Published in final edited form as:

Dev Dyn. 2010 December ; 239(12): 3235–3246. doi:10.1002/dvdy.22466.

***Gsk3 β* is required in the epithelium for palatal elevation in mice**

Fenglei He^{1,¶,□}, Anthony P. Popkie^{2,¶}, Wei Xiong¹, Lu Li¹, Ying Wang¹, Christopher J. Phiel^{2,3,*}, and YiPing Chen^{1,*}

¹Department of Cell and Molecular Biology, Tulane University, New Orleans, LA 70118, USA

²Graduate Program in Molecular, Cellular & Developmental Biology, The Ohio State University

³Center for Molecular and Human Genetics, The Research Institute at Nationwide Children's Hospital, Columbus, OH 43205, USA

Abstract

In Wnt/ β -catenin signaling pathway, *Gsk3 β* functions to facilitate β -catenin degradation. Inactivation of *Gsk3 β* in mice causes a cleft palate formation, suggesting an involvement of Wnt/ β -catenin signaling during palatogenesis. In this study, we have investigated the expression pattern, tissue-specific requirement and function of *Gsk3 β* during mouse palatogenesis. We showed that *Gsk3 β* is primarily expressed in the palatal epithelium, particularly in the medial edge epithelium (MEE) overlapping with β -catenin. Tissue-specific gene inactivation studies demonstrated an essential role for *Gsk3 β* in the epithelium for palate elevation, and disruption of which contributes to cleft palate phenotype in *Gsk3 β* mutant. We observed that expression of *Aixn2*, a direct target gene of Wnt/ β -catenin signaling, is ectopically activated in the mutant tongue, but not in the palate. Our results indicate that *Gsk3 β* is an intrinsic regulator required in the epithelium for palate elevation, and could act through a pathway independent of Wnt/ β -catenin signaling to regulate palate development.

Keywords

Gsk3 β ; cleft palate; palatal elevation; palate development

Introduction

Cleft palate is a common birth defect in human, with a prevalence ranging between 1/1000 and 1/700 (Koillinen et al., 2005). The development of mammalian secondary palate is a multiple-step process and disruption at any step, environmentally or genetically, could result in a cleft palate formation. In mice, the palatal shelves protrude from bilateral maxillary processes at embryonic day (E) 11.5, and grow vertically along the developing tongue from E12.5 through E14.0. Subsequently, they elevate, meet and fuse at the midline, ultimately form an intact palate shelf at E14.5 (Ferguson, 1988). Palatal shelf elevation is a rapid process, accompanied by extracellular matrix accumulation within the palatal shelf and movement of other craniofacial structures. Impaired palatal shelf elevation, along with defective palate growth and fusion, comprises major causes for cleft palate formation (Ferguson, 1988; Gritli-Linde, 2007). It has been proposed that palate elevation is triggered

*Corresponding authors: CJP: The Research Institute at Nationwide Children's Hospital, Columbus, OH 43205; Tel: 614-722-2700; Christopher.Phiel@nationwidechildrens.org, YC: Department of Cell and Molecular Biology, Tulane University, New Orleans, LA 70118; Tel: 504-865-5546; Fax: 504-865-6785; ychen@tulane.edu.

¶Equal first authors

□Present address: Department of Developmental and Regenerative Biology, Mount Sinai School of Medicine, New York, NY 10029

by the intrinsic erectile forces, from both palatal mesenchyme and epithelial covering, and the extrinsic influence, such as coordinated movement of adjacent craniofacial and oral structures including descending of the tongue (Ferguson, 1988). It is thought that mesenchymal cells provide erectile elevation force and the epithelial covering constrains and guides the force to appropriate direction to manage the elevation (Bulleit and Zimmerman, 1985; Ferguson, 1988). Studies using gene-targeting mice have demonstrated that failed palatal shelf elevation could be attributed either to intrinsic elevation defects or to other oral structure deformation (reviewed in Gritli-Linde, 2007). In addition, anomalous fusion of the palatal shelves with the tongue or the mandible also leads to elevation defect (Rice et al., 2004; Alappat et al., 2005; Casey et al., 2006; Xiong et al., 2009).

The β -catenin-mediated Wnt canonical (Wnt/ β -catenin) signaling pathway plays pivotal role in regulating multiple events of embryonic development. This signaling cascade is activated by Wnt ligand binding to the Frizzled (Fz) transmembrane receptors and LRP5/6 co-receptors. The binding disassembles in the cytoplasm the β -catenin destruction complex, which is composed of glycogen synthase kinase-3 β (Gsk3 β), Axin, and adenomatous polyposis coli (APC). Consequently, β -catenin accumulates and translocates into the nucleus, where it binds to LEF/TCF co-transcription factors and activates target genes expression (van Amerongen and Nusse, 2009). In the absence of Wnt ligands binding, β -catenin is phosphorylated by Gsk3 β and is further processed to degradation. Gsk3 β acts as a negative modulator of Wnt/ β -catenin signaling.

Two highly related Gsk3 protein homologues have been identified in mammals: Gsk3 α and Gsk3 β , which are encoded by two genes (Frame and Cohen, 2001; Doble and Woodgett, 2003). First recognized by its function in regulating glycogen synthase activity, Gsk3 β has been shown to play a role in a number of cellular processes, including modulating the Wnt/ β -catenin signaling (Embi et al., 1980; Frame and Cohen, 2001; Grimes and Jope, 2001; Doble and Woodgett, 2003). During mouse embryonic development, Gsk3 β is essential for liver cell survival as well as palate formation (Hoefflich et al., 2000; Liu et al., 2007). Although the specific function for Gsk3 α remains unknown, it has been shown that Gsk3 α and Gsk3 β function redundantly in regulating Wnt/ β -catenin signaling in embryonic stem cell lines (Doble et al., 2007).

Wnt/ β -catenin signaling has been implicated in the development of various tissues and organs, including the craniofacial structures (Grigoryan et al., 2008). Although a direct role for β -catenin in the regulation of palate development awaits evidence, β -catenin expression was indeed observed in the palatal epithelium and possibly in the mesenchyme as well (Martinez-Alvarez et al., 2000; Tudela et al., 2002; Nawshad and Hay, 2003; He et al., 2008). In fact, many lines of recent evidence suggest an involvement of the β -catenin-mediated Wnt canonical signaling in palatogenesis. For example, mice deficient for either *Wnt9b*, or *Lrp6*, or *Wnt5a* exhibit a cleft palate phenotype (Yang et al., 2003; Carroll et al., 2005; He et al., 2008; Song et al., 2009). *Wnt11* was found to be expressed in the palatal epithelium and its function is indispensable for palate fusion (Lee et al., 2008). In addition, mutations in *WNT5A* or *WNT3* have been correlated to a cleft palate defect in humans (Blanton et al., 2004; Niemann et al., 2004). All these lines of evidence suggest a role for Wnt canonical signaling in palatogenesis.

