal mesenchyme was grafted into the posterior region of the host explants, they displayed a directional migration towards the anterior side in 77% of grafts (Fig. 7B; Table 1). This posterior to anterior cell migration pattern coincides with the gradient \textit{Wnt5a} expression along the AP axis. However, in the anterior palate, the grafted anterior palatal cells exhibited a directional migration towards the lateral side in the majority of the cases (54%) (Fig. 7A; Table 1). In about 13% of cases, directional cell migration towards the medial side was recorded. Nevertheless, these results demonstrate the existence of active directional cell movement in the developing palate.

We next asked whether the directional cell migration could become altered in the palatal mesenchyme lacking Wnt5a. In a similar assay using E12.5, \textit{Wnt5a}–/– palatal shelves as hosts, we did not observe a constant directional cell migration in both the anterior and posterior palatal regions (Table 1). In most cases (more than 80%), the grafted cells either migrated slightly but randomly, or never moved (Fig. 7C,D), indicating a defective cell movement in the \textit{Wnt5a}–/– palate.

Ror2 mediates the chemotactic activity of Wnt5a in the developing palate

We tried to determine whether Wnt5a acts as a chemoattractant for mesenchymal cells of the palate. We coupled Dil-cell labeling/tissue grafting with bead implantation in explanted palatal shelves. As shown in Fig. 8, BSA-soaked beads (1 mg/ml) had no influence on migration direction of Dil-labeled cells or grafted \textit{Egfp}-expressing cells (26/28). By contrast, Dil-labeled cells/grafted \textit{Egfp}-expressing cells exhibited a directional migration towards Wnt5a-soaked beads (0.5 mg/ml) in 24 hours (Fig. 8B). This chemotactic effect of Wnt5a was seen in 69% of cases (25/36). We also found that cells responded to the chemotactic action of Wnt5a identically, regardless where the beads were implanted, implying that exogenous Wnt5a has overcome the endogenous gradient. To further test whether Wnt5a is essential and sufficient to drive AP cell movement in the palate, we applied Wnt5a beads onto the anterior side of E12.5

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<tr>
<th>Host palate</th>
<th>Directional migration (sample/total number)</th>
<th>Undirectional migration</th>
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<tr>
<td></td>
<td>Anterior</td>
<td>Posterior</td>
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<tr>
<td>Wild-type anterior</td>
<td>2/70</td>
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<tr>
<td>Wild-type posterior</td>
<td>23/30</td>
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<tr>
<td>Wnt5a–/– anterior</td>
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Cell movement is an essential process during embryogenesis and the development of many organs. In the developing limbs, cell migration is crucial for pattern formation and outgrowth. Abnormal cell migration or change of migration direction could cause dramatic changes in limb bud shape and outgrowth inhibition (Li and Muneoka, 1999). During palate development, palatal shelves undergo rapid outgrowth and significant changes in palate shape and position. Cell proliferation and extracellular matrix production and deposition within the palatal mesenchyme contribute to these processes significantly (Ferguson, 1988; Gritli-Linde, 2007). The morphological movement of the palatal shelf has been considered an active process of shelf elevation. However, an active directional cell migration by the mesenchymal cells during palate development has not been reported. In this paper, we demonstrate the existence of two streams of active directional cell migration: in the posterior palate, cells migrate towards to anterior aspect, whereas in the anterior palate, cells move towards the lateral aspect. Though its biological significance in palate formation is yet to be elucidated, the directional cell migration should have a crucial role in palate patterning (shaping) and elevation. The Wnt5a-/- deformed palate could be partially attributed to the aberrant cell migration. During palate shelf elevation, different means (rotation versus remodeling) are thought to be used in different regions of the palate along the AP axis (Okano et al., 2006). The distinct directions of cell migration in the anterior and posterior palate may represent a cellular process underlying these two different elevation mechanisms. Computer modeling studies will be required to model cell migration directionality associated with palate elevation.

