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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.

Keywords

Wnt/β-catenin signaling; Tgf-β3; palate fusion; cleft palate

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Research Highlights

1. The canonical Wnt signaling activity is detected in the developing palate, particularly in the MEE.
2. Epithelial inactivation of Catnb causes downregulation of *Tgfβ3* and persistent midline seam, leading to a cleft palate formation.
3. Inclusion of exogenous Tgfβ3 in organ culture rescues the palate fusion defect in the *K14Cre;Catnb*$^{F/F}$ palate.
4. Ectopic activation of the caonical Wnt signaling in the palatal epithelium results in ectopic activation of *Tgfβ3* and aberrant palate-mandible fusion.
5. Wnt/β-catenin signaling functions in the palatal epithelium to control palate fusion by regulating *Tgfβ3* expression 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 (Kaartinent 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., 2006; Liu et al., 2007a; Liu et al., 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; Liu et al., 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 (Maritinez-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 37º 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 10μm. 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 (Maritinez-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; He et al., 2010a). This result appears to argue against for an involvement of functional Wnt/β-catenin signaling in palate development. However, the concern that the *TOPGAL* transgenic allele is not sensitive enough to detect low signaling level prompted us to use a different transgenic Wnt reporter line, the *BATGAL* mice, and *Axin2* expression as an indicator of Wnt/β-catenin signaling activity. Indeed, we observed *Axin2* expression in the developing palatal shelves, primarily in the MEE region (Fig. 3C; Fig. 7G; He et al., 2010a). Consistent with this observation, we detected *LacZ* reporter expression in the MEE of the palatal shelves of *BATGAL* embryo at embryonic day 13.5 (E13.5), with a few sporadic positive cells in the anterior palatal mesenchyme and some above background positively

stained cells in the posterior palatal mesenchyme (Fig. 1A–C). At E14.5, *LacZ* reporter expression expanded to the rugae and primary palate in *BATGAL* embryo (Fig. 1D). The lack of *LacZ* reporter expression in the middle portion of the developing palate is very likely due to relatively lower level of canonical Wnt signaling that is below the sensitivity of the *BATGAL* reporter. The variation in reporter activities in the developing palatal shelves of *TOPGAL* and *BATGAL* transgenic mice is not a surprise, since similarities and differences in the patterns of reporter activities during craniofacial development in these mice have been reported previously (Brugmann et al., 2007). Nevertheless, these results demonstrate the presence of functional Wnt/β-catenin signaling in the developing palate, especially in the MEE region.

In a microarray survey of gene expression profile in E13.5 mouse palatal shelves, we identified 18 genes encoding components of Wnt signaling pathway (data not shown). We chose to examine in the developing palate the expression of some of these factors that act in the canonical Wnt/β-catenin signaling pathway, including ligands, receptors, and extracellular antagonists. At E13.5, all three canonical Wnt ligands examined show a restricted expression in the palatal epithelium, with *Wnt2* and *Wnt4* in both the anterior and posterior palate (Fig. 2A, 2A', 2C, 2C'), and *Wnt3* in the anterior palate (Fig. 2B, 2B'). Activation of Wnt/β-catenin signaling requires both Fzd receptors and LRP5/6 co-receptors. We have reported previously the expression of *Fzd4* in the developing palate (He et al., 2008), and here we show that *Fzd6*, *Lrp5* and *Lrp6* mRNAs are expressed primarily in the palatal epithelium, with *Lrp6* expression being also observed in anterior palatal mesenchyme (Fig. 2D–F'). We also confirmed by in situ hybridization the expression several Wnt signaling antagonists/regulators in the developing palate. Of these, expression of *Sfrp2* and *sFrp4* was detected dominantly in the palatal mesenchyme; both of them exhibit a gradient expression along the anterior-posterior axis, with higher level of *sFrp2* in the posterior and *sFrp3* in the anterior domain (Fig. 2G, 2G', 2H and 2H'). *Dkk1* expression was found to be restricted in the anterior palatal epithelium, consistent with its previously reported expression pattern (Lieven et al., 2010). Interestingly, almost all Wnt/β-catenin signaling components that were examined show a restricted expression in the palatal epithelium, while the extracellular antagonists sFrp2 and sFrp3but not Dkk are strongly expressed in the mesenchyme. The presence of these Wnt signaling antagonists appears to modulate and confine functional Wnt/β-catenin signaling to the MEE region, as demonstrated by *BATGAL* reporter activity and *Axin2* expression.

