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Epithelial Wnt/β-catenin signaling regulates palatal shelf fusion through regulation of *Tgfβ3* expression

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ABSTRACT

The canonical Wnt/β-catenin signaling plays essential role in development and diseases. Previous studies have implicated the canonical Wnt/β-catenin signaling in the regulation of normal palate development, but functional Wnt/β-catenin signaling and its tissue-specific activities remain to be accurately elucidated. In this study, we show that functional Wnt/β-catenin signaling operates primarily in the palate epithelium, particularly in the medial edge epithelium (MEE) of the developing mouse palatal shelves, consistent with the expression patterns of β-catenin and several Wnt ligands and receptors. Epithelial specific inactivation of β-catenin by the *K14-Cre* transgenic allele abolishes the canonical Wnt signaling activity in the palatal epithelium and leads to an abnormal persistence of the medial edge seam (MES), ultimately causing a cleft palate formation, a phenotype resembling that in *Tgfβ3* mutant mice. Consistent with this phenotype is the down-regulation of *Tgfβ3* and suppression of apoptosis in the MEE of the β-catenin mutant palatal shelves. Application of exogenous Tgfβ3 to the mutant palatal shelves in organ culture rescues the midline seam phenotype. On the other hand, expression of stabilized β-catenin in the palatal epithelium also disrupts normal palatogenesis by activating ectopic *Tgfβ3* expression in the palatal epithelium and causing an aberrant fusion between the palate shelf and mandible in addition to severely deformed palatal shelves. Collectively, our results demonstrate an essential role for Wnt/β-catenin signaling in the epithelial component at the step of palate fusion during palate development by controlling the expression of *Tgfβ3* in the MEE.

Introduction

Palate development is a unique process during mammalian embryogenesis: two secondary palatal shelves outgrow from bilateral maxillary processes *ab initio* and then, together with the primary palate, fuse to form an intact structure. Palate fusion is a characteristic and crucial step of palatogenesis. To prepare for this step, the two secondary palatal shelves have to elevate to the horizontal position above the tongue and adhere to each other with their medial edge epithelium (MEE), which then develops into a single layered medial edge seam (MES). Progressive elimination of the MES ultimately leads to fusion of the two palatal shelves that become the definite palate. Subsequently, the definite secondary palate further fuses with the primary palate and the nasal septum, forming a complete palatal structure that separates the oral and nasal cavities (Ferguson, 1988). Abnormal persistence of MES prevents palate fusion, leading to a cleft palate formation, as exemplified in *Tgfβ3* mutants (Kaartinen et al., 1995; Proetzel et al., 1995; Taya et al., 1999).

In humans, the cleft palate is a prevalent birth defect whose etiologies are still poorly understood. The mouse shares great similarity with the human in embryogenesis and its genome can be manipulated by sophisticated tools, allowing dissection of gene function in temporal and spatial manners. Recent studies have implicated complicated genetic networks in palatogenesis and demonstrated essential roles for growth factor signaling pathways in each step of this process (Gritli-Linde, 2007; Jugessur et al., 2009). For example, Bmp, Shh and Fgf signaling pathways are crucial for palate outgrowth and patterning, Pdgf signaling has a role in palate elevation, and Tgfβ1-3 engaged signaling cascade is required for MES disintegration and palate fusion (Gritli-Linde, 2007).

The canonical Wnt/β-catenin signaling pathway plays an essential role in multiple developmental processes, including craniofacial development (Grigoryan et al., 2008; Liu and Millar, 2010). Active Wnt/β-catenin signaling has been detected in the cranial neural crest cells, nasal ectoderm, taste papilla, and developing tooth (Lan et al.,

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2006; Liu et al., 2007a, 2008; Lohi et al., 2010; Mani et al., 2010). *Wnt1-Cre* mediated deletion of *Catnb*, which encodes β-catenin protein, leads to an absence of the cranial neural crest-derived structures, and epithelial specific inactivation of *Catnb* causes defective development of the tooth, hair follicle, and taste papilla (Brault et al., 2001; Huelsken et al., 2001; Liu et al., 2007a, 2008). In addition, targeted inactivation of *Lrp6*, a key receptor for Wnt/β-catenin signaling, causes severe craniofacial defects, including cleft lip and cleft palate in mice (Song et al., 2009).

Mutations in several WNT genes have been linked to cleft lip/palate defect in humans (Chiquet et al., 2008). In mice, expression of a number of Wnt ligands has been reported in the developing palate, and cleft palate phenotype has been shown in several mouse models deficient for Wnt signaling components, including Wnt5a, Wnt9b, Gsk3β, and Rspo2 (Brown et al., 2003; Lan et al., 2006; Liu et al., 2007b; He et al., 2008; Warner et al., 2009; Yamada et al., 2009; He et al., 2010a). Wnt5a was shown to regulate cell proliferation and cell migration in the developing palate via Ror2-mediated noncanonical pathway (He et al., 2008). The evidence for a direct involvement of β-catenin in palate development came from the studies in which tissue specific deletion of *Catnb* in the palatal mesenchyme produces a cleft palate defect (Chen et al., 2009). However, since functional Wnt/β-catenin signaling has not yet been evidenced in the developing palatal shelves, the requirement of β-catenin for the palatal mesenchyme could be attributed to its cell-adhesion function. In addition, despite strong β-catenin expression in the developing palatal epithelium, particularly in the MEE (Martinez-Alvarez et al., 2000; Tudela et al., 2002; Nawshad and Hay, 2003; He et al., 2008), its role in the epithelial component for palatogenesis appears elusive. This is because a cleft palate phenotype was not reported in mice carrying Cre-mediated ablation of *Catnb* in the palatal epithelium in the previous studies (Huelsken et al., 2001; Liu et al., 2008).

