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The inductive role of Wnt-β-Catenin signaling in the formation of oral apparatus

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ABSTRACT

Proper patterning and growth of oral structures including teeth, tongue, and palate rely on epithelial–mesenchymal interactions involving coordinated regulation of signal transduction. Understanding molecular mechanisms underpinning oral–facial development will provide novel insights into the etiology of common congenital defects such as cleft palate. In this study, we report that ablating Wnt signaling in the oral epithelium blocks the formation of palatal rugae, which are a set of specialized ectodermal appendages serving as Shh signaling centers during development and niches for sensory cells and possibly neural crest related stem cells in adults. Lack of rugae is also associated with retarded anteroposterior extension of the hard palate and precocious mid-line fusion. These data implicate an obligatory role for canonical Wnt signaling in rugae development. Based on this complex phenotype, we propose that the sequential addition of rugae and its morphogen Shh, is intrinsically coupled to the elongation of the hard palate, and is critical for modulating the growth orientation of palatal shelves. In addition, we observe a unique cleft palate phenotype at the anterior end of the secondary palate, which is likely caused by the severely underdeveloped primary palate in these mutants. Last but not least, we also discover that both Wnt and Shh signalings are essential for tongue development. We provide genetic evidence that disruption of either signaling pathway results in severe microglossia. Altogether, we demonstrate a dynamic role for Wnt-β-Catenin signaling in the development of the oral apparatus.

Introduction

Oral cavity forms through a series of fusion events in-between first branchial arch derivatives including maxillary, mandibular and frontal nasal prominences. A mature oral cavity is enclosed dorsally by the palate and ventrally by the floor of the mouth. Being the most anterior part of the alimentary tract, it is responsible for sensing and the initial mechanical processing of food. In mammals, the oral cavity is separated from the nasal cavity by the complete closure of the secondary palate. This separation is particularly important for swallowing, mastication and speech in humans. The palatal primordia first emerge as bilateral vertical downgrowth from maxillary prominences. As development proceeds, the two palatal shelves (PS) are elevated above the tongue into a horizontal opposition. The continuous expansion toward the midline eventually brings the two PS together which then fuse, and the remaining medial edge epithelium (MEE) is removed through apoptosis and/or epithelial–mesenchymal transition (Gritli-Linde, 2007). The mediolateral growth and midline fusion of palates is of particular interest to developmental biologists and physicians because of high incidence of cleft palates in humans (Gorlin et al., 2001), whereas the anteroposterior (A-P) development of the secondary palate is much less studied and understood. The secondary palate can be divided into two parts with distinct anatomy: the anterior hard palate which forms the maxillary processes and palatine bone, and the posterior soft palate which is composed of muscle and connective tissues. This regional difference is conferred by differential gene expression during development. Regulatory genes such as *Shox2* (Yu et al., 2005b), *Meox2* (Li and Ding, 2007), *Tbx22* (Liu et al., 2008b), *Msx1* (Zhang et al., 2002), and *Fgf10* (Welsh and O'Brien, 2009) are differentially expressed anteroposteriorly. It's noteworthy that during palatal formation, a set of specialized ectodermal appendages, termed palatal rugae, develop along the A-P axis as transversal ridges on the surface of the hard palate. Early rugae development starts with the induction of epithelial thickening termed placode, and condensation of the underlying mesenchyme. The ensuing morphogenesis includes patterning and vaulting of the mesenchyme toward the oral cavity. Fully developed rugae in adult animals host a variety of sensory cells (Nunzi et al., 2004) as well as cranial neural-crest-related stem cells (Widera et al., 2009). Recently,

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two groups independently described a unique "posterior interposition"(Pantalacci et al., 2008) in rugae development. They found that coupled with palatal extension, all but one ruga sequentially form at the position just anterior to the most posterior (R1) ruga which forms first and defines the boundary between the anterior and posterior palate. This sequential addition of the rugae also posits a connection to anteroposterior palatal growth and patterning (Pantalacci et al., 2008; Welsh and O'Brien, 2009). Despite these findings, the molecular mechanism regulating rugae formation, as well as how rugae addition contribute to the overall palatal development remains to be elucidated.

The tongue is composed of cranial neural-crest-cell (CNC)-derived fibroblasts, and mesoderm-derived muscles (Hosokawa et al., 2010). The dorsal surface of the tongue is covered by the oral epithelium where taste papillae reside. The early development of the tongue is achieved through epithelial–mesenchymal interactions whereby Shh signaling has been suggested to play a key role (Liu et al., 2004). Nonetheless, the molecular mechanism regulating tongue development is not well understood.