Epithelial inactivation of β-catenin causes cleft palate by impairing palate fusion

Despite the fact that a cleft palate phenotype was not reported in mice with K14Cre-mediated epithelial ablation of *Catnb* in previous studies (Huelsken et al., 2001; Liu et al., 2008), the presence of Wnt/β-catenin signaling activity in the MEE let us to revisit potential role of Wnt/β-catenin signaling in the epithelium during palatogenesis. We generated *K14Cre;Catnb*$^{F/F}$ mice using a *K14Cre* line that exhibits Cre activity as early as E11.5 in the craniofacial epithelium including the palatal shelves (He et al., 2010b). Our in situ hybridization assay showed that at E13.5, *Catnb* expression is completely abolished, so is the expression of the Wnt/β-catenin signaling target *Axin2*, in the mutant palatal epithelium (Fig. 3A–D). These results confirm a successful ablation of *Catnb* and Wnt/β-catenin signaling activity. A low level of *Axin2* expression remained in the palatal mesenchyme of *K14Cre;Catnb*$^{F/F}$ embryo further supports a tissue-specific inhibition of Wnt/β-catenin signaling activity. At E18.5, in general, we did not find significant alterations in craniofacial structures, but we did observe a slightly pointed head with open eyelid and hypoplastic whisker pad in *K14Cre;Catnb*$^{F/F}$ embryos (Fig. 3E–H). However, gross examination revealed a complete cleft of the secondary palate phenotype in *K14Cre;Catnb*$^{F/F}$ embryos with 82% penetrance (18/22), indicating an essential role for Wnt/β-catenin signaling in

secondary palate development (Fig. 3H). The inconsistency of the *K14* promoter activity could have contributed to this incomplete penetrance of the cleft palate defect in *K14Cre;Catnb*$^{F/F}$ mice. Since *BATGAL* expression is seen in the palatal rugae at E14.5 (Fig. 1D), suggesting a potential role for the canonical Wnt signaling in the regulation of rugal formation. In accordance with this assumption, a closer morphological examination revealed the absence of rugae in the mutant (Fig. H'). Thus possibility exists that the disrupted rugal formation may contribute to the cleft palate defect in *K14Cre;Catnb*$^{F/F}$ mice.

To characterize the cleft palate defects in *K14Cre;Catnb*$^{F/F}$ embryos, we performed histological analysis on both mutant and control embryos aging from E11.5 to E16.5. We found that the mutant palate showed structures comparable morphologically to littermate controls from E11.5 to E14.0 (Fig. 4; and data not shown). At E14.0, similar to littermate controls, the mutant palatal shelves had elevated to the horizontal position and made contact in the midline in the middle portion of the palate (Fig. 4A, 4B, 4E, 4F, 4I, and 4J). At E14.5, the MES in the control had begun to disappear (insert in Fig. E), but remained persistent in the mutant (insert in Fig. 4F). At E16.5, the control palate have completed fusion and formed an intact structure (Fig. 4C, 4E, 4K). In contrast, the mutant palatal shelves did not fuse, and stayed separately, forming a complete clefting along the anterior-posterior axis (Fig. 4D, 4H, 4L). This cleft palate phenotype appears to resemble that observed in *Tgfβ3* mutant mice (Kaartinent et al., 1995;Proetzel et al., 1995)

To investigate if deletion of *Catnb* in the palatal epithelium would alter cell proliferation rate and cause ectopic cell apoptosis in the palatal shelves, we conducted BrdU labeling and TUNEL assays, respectively, on control and mutant palate. We found that cell proliferation rate in the palatal mesenchyme remained comparable between the controls and mutants at E13.5, but the rate in the mutant palatal epithelium indeed decreased as compared to that in controls ($P < 0.05$) (Fig. 5A, 5B, 5E). Using TUNEL assay, we did not observe excessive apoptotic cells in the mutant palatal shelves at E12.5 and E13.5, as compared to controls (Fig. 5C, 5D; and data not shown). *Shh*, a known down-stream target of Wnt/β-catenin signaling in several developing organs, is expressed in the MEE of the anterior palate and rugae of the palatal shelves and acts in palatal mesenchyme to regulate cell proliferation (Zhang et al., 2002; Rice et al., 2004; Han et al., 2009; Lan and Jiang et al., 2009). Consistent with an unaltered cell proliferation rate in the palatal mesenchyme of *K14Cre;Catnb*$^{F/F}$ embryo, *Shh* expression remained unchanged in the MEE, as shown by in situ hybridization (Fig. 5F, 5G). Given the fact that the mutant palatal shelves did not exhibit an altered morphology and were able to make contact at the right time and right place (Fig. 4), it appears that the decreased cell proliferation rate in the palatal epithelium of mutants does not have significant impact on palatal growth, patterning, and elevation at the early developmental stage. However, we still cannot rule out the possibility that the decreased cell proliferation rate in the epithelium, even it is relatively mild, may contribute at certain extent to the formation of cleft palate in *K14Cre;Catnb*$^{F/F}$ mutant.