To reveal a role for the canonical Wnt/β-catenin signaling in palatogenesis, we surveyed the expression of a number of Wnt signaling molecules, receptors, and antagonists, and examined activity of Wnt/β-catenin signaling in the developing palatal shelves. We found that the canonical Wnt/β-catenin signaling is primarily activated in the palatal epithelium, particularly in the MEE, consistent with the restricted expression of several canonical Wnt ligands and receptors, and β-catenin. We used *K14Cre*-mediated gene ablation to inactivate *Catnb* function in the palatal epithelium. The conditional knockout mice (*K14Cre*;*Catnb*$^{F/F}$) exhibit a cleft palate defect due to failed palate fusion, consistent with a down-regulation of *Tgfβ3* expression and suppression of apoptosis in the MEE cells. The persistent midline seam phenotype in the mutant palate could be rescued by exogenous Tgfβ3 in organ culture. Ectopic activation of Wnt/β-catenin signaling in the palatal epithelium induces ectopic *Tgfβ3* expression, resulting in an aberrant palate-mandible fusion and ultimately a cleft palate formation. Our results indicate that functional Wnt/β-catenin signaling operates primarily in the epithelium to control palate fusion by regulating *Tgfβ3* expression during palate development.

Materials and methods

Animals

TOPGAL (DasGupta and Fuchs, 1999), *BATGAL* (Maretto et al., 2003), and *Catnb*$^{F/F}$ (Brault et al., 2001) mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Genotyping of *K14Cre* and *Catnb*$^{F(ex3)}$ mice have been described previously (Andl et al., 2004; Harada et al., 1999). To inactivate *Catnb* specifically in the embryonic epithelium, *K14Cre;Catnb*$^{F/+}$ mice were crossed to *Catnb*$^{F/F}$ mice to generate *K14Cre;Catnb*$^{F/F}$ mice. The Wnt/β-catenin signaling gain-of-function model (*K14Cre*;*Catnb*$^{F(ex3)}$) was generated by intercrossing *K14Cre* mice with *Catnb*$^{F(ex3)}$ mice. Animals and procedures used in this study were approved by the Tulane University Institutional Animal Care and Use Committee.

In vitro organ culture

For in vitro palate fusion assay, paired palatal shelves were carefully dissected from embryonic day 13.5 (E13.5) *K14Cre;Catnb*$^{F/F}$ mutant and control embryos. *K14Cre;Catnb*$^{F/F}$ embryos at this stage can be easily identified by hypoplastic limb buds (data not shown), and confirmed late by a PCR-based genotyping. Paired palatal shelves were placed on a filter paper in Trowell type organ culture and were oriented and juxtaposed with the MEE facing each other closely, as described previously (Taya et al., 1999; Zhang et al., 2002). Samples were cultured in a chemical defined medium with or without recombinant Tgfβ3 (50 ng/ml) at 37° for 72 hrs (Taya et al., 1999). Medium was changed once after 48 hrs in culture. Samples were then harvested for fixation and histological analysis.

Histology, in situ hybridization, and X-gal staining

Mouse embryos were collected from timed pregnant females in ice-cold PBS and fixed in 4% paraformaldehyde (PFA)/PBS solution at 4 °C overnight. Following dehydration through gradient ethanol, samples were embedded in paraffin and coronally sectioned at 10 μm. Slides were subjected to either Hematoxylin/Eosin staining for histological analysis or to non-radioactive in situ hybridization, as described previously (St. Amand et al., 2000). For whole mount in situ hybridization, samples were dehydrated through gradient methanol after overnight fixation in 4% PFA, and were subjected to non-radioactive in situ hybridization assay as described before (Zhang et al., 1999). Whole mount and section X-gal staining of Wnt reporter embryos were carried out as described previously (Chai et al., 2000; He et al., 2010b).

Cell proliferation and TUNEL assays

To assess the cell proliferation rate, timed pregnant female mice were injected with BrdU solution (Bromodeoxyuridine (BrdU) Labeling and Detection Kit) (Roche Diagnostics Corporation, Indianapolis) at the concentration of 1.5 ml/100 g body weight. Embryos were harvested 1 hr later, Carnoy-fixed, paraffin-embedded, sectioned, and processed for immunodetection of BrdU labeling, as described previously (Xiong et al., 2009). BrdU-positive cells were counted in an arbitrary area in the palatal mesenchyme and epithelium, respectively. Nine continuous sections from three individual samples were counted, and the outcome of BrdU labeling was presented as percentage of BrdU-positive cells among total nuclei in the fixed area. To determine the significance of difference, data were subjected to Student's *t*-test. TUNEL assay were performed to detect apoptotic cells as described previously (Alappat et al., 2005). TUNEL-positive cells were counted on palatal shelf sections from three mutant and control embryos, respectively.

Results

Wnt/β-catenin signaling activity and expression of Wnt/β-catenin signaling components in the developing secondary palate

Previous studies have shown a restricted expression of *Catnb* in the epithelium of developing palatal shelves, implicating a role for Wnt/β-catenin signaling in palatogenesis (Martinez-Alvarez et al., 2000; Tudela et al., 2002; Nawshad and Hay, 2003; He et al., 2008). However, by using the *TOPGAL* transgenic reporter mice, we failed to detect activity of the canonical Wnt/β-catenin signaling in the developing palatal shelves (He et al., 2008, 2010a). This result appears to argue against for an involvement of functional Wnt/β-catenin