Canonical Wnt signaling is a key player in mediating epithelial–mesenchymal interactions during organogenesis. It is well established that activation of Wnt signaling is the initial step in patterning and specification of ectodermal appendages such as the hair follicle (Andl et al., 2002; DasGupta and Fuchs, 1999; Huelsken et al., 2001), tooth (Liu et al., 2008a), mammary gland (Chu et al., 2004) and taste papilla (Liu et al., 2007). Wnt signaling is also obligatory in regulating organ outgrowth as it is required for limb (Barrow et al., 2003; Soshnikova et al., 2003) and genital tubercle (Lin et al., 2008) development. The involvement of Wnt signaling in craniofacial development is suggested by a spectrum of phenotypes observed in β-Catenin conditional knockout (cKO) (Brault et al., 2001) and Tcf/Lef knockout embryos (Brugmann et al., 2007). However, these abnormalities rather reflect a requirement for Wnt responsiveness in CNC-derived mesenchyme but not in the epithelial compartment. In this report, we provide genetic evidence that canonical Wnt signaling in oral epithelium plays a dynamic role in tongue and palate development. Removal of canonical Wnt effector β-Catenin using the *Shh*^{Cregfp} line (Harfe et al., 2004) which confers oral epithelial expression resulted in a complete abolishment of rugae formation, a unique cleft palate at the anterior end of the secondary palate, and microglossia. We show that canonical Wnt signaling is required for rugae induction and subsequent *Shh* induction, which may play a key role in coordinating anteroposterior extension and mediolateral growth of the hard palate. We also demonstrate that the induction of *Shh* expression by Wnt signaling in lingual epithelium is critical for tongue formation.

Materials and methods

Animal maintenance and Tamoxifen treatment

Shh^{CreGFP}, *Shh*^{Creesr}, *RosaR26LacZ*, BATGAL and *Shh*$^{c/c}$ stains were obtained from the Jackson Laboratory (Bar Harbor, MN). *β-Cat*$^{c/c}$ and *β-Cat*$^{ex3/ex3}$ mice are gifts from Dr. Fanxin Long at Washington University in St. Louis. Tamoxifen (Sigma-Aldrich, St. Louis, MO) was given to pregnant female mice by oral gavaging at a dose of 0.2 g/kg body weight.

Histology and immunofluorescence

The procedures for sample preparation and immunofluorescence analysis were previously described (Yin et al., 2006). For Monoclonal β-Catenin antibody (BD biosciences) staining, 1:300 dilution was used. For polyclonal Lef-1 (Cell Signaling) and Myf-5 antibody (Santa Cruz Biotechnology) staining, 1:100 dilution was used.

Scanning electron microscopy (SEM)

Samples were fixed in 3% glutaraldehyde and 4% paraformaldehyde (PFA) in PBS (Ph 7.4) for at least 2 days. SEM analysis was then carried out as previously described (Lin et al., 2008).

In situ hybridizations

Whole mount in situ analysis was performed using a standard protocol (Wilkinson, 1992). Briefly, palates were isolated, fixed in 4% PFA and dehydrated through graded methanol solutions. Following that, tissues were pretreated with proteinase K and hybridized with RNA probes at 65 °C for overnight.

For Dig-labeled in situ hybridization and radioactive ^{35}S in situ hybridization, palates and tongues were collected, fixed in 4% PFA, dehydrated through graded ethanol solution, and embedded in paraffin blocks. Following that, 10 μm-sections were generated using a microtome. Hybridization was then carried out following a standard protocol (Wawersik and Epstein, 2000).

Probes for *Fgf8*, *Shh*, *Ptch1*, *Wnt5a*, *Wnt3*, *Bmp7* and *Lef1* were described previously (Lin et al., 2008). Probes for *Shox2*, *Gli1*, *Wnt10b*, *Wnt10a*, *Wnt9b*, *Axin2*, and *Dkk1* were gifts from Dr. Fanxin Long in Washington University. Probes for *Barx1*, *Meox2*, *Tbx22*, and *β-Catenin* were generated by PCR amplification of specific cDNA fragment of corresponding gene.

β-Galactosidase staining

X-Gal staining was carried out as previously described (Lin et al., 2008).

Skeleton preparation

Embryonic mouse heads were skinned, fixed in 95% ethanol overnight, incubated in acetone overnight, and then stained in a solution containing 0.3% Alcine Blue and 0.3% Alizarin Red.

Statistics

Data were analyzed by unpaired Student's *t*-test. The number of independent experiments is specified in the Results section.

Results

Activation of canonical Wnt signaling in the palatal rugae

We first examined the expression of Wnt family genes in the mouse palate at embryonic day (E) 14.5. In situ hybridization revealed that both *β-Catenin* and *Lef1* mRNA was highly expressed in all palatal rugae epithelium (arrows in Figs. 1A, E). Consistently, indirect immunofluorescence analysis demonstrated that β-Catenin (arrows in Figs. 1B–D) and LEF1 (arrows in Figs. 1F–H) proteins were more abundant in rugae epithelium, with LEF1 showing clear nuclear localization in rugae placodes. We carefully examined two particular rugae at different developmental stages. The most posterior ruga R1 was the most developed and exhibited clear placode formation (Figs. 1D and H), whereas the ruga anterior to it was just forming and had not shown any epithelial thickening (Figs. 1C and G). Intriguingly, elevated β-Catenin and LEF1 expression can be found in both rugae. Consistently, we observed elevated expression of *Pitx2* and *Tcf1* (arrows in Figs. 1U, W), both modulators and direct downstream targets of Wnt-β-Catenin signaling, in rugae epithelium. Moreover, we also detected *Wnt10a* and *Wnt10b* transcripts in palatal epithelium, with *Wnt10a* having a stronger rugae expression (Supplemental Figs. S1G–H).