Wnt/β-catenin signaling is required for Tgfβ3 expression in the MEE

TGFβ signaling plays an essential role in palate fusion. Inactivation of *Tgfβ3* or its receptors *Alk2* and *Alk5* in the palatal epithelium disrupts palate fusion (Kaartinent et al., 1995; Proetzel et al., 1995; Dudas et al., 2004; Martinez-Alvarez et al., 2004; Murillo et al., 2009; Xu et al., 2006; Yang and Kaartinen, 2007). On the other hand, ectopic *Tgfβ3* activity leads to ectopic palatal epithelial cell death in both in vivo and in vitro conditions (Alappat et al., 2005; Murillo et al., 2009; Xu et al., 2006; He et al., 2010b). The similarity in cleft palate formation in *Tgfβ3* mutants and *K14Cre;Catnb*$^{F/F}$ mice and the overlapped expression of *Tgfβ3* with *BATGAL* activity and *Axin2* expression in the MEE of the developing palatal shelves (Fig. 6A, 6C) prompted us to determine if the canonical Wnt signaling functions to regulate *Tgfβ3* expression. As we expected, *Tgfβ3* expression was almost, if not completely,

abolished in the MEE of the *K14Cre;Catnb*$^{F/F}$ palatal shelves along the anterior-posterior axis, as compared to controls (Fig. 6B, 6D; Suppl. Fig. 1). To assess fusion ability of the *K14Cre;Catnb*$^{F/F}$ palatal shelves , paired palatal shelves isolated from E13.5 mutants and littermate controls were cultured in the Trowell-type organ culture set-up. After 72 hrs in culture, control palatal shelves underwent fusion, as indicated by the disappearance of the midline seam and establishment of the mesenchymal continuity (3/3) (Fig. 6E). In contrast, the mutant palatal shelves failed to fuse, evidenced by the persistent midline seam (MES) (4/4) (Fig. 6F). However, we noted that the midline seam in all cases was not fully persistent. This is very likely due to residual *Tgfβ3* expression as the consequence of inconsistency of the *K14* promoter and Cre activities in transgenic animals. Nevertheless, addition of exogenous Tgfβ3 protein in organ culture caused complete disappearance of the midline seam and rescued the fusion defect in the mutant (Fig. 6H), confirming that a down-regulation of *Tgfβ3* is responsible for the persistent midline seam phenotype. Since programmed cell death is crucial for MES elimination and palate fusion (Cuervo and Covarrubias, 2004; Nawshad, 2008), we examined cell apoptosis in mutant and the control palate. At E14.5, intensive apoptotic cells were detected by TUNEL assay in the MES of control embryos (Fig. 6I). However, we detected rare apoptotic cells, if there was any, in the mutant MES at the same stage (Fig. 6J). These results indicate that suppression of cell apoptosis in the MES attributes to failed palate fusion, leading to a cleft palate formation in *K14Cre;Catnb*$^{F/F}$ mice. While the possibility exists that the cell adhesion role of β-catenin in the epithelium may contribute palate fusion, the down-regulation of *Tgfβ3* expression and suppression of apoptosis in the MEE cells in the absence of functional Wnt/β-catenin signaling strongly support an essential role for the β-catenin-mediated signaling in palate development.

Wnt/β-catenin signaling is sufficient to induce Tgfβ3 expression

To further confirm that it is β-catenin-mediated Wnt signaling rather than its cell adhesion function that regulates *Tgfβ3* expression, we took a gain-of-function approach by ectopically activating a stabilized form of β-catenin in embryonic epithelium. We used an exon3 floxed *Catnb* allele (*Catnb*$^{F(ex3)}$), which, upon Cre-mediated recombination, produces a stabilized β-catenin, leading to ectopic activity of the canonical Wnt signaling (Harada et al., 1999). To do this, we compounded the *K14Cre* allele to the *Catnb*$^{F(ex3)}$ alelle to generate *K14Cre;Catnb*$^{F(ex3)}$ embryos. At E14.5, when the control palate had completed elevation and fused, the mutant palatal shelves appeared too short to make contact at the midline, forming a complete cleft of the secondary palate (Fig. 7A, 7B). Histological analysis revealed that in mutant the anterior palatal shelves grew horizontally similar to the controls, but the posterior palatal shelves remained in a vertical position and fused to the mandible abnormally (Fig. 7C–F). This phenotype resembles that found in mice deficient for either *Fgf10 Noggin*; in both these mutants ectopic *Tgfβ3* expression and excessive apoptotic cells were observed in the ventral palatal epithelium (Alappat et al., 2005; He et al., 2010b). This is indeed the case we found. In the palatal shelves of *K14Cre;Catnb*$^{F(ex3)}$ embryos at E13.5, we detected ectopic expression of *Axin2* as well as *Tgfβ3* in the palatal epithelium (Fig. 7G–J; Suppl. Fig. 1), indicating that ectopic Wnt/β-catenin signaling is sufficient activate *Tgfβ3* expression. In addition to aberrant palate-mandible fusion, the palatal shelves of *K14Cre;Catnb*$^{F(ex3)}$ mice also displayed several morphological abnormalities (Fig. 7D, 7F), suggesting alteration in other signaling pathways. Indeed, elevated cell proliferation rate was found in the palatal mesenchyme of *K14Cre;Catnb*$^{F(ex3)}$ mice (data not shown). We are currently investigating the underlying mechanisms. It was also noticeable that the rugae failed to form in the *K14Cre;Catnb*$^{F(ex3)}$ palatal shelves at E14.5 (Fig. 7B). At E18.5, the mutant embryo developed severely hyper-keratinized epidermis, making it impossible to see if any rugal structure forms anyway (data not shown). Taken together, mis-regulated Wnt/β-catenin signaling in the palatal epithelium disrupts multiple developmental processes during