We report here that Gsk3 β is primarily expressed in the palatal epithelium, particularly in the medial edge epithelium (MEE) region overlapping with β -catenin. To determine the tissue-specific requirement of Gsk3 β in palatogenesis, we have generated Gsk3 β conditional knockout mice and demonstrated that Gsk3 β is specifically required in the epithelium for normal palatogenesis. Mice deficient for Gsk3 β exhibit an impaired elevation of the palatal shelves, leading to a complete cleft of secondary palate. We also detected *Axin2* expression

in the developing palate, and showed that its expression is not altered in the *Gsk3 β* mutant palate. Our results support the idea that *Gsk3 β* is an intrinsic regulator of palate elevation, and its absence does not alter the Wnt/ β -catenin signaling pathway in the developing palatal shelves.

Results

Expression of *Gsk3 β* is enriched in the epithelium of developing palate

Although it was proposed that *Gsk3 β* is ubiquitously expressed in all cells, tissue specific expression of this gene has been reported (Ciaraldi et al., 2006). To investigate the role of *Gsk3 β* during palatogenesis, we first examined its expression pattern in the developing palate by in situ hybridization. Our results showed that, at E12.5, *Gsk3 β* is expressed both in the anterior and posterior palate, at a higher level in the palatal epithelium and an above background level of expression in the palatal mesenchyme (Fig. 1A, 1C). At E13.5, *Gsk3 β* is expressed in a pattern similar to that in E12.5 embryo, but its expression is mainly restricted to the medial edge epithelium (MEE) (Fig. 1B, 1D). This expression pattern overlaps with that of *Catnb*, which encodes β -catenin, the central mediator of the Wnt canonical signaling (He et al., 2008). In addition, *Gsk3 β* expression was also found in the epithelium of the developing tongue (Fig. 1). These results suggest that *Gsk3 β* may modulate Wnt/ β -catenin signaling function during palate development.

Gsk3 β is specifically required in the epithelium to regulate palate development

It was reported previously that *Gsk3 β* deficient mice exhibit a cleft palate defect, but the mechanisms underlying this phenotype still remained unknown (Liu et al., 2007). To extend the finding and examine the morphological and molecular defects that lead to the complete cleft palate formation in *Gsk3 β* mutant mice, we created by gene targeting in ES cells a *Gsk3 β* conditional allele (*Gsk3 β ^{F/+}*) in which exon 2 was flanked with the *LoxP* sequences (Fig. 2). The targeted locus in ES cells and germline transmission were confirmed by Southern blotting (Fig. 2B), mice heterozygous or homozygous for the conditional allele were genotyped by a PCR based method (Fig. 2). To create a null *Gsk3 β* allele (*Gsk3 β ^{+/-}*), we crossed *Sox2Cre* mice to *Gsk3 β ^{F/+}* mice to delete exon 2 which encodes the *Gsk3 β* protein domain responsible for the kinase activity. While mice heterozygous for *Gsk3 β* mutation appear normal and fertile, *Gsk3 β ^{-/-}* mice, obtained by intercrosses between *Gsk3 β ^{+/-}* mice, showed a complete cleft palate defect and died within 24 hours after birth (Fig. 3B), a phenotype identical to what was reported previously (Liu et al., 2007).

Histological analyses of the developing palate of *Gsk3 β ^{-/-}* mice at multiple stages revealed a normal development of the palatal shelves before E13.5, as compared to the wild type controls (Fig. 3D, 3E, 3G, and 3H). At E14.5, the palatal shelves have elevated to a horizontal position and fused in the middle line in the wild type controls (Fig. 3J). In *Gsk3 β ^{-/-}* embryo, however, the palatal shelves remained in the vertical position and failed to elevate to the position above the tongue (Fig. 3K). At E18.5, the mutant anterior palatal shelves eventually elevated to a horizontal position but failed to make contact (data not shown), and the posterior palatal shelves remained in a vertical orientation and never elevated (Fig. 3M, 3N). The delayed/failed elevation of palatal shelves is therefore the cause of cleft palate phenotype in *Gsk3 β ^{-/-}* mice.

Since *Gsk3 β* expression is detected primarily in the palatal epithelium, and in the mesenchyme as well, we next sought to address the tissue-specific requirement of *Gsk3 β* function during palate development. We chose to use two established transgenic lines *Pitx2Cre* and *Osr2Cre* to abort *Gsk3 β* function in tissue-specific manners in the developing palate. *Pitx2Cre* and *Osr2Cre* mice have been used in previous studies to delete *LoxP*-

flanked genes in the palatal epithelium and mesenchyme, respectively (Lan et al., 2007; Xiong et al., 2009). We found that *Osr2Cre;Gsk3 β ^{F/F}* mice survived postnatally and showed normal palate formation (5/5). In contrast, among fourteen *Pitx2Cre;Gsk3 β ^{F/F}* mice examined, eleven exhibited a complete cleft of the secondary palate, phenocopying the defect in the *Gsk3 β ^{-/-}* mice (Fig. 3C; and data not shown). The incomplete penetrance of cleft palate phenotype in *Pitx2Cre;Gsk3 β ^{F/F}* mice could be attributed to variation in Cre activity or expression timing by the *Pitx2Cre* transgenic allele in different embryos. Nevertheless, histological examination of *Pitx2Cre;Gsk3 β ^{F/F}* embryos revealed the development of the palatal shelves do not exhibit any malformation until E13.5 (Fig. 3F, 3I). However, at E14.5, similar to defects found in *Gsk3 β ^{-/-}* mice, the *Pitx2Cre;Gsk3 β ^{F/F}* palatal shelves failed to elevate (Fig. 3L). While the anterior palatal shelves did elevate at E18.5, the posterior palatal shelves remained at the vertical position (Fig. 3O). These results indicate that *Gsk3 β* function is required in the palatal epithelium during palate development, and *Gsk3 β* acts in the palatal epithelium to control palatal shelf elevation.

To rule out the possibility that the failure of palate shelf elevation in *Gsk3 β* mutants is due to extrinsic defects such as malformation of the tongue and mandible, we performed in vitro organ culture on a rotary apparatus. Individual embryonic head with removal of mandible of E13.5 embryos harvested from mating of *Gsk3 β ^{+/-}* mice was cultured for 24 hrs, and then processed for histological examination after genotyping. As shown in Figure 4, after 24 hrs in culture, the wild type controls exhibited elevated palatal shelves (9/9); however, the mutant palatal shelves remained at the vertical position (10/10). These results indicate that intrinsic defects in the absence of *Gsk3 β* in fact contribute to the failure of palate elevation.