Wnt5a has been implicated in cell migration in developing vertebrate embryos and in invasive cancer cells (Weeraratna et al., 2002; Jopling and den Hertog, 2005; Kim et al., 2005; Witze et al., 2008). Here, we show a defective cell migration in the Wnt5a-/- palate. Our results prove that Wnt5a is a potent and specific chemoattractant for the palatal mesenchymal cells. The gradient of Wnt5a expression along the AP axis therefore sets a chemoattractant base for the posterior-to-anterior migration of the mesenchymal cells in the palate. Such graded expression was also observed for Wnt5a in those organs and tissues that undergo outgrowth, including the limb and tailbud (Yamaguchi et al., 1999). Consistent with the graded fashion of Wnt5a expression in the distal limb mesenchyme, active proximal-distal cell migration has been reported in developing chick limb buds (Li and Muneoka, 1999). Although defective cell migration has not yet been reported in the limbs of mice lacking Wnt5a, a reduced Wnt5a dose indeed impairs cell migration in fish fins, the homolog of the tetrapod limbs (Sakahuchi et al., 2006). In addition, impaired cell migration has been reported in the developing pancreas of Wnt5a-/- embryo (Kim et al., 2005). In the developing palate, Ror2 appears to be essential for the chemotactic action of Wnt5a, as cells lacking Ror2 failed to respond to Wnt5a. It has been shown that Ror2 is required for Wnt5a-induced migration of cultured cells by mediating filopodia formation (Nishita et al., 2006). By contrast, it has recently been shown that Wnt5a can also act as a chemorepellent for neuronal cells (Keeble et al., 2007).
This chemorepulsive action of Wnt5a is mediated by Ryk. Wnt5a thus has dual roles in the regulation of cell migration, depending on the presence of specific receptor. As Ryk is also expressed in the developing palate in a punctuate pattern (Halford et al., 2000), the Ryk-expressing cells could move in a direction away from the source of Wnt5a. This potential chemorepulsive action of Wnt5a may account partially for the lateral directionality of mesenchymal cells migration in the anterior palate, which warrants future investigation.

Many growth factors and receptors are expressed in a specific temporal and spatial manner in the developing palate (Gritti-Linde, 2007). Of them, some are proven chemotactants. We demonstrate in this study that, besides Wnt5a, Fgf10 is also a potent chemoattractant for mesenchymal cells of the palate. Fgf10 is expressed in the lateral region of the anterior palate at E12.5 and E13.5 (Alappat et al., 2005), which could at least partially account for the lateral migration of mesenchymal cells. As the primary Fgf10 receptor Fgfr2b is only expressed in the palatal epithelium (Rice et al., 2004), this chemotactic action of Fgf10 is probably mediated by other Fgf receptor(s) in the mesenchyme. This idea is supported by the fact that exogenous Fgf10 inhibits cell proliferation in the anterior palatal mesenchyme (Yu et al., 2005). As multiple chemoattractants and chemorepellents are likely to be present in the developing palate, the directionality of cell migration is probably controlled by a combinatorial effect of multiple factors.

The gradient expression of Wnt5a and its receptors Fz4 and Ror2 correlates with the tissue heterogeneity along the AP axis of the developing palate. We observed in the Wnt5a−/− palate altered expression of several genes crucial for palate development. However, a closer examination of gene expression reveals distinct or even opposite responses of the anterior and posterior palatal tissues to the absence of Wnt5a. For example, Shh and Bmp4 were downregulated in the anterior palate, but were ectopically activated in the posterior region. In addition, opposite cellular responses (cell proliferation) were found in the anterior and posterior palatal mesenchyme lacking Wnt5a, with an increased level of cell proliferation in the anterior but a decreased level in the posterior palate. Shh has been shown to positively regulate cell proliferation in the palatal mesenchyme (Zhang et al., 2002). However, the altered Shh expression patterns do not correlate with the changes in cell proliferation rate in the Wnt5a−/− palate. One would expect a decreased cell proliferation rate in the anterior region of the Wnt5a−/− palate where Shh is downregulated. However, a hyperproliferation of mesenchymal cells was instead observed, suggesting an involvement of multiple signaling pathways in cell proliferation regulation in the anterior palate where Wnt5a-mediated signaling acts as a negative regulator. By contrast, the proliferation rate in the posterior palate of Wnt5a mutant is significantly decreased when compared with the controls, revealing a positive role for Wnt5a in cell proliferation regulating in the posterior palate. As the number of migrating mesenchymal cells in the palate appears small, the altered expression of these crucial morphogenetic genes and the aberrant cell proliferation appear to have major impact on cleft palate formation in Wnt5a mutants.