palatogenesis, leading to cleft palate formation, but the aberrant palate-mandible fusion phenotype appears to be resulted from the ectopic activation of *Tgfβ3* in the ventral palatal epithelium.

Discussion

Mutations in several *WNT* genes have been associated with cleft lip/palate in humans; however, the function of these genes and their engaged signaling pathways in palate development still remains to be elucidated (Chiquet et al., 2008). We previously reported that Wnt5a-activated noncanonical Wnt signaling is required for directional cell migration and normal cell proliferation during palate development (He et al., 2008). However, despite of increasing evidence, the lack of evidence for the presence of functional Wnt/β-catenin signaling in the developing palate makes it elusive as if the canonical Wnt/β-catenin signaling plays a role in palate development. In this paper, we show that the canonical Wnt/β-catenin signaling does operate in the palatal epithelium and has an essential role in palate fusion through regulating *Tgfβ3* expression in the MEE.

β-catenin constitutes the core mediator of the canonical Wnt signaling, but it also has an important role in cell adhesion by linking actin-cytoskeleton to E-cadherin (Nelson and Nusse, 2004; Brembeck et al., 2006). Thus, the cleft palate defect in *K14Cre;Catnb*$^{F/F}$ could be attributed to either Wnt/β-catenin signaling deficiency or an defective adhesion, or both in the palatal epithelium. Taking account of our results and previously reported evidence, we tend to support a signaling role for β-catenin in palate development: 1) functional Wnt/β-catenin signaling is present in the palatal epithelium, especially in the MEE, overlapping with *Tgfβ3* expression; 2) mice deficient for both the Wnt signaling effectors *Tcf4 and Lef1* exhibit persistent palatal MES, resembling the phenotype observed in *K14Cre;Catnb*$^{F/F}$ mice (Brugmann et al., 2007); 3) mutation in *Lrp6* leads to a cleft palate formation (Song et al., 2009); 4) while *E-cadherin* is expressed in the palatal epithelium, epithelial ablation of its function does not disrupt palate development (Luning et al., 1994; Tunggal et al., 2005). However, the cell adhesion function of β-catenin may still contribute to normal palate development. This is exemplified by the observation that inactivation of *Catnb* in the palatal mesenchyme where Wnt/β-catenin signaling activity is present at a relatively lower level causes a cleft palate formation (Chen et al., 2009).

Numerous previous studies have implicated a crucial role for β-catenin has been in regulation of cell proliferation and apoptosis. Deletion of β-catenin reduces proliferation rate and enhances cell apoptosis. In line with the role of β-catenin in regulating cell proliferation, a decreased level of cell proliferation was found in the palatal epithelium of *K14Cre;Catnb*$^{F/F}$ mice. However, cell proliferation rate remained unaltered in the palatal mesenchyme of *K14Cre;Catnb*$^{F/F}$ mice. *Shh* shares an overlapped expression with β-catenin in the palatal epithelium, and acts in the palatal mesenchyme to regulate cell proliferation (Zhang et al., 2002: Rice et al., 2002; Han et al., 2009; Lan and Jiang, 2009). Despite being a downstream target of the Wnt/β-catenin signaling in the hair follicle, tooth germ, and taste papilla (Huelsken et al., 2001; Silva-Vargas et al., 2005; Liu, et al., 2007a; Liu et al., 2008), inactivation of β-catenin did not affect *Shh* expression in the MEE, consistent with an unchanged level of cell proliferation in the palatal mesenchyme. Interestingly, we did not detect excessive cell death in the palatal epithelium of *K14Cre;Catnb*$^{F/F}$ mice, suggesting differential roles for β-catenin in different developing organs and at different developmental stages.