Gsk3 β is required for normal cell proliferation and palatal epithelial cell survival

Defects in cell proliferation and/or survival have been shown to impair normal development of the palatal shelves, leading to the cleft palate formation (Gritli-Linde, 2007). To determine if these cellular processes were affected in *Gsk3 β ^{-/-}* palate, we performed BrdU labeling and TUNEL assays on E12.5 and E13.5 palatal shelves of wild type controls and mutants. Our found comparable cell proliferation rates ($P > 0.05$) in the mesenchyme between the controls and mutant at E12.5 and E13.5 (Fig. 5A, 5B; and data not shown). In contrast, cell proliferation rates in the epithelium showed significant difference statistically, with a enhanced rate at E12.5 and a decreased level at E13.5 (Fig. 5G). This altered cell proliferation rate in the mutant epithelium is consistent with *Gsk3 β* expression domain in the developing palatal shelf. In additional we also observed significantly increased apoptotic cells in the palatal epithelium, including the MEE of the *Gsk3 β ^{-/-}* palate at E13.5 (Fig. 5D, 5F). These results indicate that *Gsk3 β* is implicated in the regulation of cell proliferation and survival during palatogenesis.

Gsk3 β does not regulate palate elevation through Shh, Osr2 and PDGF signaling

To determine the molecular defects in the *Gsk3 β ^{-/-}* palate, we examined the expression of several genes that either exhibit an overlapped expression pattern with *Gsk3 β* in the developing palate or have been shown to regulate palate elevation. Among them, *Shh* is expressed in the MEE and oral palatal epithelium (Fig. 6A, 6E) (Zhang et al., 2002; Rice et al., 2004). Epithelial-specific inactivation of *Shh* results in abnormal cell proliferation and apoptosis, as well as failed palatal shelf elevation (Rice et al., 2004; Gritli-Linde, 2007). Our in situ hybridization results showed that the expression of *Shh*, as well as that of its direct target *Ptch1*, was not altered in the *Gsk3 β ^{-/-}* palate (Fig. 6B, 6F; and data not shown), suggesting that the Shh signaling functions upstream of or in parallel to *Gsk3 β* during palate development. *Osr2* is expressed in the oral side palatal mesenchyme and inactivation of *Osr2* leads to a defect in palatal shelf elevation in mice (Fig. 6C, 6G; Lan et al., 2004). We found that *Osr2* expression was not changed in the *Gsk3 β ^{-/-}* palate (Fig. 5D, 5H). The

platelet-derived growth factor (PDGF) signaling has been shown to regulate palate development (Soriano, 1997; Tallquist and Soriano, 2003; Ding et al., 2004). *Pdgfa* and *Pdgfc*, encoding two of the PDGF ligands, were specifically expressed in the palatal epithelium overlapping with *Gsk3 β* (Fig. 6I, 6K; Ding et al., 2004). Inactivation of *Pdgfc* in mice causes delayed palatal shelf elevation, and *Pdgfa*;*Pdgfc* double knockout mice exhibit a more severe cleft face phenotype, indicating an essential role for these two genes in craniofacial development (Ding et al., 2004). We therefore examined the expression of *Pdgfa* and *Pdgfc* in the *Gsk3 β ^{-/-}* palate. As a result, we did not observe an altered expression of either gene (Fig. 6J, 6L, 6N, 6P). We concluded that *Gsk3 β* did not regulate expression of these genes during the palatal shelf elevation.

Inactivation of *Gsk3 β* does not alter activity of Wnt/ β -catenin signaling in developing palate

It was reported previously that *Catnb* expression is detected in the palatal epithelium, overlapping with that of *Gsk3 β* (Martinez-Alvarez et al., 2000; Tudela et al., 2002; Nawshad and Hay, 2003; He et al., 2008). It is thus intriguing to determine if *Gsk3 β* modulates the activity of Wnt/ β -catenin signaling, and if Wnt/ β -catenin signaling is involved in palate development. To this end, we have taken advantage of *TOPGAL* transgenic reporter line, which has been widely used to detect the activity of Wnt/ β -catenin signaling (DasGupta and Fuchs, 1999). To detect potentially enhanced and/or ectopic Wnt/ β -catenin signaling activity in the *Gsk3 β ^{-/-}* palate, we compounded the *TOPGAL* transgenic allele onto the *Gsk3 β ^{-/-}* background, and compared the β -galactosidase reporter activity in the mutant mice with that in the *TOPGAL* controls. In the *TOPGAL* transgenic control at E13.5, while intensive *LacZ* expression was detected in the lip and incisor regions, we did not observe any β -galactosidase activity in the developing palate (Fig. 7A), consistent with the previous report (He et al., 2008). If *Gsk3 β* regulates palate development by modulating Wnt/ β -catenin signaling embryos, one would expect to see an ectopic activity of the Wnt canonical signaling in the palatal epithelium. Surprisingly, we observed neither enhanced β -gal staining in the lip and incisor regions nor ectopic *LacZ* expression in the developing palatal shelves (Fig. 7B). These results suggest that either Wnt/ β -catenin signaling is not operating in the developing palate or the *TOPGAL* transgenic reporter has a limited sensitivity. To address these concerns, we chose to examine the expression of *Axin2*, a direct target gene of Wnt/ β -catenin signaling and a reliable indicator of active Wnt/ β -catenin signaling (Jho et al., 2002; Ontiveros et al., 2008; Wang et al., 2008). In the wild type controls, we observed a specific *Axin2* expression in the MEE, with some expression in the immediately adjacent mesenchyme (Fig. 7C). The expression of *Axin2* in the MEE overlaps with that of *Catnb* and *Gsk3 β* , indicating the presence of Wnt/ β -catenin signaling activity in the MEE region. At the level of Wnt/ β -catenin signaling activity in the developing palatal shelves, the *TOPGAL* transgenic allele appears to have limited sensitivity. Interestingly, we did not observe an elevated or ectopic *Axin2* expression in the *Gsk3 β ^{-/-}* palate (Fig. 7D). However, ectopic *Axin2* expression was detected in the mesenchyme adjacent to the epithelium in the developing tongue of *Gsk3 β ^{-/-}* mice (Fig. 7D). These results thus suggest that the function of *Gsk3 β* in modulating Wnt/ β -catenin signaling is compensated in the palate but not in the tongue. Since *Gsk3 α* has been shown to have functional redundancy with *Gsk3 β* in modulating the Wnt/ β -catenin signaling in stem cell lines (Doble, et al., 2007), we reasoned that *Gsk3 α* could have an overlapped expression pattern with *Gsk3 β* in the developing palate where they function redundantly. In situ hybridization assays showed that *Gsk3 α* was expressed in the palatal epithelium, primarily in the MEE and the oral side epithelium, (Fig. 7E). This expression pattern in the developing palate overlapped almost perfectly with that of *Gsk3 β* (Fig. 1). In the developing tongue, *Gsk3 α* expression was detected only in the mesenchyme and the epithelium in the ventral-lateral region, complementary to the ectopic *Axin2* expression domain in the *Gsk3 β ^{-/-}* tongue (Fig. 7D, 7E). In the developing palate and

tongue of *Gsk3 β* mutant, we did not observe an altered expression of *Gsk3 α* (Fig. 7F). These data suggest that *Gsk3 α* may compensate for the loss of *Gsk3 β* function in modulating Wnt/ β -catenin signaling in the developing palate. We thus concluded that a Wnt/ β -catenin signaling independent pathway regulated by *Gsk3 β* is essential for palate elevation during normal palate development.