Wnt5a has been previously shown to exert distinct effects on cell proliferation in different developing organs. Inactivation of Wnt5a results in reduced proliferation rate of the progenitor cells in the developing limbs, but leads to an increased level of cell proliferation during distal lung morphogenesis and mammary tissues (Yamaguchi et al., 1999; Li et al., 2002; Li et al., 2005; Roarty and Serra, 2007). Wnt5a has also been shown to inhibit B cell proliferation and functions as a tumor suppressor in hematopoietic tissue (Liang et al., 2003). However, in the developing palate, Wnt5a appears to exert opposite roles in the regulation of gene expression and cell proliferation in different regions. The differential expression of Wnt5a receptors (such as Ror2 and Fz4) in the palatal shelf along the AP axis could account for the distinct functions of Wnt5a. It is possible that other unidentified Wnt5a receptors are expressed and involved in mediating Wnt5a signaling in the palate. However, several signaling pathways initiated by other growth factors, including BMP, FGF and TGFβ, have been proven to differentially operate and exert their distinct effects on cell proliferation regulation in different domains of the developing palate along the AP axis. A combinatorial effect of, and a finely tuned balance between, these signaling pathways appear essential for the precise control of normal palate growth. The absence of Wnt5a could disrupt the closely linked signaling network, leading to dysregulated cell proliferation rate.

Wnt5a has dual roles in signaling: activating either the Wnt canonical or noncanonical signaling pathways. At least two known Wnt5a receptors, Fz4, which mediates the activation of the canonical pathway by Wnt5a, and Ror2, which mediates the noncanonical signaling, are expressed in an overlapping pattern with Wnt5a in the developing palate. However, activities of the canonical Wnt signaling were not detected in the developing palate in wild-type and Wnt5a−/− mice, excluding an involvement of the canonical Wnt signaling in palate development. However, we cannot rule out the possibility that the TOPGAL transgenic allele is not sensitive enough to detect a low signaling level. Wnt5a appears to regulate palatogenesis via a noncanonical Wnt pathway that does not antagonize the canonical signaling. The fact that Fz4-null mice do not exhibit a cleft palate defect and that the inactivation of both Lef1 and Tcf4, the essential nuclear mediators of the canonical Wnt signaling, does not cause an abnormal palate formation, further supports this conclusion (Hsieh et al., 2005; Brugmann et al., 2007). Several lines of evidence support the conclusion that Ror2 mediates the function of Wnt5a in regulating palatogenesis: (1) Wnt5a and Ror2 are co-expressed in the anterior palatal mesenchyme; (2) Wnt5a and Ror2 exert an epistatic effect in palate development; (3) the similar cleft palate phenotype, including elevated cell proliferation rate and altered expression pattern of downstream genes, are seen in both Wnt5a and Ror2 mutants; (4) exogenous Wnt5a protein fails to rescue (reduce) cell proliferation rate in Ror2−/− palatal mesenchyme; (5) exogenous Wnt5a protein fails to exert chemotactic effect on the palatal cells lacking Ror2. In fact, Ror2-mediated Wnt5a signaling can activate the Wnt-JNK pathway and/or inhibit Wnt canonical signaling cascade (Oishi et al., 2003; Mikels and Nusse, 2006; Schambony and Wedlich, 2007). Although the activity of Wnt/β-catenin canonical signaling is not detected or elevated in Wnt5a−/− palate, it still remains unknown whether Wnt5a/Ror2 acts through the JNK pathway to regulate palatogenesis.

The authors thank Drs Manjong Han and Ken Muneoka for providing Dil reagent and protocols, and Dr Philippa Francis-West for her critical reading and suggestions on the manuscript. This work was supported by NIH grants R01DE12329 and R01 DE14044 to Y.P.C., and by a subcontract of P50 DE16215 to J. Murray (University of Iowa), which supported Y.P.C.

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