Tgfβ3 is expressed in the MEE and its essential role in palate fusion is widely accepted. Inactivation of *Tgfβ3* or its receptors leads to cleft palate defect due to persistence of the MES, which is disintegrated during normal palate formation (Dudas et al., 2004; Martinez-

Alvarez et al., 2004; Xu et al., 2006). While many studies have been conducted to look into molecular mechanism and downstream cascade of *Tgfβ3* action in palate fusion (Martinez-Alvarez et al., 2004; Yang and Kaartinen, 2007; Nawshad, 2008), upstream regulators of *Tgfβ3* are less understood. We have previously demonstrated that both *Fgf10* and *Noggin* function to repress *Tgfβ3* transcription in the palatal epithelium at ventral side (Alappat et al., 2005; He et al., 2010); however, upstream activators that regulate *Tgfβ3* expression in the MEE remain unknown. It was reported previously that a TCF binding site exists in the *Tgfβ3* upstream region, and ChIP assay further confirmed binding of β-catenin and TCF4 to this site in an in vitro system, suggesting that Wnt/β-catenin signaling regulates *Tgfβ3* transcription directly (Medici et al., 2008). In this study, we show a co-localization of functional Wnt/β-catenin signaling and *Tgfβ3* expression in the MEE. We further used both in vivo loss-of- and gain-of-function approaches to show that Wnt/β-catenin is essential and sufficient for activation of *Tgfβ3* in the palatal epithelium. Based on these lines of evidence, we conclude that Wnt/β-catenin signaling regulates palate fusion by controlling *Tgfβ3* expression in the MEE, most likely through a direct transcription activation mechanism.

Prior to our study reported here, mouse models with *K14Cre*-mediated ectodermal deletion of *Catnb* have been generated for studies of ectodermal organ development (Huelsken et al., 2001; Liu et al., 2007a; Liu et al., 2008). However, a palate phenotype was not reported these studies. Either the phenotype was ignored or not described, or did not exist at all. In one of these previous studies, *K14Cre;Catnb*$^{F/F}$ mice apparently survived to adulthood showing skin defect (Huelsken et al., 2001). The different outcome of ectodermal β-catenin deletion could attribute to the different efficiency and versatility of activation stage of different *K14-Cre* lines. In this study, we use a *K14-Cre* line whose Cre activity in the palatal epithelium could be detected as early as at E11.5, coinciding with onset of palate development (He et al., 2010b). Even though, this *K14-Cre* line could also exhibit inconsistent promoter activity to certain extent. This explains the incomplete penetrance of the cleft palate defect in *K14Cre;Catnb*$^{F/F}$ mice as well as the lack of fully persistent MES in cultured mutant palatal shelves.

In conclusion, in this study, we have examined the activity of Wnt/β-catenin signaling in the developing palate and demonstrated an essential role for Wnt/β-catenin signaling in palate fusion by regulating *Tgfβ3* expression. Together with our previous finding that the Wnt5a/Ror2-mediated noncanonical signaling regulates cell proliferation and migration in palatal mesenchyme, we conclude that both the canonical and noncanonical Wnt signaling pathways are essential for palatogenesis, but regulate different steps of palate development, which could provides biological basis for the development of effective prevention and therapeutic treatments of cleft palate defects in humans with altered Wnt signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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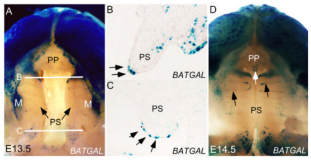

Figure 1.

Detection of Wnt/β-catenin signaling activity in the palatal epithelium. (A) Oral view of an E13.5 *BATGAL* reporter embryonic head (mandible removed) shows *LacZ* staining in the upper lip, developing molar, and the secondary palate, but not in the primary palate. Arrows point to the secondary palatal shelves. While lines indicate section levels shown in panel B and panel C, respectively. (B, C) Coronal sections of an E13.5 *BATGAL* embryo show that Wnt/β-catenin signaling activity is largely restricted to the MEE cells (arrows) in both the anterior (B) and posterior palatal shelf (C). Sporadic *LacZ*-positive cells are found in the anterior palatal mesenchyme. (D) Oral view of an E14.5 *BATGAL* reporter embryonic head with removal of mandible reveals that *LacZ* activity has expanded to the primary palate (white arrow) and rugae (black arrows) in addition to the patterns observed at E13.5. M, Molar; PP, primary palate; PS, secondary palatal shelf.