Discussion

The β -catenin mediated Wnt canonical signaling has been implicated in many developmental events. However, direct evidence for an involvement of this signaling pathway in palate development is still lacking. In this paper, we reported that *Gsk3 β* is primarily expressed in the palatal epithelium, and its major function in palate development is to regulate palatal shelf elevation. Our *Gsk3 β* conditional knockout mice enable us to determine that *Gsk3 β* is specifically required in the palatal epithelium for palatal shelf elevation. We further demonstrate the presence of Wnt/ β -catenin signaling activity in the normal developing palatal shelves, and the activity remains unaltered in the *Gsk3 β* mutant palate. Our studies thus support the idea that *Gsk3 β* is an intrinsic epithelium-expressed regulator of palatal shelf elevation and functions independently of Wnt/ β -catenin signaling.

Gsk3 β is an epithelially-expressed intrinsic regulator of palatal shelf elevation

The phenomenon of palatal shelf elevation has been recognized for a long time, but the mechanism of this process remains far from well understanding. It is now widely accepted that palatal shelf elevation is a rapid process, undergoing in an anterior to posterior sequence and being controlled by both intrinsic erectile force and extrinsic influences from other craniofacial structures (Lazzaro, 1940; Ferguson, 1988). A number of mechanisms have been proposed for this process. Considering the intrinsic factors, it has been suggested that hyaluronan differential accumulation, type I collagen distribution, mesenchymal cell alignment, and epithelial covering all provide elevation force during palatal shelf elevation (reviewed in Ferguson, 1988). Although the mesenchymal tissue was thought to provide major driving force for the elevation, removal of oral epithelium was shown to inhibit the reorientation of palatal shelves in vitro. The palatal epithelium is thus considered to constrain and direct the elevation force generated by the mesenchymal core during palatal shelf elevation (Bulleit and Zimmerman, 1985). Temporally and spatially, palatal shelf elevation occurs coincidentally with the descending of the tongue, and the growth of the mandible and basicranium. Malformation of these structures therefore could also prevent palatal shelf elevation as extrinsic factors (Ferguson, 1988).

Corresponding to the above proposed mechanisms for palatal shelf elevation, defects in palatal shelf elevation in gene-targeting mice could be categorized into three major classes. First, the palatal shelf elevation can be impaired by intrinsic defects, as seen in *Osr2* or *Pdgfc* mutant mice (Ding et al., 2004; Lan et al., 2004). Second, palatal shelf elevation could be hindered by abnormal fusion between palatal shelf and adjacent oral structures. For example, in mice deficient for *Jagged2*, or *Fgf10*, or *Hand2*, the palatal shelves abnormally fuse with the developing tongue or the mandible, preventing palatal shelf elevation and causing cleft palate formation (Alappat et al., 2005; Casey et al., 2006; Xiong et al., 2009). Third, malformation of the tongue or mandible could physically block palatal shelf elevation, as it is seen in *Hox2a* and *Gli3* mutant mice (Barrow et al., 1999; Huang et al., 2008). In this study, we show that *Gsk3 β* is expressed and functions in the palatal epithelium. Inactivation of *Gsk3 β* impairs palatal shelf elevation without obvious extrinsic defects (for example, tight contact with the tongue or malformation of the mandible) or abnormal fusion with adjacent tissues. Moreover, the observation that the failure of palatal shelf elevation in the mutant head without mandible in organ culture rules out the possibility

of extrinsic effects. These results support the conclusion that *Gsk3 β* is an intrinsic regulator of palatal shelf elevation.

Gsk3 β has been implicated in cell proliferation regulation (Force and Woodgett, 2008; Kerkela et al., 2008). The absence of *Gsk3 β* results in cardiomyocyte hyperproliferation without influence on β -catenin signaling (Kerkela et al., 2008). Consistent with this line of discoveries, we found an altered cell proliferation rate in the mutant palatal epithelium where Wnt/ β -catenin signaling activity remained unaltered. It appears that the role of *Gsk3 β* on cell proliferation regulation in the palatal shelves depends largely on developmental stages. We certainly cannot rule out the possibility that this altered cell proliferation rate, together with enhanced cell apoptosis in the palatal epithelium, may also contribute to the cleft palate formation in *Gsk3 β* mutants.

Gsk3 β has been shown to modulate activities of several signaling pathways (Frame et al., 2001). Among them is Shh signaling, which is critical for palate elevation (Gritli-Linde, 2007; Lan and Jiang, 2009). Our findings show that *Gsk3 β* is expressed overlapping with *Shh* in MEE (Fig. 1A, C) and the mutants also exhibit palate elevation defect (Fig. 3K, N). Shh signaling is thus potential related to the defective palate development of the *Gsk3 β* mutant. However, our in situ hybridization studies showed an unaltered expression of *Shh* and its transcriptional target *Patch1* in the mutant palate, indicating that *Gsk3 β* does not regulate palatal shelf elevation by controlling Shh signaling activity. The expression of *Osr2*, *Pdgfa* and *Pgdfc* is neither affected in the *Gsk3 β* mutant palatal shelves. Future studies comparing gene expression profiles in the wild type and *Gsk3 β* mutant palatal shelves would help to identify downstream targets of *Gsk3 β* in the developing palate.

The role of *Gsk3 β* in palate development seems to vary slightly in different genetic background. In the present study, we have shown that *Gsk3 β* controls palate elevation in an inbred C57BL/6 background. In an outbred CD-1 background, however, *Gsk3 β* deficiency does not affect palate elevation, although it also causes cleft palate defect in the null mutant (Liu et al., 2007). Interestingly, in another study by Hoeflich and colleagues (2000), *Gsk3 β* homozygous null mice were reported to die during mid-gestation, a phenotype we never observed in our studies. Thus genetic background appears to exert influence on phenotype severity in the absence of *Gsk3 β* . Such correlations between genetic background and phenotype have been reported for other genes (Dansky et al., 1999; Yang et al., 2005).