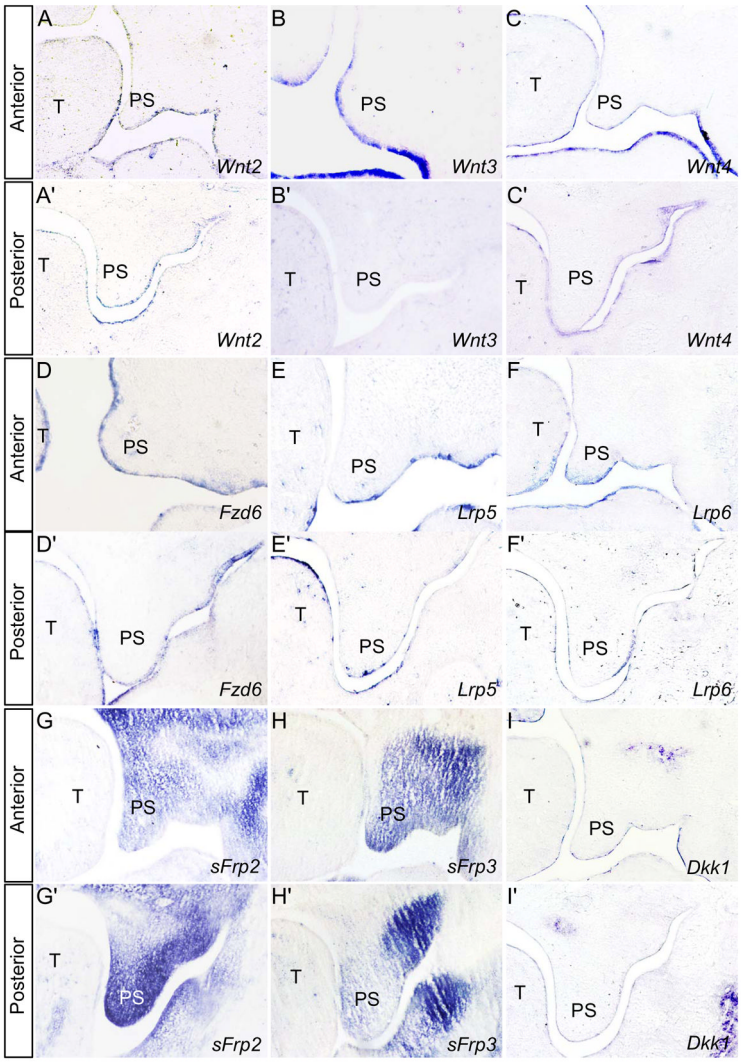

Figure 2.

Expression of Wnt/β-catenin signaling components and antagonists in E13.5 developing palate. (A–C') *Wnt2* (A, A') and *Wnt4* (C, C') are expressed in the epithelium of both anterior and posterior palate (A, A', C, C'), respectively, while *Wnt3* is expressed only in the anterior palate epithelium (B, B'). (D–F') Wnt receptors *Fzd6* (D, D'), *Lrp5* (E, E'), and *Lrp6* (F, F') are expressed in palatal epithelium, and *Lrp6* is also expressed in the anterior palatal mesenchyme (F). (G, G') *Sfrp2* expression is detected strongly in the palatal mesenchyme, forming an expression gradient from the posterior to the anterior domain. (H, H') *Sfrp4* expression in the palatal mesenchyme also forms a gradient with the highest level in the anterior palate. (I, I') *Dkk1* is expressed in the palatal epithelium. T, tongue; PS, palatal shelf.

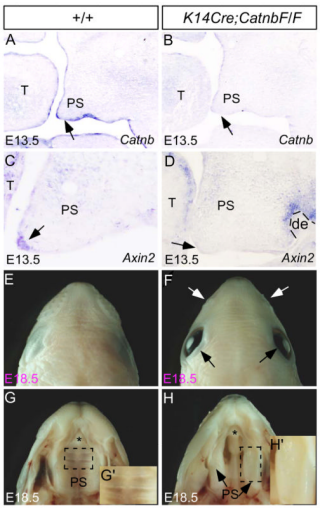

Figure 3.

Epithelial ablation of *Catnb* generates a cleft palate defect. (A) At E13.5, *Catnb* is expressed in the palatal epithelium, including the MEE (arrow). (B) *Catnb* expression is undetectable in the palatal epithelium (arrow) as well as oral epithelium of an E13.5 *K14Cre;Catnb*$^{F/F}$ embryo. (C) *Axin2* expressed is detected in the MEE (arrow) and in the palatal mesenchyme at a low level of an E13.5 control palate. (D) An E13.5 *K14Cre;Catnb*$^{F/F}$ palatal shelf show absent *Axin2* expression in the MEE (arrow), but a weak expression in the palatal mesenchyme and a strong expression in the dental mesenchyme remain. (E, F) A dorsal view of an E18.5 *K14Cre;Catnb*$^{F/F}$ head (F) shows open eyelid (black arrows) and hypoplastic whisker pad (while arrows), as compared to a littermate control. (G, H) An E18.5 wild type embryo develops an intact palate (G) while the mutant exhibits a complete cleft secondary palate. (G', H') High magnification images of E18.5 wild type (G') and mutant (H') palate show rugae in the wild type control and the lack of rugal formation in the mutant. T, tongue; de, dental epithelium; PS, palatal shelf. Asterisk indicates the primary palate.

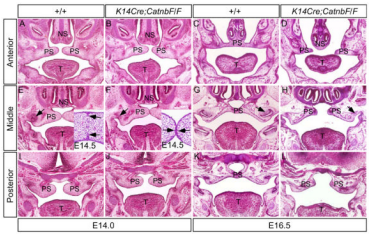

Figure 4.