Implication of the Wnt/ β -catenin signaling in palate development

The Wnt/ β -catenin signaling has been implicated in the formation of many organs (Grigoryan et al., 2008). Several Wnt ligands, including Wnt9b, Wnt5a and Wnt11, have been shown to play a role in palate development (Yang et al., 2003; Carroll et al., 2005; He et al., 2008; Lee et al., 2008). However, these lines of evidence do not support a direct involvement of the Wnt/ β -catenin signaling. This is because that in *Wnt9b* mutant mice, the cleft palate phenotype is a secondary defect (Lan et al., 2006). Wnt5a was shown to act through a β -catenin independent signaling pathway to regulate palate development (He et al., 2008). Stronger evidence, though still not direct, came from a recent publication in which cleft lip and cleft palate defects are observed in *Lrp6* mutant mice (Song et al., 2009). While the *TOPGAL* reporter activity is not detectable in the developing palate (He et al., 2008; this study), here we show that *Axin2*, a direct target gene of Wnt/ β -catenin signaling and a sensitive indicator of active Wnt/ β -catenin signaling (Jho et al., 2002; Ontiveros et al., 2008; Wang et al., 2008), is expressed in the palatal epithelium and restricted to the MEE region. This expression pattern is overlapped with that of *Gsk3 β* and β -catenin (Fig. 1; He et al., 2008). These results demonstrate the presence of active Wnt/ β -catenin signaling in the developing palate. Together with the cleft palate phenotype found in *Lrp6* knockout mice, these observations support a role for the Wnt/ β -catenin signaling in palate development.

Functional redundancy of *Gsk3 β* and *Gsk3 α* in palate development

The mammalian *Gsk3* genes have two homologues, *Gsk3 α* and *Gsk3 β* . Although *Gsk3 β* alone is able to modulate the Wnt/ β -catenin signaling, in some cases, inactivation of *Gsk3 β* does not necessarily lead to Wnt/ β -catenin signaling up-regulation. For instance, in embryonic stem cell lines, *Gsk3 β* and *Gsk3 α* have been shown to function redundantly in modulating Wnt/ β -catenin signaling (Doble et al., 2007). Wnt/ β -catenin signaling activity, which is assessed by the expression of *Axin2* transcripts and TCF reporter, is not significantly elevated until three of the four *Gsk3* alleles are depleted (Doble et al., 2007). In mouse embryonic fibroblasts, on the other hand, loss of *Gsk3 β* function itself enhances Wnt/ β -catenin signaling activity (Kapoor et al., 2008). The differential outcome of *Gsk3 β* ablation in the embryonic stem cells and fibroblasts may reflect the differential expression of *Gsk3 α* in these two types of cells. Interestingly, we found that *Gsk3 α* is expressed in a pattern overlapped with that of *Gsk3 β* in the developing palate, but not in the tongue. Consequently, an ectopic Wnt/ β -catenin signaling activity is found in the *Gsk3 β ^{-/-}* tongue, but not in the *Gsk3 β ^{-/-}* palate. These observations suggest a redundant role for *Gsk3 β* and *Gsk3 α* during palate development. The absence of *Gsk3 β* does not alter the Wnt/ β -catenin signaling, suggesting that *Gsk3 β* could also mediate a signaling pathway independent of Wnt/ β -catenin signaling during palate development.

Experimental Procedures

Generation and genotyping of *Gsk3 β* conditional knockout mice

Targeting construct for *Gsk3 β* was created using a recombineering strategy (Liu et al, 2003). Briefly, a BAC clone containing the murine *Gsk3 β* locus, from Children's Hospital of Oakland Research Institute (CHORI), was transformed into EL350 cells. PCR was used to retrieve about 400-bp homology arms flanking exon 2 of *Gsk3 β* by 5-kb on each side. Deletion of this exon has been shown to result in a non-functional kinase (Hoeflich, et al, 2000). After cloning homology arms into a retrieval vector (pBluescript modified to contain a thymidine kinase (TK) cassette), this construct was transformed into the EL350 BAC-containing cells, and induced homologous recombination via gap repair, resulting in a 10-kb fragment of *Gsk3 β* being retrieved into the pBluescript derivative. Next, mini-targeting vectors were created by introducing identical 34 base pair loxP sites on both sides of a neomycin resistance cassette (neomycin phosphotransferase gene; Neo^R). The loxP/Neo^R cassette was then introduced into gap repaired retrieval plasmids using a similar approach as just described above for retrieving the *Gsk3 β* fragment. Transformation of these constructs into bacteria with arabinose-inducible Cre allowed recombination, removing the Neo^R cassette and one loxP site. An additional round of this cloning approach was used to introduce a second loxP/Neo^R cassette, now flanked by FRT sites, on the 3' side of exon 2. Plasmids were linearized with NotI, gel purified, and electroporated into mouse ES cells by the ES Cell and Transgenic Core Facility at The Research Institute at Nationwide Children's Hospital. Targeted ES cells were grown in the presence of G418 (350 μ g/ml) and FIAU (5-iodo-2'-fluoro-2'-deoxy-arabinouridine; 200 nM) for the selection of cells which contained correct gene targeting.

Southern blotting of G418/FIAU-resistant clones revealed 11 out of 288 colonies (3.8%) contained a correctly targeted *Gsk3 β* floxed allele. Two clones were selected for injection into blastocysts, from which we obtained several male chimeric mice. These mice were mated to either ROSA26-FLPe mice, a well-characterized transgenic mouse that ubiquitously expresses Flp at high levels (Farley et al., 2000) resulting in the highly efficient removal of the Neo^R cassette, or wild-type C57BL/6 females. Primer information for PCR genotyping is available upon request.

Other animals

The conventional *Gsk3 β* mutant (*Gsk3 β ^{+/-}*) mice were generated by intercrossing *Gsk3 β* conditional (*Gsk3 β ^{F/F}*) mice with the *Sox2Cre* transgenic mice. *Sox2Cre* and *TOPGAL* mice were obtained from the Jackson Laboratories (Bar Harbor, Maine, USA) and were genotyped as described previously (DasGupta and Fuchs, 1999; Hayashi et al., 2002). The generation and genotyping of *Pitx2Cre* and *Osr2Cre* transgenic mice have been reported previously (Lan et al., 2007; Xiong et al., 2009). All the genetically engineered mice used in this study were maintained on the B6/C57 background. The age of embryos was defined as embryonic day 0.5 (E0.5) in the morning of the day when a vaginal plug was discovered. All animal studies were approved by the Tulane University Institutional Animal Care and Use Committee.

In vitro organ culture

E13.5 embryos were harvested from *Gsk3 β ^{+/-}* crosses and decapitated. After removal of mandible, each embryonic head was placed in 2 ml DMEM culture medium supplemented with 20% fetal calf serum in a 20 ml glass bottle. Bottles were fixed in vertical position on a rotary apparatus rotating at a speed of 4 rpm in an incubator at 37°C and 5% CO₂. Samples were harvested after 24 hrs in culture, and processed for histological examination.