K14Cre;Catnb$^{F/F}$ palate shelves exhibit normal outgrow and elevation. (A, E, I) At E14.0, the wild type palate shelves have elevated horizontally and made contact at the middle position. (B, F, J) At the same stage, mutant palate shelves develop comparably to the control. Note molar tooth in mutant is arrested at the lamina stage (arrow in F). Insert in (E) shows disappearance of the midline seam at E14.5. Only residual epithelial seam (arrows) is present at this stage. Insert in (F) shows the persistent midline seam (arrows) in the E14.5 mutant palate (C, G, K) At E16.5, the wild type palate has fused to form an intact structure, and the molar has developed to the bell stage (arrow in G). (D, H, L) At E16.5 in mutant, the palatal shelves are separated, forming a complete cleft of the secondary palate, and the mutant molar development remains at the lamina stage (arrow in H). T, tongue; NS, nasal septum; PS, palatal shelf.

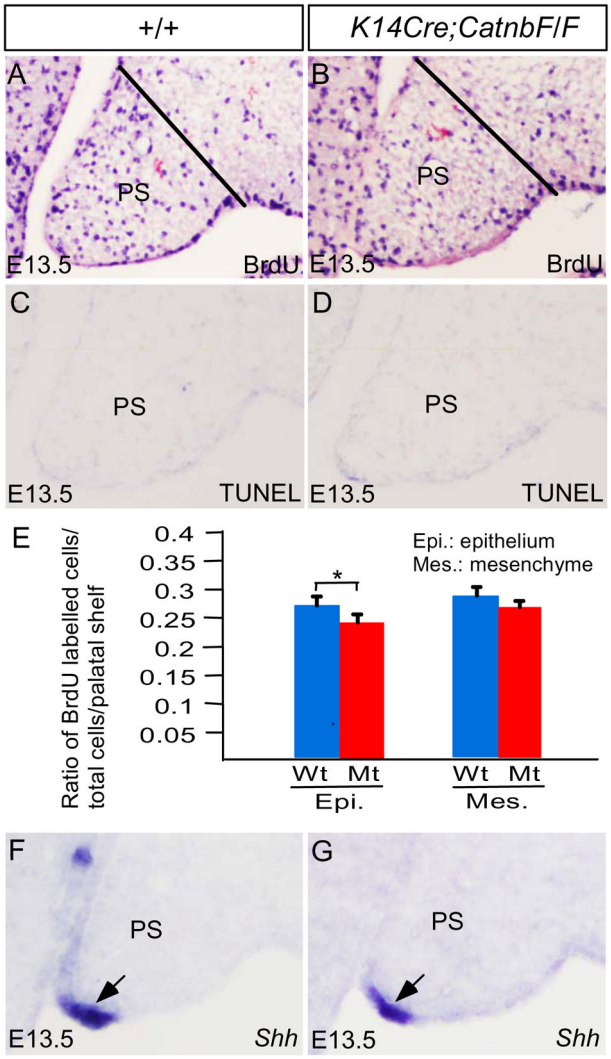

Figure 5.

Cell proliferation, apoptosis, and *Shh* expression in *K14Cre;Catnb*$^{F/F}$ palatal shelves. (A, B) E13.5 palatal shelves from control (A) and mutant (B) show BrdU labeling. Black lines demarcate the palatal region for counting of BdrU-positive cells in the epithelium and mesenchyme, respectively. (E) Comparison of BrdU-labeled cells in epithelium and mesenchyme of fixed area in control and mutant palatal shelves. *: $P < 0.05$. (F, G) *Shh* expression (arrow) is detected in the MEE of the palatal shelves from E13.5 control (F) and mutant (G). PS, palatal shelf.

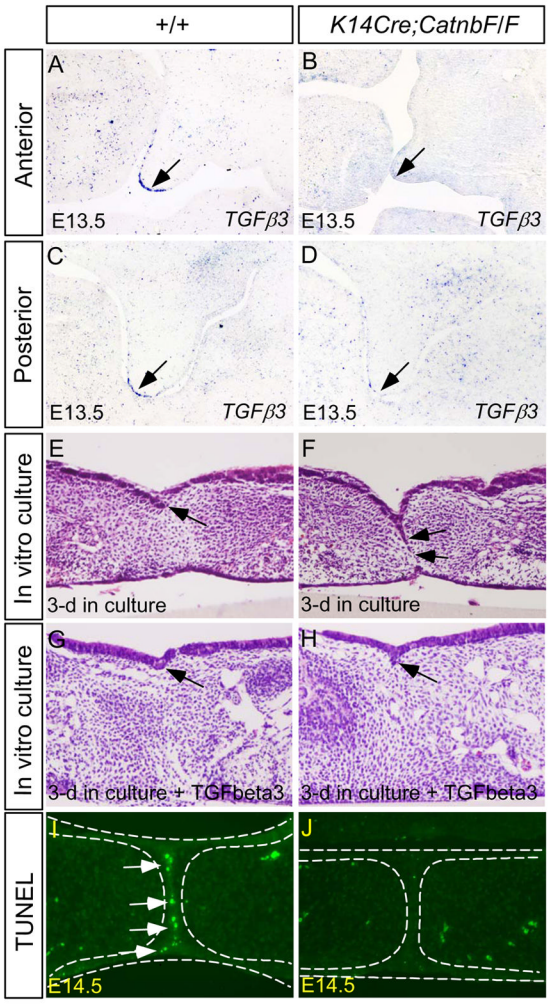

Figure 6.

Palate fusion in *K14Cre;Catnb*$^{F/F}$ embryo is disrupted by abnormal MES persistence due to downregulation of *Tgfβ3* and failed cell apoptosis. (A, C) In E13.5 control palatal shelf, *Tgfβ3* is expressed in the MEE cells (arrow) in the anterior (A) and posterior palatal shelf (B). (B, D) *Tgfβ3* expression is not detectable in the MEE of the anterior (B) and posterior (D) palatal shelf of E13.5 mutant. (E) Palatal shelves from E13.5 wild type control have successfully fused after 3-day in culture. Arrow points to the remainder of the midline seam. (F) Palatal shelves from E13.5 mutant show limited fusion and the majority of MES is persistent (arrows). (G, H) Histological sections show disappearance of the midline seam in both control (G) and mutant (H) palatal shelves after 3-day in culture in the presence of exogenous Tgfβ3. Arrows point to the remainders of the midline seam. (I) TUNEL assay reveals extensive apoptotic cells in the palatal MES of E14.5 wild type embryo. (J) Apoptotic MES cells are hardly detected in the mutant MES at E14.5

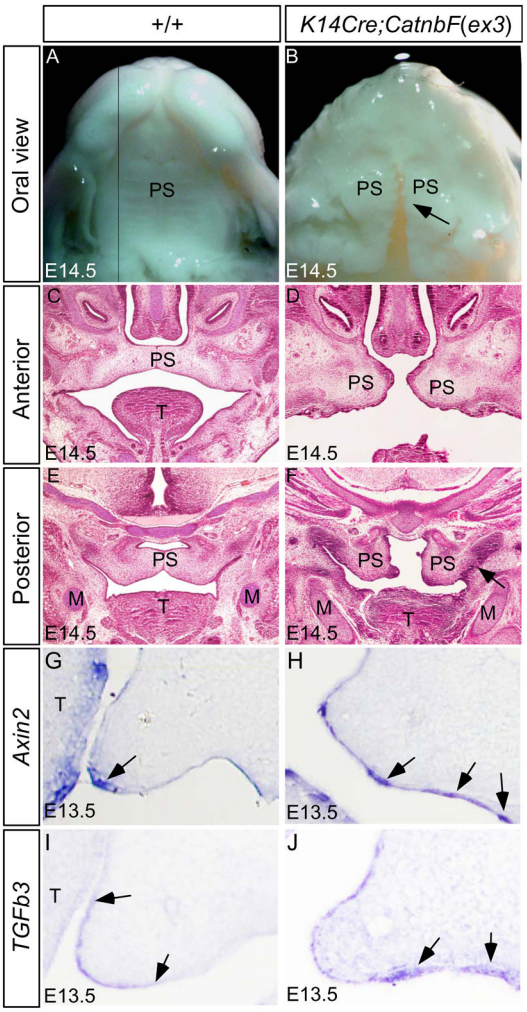

Figure 7.

Ectopic Wnt/β-catenin signaling activity in the palatal epithelium activates ectopic *Tgfβ3* expression and causes cleft palate defect. (A, B) At E14.5, wild type embryo develops an intact palate with visible rugal formation (A), but the palatal shelves remain separately in *K14Cre;Catnb*$^{F(ex3)}$ embryo, forming a clefting (arrow), and do not develop rugae (B). (C, E) Histological analysis reveals that the wild type palate has elevated and fused at E14.5, in both the anterior (C) and posterior region (E). (D, F) In E14.5 *K14Cre;Catnb*$^{F(ex3)}$ embryo, the anterior palatal shelves are positioned at the horizontal level, but are severely deformed and are too short to make contact at the midline (D); the posterior palate shelves fail to elevate and exhibit aberrant fusion (arrow) with the mandible (F). (G, H) At E13.5, *Axin2* expression (arrow) is restricted to the MEE of wild type palate (G), but its expression is ectopically activated in the mutant palatal epithelium, particularly in the ventral palatal epithelium (arrows) (H). (I) *Tgfβ3* expression is detected in the MEE of E13.5 control palate. Arrows define the expression domain in the palatal shelf. (J) Ectopic *Tgfβ3* expression (arrows) is detected in the E13.5 *K14Cre;Catnb*$^{(ex3)}$ palatal epithelium. M, Meckel's cartilage; T, tongue; PS, palatal shelf.