Histology and in situ hybridization

Staged embryos were harvested in ice-cold PBS, and embryonic heads were removed and fixed in 4% paraformaldehyde (PFA)/PBS overnight at 4°C prior to dehydration through ^a graded ethanol series and embedded in paraffin. Samples were processed into 10- μ m coronal sections for histological analysis by standard Hematoxylin/Eosin staining or for non-radioactive in situ hybridization, as described previously (St. Amand et al., 2000). For the expression of each gene, three independent experiments of in situ hybridization were performed.

X-gal staining

Embryos were collected, and embryonic heads were dissected in ice-cold PBS. The mandible of each head was carefully removed with a pair of fine forceps and discarded. Samples were washed in PBS, and fixed in 1% glutaraldehyde/PBS at 4°C for 1 hr. After three washes in PBS, samples were stained in X-gal solution (400 μ g/ml, with potassium ferricyanide and potassium ferrocyanide) overnight at room temperature for β -galactosidase activity.

Cell proliferation and TUNEL assays

Cell proliferation rate was measured using Bromodeoxyuridine (BrdU) Labeling and Detection Kit (Roche Diagnostics Corporation, Indianapolis). Briefly, timed pregnant female mice were injected intraperitoneally with BrdU solution (1.5ml/100g body weight) one hour before embryos were harvested. Embryonic heads were fixed in Carnoy's fixative, dehydrated, embedded in paraffin and sectioned at 5- μ m for immunostaining according to manufacturer's instruction (He et al., 2008). BrdU-labeled cells were counted within arbitrarily defined areas and presented as percentage of total nuclei of the same area. With data collected from nine sections of three individual samples of wild type controls and mutants, respectively, were subjected to Student's *t*-test to determine the significance of differences. For TUNEL assays, samples were fixed in 4% PFA/PBS, dehydrated and embedded in paraffin. Coronal sections were made at 5- μ m thickness. Apoptotic cells were detected as described previously (Alappat et al., 2009).

Acknowledgments

We thank Dr. Hao Ding of University of Manitoba, Canada and Dr. Andras Nagy of Mount Sinai Hospital, Canada, for providing *Pdgfa* and *Pdgfc* probes. We thank Dr. Rulang Jiang of the University of Rochester for providing *Osr2-Cre* mice. This work was supported by NIH grants (R01 AG031883 to C.J.P. and R01 DE14044 to Y.C.)

References

- Alappat SR, Zhang Z, Suzuki K, Zhang X, Liu H, Jiang R, Yamada G, Chen Y. The cellular and molecular etiology of the cleft secondary palate in *Fgf10* mutant mice. *Dev Biol*. 2005; 277:102–113. [PubMed: 15572143]
- Barrow JR, Capecchi MR, Rossel M. Compensatory defects associated with mutations in *Hoxa1* restore normal palatogenesis to *Hoxa2* mutants. *Development*. 1999; 126:5011–5026. [PubMed: 10529419]
- Blanton SH, Bertin T, Serna M, Stal S, Mulliken JB, Hecht JT. Association of chromosomal regions 3p21.2, 10p13, and 16p13.3 with nonsyndromic cleft lip and palate. *Am J Med Genet*. 2004; 125A: 23–27. [PubMed: 14755462]
- Bulleit RF, Zimmerman EF. The influence of the epithelium on palate shelf reorientation. *Development*. 1985; 88:265–279.
- Carroll TJ, Park J-S, Hayashi S, Majumdar A, McMahon AP. Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Dev Cell*. 2005; 9:283–292. [PubMed: 16054034]
- Casey LM, Lan Y, Cho ES, Maltby KM, Gridley T, Jiang R. Jag2-Notch1 signaling regulates oral epithelial differentiation and palate development. *Dev Dyn*. 2006; 235:1830–1844. [PubMed: 16607638]
- Ciaraldi TP, Oh DK, Christiansen L, Nikoulina SE, Kong APS, Baxi S, Mudaliar S, Henry RR. Tissue-specific expression and regulation of GSK-3 in human skeletal muscle and adipose tissue. *Am J Physiol Endocrinol Metab*. 2006; 291:E891–E898. [PubMed: 16757548]
- Dansky HM, Charlton SA, Sikes JL, Heath SC, Simantov R, Levin LF, Shu P, Moore KJ, Breslow JL, Smith JD. Genetic Background Determines the Extent of Atherosclerosis in ApoE-Deficient Mice. *Arterioscler Thromb Vasc Biol*. 1999; 19:1960–1968. [PubMed: 10446078]
- DasGupta R, Fuchs E. Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development*. 1999; 126:4557–4568. [PubMed: 10498690]
- Ding H, Wu X, Bostrom H, Kim I, Wong N, Tsoi B, O'Rourke M, Koh GY, Soriano P, Betsholtz C. A specific requirement for PDGF-C in palate formation and PDGFR- α signaling. *Nat Genet*. 2004; 36:1111–1116. [PubMed: 15361870]
- Doble BW, Patel S, Wood GA, Kockeritz LK, Woodgett JR. Functional Redundancy of GSK-3 α and GSK-3 β in Wnt/ β -Catenin Signaling Shown by Using an Allelic Series of Embryonic Stem Cell Lines. *Dev Cell*. 2007; 12:957–971. [PubMed: 17543867]
- Doble BW, Woodgett JR. GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci*. 2003; 116:1175–1186. [PubMed: 12615961]
- Embi N, Rylatt DB, Cohen P. Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *Eur J Biol*. 1980; 107:519–527.
- Farley FW, Soriano P, Steffen LS, Dymecki SM. Widespread recombinase expression using FLP α (flipper) mice. *Genesis*. 2000; 28:106–110. [PubMed: 11105051]
- Ferguson MWJ. Palate development. *Development*. 1988; 103 suppl.:41–60. [PubMed: 3074914]
- Force T, Woodgett JR. Unique and overlapping functions of GSK-3 isoform in cell differentiation and proliferation and cardiovascular development. *J Biol Chem*. 2008; 284:9643–9647. [PubMed: 19064989]
- Frame S, Cohen P. GSK3 takes centre stage more than 20 years after its discovery. *Biochem J*. 2001; 359:1–16. [PubMed: 11563964]

- Grigoryan T, Wend P, Klaus A, Birchmeier W. Deciphering the function of canonical Wnt signals in development and disease: conditional loss- and gain-of-function mutations of beta-catenin in mice. *Genes Dev.* 2008; 22:2308–2341. [PubMed: 18765787]
- Grimes CA, Jope RS. The multifaceted roles of glycogen synthase kinase 3 β in cellular signaling. *Prog Neurobiol.* 2001; 65:391–426. [PubMed: 11527574]
- Gritli-Linde A. Molecular control of secondary palate development. *Dev Biol.* 2007; 301:309–326.
- Hayashi S, Lewis P, Pevny L, McMahon AP. Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain. *Mech Dev.* 2002; 119:S97–S101. [PubMed: 14516668]
- He F, Xiong W, Yu X, Espinoza-Lewis R, Liu C, Gu S, Nishita M, Suzuki K, Yamada G, Minami Y, Chen YP. Wnt5a regulates directional cell migration and cell proliferation via Ror2-mediated noncanonical pathway in mammalian palate development. *Development.* 2008; 135:3871–3879. [PubMed: 18948417]
- Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, Woodgett JR. Requirement for glycogen synthase kinase-3 β in cell survival and NF- κ B activation. *Nature.* 2000; 406:86–90. [PubMed: 10894547]
- Huang X, Goudy SL, Ketova T, Litingtung Y, Chiang C. Gli3-deficient mice exhibit cleft palate associated with abnormal tongue development. *Dev Dyn.* 2008; 237:3079–3087. [PubMed: 18816854]
- Jho EH, Zhang T, Domon C, Joo CK, Freund JN, Costantini F. Wnt/ β -Catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol Cell Biol.* 2002; 22:1172–1183. [PubMed: 11809808]
- Kerkela R, Kockeritz L, Macaulay K, Zhou J, Doble BW, Beahm C, Greytak S, Woulfe K, Trivedi CM, Woodgett JR, Epstein JA, Force T, Huggins GS. Deletion of GSK-3 β in mice leads to hypertrophic cardiomyopathy secondary to cardiomyoblast hyperproliferation. *J Clin Invest.* 118:3609–3618. [PubMed: 18830417]
- Lan Y, Ovitt CE, Cho ES, Maltby KM, Wang Q, Jiang R. Odd-skipped related 2 (Osr2) encodes a key intrinsic regulator of secondary palate growth and morphogenesis. *Development.* 2004; 131:3207–3216. [PubMed: 15175245]
- Lan Y, Jiang R. Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth. *Development.* 2009; 136:1387–1396. [PubMed: 19304890]
- Lan Y, Ryan RC, Zhang Z, Bullard SA, Bush JO, Maltby KM, Lidral AC, Jiang R. Expression of Wnt9b and activation of canonical Wnt signaling during midfacial morphogenesis in mice. *Dev Dyn.* 2006; 235:1448–1454. [PubMed: 16496313]
- Lan Y, Wang Q, Ovitt CE, Jiang R. A unique mouse strain expressing Cre recombinase for tissue-specific analysis of gene function in palate and kidney development. *Genesis.* 2007; 45:618–624. [PubMed: 17941042]
- Lazzaro C. Ccanismo di chiusura del palato secondario. *Monit Zool Ital.* 1940; 51:249–273.
- Lee JM, Kim JY, Cho KW, Lee MJ, Cho SW, Kwak S, Cai J, Jung HS. Wnt11/Fgfr1b cross-talk modulates the fate of cells in palate development. *Dev Biol.* 2008; 314:341–350. [PubMed: 18191119]
- Liu KJ, Arron JR, Stankunas K, Crabtree GR, Longaker MT. Chemical rescue of cleft palate and midline defects in conditional GSK-3 β mice. *Nature.* 2007; 446:79–82. [PubMed: 17293880]
- Liu P, Jenkins NA, Copeland NG. A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res.* 2003; 13:476–484. [PubMed: 12618378]
- Martinez-Alvarez C, Bonelli R, Tudela C, Gato A, Mena J, O’Kane S, Ferguson MW. Bulging medial edge epithelial cells and palatal fusion. *Int J Dev Biol.* 2000; 44:331–335. [PubMed: 10853831]
- Nashad A, Hay ED. TGF β 3 signaling activates transcription of LEF1 gene to induce epithelial mesenchymal transformation during mouse palatal development. *J Cell Biol.* 2003; 163:1291–1301. [PubMed: 14691138]
- Niemann S, Zhao C, Pascu F, Stahl U, Aulepp U, Niswander L, Weber J, Muller U. Homozygous WNT3 Mutation Causes Tetra-Amelia in a Large Consanguineous Family. *Am J Hum Genet.* 2004; 74:558–563. [PubMed: 14872406]
- Ontiveros CS, Salm SN, Wilson EL. Axin2 expression identifies progenitor cells in the murine prostate. *Prostate.* 2008; 68:1263–1272. [PubMed: 18563716]

- Rice R, Spencer-Dene B, Connor EC, Gritli-Linde A, McMahon AP, Dickson C, Thesleff I, Rice DPC. Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. *J Clin Invest.* 2004; 113:1692–1700. [PubMed: 15199404]
- Song L, Li Y, Wang K, Wang YZ, Molotkov A, Gao L, Zhao T, Yamagami T, Wang Y, Gan Q, Pleasure DE, Zhou CJ. Lrp6-mediated canonical Wnt signaling is required for lip formation and fusion. *Development.* 2009; 136:3161–3171. [PubMed: 19700620]
- Soriano P. The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. *Development.* 1997; 124:2691–2700. [PubMed: 9226440]
- St. Amand TR, Zhang Y, Semina EV, Zhao X, Hu Y, Nguyen L, Murray JC, Chen YP. Antagonistic signals between BMP4 and FGF8 define the expression of Pitx1 and Pitx2 in mouse tooth-forming anlage. *Dev Biol.* 2000; 217:323–332. [PubMed: 10625557]
- Tallquist MD, Soriano P. Cell autonomous requirement for PDGFR α in populations of cranial and cardiac neural crest cells. *Development.* 2003; 130:507–518. [PubMed: 12490557]
- Tudela C, Formoso MA, Martinez T, Perez R, Aparicio M, Maestro C, Del Rio A, Martinez E, Ferguson MW, Martinez-Alvarez C. TGF-beta3 is required for the adhesion and intercalation of medial epithelial cells during palatal fusion. *Int J Dev Biol.* 2002; 46:333–336. [PubMed: 12068957]
- van Amerongen R, Nusse R. Towards an integrated view of Wnt signaling in development. *Development.* 2009; 136:3205–3214. [PubMed: 19736321]
- Wang BE, Wang XD, Ernst JA, Polakis P, Gao WQ. Regulation of epithelial branching morphogenesis and cancer cell growth of the prostate by Wnt signaling. *PlosOne.* 2008; 3(5):e2186.
- Xiong W, He F, Morikawa Y, Yu X, Zhang Z, Lan Y, Jiang R, Cserjesi P, Chen YP. Hand2 is required in the epithelium for palatogenesis in mice. *Dev Biol.* 2009; 330:131–141. [PubMed: 19341725]
- Yang T, Huang YG, Ye W, Hansen P, Schnermann JB, Briggs JP. Influence of genetic background and gender on hypertension and renal failure in COX-2-deficient mice. *Am J Physiol Renal Physiol.* 2005; 288:F1125–F1132. [PubMed: 15613621]
- Yang Y, Topol L, Lee H, Wu J. Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. *Development.* 2003; 130:1003–1015. [PubMed: 12538525]
- Zhang Z, Song Y, Zhao X, Zhang X, Fermin C, Chen YP. Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. *Development.* 2002; 129:4135–4146. [PubMed: 12163415]

Figure 1.

Gsk3β expression pattern in the developing palate. (A, C) At E12.5, *Gsk3β* expression is primarily detected in the epithelium of both the anterior (A) and posterior (C) palatal shelf. (B, D) In the E13.5 palatal shelf, *Gsk3β* expression remains in the palatal epithelium, particularly in the medial edge epithelium (MEE), of the anterior (B) and posterior (D) regions. In addition, *Gsk3β* is also expressed in the epithelium of the developing tongue. PS, palatal shelf; T, tongue.

Figure 2.

Creation of *Gsk3β* conditional mice. (A) Targeting scheme. Exon 2 of *Gsk3β* was flanked by loxP sites (closed arrowheads). PGK-Neo cassette used for isolation of clones was flanked by FRT sites (open arrowheads), and removed by in utero exposure to Flp recombinase. Homology arms used for recombineering are shaded boxes. Primers used for genotyping are denoted as F1, F2, F3, R1. (B) Southern blot of correctly targeted ES cell clones. Genomic DNA was digested with *SpeI*. WT locus – 12 kb; targeted locus – 6 kb. Targeted clones are denoted by asterisks (*). (C) PCR genotyping of *Gsk3β* floxed mice after breeding to *Sox2Cre* mice. Product for WT allele (using F1/R1 primer pair) is present in all animals. Animals transgenic for Cre recombinase results in loss of F2/R1 fragment, while generating F3/R1 fragment, demonstrating loxP sites are functional in vivo. MW: molecular weight ladder; +/-: *Gsk3β* heterozygote; +/loxP: WT mouse containing one floxed allele.

Figure 3.

Epithelial inactivation of *Gsk3β* leads to cleft palate formation. (A) A wild type mouse embryo shows an intact palate at E18.5. (B, C) Global inactivation of *Gsk3β* (B) or epithelial-specific ablation of *Gsk3β* (C) generates identical cleft palate phenotype in mice at E18.5. (D, G) Coronal sections of an E13.5 wild type embryo show the secondary palatal shelves growing vertically along the tongue at the anterior (D) and posterior (G) level. (E, F, H and I) *Gsk3β*^{-/-} and *Pitx2Cre;Gsk3β*^{F/F} palatal shelves exhibit morphology comparable to the wild type controls at E13.5 in both the anterior and posterior domains. (J) At E14.5, the wild type palatal shelves have elevated and fused to form an intact palatal shelf. (K, L) At the same stage, however, the palatal shelves in *Gsk3β*^{-/-} (K) and *Pitx2Cre;Gsk3β*^{F/F} (L) remain vertically oriented and fail to elevate. (M) Coronal section of an E18.5 wild type palatal shelf at the posterior level. (O, N) At E18.5, the posterior palatal shelves of both *Gsk3β*^{-/-} and *Pitx2Cre;Gsk3β*^{F/F} still do not elevate. NS, nasal septum; PS, palatal shelf; T, tongue; asterisks highlight clefting.

Figure 4. *Gsk3β* is an intrinsic regulator of palatal shelf elevation. (A, B) Coronal sections of E13.5 wild type (A) and mutant (B) heads before culture show palatal shelves at the vertical position. (C) Coronal section of an E13.5 wild type heads after 24 hrs in organ culture shows elevated palatal shelves. (D) Coronal section of an E13.5 mutant head after 24 hrs in organ culture shows failure of palatal shelf elevation. Scale bar = 200 μ m.

Figure 5.

Gsk3 β is required for normal cell proliferation and epithelial cell survival during palate development. (A, B) BrdU labeling shows cell proliferation levels in the wild type controls (A) and *Gsk3 β ^{-/-}* palatal shelves (B). Black line demarcates the palatal shelf region for counting BrdU positive cells and apoptotic cells. (C–F) Increased apoptotic cells are detected in the *Gsk3 β ^{-/-}* palatal region, including the MEE, at E12.5 (D) and E13.5 (F) as compared to the wild type controls (C, E). (G) Comparison of the ratio of BrdU-labeled cells in the epithelium of developing palatal shelves in E12.5 and E13.5 wild type controls and *Gsk3 β* mutants. *: $P < 0.01$; **: $P < 0.005$. (H) Comparison of numbers of apoptotic cells found in the palatal epithelium in wild type controls and mutants at E12.5 and E13.5.

Figure 6.

Gsk3β does not regulate *Shh*, *Osr2*, *Pdgfa* and *Pdgfc* expression during palate development. At E13.5, *Shh* is expressed in the MEE and oral epithelium in the wild type palate (A, E). In the *Gsk3β*^{-/-} palate, *Shh* is expressed comparably to that in the wild type controls (B, F). *Osr2* is expressed in the oral side palatal mesenchyme of the wild type palatal shelf along the anterior-posterior axis (C, G), and its expression is not unaltered in the *Gsk3β*^{-/-} palate (D, H). *Pdgfa* and *Pdgfc* are expressed throughout palatal and oral epithelium in the wild type controls (I, M, K and O), the expression of these genes remains unaltered in the *Gsk3β*^{-/-} palate (J, N, L and P). PS, palatal shelf; T, tongue.

Figure 7.

The Wnt/ β -catenin signaling activity is not affected in the *Gsk3β*^{-/-} palate. (A) At E13.5, the *TOPGAL* reporter activity is detected in the lip and the upper incisors (blank arrows), but not in the palatal shelves (arrow). (B) The *TOPGAL* activity is also not detected in the *Gsk3β*^{-/-}; *TOPGAL* palate (arrows). (C) In the E13.5 wild type embryo, *Axin2* is expressed in the developing palatal shelves, particularly in the MEE and underlying mesenchyme, as well as the mesenchyme immediately adjacent to the epithelium in the ventral-lateral region of the developing tongue (arrow). (D) In the E13.5 *Gsk3β*^{-/-} embryo, *Axin2* expression is detected in the developing palatal shelves, with expression pattern and level comparable to that in the wild type control. However, ectopic *Axin2* expression is found in the

mesenchyme adjacent to the epithelium in the developing tongue (red arrows). (G) *Gsk3α* is expressed in the palatal epithelium including the MEE but not in the mesenchyme in the dorsal-lateral region of the tongue (arrow) in the wild type control. (H) *Gsk3α* expression is unaltered in the *Gsk3β*^{-/-} palate. PS, palatal shelf; T, tongue; U, upper incisor.